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IMMUNOBIOLOGICAL AND MOLECULAR GENETIC PROPERTIES OF NON-HEMADSORBING AFRICAN SWINE FEVER VIRUS STRAINS
(review)

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Abstract

African swine fever virus (ASF, *Asfivirus*, *Asfarviridae*) is the most serious problem for the swine industry worldwide. The proposed review presents the results of the study of non-hemadsorbing strains of the African swine fever virus (ASF, African swine fever virus). According to published data, most of the non-hemadsorbing strains of the ASF virus isolated in nature or obtained in laboratory conditions are weak or avirulent and have the property of forming immunological protection against homologous virulent hemadsorbing isolates or strains in subsequent infection of pigs (J.D. Vigário et al., 1970). On the African continent, avirulent non-hemadsorbent strains of ASF virus were usually isolated from persistently infected warthogs (*Phacochoerus* spp.), bush pigs (*Potomochorus porcus*) and soft mites *Ornithodoros moubata* (A. Pini, 1976; G.R. Thomson et al., 1979). In Europe (Portugal, Spain) and Asia (China) − from persistently infected domestic pigs (*Sus scrofa domesticus*), wild boars (*Sus scrofa*) and from *Ornithodoros erraticus* (*marocanus*) ticks (F.S. Boinas et al., 2004; C. Gallardo et al., 2019; Sun E. et al., 2021). The review focuses on the use of non-hemadsorbing strains in order to study immunological mechanisms of protection against ASF. In experiments with the OURT88/3 strain, CD8+ T-cells were shown to have an important role in immunological protection against ASF. The cross-protection induced by the OURT88/3 strain against infection with virulent isolates of unrelated genotypes correlated with the ability of these isolates to specifically stimulate the production of IFNγ by lymphocytes of the immunized pigs (C.C. Abrams et al., 2013). Experiments with the non-hemadsorbing strain NH/P68 demonstrated that a high levels of specific antibodies to the ASF virus is characteristic to the chronic form of the disease, while low levels of antibodies were noted in asymptomatic pigs after intranasal and intramuscular immunization (A. Leitão et al., 2001; C. Gallardo et al., 2019). The low pathogenicity of non-hemadsorbing isolates is associated with the loss of virulence factors due to large deletions close to the left end of the genome or smaller deletions and substitutions in genes encoding virulence factors elsewhere in the genome (F.S. Boinas et al., 2004). The loss of the hemadsorbing properties of the ASF virus is associated with deletions and/or a shift in the reading frame in the *EP402R* gene (R.J. Rowlands et al., 2009; R. Portugal et al., 2015; K.A. Mima et al., 2015). In terms of possible practical application of non-hemadsorbing strains this paper presents results on reducing adverse clinical reactions in pigs inoculated with deletion mutants of strains OURT88/3 and NH/P68 (M.L. Nogal et al., 2001; C. Hurtado et al., 2004; A.G. Granja et al., 2009). Naturally attenuated non-hemadsorbent strains of the ASF virus are used in research focused on the creation of candidate live vaccines. In experiments conducted with their use, up to a 100 % protection against homologous virulent isolates and strains of ASF virus was obtained in domestic pigs (K. King et al., 2011; P.J. Sánchez-Cordyn et al., 2017; C. Gallardo et al., 2018; C. Gallardo et al., 2019; P.J. Sanchez-Cordon et al., 2020) and wild boars (J.A. Barasona et al., 2019).

Keywords: African swine fever, non-hemadsorbing isolates, non-hemadsorbing strains, candidate vaccines

African swine fever (ASF) is a cruel infectious diseases of pigs and wild boars. ASF is currently the most serious problem for the pig industry worldwide.

Due to the lack of commercial vaccines [1-3], the only way to combat ASF remains the total destruction of domestic pigs and wild boars in the foci of infection [4, 5]. Due to the severe economic impact on the international trade in pigs and pork products, ASF is on the list of notifiable diseases.

The disease is caused by a single member of the *Asfarviridae* family (*Asfar*, *African swine fever virus*), a large DNA-containing virus that infects domestic and wild pigs (*Suidae*) [6-8]. In southeastern and southern Africa, ASF is maintained in a sylvatic transmission cycle between warthogs (*Phacochoerus* spp.), bush pigs (*Potomochorus porcus*) and soft mites (*Ornithodoros moubata*) [9]. Typically, in natural hosts, including ticks, ASFV causes a subclinical chronic or inapparent form of infection [10, 11]. Isolates and strains of the ASF virus differ in pathogenic, antigenic, haemadsorbing, genetic properties [12-14]. In particular, based on the 3'-terminal sequences of the B646L gene encoding the main p72 capsid protein, 24 ASFV genotypes have been identified in Africa [15-17]. ASF genotype I virus caused outbreaks of ASF outside the African continent from 1957 to 1991 in Portugal, Spain, France, the Netherlands, on the island. Madeira, in Italy, Cuba, Malta, about. Sardinia (Italy), in Brazil, the Dominican Republic, Haiti and the USSR [18-20]. All of them were eliminated (with the exception of the outbreak on the island of Sardinia). In 2007, the ASF virus of genotype II was introduced from Africa to Georgia, which received the name Georgia 2001/1 strain, the derivatives of which spread to other countries of the Caucasus and Europe [21-23]. The general picture of the development of the incidence indicates that ASF has become epizootic with the involvement of populations of both domestic and wild pigs [24-26]. In 2018, Georgia 2001/1-like ASFV genotype II was isolated in China and spread to 15 other Asian countries within three years [27-29]. However, along with the emergence of low-virulence genotype II isolates, two strains of genotype I ASF virus were isolated from diseased domestic pigs in China in mid-2021 [30-31]. Special attention of specialists was caused by the fact that both strains were characterized as non-hemadsorbing [31).

This review analyzes studies of non-hemadsorbing strains of the ASF virus, considers the features of their immunobiological and molecular genetic properties, and their use in fundamental and applied scientific research. In a number of works, when describing the viruses under study, the term “isolate” is more often used. In the context of this article, we mainly used the term “strain”, based on the fact that a strain is a local population of a virus identified by modern classification tests with original, stable properties (features), and an isolate is a virus isolated from a specific source.

Immunobiological properties of non-hemadsorbing strains of the ASF virus. The vast majority of ASF virus isolates are characterized as virulent and haemadsorbing. In 1968, L. Coggins reported the isolation of non-haemadsorbing subpopulations of the ASF virus [32]. Further studies have shown that the loss of the ability to induce hemadsorption for the ASF virus during reproduction in cell culture is a common phenomenon [33-35]. A number of researchers have noted that non-hemadsorbing strains of the ASF virus isolated in nature or obtained under laboratory conditions have low virulence and the ability to form immune protection against subsequent infection of pigs with homologous virulent hemadsorbing isolates [36, 37]. On the African continent, low-virulence or avirulent non-hemadsorbing isolates of the ASF virus, as a rule, were isolated from persistently infected warthogs, bush and domestic pigs, ticks of the genus *Ornithodoros* [38-40]. According to the EU Commission, 1% of samples obtained from domestic pigs in the Iberian Peninsula between 1968 and 1976 contained non-haemadsorbing ASF viruses [41]. Researchers who have attenuated ASFV in the laboratory to obtain candidate live vaccines have empirically arrived at a

method for selecting avirulent strains based on their reduced ability to induce haemadsorption during in vitro reproduction [42]. It should be noted that virulent non-hemadsorbing isolates have sometimes been isolated in nature. For example, of the two non-hemadsorbing isolates tested, one (Lillie-148) was virulent, while the other (Zaire) caused disease and death in only 33% of pigs [40].

The first naturally occurring non-hemadsorbing isolates of ASF virus were obtained from pigs in southern Portugal, where most of the pig herds were seropositive [43, 44]. This was preceded by outbreaks of ASF in the Iberian Peninsula in 1957 and 1960, which served as the beginning of an epizootic that lasted until the 1990s and still persists on the island. Sardinia in Italy. From the virulent haemadsorbing strain Lisbon 60 isolated during the second outbreak, a haemadsorbing "vaccine" strain 1455 was obtained as a result of 150 passages in primary culture of pig bone marrow cells. vaccinated animals, 7% of pigs developed unacceptable post-vaccination reactions, including pneumonia, movement disorders, skin ulcers, abortions and death of animals [45, 46]. More recently, a non-pathogenic, non-hemadsorbing strain of ASF virus NH/P68 (NHV, NHA2) was isolated from a pig with chronic ASF in Portugal. Also in Spain, 206 non-hemadsorbing isolates were obtained between 1965 and 1974 (47). It was noted that it was more difficult to isolate non-hemadsorbing ASFV isolates than hemadsorbing isolates, since the viremia caused by them was sporadic, and the virus accumulated in the organs of pigs in small quantities. Experiments have shown that non-pathogenic non-hemadsorbing isolates were less efficiently transmitted via contacts than virulent hemadsorbing isolates (40-50% vs. 100%). Infection of pigs with non-pathogenic non-hemadsorbing isolates could be the reason for the seropositivity of some herds in the absence of clinical symptoms in pigs. Based on the fact that inoculation of the NH/P68 strain resulted in protection against subsequent lethal challenge with the virulent Lisbon 60 strain, it was concluded that they are antigenic related [48].

Twenty years later, two types of ASF virus isolates of different pathogenicity were isolated from *O. erraticus* (*marocanus*) ticks collected from pigs and piggeries in southern Portugal. Isolates of the first type caused 100% death of pigs from the acute form of ASF, the second, in particular OURT88/3, did not cause clinical signs of disease and death of animals, although antibodies against the ASF virus were detected in all infected pigs [49]. It is believed that the direct ancestor of the OURT88/3 strain was the NH/P68 strain, which also replicates in ticks and is well adapted to the natural transmission cycle [50]. Two assumptions have been put forward regarding the origin of non-pathogenic non-hemadsorbing isolates in the Iberian Peninsula: they were obtained either from an attenuated vaccine strain or from an initial virulent isolate capable of persisting in the domestic pig—tick—wild boar cycle. Pigs inoculated with the non-haemadsorbing strain OURT88/3 were protected from death after infection with the related pathogenic haemadsorbing strain OURT88/1. Less effective protection was achieved when recovered pigs were injected with more distantly related isolates or strains of the ASF virus. It has been noted that after infection of immune pigs with the Lisbon 57 strain isolated during the first outbreak of ASF in Portugal in 1957 and the African isolate Malawi LIL20/1, the animals died, although the onset of clinical manifestation of the disease was delayed in time [51-53].

A comparison was made between the intramuscular and intranasal routes of immunization of pigs with different doses of the non-haemadsorbing ASF virus strain OURT88/3 [54]. With intranasal administration, two clinical groups were formed: pigs that developed intermittent clinical manifestations (10^3 and 10^4 $TCID_{50}$, 100% protection against OURT88/1), and animals that developed

chronic ASF (a dosage of 10^5 $TCID_{50}$, 66% protection. Pigs immunized intramuscularly with low and medium doses (10^3 and 10^4 $TCID_{50}$) showed a lower percentage of protection (50 and 66%). In blood samples throughout the study period, a low content of the virus genome was found. Interestingly, intramuscular immunization did not result in signs of chronic ASF in protected pigs. Viremia was not detected as early as 7 days after virus inoculation. These results indicated that the route of administration and the dose of virus determined the outcome of immunization with the naturally attenuated OURT88/3 strain. In studies with the low virulent NH/P68 strain, intranasal immunization also induced higher protection than intramuscular immunization [53]. A correlation was established between late viraemia after NH/P68 immunization (14 days after virus inoculation) and the appearance of pigs with chronic ASF. This ratio was not observed in protected pigs immunized intranasally with OURT88/3 strain, where late viremia has been described in animals without chronic ASF [48, 55].

It has been established that the OURT88/3 strain induces a high degree of protection against lethal infection by related virulent isolates of the ASF virus [49, 56, 57]. Experimental immunization of pigs with OURT88/3 followed by challenge with the closely related virulent strain OURT88/1 induced protective immunity in European domestic pigs against challenge with two virulent African isolates of ASFV: Benin 97/1 genotype I from West Africa (85.7%) and Uganda 1965 genotype X from East Africa (100%). More than 78% of pigs infected with Benin 97/1 and 50% infected with Uganda 1965 showed no signs of disease or development of viremia [57].

In the Democratic Republic of the Congo, the non-hemadsorbing strain Mfuati-79 (immunotype II, genotype I) was isolated from domestic pigs in 1979. Fifteen days after intramuscular immunization with the Mfuati-79 strain at $10^{3.0}$-$10^{4.0}$ TCD_{50}, pigs developed resistance to intramuscular infection with the homologous virulent haemadsorbing strain Congo-49 (immunotype II, genotype I) at $10^{5.5}$-$10^{7.5}$ HAU_{50} [58].

In 2017, a non-hemadsorbing ASF virus isolate Lv/17/WB/Rie1 genotype II was isolated in Latvia (59). In pigs inoculated intramuscularly with the Lv/17/WB/Rie1 isolate, an asymptomatic form of infection was observed, periodic and weak viremia was manifested, and a high content of virus-specific antibodies was noted in blood sera. In addition, 2 months after primary infection with the Lv17/WB/Rie1 isolate, two pigs infected with the virulent hemadsorbing Latvian isolate survived. Despite the fact that the number of animals was small, these results, according to the authors, open the prospect of using the Lv17/WB/Rie1 isolate as an object for the development of live attenuated vaccines, as is the case with the NH/P68 and OURT88/3 strains. Importantly, the study illustrates the natural evolution of the ASF virus, including the emergence of less avirulent non-haemadsorbing isolates over time in the absence of *Ornithodoros* ticks.

For the first time in 14 years of global ASF panzootic caused by strains similar to Georgia 2007/1 genotype II, outside of Africa and about. Sardinia in Henan and Shandong provinces of the PRC isolated two strains of genotype I (HeN/ZZ-P1/21 and SD/DY-I/21) from domestic pigs, which were non-hemadsorbing and caused chronic ASF disease [31]. Phylogenetic analysis showed some differences between the strains HeN/ZZ-P1/21 and SD/DY-I/21 and their commonality with the Portuguese strains NH/P68 and OURT88/3.

It has been known for many years that pigs that recover from infection with ASF virus can be protected from disease and/or death when subsequently infected with related virulent isolates of the virus [60-62]. In addition, pigs inoculated with naturally attenuated or laboratory-selected cell culture passaged ASF viruses may also be protected from challenge by homologous virulent isolates [49,

63]. The non-hemadsorbing strains NH/P68 and OURT88/3 of the ASF virus are effectively used in the study of the mechanisms of formation of immune defense and to determine the significance of various genes in the pathogenicity of the virus. In experiments with the OURT88/3 strain, an important role of CD8+ T cells in the immune defense against ASF was established. Monoclonal antibody depletion of this cell subpopulation abolished the protection induced by the OURT88/3 strain against infection with the virulent OURT88/1 isolate [56]. The possible role of antibodies in protection against ASF has been shown in experiments on the passive transfer of antibodies from immunized to intact pigs [64-66]. It has been found that neutralizing antibodies are not effective enough, but other antibody-mediated protective functions are possible, in particular antibody-dependent cytotoxicity [64, 67].

Experiments with the NH/P68 strain have shown that a high level of specific antibodies to the ASF virus is characteristic of the chronic form of the disease, while a low level is noted in asymptomatic pigs after intranasal and intramuscular immunization [46, 57]. Regardless of doses and methods of immunization with strain OURT88/3, high levels of antibodies against the structural protein p72 were observed in pigs both with signs of chronic ASF and without them, and even in 50% of immunized pigs that were not protected from infection with a homologous virulent isolate [68]. Cross-protection induced by strain OURT88/3 against infection with virulent isolates of ASF virus from unrelated genotypes correlated with the ability of these isolates to specifically stimulate the production of IFNγ by lymphocytes of immunized pigs [57, 69].

Studies measuring the activity of NK cells in pigs inoculated with the NH/P68 strain revealed the functional role of this subset of lymphocytes in antiviral defense. In animals that remained healthy after the introduction of the NH/P68 strain and became resistant to infection with the Lisbon 60 strain, an increased number of NK cells was noted on the 7th day after inoculation. In some pigs, high NK cell activity was observed throughout the experiment. In contrast, in animals that developed chronic ASF after inoculation with NH/P68, NK cell activity was similar to or slightly higher than that of control animals. Virulent ASF virus isolates suppressed NK cell activity in pigs [70]. In vitro NK activity of porcine mononuclear cells was suppressed by both low- and high-virulence ASF virus isolates [71]. These data support the notion that immunity to ASFV depends, at least in part, on cellular mechanisms, in particular NK cells [48].

Structural, functional, comparative genomics of non-hemadsorbing ASF virus strains. In the ASF defense strategy, the use of a live attenuated vaccine is considered to be preferable because it elicits immune responses against all viral antigens that the host normally encounters during infection [72, 73]. With this in mind, studies mainly use naturally attenuated non-hemadsorbing strains OURT88/3 and NH/P68. Including the possibility of reducing adverse clinical reactions in pigs inoculated with deletion mutants of strains OURT88/3 and NH/P68 while maintaining high protection against homologous virulent isolates of the ASF virus [74-76].

It is known that the deletion of the DP96R gene in the DNA of the virulent E70 strain did not affect the growth characteristics of the ASF virus in macrophage cell cultures in vitro, but the degree of viremia in pigs inoculated with a deletion mutant of the virus was reduced 100-1000-fold [77, 78]. Similar deletions of the *DP71L* and *DP96R* genes from the DNA of the OURT88/3 strain also did not reduce the replication of the deletion mutant OURT88/3ΔDP2 in primary porcine macrophages in vitro compared to the parent strain OURT88/3. However, two of the six pigs inoculated with the deletion OURT88/3ΔDP2 virus were not protected from subsequent challenge with the virulent OURT88/1 strain, while all

six pigs inoculated with the parent strain OURT88/3 were protected (69). Deletion of the *A224L* gene encoding an apoptosis inhibitor from the genome of strain NH/P68 did not affect the ability of the deletion mutant NH/P68DA224L to protect pigs from the homologous virulent Lisbon 60 strain. one pig immunized with NH/P68DA224L). In contrast, the parental NH/P68 virus completely protected the animals after infection with Arm07. Not only did the pigs not show any noticeable clinical signs after infection with Arm07, but no virus was found in their blood or tissues. Interestingly, from pigs immunized with the parent non-hemadsorptive strain NH/P68, the virus was effectively transmitted to controls within 3-4 weeks after initial infection. However, transmission of the virulent Arm07 virus to control pigs was not observed [72].

The question is obvious: what is the difference between the genomes of virulent hemadsorbing and attenuated non-hemadsorbing strains of the ASF virus? The genomes of two non-hemadscoping strains of the ASF virus, NH/P68 and OURT88/3, have been well studied. In the first one, the genome consists of 172051 bp, in the second, of 171719 bp. 158 open reading frames (ORFs) are encoded in their genomes, the similarity is 99.98% [69, 79].

Variants of the ASF virus genomes are mostly the result of the presence of a different number of genes of multigene families (MGF) on the left and right variable regions (LVR and RVR). MGFs are specific to the ASF virus and have no obvious homology with other known genes. Depending on the size of proteins, they are divided into 5 families: *MGT-100*, *MGT-110*, *MGT-300*, *MGT-360*, and *MGF-505* [79, 80]. It is known that MGF proteins play an important role at different stages of viral infection and modulate transcription and translation in host cells. For example, the *MGF-360* and *MGF-505* genes have been shown to be important for the propagation of the ASFV strain BA71V in macrophages [80, 81]. However, the properties of most MGF proteins still remain unexplored.

The low pathogenicity of non-hemadsorbing isolates may be due to the loss of virulence factors due to large deletions close to the left end of the genome or smaller deletions or substitutions in genes encoding virulence factors elsewhere in the genome [62]. When strains NH/P68 and OURT88/3 were compared with strain Lisbon 60, the main differences were found in the left part of the genome (Fig.) [82]. Discrepancies were also noted in the central and right parts of the genome. The main differences were established in the genes of MGF proteins.

Comparison of the genome of the African swine fever virus strain Lisbon 60 (L60) with the genomes of strains NH/P68 (NHV) and OURT88/3. The bold black line shows homology regions of the NHV and OURT88/3 genomes with the Lisbon 60 genome. The thin black line shows deletions in the NHV and OURT88/3 genomes; the vertical black lines show insertions that are present in NHV and OURT88/3.

The LVR of the genomes of strains NH/P68 and OURT88/3 is 10 kb shorter than the LVR of the genome of strain Lisbon 60. Notably, in addition to the deletion, there is a 4458 bp insert located between *MGF 110*-2L and *110-13L*. The insert contains genes for proteins *MGF 110-4L*, *110-5L*, *110-9L*, *100-1R*, *ORFs 285L* and *86R*. Unfortunately, the functions of the proteins encoded by these genes are unknown. The next difference is the absence of nucleotides at positions 7244-8632 in the genome of the NH/P68 strain compared to the Lisbon 60 strain. This deletion results in the absence of the *MGF 110-11L*, *110-12L*, and *110-13L* genes. As a result of another deletion 2173 bp long, the NH/P68 strain lost the *MGF 360-6L* gene. It should be noted that this gene is also absent in the

non-pathogenic strain BA71V. The third region that is absent in the genome of the NH/P68 strain is fragment 19809-29877 of the Lisbon 60 strain genome. This deletion results in the loss or damage of the genes *MGF 360-9L*, *360-10L*, *360-11L*, *360-12L, 360-13L*, *360-14L*, *505-1R*, *505-2R*, *505-3R*. Some of them are involved in macrophage replication, virulence, tick infection, and type I IFN immune response [82-84] (Table). The RVR regions of NH/P68 and Lisbon 60 also have differences. Basically, these are short insertions or deletions in the genes encoding MGF proteins. The protein of the virulent strain, encoded by the *MGF 360-16R* gene, is two amino acids shorter than its non-hemadsorbing phenotype homologue. The MGF 505-11L protein in the NH/P68 and OURT88/3 strains has an insert (its length is four amino acids). Non-hemadsorbing strains also have a mutation in the *MGF 100-2L* gene, which leads to a frameshift and the appearance of a stop codon [85, 86].

MGF families in the LVR and RVR genomes of the virulent hemadsorbing strain of African swine fever virus Lisbon 60 and the avirulent non-hemadsorbing strain NH/P68 (NHV) [86]

Region of the genome	*MGF* family	Strains	
		Lisbon 60	NH/P68
LVR (left)	*MGF 100*	-	1R
	MGF 110	1L, 13L+2L, 11L, 12L, 13L, 14L	1L, 2L, 4L, 5L, 9L, 13L§, 14L
	MGF 300	1L, 2R, 4L	1L, 2R, 4L
	MGF 360	1L, 2L, 3L, 4L, 6L, 8L, 9L, 10L, 11L, 12L, 13L, 14L	1L, 2L, 3L, 4L, 8L, 9L‡
	MGF 505	1R, 2R, 3R, 4R, 5R, 7R, 8R, 9R, 10R	3R‡, 4R, 5R, 7R, 8R, 9R, 10R
RVR (right)	*MGF 100*	2L	2L‡
	MGF 360	16R, 18R	16R, 17R‡, 18R
	MGF 505	11L	11L

N o t e. A dash means no members of the *MGF* family of the specified type. ‡ is a truncated version of the gene; § is different from the same gene in the OURT88/3 strain.

The CD2v glycoprotein, which is encoded by the *EP402R* gene, is responsible for the hemadsorption phenomenon during ASF virus reproduction [87, 88]. This glycoprotein is homologous to the mouse, human, and porcine T cell adhesive receptor CD2. Deletion of the *EP402R* gene from the ASF virus genome deprived it of its ability to induce the adsorption of porcine erythrocytes on the surface of infected cells, but did not affect the rate of its reproduction in vitro. Expression of CD2v on the surface of viral particles correlates with the association of virions with erythrocytes in the blood of infected pigs [88]. The calculated molecular weight of the CD2v glycoprotein polypeptide is about 45 kDa, the mature glycoprotein, taking into account carbohydrate chains, is 105-110 kDa [89, 90]. According to its hydrophilic profile, it is a typical transmembrane glycoprotein consisting of four differentiated regions: a hydrophobic leading region at the N-terminus of 20 amino acids, a hydrophilic extracellular part of 183 amino acids with 15-16 N-glycosylation sites, a transmembrane region of 25 amino acids and a proline-rich cytoplasmic C-terminal part of 174 amino acids. Comparative genomics has shown that the *EP402R* open reading frame is one of the most variable in the ASFV genome [91]. Genotyping for the genetic locus encoding CD2v coincides with the grouping of ASF virus strains according to seroimmunogroups [92, 93]. In the ASF virus strains 26544/OG10, Benin 97/1 and BA71V, the polypeptide determined by the EP402R gene consists of 428 amino acids, in the E75 strain it consists of 420 amino acids, in the non-hemadsorbing strains OURT/88 and NHV/P68 it consists of 330 [82].

It has been established that the interaction between CD2v and its ligand on erythrocytes is stabilized through the expression of a virus-specific lectin-like C-type glycoprotein encoded by the *ORF EP153R*, since the deletion of the *EP153R* gene led to a decrease in hemadsorption around ASF virus-infected cells

[85, 94].

There is an opinion that hemadsorption is not directly related to virulence, since non-hemadsorbing virulent isolates are known [95, 96]. Deletion of the *EP402R* or *EP153R* genes from the Malawi LIL20/1 isolate genome did not reduce its virulence in domestic pigs [85]. Interestingly, the haemadsorbing phenotype favors increased ASF virus replication in ticks. This was observed after recovery of haemadsorbing activity in the NHV/P68 strain, with no recovery of virulence in pigs [55]. Both glycoproteins also perform other functions: CD2v has immunosuppressive activity (28), C-lectin-like glycoprotein suppresses apoptosis and expression of the histocompatibility antigen SLA I on the plasma membrane [92].

The question is natural of the molecular mechanisms of the origin of non-hemadsorbing strains of the ASF virus. Naturally isolated strains not capable of haemadsorption showed changes in the *EP402R* gene sequence. The loss of haemadsorbing properties in some strains of the ASF virus is associated with deletions and/or frameshifts in the *EP402R* gene [86, 97]. It can be assumed that, along with the deprivation of the ability to induce hemadsorption, the ability of non-hemadsorbing isolates to form immunotype-specific protection should be lost. In fact, this is not the case [98]. Most non-hemadsorbing isolates and laboratory strains retain the ability to induce immunotype-specific protection [99-101].

Thus, populations of African swine fever (ASF) virus and susceptible animals in Africa represent a co-evolutionary biological system. Virus isolates differ in genetic and immunobiological characteristics. To date, 24 genotypes and 9 immunoserotypes of the ASF virus have been established, as well as a variety of isolates in terms of virulence, as well as the ability to induce hemadsorption. Non-hemadsorbing isolates and strains are a natural element of the phenotypic heterogeneity of the ASF virus. The absence of haemadsorbing properties is explained by deletions and/or frame shifts in the *EP402R* gene encoding CD2v envelope glycoprotein. As a rule, isolates isolated from nature or obtained under laboratory conditions, non-hemadsorbing strains of ASF virus are characterized by low virulence up to the absence of clinical symptoms after inoculation in pigs, as well as the ability to form an immune defense against subsequent infection with homologous virulent hemadsorbing isolates. Therefore, non-hemadsorbing natural, laboratory-selected and recombinant strains of the ASF virus are used both to obtain fundamental knowledge about the mechanisms of protective immunity formation and to develop promising live vaccines against ASF.

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MICROBIOTA AND REPRODUCTION IN AGRICULTURAL MAMMALS
(review)

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Abstract

The use of specialized animal breeds of agricultural species is often accompanied by a decrease in reproductive success. In dairy cattle breeding, the number of service-period days, artificial insemination procedures per pregnancy, and the frequency of pregnancy losses are increasing (S.V. Guskova et al., 2014). Accumulated data on obtaining embryos by *in vivo* and *in vitro* methods and their transplantation indicate a significant level (30-60 %) of embryo losses (P.J. Hansen, 2020). The reasons for low rates in reproductive technologies are diverse and associated with both biotic and abiotic factors, and one of the key factors of embryo losses may be the imbalance of microbial communities in the reproductive system sections of both female donors and recipients. The study of the microbiota composition of various departments and systems of the multicellular organism has recently become an increasingly dominant topic in the scientific literature. Modern methods of microbial identification, e.g., metagenomic sequencing, reveals great microbial diversity in various anatomical departments of macroorganisms. The accumulated data show the microbial composition, dynamics in the organs of the reproductive system, and its relationship with the reproduction of mammals, reproductive success, the course of pregnancy, the prognosis of the possibilities of pathological processes. The review focuses on the impact of microbiota on the success of reproductive technologies, e.g., in vitro fertilization, embryo transplantation, and artificial insemination. For example, F. Marco-Jiménez et al. (2020) discuss the effect of symbiotic bacteria on fertility and semen quality. The understudied nature of this area for mammals and the extreme need for additional research on the microbiota of the reproductive tract of farm animals, the results of which will provide insight and insight into the unsuccessful and positive outcomes of reproduction, are noted. At the same time, the practical application of this information will increase the chances of success in reproductive biotechnology, reduce the costs associated with reproduction and therapeutic interventions in the treatment of pathological processes of the reproductive system, and open up the possibility of developing and implementing new methods such as microbial therapy. Thus, it can be concluded that the microbiota of mammalian reproductive system and organs influence the physiological processes of reproduction (R. Koedooder et al., 2019). It is clear that by being able to manage microbial communities, humans can increase the chances of reproductive success in the reproduction of highly specialized breeds of farm animals (P.J. Hansen, 2020; R.W. Hyman et. al., 2012; D.E. Moore et. al., 2000).

Keywords: endometrium, microbiota, microbiome, reproductive system, sperm, uterus, reproductive technology

Livestock reproduction at any livestock is the main technological stage in ensuring the success of anima husbandry. The development of progressive technologies, market economy, and competition provide the transition of most modern agricultural enterprises from extensive to intensive development. For this purpose, herds are created, consisting of specialized highly productive breeds, and in almost every branch of animal husbandry, reproductive biotechnologies, e.g. artificial insemination, obtaining embryos by in vivo and in vitro methods, embryo transplantation, etc., are used to reproduce livestock. An increase in the specialization of animals in the direction of productivity inevitably leads to a decrease in the potential of their biological characteristics, such as adaptive qualities, reproductive longevity, and reproductive success [1, 2]. Recent works aimed at studying the

microbial communities of organs and systems of a multicellular organism indicate that an imbalance in the composition of the microbiota can lead to negative phenomena and manifest itself in the form of acute pathological processes or a functional disorder in one or more physiological systems of the body.

All tissues and organs of a multicellular organism are colonized by a coexisting microbial community, which includes bacteria, viruses, fungi, yeasts, archaea and protozoa [3]. The diversity of microorganisms within a particular physiological system of a macroorganism is defined as microbiota, i.e. the species composition of the microbial community [4]. The prevailing microorganisms is called dominant, and each organ or system in a macroorganism has its own characteristic composition of microbial associations. Diversity of microorganisms is referred to as alpha and beta diversity. Alpha diversity characterizes the average species diversity in a sample of interest, while beta diversity reflects the diversity between different samples [5]. Components of the microbiota affect both the macroorganism and each other. The relationship between them can be mutualistic (mutually beneficial), commensal and parasitic. The totality of the genomes of these communities is defined as the microbiome [3, 4]. With the advent of methods for sequencing the conserved bacterial 16S rRNA gene (6, 7), next-generation sequencing (NGS) [8-10], whole genome sequencing (WGS) [11, 12], quantitative PCR (qPCR) [13, 14], a large amount of data on new genes, genome organization, and bacterial community structures has been obtained. Bioinformatic resources such as mothur [15] and Quantitative Insights Into Microbial Ecology (QIIME) [16] are available for processing such data. The main function of these information systems is to combine the obtained DNA sequences into operational taxonomic units (OTU) by various methods [17, 18] using external reference databases - Greengenes [19], SILVA [20], Ribosomal Database Project [21]. However, the shortcomings associated with the methodological features of research, analysis and interpretation of the obtained data can adversely affect the objectivity and quality of the results [22-25].

At present, the microbiome of various human systems and organs has been most studied [5], while the microbial communities of the organs of the reproductive system of agricultural mammals have not been studied enough.

The purpose of our review is to analyze current publications on the microbiota of the reproductive tract, describing the composition of microbial communities in various anatomical regions (vagina, cervix, endometrium and placenta) of mammals, and to consider the proposed mechanisms of the relationship between the abnormal composition of the microbiota of the reproductive organs and reproductive success in mammalian agricultural species. .

Microbiota and multicellular organism. The accumulated information about the microbiota of various species and breeds helps to expand our understanding of the processes of evolution and domestication. Thus, data have been obtained indicating that the most universal indicators of the domestication syndrome. i.e. a change in behavioral characteristics (decrease in aggressiveness, increase in socialization) [26, 27] and a significant expansion of phenotypic and population genetic variability [28] can manifest themselves and be closely related to each other. with a friend due to changes in the microbiota of animals formed in the same ecological niche together with humans [26]. The microbiota of the mammalian gastrointestinal tract has been called the “forgotten organ” [29], and its study has become the basis of the theory developed in recent years about the role of the microbiota in evolutionary processes [30]. Each mammalian species contains an intestinal microbiota, the variability of which is associated with the processes of adaptation and diversification of animals, contributing to the possibilities of changing the type of nutrition, phenotypic plasticity, and the work of

innate and adaptive immunity. The gut microbiota serves as an important target for environmental factors and as a selective agent shaping the adaptive evolution of the mammalian diet, phenotypic plasticity, gastrointestinal tract morphology, and immunity [30]. The concept of the role of interactions between a multicellular organism and microbiota in the process of evolution has been called the hologenomic theory of evolution, in which the relationship between them is considered as the main target of genomic transformations under the influence of environmental factors [31]. Comparative analysis of the microbiota of wild and closely related domesticated species provides insight into how domestication may have affected the composition of microbial associations in farm animals. For example, a comparative study of the microbiota of domestic pigs and wild boars showed, in particular, that some representatives of *Enterobacteriaceae*, which are considered the dominant bacterial groups in the intestinal microbiota of pigs, do not occur in wild boars. Interestingly, in recently domesticated wild boars, a corresponding shift in the species representation of *Enterobacteriaceae* was found. Taken together, this suggests that the composition and structure of the gut microbiota of domestic pigs may reflect the management practices of this livestock sector. It has also been shown that in cattle, inoculation with bison rumen content increases protein digestibility and nitrogen retention, but not fiber digestibility, which suggests the ability of microbial communities in the gastrointestinal tract of farm animals' ancestors to use nitrogen from plant feed mass for amino acid biosynthesis [32].

Ongoing microbiome research aims to reveal missing details in pathophysiological processes and to explain seemingly random variations in disease severity and phenotypic manifestations due to, for example, environmental-geographical and forage factors. Thanks to advances in the study of microbial communities, important information has been obtained that bacterial dysbiosis can lead, in particular, to disturbances in the functioning of the nervous system [33, 34]. There is evidence suggesting a role for the microbiota in many complex disorders such as obesity, cancer and inflammatory bowel disease [35]. It is now known that the gut microbiota significantly influences overall host metabolism and immune responses [36]. External factors (antibiotics, diet, and geographic location) can have a critical impact on the composition of the gut microbiota [37].

A similar trend is observed in the microbiota of the reproductive system under both physiological and pathological conditions [38]. The reproductive system of multicellular organisms is the main structure that determines the reproduction of a biological object. When studying the microbial communities of the reproductive organs, in particular in humans, it was shown that the differences between biosamples within the same physiological system (beta diversity) were significantly greater than the differences between samples obtained from the same organ (alpha diversity). The vaginal microbiota in mammals was characterized by the least alpha diversity with relatively low beta diversity at the genus level, but very high diversity among taxonomic units studied due to the predominance of lactobacilli.

It has been established that a symbiotic relationship between the host and microorganisms is necessary and disruption of this relationship can lead to a dysbiotic state [39, 40]. For example, bacterial vaginosis is characterized by a shift from a healthy, low pH values in a lactobacillus-dominated community to an elevated pH and a more diverse microbial community [41]. However, shifts between symbiosis and dysbiosis and vice versa are still not well understood.

In farm animals, these issues are very important because they are related to reproduction and therefore have significant economic importance, directly affecting the efficiency of livestock production [42].

Microbial communities of the reproductive system in female mammals. In mammals, the reproductive organs of both females and males are systems separated by anatomical or physiological barriers. In females, the reproductive tract consists of the following sections: vagina, cervix and uterine cavity, uterine horns, oviducts and ovaries. More and more evidence is accumulating, indicating that certain bacterial communities have an unequal impact on reproductive health and reproductive success. Thus, a specific microbial composition has been found in humans, which differs in the parts of the reproductive system [3, 43]. It has been found that the number of bacteria localized in the endometrium is significantly lower compared to their number in the vagina, suggesting that the cervix acts as a protective barrier to the ascending microbiota [44].

The vaginal microbiota can be divided into five (I-V) community state types (CSTs), four of which are dominated by lactobacilli. Group I is dominated by Lactobacillus crispatus (26.2%), group II by L. gasseri (6.3%), group III by L. iners (34.1%), and group V by L. jensenii (5.3%) [45, 46]. In group IV, there is no dominance of lactobacilli, but there are many more severe anaerobes [47]. CST IV-A is characterized by the presence of some species of *Lactobacillus* spp. and a variety of strictly anaerobic bacteria, the IV-B community combines representatives of the genera *Atopium*, *Prevotella*, *Sneathia*, and *Gardnerella* [48]. A.Y.K. Albert et al. [49] expanded our understanding of the range of bacterial communities. The authors, by changing the methodological approach, found that *Gardnerella* subgroups (CST IV-C and IV-D) predominate in the communities [49].

It was found that the vaginal microbiota is dynamic, as the species composition of communities undergoes modifications over time. It is known that CST IV-B often changes to CST III, but rarely to CST I, CST I often changes to CST III or CST IV-A, CST III changes 2 times more often to CST IV-B compared to CST IV- A, CST II rarely changes, with no change from CST I to CST II observed, and CST II is relatively stable compared to CST IV-A.

Differences in microbial composition are also reflected in vaginal pH. CST I appears to have the lowest median pH (4.0±0.3), while CST IV has the highest pH (5.3±0.6). The difference in pH between different CSTs is most likely due to the specific dominance of lactobacilli and the ability of each lactobacillus to produce lactic acid [50].

The vaginal microbiota of non-pregnant healthy women may change depending on a number of characteristics: the periods of the sexual cycle (estrus, ovulation, etc.), ethnic origin, ecological and geographical factors x47, 48, 51-53].

Hormonal status has a significant impact on the composition of the microbiota; for example, changes in microbial composition during pregnancy have been shown to be a response to increased estrogen levels [54].

During pregnancy, the abundance and biodiversity of the vaginal microbiota decreases, while closer to childbirth, it returns to a state characteristic of non-pregnant women [54, 55]. The predominance of *Lactobacillus* spp. during pregnancy [54, 56] reduces the risk of preterm birth [57] and protects against bacterial vaginosis [47]. Without the *Lactobacillus* spp. dominance, the opportunistic microbiota such as *Gardnerella* or *Ureaplasma* become abundant, which may increase the risk of preterm birth [58].

An important property of lactobacilli, which is associated with their ability to inhibit the growth of other bacteria, is the production of bacteriocins [57). As already noted, lactobacilli synthesize both D- and L-isomers of lactic acid, while the macroorganism itself is capable of producing only the L-isomer [48, 50, 59]. The main beneficial effect of D-lactic acid is to reduce the activity of matrix metalloproteinase (MMP)-8, which allows the cervical plug to maintain integrity

and thereby limits the vertical transmission of vaginal bacteria to the uterus. Dactobacteria act as a mechanical barrier, binding to the surface of epithelial cells to prevent other bacteria from attaching [60].

Microbiota and reproductive health. The accumulated data suggest that the species and quantitative composition of microbial communities affects reproductive health in mammals. For example, infertility problems are often associated with a decrease in the abundance of lactobacilli in the cervix [61]. The presence of certain bacteria (particularly *Atopobium vaginae*, *Ureaplasma vaginae*, *U. parvum*, *U. urealyticum*, and gardnerella) and the reduced frequency of *Mycoplasmateceae* species compared with the microbiota in healthy individuals have been shown to result in a high prevalence of asymptomatic bacterial vaginosis [62, 63].

In infertility due to infection [61], a decrease in the number of lactobacilli and a higher diversity of microorganisms in the cervix were found, with a significant increase in the number of detections of *Gardnerella vaginalis*, *Prevotella* spp., *Leptotrichia*, *Sneathia* compared with controls in normal fertility [61, 64]. It has been established that bacterial vaginitis is the most common disease of the vagina of microbial etiology, described as polybacterial dysbiosis [65], affecting 30% of women of reproductive age [66]. Anaerobes, in particular *Gardnerella vaginalis*, *Atopobium vaginae*, *Mobiluncus*, *Mollicutes*, *Dialister invisus*, *Sneathia*, *Prevotella* spp., are considered as possible pathogens in bacterial vaginitis [67]. It is noted that in this pathology, the bacterial composition of the vaginal microbiota is more diverse [68]. It is important to note that bacterial vaginitis is associated with adverse reproductive outcomes such as infertility, miscarriage [69], recurrent pregnancy loss [70] and preterm birth [67].

Microbiota of the reproductive system of farm animals on the example of cattle. The study of the microbiota of the reproductive system of farm animals is important for understanding the role of microorganisms in pathological processes associated with reproduction. For example, in a study of cows with purulent uterine discharge, a significant positive correlation was found between the presence of *Trueperella pyogenes* and clinical endometritis, and between *Escherichia coli*, *Fusobacterium necrophorum*, *Prevotella melaninogenica*, *Bacteroides* spp. and metritis. In healthy cows, *Streptococcus* spp., *Staphylococcus* spp. and *Bacillus* spp. are commonly detected. A study of the bovine uterine microbiota has shown that the *Porphyromonadaceae*, *Ruminococcaceae* and *Lachnospiraceae* families are the most abundant, and the cow can carry a pregnancy despite the presence of potentially pathogenic bacteria in the uterus [71].

A study of the effect of *Trueperella pyogenes* on the reproductive function of cows showed that with endometritis caused by this pathogen, the frequency of successful pregnancy is 47% lower, and the average time for its onset is 57 days longer than in healthy cows [72]. Using PCR analysis, it was found that in the microbiota of the reproductive organs of cows with metritis and clinical endometritis, *Escherichia coli* acts as a precursor pathogen that predisposes cows to infection with *F. necrophorum* associated with metritis and *T. pyogenes* associated with clinical endometritis.

Metagenomic sequencing has expanded the knowledge of the bovine uterine microbiota. It has been established that in cows, bacteria are present in the uterus even before calving. In animals with developing metritis and healthy individuals, the structure of microbial associations is identical until the 2nd day of the postpartum period, after which the microbial community of the uterus of individuals with metritis changes towards a larger relative abundance of representatives of *Bacteroidetes* and *Fusobacteria* and a smaller one of *Proteobacteria* and *Tenericutes*. A potential route of infection by uterine pathogens has been found to be

hematogenous and that metritis is associated with a dysbiosis of the uterine microbiota characterized by reduced diversity and increased abundance of *Bacteroidetes* and *Fusobacteria*, especially *Bacteroides*, *Porphyromonas* and *Fusobacterium* [73-75]. In addition, the study of bovine endometritis revealed a significant effect of microbiota structure variability on immunity and general resistance of animals to adverse biotic and abiotic environmental factors. In endometritis, the inflammatory reaction caused by Gram-negative *E. coli* has been found to affect the expression of microRNA (miRNA) involved in the regulation of innate immunity [76]. It is interesting to note that the dominance of lactobacilli in the composition of the vaginal microbiota is unique for humans as a biological species, while in other mammals (including primates) the vaginal microbiota is rarely characterized by the dominance of lactobacilli, while the pH of the vagina in women is always lower than in females of other mammals [77]. J.D. Swartz et al. [78) emphasized that lactobacilli were common and found in vaginal samples in 80% of cows (16 individuals in a sample of $n = 20$) and 90% of sheep (18 individuals in a sample of $n = 20$), while lactobacilli always had low relative abundance (0.36 ± 0.66 and 0.53 ± 0.65% of the population, respectively, as assessed by the 16S rRNA gene) and pH was almost always neutral [78].

Microbiota and reproductive technologies. The study of microbial communities of the reproductive system is important for the successful application of reproductive biotechnologies, e.g., in vitro fertilization (IVF), embryo transplantation, etc. Thus, the dominance of lactobacilli (*L. crispatus*, *L. iners*, *L. jensenii*, *L. gasseri*, or other lactobacilli species) in the vaginal microbiota in the pre-transplant cycle is associated with a positive outcome of the procedure [79, 80]. However, some vaginal microbial communities adversely affect pregnancy [79, 81]. An increase in the number of opportunistic microflora in the genital tract always correlates with a decrease in the frequency of lactobacilli species, which decreases the success of reproductive biotechnology methods [82, 83].

As a reason for an unfavorable result in the IVF procedure, the possibility of colonization of the follicular fluid by microorganisms during egg retrieval is considered. Negative pregnancy outcomes were noted in the presence of *Actinomyces* spp., *Bifidobacterium* spp., *Propionibacterium* spp. in the ovaries. and *Streptococcus* spp. and, conversely, positive outcomes occurred when *Lactobacillus* spp. are detected in the ovaries [80]. It has been previously demonstrated that the presence of *Streptococcus viridans* on embryo transfer instruments is associated with adverse outcomes of the procedure [80].

The microbiota of the upper reproductive tract is associated with the likelihood of conception both in vivo and with the use of reproductive technologies. Problems with conception may be due to changes in the structure of the microbiota due to the penetration of pathogens from the vagina into the upper reproductive system, which leads to an imbalance in the intrauterine environment (82). Lactobacilli contribute to the creation of favorable conditions for embryo implantation and pregnancy due to their protective and supportive properties [48, 50, 59, 85]. Recent studies have associated reproductive success with a predominance (>90%) of lactobacilli in the microbial profile of the endometrium [86]. The dominance of the genera *Gardnerella* (family *Bifidobacteriaceae*) and streptococci (family *Streptococcaceae*) in the endometrium is associated with a significant decrease in the likelihood of implantation and favorable delivery [86].

Endometrial microbiome profiles have been described that may be associated with chronic endometritis [87, 88] associated with a predisposition to infertility in endometriosis [87] and determine repeated implantation failures [87, 88]. There is evidence of a limited role for the microbial landscape of the cervical canal

and endometrium during embryo transfer and the absence of a significant microbial effect on the likelihood of pregnancy [85, 87]. Subsequent pregnancy rates have not been reported to be affected by prophylactic antibiotic use [92]. A number of studies [93] also found no statistically significant difference in reproductive success rates between those treated with antibiotics prior to embryo transfer and those who did not receive antibiotics.

Microbiota and pregnancy. The microbiota of the reproductive tract continues to play a role after pregnancy setting [94]. Dysbacteriosis in the vagina, endometrium or placenta can lead to an unfavorable pregnancy outcome.

At the end of the 1st trimester, the vaginal microbiota is mainly composed of lactobacilli — *L. crispatus*, *L. iners*, *L. gasseri* or *L. jensenii* [95]. Premature birth (before 34 weeks) is highly likely to be associated with the dominance of *L. iners* at the 16th week of pregnancy, while the predominance of *L. crispatus* presumably serves as an indicator of a successful pregnancy outcome.

Abnormal colonization of the vagina by *Klebsiella pneumonia* in the 2nd trimester increases the risk of preterm birth (before 28 weeks), and colonization of *Streptococcus agalactiae* in the 2nd trimester leads to an increased likelihood of late miscarriages [96, 97].

Embryonic development and growth are largely dependent on the function of the placenta. Once thought to be sterile, the placenta has been found to have its own unique microbiota. In humans, a significant presence of non-pathogenic commensal microbiota of the *Firmicutes*, *Tenericutes*, *Proteobacteria*, *Bacteroidetes*, and *Fusobacteria* phyla has been found in the uterus and placenta [98].

Manipulations that alter the structure of the uterine microbiota can help modulate the local immune system in preparation for embryo implantation and placenta formation [99], which can directly influence the development of preeclampsia [96].

Sperm microbiota and reproductive health. In males, the reproductive system is represented by the external part (penis and scrotum) and the internal part (testes, accessory glands, vas deferens and urethra).

Recent analyzes have shown that the seminal microbiota is most likely formed by the association of microbial communities from all parts of the male reproductive tract. The NGS method showed that the bacterial communities of the seed are divided into three groups, in which either lactobacilli, or Pseudomonas aeruginosa, or Prevotella predominate. It is important to note that 80% of the quality semen samples belonged to the group dominated by lactobacilli [100]. It has been established that low concentration and abnormal morphology of spermatozoa are associated with the presence of *Mycoplasma* spp. [101, 102]. The incidence of *Mycoplasma hominis* is significantly higher in infertile men than in fertile men, and antibiotic therapy has also been shown to improve sperm quality in infertile men [103].

Similar to the female reproductive tract, in male reproductive diseases, the abundance of lactobacilli will decrease with a higher species diversity of the microbial community [104]. An increase in abundance of *Neisseria*, *Klebsiella* and *Pseudomonas aeruginosa* and a decrease in the number of lactobacilli has been associated with increased seminal fluid viscosity and oligoasthenoteratozoospermia [105], therefore, sexually transmitted diseases reduce not only female but also male fertility. When studying the effect of the seed microbiota on reproductive success in rabbits, it was found that *Lysinibacillus* and *Flavobacterium* can act as markers of potential fertility [106].

Clearly, the microbiota of both sexes influence each other and appear to interact. Comparison of seminal and vaginal microbiota in surveyed couples revealed a large number of common DNA markers for microbiota components

[107]. Among the common microbiota components, the most common genera were *Lactobacillus*, *Veillonella*, *Streptococcus*, *Porphyromonas*, *Atopobium vagine*. Although the microbial communities of the semen were more diverse, the overall concentration of bacteria in the semen was lower than in the vaginal communities. The sperm microbiota significantly, albeit temporarily, influences the vaginal microbiota [108]. Earlier studies showed no effect on vaginal lactobacilli and pH 8-12 h post-coitus, with significantly more *E. coli* found in the vagina [109]. It is hypothesized that the physiological postcoital transient state of the vaginal microbiome, in which vaginal lactobacilli are replaced by *Gardnerella vaginalis* under the influence of ejaculate, leads to a change in pH [110]. Extensive rodent studies have shown that exposure to seminal fluid induces a spectrum of cytokines in the female reproductive tract, altering endometrial receptivity and pre-implantation developmental dynamics of the embryo [111]. Unfortunately, the interaction and influence on each other between the microbiota of the reproductive systems of male and female mammals is still poorly understood. When analyzing the influence of microbiota on reproductive success, one of the directions may be to study the temporary combined microbial community of the female and male reproductive systems, which is formed in the postcoital period and, possibly, even persists in the preimplantation period, which can contribute to successful conception and pregnancy.

So, it becomes obvious that the microbiota serves as a factor that unites all the physiological systems of the body. Any changes in microbial communities at the level of a system or even an organ lead to the emergence of pathological processes. At present, the accumulation of data on the microbiomes of agricultural animals is of both theoretical and practical importance. This scientific direction remains relevant and promising, since the productivity and adaptive potential of valuable agricultural species and animal breeds can be improved by purposeful changes in the qualitative and quantitative composition of their microbial communities. The study of the microbial communities of the reproductive system organs in farm animals will provide new data on the physiology of reproduction, increase the likelihood of reproductive success in the application of methods of reproductive biotechnology, as well as reduce the associated costs and apply new methods of treatment, such as microbial therapy. Today, the accumulated materials indicate the need to strengthen the biological control of the microbiota in seed production, the use of in vitro and in vivo technologies to obtain embryos of agricultural animals. In addition, important scientific areas include the study of the composition of microbial communities of the reproductive organs of producers and the possibility of reducing the predicted loss of embryos during preliminary colonization of certain types of bacteria in the parts of the reproductive system of female donors and female recipients.

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MOLECULAR MECHANISMS AND GENETIC DETERMINANTS OF RESISTANCE TO ANTIBACTERIAL DRUGS IN MICROORGANISMS
(review)

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Abstract

The emergence of antibiotic resistance is a serious public health problem, since antibiotic-resistant bacteria that develops in conditions of agro-industrial enterprises can easily transmit to humans through products and raw materials of animal origin and contaminate the environment with agricultural waste. Several reviews cover the problem (C. Manyi-Loh et al., 2018; A.N. Panin et al., 2017). A significant number of publications describe the mechanisms of antibiotic resistance, including modification of the target affected by the drug; the acquisition of metabolic pathways alternative to those inhibited by an antimicrobial agent; overproduction of the target enzyme; enzymatic inactivation and active efflux of the antibiotic (it's excretion outside the microbial cell). These mechanisms can be natural for some microorganisms or acquired from other microorganisms (M.F. Varela et al., 2021; W.C. Reygaert, 2018; A.L. Bisekenova et al., 2015). Understanding these mechanisms will allow us to choose the best treatment option for each specific infectious disease and develop antimicrobial drugs that prevent the spread of resistant microorganisms. The most clinically significant antibiotic resistance genes are usually located on different mobile genetic elements (MGE) that can move intracellularly (between the bacterial chromosome and plasmids) or intercellularly (within the same species or between different species or genera) (C.O. Vrancianu et al., 2020). Among the three main mechanisms involved in horizontal gene transfer, transformation of antibiotic resistance genes between bacterial species happens rarely. However, conjugation with the participation of mobile genetic elements, such as transposons and plasmids, is the most effective and important method of spreading antibiotic resistance (J.M. Bello-López et al., 2019). The purpose of this review is to describe antibiotic resistance genes distinctive for the microbiota of farm animals under the conditions of the agro-industrial complexes, as well as the mechanisms of the formation of antibacterial resistance to antimicrobial drugs used in veterinary medicine. In addition, this report covers the direct localization of the genetic determinants of antibiotic resistance, outlines the main measures to control antibiotic resistance, which include i) reducing the use of antibiotics due to improving animals' welfare and living conditions and ii) monitoring and supervision of the spread of antibiotic-resistant bacteria.

Keywords: antibiotic resistance, livestock sector, mechanisms of resistance, antibiotic drugs, mobile genetic elements, genetic determinants, microorganisms

The spread of antibiotic-resistant bacteria is a growing problem worldwide [1]. With the discovery of penicillin in 1928, many life-threatening or even fatal diseases became curable, which brought obvious benefits to specialists in the field of veterinary medicine and animal science. Since the 1960s, however, antibiotics have been widely used as growth promoters in farm animals [2]. In 2017, the World Health Organization (WHO) published a list of bacteria that require the

development of new antibiotics. According to the WHO, antimicrobial resistance causes 25,000 deaths per year in the European Union (EU) and 700,000 worldwide. With the current unfavorable trend, by 2050 antibiotic-resistant bacteria may cause more deaths than cancer [1, 3].

The purpose of this review was to describe the genes for antibiotic resistance of bacteria that persist in the agro-industrial complex and are characteristic of the microbiota of farm animals, as well as the mechanisms for the formation of antibacterial resistance to antimicrobial drugs used in veterinary medicine.

The uncontrolled use of antibiotics leads to the accumulation of low sub-inhibitory concentrations in the tissues and intestines of treated animals and in the environment, which promotes the selection of antibiotic-resistant bacteria, enhances their growth, the mutation process occurs and the introduction of de novo mutations (4). In addition, the presence of antibiotics can stimulate biofilm formation and horizontal gene transfer (HGT) in some bacteria. For example, transfer of resistance to azithromycin, ciprofloxacin or tigecycline has been observed in *Enterococcus faecalis* and *Pseudomonas aeruginosa* [5].

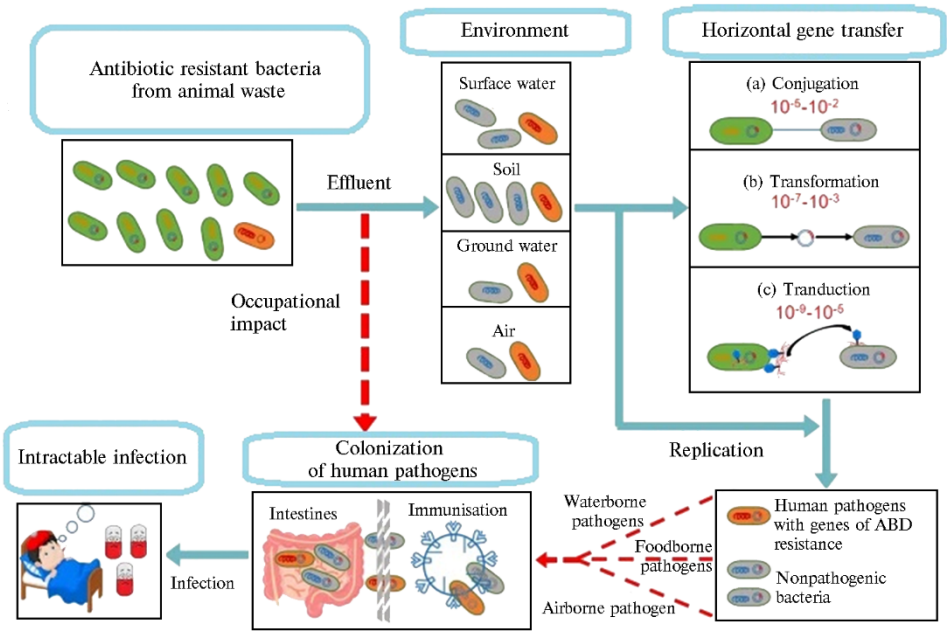
The main transfer of antibiotic resistance genes occurs through HGT, that is, through the exchange of transposable genetic elements (TGEs), such as plasmids or transposons encoding antibiotic resistance genes, between bacterial species, even if they are not closely related (6). Some countries have officially restricted the use of antibiotics in animal husbandry to therapeutic use only (eg the EU in 2006 under legislation 1831/2003/EC). However, antibiotics are still overused in areas with high livestock intensity: the US, Russia, India, China and South Africa [7]. In the US, antimicrobial treatment of animals used for food production accounts for approximately 80% of the total annual volume. However, the vast majority of antibiotics are essential drugs used to treat common infections or are required for surgery, organ transplantation or chemotherapy in humans [8]. In the Russian Federation, from March 1, 2022, an order came into force on the approval of the list of antibacterial drugs intended for the treatment of infectious and parasitic diseases of animals caused by pathogenic and opportunistic microorganisms, in respect of which restrictions are imposed on the use for therapeutic purposes, including for treatment of farm animals. This list divides antibacterials into group A (prohibited for all animals; prohibited for food producing animals), group B (second choice drugs) and group C (first choice drugs) [9].

There are a number of mechanisms that contribute to the development of resistance of a bacterial cell to one or more antimicrobial drugs: a decrease in the accumulation of an antimicrobial drug inside the cell through a decrease in wall permeability and/or active efflux (removal) of the antimicrobial drug from the bacterial cell; enzymatic modification or degradation (inactivation) of the antimicrobial agent; acquisition of alternative metabolic pathways to existing ones (formation of a metabolic shunt); modification or protection of the antimicrobial target; overproduction of the target enzyme [11-13].

Reducing the accumulation of antimicrobial drug in the bacterial cell by reducing the permeability of the cell wall and active efflux. Efflux pumps are protein carriers localized in the cytoplasmic membrane of all cell types that require a source of chemical energy to perform their function. Some of them are primary active transporters, using the hydrolysis of adenosine triphosphate as an energy source, while others are secondary active transporters (uniporters, symporters, or antiporters), in which transport is associated with an electrochemical potential difference created by the removal of hydrogen or sodium ions outside the cell [14]. The change in permeability that

occurs in the outer membranes of bacterial cells causes a decrease in the entry of the antibiotic into the cell; at the same time, efflux pumps are activated, and the rest of the drug is excreted outside the cell. These resistance mechanisms correlate with each other and always have an cumulative effect on drugs [15]. Cell permeability undergoes changes through the acquisition of mutations in porins (protein channels that pass through the cell membrane). These mutations include loss of the porin, alteration in the size or conductivity of the porin channel, or lower porin expression [16]. As for efflux pumps, some of them are constantly expressed, mediating the innate resistance of bacteria, while others are expressed under the influence of an inducer. In addition, overexpression of efflux pumps may contribute to higher resistance. Overexpression can be transient and occur in the presence of an effector (phenotypic resistance) or be permanent when mutants are selected for regulatory elements of efflux pump expression (acquired or secondary resistance) [17].

In reviews by S. Hernando-Amado et al. [18] and W.C. Reygaert [19] efflux pumps are grouped into five main structural superfamilies — resistance-nodulation-division (RND), small multidrug resistance (SMR), multidrug and toxin extrusion (MATE), major facilitator superfamily (MFS) and ATP-binding cassette (ABC) [18, 19]. This classification is based on three criteria: amino acid sequence identity, substrate specificity, and the source of energy needed to drive efflux [20]. A.E. Ebbensgaard et al. [21] also mention the family of proteobacterial antimicrobial compound efflux (PACE). While RND and PACE are unique to Gram-negative bacteria, SMR, MATE, MFS and ABC are found in both Gram-positive and Gram-negative members of the microbial community [21].



Possible pathways for the transfer of antibiotic resistance genes from animal waste to human pathogens. Bacteria containing antibiotic resistance genes move through drainage, treated wastewater, and solid waste from livestock enterprises to various host environments. Horizontal gene transfer between antibacterial drug-resistant bacteria (ARB) and autochthonous microflora occurs through three main mechanisms: conjugation (involving plasmids), transformation (involving free DNA) and transduction (involving bacteriophages) with indicated frequencies based on data literature. Pathogenic bacteria are able to penetrate into the human body through the alimentary route, as well as as a result of occupational exposure. The bacteria then multiply in the body (especially in the gut), causing endogenous or exogenous infections [10].

ABC transporters are functionally diverse and mediate ATP-dependent

import or export of solutes. ABC transporters contain transmembrane domains (TMDs) that are able to recognize substrates and transfer these substances through the membrane through conformational switches, and nucleotide binding domains (NBDs) that bind and hydrolyze ATP, controlling the transport cycle [22, 23]. The mechanism for the transfer of substances of this family operates on the principle of variable access, such a structure has three states: open inward, closed or open outward to move substrates through the membrane.

The MFS Group is the largest and most diverse conveyor family. It includes uniporters (provide the movement of substrates through the lipid bilayer in one direction along the concentration gradient, regardless of other molecules), symporters (transport of matter and ions in one direction outside the cell) and antiporters (movement of ions and substances in opposite directions). Most members of this family function as separate monomeric units. They are 400 to 600 amino acid residues in length and have 12 or 14 transmembrane helices organized as two domains, each consisting of six helical bundles. The variable access switching mechanism for MFS proteins has two states: open to the inside of the cell or open to the outside during the transport cycle.

Other proteins of the MATE family use H^+ and/or Na^+ transmembrane gradients for transport. All currently known structures of MATE transporters are fixed in the external state (open to the outside). Probably, the state of "open inside the cell" is achieved due to the transfer of lipids with the help of flippase to the cytoplasmic side of the membrane [24].

RND-type efflux systems consist of three components: integral membrane protein (IMP), periplasmic membrane fusion protein (MFP), and outer membrane protein (OMP). The H^+ transmembrane electrochemical potential drives the drug efflux associated with RND. All RND transporters have a rather atypical structure for a group of secondary transporters. Most transport systems of the RND superfamily consist of large polypeptide chains containing 12 transmembrane domains. The large periplasmic domain is involved in substrate recognition and forms a cavity that can accept multiple drugs at the same time [25].

Proteins of the SMR family consist of only four transmembrane helices, but function as homodimers or heterodimers. The first three transmembrane helices form an active cavity for substrate binding, while the fourth helix is mainly involved in dimerization. During transport, two states of the pump alternate: inward-facing or outward-facing through a conformational exchange of two protomers [24].

Transport proteins of the PACE family form resistance to a number of biocides used as disinfectants and antiseptics. The range of functions and transport mechanisms operating in these proteins are not well understood. PACE proteins are known to have several conserved amino acid motifs that likely play a role in substrate transport. PACE proteins also have a conserved region between the N- and C-terminal amino acids. They probably evolved as a result of duplication of an ancestral protein consisting of two transmembrane helices [26].

These mechanisms of formation of bacterial resistance have been identified for tetracyclines, macrolides, quinolones and amphenicols. Approximately 30 tetracycline resistance genes, such as *tet* (A, B, C, D, E, G, H, J, K), encode a tetracycline-specific efflux pump that both Gram-positive and Gram-negative bacteria have and is usually encoded by transposons. and integrons. In addition, several *mef* genes encode an efflux pump specific for macrolides, which reduces their intracellular concentration. Unlike the *erm* genes, the *mef* genes can only protect against macrolides, resulting in an M phenotype. There is evidence that although the efflux mechanism mediating quinolone resistance is sensitive to reserpine, this phenotype is more of a multidrug resistance phenotype established

by non-specific efflux [11]. The *cml* and *flo* genes encode specific efflux pumps found mainly in Gram-negative bacteria.

Enzymatic modification or degradation (inactivation) of an antimicrobial agent. Bacteria are able to synthesize enzymes that chemically modify the antibiotic target by adding additional chemical groups. For example, the mph genes are mostly found in Gram-negative bacteria and mediate macrolide inactivation, which limits their clinical relevance. The vat genes, which also code for enzyme inactivation, have been found in *Enterococcus* spp. and *Staphylococcus* spp. [11]. The second type of enzyme chemically modifies the antibiotic itself, which prevents the antibiotic from binding to its target site. This mechanism of antibiotic resistance can be provided by enzymes that modify aminoglycosides, in particular N-acetyltransferases, which add an additional acetyl group (CH3CO−) to aminoglycoside antibiotics, such as kanamycin. Binding to the ribosome is disrupted and bacteria become resistant [27]. Most of these enzymes have a narrow spectrum of activity. For example, ANT(2″)-I can only inactivate gentamicin, tobramycin, and kanamycin. Bifunctional phosphotransferases and/or acetyltransferases found in Gram-positive cocci inactivate most aminoglycosides. The genes for these enzymes are often found in plasmids and transposons and can be mobilized as gene cassettes between integrons. In addition, some of the 1000 different β-lactamases known to date are only able to hydrolyze a few substrates, while others can also inactivate third-generation cephalosporins (extended-spectrum β-lactamases, ESBLs) and β-lactamase inhibitors, such as clavulanic acid. acid. β-Lactamases are widely dispersed in bacterial groups and can be encoded by chromosomal or plasmid genes. A recently discovered modified aminoglycoside resistance enzyme AAC(6′)-lb-cr is able to inactivate ciprofloxacin. The enzyme is quite common in clinical practice in isolates of intestinal bacteria with reduced sensitivity to ciprofloxacin. Bacteria producing chloramphenicol acetyltransferase, which is encoded by multiple cat genes and has the ability to inactivate chloramphenicol, become resistant to the antibiotic. The *cat* genes have been found in both Gram-positive and Gram-negative bacteria [11]. Table 1 shows the main types of enzymes that modify antimicrobials.

1. Main classes of enzymes that modify antimicrobials (27)

Classes	Enzymes	Inactivated antibiotics
Hydrolases	β-Lactamases (penicillinases, cephalosporinases, carbapenemases)	Penicillins, cephalosporins, carbapenems and monobactams
	Esterases	Macrolides
	Epoxide hydrolases	Phosphomycin
Transferases	Acetyltransferases	Aminoglycosides, fenicols, quinolones, streptogramin A
	Phosphotransferases	Macrolides, rifamycins, phosfomycin, aminoglycosides
	Nucleotidyltransferase	Aminoglycosides, lincosamides
	Glycosyltransferase	Macrolides, rifamycins
	ADP-ribosyl transferases	Rifamycins
	Glutathione-S-transferase	Phosfomycin
Redox enzymes	Monooxygenases	Tetracyclines, rifamycins
	Liase	Streptogramin B

Acquisition of alternative metabolic pathways (formation of a metabolic shunt). This mechanism of resistance is quite specific, most often associated with the acquisition of new genes by bacteria, which make it possible to produce an alternative target (usually an enzyme) that is resistant to the action of an antibiotic. Bacteria also synthesize the original target, which is sensitive to antibiotics. An alternative target mediates the development of resistance in bacteria, taking on the role of the original target, i.e., a metabolic shunt is formed [28, 29]. A striking example of such a mechanism of resistance to quinolone

antibiotics is the imitation of a target molecule by a protein of the MfpA family of pentapeptide repeats. It was noted that when MfpA was expressed on the pGADIV plasmid in *Mycobacterium smegmatis* or *Mycobacterium bovis*, the minimum inhibitory concentration (MIC) for all fluoroquinolones increased 2-8-fold, and when mfpA was eliminated from the chromosome of *M. smegmatis*, the MIC decreased by 2-4 times (30). The MfpA protein mimics the structure of DNA and interacts with DNA gyrase or topoisomerase IV, thereby protecting them from the inhibitory effect of quinolones that bind to these target enzymes [31].

Another class of antibiotics, glycopeptides, bind the terminal residues of D-alanyl-D-alanine to the cell wall of pentapeptide precursors, blocking the next stages of cell wall synthesis (trans-glycosylation and transpeptidation). The van genes alter peptidoglycan synthesis pathways such that D-alanyl-D-lactate or D-alanyl-D-serine is formed instead of D-alanyl-D-alanine. Clusters of van genes (five or more genes) are required to achieve glycopeptide resistance, so the entire cluster must be moved horizontally, probably by conjugation. Some *van* genes, apparently derived from vancomycin-producing organisms, were passed on to members of the genus Streptomyces and then to Gram-positive cocci [32, 33].

The action of β-lactam antibiotics is based on the suppression of several enzymes responsible for the synthesis of bacterial cell walls (penicillin-binding proteins, PBP). The acquisition of alternative enzymes promotes the development of cell resistance to many or all β-lactams. Thus, *Staphylococcus aureus* becomes resistant to most β-lactam antibiotics, in particular to penicillin. Methicillin-resistant *Staphylococcus aureus* (MRSA) acquires resistance to β-lactam antibiotics by obtaining an additional copy of penicillin-binding protein 2a (PBP2a), which serves as a target for β-lactam antibiotics and retains its functionality in their presence [33]. Based on B.A. Wall et al. (11) and D.M. Boothe (33) reports, it is known that altered PBPs underlie penicillin resistance in Streptococcus pneumoniae acquired through transformation, accumulation of repeated point mutations, or through recombination between PBP genes in related streptococcal species. Organisms without a cell wall, such as *Mycoplasma*, are inherently resistant to β-lactams and to all antimicrobials that act to inhibit or interfere with cell wall synthesis of target bacteria [34]. The phenotypic form of resistance can occur in the presence of spheroplasts (incomplete cell wall) or protoplasts (no cell wall). These L-forms require a hyperosmotic environment (eg, renal medulla) to survive or they will be lysed [33].

Modification or protection of the antimicrobial target. One of the common mechanisms used by bacteria to acquire resistance to antibacterial drugs is to change or protect the antibiotic target. As bacteria grow and reproduce, they copy their genome. Sometimes errors occur in the DNA sequence during the copying process (for example, adenine is replaced by cytosine). By themselves, such events are rare, but large population sizes greatly increase their frequency. If one of these mutations occurs at the location of the gene encoding the protein that is the target of the antibiotic, then the latter can no longer bind to the target. There is a selection of bacteria resistant to the action of antimicrobial agents. This is the general mechanism of penicillin resistance in Streptococcus pneumoniae when mutations in penicillin-binding proteins (PBPs) are acquired.

P. Valderrama-Carmona et al. [35] reported about 16S rRNA methylases which modify the nucleic acid molecule, which alters the structure of the ribosome to prevent binding of aminoglycosides, in the intestinal bacteria *Pseudomonas* spp. and Gram-positive cocci [35]. Ribosomal mutations can also make ribosomes insensitive to aminoglycosides. The erm gene (erythromycin ribosomal methylase gene family) provides resistance to macrolide antibiotics such as erythromycin. Methylation occurs in the portion of the ribosome that is targeted by

erythromycin, erythromycin loses its ability to bind to the target, and bacteria can continue to grow in the presence of the antibiotic [27]. This modification protects the ribosome from other chemically unrelated antimicrobials such as lincosamides and streptogramins. The so-called macrolide-lincosamide-streptogramin B (MLSB) phenotype is a clear example of cross-resistance. The erm genes are often localized on transposable genetic elements; *erm*(B) and *tet*(M) are located in Tn1545, the streptococcal conjugative transposon [36]. Sulfonamides, in turn, are able to inhibit the enzyme dihydropteroate synthetase (DHPS), and trimethoprim can inhobit dihydrofolate reductase (DHFR).

Of particular importance is the *sul/I* gene encoding DHPS. It serves as part of the conserved region of class 1 integrons. By acquiring (via horizontal transfer) genes for DHPS enzymes and/or DHFR variants that are not inhibited by these drugs, bacteria become resistant [11]. Mutations in the *gyr* and/or *par* genes encoding DNA gyrase and topoisomerase IV, respectively, allow these enzymes to complete the three-step process of DNA supercoiling in the presence of quinolones [37]. A single mutation can make a bacterial cell resistant to nalidixic acid, but two or more mutations are needed to achieve resistance to fluoroquinolones (e.g., ciprofloxacin and enrofloxacin). Although these mutations are recessive in nature (with presumably limited capacity for horizontal mobilization), transmission through transformation has been reported in streptococci because the newly acquired gene replaced the wild-type gene through recombination.

The *cfr* gene encodes RNA methyltransferase, as a result of which the ribosome is modified, preventing the binding of florfenicol to it, which leads to the emergence of resistance. Currently, the use of chloramphenicol in medicine is limited, and florfenicol is used only in veterinary medicine. However, *cfr* genes are of public health importance because the produced methylase also protects bacterial ribosomes from the action of linezolid, a class of oxazolidinones considered as a "last resort" against infections associated with *Staphylococcus aureus* and resistant enterococci in humans. The *cfr* genes have been found worldwide in clinical isolates resistant to linezolid.

Approximately 10 genes, including the tetracycline resistance *tet* genes (M, O, Q, S, T), encode proteins that interact with ribosomes, protecting them from binding to tetracyclines. In addition to enteric bacteria, tet genes, especially *tet*(M), are commonly found together with macrolide resistance genes in the same transposon in Gram-positive cocci, and can also be found in anaerobes.

The *qnr* gene group encodes a protein that protects enzymes of the topoisomerase group from the action of quinolones. These genes were first mentioned as a unique plasmid-mediated quinolone resistance mechanism found in enteric bacteria. Later, they were found in the chromosomes of many other organisms, along with related mdp genes of a similar nature. They code for low resistance to quinolones, often below the breakpoints for development of complete resistance in the clinical setting [11].

Overproduction of the target enzyme. Bacteria can also oversynthesize antibiotic targets, i.e. the concentration of the target protein exceeds the concentration of the antibiotic itself [38]. Therefore, the target protein is sufficient to continue its role in the cell in the presence of antibiotics. This is due to the mechanism of resistance to trimethoprim in *Escherichia coli* and *Haemophilus influenzae*. Trimethoprim is usually used with sulfamethoxazole (a combination known as co-trimoxazole, or SXT). Overexpression is sometimes found in association with mutations that reduce the ability of an antibiotic to bind to its target. Mutants overexpressing DHPS and/or DHFR are able to overcome the inhibitory ability of antifolate drugs at therapeutic concentrations and become resistant [32].

The role of mobile genetic elements in the spread of antibiotic resistance

genes. Researchers have yet to determine the involvement of transposable genetic elements (TGEs) in the spread of antibiotic resistance [39].

Plasmids are involved in the acquisition of resistance to most classes of antibiotics, including β-lactams, aminoglycosides, tetracyclines, sulfonamides, trimethoprims, macrolides, polymyxins, and quinolones, mainly in Gram-negative bacteria [28, 40]. Multidrug resistance plasmids are usually conjugative, capable of initiating not only their own transfer but also the transfer of other plasmids, and have mechanisms to control their cellular copy number and/or ability to replicate. Plasmids ensure the transfer of antibiotic resistance genes through various mechanisms such as active separation systems, random segregation, or post-segregation killing. S. Nolivos et al. [41] found that tetracycline-resistant *Escherichia coli* strains transferred their resistance to more than 70% of initially tetracycline-susceptible *Escherichia coli* strains in as little as 3 h by conjugative transfer of a transmissible plasmid, the *E. coli* fertility factor (F), carrying an insertion in the Tn10 transposon. In addition to conjugative plasmids, there are mobilizable plasmids that are smaller and do not self-transport, but can transport DNA to a specific host in the presence of conjugative plasmids. This transfer occurs both vertically and horizontally [39].

Insertion sequences (IS) are the smallest (0.7-2.5 kb) and simplest mobile genetic elements (MGEs) found in bacteria. They are flanked by short, mostly inverted, repeats that sometimes generate direct target repeats (DR) during integration into the target DNA [42]. Currently, more than 4500 ISs are described in the specialized database ISFinder (http://www-is.biotoul.fr) [43]. The role of IS in antibiotic resistance has been emphasized many times, especially in studies looking at resistance to colistin and carbapenem [39). Unlike complex transposons, which exist only as a single copy in a specific replicon, ISs can be present in multiple copies, thereby contributing to the accumulation of antibiotic resistance genes (ARGs) [32].

Transposons (Tn) are a category of MGEs carrying antibiotic resistance genes. Many Tn have the ability to move to different parts of the genome (both intra- and intermolecular), mediating ARG mobility [44]. Bacterial Tn can be divided into two types: composite (two IS elements flanking the central gene) and complex (containing the *tnpA* gene encoding transposase and the *tnpR* gene encoding resolvase). The predominant ARG-containing transposons are Tn5 thaat encodes neomycin and kanamycin resistance in *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, Tn10 that encodes tetracycline resistance, Tn9, Tn903, Tn1525, and Tn2350 [40].

Integrons are MGEs capable of accumulating gene cassettes, including ARGs, and distributing them through other mobile genetic elements. They are most commonly found in Gram-negative bacteria, but they are also present in Gram-positive bacteria. Integrons contain the integrase gene (*intI1*), an enzyme that performs site-specific recombination, which leads to the insertion of one or more gene cassettes into the integron platform. Integrons are divided into several classes (1st, 2nd, and 3rd) depending on the amino acid sequence of the IntI enzyme [36]. Class 1 integrons, which are commonly associated with plasmids, are most commonly found in hospital clinical isolates and have also been found in bacterial pathogens detected in food production (e.g., at livestock farms) [45].

Genomic islands are a category of integrative and conjugative elements capable of mediating their own excision. The study of multiple genomic islands has revealed several common and significant characteristics of these chromosomal regions. They are 10-200 kb DNA fragments that are inserted into tRNA genes [46]. Genomic islands contain repetitive recognition sequences and cryptic genes encoding factors that are involved in integration, insertion or transfer [32].

Integrative and conjugative elements (ICE) are responsible for the horizontal transfer of most resistance and virulence factors. ICEs are 18-600 kb in size and are similar to genomic islands in having an insertion at a specific site, associations with phage integrase genes, and flanking with inverted repeats. The excision and integration of ICE is accomplished by a recombinase, often referred to as an integrase. ICE-associated integrases are tyrosine or serine recombinases. Integrative and conjugative elements have the ability to mobilize neighboring sequences, including genomic islands or composite transposons carrying ARGs [39].

Table 2 shows the main types of genetic determinants of resistance to antibacterial drugs in microorganisms.

2. Examples of genetic determinants of antibiotic resistance in microorganisms

Resistance marker gene (bacteria - carriers of genes)	Gene localization	Encoded traits	Mechanism of antibiotic resistance	References
aac(6′)-Ib (*Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Escherichia coli*)	Gene cassettes are located in the first position in class 1 integrons and are associated with weak variants of the PC promoter (PCW or PCH1)	Aminoglycoside resistance (gentamicin)	Enzymatic modification of aminoglycoside antibiotics, namely O-phosphorylation, O-nucleotidylation and N-acetylation, is catalyzed by aminoglycoside phosphotransferases (APH), aminoglycoside nucleotide diltransferases (ANT) and aminoglycoside acetyltransferase (AAC)	[47-49]
aac(6′)-Ib-cr (*P. aeruginosa*, *E. cloacae*, *K. pneumoniae*, *E. coli*)		Resistance to aminoglycosides (gentamicin) and fluoroquinolones (ciproflox-cinofloxacin)	Enzymatic modification of aminoglycoside antibiotics, namely O-phosphorylation, O-nucleotidylation and N-acetylation, is catalyzed by aminoglycoside phosphotransferases (APH), aminoglycoside nucleotide diltransferases (ANT) and aminoglycoside acetyltransferase (AAC), gene mutations (targets); efflux pump	[47-49]
strB, или *aph(6)-Id*, *strA,* или *aph(3′′)-Ib* (*E. coli*, *K. pneumonia*, *Enterococcus faecium*, *P. aeruginosa*)	In non-conjugative plasmid RSF1010 of a wide range of hosts; in transposon Tn5393 with insertion sequence IS1133	Aminoglycoside resistance (streptomycin)	Enzymatic modification of antibiotics, catalyzing the modification of —OH or —NH2 groups in the core of 2-deoxystreptamine or sugar fragments	[47, 50, 51]
aphA, или *aph(3′)IIa* (*E. coli*, *S. aureus*)	In Tn5	Resistance to aminoglycosides (neomycin, kanamycin)	Enzymatic modification of antibiotics	[47, 48, 51]
bla$_{CMY-2}$ (*Citrobacter freundii*, *E. coli*, *K. pneumoniae*, *Salmonella enterica*, *Proteus mirabilis*)	In conjugative/non-conjugative plasmid IncA/C; insertion sequence ISEcp1	Resistance to β-lactams (ampicillin, cefoxitin, ceftriaxon, amoxicillin, ceftiofur)	Pproduction of β-lactamase class C. Resistance is due to formation of a stable acyl enzyme intermediate; due to the high affinity of the antibiotic and the enzyme, the antibiotic is "trapped" and does not reach the target	[47, 50, 52]
bla$_{TEM-1}$ (*K. pneumoniae*)	In pBR322 plasmid	Resistance to β-lactams (ampicillin)	Target modification	[47, 50, 53]
bla$_{CARB-3}$ (*P. aeruginosa*, *E. coli*)	Mobile gene cassettes are located in integrons 1st class	Resistance to β-lactams (ampicillin)	Target modification	[47, 50, 53]
bla$_{CTX-M}$ (*E. coli*, *K. pneumoniae*, *Proteus* spp., *Enterobacter* spp., *Citrobacter* spp., *Salmonella* spp., *P. aeruginosa*)	On plasmids of the IncF family	Resistance to β-lactams (1st generation: cephalosporins: cefazolin, cephalothin, cephalexin; 2nd generation: cefu-roxime, cefaclor; 3rd generation: cefo-taxime, ceftriaxone, ceftazidime, cefix-sim	Target modification	[50, 55]

Contonued Table 2

AmpC (*E. coli*, *K. pneumoniae*, *Proteus* spp., *Enterobacter* spp., *Serratia* spp., *Citrobacter* spp., *Shigella* spp., *Salmonella* spp., *P. aeruginosa*)	Single nucleotide polymorphism, a gene conferring resistance to antibiotics; AmpC enzymes are encoded by chromosomal, plasmid genes and move between chromosomes and plasmids	Resistance to β-lactams (4th generation cephalosporins: cefepime)	Target modification	[50]
blaVIM-1 (*E. coli*, *K. pneumoniae*, *Proteus* spp., *Enterobacter* spp.)	In integrons of the 1st class, which are derivatives of Tn402 (also called Tn5090), a transposon characterized by the presence of a transposition module that includes a set of four genes (*tniR*/*tniC*, *tniQ*, *tniB* and *tniA*). Some of the Tn402 elements have been found in various Tn3-like transposons. In *Enterobacteriaceae* isolates, blaVIM-1 is part of the integrons located either in the In2-Tn402 element associated with Tn21 or in the Tn402 transposon, which is associated with the IncHI2 or IncI1 plasmid, respectively.	Resistance to β-lactams (carbapenems: meropenem, imipenem, doripenem)	Production of carbapenemases, enzymes capable of hydrolyzing almost all β-lactams, or through modifications to the outer cell membrane, in particular, by reducing the permeability of the cell membrane as a result of porin modification and/or production of an efflux pump	[50, 56, 57]
blaKPC-2 (*E. coli*, *Proteus* spp., *Enterobacter* spp., *P. aeruginosa*)	Located in the Tn3-related transpozone Tn4401, capable of high transposition frequency	Resistance to β-lactams (carbapenems: meropenem, imipenem, doripenem)	Production of β-lactamases (carbapenemases), presence of efflux pumps and mutations that alter the expression and/or function of porins and penicillin-binding proteins (PBPs)	[50, 58, 59]
tetA, *tetR*, *tetB*, *tetC*, *tetG* (*E. coli*, *K. pneumoniae*)	Located in the Tn10	Tetracycline resistance (tetracycline)	Efflux by tetracycline-specific pumps: the drug is actively pumped out of the bacterial cell unchanged	[47, 50, 60]
tetM (*Clostridium difficile*, *E. faecalis*, *E. faecium*, *S. aureus*, *E. coli*)	Located in the Tn916 и Tn1545		The ribosome can function at a high drug content inside the bacterial cell due to complex interactions with other bacterial proteins; proteins prevent tetracycline from binding to the ribosome and provide some degree of protection against it	[47, 50, 60]
sul1, *sul2*, *sul3* (*Enterococcus* spp., *C. freundii*, *E. coli*, *Klebsiella oxytoca*)	Located in small conjugative plasmids or large transimisive plasmids with multiple resistance in class 1 integrons	Resistance to sulfonamides (sulfanilamide)	Antibiotic target modification	[47, 50, 61]
floR, *cmlA* (*E. coli*, *K. pneumoniae*)	Mobile gene cassettes are located in integrons 1st class	Resistance to phenicols (chloramphenicol)	Active efflux: resistance to antibiotics due to the transport of antibiotics outside the cell	(47, 49, 62)
cat1 (*C. freundii*, *E. coli*, *Proteus vulgaris*)	Located in the Tn9	Resistance to fenicolas (chloramphenicol, florfenicol)	Enzymatic inactivation of an antibiotic causing drug resistance	[47, 50]
cfr (*E. faecalis*, *E. coli*, *S. aureus*)	Located in the plasmids pEF-01, pEC-01, pSCFS3	Resistance to oxazolidinones, streptogramin, lincosamide, fenicols	Mutational change or enzymatic modification of an antibiotic target leading to antibiotic resistance	[47, 63, 64]
fosA1 (*Serratia marcescens*)	Licated in the Tn2961	Fosfomycin resistance	Enzymatic inactivation of an antibiotic causing drug resistance	[47, 50, 65]

Contonued Table 2

mphA (*C. freundii*, *E. coli*)	Insertion sequence IS 26 in transposon Tn6242 in integrals of the 1st class	Resistance to macrolides (azithromycin, erythromycin)	Enzymatic inactivation of an antibiotic causing drug resistance	[47, 50, 56]
ErmB (*E. faecium*, *S. aureus*)	In a transposon Tn1546 located on plasmid pMCCL2	Macrolide resistance (erythromycin)	Mutational change or enzymatic modification of an antibiotic target leading to antibiotic resistance	[50, 67]
arr2 (*P. aeruginosa*, *C. freundii*, *E. coli*, *K. pneumoniae*)	Gene cassette localized in class 1 integron — In53, located on a composite trans-poson or plasmid	Resistance to rifampicin	Enzymatic inactivation of an antibiotic causing drug resistance	[47, 50, 68]
VanA (*E. faecium*, *E. faecalis*)	In transposon Tn5281 on plasmid pBEM10; in transposon Tn1546 on plasmid pIP816	Glycolepid resistance (teicoplanin, vancomycin)	Mutational change or enzymatic modification of an antibiotic target leading to antibiotic resistance	[47, 69]
GyrA, *ParC* (*Streptococcus* spp., *P. aeruginosa*, *Enterobacteriaceae*, *K. pneumoniae*)	Localization not clear	Resistance to fluoroquinolones (ciprofloxacin, ofloxacin, levofloxacin, cefixime)	Mutational change or enzymatic modification of the antibiotic target leading to antibiotic resistance; active efflux: resistance to antibiotics due to the transport of antibiotics outside the cell	[50, 70]

Control and containment of the spread of antibiotic resistance in animal husbandry. The spread of antimicrobial resistance among human and animal pathogens poses a huge threat to global public health. The use of antimicrobials in human and veterinary medicine, especially the use of large amounts of antibacterial agents in animal husbandry to promote animal growth, reinforces the unfortunate trend of the emergence and spread of antimicrobial-resistant bacteria [71, 72], which exacerbates the need for the rational use of antibiotics.

The World Health Organization (WHO) classifies fluoroquinolones, 3rd and 4th generation cephalosporins, macrolides, glycopeptides, and polymyxins as primary and critical antibacterial agents for human and veterinary use [73]. However, penicillin antibiotics, macrolides, and fluoroquinolones are used only in humans, while tetracyclines, penicillins, and sulfonamides are used only in animals.

First of all, reserve antibiotics in animal husbandry (erythromycin, oleandomycin, chloramphenicol, neomycin, monomycin, kanamycin, gentamicin, vancomycin, ciprofloxacin) should be used with caution [74]. The use of avoparcin as a feed additive has led to the emergence of bacteria resistant to vancomycin, an antibiotic of reserve for combating life-threatening infections caused by Gram-positive bacteria [75]. Recently, the veterinary service of molecular genetics (Servei Veterinari de Genиtica Molecular, SVGM) of the Universitat Autтnoma de Barcelona (UAB) analyzed and sequenced faecal samples from the owner of the farm and the animals kept there (cattle, pigs). The results showed that the isolated culture of *E. coli* in the studied samples from calves and pigs, as well as from the farmer, carried genes for resistance to colistin, the antibiotic of last resort in medicine. The experts concluded that the *mcr-1* resistance gene was transferred from animals to humans through horizontal transfer and plasmid exchange between *E. coli*, since calves and pigs received the antibiotic for preventive and therapeutic purposes, and the farm owner never received therapy with this drug [76].

One of the main challenges is to reduce the use of antibiotics in livestock production by improving the quality of life and animal welfare. In this regard, it is recommended that good practices for keeping and handling animals in livestock establishments and when transporting animals are recommended; improving

animal welfare (e.g., providing an optimal microclimate, quality water, adequate ventilation and space allocation) at all stages, including production, transport and slaughter; using locally adapted breeds that are more resistant to disease and stress, or animals selected for disease resistance (resistant animals will require fewer antimicrobial treatments); compliance with veterinary and sanitary, sanitary and hygienic rules, biosafety measures at agribusiness enterprises to prevent the use of medicines; adherence to strict disease control measures (eg vaccinations); the use of feed ingredients/additives that increase the efficiency of feed conversion to eliminate the use of antibiotics as growth stimulants (feed enzymes, competitive probiotics, prebiotics, acidifiers, plant extracts, nutraceuticals, essential oils, yeast, etc.); avoiding food ingredients with anti-nutritional properties (such as lectins and protease inhibitors); application of modern methods of waste disposal. Particular attention needs to be paid to primary production (specific supply chains) and the planning of practical actions that can be taken to reduce the need for antimicrobials and control the spread of antimicrobial-resistant organisms in the environment [11, 77].

In addition, it is necessary to organize and conduct monitoring and surveillance of the spread of antibiotic-resistant bacteria, including the assessment and identification of trends and sources of antimicrobial resistance in bacteria; discovery of new mechanisms of antimicrobial resistance; providing data necessary for the analysis of risks in relation to animal and human health; providing a basis for practical advice on animal and human health; providing information for monitoring antimicrobial prescribing in agricultural organizations and judicious use of recommendations; evaluation and determination of the effectiveness of measures to combat antibiotic resistance [78-80]. Because antibiotic resistance occurs as part of an irreversible process, it can be slowed down but not stopped. Therefore, there will always be a need to develop new antibiotics and diagnostic tests to combat the development of resistance [81].

Thus, various mechanisms of antibiotic resistance and ways of acquiring them by bacteria significantly complicate the process of selecting effective antibiotic therapy both in agro-industrial organizations and in medical institutions. The mechanisms of acquired and natural antibiotic resistance are inherently complex and vary from species to species, from strain to strain of microorganisms. Basically, intraspecific and interspecific acquisition of antibiotic resistance genes is carried out through horizontal transfer (conjugation, transformation, transduction). Key measures to combat antibiotic resistance include reducing the use of antibiotics by improving the quality of life and animal welfare; organizing and conducting monitoring and supervision of the spread of antibiotic-resistant bacteria; development of new antibiotics and test systems for diagnosing antibiotic resistance in bacteria.

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LACTATION CURVES AS A TOOL FOR MONITORING THE HEALTH AND PERFORMANCE OF DAIRY COWS — A MINI-REVIEW

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Abstract

According to the estimation of FAO, worldwide milk production increased from 694 million tons in 2008 to 914.3 million tons in 2020. Currently, animal breeding for high milk yield continues. However, some authors consider high milk productivity the main reasons of milk quality and health deterioration, including fertility. Therefore, monitoring of animal physiological state and productivity becomes especially important. Lactation curve modeling is one of the most effective methods for predicting component milk composition, yielding capacity and animal health. It is a tool for early diagnostics of certain diseases, which can help reduce treatment costs and improve the disease course prognosis. Thus, predicting the evolution of milk yields is an important stage of management and breeding decisions; it is widely used for diagnostic purposes. The article provides a brief overview of mathematical methods for modeling lactation curves (for milk yield, percentage of fat, fat yield and protein). Classic Wood's model (P.D.P. Wood, 1967), Ali and Schaeffer (T.E. Ali and L.R. Schaeffer, 1987), Wilmink parametric models (J.B.M. Wilmink, 1987) and models based on the machine learning algorithms are considered here. It should be mentioned that there is no universal model for describing lactation curves. Most attention is paid to Wood's model used for constructing lactation curves described by A.S. Emelyanov (1953). This equation application for prediction milk yield curves and milk components has shown good prediction accuracy. In this study, it was found that most of the models are unstable under decreasing input data that makes their use almost impossible for farms unable to accurately collect data of the lactation activity on a regular basis. It is shown that deviations of the observed milk yield from a prediction made by a well-fitted model are clear indicator of animal's diseases and can be used to prevent and detect udder diseases such as mastitis.

Keywords: lactation curve, Wood's model, cattle, mammary gland, milk production, environmental influence

The annual population growth entails an increase in the demand for dairy products. The dairy industry is showing impressive results in increasing the productivity of cattle. Directed selection taking into account genetic and breeding data, improved feed quality and housing conditions, effective management contributed to an increase in milk yield by more than 20%. According to FAO estimates [1], from 2008 to 2021, world milk production increased from 694.0 to 914.3 million tons per year [2].

Studies show that highly productive cows spend most of the nutrients received precisely on the synthesis of milk components. According to data, on average, about 31% of metabolic energy was spent on this in 1944, 65% in 2016. The current world record holding Holstein cow, My Gold, uses 84% of her metabolic energy to produce milk [3]. However, such an increase in productivity entails metabolic stress, which affects the quality of products and animal health, including reproductive function. Currently, selection for increasing milk production continues. According to some authors, the resulting metabolic problems indicate that every day in the process of selection we are approaching the limit of the adaptive capabilities of the animal [4]. As a result, means of timely monitoring of animal health and productivity are of particular importance. One of these tools is the modeling of lactation curves, which are a graphical representation of the dynamics of milk production of cattle and other domesticated animal species over the lactation period. The lactation curve is a graph, the abscissa of which marks the time since calving, the ordinate shows one of the productivity parameters: milk yield, fat and protein composition, and the ratio of milk components [5]. Thus, the value of the ratio of fat and protein in milk can serve as an indicator of the ability of the animal to adapt to the period of feeding, as well as an indicator of the rate of recovery of reproduction efficiency [6]. After calving, during a period of negative energy balance, high fat:protein ratios have been associated with reduced dry matter intake and increased fat mobilization [7]. The fat and protein content has an inverse curve with respect to milk yield, which is probably due to the effect of dilution [8].

In female mammals, milk production during lactation is usually characterized by two phases, which correspond to the change in the milk requirement of the offspring [9]. In cows, the phase of increasing milk production continues from 5-6 days from birth to the peak of productivity (2-3 months), which reflects the increase in the calf's need for milk, the phase of decreasing secretion from the peak to the start of the dry period (approximately day 305 of lactation) is associated with an expansion in the offspring's diet and, consequently, a decrease in the importance of milk as the sole source of nutrients [10].

Physiologically, the evolution of milk production, and thus the shape of the lactation curve, can be explained by the number and activity of milk-secreting cells [11]. The increase in milk yield from the beginning to the peak of lactation is explained by the increasing secretory activity of mammary epithelial cells (MECs), while their proliferation remains relatively stable throughout the lactation period [12]. The decrease in milk production between the 8th and 23rd weeks of lactation is due to a decrease in the MEC population due to an increase in the rate of apoptosis, and, at a later stage, also to a decrease in the secretory activity of cells. During the involution phase, the rate of apoptosis significantly exceeds the rate of proliferation [13], and thus the number of mammary epithelial cells is mainly modulated by the rate of apoptosis [14].

Breast development and milk synthesis are tightly controlled by the endocrine system, various growth factors, and other genetic and epigenetic factors [15, 16]. In addition, environmental factors such as nutrition, climate, calving season, photoperiod and circadian rhythms, heat stress, milking system and type of housing play an important role in the realization of genetic potential [17]. Animal age and health are also important in predicting expected production [11]. All of these factors cause changes in milk composition and milk yield and therefore should be taken into account when modeling the lactation curve [18].

The lactation curve can be described using mathematical models, which makes it possible to predict lactation activity. These models can predict a range of key milk parameters based on cow management parameters and data from previous

lactations [8].

We present a brief review of mathematical methods for modeling lactation curves, described in domestic and foreign special literature. One of the most popular methods (Wood's method, P.D.P. Wood, 1967) as applied to the classical classification of lactation curves given by A.S. Emelyanov [19]. Curve models are considered both for milk yield and for other key parameters, such as the percentage of fat and protein, as well as the yield of fat and protein.

Predicting the evolution of milk yield is an important step in making managerial and breeding decisions on herd management and is widely used for diagnostic purposes [20]. Based on the analysis of the simulated functions, the relationship between their mathematical characteristics and parameters describing the state of health of an individual is traced. Thus, lower values of derivatives in the vicinity of the maximum and the presence of a single extremum characterize an individual as healthier, with stable fertility, and more efficient use of cheaper feed [21, 22]. Therefore, milk composition data can be used to adjust diets [23] and to detect diseases before clinical signs appear, helping to reduce treatment costs and improve disease prognosis [24, 25].

For dairy production, cows with an even type of lactation are considered the most valuable [26]. It is more economically beneficial when a cow produces a moderate amount of milk throughout the entire lactation period than when she quickly reaches the peak of productivity, after which milk yield drops sharply. The stability of lactation depends on genetic factors and thus may vary between breeds, as well as depending on physiological conditions (eg pregnancy) and external influences [27, 28]. The economic feasibility of genomic selection for traits of milk production to achieve the desired curve has been demonstrated in a number of works [29-31].

Physiological bases of the lactation cycle of cows. Reproduction of offspring and providing it with milk are two interrelated processes. It is known that during pregnancy, cows develop mammary tissue, which continues to grow until the number of mammary cells reaches a maximum. Shortly after birth, there is a decrease in the number of cells due to apoptosis (programmed death) of secretory cells. This decrease is usually observed before the end of the lactation period. Milk in a cow begins to be secreted during or immediately before calving. The entire lactation cycle can be divided into two periods: a rise period (calving to peak og milk production) and a decline period (from peak to dry period) [32]. An increase in milk yield at the beginning of lactation is directly related to the rate of cell differentiation, and a decrease in milk yield at the end of the lactation period is directly related to the apoptosis of these cells. Thus, it is quite logical to combine three processes into one model: differentiation of secretory cells, their apoptosis, and the rate of milk secretion per cell.

In cattle, the lactation period averages 10 months (305 days) and includes several phases. The first phase covers up to 70-100 days from calving, i.e., from calving to reaching the peak of productivity. The second phase lasts from the peak of lactation to the middle of lactation (up to 150-200 days from calving), the third phase from the middle to the end of lactation (up to 305-320 days from calving), and the fourth phase is the dry period (45-60 days from launch) [33].

Preparation for a new reproductive cycle (service period) can last 65-80 days. This period is considered optimal for preparing for insemination. The duration of pregnancy in cattle is 285 days (Fig. 1).

In dairy cattle, pregnancy, as a rule, occurs against the background of lactation. A significant decrease in milk secretion is recorded between day 100 and day 200 of pregnancy and does not have a pronounced dependence on the timing

from the start of lactation [34]. By studying the relationship of these two states at the molecular level, J.V. Nшrgaard et al. [35] concluded that hormones produced by the fetoplacental system reduce the activity of mammary gland cell proliferation during lactation and do not affect apoptosis.

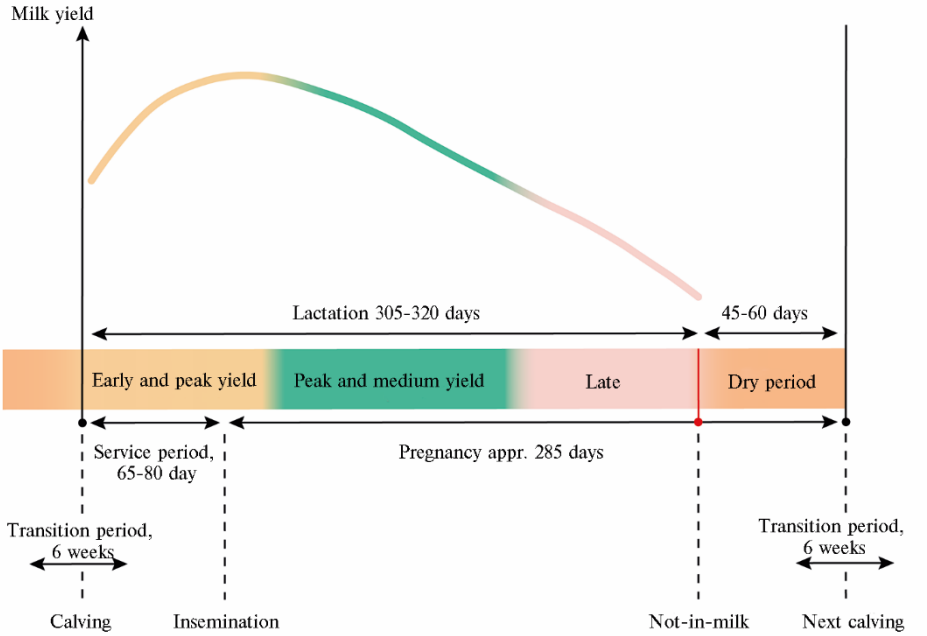

Fig. 1. The relationship between lactation and reproduction cycles in highly productive cows.

The shape of the lactation curve demonstrates the calf's need for milk: increasing milk secretion up to the peak of lactation reflects an increased demand for milk as the only source of nutrition, while a phase of declining milk production indicates an expansion of the diet and, as a result, a decrease in the proportion of milk consumed [9].

Approaches to the construction of models of lactation activity. Mathematical modeling of lactation curves makes it possible to predict milk yield depending on the time elapsed since the birth of a calf. Models can accept additional parameters such as lactation number, breed, feed-related parameters, etc. [36]. The lactation curve most often has a Λ-shape, rapidly increasing for several weeks, after which it passes into a decreasing phase with a much smaller derivative. With daily measurements of milk yield, there is usually a beating of the milk yield around the trend [19]. The highest productivity occurs between 4 and 8 weeks after calving. Over the long history of research, models of various types have been proposed - linear, exponential, polynomial, and many others [21].

The first attempts to describe the results of lactation were made as early as the 1920s, and since then the complexity and accuracy of the descriptions has only increased [37]. The models differ in the degree of dependence on the parameters of the medium and time (linear, exponential, polynomial), the number of these parameters, and the methods of describing the main characteristics of the curves (extremum points, values at the extremum points, the degree of decrease in the descending phase, etc.). In most cases, lactation curves are based on the amount of milk yield during the entire lactation period for a cow, with the idea of predicting subsequent ones. To increase the accuracy and stability of the results of the model of lactation activity, it is possible to take into account the change in the parameters of keeping animals over time [38].

Simple mathematical models predict the lactation activity of a cow at the beginning of lactation based on a fixed amount of data from the previous lactation. There are also studies that propose a model for predicting the entire curve at once by building deep learning structures based on all historical data about a cow. Such models make it possible to find at first glance implicit patterns. For example, in the work of A. Liseune et al. [39] showed that such a scheme can exceed the standard models in accuracy during the first 26 days of lactation. It can be used to detect deviations in milk yield from the norm, which reduces the time for detection of diseases. These forecasts are also useful when planning a farm budget.

Mathematical models of lactation curves. The lactation curve for milk is the function

$$y = f(t), \quad (1)$$

where t is the time since the birth of the calf, days; y is the amount of milk produced during the t-th day, kg. Perhaps the most popular model for describing lactation curves is Wood's empirical model (P.D.P. Wood, 1967), where milk yield, percentage of fat and protein, fat and protein yields by weight act as milk characteristics [18, 21, 40]. In general, the model looks like this:

$$y_t = at^b e^{ct}, \quad (2)$$

where y_t is the milk quality parameter on day t; e is the Euler number, the parameters b, c determine the slopes of the curve before and after the extremum (the function in the standard form has one maximum), the parameter a is the coefficient associated with the absolute values. The maximum productivity is determined at the point

$$t_{max} = \frac{b}{c}, \quad (3])$$

that is, the time to reach the peak of productivity does not depend on the parameter a. For a standard lactation curve, parameter b takes positive values, and parameter c takes negative values. Variations in b and c give four different configurations of lactation curves: standard iso-form for $b > 0$, $c < 0$; increasing isoform for $b > 0$, $c > 0$; decreasing isoform for $b < 0$, $c < 0$; reverse isoform for $b < 0$, $c > 0$.

The reliability of the model is assessed using the coefficient of determination. The coefficient of determination $R^2_{(adj)}$ shows what proportion of the variance of the resulting feature is explained by independent variables. The standard milk yield curve showed the highest average level of accuracy, for 64.7% of the points $R^2_{(adj)} > 0.75$. The use of the same form to describe the percentage of milk fat turned out to be the least accurate, only for 18.7% of the points $R^2_{(adj)} > 0.75$. These facts indicate that the same configuration of coefficients cannot be used to describe all milk parameters. Milk yield, protein yield and fat yield are well modeled by the standard form, while fat and protein percentages are more accurately described by the inverse form.

A number of studies have considered other characteristics of milk. For example, the ratio of the percentage of fat content of milk to the percentage of protein in milk (Fat Protein Ratio, FPR) [41]. The assumption is checked that this parameter plays a key role in predicting the overall productivity of cows. A variety of models have been built, e.g., Wilmink (J.B.M. Wilmink, 1987), Ali-Schaeffer (T.E. Ali and L.R. Schaeffer, 1987), Guo (Guo) and Salvador (Salvador), approximations using Legendre polynomials. The Bayesian information criterion and Akaike information criterion were used to assess the accuracy of the models, and in some cases, the correlation between the theoretically expected and actual values of the FPR parameter [41] was considered for this. The highest accuracy was shown by the function proposed by T.E. Ali and L.R. Schaeffer [42] in both random and fixed modifications. The study showed that FPR correlates

with the energy status of animals, especially at the initial stage of lactation. According to the totality of studies, all considered models for constructing lactation curves gave almost identical predictions. The main factor in the decrease in accuracy was the reduction in the volume of input data on the dependence of daily milk yield on time. Models based on empirical approximation (Wood, Wilmink and Ali-Schaeffer models) turned out to be the least resistant to increasing intervals between measurements. Of the earlier works, it is worth noting the study conducted in 1953 by A.S. Emelyanov [19], where a detailed study of lactation curves was carried out with a high frequency of measurements throughout the entire lactation period and a differential mathematical model was proposed.

*Application of Wood's model to different types of lactation curve*s. M*odeling lactation curves of milk yield.* The constructed lactation curve reflects the individual characteristics of the cow, such as the state of health, the tendency to milk, and the stability of the volume of milk produced throughout the cycle. To demonstrate, let us take a closer look at the demonstrative study of A.S. Emelyanov, mentioned above [19, 43]. For curves according to milk yield, he proposed the division of all individuals into groups according to the type of lactation activity (that is, according to the type of lactation curves that differ in height and stability): high stable, two-top, high unstable, low stable. It should be noted that these types were identified among individuals of different breeds under the same conditions of detention.

All these curves are quite accurately described by A.S. Emelyanov using the formula

$$y = \frac{A}{(t-1)} B(t-1) + 100, \quad (4)$$

where y is the percentage of increase in milk yield on the next day vs the previous one, t is the time from the birth of the calf, A is the percentage of lactation acceleration, that is, by what percentage does the milk yield increase in the first two periods of lactation compared to the first period, and B is the degree of decrease in the percentage growth. This formula is inherently differential when passing to the limit to infinitely small measurement intervals for the coefficients A and B. However, integrating this equation to obtain a lactation curve is laborious, so constructive models, such as the Wood model above, have become more common.

When selecting breeding pairs, preference is given to cows whose curves show the greatest resistance. Lifetime milk yield and the number of possible lactations are usually higher.

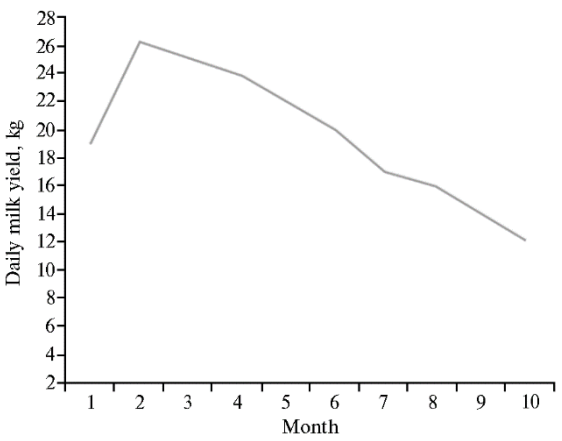

Fig. 2. Milk yield curve for cows with high productivity and lactation stability. Adapted from the work of A.S. Emelyanov [19]

I. High stable type. A group of individuals of this type is characterized by a low rate of decline in milk yield by the end of the lactation period. Lactation stably persists for all 300-305 days. Changes in feeding have little effect on milk output, animals are able to maintain an intensive metabolism for a long time, they have stable cardiovascular and nervous systems. As a rule, the integral milk yield of such cows is one of the best in the herd. Such individuals are highly desirable for selection (Fig. 2) [43].

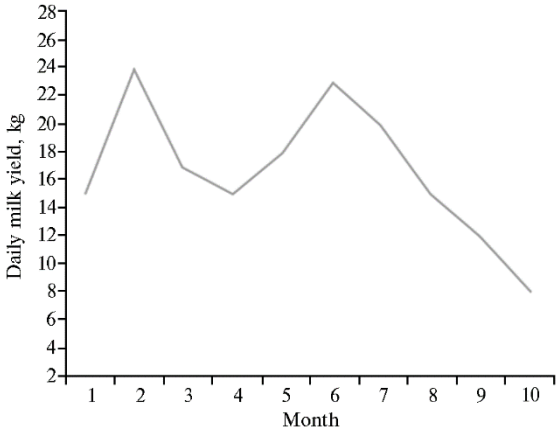

Fig. 3. Two-peak lactation curve. Adapted from the work of A.S. Emelyanov [19].

These curves are described with good accuracy by the standard form of the Wood model discussed above. To evaluate according to the lactation curve, Wood proposed a stability coefficient expressed by the formula

$$p = -(b + 1)\ln(c), \quad (5)$$

where b and c are the coefficients introduced in (2). The first type is characterized by high values of the stability parameter $p > 2.2$ [21].

II. Double top type. In this group (Fig. 3), the milk yield has several significant periods of rise and fall. From the point of view of lactation activity, animals are similar to type 1 cows, but in general they have poorer health, which does not allow them to maintain a constant level of metabolism, as a result, alternation of periods of rest and intensive milk production is observed on the lactation curve. Fluctuations in lactation in this case are not related to the quality of feeding, since the conditions of detention are the same. Full 300-day lactation is considered acceptable, integral milk yields are slightly lower than in cows with the 1st type of lactation. It is believed that these animals can be involved in selection.

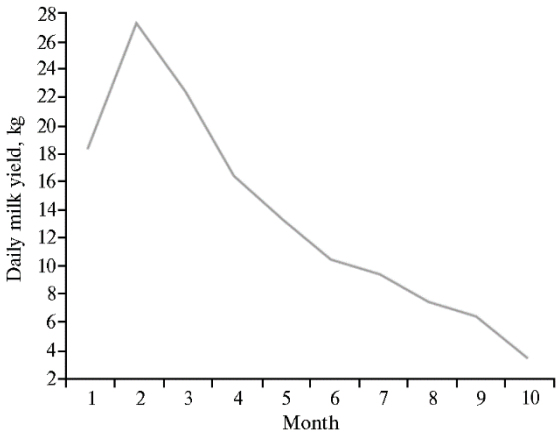

Рис. 4. Milk yield curve in cows with high productivity and unstable lactation. Adapted from the work of A.S. Emelyanov [19].

With the help of most standard mathematical models, modeling such curves is impossible. In such cases, the lactation curve can be described by polynomial series, taking into account a larger range of parameters [45, 46].

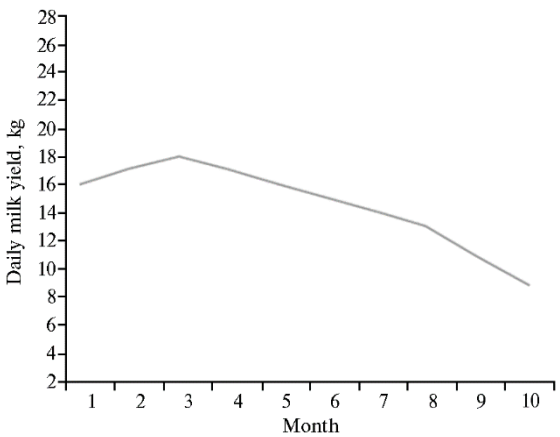

Рис. 5. Milk yield curve for cows with low productivity and stable lactation. Adapted from the work of A.S. Emelyanov [19].

III. High unstable type. In the first months of lactation, these cows have high milk yields, but from the 5-6th month they quickly fall. The preferred lactation period is 250 days, while the integral milk yield is almost equal to that of cows of the 1st type. The cardiovascular system of such animals cannot cope with the long-term stress of metabolism. Suitable for selection in the second turn, also have a limited period of fertilization (Fig.

4) [43]. Such curves are also well estimated using the Wood model, but have a significantly lower stability coefficient $p < 1.6$.

IV. Low resistant type. Almost the entire period of lactation, the milk yield curve reflects consistently low rates. Individuals are highly resistant, have a low integral milk yield, and are stable (Fig. 5) [43]. Animals of this type are mostly low-milk, have a weak metabolism, deficiencies in the structure of the mammary gland, problems with the cardiovascular system and digestion. If this type of lactation activity is detected in individuals of highly productive breeds, their use in breeding work is not recommended. However, one should not forget that this type is often found in representatives of native breeds and is the norm in the selection of animals for further breeding. Lactation curves of this type can be described by the Wood model with a stability coefficient $p \approx 2$.

Modeling lactation curves for milk components. I. Construction of lactation curves for fat and protein. As noted above, Wood's model is also used to describe fat percentage and protein content. It is noteworthy that the stability coefficient is calculated using the same formula. The curves describing the content of fat in milk do not have sharp drops, however, there is usually a local minimum at the time point of maximum milk yield. During the first 5 weeks (colostrum) of lactation, milk tends to have the highest fat content and highest protein content. Fat percentage is influenced by the same set of factors as milk yield, the curves for milk yield and for fat content have extremes at the same points, which means they are directly correlated [47)]. After the first 2 weeks of lactation, the short-term hormonal changes caused by the birth of a calf stabilize, and the volume of milk ceases to vary greatly over time.

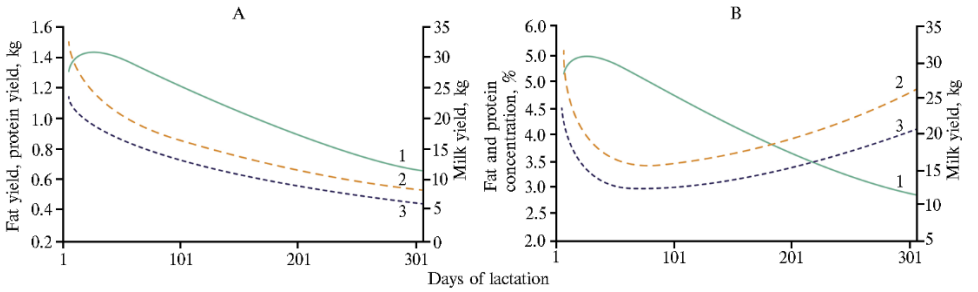

Рис. 6. Schematic representation of Wood's models for indicators of fat (2) and protein (3) in relation to milk yield (1) on the same coordinate plane: A — yield of protein and fat, B — percentage of protein and fat content. The data of milk productivity of individuals of the Holstein breed of cows were used. Adapted from A.M. Silvestre et al. [18].

As can be seen from the graphs (Fig. 6, A, B), these same processes almost do not affect the main components of milk - protein and fat, therefore, at the beginning of lactation (the first day), their abnormally high percentage in milk is possible. The yield of protein and fat in kilograms decreases in proportion to milk yield after passing the maximum point, which is associated with a general decrease in metabolic rate [48]. However, the drop in milk yield is always faster, so milk by the end of lactation becomes somewhat fatter and more saturated with protein [5, 18, 48].

Lactation curves as a tool for timely diagnosis of the physiological state of animals. Parameter "fat:protein ratio". As is known, the FPR coefficient has a high heritability and correlates with the energy state of the animal.

It has been reported that this indicator can be used for early detection of udder diseases. Also, FPR can be used to assess the general condition of an individual in order to make decisions about its further maintenance. For example, an important characteristic is the energy balance (EB), which allows you to adjust

the diet and more accurately select feed. Correlations have been found between FPR and EB, which provide data on the deficiency or excess of certain dietary components on the farm during lactation (Fig. 7) [41].

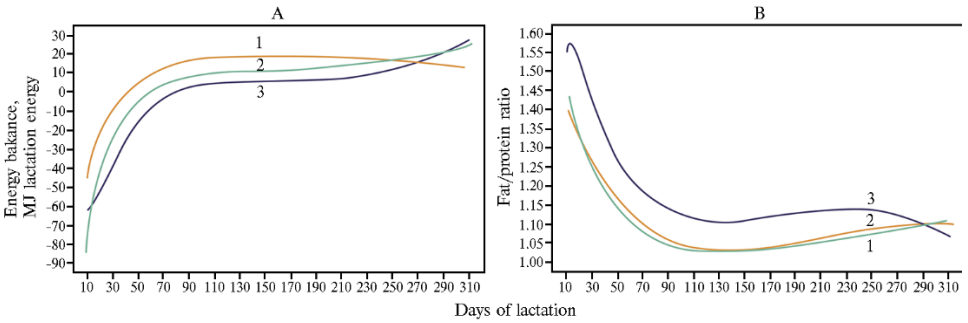

Fig. 7. Negative correlations of energy balance (EB, NEL — lactation net energy, A) **and fat:protein ratio** (FPR, B) **in the 1st** (1)**, 2nd** (2) **and 3rd or subsequent** (3) **lactations.** The data of milk productivity of individuals of the German Holstein breed of cows were used. Adapted from N. Buttchereit et al. [41].

Prediction of diseases by deviations of lactation curves from predicted values. The models built can be used to prevent and detect udder diseases such as mastitis. For example, you can build a model for individual parts of the udder, comparing deviations from which you can see the effects of the disease. Based on the milk yield for each quarter of the udder, a special improved Wood model is built. Based on the curves of these models and the actual readings of milk yield for parts of the udder, a graph of their difference (QL) is built. Figure 8 shows the curves for clinical mastitis in the case of acute QL_CM1 and more mild disease QL_CM2 compared with the healthy part of the udder QL_NI1 and QL_NI2, respectively. It can be seen that for QL_CM1 and QL_NI1 there is a common peak in the decline in productivity, and after several milkings it becomes possible to find out which particular quarter of the udder is infected. For QL_CM2 and QL_NI2, the productivity of the affected quarter is gradually decreasing. Notably, in this case, the healthy part of the udder compensated for the loss of milk (probably due to redistribution of nutrients) (see Fig. 8) [49].

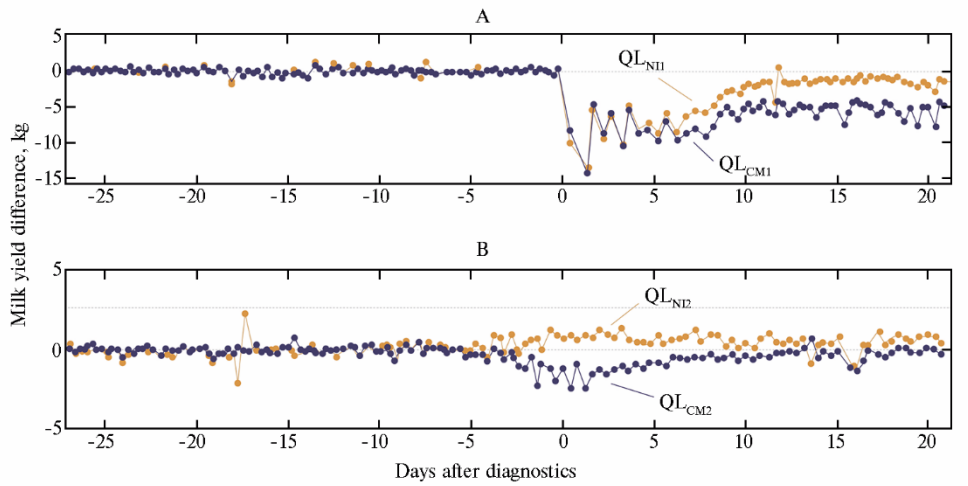

Fig. 8. The difference between expected and observed milk yields (QL) **in case of clinical mastitis for the diseased and healthy part of the udder of cows in the case of acute** (A) **and moderately severe** (B) **course of the disease.** Adapted from I. Adriaens et al. [49].

Modeling lactation curves to assess breeding value and take into account

the influence of environmental factors. Improvement of models of lactation curves continues. In the studies of A.B. Abdelkrim et al. [38] used a sophisticated modeling approach to refine milk loss as a function of various factors affecting the animals. The approach uses a superposition of perturbation signature plots and the previously mentioned Wood's function. It is assumed that there is a certain reference curve that depends solely on genetic traits, on which perturbations caused by the imperfection of the habitat are superimposed (Fig. 9). The effects of a wide variety of parameters were taken into account, from lactation number to seasonality. This approach allows not only to calculate the influence of environmental factors on the integral milk yield with high accuracy, but also to assess the genetic potential of animals for further selection [38].

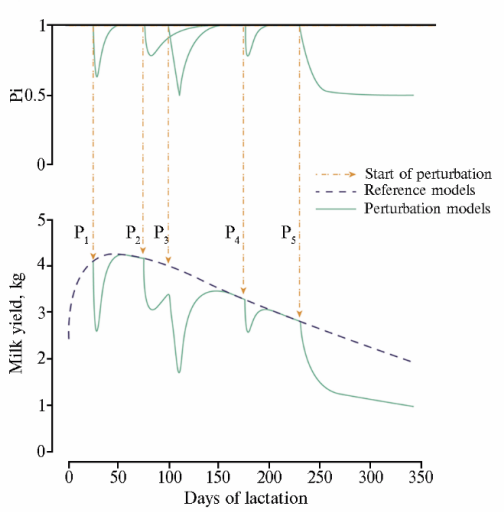

Fig . 9. Translation of the superposition of perturbation functions (P1-P5, top) **onto the milk yield curve** (bottom) **in the Wood model for cows by lactation.** The individual dynamics of disturbances is expressed as the proportion of the undisturbed lactation curve (P1-P5). The graph of milk yield shows the unperturbed and perturbed dynamics of milk yield. The data on the milk productivity of goats of the Alpine and Saanen breeds were used. Adapted from A.B. Abdelkrim et al. [50].

Genomic selection is another important way of using these lactation curves. To do this, animals with different types of lactation curves must be genotyped for the maximum set of markers associated with milk production and milk quality in order to identify correlations between the haplotype and the type of lactation curve. In the future, the selection of suitable animals can be carried out immediately after birth or at the stage of selection of pairs during breeding. This approach will significantly reduce the cost of growing and keeping animals that are unpromising for milk production. Animals whose lactation activity is characterized by lower peaks and a more persistent lactation curve experience fewer health and reproductive problems and can efficiently use cheaper feed [22]. Predicting the evolution of milk yield can be widely used for diagnostic purposes, being an important tool in choosing a management strategy and breeding policy for herd management [20].

So, forecasting the evolution of milk yield is widely used by farms for making managerial and breeding decisions. For dairy production, cows with an even type of lactation are considered the most valuable. Modeling of lactation curves is one of the most effective methods for predicting various parameters of milk based on a fixed amount of data at the beginning of lactation following lactation with measured indicators. Accounting for hereditary and non-hereditary factors affecting lactation activity is important to obtain a more accurate prognosis. In addition, the reliability of the model should be assessed based on its stability when modeling specific parameters. Some components of milk, as well as their ratios, can act as very accurate markers of the physiological state of animals. Thus, the modeling of lactation curves is an important tool for timely monitoring of the health status of animals and their productivity. Identification of diseases before the appearance of clinical signs helps to reduce the cost of treatment and improve the prognosis of the course of the disease. The main difficulty of regular monitoring of lactation activity lies in the difficulty of making accurate measurements and their subsequent analysis, the cost of these procedures, as well

as the availability of technical capabilities. Recently, deep learning algorithms have begun to be used to model lactation curves, which can surpass standard models in accuracy (for example, in predicting productivity in the first day of lactation). When training, such models take into account the statistics of milk yield for previous lactations, health parameters (in an implicit form), including fertility, and use these data when modeling the entire curve at once. It is advisable to use these forecasts when planning the budget of the economy.

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GENETIC STRUCTURE OF THE LOCAL YAKUTIAN HORSE POPULATION FOR GENES *MC1R*, *ASIP*, *DMRT3*, AND *MSTN*

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Abstract

The Yakutian horse is believed to be one of the oldest breeds. The breed has unique morphological characteristics and is well adapted to survive within the Arctic Circle. Yakutian horses have compact body conformation and extremely thick winter coats with long mane and tail. In the Yakutian breed dominate light coat colours: gray and dun. The gray and dun coat colours of Yakutian horses are their natural camouflage. The Yakutian horse is multipurpose breed, because the local horses have been used by people not only for the production of milk and meat, but also as transport animals. In this paper, the genetic structure of the native Yakutian breed was characterized using markers of four genes that are associated with important selected traits in different modern populations of domestic horses (Equus caballus). The aim of our study was to investigate the polymorphism of the *ASIP* and *MC1R* genes that determine skin and hair pigmentation, as well as to assess the occurrence of mutations in the *MSTN* (g.66493737C>T) and *DMRT3* (g.22999655C>A) genes associated with athletic performance and locomotion in domestic horses. Hair samples were collected from 45 adult purebred Yakutian horses (*Equus caballus*), including 11 samples from animals of the indigenous type and 34 samples from animals of the Yana type. DNA was isolated using ExtraGene™ DNA Prep 200 reagents (Isogen Laboratory, Russia). Genotyping for the SNP marker C>T of the *MC1R* gene was carried out using the PCR-RFLP (PCR-restriction fragment length polymorphism) method according to L. Marklund et al. (1996). Detection of 11 bp deletion in the *ASIP* gene was carried out according to the method described by S. Rieder et al. (2001). Allele nomenclature was used according to M. Reißmann (2009): *E* — dominant wild-type allele, *e* — recessive (mutant) allele (*MC1R*); *A* — dominant wild-type allele, *a* — recessive (mutant) allele (*ASIP*). The SNP mutation in the *MSTN* gene (g.66493737C>T) was detected by the amplification-created restriction site-PCR (ACRS-PCR) method described by M. Gábor et al. (2014). Genotyping of DNA samples for the SNP marker of the *DMRT3* gene (g.22999655C>A) was performed by PCR-RFLP method, C>A polymorphism was detected using restriction endonuclease HpyF3I (Thermo Scientific, Lithuania). Frequencies of alleles, frequencies of genotypes in the population and observed heterozygosity were calculated. Polymorphism of the *ASIP* and *MC1R* genes observed in Yakutian horses demonstrated a predominance of allelic variants that determine the synthesis of eumelanin, the darker type of the pigment. In the studied group of horses the frequency of the dominant *E* allele of the *MC1R* gene that determines the production of the black pigment eumelanin, was 0.711. The number of homozygous carriers of the recessive mutation of the *MC1R* gene (*e* allele) that determines production of red pigment pheomelanin was 13.3 %. The frequency of the dominant *A* allele of the *ASIP* gene that limits the synthesis of the black pigment eumelanin and affects the character of its distribution was 0.400. The number of homozygous carriers of the recessive mutation of the *ASIP* gene (*a* allele) among the tested Yakutian horses was 40 %. This is relatively high value, because in the most of modern horse breeds, the recessive *a* allele of the *ASIP* gene is rather rare. In total, eight different genotypes were identified for two key genes affecting skin and hair pigmentation. The most typical genotypes for Yakutian horses were *E/E-A/a* and *E/E-a/a*. The character of skin and hair pigmentation in the Yakutian horses could have an adaptive meaning for survival within the Arctic Circle. The frequency of the mutant variants of genes *DMRT3*

(g.22999655C>A) and *MSTN* (g.66493737C>T) in the tested horses were 0.011 and 0.022, respectively. Obviously, being presented in the population at a low frequency, the mutant variants of the *DMRT3* and *MSTN* genes have no selection value, because historically, the Yakutian horse has served people as a transport animal in the forest and swampy areas, where only riding is suitable and the most convenient gait is walk.

Keywords: horses, Yakutian breed, DNA markers, polymorphism, *MC1R*, *ASIP*, *DMRT3*, *MSTN*, eumelanin, pheomelanin, performance traits

The Yakut horse is the northernmost breed in the world, which differs from others in its unique morphological and physiological characteristics [1]. This ancient aboriginal breed was formed in the extreme natural and climatic conditions of Yakutia, one of the coldest places on Earth. Since time immemorial, the indigenous population of Yakutia has been breeding herd horses, which were kept on pastures, independently extracting pasture [2]. As a result of centuries of selection, local horses have successfully adapted to year-round open-air keeping on natural pastures, using vegetation that is under snow cover during the long winter period. The compact physique of the Yakut horses, their extremely dense hairline and metabolic features contribute to survival in the extreme conditions of the Subarctic. The Yakut horse received the status of an independent breed in 1987 [3]. The breed is universal, Yakut horses are used in agricultural work, as transport animals, in sports, as well as for the production of meat, koumiss, leather and fur raw materials. Several intrabreed types are distinguished in the breed [4].

There are several hypotheses about the origin of the native Yakut horse [5]. P. Librado et al. [6] sequenced and analyzed the complete genomes of 11 Yakut horses, including nine modern animals and two fossils (one sample dated to the early 19th century, the second to 5200 BC). The authors came to the conclusion that, most likely, modern Yakut horses are the descendants of horses brought by the Yakut people who migrated to this region in the 13th-15th centuries. The main mechanism that ensured the relatively rapid adaptation of animals to existence in the subarctic conditions was cis-regulatory changes in the genome. Unlike mutations in the coding region, which can lead to a change in the structure of the encoded protein, cis-regulatory changes contribute to the adaptation of the animal population to extreme conditions by fine-tuning gene expression [6].

The development of molecular genetics technologies has provided new opportunities for a detailed study and comparative analysis of the genomes of modern and fossil horses, making it possible to reconstruct the history of animal domestication and the formation of individual breeds. A study of ancient genomes on the polymorphism of genes affecting hair pigmentation showed that during the process of domestication of the horse, the diversity of colors found in animal populations rapidly increased [7]. Genotyping of DNA samples of fossil horses for 8 mutations in 6 genes that determine the color showed that wild horses of ancient populations were characterized by the same type of hair color. On the contrary, a rapid and significant increase in the diversity of colors among ancient horses was observed both in Siberia and in Eastern Europe, starting from the 5th millennium BC, which is associated with a period of domestication [7].

Color is one of the most significant morphological features of domestic horses [8]. The color of hair and skin is determined by the pigment melanin, represented by two main forms - eumelanin (black pigment) and pheomelanin (red-yellow pigment). The color of a horse is determined both by the amount of melanin and by the distribution of its types in the covering and guard hairs [9]. Skin and hair pigmentation in mammals is a polygenic trait determined by the combined action of a large number of genes, with the genes of the *MC1R* and *ASIP* loci playing a key role [10].

The *MC1R* gene encoding the type 1 melanocortin receptor is localized on chromosome 3 of the horse and has two main alleles: the dominant allele *E* determines the production of the eumelanin pigment, the recessive allele e in the homozygous state suppresses the synthesis of eumelanin and causes the synthesis of predominantly red-yellow pigment pheomelanin [9]. Animals homozygous for the recessive allele of the *MC1R* gene have a red color. L. Marklund et al. [11] found that the recessive mutation that determines the red color in domestic horses is a C>T single nucleotide substitution in the *MC1R* gene [11].

The *ASIP* gene encoding the agouti-signaling protein affects the production and distribution of the black eumelanin pigment, while the wild-type dominant allele *A* limits the synthesis of eumelanin and allows it to accumulate only in certain parts of the body (in the hair of the legs, mane and tail), determining the bay color [9]. In 2001, S. Rieder et al. [12] found that the recessive mutant allele a of the *ASIP* gene, which does not affect the distribution of eumelanin and determines the black suit, is an 11 bp deletion.

Due to the combined action of the *MC1R* and *ASIP* genes, as well as a number of modifier genes that can cause a decrease in pigmentation and the appearance of an admixture of white or dark hair in the coat, the colors of modern domestic horses are characterized by exceptionally wide variability [10]. In some modern commercial breeds, the color is one of the main traits selected by man, while animals of rare original colors are in high demand among buyers. The genes that determine the colors of native horses, bred for centuries by year-round herd keeping, were significantly influenced by natural selection. In the Yakut breed of horses, as a result of natural selection, gray and savras colors predominate [4, 13]. M.F. Gabyshev [13] notes that under polar conditions, the light gray color serves as a natural protection for animals, making them less noticeable to predators against the background of winter nature. Gray color in horses is an autosomal dominant trait in which there is a progressive "graying" of the integumentary and guard hairs, while the skin remains pigmented [10]. In 2008, it was found that the gray coat phenotype in the domestic horse is determined by a 4.6 bp duplication in intron 6 of the *STX17* gene, which is a cis-regulatory mutation [14]. Savrasaya color is caused by the dominant *TBX3* gene, which causes a decrease in the intensity of pigmentation of the hairline [15]. The phenotype of saurian colors also contributes to the effective visual camouflage of animals against the background of natural landscapes.

The use of horses as working and transport animals has had a huge impact on the development of human civilization. For thousands of years, domestic horses have been used for riding, and in many cultures, animals that are able to move in comfortable gaits (pacing) are especially valued [16, 17]. Nowadays, in many countries of the world, breeds of horses capable of alternative gaits are very popular.

L. Andersson et al. [18] found that the single nucleotide substitution C>A (chr23:22999655) in the *DMRT3* (doublesex and mab-3 related transcription factor 3) gene has a key effect on locomotion characteristics in domestic horses [18]. A high frequency of occurrence of the *DMRT3* gene mutation is observed in stud and native breeds, which are characterized by alternative gaits [19]. According to E.A. Staiger et al. [20], the *DMRT3* gene mutation (g.22999655C>A) could have appeared either immediately before domestication, or, more likely, some time after horse domestication and subsequently spread widely throughout the world due to intensive artificial selection [20]. It was established that in a highly specialized standardbred breed, bred exclusively for participation in hippodrome races of pacers and trotters, the mutant allele was completely fixed by selection [19].

Unlike the Standardbred breed, the Thoroughbred Saddlebred is a highly

specialized factory breed, the evolution of which took place under the pressure of intensive artificial selection of animals for the ability for outstanding gallop agility [21]. Exploring the genome of thoroughbred riding horses, E.W. Hill et al. [22] revealed a mutation — a single nucleotide substitution in the first intron of the myostatin gene (*MSTN*, g.66493737C>T), associated with high agility of racehorses over short distances. To win in races over short distances, the horse is required to develop maximum speed right from the start. It has been established that thoroughbred riding horses with the *C/C* genotype are predisposed to the manifestation of outstanding sprinting abilities, and the *T/T* genotype is characteristic of stayer horses [22]. M.A. Bower et al. [23] showed that the *C* allele became widespread in Thoroughbred horse breeds in the second half of the 20th century, which is explained by the growing popularity of sprint races during this period. It has been established that under the influence of one-sided artificial selection for agility over short distances, the mutation of the *MSTN* gene (g.66493737C>T) was completely fixed among racehorses in the American quarter-mile breed [24]. Interestingly, the *C* allele occurs not only in half-bred breeds bred using thoroughbred sires, but also in most aboriginal horse breeds of various geographic origins [23], which indicates the antiquity of the origin of this allele.

The obtained data on DNA polymorphism in horses of various breeds and directions of use provide basic insight into the mechanisms of evolution of breeds and intrabreed groups of horses. Over the centuries, human activity has selectively affected different populations of horses. Studies of the horse genome have shown that populations of ancient animals were characterized by significant genetic diversity. Artificial selection has shifted the average characteristics of different populations over time to form breeds [25].

In this paper, for the first time, the genetic structure of the native Yakut breed is characterized by four DNA markers that are of breeding importance in specialized breeds of horses for various purposes.

Our goal was to study the polymorphism of the *ASIP* and *MC1R* genes that determine skin and hair pigmentation, as well as to assess the occurrence of mutations in the *MSTN* (g.66493737C>T) and *DMRT3* (g.22999655C>A) genes associated with the working qualities of domestic horses.

*Materials and method*s. The material for the study was hair samples with bulbs from the mane, taken in 2014 in the horse breeding farms of the Republic of Sakha (Yakutia) from 45 adult purebred Yakut horses (*Equus caballus* L.), including 11 samples from indigenous animals and 34 samples from animals yang type.

DNA was isolated from hair follicles using ExtraGene™ DNA Prep 200 reagents (Isogen Laboratory, Russia). Genotyping of biological samples was performed using commercial kits of reagents GenPak® PCR Core (Isogen Laboratory, Russia) in accordance with the manufacturer's recommendations.

Genotyping for the SNP marker C>T of the *MC1R* gene was performed using the PCR-RFLP (PCR-restriction fragment length polymorphism) method as described by L. Marklund et al. [11] using published primer sequences [26]: 5'-CCTCGGGCTGACCACCAACCAGACGGGGCC-3', 5'-CCATGGAGCCGC-AGATGAGCACAT-3'. Amplification was carried out in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Inc., USA) according to the following scheme: 10 min at 95 °C; 30 s at 95 °C, 40 s at 60 °C, 1 min 30 s at 72 °C (35 cycles); 30 min at 72 °C (final elongation). Detection of C>T polymorphism in the amplified DNA fragment was carried out using TaqI restriction endonuclease (Thermo Scientific, Lithuania) according to the manufacturer's recommendations with further separation of the resulting fragments by electrophoresis in 2%

agarose gel.

11 bp deletion detection in the *ASIP* locus was carried out according to the method of S. Rieder et al. (12) using primer sequences: 5'-CTTTTG-TCTCCTTTGAAGCATTG-3', 5'-GAGAAGTCCAAGGCCTACCTTG-3'). The amplification mode was as follows: 10 min at 95 °C; 30 s at 95 °C, 40 s at 55 °C, 1 min 30 s at 72 °C (35 cycles); 30 min at 72 °C (final elongation). The resulting amplicons were separated by electrophoresis in 3% agarose gel.

Designations of allelic variants of the *MC1R* and *ASIP* genes corresponded to the nomenclature of M. Reißmann [9]: *E* — dominant wild-type allele, *e* — recessive (mutant) allele (*MC1R*); *A* is the dominant allele of the wild type, *a* is the recessive (mutant) allele (*ASIP*).

The SNP mutation in the *MSTN* gene (g.66493737C>T) was detected by the amplification-created restriction site-PCR (ACRS-PCR) method proposed by M. Gábor et al. [27], using published primer sequences: 5'-GAGAAGG-CATGACACGGAAG-3', 5'-TTGATAGCAGAGTCATAAAGGAAAAGTA-3'. PCR was carried out according to the scheme: 10 min at 95 °C 30 s at 95 °C, 40 s at 56 °C 1 min 30 s at 72 °C (35 cycles); 30 min at 72 °C (final elongation). The polymorphism of the obtained fragments was detected using restriction endonuclease RsaI (Thermo Scientific, Lithuania) in accordance with the manufacturer's recommendations with electrophoresis in 3% agarose gel.

Genotyping of DNA samples for the SNP marker of the *DMRT3* gene (g.22999655C>A) was carried out by the PCR-RFLP method, as described earlier, using the original primers 5'-AGCTTGAAAGCCAACAGACC-3', 5'-CAAAGA-TGTGCCCGTTGGA-3'. They were designed using the Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/pri-mer-blast/) and PerlPrimer (http://perl-primer.sourceforge.net/) programs using the reference the DNA sequence of the domestic horse *Equus caballus* published in the NCBI database (NC_009166.2). Amplification was carried out according to the scheme: 10 min at 95 °C; 30 s at 95 °C, 40 s at 60 °C, 1 min 30 s at 72 °C (35 cycles); 30 min at 72 °C (final elongation). Detection of C>A polymorphism in the amplified DNA fragment was carried out using restriction endonuclease HpyF3I (Thermo Scientific, Lithuania) in accordance with the manufacturer's recommendations, followed by separation of the obtained fragments in 3% agarose gel [28].

Fig. 1. A stallion of the Yakut breed at the experimental stable of the All-Russian Research Institute of Horse Breeding (Ryazan Province).

Genetic and population analysis was carried out with the determination of the frequency of occurrence of allelic variants of the studied genes, the frequency of occurrence of genotypes in the population, and the observed heterozygosity (H_o). Statistical processing of the obtained results was performed using Microsoft Excel 2010 software.

Results. When genotyping Yakut horses (Fig. 1) for the *MC1R* gene, we identified three genotypes: 25 horses had the *E/E* genotype homozygous for the dominant wild-type allele, 6 horses were homozygous for the recessive mutant allele (*e/e* genotype), and 14 horses were heterozygous genotype *E/e*. Most of the studied animals (39 animals) turned out to be carriers of the dominant allele *E* which determines the production of the black eumelanin pigment. The frequency of occurrence of the C>T mutation in the *MC1R* gene (allele *e*) which determines the suppression of the synthesis of the eumelanin pigment, was 0.289 in the studied group (Table 1).

A study of Yakut horses for the ASIP gene showed that the frequency of occurrence of the mutant allele a in the population was 0.600, including 18 animals (40% of the population) were its homozygous carriers. This is a relatively high figure, since the recessive allele a in the *ASIP* gene is quite rare in most modern horse breeds [10]. For example, the frequency of allele a in horses of the Vladimir breed is 0.252, while in purebred Arabian horses of the Russian population it is 0.100 [29, 30]. Consequently, the genes that determine the synthesis of eumelanin predominate in the Yakut horse population.

1. Characteristics of the population of horses (*Equus caballus* L.) of the Yakut breed according to the frequency of occurrence of alleles of the *MC1R* and *ASIP* genes that determine skin and hair pigmentation (n = 45, Republic of Sakha-Yakutia, 2014)

Gene	Allele	Frequency	Observed heterozygosity
MC1R	*E*	0.711	0.311
	e	0.289	
ASIP	*A*	0.400	0.400
	a	0.600	

In general, we identified 8 variants of genotypes in 45 Yakut horses based on the two studied genes that determine pigmentation (Table 2). Interestingly, the most typical for Yakut horses were the E/E-A/a and E/E-a/a genotypes, which were not found in a group of 80 purebred Arabian horses (30) (Fig. 2).

2. Genotypes for the *MC1R* and *ASIP* genes encoding skin and hair pigmentation in horses (*Equus caballus* L.) of the Yakut breed (n = 45, Republic of Sakha-Yakutia, 2014)

Genotypes	Число лошадей		Frequency
	Yang type (n = 34)	root/native type type (n = 11)	
E/E-A/A	4	0	0.089
E/E-A/a	11	0	0.244
E/e-A/A	1	2	0.067
E/e-A/a	6	1	0.156
E/E-a/a	6	4	0.222
E/e-a/a	2	2	0.089
e/e-A/A	2	0	0.044
e/e-a/a	2	2	0.089

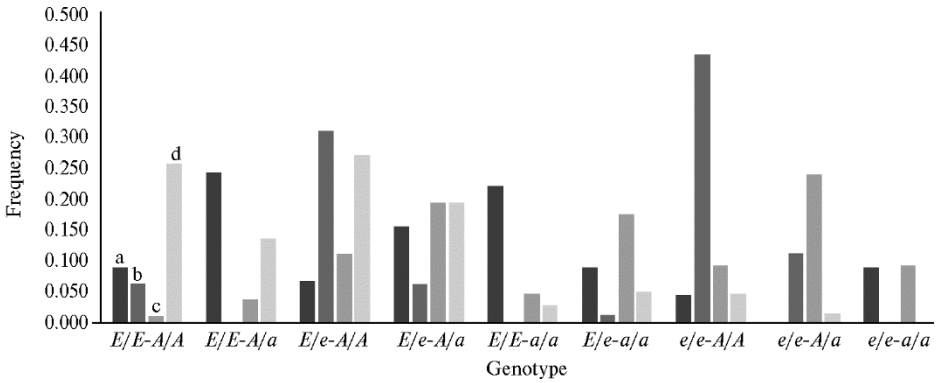

Fig. 2. Comparative characteristics of four breeds of horses (Equus caballus L.) according to the frequency of occurrence of different genotypes for the MC1R and ASIP genes: a — Yakut breed (n = 45, present study, Republic of Sakha-Yakutia, 2014); b — Arabian horses (n = 80) [30]; c — native horses from Jeju Island (Jeju) (n = 108) [31], d — Vladimir breed (n = 220) [29**].**

According to N.-Y. Kim et al. [31], genotypes *E/E-A/a* and *E/E-a/a* are rare for Korean native horses from Jeju Island [31]. In the Vladimir horses, the *E/E-A/a* genotype is not frequent (0.136), and the *E/E-a/a* genotype is rare [29]. It is likely that the predominance of allelic variants of the *MC1R* and *ASIP* genes,

which determine the synthesis of eumelanin, in Yakut horses may be of adaptive importance for the survival of animals in Subarctic conditions.

Many genes that control skin and hair pigmentation in domestic horses are known to have a pleiotropic effect [32]. L.N. Jacobs et al. [33] investigated the dependence of temperament characteristics in Tennessee pleasure horses on *MC1R* and *ASIP* genotypes and found such a correlation for the *ASIP* gene. Interestingly, according to the results obtained by the authors, Tennessee horses with the *a/a* genotype were more independent and independent.

Yakut horses are adapted to year-round herd keeping, which suggests the selection significance of certain behavioral responses. Of great interest may be a detailed study of the dependence of the neurohumoral mechanisms that regulate the behavior of herd horses on the polymorphism of genes that control skin and hair pigmentation.

When analyzing the genetic structure of the Yakut breed using SNP markers associated with the working qualities of horses, one indigenous horse was identified, a heterozygous carrier of the *DMRT3* gene mutation (g.22999655C>A) and two horses of the Yang type with a heterozygous MSTN genotype (g .66493737C>T) (Table 3). The frequency of occurrence of mutant variants of the *DMRT3* and *MSTN* genes in the studied group of Yakut horses was 0.011 and 0.022, respectively. It is obvious that, being present in the population with a low frequency of occurrence, these mutations have no breeding value, since the Yakut horse historically served people as a transport animal in the forest and swampy areas [13].

3. Genotypes of Yakut horses (*Equus caballus* L.) for the *MSTN* and *DMRT3* loci (n = 45, Republic of Sakha-Yakutia, 2014)

Gene	Genotype	Number of animals	
		root/native type	Yang type
	T/T	11	32
MSTN (g.66493737C>T)	*C/T*	0	2
	C/C	0	0
DMRT3 (g.22999655C>A)	*C/C*	10	34
	A/C	1	0
	A/A	0	0

Numerous studies have shown that mutations in the *DMRT3* and *MSTN* genes associated with key breeding characteristics of today's highly specialized commercial breeds occur in many geographically distinct populations of native horses [19, 20, 23]. According to the hypothesis of P. Librado et al. [25], genetic polymorphisms associated with the desired phenotypes of modern prize horses existed in populations of ancient animals. In the process of horse domestication and subsequent breed formation, selection for the most important breeding traits occurred not by de novo mutations, but by genetic variations present in the domesticated stock of ancient populations [25].

Thus, the study of the polymorphism of the *MC1R* and *ASIP* genes which determine skin and hair pigmentation in the native Yakut breed showed that the frequency of occurrence of the dominant allele *A* of the *ASIP* gene and the dominant allele *E* of the *MC1R* gene was 0.400 and 0.711, respectively. In the studied population, allelic variants of genes that determine the predominant synthesis of eumelanin prevailed. In the tested horses, the most common genotypes were *E/E-A/a* (24.4%) and *E/E-a/a* (22.2%) where *E* is the dominant *MC1R* wild-type allele (no C>T mutation); *A* and *a* are the dominant wild-type allele and the recessive (mutant) *ASIP* allele (with an 11 bp deletion), respectively. The frequency of the mutant allele *A* of the *DMRT3* gene (g.22999655C>A) and the mutant allele *C* of the *MSTN* gene (g.66493737C>T) was 0.011 and 0.022,

respectively. Obviously, the mutant variants of the *DMRT3* and *MSTN* genes in Yakut horses have no breeding value.

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SOME PROBLEMS OF IDENTIFICATION OF HONEYBEE SUBSPECIES AND THEIR SOLUTION ON THE EXAMPLE OF STUDYING THE *Apis mellifera* IN SIBERIA

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Abstract

Studies of the honeybee *Apis mellifera* L. are carried out by both classical morphometric and molecular methods, including whole genome sequencing. Morphometric analysis has revealed thirty subspecies of *A. mellifera* for four evolutionary lineages that corresponded to their geographical origin. mtDNA analysis, e.g., for the variability of COI-COII locus (the sequence between the cytochrome oxidase I and cytochrome oxidase II genes), has identified three major evolutionary lineages, A, M, and C. However, this method has a limitation associated with maternal inheritance of the mitochondrial genome. The honeybee does not have sex chromosomes, so information about inheritance in the paternal line (as well as in the maternal line) can only be obtained from the analysis of autosomal loci, such as SNP (single nucleotide polymorphism) and SSR (simple sequence repeats) markers. The present work, for the first time, evaluates the informativity of morphometric and molecular methods for the identification of *A. mellifera* subspecies inhabiting Siberian apiaries. We have shown that the analysis of the variability of the main parameters of the wing (cubital index, hantel index, and discoidal shift) and the mtDNA COI-COII locus accurately detect the origin of the bee colony. We also studied for the first time the genetic diversity of the *A. mellifera mellifera* Siberian populations for microsatellite loci. Diagnostic alleles specific for subspecies and ecotypes of the honeybees have been identified to differentiate the *A. m. mellifera* subspecies and its ecotypes, as well as subspecies of southern origin (Carpathian bee, Carnica). This work aimed to evaluate prospects for morphometric and molecular analysis methods in differentiation of *A. mellifera* subspecies reared in Siberia. Honeybees from 92 apiaries in 69 settlements located in five regions of Siberia (Tomsk and Kemerovo regions, Krasnoyarsk Territory, Altai Territory, and the Altai Republic) were studied. The first stage was the investigation of worker bees from 414 bee colonies using the morphometric method and analysis of the mtDNA COI-COII locus variability. The second stage was the identification of the *A. m. mellifera* colonies based on a complex of SSR markers. We examined the variability of 31 microsatellite loci. To search for unique or specific SSR markers for different honeybee subspecies, we also examined the genetic diversity of two southern subspecies, *A. m. carpathica* and *A. m. carnica* (a comparison group). Population genetic parameters (allele frequency, observed and expected heterozygosity H_o and H_e) were calculated using GenAlEx 6.5 software (https://biology-assets.anu.edu.au/GenAlEx/). Introgression of the genes of the evolutionary lineage C into the lineage M was assessed based on the microsatellite loci polymorphism data using the STRUCTURE 2.3.4 program (https://web.stanford.edu/group/pritchardlab/home.html). It is shown that three wing parameters, i.e., cubital index, hantel index, and discoidal shift, together with mtDNA polymorphism analysis data, are necessary and sufficient for differentiation of *A. mellifera* subspecies. The discoidal shift parameter is one of the first morphometric trait to deviate from the breed standard values in the honeybee hybridization. Microsatellite analysis revealed loci that differentiate both subspecies of different evolutionary lineages (M and C) and different *A. m. mellifera* ecotypes. Loci A043, Ap081, Ap049, AT139, A113, *mrjp3*, etc. can be considered as diagnostic (subspecies-specific) loci the composition and frequency of the prevailing alleles of which differ in *A. m. mellifera* subspecies (lineage M) and two subspecies of southern origin (*A. m. carpathica* and *A. m. carnica*, lineage C). In *A. m. mellifera* honeybees, alleles 128 bp at the A043 locus, 124 bp at the Ap081 locus, 127 bp at the Ap049 locus, 190 bp at the AT139 locus, 218 bp at the A113 locus, and 529 bp at the *mrjp3* occur in high frequency (0.54-0.99). In honeybees of southern origin (*A. m. carpathica* and *A. m. carnica*), these alleles are rarer (0.01-0.27). The microsatellite locus A008 is the most promising molecular

marker to differentiate *A. m. mellifera* ecotypes from Siberia, the Urals and Europe (eco-specific locus). Based on the genetic diversity of Siberian honeybees for microsatellite loci, a diagnostic panel of molecular markers has been developed to differentiate subspecies and ecotypes of honeybees belonging to the evolutionary M and C lineages (*A. m. mellifera*, *A. m. carpathica*, and *A. m. carnica*).

Keywords: honeybee, *Apis mellifera*, morphometric signs, molecular genetic methods, mtDNA, microsatellite loci, COI-COII, DNA markers, Siberia

Currently, in the study of the honey bee *Apis mellifera* L., both classical morphometric [1, 2] and molecular genetic methods [3-5], including whole genome sequencing [6], are used. Morphometry and mitochondrial DNA (mtDNA) polymorphism analysis are most commonly used to identify honey bee subspecies [1, 7, 8].

The morphometric method involves the analysis of about 40 qualitative traits (coloration of tergites, the shape of the posterior border of the wax mirror, characteristics of the hair border on the abdomen of worker bees, etc.) and quantitative (tarsal index, cubital index, area of the wax mirror, etc.) traits of honeybee [9, 10]. Based on morphometric parameters, 30 subspecies of *A. mellifera* were assigned along four evolutionary lines that corresponded to their geographical origin. These are subspecies of the African continent — the line A (African); subspecies of the western Mediterranean and northwestern Europe — the line M (Mellifera); subspecies of southeastern Europe and the eastern Mediterranean— the line C (Carnica); subspecies of the Middle East and western Asia — the line O (Oriental) [10].

The study of mtDNA polymorphism, for example, the COI-COII locus (the sequence between the genes for cytochrome oxidase I and cytochrome oxidase II), allows three main evolutionary lines, the A, M, and C to be identified [7, 11, 12]. However, mtDNA variants do not always agree with the systematics based on morphology. For example, subspecies of C and O morphological lines are characterized by the same mtDNA variant [12]. According to mtDNA analysis, the C subspecies had the shortest sequence of the COI-COII locus, or Q variant. Conversely, the M and A subspecies had a longer sequence that included at least one Q element, as well as a P element (variants PQ, PQQ, PQQQ, PQQQQ or PQQQQ) [13, 14].

However, the method has a limitation caused by the maternal inheritance of the mitochondrial genome. Since the honey bee does not have sex chromosomes, information about inheritance in the drone line (as well as in the line of the uterus) can only be obtained based on the analysis of autosomal loci, such as SNP (single nucleotide polymorphism, single nucleotide polymorphism) and SSR (simple sequence repeats, microsatellite loci) markers. Thus, microsatellite loci are widely used in honey bee studies, for example, to characterize the population genetic structure [15-17], identify hybridization between different subspecies [5, 18, 19], differentiate subspecies [20, 21] and assess the adaptive potential of bees of different origin and evolutionary lines [22-24]. However, the genetic diversity of different subspecies of the honey bee for microsatellite loci and other nuclear markers has not been studied enough, reference materials have not been developed. Based on the data of polymorphism of SNP markers in bees of 14 subspecies, groups were identified that largely correspond to morphological evolutionary lines. Additional lines of African origin have also been established (e.g., the line Y — Yemenitica, Ethiopia) [7, 25].

In Russia, due to the diversity of natural and climatic conditions, three subspecies of bees are recommended for breeding [23]: the dark forest bee, or the Central Russian breed (*Apis mellifera mellifera* L.), the Caucasian breed (*A. m. caucasica* Gorb.) and the Carpathian breed (*A. m. carpathica*, derived from *A. m. carnica* Poll. *A. m. carnica*, the Italian bee *A. m. ligustica* Spin. and the Far Eastern

bee (the product of unsystematic crossing of Ukrainian steppe *A. m. sossimai* Engel and Central Russian bees with the participation of Caucasian and Italian bees) are also bred. In Siberia, the Central Russian bee (*A. m. mellifera*) brought 230 years ago to the Tomsk province was initially reared. The Central Russian bees have adapted well to the local harsh climatic conditions and plants but the wintering of bee colonies is under human' control. Since the end of the 20th century, active importation of bees of southern origin has been observed in Siberia, which led to mass hybridization [26].

This research, for the first time, assesses the evaluability of morphometric and molecular genetic methods for the identification of subspecies of *A. mellifera* inhabiting the apiaries of Siberia. It was shown that the analysis of the variability of the main parameters of the wing (cubital index, dumbbell index, discoidal displacement) and the mtDNA COI-COII locus provides identification of the bee colony origin. This is the first study of the genetic diversity of the Central Russian bees from Siberian populations using microsatellite loci. Breed- and eco-specific diagnostic alleles have been identified, which make it possible to differentiate the Central Russian breed and its ecotypes, as well as subspecies of southern origin (Carpathian breed, carnica).

The purpose of this work is to evaluate the information content of morphometric and molecular genetic methods for the identification of subspecies of *Apis mellifera* in Siberia.

Materials and methods. In the research carried out in 2008-2018, honey bees were obtained from 92 apiaries in 69 settlements located in five regions of Siberia, including Tomsk region (52 points/71 apiaries/340 bee colonies), Kemerovo region (4/5/16), Krasnoyarsk region (6/6/25), Altai Territory (6/9/31) and the Altai Republic (1/1/2). To study the breed composition of honey bees in Siberian apiaries, worker bees from 414 bee colonies were initially studied using the morphometric method and mtDNA analysis.

At the second stage, the identified bee colonies of the Central Russian breed (*A. m. mellifera*) were studied using a complex of SSR markers. The variability of 31 microsatellite loci was studied. In order to search for unique or specific SSR markers for different subspecies of the honey bee, the genetic diversity of bees of two subspecies, *A. m. carpathica* and *A. m. carnica* of southern origin, was investigated (a comparison group).

For morphometric analysis, we studied the main breed-determining parameters of the wing (discoidal displacement, cubital and dumbbell indices) and the color of tergites in 25-30 workers from each family [27]. The obtained morphometric data was compared to the breed standards [28].

DNA was isolated from the pectoral muscles of worker bees using the DNA-Extran-2 kit (ZAO Sintol, Russia) and analyzed by polymerase chain reaction (PCR) using the BioMaster HS-Taq PCR-Color reagent kit (OOO Biolabmix, Russia) on a BioRad T100 cycler (Bio-Rad Laboratories, Inc., USA).

Analysis of mtDNA COI-COII locus variability was performed using the primers F-5'-CACATTTAGAAATTCSSATTA-3', R-5'-ATAAATATGAATCATGTGGA-3' [29] and PCR protocol as follows: 5 min at 95 °C (initial denaturation); 1 min at 95 °C (denaturation), 2 min at 57 °C (primer annealing), 2 min at 72 °C (chain elongation) (35 cycles); 7 min at 72 °C (final elongation). PCR products were separated in 1.5% agarose gel and analyzed using Image LabTM Software (Bio-Rad Laboratories, Inc., USA). In the Central Russian honey bees, the COI-COII locus contains the PQQ and PQQQ alleles, having sizes of 600 and 800 bp, respectively, while the breeds of southern origin (Carpathian breed, carnica, etc.) had the Q allele 350 bp in size. Five workers from each bee colony were

tested.

For SSR analysis, the previously described primer sequences and amplification conditions were used [30-32]. The variability of microsatellite loci A008, Ap049, A043, AC117, Ap243, H110, A024, A113, SV185, Ap066, Ap081, A088, A007, A028, 6339, SV220, K0457B, K1168, Ap033, K0820, Ap06, K1615, K0711, SV167, Ap249, Ap226, A056, AT139 and *mrjp3* was studied. One primer from each pair had a fluorescent label. Genotyping was performed on an ABI Prism 3730 genetic analyzer (Applied Biosystems, Inc., USA; Medical Genomics Center for Collective Use, the Research Institute of Medical Genetics of the Tomsk Scientific Research Center RAS). The GeneScan500-ROX DNA length standard was used under the conditions recommended by the manufacturer. Fragment size was analyzed using GeneMapper Software (Applied Biosystems, Inc., USA). In each sample of bees, a minimum of 27 and a maximum of 534 workers were studied for definite microsatellite loci. Microsatellite analysis was not performed for loci A028, A088, A056, K0711, Ap068, K1615, and Ap249 in bees from the Altai population and for loci Ap033 and SV167 in bees from the Tomsk population.

Population genetic parameters (allele frequency, observed and expected heterozygosity, H_o and H_e) were calculated using GenAlEx 6.5 (https://biology-assets.anu.edu.au/GenAlEx/) [33]. Statistically significant differences between observed and expected heterozygosity were identified using Student's *t*-test. The degree of introgression of genes of the evolutionary line C (Carpathian breed *A. m. carpathica* and Carnica *A. m. carnica*) in the nuclear genome of Central Russian bees (evolutionary line M) from different populations of Siberia was assessed according to the polymorphism of microsatellite loci using STRUCTURE 2.3.4 (https://web.stanford.edu/group/pritchardlab/home.html) based on Bayesian analysis. The Monte Carlo Markov Chain (MCMC) clusterisation was used for a given number of clusters K = 2, K = 3 and K = 5 using the admixture model and 500,000 MCMC iterations [34]. Preliminary data processing was carried out in Microsoft Excel 2010; statistical calculations were performed in StatSoft STATISTICA 8.0 for Windows (StatSoft, Inc., USA). The tables show mean values (*M*) and standard errors of means (±SEM).

Results. According to the data obtained in the study of morphometric parameters and the mitochondrial genome of honey bees, the majority of bee colonies living in Siberia were represented by hybrids of Central Russian and Carpathian breeds (Fig. 1). Moreover, more than 60% of families on the maternal side descended from the Central Russian bee, according to mtDNA analysis data [35].

A pronounced heterogeneous pattern was observed in regions with well-developed beekeeping (Kemerovo region, southern regions of the Tomsk region, Altai Territory), where bees of southern origin (mainly Carpathian breed, carnica) and hybrids are actively imported. For example, in the Kemerovo region, in most apiaries, hybrids of the Central Russian bee and breeds of southern origin were found, but there were also commercial apiaries where breeds of southern origin were reared (*A. m. carpathica*, *A. m. carnica*). We did not find apiaries where purebred Central Russian bees were bred.

In more remote regions of Siberia (the northern regions of the Tomsk region, the Krasnoyarsk Territory, the Republic of Altai), territories were found with a homogeneous array of bees descending from the Central Russian bee *A. m. mellifera*. Of considerable interest were the populations of the Central Russian breed in the Krasnoyarsk Territory (Yenisei population), in the north of the Tomsk region (Ob population) and in the mountainous regions of the

Altai Republic (Altai population), as well as some commercial apiaries in the Altai Territory (Zmeinogorsky and Charyshsky districts) where *A. m. mellifera* was reared (see Fig. 1).

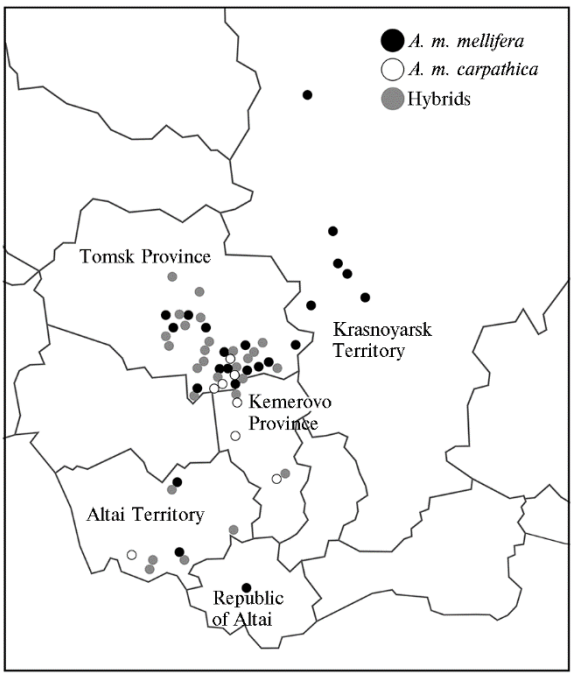

Fig. 1. Distribution map of subspecies *Apis mellifera mellifera* and *A. m. carpathica*, as well as hybrids between these subspecies in the apiaries of the Siberian region of the Russian Federation according to the morphometric study and mtDNA analysis (2008-2018).

Table 1 shows the main parameters of the wing and the variant of the mtDNA COI-COII locus in bees from 34 purebred and hybrid families from Siberia. For some families of the Central Russian breed from isolated apiaries (e.g., family No.3 from the apiary in the village of Ostyatskoe and family No. 1 from the apiary in the vicinity of the village of Turukhansk, Krasnoyarsk Territory; family No. 1 from the apiary in the vicinity of the village of Ongudai of the Altai Republic), a deviation is shown from the breed standard for the cubital index. This may indicate in favor of genetic drift as a result of long-term isolation of families. At the same time, the deviation of some morphometric parameters from the breed standard, for example, in colonies from the apiary of the village of Zarechny (the presence in the family of 27% of individuals with a neutral discoidal displacement), could indicate the influence of subspecies of southern origin (see Table 1). The apiary in the village of Zarechny is located in the southern region of the Tomsk region, where bees of various origins are bred in closely spaced apiaries.

Thus, Siberian honey bee populations are characterized by a complex and mosaic genetic structure. We have identified both purebred bee colonies of various origins and hybrids. Bee colonies *A. m. mellifera* can be considered as a unique material for the conservation and restoration of the Central Russian bee gene pool in Russia.

The genetic diversity of honey bees of the Central Russian breed was studied using 31 microsatellite loci. As a comparison group, we used honey bees from the families of the southern subspecies *A. m. carpathica* and *A. m. carnica*. Among the studied loci, 22 polymorphic SSR markers were selected, which are the most informative for the differentiation of subspecies and ecotypes of the honey bee (Table 2). For the remaining 9 microsatellite loci, a similar composition and frequency of alleles were noted, including those prevailing in bees of different origin (in Table 2, AC117, SV185, and H110 are given as an example of such loci).

The comparative analysis of the variability of microsatellite loci in Central Russian, Carpathian and Carnica bees, differences in the composition and/or frequency of alleles between subspecies were found for some SSR markers.

1. Morphometric parameters and variant of the mtDNA COI-COII locus in bees (*Apis mellifera* L.) of some purebred and hybrid families living in Siberia (2008-2018)

Locality	Family No.	mtDNA COI-COII locus	Cubital index		Dumb-bell index		Discoidal shift, %		
			min-max	*M*±SEM	min-max	*M*±SEM	–	0	+
Central Russian bees (Tomsk population)									
Settlement Chalkovo	1	PQQ	1.30-2.00	1.75±0.04	0.720-0.871	0.811±0.010	100.0	0.0	0.0
	2	PQQ	1.32-2.00	1.57±0.04	0.712-0.851	0.789±0.010	100.0	0.0	0.0
Village Teguldet	1	PQQQ	1.44-2.10	1.75±0.03	0.692-1.000	0.854±0.011	100.0	0.0	0.0
	2	PQQQ	1.26-2.22	1.74±0.04	0.701-0.914	0.825±0.010	100.0	0.0	0.0
Village Mogochino	1	PQQ	1.36-2.00	1.73±0.02	0.693-0.923	0.821±0.006	100.0	0.0	0.0
	2	PQQ	1.57-2.82	1.88±0.04	0.770-1.000	0.855±0.015	92.0	8.0	0.0
Settlement Zarechny	1	PQQQ	1.39-2.33	1.65±0.04	0.712-0.932	0.825±0.009	73.5	26.5	0.0
	2	PQQQ	1.19-2.05	1.64±0.06	0.679-1.000	0.865±0.012	73.1	26.9	0.0
Central Russian bees (Keasnoyarsk population)									
Village Ostyatskoe	1	PQQ	1.24-2.00	1.61±0.04	0.675-0.892	0.795±0.011	100.0	0.0	0.0
	2	PQQ	1.25-1.76	1.60±0.04	0.722-0.849	0.812±0.011	100.0	0.0	0.0
	3	PQQ	1.26-1.79	1.51±0.04	0.743-0.910	0.849±0.012	97.0	3.0	0.0
Village Kolmogorovo	1	PQQ	1.32-2.10	1.60±0.05	0.724-0.900	0.820±0.009	97.0	3.0	0.0
	2	PQQ	1.28-1.76	1.58±0.05	0.716-0.919	0.820±0.012	93.0	7.0	0.0
	3	PQQ	1.28-1.86	1.56±0.04	0.746-0.985	0.810±0.011	97.0	3.0	0.0
Village Ozernoe	1	PQQ	1.02-2.00	1.62±0.04	0.746-1.000	0.845±0.011	100.0	0.0	0.0
	2	PQQ	1.45-1.95	1.65±0.04	0.768-1.000	0.867±0.010	93.3	6.7	0.0
	3	PQQ	1.35-2.05	1.65±0.04	0.716-0.951	0.806±0.010	100.0	0.0	0.0
Settlement Yaksha	1	PQQ	1.43-1.82	1.65±0.04	0.803-0.915	0.859±0.011	100.0	0.0	0.0
Village Yartsevo	1	PQQ	1.31-1.85	1.59±0.02	0.711-0.846	0.775±0.008	100.0	0.0	0.0
Village Турухансk	1	PQQ	1.15-1.89	1.52±0.04	0.779-0.919	0.849±0.011	100.0	0.0	0.0
Central Russian bees (Altai population)									
Village Baranovka	1	PQQ	1.42-2.10	1.75±0.04	0.732-0.883	0.833±0.012	100.0	0.0	0.0
The vicinity of the village Ongudai	1	PQQ	1.00-1.67	1.44±0.04	0.740-0.919	0.840±0.012	100.0	0.0	0.0

Continued Table 1

Carpathian bee breed									
Siveria, various regions	1	Q	2.20-3.40	2.80±0.10	0.919-1.208	0.972±0.009	0.0	23.3	76.7
	2	Q	2.00-3.50	2.93±0.07	0.967-1.230	1.118±0.015	0.0	0.0	100.0
	3	Q	2.11-4.10	3.12±0.10	0.905-1.205	1.048±0.015	3.4	10.4	86.2
	4	Q	1.68-3.64	2.51±0.06	0.867-1.210	1.050±0.010	4.0	20.0	76.0
	5	Q	2.00-5.56	3.41±0.02	0.905-1.310	1.125±0.016	0.0	3.0	97.0
Hybrid faminies (apiaries from Tomsk Province)									
Settlement Kurlek	1	PQQQ	1.74-3.29	2.14±0.07	0.857-1.053	0.937±0.100	32.14	57.14	10.72
	2	Q	1.30-2.29	1.66±0.04	0.735-0.965	0.878±0.011	72.40	27.60	0.00
Village Krivosheinj	1	PQQ	1.00-2.67	1.79±0.07	0.707-1.000	0.884±0.018	27.27	18.18	54.55
Settlement Sinii Utes	1	Q	1.83-2.87	2.37±0.06	0.815-1.053	0.931±0.012	6.70	76.70	16.60
Village Podgornoe	1	Q	1.35-2.87	2.19±0.10	0.797-1.100	0.910±0.010	34.30	45.70	20.00
	2	Q	1.72-3.01	2.53±0.06	0.852-1.178	0.977±0.011	51.40	14.30	34.30
The vicinity of Tomsk	1	Q	1.64-2.69	2.12±0.05	0.722-0.946	0.895±0.011	90.00	6.70	3.30
Breed standards									
A. m. mellifera [a]	PQQ/PQQQ		1.30-2.10	1.70	0.600-0.923	–	–	–	–
A. m. mellifera [b]			1.30-1.90	1.5-1.7	0.600-0.923	–	91-100	5-10	0
A. m. carpathica [a]	Q		2.30-3.00	2.65	–	0.925	0-5	0-20	80-100

Note. [a] — the European standard for bee subspecies, developed on the basis of the values of the cubital and dumbbell indices [28], [6] — the breed standard of bees adopted in Russia. At least 25 individuals from the family were studied morphometrically; mtDNA analysis (variability of the COI-COII locus) was carried out for 5 individuals from the bee colony. Dashes indicate no data.

2. Parameters of genetic diversity of 25 microsatellite loci in honey bees (*Apis mellifera* L.) of different origin living in Siberia (2008-2018)

Parameter	Subspecies of *A. mellifera*				
	mellifera			*carpathica*	*carnica*
	evolutionary line M (PQQ, PQQQ of mtDNA locus COI-COII)			evolutionary line C (Q of mtDNA locus COI-COII)	
	Tomsk Province	Krasnoyars Territory	Altai	femilied introduced to Siveria from bee nurseries	
Locus Ap066					
The numer					
of bees	206	323	36	196	97
of alleles	6	7	5	6	3
Prevailing allele frequency:					
90 bp	0.325±0.023[a]	0.130±0.013	0.306±0.054[a]	0.933±0.018[a]	0.921±0.014a
96 bp	0.187±0.019	0.370±0.019[a]	0.417±0.058[a]	0.046±0.015	0.041±0.010
98 bp	0.347±0.024[a]	0.252±0.017	0.222±0.049	0	0
Ho	0.782±0.029	0.625±0.027***	0.694±0.077	0.148±0.025	0.113±0.032
He	0.726±0.010	0.728±0.007	0.682±0.026	0.150±0.024	0.127±0.032
Locus A024					
The numer					
of bees	307	534	34	183	106
of alleles	6	8	4	6	9
Prevailing allele frequency::					
92 bp	0.660±0.019[a]	0.473±0.015[a]	0.779±0.050[a]	0	0
96 bp	0.007±0.003	0.156±0.011	0	0	0
98 bp	0.011±0.004	0.028±0.005	0.029±0.021	0.219±0.022	0.099±0.021
100 bp	0.186±0.016	0.126±0.010	0.162±0.045	0.301±0.024[a]	0.448±0.034[a]
102 bp	0.007±0.003	0.029±0.005	0	0.156±0.019	0.297±0.031
104 bp	0	0.059±0.007	0	0.169±0.020	0
106 bp	0.130±0.014	0.085±0.009	0	0.101±0.016	0.057±0.016
Ho	0.505±0.029	0.468±0.022***	0.265±0.076	0.563±0.037***	0.566±0.048*
He	0.513±0.020	0.722±0.012	0.365±0.065	0.798±0.009	0.693±0.021
Locus Ap081					
The numer					
of bees	150	372	33	193	137
of alleles	5	4	3	6	4
Prevailing allele frequency:					
124 bp	0.820±0.022[a]	0.973±0.006[a]	0.955±0.026[a]	0.204±0.024	0.042±0.010
130 bp	0.037±0.011	0.009±0.004	0.030±0.021	0.762±0.022[a]	0
132 bp	0	0	0	0.101±0.015	0.708±0.028[a]
Ho	0.120±0.027***	0.032±0.009	0.091±0.050	0.337±0.034	0.489±0.043
He	0.318±0.034	0.053±0.011	0.088±0.047	0.404±0.030	0.452±0.031
Locus A043					
The numer					
of bees	305	418	33	177	106
of alleles	4	4	3	4	3
Prevailing allele frequency:					
128 bp	0.831±0.015[a]	0.974±0.006[a]	0.864±0.042[a]	0.071±0.014	0.222±0.029
132 bp	0.012±0.004	0.006±0.003	0.121±0.040	0.020±0.007	0
138 bp	0	0	0	0.105±0.016	0.009±0.007
140 bp	0.156±0.015	0.014±0.004	0.015±0.015	0.805±0.021[a]	0.769±0.029[a]
Ho	0.279±0.026	0.022±0.007*	0.030±0.030**	0.266±0.033	0.443±0.048
He	0.285±0.021	0.052±0.011	0.239±0.063	0.336±0.030	0.360±0.032
Locus A007					
The numer					
of bees	206	381	36	193	114
of alleles	3	6	3	9	8
Prevailing allele frequency:					
104 bp	0.063±0.012	0.154±0.013	0.444±0.059[a]	0.005±0.004	0.081±0.019
108 bp	0.820±0.019[a]	0.810±0.014[a]	0.542±0.059[a]	0.357±0.025[a]	0.257±0.030
112 bp	0.117±0.016	0.013±0.004	0	0.378±0.025[a]	0.114±0.022
114 bp	0	0.008±0.003	0.014±0.014	0.114±0.016	0.252±0.030
131 bp	0	0	0	0.082±0.014	0.200±0.028
Ho	0.199±0.028**	0.297±0.023	0.806±0.066	0.609±0.036*	0.495±0.049***
He	0.309±0.027	0.320±0.020	0.509±0.020	0.708±0.013	0.808±0.011
Locus A008					
The numer					
of bees	306	451	34	193	135
of alleles	6	6	3	9	7
Prevailing allele frequency:					
162 bp	0.868±0.014[a]	0.938±0.008[a]	0.868±0.041[a]	0.106±0.016	0.315±0.028[a]
170 bp	0	0	0.118±0.039	0.065±0.013	0.067±0.015

Continued Table 2

174 bp	0.023±0.006	0.009±0.003	0.015±0.015	0.443±0.025[a]	0.415±0.030[a]
178 bp	0	0	0	0.153±0.018	0.096±0.018
H_o	0.226±0.024	0.093±0.014	0.265±0.076	0.580±0.036***	0.607±0.042*
H_e	0.243±0.023	0.119±0.015	0.233±0.062	0.751±0.018	0.710±0.017
		Locus AC117			
The number					
of bees	301	497	34	185	137
of alleles	4	4	3	4	4
Prevailing allele frequency:					
177 bp	0.098±0.012	0.139±0.011	0.029±0.021	0.014±0.006	0.157±0.022
181 bp	0.292±0.019	0.163±0.012	0.044±0.025	0.203±0.021	0.190±0.024
185 bp	0.517±0.020[a]	0.687±0.015[a]	0.927±0.032[a]	0.778±0.022[a]	0.650±0.029[a]
H_o	0.389±0.028***	0.302±0.021***	0.147±0.061	0.297±0.034	0.219±0.035***
H_e	0.629±0.014	0.482±0.016	0.139±0.055	0.353±0.026	0.517±0.028
		Locus AT139			
The number					
of bees	38	162	34	129	135
of alleles	3	3	2	7	5
Prevailing allele frequency:					
177 bp	0.211±0.047	0.080±0.015	0.015±0.015	0.124±0.021	0.656±0.029[a]
179 bp	0	0	0	0.349±0.030[a]	0
182 bp	0	0	0	0.349±0.030[a]	0.230±0.026
186 bp	0.118±0.037	0	0	0.012±0.007	0
190 bp	0.671±0.054[a]	0.880±0.018[a]	0.985±0.015[a]	0.008±0.006	0.022±0.009
H_o	0.500±0.081	0.154±0.028	0.029±0.029	0.674±0.041	0.430±0.043
H_e	0.491±0.053	0.218±0.029	0.029±0.028	0.718±0.013	0.510±0.028
		Locus Ap243			
The number					
of bees	212	316	32	198	107
of alleles	8	8	8	4	7
Prevailing allele frequency:					
252 bp	0.014±0.006	0.137±0.014	0.141±0.044	0.970±0.009[a]	0.869±0.023[a]
255 bp	0.427±0.024[a]	0.242±0.017	0.031±0.022	0	0.037±0.013
257 bp	0	0.008±0.004	0.563±0.062[a]	0	0
263 bp	0.330±0.023[a]	0.390±0.020[a]	0.125±0.041	0.005±0.004	0.033±0.012
H_o	0.439±0.034***	0.468±0.028***	0.500±0.088	0.020±0.010*	0.140±0.034
H_e	0.694±0.014	0.753±0.010	0.642±0.058	0.059±0.016	0.241±0.039
		Locus SV185			
The number					
of bees	243	278	36	57	30
of alleles	5	5	3	4	5
Prevailing allele frequency:					
263 bp	0.288±0.021	0.158±0.016	0.417±0.058[a]	0.149±0.033	0.100±0.039
266 bp	0.117±0.015	0.385±0.021[a]	0.097±0.035	0.149±0.033	0.300±0.059[a]
269 bp	0.578±0.022[a]	0.421±0.021[a]	0.486±0.059[a]	0.386±0.046[a]	0.233±0.055
272 bp	0.010±0.005	0.002±0.002	0	0.316±0.044[a]	0.350±0.062[a]
H_o	0.527±0.032	0.550±0.030**	0.583±0.082	0.439±0.066***	0.867±0.062*
H_e	0.569±0.017	0.649±0.009	0.581±0.027	0.707±0.019	0.723±0.023
		Locus Ap049			
The number					
of bees	309	442	36	183	85
of alleles	5	7	4	9	5
Prevailing allele frequency:					
120 bp	0.123±0.013	0.002±0.002	0	0.227±0.022	0.194±0.030
127 bp	0.673±0.019[a]	0.761±0.014[a]	0.542±0.059[a]	0.030±0.009	0.035±0.014
130 bp	0.175±0.015	0.154±0.012	0.250±0.051	0.160±0.019	0.235±0.033
139 bp	0.023±0.006	0.042±0.007	0	0.511±0.026[a]	0.453±0.038[a]
152 bp	0	0.008±0.003	0.181±0.045	0.055±0.012	0.082±0.021
H_o	0.447±0.028	0.378±0.023	0.389±0.081*	0.564±0.037*	0.506±0.054**
H_e	0.501±0.020	0.394±0.019	0.611±0.040	0.658±0.019	0.694±0.022
		Locus A113			
The number					
of bees	290	509	33	194	136
of alleles	11	4	2	9	6
Prevailing allele frequency:					
212 bp	0.107±0.013	0.048±0.007	0	0.874±0.017[a]	0.599±0.030[a]
218 bp	0.571±0.021[a]	0.818±0.012[a]	0.879±0.040[a]	0.034±0.009	0.272±0.027
220 bp	0.255±0.018	0.129±0.011	0.121±0.040	0.039±0.010	0.015±0.007
H_o	0.521±0.029*	0.236±0.019**	0.000±0.000***	0.180±0.027	0.456±0.043*
H_e	0.597±0.017	0.312±0.017	0.213±0.060	0.233±0.029	0.562±0.025

Continued Table 2

Locus H110					
The number					
of bees	282	439	36	195	77
of alleles	3	5	5	5	6
Prevailing allele frequency:					
154 bp	0	0	0	0.005±0.004	0.110±0.025
158 bp	0	0.040±0.007	0.014±0.014	0.126±0.017	0.013±0.009
162 bp	0.789±0.017[a]	0.452±0.017[a]	0.111±0.037	0.385±0.025[a]	0.097±0.024
166 bp	0.027±0.007	0.126±0.011	0.722±0.053[a]	0.413±0.025[a]	0.630±0.039[a]
170 bp	0.184±0.016	0.342±0.016[a]	0.028±0.019	0.072±0.013	0.143±0.028
H_o	0.333±0.028	0.421±0.024***	0.333±0.079	0.631±0.035	0.584±0.056
H_e	0.343±0.022	0.660±0.009	0.450±0.065	0.661±0.012	0.561±0.040
Locus A028					
The number					
of bees	126	343	0	170	109
of alleles	3	4	No data	5	2
Prevailing allele frequency:					
126 bp	0.770±0.027[a]	0.848±0.014[a]		0.071±0.014	0.243±0.029
132 bp	0.167±0.024	0.054±0.009		0.829±0.020[a]	0.757±0.029[a]
H_o	0.397±0.044	0.274±0.024		0.341±0.036	0.431±0.047
H_e	0.376±0.034	0.268±0.021		0.301±0.031	0.368±0.030
Locus Ap226					
The number					
of bees	120	345	55	167	100
of alleles	5	3	2	5	5
Prevailing allele frequency:					
227 bp	0.033±0.012	0.197±0.015	0.200±0.127	0.087±0.015	0.065±0.017
233 bp	0.871±0.022[a]	0.801±0.015[a]	0.800±0.127[a]	0.051±0.012	0.260±0.031
235 bp	0.004±0.004	0	0	0.210±0.022	0.090±0.020
237 bp	0.017±0.008	0	0	0.608±0.027[a]	0.400±0.035[a]
247 bp	0	0	0	0.045±0.011	0.185±0.028
H_o	0.225±0.038	0.035±0.010***	0.000±0.000*	0.473±0.039	0.620±0.049
H_e	0.235±0.035	0.319±0.018	0.320±0.143	0.575±0.025	0.726±0.017
Locus A088					
The number					
of bees	82	236	0	82	77
of alleles	2	2	No data	2	3
Prevailing allele frequency:					
141 bp	0.927±0.020[a]	0.998±0.002[a]		0.098±0.023	0.234±0.034
150 bp	0	0		0.902±0.023[a]	0.753±0.035[a]
H_o	0.122±0.036	0.004±0.004		0.024±0.017*	0.260±0.050
H_e	0.136±0.035	0.004±0.004		0.176±0.037	0.378±0.037
Locus A056					
The number					
of bees	39	41	0	146	110
of alleles	3	2	No data	5	4
Prevailing allele frequency:					
280 bp	0.026±0.018	0		0.188±0.023	0.336±0.032[a]
282 bp	0.667±0.053[a]	0.720±0.050[a]		0.089±0.017	0.127±0.023
284 bp	0.256±0.049	0.281±0.050		0.630±0.028[a]	0.473±0.034a
H_o	0.013±0.013	0.463±0.078		0.473±0.041	0.536±0.048*
H_e	0.039±0.022	0.404±0.044		0.555±0.029	0.643±0.019
Locus K0711					
The number					
of bees	82	170	0	127	53
of alleles	3	3	No data	5	3
Prevailing allele frequency:					
212 bp	0.976±0.012[a]	0.918±0.015[a]		0.020±0.009	0
219 bp	0.018±0.011	0.071±0.014		0.815±0.024[a]	0.868±0.033[a]
222 bp	0	0		0.130±0.021	0.113±0.031
H_o	0.049±0.024	0.094±0.022		0.299±0.041	0.264±0.061
H_e	0.048±0.023	0.153±0.025		0.318±0.035	0.234±0.050
Locus Ap068					
The number					
of bees	125	242	0	157	55
of alleles	10	5	No data	7	9
Prevailing allele frequency:					
146 bp	0.020±0.009	0.074±0.012		0.255±0.025	0.118±0.031
150 bp	0.008±0.006	0		0.242±0.024	0.073±0.025
152 bp	0.092±0.018	0		0.303±0.026[a]	0.209±0.039
154 bp	0.036±0.012	0.008±0.004		0.045±0.012	0.227±0.040
158 bp	0.392±0.031[a]	0.552±0.023[a]		0.061±0.014	0.173±0.036
162 bp	0.212±0.026	0.337±0.022[a]		0.083±0.016	0.073±0.025

Continued Table 2

164 bp	0.128±0.021	0		0	0
H_o	0.816±0.035	0.488±0.032*		0.605±0.039***	0.782±0.056
H_e	0.769±0.018	0.576±0.015		0.772±0.010	0.841±0.013
		Locus Ap033			
The number					
of bees	0	274	35	90	84
of alleles	Нет данных	6	6	11	9
Prevailing allele frequency:					
221 bp		0.113±0.014	0.300±0.055[a]	0.006±0.006	0
225 bp		0.007±0.004	0.286±0.054	0.017±0.010	0.131±0.026
227 bp		0	0	0.233±0.032	0.262±0.034
229 bp		0	0	0	0.113±0.024
231 bp		0	0	0.356±0.036[a]	0.107±0.024
233 bp		0.016±0.005	0.100±0.036	0.200±0.030	0.030±0.013
235 bp		0.312±0.020[a]	0.229±0.050	0.061±0.018	0.262±0.034
239 bp		0.146±0.015	0.043±0.024	0	0
241 bp		0.405±0.021[a]	0.043±0.024	0	0
H_o		0.631±0.029*	0.857±0.059	0.489±0.053***	0.607±0.053***
H_e		0.704±0.010	0.762±0.021	0.770±0.017	0.816±0.014
		Locus SV167			
The number					
of bees	0	251	36	80	82
of alleles	Нет данных	4	3	8	8
Prevailing allele frequency:					
198 bp		0.492±0.022[a]	0.458±0.059[a]	0	0
201 bp		0.400±0.022[a]	0.444±0.059[a]	0.475±0.040[a]	0.482±0.039[a]
204 bp		0	0	0	0.177±0.030
207 bp		0.104±0.014	0.097±0.035	0.188±0.031	0.037±0.015
H_o		0.558±0.031	0.444±0.083	0.475±0.056***	0.512±0.055**
H_e		0.587±0.011	0.583±0.026	0.716±0.030	0.711±0.030
		Locus SV220			
The number					
of bees	81	362	36	82	81
of alleles	7	7	5	7	6
Prevailing allele frequency:					
176 bp	0.062±0.019	0.003±0.002	0	0.117±0.025	0.277±0.037
179 bp	0.031±0.014	0	0.028±0.019	0.599±0.039[a]	0.453±0.041[a]
182 bp	0	0.354±0.018[a]	0.097±0.035	0.031±0.014	0.014±0.010
185 bp	0.617±0.038[a]	0.403±0.018[a]	0.681±0.055[a]	0.037±0.015	0.047±0.017
188 bp	0.173±0.030	0.079±0.010	0.167±0.044	0.043±0.016	0.101±0.025
191 bp	0.090±0.023	0.006±0.003	0	0.173±0.030	0.095±0.024
H_o	0.432±0.055*	0.503±0.026***	0.528±0.083	0.500±0.058**	0.519±0.056
H_e	0.574±0.039	0.693±0.010	0.498±0.061	0.697±0.026	0.594±0.037
		Locus K1615			
The number					
of bees	42	282	0	49	40
of alleles	5	4	No data	5	5
Prevailing allele frequency:					
208 bp	0.512±0.055[a]	0.910±0.012[a]		0.010±0.010	0.025±0.018
210 bp	0	0		0	0.175±0.043
212 bp	0	0		0.796±0.041[a]	0.700±0.051[a]
214 bp	0.429±0.054[a]	0.016±0.005		0.102±0.031	0
H_o	0.667±0.073	0.181±0.023		0.367±0.069	0.250±0.069*
H_e	0.553±0.026	0.170±0.021		0.351±0.058	0.473±0.059
		Locus Ap249			
The number					
of bees	114	247	0	159	85
of alleles	6	4	No data	9	8
Prevailing allele frequency:					
207 bp	0	0.020±0.006		0.110±0.018	0.038±0.015
213 bp	0.013±0.008	0.010±0.005		0.211±0.023	0.294±0.036
215 bp	0.013±0.008	0		0.289±0.025	0.019±0.011
219 bp	0.254±0.029	0.004±0.003		0.233±0.024	0.469±0.040[a]
221 bp	0.544±0.033[a]	0.966±0.008[a]		0.098±0.017	0.069±0.020
H_o	0.588±0.046	0.061±0.015		0.679±0.037**	0.663±0.053
H_e	0.624±0.026	0.067±0.016		0.795±0.009	0.682±0.027
		Locus *mrjp3*			
The number					
of bees	89	244	27	145	129
of alleles	4	5	3	9	8
Prevailing allele frequency:					
391 bp	0.034±0.014	0.041±0.009	0.370±0.066[a]	0.110±0.018	0.043±0.013
406 bp	0	0	0	0.486±0.029a	0.399±0.031[a]

Continued Table 2

464 bp	0.084±0.021	0.027±0.007	0	0.097±0.017	0.194±0.025
518 bp	0	0	0	0.197±0.023	0.194±0.025
529 bp	0.832±0.028[a]	0.812±0.018[a]	0.574±0.067[a]	0.041±0.018	0.019±0.009
H_o	0.067±0.027***	0.283±0.029	0.333±0.091	0.621±0.040	0.550±0.044***
H_e	0.298±0.043	0.329±0.024	0.530±0.039	0.700±0.022	0.753±0.017

N o t e. The subspecies of the honey bee were established according to the data of morphometric study and mtDNA analysis. H_o — observed heterozygosity, H_e — expected heterozygosity; [a] — predominant alleles the frequency of which is more than 30%.

*, **, *** Differences between observed and expected heterozygosity are statistically significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Thus, for loci Ap081, A008, A043, A139, A113, Ap243, Ap049, A024, A088, Ap226, K0711, SV220, K1615, and *mrjp3*, the predominant alleles (the frequency of occurance more than 0.40) were identified, the composition of which differed in bees of the Central Russian breed (line M) and southern subspecies (line C). For the A043 locus, in *A. m. mellifera* (line M), a 128 bp allele prevailed with the frequency over 0.83, while in *A. m. carpathica* and *A. m. carnica* (line C), 140 bp allele predominated with the frequency more than 0.76. For the A113 locus, the predominant allele in *A. m. carpathica* and *A. m. carnica* had a size of 212 bp, in A. m. mellifera — 218 bp (the frequency more than 0.57). For a number of loci (e.g., Ap066 and A007), common predominant alleles were identified, but with different frequencies of occurrence in the subspecies of lines M and C (see Table 2).

The characterization of genetic diversity based on heterozygosity indicators revealed similar results for most of the studied loci in different bee subspecies: lower values of observed heterozygosity (H_o) compared to expected heterozygosity (H_e) were shown. For some SSR markers, e.g., the Ap066 locus in Central Russian bees and the A043 and SV185 loci in Carnica bees, the H_o value was higher than H_e. Among the groups of Central Russian bees, this was most typical for the Altai population which may be due to the small number of individuals in the sample.

We found statistically significant differences between H_o and H_e values for different samples for most loci, except for AT139, A028, and K0711. Thus, statistically significant differences between H_o and H_e were characteristic: in Central Russian bees (Yenisei population) for loci Ap066, A024, AC117, Ap243, H110, Ap226 and SV220 ($t \geq 3.69$, $p < 0.001$), SV185 and A113 ($t \geq 2.98$, $p < 0.01$), A043, Ap033 and Ap068 ($t \geq 2.30$, $p < 0.05$); in the Tomsk population for Ap081, AC117, Ap243, *mrjp3* ($t \geq 4.55$, $p < 0.001$), A007 ($t = 2.83$, $p < 0.01$), A113 and SV220 ($t \geq 2.26$, $p < 0.05$); in the Altai population for A113 ($t = 3.55$, $p < 0.001$), A043 ($t = 3.00$, $p < 0.01$), Ap049 and Ap226 ($t \geq 2.46$, $p < 0.05$); in *A. m. carpathica* for A024, A008, SV185, SV167, Ap068, Ap033 ($t \geq 3.90$, $p < 0.001$), SV220 and Ap249 ($t = 3.10$, $p < 0.01$), A007, Ap243, Ap049, A088 ($t \geq 2.07$, $p < 0.05$); in *A. m. carnica* for A007, AC117, Ap033, *mrjp3* ($t \geq 4.30$, $p < 0.001$), Ap049 and SV167 ($t \geq 3.18$, $p < 0.01$), A113, A008, A024, SV185, A056 and K1615 ($t \geq 2.13$, $p < 0.05$) (see Table 2).

To assess the introgression of the genes of breeds of southern origin (Carpathian breed, carnica, evolutionary line C) into the M line (Central Russian breed), as well as to identify the boundaries of A. m. mellifera, we conducted a comparative study of the genetic diversity of the Central Russian breed, the Carpathian breed, and the Carnica breed according to the complex of nuclear genome markers (Fig. 2). The histogram constructed on the basis of data on the variability of 24 microsatellite loci, on the one hand, clearly shows the low degree of introgression of the C line genes into the M line, that is, the purebred Central Russian bees of Siberian populations. On the other hand, an important characteristic of the Siberian populations of the Central Russian bees was genetic polymorphism,

with the greatest diversity found for the bees of the Tomsk population (apiaries of the northern and southern regions), and the bees of the Altai population turned out to be more homogeneous (mainly from the reproducer of the Central Russian breed, as well as an isolated apiary of the Altai Republic). There was an overlap of genetic variants in Central Russian bees from different populations, which is probably associated with the movement of bee colonies, which beekeepers carry out in Siberia (for example, from isolated apiaries of the Krasnoyarsk Territory or the Altai Republic to the territory of the Tomsk Region). Finally, high genetic relatedness is shown for the subspecies A. m. carnica and A. m. carpathica (a derivative of carnica), despite the different habitat conditions (the Carpathian breed was cultivated for a long time in the conditions of the Carpathians).

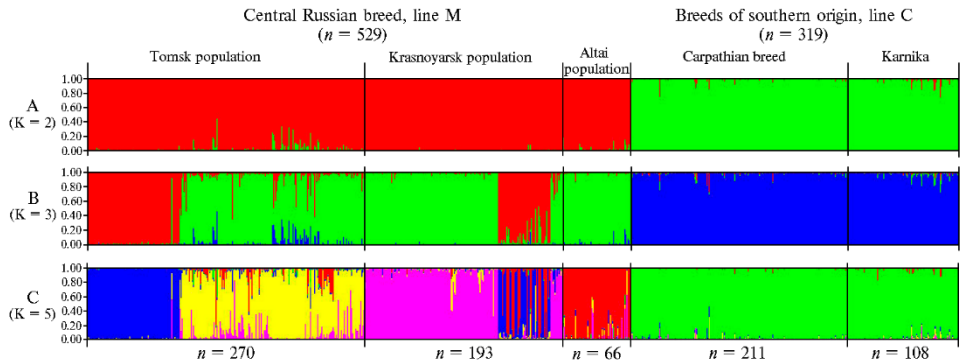

Fig. 2. Histogram constructed in the STRUCTURE 2.3.4 program based on data on the variability of 24 microsatellite loci in honey bees (*Apis mellifera* L.) of the Central Russian breed and breeds of southern origin living in Siberia (2008-2018). The vertical lines represent the proportion of the individual's membership in the color-coded cluster.

A (K = 2): green color reflects the representation of nuclear genes characteristic of breeds of southern origin (Carpathian A. m. carpathica and A. m. carnica), red color - genes of the Central Russian breed A. m. mellifera.

B (K = 3): blue color reflects the representation of nuclear genes characteristic of breeds of southern origin, the other colors - genes of the Central Russian breed.

B (K = 5): green color reflects the representation of nuclear genes characteristic of breeds of southern origin, the other colors - genes of the Central Russian breed.

When studying honey bees from Siberian apiaries, we encountered some problems in identifying bee subspecies and identifying hybrids, which led to the need to develop a research algorithm and select the most informative markers to differentiate different breeds, primarily the Central Russian bee.

The morphometric method, on the one hand, is simple and quite economical, but on the other hand, it is a laborious approach that involves the analysis of a large number of morphological features, which greatly complicates and lengthens the procedure for studying bee colonies [6]. To simplify morphometric analysis, geometric morphometry is used (analysis of the wing shape instead of measuring the angles and distances of cells) [6, 36, 37], special programs for measurements [38, 39], as well as approaches that reduce the number of bee morphometric parameters to be assessed [27] or use a single wing cell [40]. In Russia, the morphometric method is widely used to identify breeds (subspecies) of bees, often with the use of computer programs [41, 42]. However, to eliminate errors in the interpretation of the results, verification of the obtained data is required.

When using the morphometric method, it is also important to take into account the fact that environmental factors can influence the variability of some morphological characters, such as proboscis length [9]. Finally, the morphometric method is not sufficiently informative in assessing hybrid families [43], and additional markers, such as the mtDNA locus, must be studied [27]. Therefore, the

question of both the choice of the most informative morphometric markers and the algorithm for studying bees as a whole is relevant.

In this paper, we have shown that among the morphometric parameters, three indicators of the wing (discoidal displacement, cubital and dumbbell indices) are highly informative and sufficient (minimally necessary) to identify subspecies of *A. mellifera* [26, 27]. It was found that some bee colonies in some respects do not meet the morphometric standards of honey bee subspecies. Moreover, this situation is observed not only in the zones of hybridization of bees, but also in isolated apiaries (see Table 1). In addition, some bee colonies that corresponded to the breed standard in terms of morphological parameters (e.g., the standard of the Central Russian breed) had maternal origin from breeds of southern origin, according to mtDNA analysis (the Q variant of the COI-COII locus was detected), that is, they are "changeling families".

Therefore, the use of the mtDNA COI-COII locus as an additional marker allowed us to refine the data of morphometric analysis, despite the fact that the analysis of the mitochondrial genome evaluates the genetic contribution only along the uterine line. The research algorithm used in the study of the breed composition of honey bees living in Siberia includes i) mtDNA analysis (detection of variability of the COI-COII locus) to establish the origin of the family on the maternal line, ii) evaluation of morphometric parameters of the wing (cubital index, dumbbell index, discoidal displacement), and iii) identification of compliance of morphometric and mtDNA analysis data with the breed standard. Thus, an integrated approach using the morphometric method and mtDNA analysis allowed us to significantly simplify the morphometric analysis of bees and to accurately identify subspecies and hybrids of honey bees living in Siberia.

Using data on the variability of 31 SSR markers, a database was created on microsatellite loci (standard allelic ladder) for Central Russian bees of Siberian populations, and a search was made for molecular markers that are informative for differentiating subspecies of bees of evolutionary lines M and C. The most promising microsatellite loci, which can be included in the diagnostic DNA panel to differentiate the subspecies *A. m. mellifera* (line M) and two subspecies of southern origin *A. m. carpathica* and *A. m. carnica* (line C) turned out to be A043, Ap081, Ap049, AT139, A024, A113, A088, A028, A008 and *mrjp3.* In general, among the studied microsatellite loci, three classes of markers can be distinguished, differing in their information content for diagnosing honey bee breeds and ecotypes.

The first class includes loci for which breed-specific alleles have been found (e.g., A043, Ap081, Ap049, AT139, A024, A113, A088, A028, A008 and *mrjp3*). For these loci, we found predominant allele (alleles) in bees of the Central Russian breed (*A. m. mellifera*) but not in *A. m. carpathica* and *A. m. carnica* where this allele or alleles, if occurred, had a low frequency (see Table 2). For example, for the A043 locus, the 128 bp allele dominated in *A. m. mellifera* from different Siberian populations (the frequency over 0.83), the Burzyanskaya population (Bashkortostan) and most European populations (frequency of occurrence from 0.68 to 0.90) [11, 12, 21], while for the southern subspecies it was a 140 bp allele is characteristic (frequency of occurrence more than 0.76). For *mrjp3*, different predominant alleles were also identified in bees of evolutionary lines M and C. A 529 bp allele was registered with a high frequency (more than 0.57) in the Central Russian bees, while in the Carpathian bees and bees of the *carnica* subspecies it was rare (the frequency less than 0.05). On the contrary, the 406 bp allele dominated in *A. m. carpathica* and *A. m. carnica* (the frequency 0.49 and 0.40, respectively) and was not found in Central Russian bees of Siberian populations. A similar situation was observed for loci Ap081, Ap049, AT139, A024, A113 (see Table

2). In addition, for some loci (Ap081, AT139, etc.), differences in the composition and frequency of dominant alleles in *A. m. carpathica* and *A. m. carnica*, which may be informative for differentiating the two southern subspecies.

The second class included loci for which eco-specific alleles were found, that is, different composition and frequency of alleles were registered in *A. m. mellifera* from different populations of Russia and Europe. The microsatellite locus A008 is of considerable interest. In Central Russian bees of Siberian populations, the162 bp allele occurred at a high frequency (more than 0.86). In bees of the *carniaca* subspecies, this allele was also registered, but with a lower frequency (less than 0.32). In this regard, the A008 locus is of interest for differentiation not so much of subspecies as of ecotypes of *A. m. mellifera*: differences in the composition of alleles between the bees *A. m. mellifera* from different populations of Russia and Europe. So, if for the Central Russian bees of Siberia the 162 bp allele was predominant, then in *A. m. mellifera* of the Ural and European populations, shorter alleles of 154 and 148 bp, respectively, were dominant [11, 12, 21]. In the Central Russian bees of the Siberian populations, the 148 bp allele was not found at all. Since the bees *A. m. mellifera* from different populations of Russia and Europe revealed a geographic gradient in the size of the dominant allele (148 bp−154 bp−162 bp) in the west-east direction, it can be assumed that the A008 locus is associated geographical/ecological conditions of bee habitat. The problem of genetic specificity of different subspecies/ecotypes of bees to local environmental conditions is actively discussed in scientific publications [7, 44-46].

Nonspecific loci (for example, AC117, H110, SV185), belonging to the third class, are markers for which a similar composition and close allele frequencies have been shown in bees of different origin and/or geographical localization.

In general, among microsatellite loci, the variability of which has been studied in Siberian bee populations, a number of markers can be used to establish the origin of subspecies and/or ecotypes of the honey bee. Despite the existing limitations in the use of DNA markers of the nuclear genome (the absence or inaccessibility of a database and reference materials on the variability of SSR markers in bees of different populations/ecotypes/subspecies), in some cases microsatellite loci are highly informative and widely used in assessing the introgression of genes from one evolutionary line into another and identifying traces of hybridization [5, 19-21].

Thus, at present, there is no universal method or diagnostic marker (morphometric, molecular) for identifying subspecies of *A. mellifera*, but with the complex application of different methods, morphometric and DNA markers complement each other.

The importance of an integrated approach is noted in many works on the taxonomy and phylogeny of animals. Thus, when describing new species of wasps (*Hymenoptera*), it was shown that, along with the morphological approach, molecular genetic, cytogenetic, and other methods are important [47]. Comprehensive analysis of nuclear and mitochondrial markers is the most informative for identifying hybrids and assessing introgression or gene flow [3, 21].

The choice of a reliable DNA marker for molecular genetic studies, as well as the search for a morphological trait, are difficult and poorly developed [48, 49]. A good molecular marker implies the presence of a sufficient number of informative sites, a low degree of homoplasia, and a relatively uniform rate of evolution within the analyzed group of organisms [50]. If the analysis of large taxa characterized by significant divergence (tribes, families) requires a DNA marker with a low degree of variability, then when studying groups of a low taxonomic level (species), it is desirable to use a rapidly evolving marker with variability that has not reached the saturation limit [49]. Finally, before using a new DNA locus

as a phylogenetic marker, it is necessary to determine the degree of its variability and informativeness, for example, in a group of organisms with a well-studied taxonomy and evolutionary history [50].

It should be emphasized that various approaches, such as morphological and molecular genetic methods used in the systematics and phylogeny of organisms, complement each other and are not competing or mutually exclusive, especially since molecular genetic methods will not replace morphological species identification system [51]. The optimal and most informative for identifying species and other taxa, as well as establishing the boundaries between species and describing species diversity is an integrated approach involving a wide arsenal of methods and taking into account data from various disciplines (comparative anatomy, ecology, ethology, population genetics, philogeography) [52]. Particularly credible studies are those in which the phylogenetic hypothesis is substantiated by the analysis of several independently evolving molecular markers [52, 53] or the variability of a molecular marker is preliminary studied and its information content and reliability are shown to resolve phylogenetic relationships at a given taxonomic level [48, 50, 54].

So, in the study of honey bees in Siberia, we used an integrated approach, including the analysis of morphometric traits, markers of the mitochondrial and nuclear genomes, and assessed the information content of different methods. The optimal research algorithm, with regards to the differentiating ability, information content and efficiency of the methods used, can be as follows: i) analysis of the origin of the family on the maternal line using markers of the mitochondrial genome; ii) study of morphometric features and assessment of their compliance with mtDNA analysis data; iii) microsatellite analysis to clarify the breed affiliation of families, the origin of hybrids, as well as to identify the genetic diversity of bees of different evolutionary lines. This approach allowed us to identify honey bee subspecies, identify populations of the Central Russian breed Apis mellifera mellifera and characterize their genetic diversity, evaluate the genotypic composition of bee colonies, and determine bee hybridization zones. The results obtained in this work are a scientific basis for the genetic certification of bees and breeding work on the selection of purebred families with the necessary biological and economically significant traits, which is an important condition for the conservation and rational use of native breeds/ecotypes.

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FEATURES OF THE RUMEN MICROBIAL GENE EXPRESSION IN DRY AND LACTATING COWS

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Abstract

The quality and quantity of feed consumed by lactating and dry cows varies greatly. Dry cows are usually fed a high in roughage and low in compound feed diet, which slows down the rate of fermentation in the rumen. Immediately after calving, cows are fed with low in fiber and high in compound feed diets, which usually have a high fermentation rate due to the high content of easily digestible polysaccharides such as starch. In the present work, for the first time it was established that a change in dairy cows diet, associated with an increase in the proportion of starch, leads to changes in the expression of numerous genes of rumen microorganisms, especially the L-lactate dehydrogenase gene. Our goal was to analyze the expression of genes involved in the key reactions of rumen metabolism depending on the physiological period of the animal and the crude fiber content in the diet. Samples were taken in 2020 at Agrofirma Dmitrova Gora (Tver region) from 15 dairy cows (*Bos taurus*) of the black-and-white Holsteinized breed of the 2nd-3rd lactation. Animals were kept in the same conditions on a tie-up housing. Six cows were selected for the experiment and two groups of animals ($n = 3$) were formed: group I — dry cows (on average, 30 days before calving), group II — cows in lactation (day 208 of lactation). Chyme samples (30-50 g from each cow) were taken from the upper part of the ventral rumen sac manually with a sterile probe. Total DNA was isolated from the studied samples using the Genomic DNA Purification Kit (Fermentas, Inc., Lithuania). The rumen bacterial community was analysed by NGS sequencing on the MiSeq platform (Illiumina, Inc., USA) using primers for the V3-V4 region of 16S rRNA. Bioinformatic data analysis was performed using Qiime2 ver. 2020.8 (https://docs.qiime2.org/2020.8/). Taxonomy was analyzed using the Silva 138 reference database (https://www.arbsilva.de/documentation/release-138/). Total RNA was isolated from cicatricial samples using the Aurum Total RNA kit (Bio-Rad, USA). cDNA was obtained on an RNA template (iScript RT Supermix kit, Bio-Rad, USA). The relative expression of genes was analyzed by quantitative PCR, which was carried out on a detection amplifier DT Lite-4 624 (DNA-Technology, Russia). It was shown that a change in the diet of cows, associated with an increase in the proportion of starch, contributed to a decrease in the proportion of cellulolytic bacteria of the families *Ruminococcaceae* and *Lachnospiraceae* and an increase in the number of bacteria of the family *Prevotellaceae* associated with the decomposition of starch. Changes in the expression of bacterial genes depending on the diet have also been shown. Thus, the expression of the L-lactate dehydrogenase gene increased in the group of lactating cows ($p \leq 0.05$) receiving a high-starch diet. This is probably due to the high content of lactate in the rumen of cows consuming high concentrations of easily digestible carbohydrates and to

the formation of adaptive mechanisms in the microbial community of the rumen. Also, in lactating cows, the expression of the phosphofructokinase gene ($p \leq 0.05$), one of the regulatory enzymes of glycolysis, increased. Improving the accessibility of monosaccharides from compound feed contributes to the intensification of the process of glycolysis by rumen microorganisms. In this regard, the *Ldh-L* gene can be considered as a candidate for biomarkers that can give an idea of the activity of lactic acid synthesis processes and, as a result, a decrease in pH in the rumen of cows.

Keywords: rumen, gene expression, microorganisms, physiological period, cattle

The productivity of a dairy farm is made up of the proper management and control of the production process. Numerous studies and practical observations confirm that obtaining the maximum milk yield is possible with constant monitoring of lactation indicators, starting from the dry period. In addition, the successful organization of the lactation cycle of cows contributes to the disclosure of the genetic potential for milk productivity while maintaining the reproductive health of the animal, which is reflected in the economic component of livestock farming. During the lactation cycle, special attention must be paid to the transition period of 21 days before and after calving. According to some data, the transition period can be seen as an opportunity to establish lactation and ensure good health and reproduction [1].

The quality and quantity of feed consumed by lactating and dry cows varies greatly. Dry cows are typically fed a diet high in roughage and low in compound feed, resulting in a slower rate of rumen fermentation [2]. Immediately after calving, cows are fed diets low in coarse fiber and high in compound feed, which are characterized by a high rate of fermentation [3]. Obviously, the type and amount of roughage and concentrates in the diets consumed by cows determine the microbial composition and activity of the rumen [4], as well as affect physiological characteristics, mainly pH and fermentation [5-7] which, in turn, may affect the epithelium of the gastrointestinal tract. A number of studies have reported that rumen epithelium in calves [8], dry cows [9] and even in transition cows [10] depended on the type of diet offered.

According to J.W. Schroeder [11], during the transition period, special attention should be paid to the consumption of animal feeds and concentrates in order to prepare the rumen wall and its microflora for the upcoming consumption of feed with a high content of cereals. M.S. Jolicoeur et al. [12] showed that the fewer times the prepartum diet is changed, the easier the rumen adapts to the diet and improves postpartum energy balance. D.W. Pitta et al. [13] indicate that the rumen microbiome changes as dairy cows transition from non-lactating to lactating due to dietary changes. According to the analysis of the rumen content of animals during the transition period, the most numerous observed types in all communities were *Bacteroidetes* and *Firmicutes*. When cows entered lactation, the ratio of *Bacteroidetes* to *Firmicutes* increased from 6:1 to 12:1 ($p < 0.05$) and was greater in primiparous than in multiparous cows ($p < 0.05$). The data obtained by A. Bach et al. (14) indicate that, before calving, the relative proportion of fiber-degrading bacteria is higher than bacteria that feed on rapidly fermentable carbohydrates. After calving, there is a rapid shift towards an increase in the proportion of bacteria that degrade rapidly fermentable carbohydrates. Animals had a higher dry matter intake after calving, resulting in an increase in non-fibrous carbohydrate intake of 1.21 kg/day, which would negatively affect rumen pH and microbial balance.

Despite a number of studies focused on the study of the rumen microbiota in different periods of the lactation cycle, it remains relevant to assess the microbial component of the rumen of dairy cows of different productivity in the transitional period of the lactation cycle, depending on the productivity of animals and the organization of feeding in livestock farms.

Most studies of rumen gene expression have focused on changes in rumen epithelial gene expression in cows [15]. Little information has been obtained on the possible relationship between the type of diet and changes in gene expression in the epithelium and microbiome of the rumen [10]. Considering the differences in dietary components at different physiological stages, similar changes should occur in the expression of genes involved in the metabolism of volatile fatty acids, carbohydrates, etc. They may be associated with changes in both the qualitative composition of microbial communities and individual transcriptional profiles of microorganisms.

In this work, it was found for the first time that an increase in the proportion of starch in the diet of dairy cows leads to changes in the expression of a number of genes by ruminal microorganisms, especially the L-lactate dehydrogenase gene.

Our goal was to analyze the expression of genes involved in the key reactions of rumen metabolism, depending on the physiological period of the animal and the content of crude fiber in the diet.

Materials and methods. Samples were collected in 2020 at Agrofirma Dmitrova Gora JSC (Tver Province) from 15 black-and-white Holsteinized dairy cows (*Bos taurus*) of the 2nd-3rd lactation. Animals were kept in the same conditions on a tether. Of six cows selected for the experiment, two groups were formed (n = 3 each), the dry cows 30 days before calving (group I) and animals during lactation (day 208 of lactation) (group II).

Dry cows were selected by expected calving date. The number of animals in groups was consistent with that in previously published studies [16, 17]. The average live weight of animals in group I was 703 kg, in group II 667 kg. Chyme samples (30-50 g from each cow) were taken from the upper part of the ventral rumen sac manually with a sterile probe using aseptic conditions as possible.

Total DNA was isolated from the samples using the Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) according to the attached instructions. The analysis is based on the selective precipitation of DNA from the substrate by the detergent method using solutions for cell wall lysis, DNA precipitation, 1.2 M sodium chloride solution, chloroform.

The rumen bacterial community was studied by NGS sequencing on the MiSeq platform (Illiumina, Inc., USA) with primers for the V3-V4 region of the 16S rRNA gene (forward primer 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCG-3', reverse primer 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGTATCTAATCC-3′. Nextera® XT IndexKit (Illiumina, Inc., USA) was used for preparation of libraries, Agencourt AMPure XP (Illiumina, Inc., USA) for purification of PCR products and MiSeq® ReagentKit v2 (500 cycle) (Illiumina, Inc., USA) for sequencing. The maximum length of the obtained sequences was 2×250 bp. Bioinformatic analysis was performed using Qiime2 ver. 2020.8 software (https://docs.qiime2.org/2020.8/). After the initial import of the sequences into the Qiime2 format, the paired rows of reads were aligned. The sequences were filtered by quality (default settings). The tests were performed with the Deblur method (the maximum length of the pruning sequence is 250 bp) (https://msys-tems.asm.org/content/msys/2/2/e00191-16.full.pdf). The de novo phylogeny was constructed using the MAFFT software package (https://mafft.cbrc.jp/align-ment/software/), followed by masked sequence alignment. The reference database Silva 138 (https://www.arbsilva.de/documentation/release-138/) was used for taxonomy analysis.

Total RNA was isolated from rumen contents using the Aurum Total RNA kit (Bio-Rad, USA) according to the manufacturer's instructions. cDNA was obtained on an RNA template (iScript RT Supermix kit, Bio-Rad, USA).

The relative expression of bacterial genes was analyzed by quantitative PCR (a detecting cycler DT Lite-4 624, LLC NPO DNA-Technology, Russia). Amplification conditions: 1 min at 95 °C (1 cycle); 15 s at 95 °C, 1 min at 50 °C (45 cycles). The amplification reaction mixture from the SsoAdvanced Universal SYBR Green Supermix kit (Bio-Rad, USA) was prepared according to the manufacturer's protocol. Relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method [18]. The primers for the genes analyzed in this work were as follows:

Primer	Nucleotide sequence (5′→3′)	Reference
Phosphofructokinase (PFK)	F: ATCGGTGGTGACGGTTCTTAT R: GATATCWCCAGCRTKACGTCCCAT	[18]
Phosphoenolpyruvate carboxykinase (PEPK)	F: AAGGKATGTTCTCWATSATGAACTAC R: TAGATMGGRTAAGAAACACGAGT	[18]
Methylmalonyl-CoA mutase (MCM)	F: GGCSATYGGCAYSAACTTCTWCATGGA R: GTCGGTSGGCAGMGCGATSGCCTCGTC	[18]
CLA-reductase	F: CATTCGCACTTGGTACATCTCAGC R: ACGTACACGTGGTACTTCCTCAAG	[18]
L-lactate dehydrogenase (L-LDG)	F: CATCAAAAAGTTGTGTTAGTCGGCG R: TCAGCTAAACCGTCGTTAAGCACTT	[19]
D-lactate dehydrogenase (D-LDG)	F: CTGGGATCCGTTGAGGGAGATGCTTAAG R: TCCGAAGCTTTTAGTTGACCCGGTTGAC	[20]
Guanine aminohydrolase (GAH1)	F: ATTGCYTTCTGYCCGACYTCCAACCT R: TTGTAKGCYTCGTTSAGCGTYTGCAG	[18]
16S rRNA (Bac)	F: AGGCCTTCGGGTTGTAAAGT R: CGGGGATTTCACATCTCACT	[21]

The universal gene encoding the 16S ribosomal subunit of prokaryotes (F: 5′-AGGCCTTCGGGTTGTAAAGT-3′, R: 5′-CGGGGATTTCACATCTCACT-3′) served as a reference.

Mathematical and statistical processing was carried out using the one-way analysis of variance (ANOVA) in Microsoft Excel XP/2003, R-Studio (Version 1.1.453) (https://rstudio.com). Tukey's HSD test (https://www.rdocument-ation.org/packages/stats/versions/3.6.1/topics/TukeyHS) was used to correct for type 1 error. The results are shown as means (M) and standard errors of the means (±SEM). Statistically significant differences between the means were assessed using the Student's t-test at $p \leq 0.05$.

Results. The two diets consumed by the control and experimental groups of cows differed significantly in the content of easily digestible polysaccharides (starch) and fiber (Table 1). The diet of dry cows contained more acid detergent and neutral detergent fiber (68.3%) vs. the diet of dairy cows (46.0%). Acid detergent fiber (ADF) includes cellulose, lignin and insoluble salts. The lower the proportion of ADF, the more feed the animal is able to consume and digest. Neutral detergent fiber (NDF) which serves as a material for the plant cell walls includes hemicellulose, cellulose, lignin, and insoluble ash. The lower the percentage of dietary NDF, the more feed the animal can consume and digest. The feed of dry cows was only 16.2% starch, and the diet of dairy cows was 26.6% starch.

1. The composition of the diets of cows (Bos taurus) of the black-and-white Holsteinized breed in the dry and milking physiological periods (JSC Agrofirma Dmitrova Gora, Tver Province, 2020)

Ingredient, kg	Dry cows (group I)	Lactation cows (group II)
Straw	0.5	–
Compound feed	1.8	4.55
Sunflower meal	0.3	–
Soybean meal	0.53	2.6
Corn	0.53	4.0
Wheat	0.29	1.2
Beet pulp	1.97	0.6
Corn silage	5.9	6.4
Syrup	–	0.61
Stillage alcohol	–	1.0
Cereal-bean haylage	–	3.5

Continued Table 1

NDF, % of DM	41.63	28.28
ADF, % of DM	26.64	17.75
Starch, %	16.22	26.64

N o t e. NDF — neutral detergent fiber, ADF — acid-detergent fiber, DM — dry matter. A dash means that the component was absent from the diet.

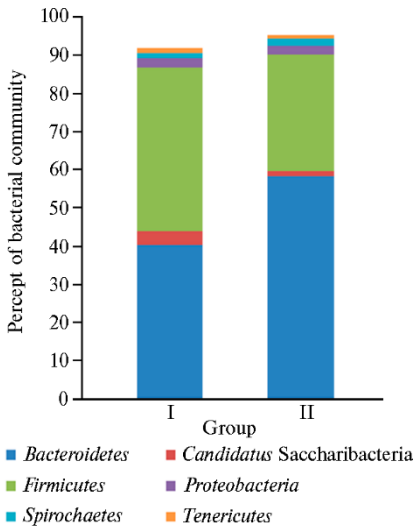

Fig. 1. Taxonomic composition of the microbial community of the rumen in cows (*Bos taurus)* of black-and-white Holsteinized breed in the dry (group I) and milking (group II) physiological periods according to the NGS-sequencing of the 16S rRNA gene fragment (AO Agrofirma Dmitrova Gora, Tver Province, 2020).

We determined the taxonomic composition (Fig. 1) and transcription features of a number of key metabolic genes of ruminal microorganisms involved in the processes of glycolysis and gluconeogenesis, lactate and fatty acid metabolism. In the rumen of dry cows, representatives of the phylum Firmicutes (43.9%) reached the highest abundance, bacteria of the phylum Bacteroidetes prevailed in dairy cows (58.3%). These two phyla were dominant in the rumen of both groups of cows, which is considered normal for the rumen and gastrointestinal communities [23]. The phylum *Firmicutes* was highly abundant with anaerobic and amylolytic bacteria. Therefore, fluctuations in the ratio of representatives of these phyla may indicate changes in the microbial community of the rumen associated with adaptation to dietary characteristics [24].

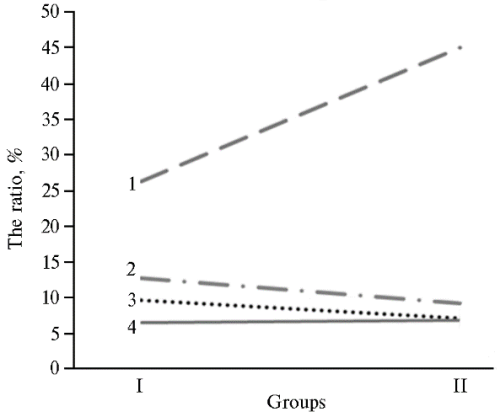

Fig. 2. The ratio of some groups of microorganisms of the rumen community in cows (Bos taurus) of black-motley Holsteinized breed depending on the diet consumed in the dry (I group) and milking (II group) physiological periods: 1 — Prevotellaceae, 2 — Ruminococcsceae, 3 — Lachnospiraceae, 4 — Succiniclasticum ruminis (JSC Agrofirma Dmitrova Gora, Tver Province, 2020).

The composition of the diet is one of the main factors influencing the change in the rumen microbiota, along with environmental influences [25]. This is confirmed by D.W. Pitta et al. [13] who showed that the rumen microbiome changes as dairy cows transition from non-lactating to lactating due to dietary changes. According to our data on the rumen contents in animals during the transition period, the ratio of *Bacteroidetes* to *Firmicutes* increased from 6:1 to 12:1 ($p < 0.05$). A. Bach et al. [14] also indicate that before calving the relative proportion of fiber-degrading bacteria is higher than that of bacteria that feed on rapidly fermentable carbohydrates. After calving, there is a rapid shift towards a higher proportion of bacteria that degrade rapidly fermentable carbohydrates.

Changes in the ratio of easily and hardly digestible carbohydrates in the diet led to a change in the ratio of different groups of microorganisms in the rumen (Fig. 2). With an increase in the proportion of starch and a decrease in roughage,

there was an increase in the abundance of the family *Prevotellaceae* and a decrease in the families *Ruminococcsceae* and *Lachnospiraceae*. That is, in the rumen of dairy cows, there was a decrease in the number of cellulolytic bacteria and an increase in the number of bacteria with amylolytic activity. *Succiniclasticum ruminis* is a rumen dweller capable of converting succinate to propionate as its sole energy production mechanism. *Succiniclasticum ruminis* is considered the main microorganism involved in this process, the importance of which is determined primarily by the participation of propionate in the process of gluconeogenesis in the animal's liver. Since propionate is the only gluconeogenic volatile fatty acid (VFA) in the rumen that provides the host with more ATP comaperd to any other VFA produced in the rumen, its importance is clear [25]. A change in the dietary easily and hardly digestible carbohydrates did not affect the abundance of *Succiniclasticum ruminis*.

To study the functional features associated with different amounts of starch and coarse fiber in the diets, we isolated mRNA from the rumen contents and studied the transcription features of a number of key metabolic genes of ruminal microorganisms involved in the processes of glycolysis (phosphofructokinase, phosphoenolpyruvate carboxykinase), lactate metabolism (lactate dehydrogenase), fatty acids (methyl melonyl-CoA mutase, CLA reductase).

Figure 3 illustrates expression of the bacterial genes bacterial gene associated with the synthesis of phosphofructokinase (PFK), phosphoenolpyruvate carboxykinase (PEPK), conjugated linoleic acid reductase (cla-r), L-lactate dehydrogenase (Ldh-L), D-lactate dehydrogenase (Ldh-0813), methylmalonyl-CoA mutase (MSM), guanine aminohydrolase (GAH) in the rumen.

Phosphofructokinase and phosphoenolpyruvate carboxykinase are important participants in carbohydrate metabolism, while linoleic acid reductase is associated with fatty acid metabolism. Phosphofructokinase (EC 2.7.1.11) serves as one of the regulatory enzymes of glycolysis, which is responsible for the transfer of a phosphate group from an ATP molecule to fructose-6-phosphate, which leads to the formation of fructose-1,6-bisphosphate and ADP. Glycolysis is a universal pathway for glucose catabolism and the most common of the three (there are also the pentose phosphate pathway and the Entner-Doudoroff pathway) glucose oxidation pathways found in living cells. An increase in PFK gene expression during lactation by 2.83 times ($p \leq 0.05$) could be associated with the stress of carbohydrate metabolism in the rumen during lactation and the adaptation of microorganisms to modifications of nutrients available in the rumen. During this period, the amount of available sources of glucose, the starch and monosaccharides increases in the diet. An increase in the expression of genes of bacterial phosphofructokinases can have negative consequences for the metabolism of the macroorganism. The result of glucose utilization in the rumen and, as a result, its low content in blood plasma, is the activation of physiological mechanisms for overcoming energy deficit: the body of animals actively mobilizes triglycerides from adipose tissue in an attempt to satisfy the need for a large amount of energy [27]. Such an effect seems to be natural, since the predominance of easily digestible starch in the diet should intensify the process of glycolysis in the rumen, which leads to the competitive displacement of cellulolytic bacteria by starch-consuming *Prevotellaceae*. In addition, obligate homofermentative and facultative heteroenzymatic lactic acid bacteria that are undesirable for the rumen, as a rule, ferment glucose to pyruvate through glycolysis. Further lactic acid fermentation reactions lead to the formation of a significant amount of lactate [28] and, consequently, a decline in pH in the rumen.

Interestingly, a 1.73-fold increase in phosphoenolpyruvate carboxykinase

(PEPK) gene expression in the rumen during lactation may also be due to activation of pathogenetic processes. Phosphoenolpyruvate carboxykinase is an enzyme in the glucose synthesis pathway from non-carbohydrate compounds (gluconeogenesis) [21]. This anabolic pathway is associated with the manifestation of virulence in a number of intracellular bacterial pathogens, for example, in *Mycobacterium tuberculosis* [29, 30].

Lactate is synthesized as a result of lactic acid fermentation from precursors by the action of two different forms of NAD-linked lactate dehydrogenases: one of them (EC 1.1.1.27) produces the L(+)-lactate L-LDG isomer, the other (EC 1.1.1.28) produces the D(–)-lactate D-LDG.

According to the report [31], the D(–)-lactate isomer significantly differs from L-lactate in its action. An important difference between the isomers is the possibility of their renal excretion, which is lower for D-lactate, which determines its main role in provoking metabolic acidosis [32]. In this regard, data on the expression of the *Ldh-L* and *Ldb 0813* genes can give an idea of the activity of lactic acid synthesis and, as a result, a decrease in pH in the rumen. The data obtained are consistent with the generally accepted opinion [33] that the transition to a highly concentrated diet provokes the formation of metabolic disorders in the rumen. Against the background of stressful situations (calving, lactation) and a negative energy balance, cows are at high risk of metabolic disorders associated with a decrease in pH.

We did not find statistically significant differences between the groups in terms of the level of expression of the D-lactate dehydrogenase gene. Nevertheless, L-lactate dehydrogenase gene expression increased in the group of lactating cows by 4.8 times ($p \leq 0.05$). This indicates that the organism of animals during the period of milk production provided more effective resistance to stress factors than the organism of dry cows. This is probably due to the formation of adaptive mechanisms in the microbial community of the rumen.

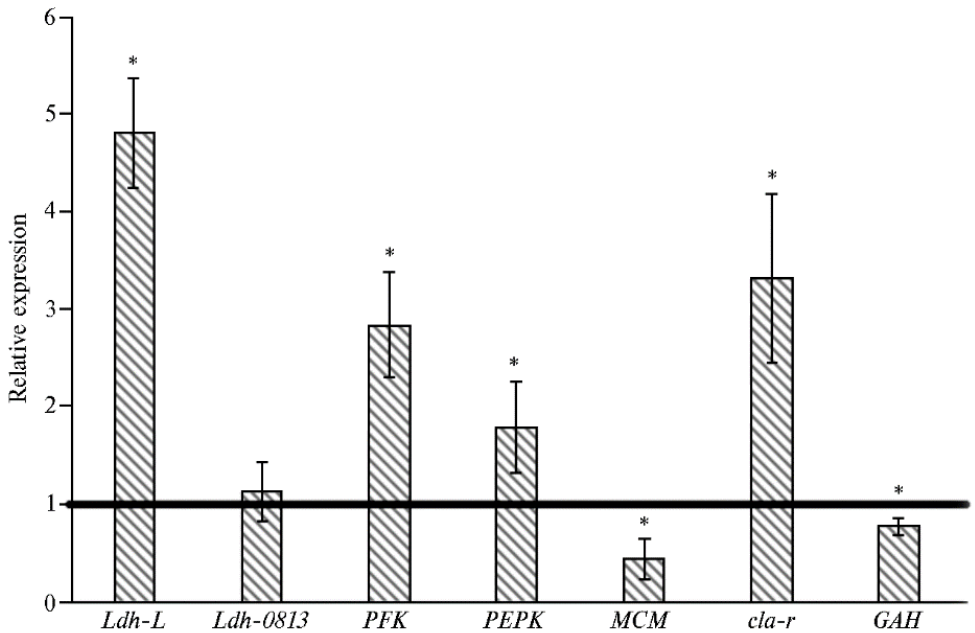

Fig. 3. Relative gene expression of the microbial community of the rumen in cows (*Bos taurus*) of black-and-white Holsteinized breed depending on the diet consumed in the dry (horizontal line) **and milking** (diagram) **physiological periods** (JSC Agrofirma Dmitrova Gora, Tver region, 2020).
* Differences with indicators in the dry period are statistically significant at $p \leq 0.05$.

In our opinion, the genes *Ldh-L* and *Ldh 0813* are important candidates for biomarkers that can give an idea of the activity of lactic acid synthesis processes and, as a result, a decrease in pH in the rumen of cows. The data obtained are consistent with the generally accepted opinion [34] that an abrupt transition to a highly concentrated diet can provoke the development of metabolic disorders in the rumen.

In group II compared to group I, there was a statistically significant ($p \leq 0.05$) decrease in the expression of the *MCM* gene associated with the synthesis of methylmalonyl-CoA mutase which activates the conversion of methylmalonyl-CoA to succinyl-CoA. It is known [35] that succinyl-CoA is the most important link in the Krebs cycle. The tricarboxylic acid cycle (Krebs cycle, citric acid cycle) undoubtedly plays a central regulatory role in the body. It is a complex, multi-step sequence of reactions supplying energy and plastic equivalents, reduced and phosphorylated cofactors of major biosynthetic pathways. The intensity of almost all processes in the body is regulated by the ratio of reduced and oxidized adenyl and flavin nucleotides, ATP/ADP, ATP/AMP and ATP/inorganic phosphate.

The MCM enzyme is widely distributed in all living organisms except plants. It has been studied, isolated and crystallized from the Gram-positive bacteria *Propionibacterium freudenreichii* var. *shermani* in which it is involved in the conversion of pyruvate to propionate. The enzyme has been described as a heterodimer consisting of large (α) and small (β) subunits, forming a 150 kDa protein one domain of which binds to acyl-CoA and the other to coenzyme B_{12} [36].

In addition, the role of the tricarboxylic acid cycle, which is central to energy metabolism, is not limited to energy production and storage [37]. Four- and five-carbon intermediates serve as precursors for the synthesis of many compounds in the rumen, including citrate for lipid synthesis, oxaloacetate for aspartate production.

The expression of the *GAH1* gene associated with the synthesis of the guanine aminohydrolase enzyme decreased in the rumen during lactation ($p \leq 0.05$) compared to dry cows. This could adversely affect the synthesis of a valuable microbial protein, since guanine aminohydrolase catalyzes purine catabolism reactions [38]. An important process of protein metabolism in ruminants is the degradation of nitrogen-containing feed compounds, in particular purines, and the synthesis of microbial protein [39]. A decrease in the *GAH1* gene expression in the rumen of cows during milking and stabilization of lactation could be associated with asynchronous consumption of carbohydrates and proteins against the background of highly concentrated feeding, as well as stress associated with the lactation process, and, as a result, a negative energy balance.

The *cla-r* gene is responsible for the synthesis of conjugated linoleic acid reductase and leads to the formation of conjugated linoleic acid (CLA) which is formed as one of the metabolic intermediates in the rumen of ruminants [40]. *Butyrivibrio fibrisolvens* has the highest potential for CLA products [41]. The increase in the *cla-r* gene expression in group II by 3.3 times ($p \leq 0.05$) could be due to an increase in the abundance of a typical rumen inhabitant, the bacterium *Butyrivibrio fibrisolvens* of the phylum *Firmicutes*. This compound has attracted significant attention from researchers as a substance that has a beneficial effect on human and animal health. The main source of CLA for humans is dairy products [42].

Thus, in our study, a change in the diet of dry and dairy cows, associated with an increase in the proportion of starch, contributes to a decrease in the content of cellulolytic bacteria of the families *Ruminococcaceae* and *Lachnospiraceae*

in the rumen and an increase in the abundance of bacteria of the family *Prevotellaceae* involved in the decomposition of starch. Changes in gene expression by rumen microorganisms occurres when diets contain different amounts of fiber and easily digestible polysaccharides. The L-lactate dehydrogenase gene expression increased in the group of lactating cows. This is probably due to the higher lactate content in the rumen of animals consuming high concentrations of easily digestible carbohydrates from compound feeds, as well as to the formation of adaptive mechanisms in the microbial community of the rumen. This is indirectly confirmed by an increase in the expression of the phosphofructokinase gene, one of the regulating enzymes of glycolysis in lactating cows. An increase in the availability of carbohydrates in mixed feed contributes to the intensification of the process of glycolysis by rumen microorganisms. Thereof, the L-lactate dehydrogenase gene, in our opinion, can be a candidate biomarker of the activity of lactic acid synthesis processes and a decrease in pH in the rumen of cows.

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ECONOMIC AND BIOLOGICAL CHARACTERISTICS OF THE FIRST-CALVING HOLSTEIN HEIFERS OF DIFFERENT ORIGIN DURING ACCLIMATIZATION ON A FARM IN CENTRAL RUSSIA

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Abstract

Making the most of the genetic potential of dairy cattle imported to the Russian Federation is an important and urgent task which requires a detailed study of animals' acclimatization and adaptation under the conditions at regional livestock enterprises. The aim of the work was to assess body scores, productive and reproductive performance, blood biochemical parameters and composition in the Holstein black-and-white first calving cows (a herd of OOO Molochnik, Bolshesoldatsky District, Kursk Province, 2019-2020). We compared performance of 15 imported heifers of European selection from Denmark (group 1) and 15 heifers from pre-adapted mother cows born on the farm (group 2). The groups were in identical feeding and housing conditions. On average, the cows calved in 23.6 (group 1) and 24.6 months (group 2), having bodyweight of 509.2 and 516.9 kg, respectively. For 305-day lactation, total milk production was 8667 kg with 3.73 % fat for group 1 and 121 kg more with 3.80 % fat for group 2 (the differences are insignificant). Milk yield adjustment to 3.4 % milk fat increased the difference to 314 kg ($P > 0.95$). In group 1, the milk fat yield was 323.6 kg, or 10.3 kg less compared to group 2 (the differences are insignificant). The difference in milk proteins was also small (3.27 vs. 3.28 %). Total milk protein yield was 283.4 kg vs. 288.2 kg, the milk production coefficients (i.e., fat-corrected milk yield per unit bodyweight) was 1867 kg vs. 1900 kg. Therefore, these findings confirm 93.1 % vs. 93.0 % realization of genetic potential for milk production, 99.5 % vs. 100.7 % for milk fat, and 100.0 % vs. 99.3 % for milk protein. All cows were quite tall, their height at the withers averaged 137.5 cm vs. 135.4 cm, at the sacrum 145.3 vs. 142.4 cm. The total exterior scores, including strong body constitution, well-developed milk traits, and leg condition, in group 1 were higher ($P > 0.95$) compared to group 2. According to a 100-point evaluation, the cows of group 1 had a slight advantage. Of the classification traits, the score of trunk volume was 0.9 points higher, of udder — 0.9 points higher, of general appearance — 0.8 points higher compered to group 2 (the differences are insignificant). The animals of both group had body type Good+ with 83.0 points vs. 81.7 points for five classification traits compared (the difference is insignificant). At month 6 of lactation, the total blood protein level averaged 83.11 g/l vs. 83.78 g/l. Other biochemical blood parameters (albumin, globulins, glucose, cholesterol, calcium, phosphorus, magnesium, activity of transamination enzymes and alkaline phosphatase) were within the physiological limits. An increased counts of blood leukocytes occurred in the European cows. The hematocrit index in group 1 was significantly higher than in group 2 ($P > 0.95$), which is apparently due to intensified metabolism. Thus, in the conditions that meet the biological needs of animals, the acclimatization of European breeding cows is quite successful.

Keywords: Holstein cows, first-calf heifers, genetic potential, exterior, body scores, linear body measurements, blood biochemical parameters, total protein, albumin, globulins, aminotransferases, alkaline phosphatase

The study of the implementation of the genetic potential of growth, development and productivity, acclimatization and adaptive qualities of dairy

cattle imported to the Russian Federation from abroad remains an important and urgent problem [1, 2]. In connection with the culling of animals for various reasons [3-6], the selection of cows of the first calving for herd repair is an important direction in selection and breeding work both in Russia and abroad. For example, in Canada, 34-36 replacement heifers are raised annually per 100 cows [7, 8].

The import of breeding stock does not completely solve the problems of dairy cattle breeding. There are risks during transportation and quarantine of animals, difficulties in adapting to new technological conditions, as a result, the duration of the productive use of cows is significantly reduced, their genetic potential is not fully realized [9, 10]. As per H.A. Amerkhanov [11], internal reserves for increasing the milk productivity of cows are the full realization of the genetic potential of animals, the improvement of feeding regimes, the use of innovative technologies for keeping and reproducing herds.

Russia ranks 6th in the world in terms of milk production. In January-September 2020, gross milk yield in farms of all categories amounted to 24.9 million tons and increased by 2.7% compared to 2019. At agricultural enterprises, milk yield per cow amounted to 6156.0 kg, or 6.5% more compared to the same period in 2019. The intensification of domestic dairy cattle breeding in Russia is carried out on the basis of a qualitative transformation of domestic cattle breeds and the creation of highly productive dairy herds that meet the requirements of modern milk production technologies. For this purpose, highly productive animals of the Holstein breed, which have a high genetic potential, are imported into the Russian Federation from Europe and North America [12]. According to the Ministry of Agriculture of Russia, for the period from 2008 to 2018, the number of Holstein cattle in Russia increased by 4.3 times, from 121.23 thousand animals, or 3.4% of probonitated individuals to almost 525 thousand heads. With loose housing and balanced feeding, the yield of Holstein cows is 8000-10000 kg of milk with a mass fraction of fat of 3.5-3.6% [13, 14]. When breeding dairy cattle, much attention is paid to the assessment of animals according to their exterior and constitutional features [15-17].

Here, we present the results of assessing the acclimatization qualities of animals imported and born on the farm, based on a comprehensive comparison of their conformation, physiological and productive indicators. Under conditions that meet the biological needs of animals, the successful acclimatization of first-calf heifers of European selection has been confirmed.

The purpose of the work is to study the economic and biological characteristics of cows of the first calving of the black-and-white Holstein cows of different origin in a dairy farm.

Materials and methods. The studies were carried out in 2019-2020 on a population of highly productive black-and-white Holstein cows (Molochnik LLC, Bolshesoldatsky District, Kursk Province). The dairy herd of the enterprise was completed with the livestock of heifers imported from the breeding farms of European countries and the USA. Information about the productive indicators of animals, their production use was borrowed from the card index of breeding cows of the archive of the program for zootechnical and breeding accounting AWP "SELEKS" (LLC Regional Center for Information Support of Pedigree Livestock Breeding of the Leningrad Region, PLINOR). For the study, we used two test groups, each of 15 randomly assigned first-calf heifers. Group 1 included imported animals of European selection (Denmark), group 2 consisted of animals that descended from mothers born on the farm and passed adaptation. Animals during the experiment were in identical conditions of feeding and maintenance.

The coefficient of milk yielding (MY, kg) was determined by the formula reducing the milk yield to the basic rate of milk fat content equal to 3.4%:

$$MY = (Y_{305} \times MFF) \times 3.4^{-1} \times LW^{-1} \times 100,$$

where Y_{305} is milk yield for 305 days, kg; MFF is mass fraction of fat, %; LW is live weight, kg.

The degree of realization of the genetic potential (RGP, %) of animals was calculated as $RGP = OP \times EP^{-1} \times 100\%$, where OP is observed productivity, EP is expected productivity according to the parental index of cows (PCI), kg. PCI was calculated by the formula of N.A. Kravchenko (1969):

$$PCI = \frac{1}{4} (2M + MM + FM),$$

where M is mother's productivity, kg, MM is productivity of mother's mother, kg, FM is productivity of father's mother, kg.

The measurements of the main body parts of the animals were determined on the 3rd-5th month after calving, body indexes were calculated based on the ratio of the corresponding measurements [18]. To study the physique, a linear assessment method was used, which makes it possible to obtain an objective assessment of individual animals, groups of animals and herds as a whole, to conduct a corrective selection to eliminate the identified shortcomings in the exterior of animals and thus influence the type of physique. Each of the features used in linear estimation and has an independent value and was evaluated separately from others on a scale from 1 to 9 points: the average value of the trait is 5 points. In assessing the trait, biological extremes (–, +) of development were taken into account. Seventeen exterior traits were evaluated, and in the complex assessment of individuals on a 100-point scale, 5 traits were used. In addition to the traits included in the linear type score, conformation deficiencies that affect health and milk production were accounted [17, 19-21].

The health and metabolic state of the animals was assessed by morphological and biochemical parameters of blood. In 5 individuals from each group, at the 6th month of lactation, blood samples were taken into vacuum tubes (from the caudal vein in the morning before feeding). Total protein and its fractions, glucose, total cholesterol, enzymes alanine aminotransferase (AlAT), aspartate aminotransferase (AsAT), alkaline phosphatase, calcium, phosphorus, magnesium were determined in blood serum in accordance with the manufacturer's instructions (a biochemical automatic analyzer BioChem FC 120 and the supplied reagents, High Technology, Inc., USA). Complete blood count was performed on a Mindray BC-2800 Vet automatic hematology analyzer with Vet 2.3 software for animals (Mindray Medical International, Ltd., China). The resulting digital material was subjected to biometric processing [22] using the standard package of the Data Analysis program in the Microsoft Excel system for WINDOWS. Means (*M*), standard errors of means (±SEM), coefficients of variation (Cv, %) are presented. To assess the significance of differences between groups, the Student-Fisher test was used. Differences were statistically significant at $P > 0.95$.

Results. The intensity of animal rearing to a certain extent affects the completeness of the implementation of their genetically determined productive qualities. For optimal formation of glandular tissue in the udder, heifers should receive 300-350 kg of milk during the milking period, feeding on balanced, complete diets with a moderate amount, but high quality of feed [23]. Analyzing the data, it can be stated that in LLC Molochnik, rearing of replacement young animals is well-organized. Heifers from group 2 were effectively inseminated at the age of 15.5±2.8 months upon reaching a live weight of 391.8±20.9 kg. It should be noted that the animals that came from Europe were also grown intensively. So, in heifers of group 1, the age of the first insemination was 29 days less than in the animals of group 2, and the live weight was 0.4 kg more, the animals calved on average at about the same age (Table 1). The live weight of cows from group 2 at the first calving was 7.7 kg more than that of cows from group 1 (differences are not

significant).

1. Growth and milk yield in black-and-white Holstein replacement heifers of various origin (*M*±SEM, LLC Molochnik, Kursk Province, 2020)

Index	Group 1 (n = 15)	Group 2 (n = 15)
Age of the 1st insemination, months	14.6±1.5	15.5±2.8
Live weight at the 1st insemination, kg	392.2±15.7	391.8±20.9
Age at 1st calving, months	23.6±1.4	24.6±2.7
Live weight at the 1st lactation, kg	509.2±15.4	516.9±25.1
Milk yield for 305 days of lactation, kg	8667±94	8788±128
Milk yield adjusted for 3.4% fat content, kg	9508±86*	9822±97
Mass fraction of fat (MFF),%	3.73±0.11	3.80±0.07
Milk fat, kg	323.6±19.1	333.9±16.8
Mass fraction of protein (MFP), %	3.27±0.06	3.28±0.04
Milk protein, kg	283.4±17.4	288.2±4.4

N o t e. Imported animals of European selection (Denmark) were group 1, heifers derived from mothers born at the farm and subjected to adaptation were group 2.
* Differences between groups are statistically significant at P > 0.95.

The evaluation of the productive indicators of first-calf heifers indicates a high genetic potential of animals, that is, all individuals are selected for abundant milk production. The milk yield of cows for 305 days of lactation in group 1 averaged 8667±94 kg, in group 2 it was 121 kg higher. When the milk yield was adjusted to the normalized milk fat content, the difference increased to 314 kg and became significant (P > 0.95). At the first calving, the cows from group 2 also differed from the imported peers by a higher content of fat in milk (3.80±0.07 vs. 3.73±0.11%). As a result, the difference between the groups in milk fat yield was more significant. So, in group 2, the milk fat yield was 10.3 kg more than in group 1 (differences are not significant). We did not reveal any noticeable difference in the mass fraction of protein in milk (see Table 1). The yield of milk protein in cows in group 2 was 4.8 kg more than in group 1 (differences are not significant) (see Table 1). In group 1, the coefficient variability was 27.2% for milk yield, 2.8 and 1.6% for the mass fraction of fat and protein, respectively, and 15.7% for milk fat; in group 2, these indicators accounted for 22.4%, 2.9% and 1.8%, 22.1%, respectively.

The live weight of dairy cows is an important breeding trait that characterizes the development of animals and is associated with their productive qualities. One of the objective indicators in assessing the milk productivity of cows is the milk yield coefficient, which shows the amount of milk produced per lactation per 100 kg of live weight. The coefficient of milk production makes it possible to judge the constitutional orientation of animals. In our studies, animals in group 2 were had a higher indicator, 1900±30.7 kg vs. 1867±23.5 kg. Given these data, it can be concluded that in terms of productivity, all experimental heifers belonged to the dairy type.

2. Values of PCI (parental indices of cows) **and RGP** (realization of genetic potential) **in replacement black-and-white Holstein replacement heifers of various origin** (*M*±SEM, Molochnik LLC, Kursk Province, 2020)

Index	Group 1 (n = 15)	Group 2 (n = 15)
PCI for milk yield, kg	9309±171	9437±134
PCI by mass fraction of fat (MFF),%	3.75±0.14	3,77±0,11
PCI by mass fraction of protein (MFP), %	3.27±0.05	3,30±0,07
Actual milk yield (MY), kg	8667±94	8788±128
Actual MFF, %	3.73±0.11	3,80±0,07
Actual MFP, %	3.27±0.06	3,28±0,04
RGP for milk yield, %	93.1	93,0
RGP for MFF, %	99.5	100,7
RGP for MFP, %	100.0	99,3

N o t e. Imported animals of European selection (Denmark) were group 1, heifers derived from mothers born at the farm and subjected to adaptation were group 2.

Animals of groups 1 and 2, as descendants of highly valuable parents, inherited high productive indicators. The calculation of parental indices of mothers of experimental cows showed that, with the exception of milk yield, they differed insignificantly in other traits, that is, the phenotypic realization of their genetic inclinations turned out to be approximately at the same level. Realization of the genetic potential was somewhat higher by MFF (100.7%) in first-calf heifers of group 2, by MFP in group 1 (100.0%) (Table 2).

The genetic potential of animal productivity is realized under the influence of paratypic factors in specifically created conditions for growing, keeping, feeding and exploitation. The body type of Holstein cattle, along with productive indicators, is one of the selected traits used in the selection improvement of animals. The practice of breeding dairy breeds has shown the existence of a positive relationship between the productivity and duration of the economic use of cows with a well-developed physique.

3. Linear scores of exterior in replacement black-and-white Holstein replacement heifers of various origin (*M*±SEM, Molochnik LLC, Kursk Province, 2020)

Linear trait	Group 1 (n = 15)		Group 2 (n = 15)	
	M±SEM	*Cv*, %	*M*±SEM	*Cv*, %
Linear score A, points				
Trunk depth	5.7±0.3	20.2	5.4±0.3	22.5
Fortress physique	5.3±0.2*	22.1	4.6±0.2	21.8
Dairy cow stature	5.6±0.2	22.8	5.2±0.2	22.0
Sacrum length	4.5±0.2	25.6	4.2±0.2	26.5
Pelvic position	5.2±0.3	14.4	4.8±0.2	23.2
Pelvis width	5.4±0.3	21.0	5.1±0.2	21.4
Muscularity	5.0±0.2	17.7	4.8±0.1	16.8
Position of the hind legs	4.8±0.2	24.7	5.0±0.1	24.2
Hoof angle	4.8±0.2	15.0	4.3±0.	17.0
Attachment of the front udder	4.8±0.2	20.7	4.5±0.2	22.8
Length of front udder	5.4±0.2	21.6	5.1±0.1	28.3
Height of udder attachment	5.3±0.3	21.5	4.9±0.2	21.6
Rear udder width	5.7±0.3	20.8	5.3±0.2	19.4
Udder furrow	5.4±0.3	24.2	5.3±0.1	18.6
Udder bottom position	5.9±0.2	19.5	5.6±0.2	20.4
Location of the anterior nipples	5.1±0.2	24.6	4.9±0.1	27.1
Nipple length	5.3±0.1	15.8	5.3±0.1	20.7
Comprehensive assessment according to system B (100-point scale)				
Body volume	83.1±0.5	5.2	82.2±0.6	8.1
Expression of milk traits	83.8±0.7*	6.4	81.7±0.5	7.8
Limbs	84.9±0.4*	5.1	83.5±0.4	7.2
Udder	82.5±0.6	5.5	81.6±0.5	7.5
General appearance	82.7±0.5	7.0	81.9±0.5	6.4
Overall score	83.0±0.4	3.7	81.7±0.5	3.6

Note. Imported animals of European selection (Denmark) were group 1, heifers derived from mothers born at the farm and subjected to adaptation were group 2.
* Differences between groups are statistically significant at P > 0.95.

A linear assessment of animal physique [24, 25] objectively determines the individual constitution in dairy cattle based on independent indicators for each parameter [26-28]. Table 3 shows linear measurements of the exterior of first-calf heifers. We found significant differences in favor of animals of group 1 for body strength by 0.7 points (significance criterion td = 2.13), milking characteristics by 1.1 points (td = 2.13), limbs by 1.4 points (td = 2.13). The angle of the hoof formed by the front wall of the hoof of the hind limb with the floor plane was close to ideal (5 points, 45°). An acute angle leads to rapid wear of the heel of the hoof, a blunt (more than 50°, "butt hoof") poorly amortizes the load on the joints of the hind legs. It should be noted that other indicators, except for the setting of the hind legs, were higher in the animals of group 1, although the observed differences were not significant.

Cows of group 1 showed high variability for the sacrum length (25.6%), setting of the hind legs (24.7%), location of the front teats (24.6%), udder furrow

(24.2%), of group 2 — for the length of the anterior udder lobes (28.3%), the location of the anterior nipples (27.1%), the sacrum length (26.5%), the position of the pelvis (23.2%).

As per the set of features in the 100-point system, animals of group 1 had a slight advantage (see Table 3). The volume of the body was higher by 0.9 points, the classification score of the udder was higher by 0.9 points, and the general appearance was higher by 0.8 points vs those in cows of group 2 (differences are not significant). According to five classification features, animals of group 1 had a total score of 83.0 points, of roup 2 81.7 points. A difference of 1.3 points tended to be close to significant (td = 1.75, P > 0.90). The complex assessment data showed that all animals were of the good+ body type, the difference between the groups was not significant, and the variability of the indicators was low.

4. Body measurements in replacement black-and-white Holstein replacement heifers of various origin (*M*±SEM, Molochnik LLC, Kursk Province, 2020)

Measurement, cm	Group 1 (*n* = 15)		Group 2 (*n* = 15)	
	M±SEM	*Cv*, %	*M*±SEM	*Cv*, %
Height at the withers	137.5±1.3*	3.00	135.4±2.1	2.77
Rump height	145.3±1.4*	2.81	142.4±1.7	3.02
Chest depth	73.8±0.7	2.97	73.7±0.4	4.88
Chest width	46.4±0.4*	8.06	43.3±0.4	8.00
Width in hook bones	54.4±0.8*	4.34	51.1±0.9	3.66
Width at ischial tuberosities	37.1±0.5*	6.12	35.1±0.4	5.89
Oblique torso length with a stick	164.4±0.5	4.08	163.1±0.4	3.87
Chest girth	197.5±0.8	5.07	195.7±1.1	5.75
Pastern girth	19.2±0.4	6.00	19.2±0.4	5.68

N o t e. Imported animals of European selection (Denmark) were group 1, heifers derived from mothers born at the farm and subjected to adaptation were group 2.
* Differences between groups are statistically significant at P > 0.95.

Comparative analysis of body measurements revealed differences between groups (Table 4). At the age of the 1st calving, the animals were quite tall, in group 1 and group 2, the height at the withers averaged 137.5±1.29 cm and 135.4±2.09 cm, respectively, in the sacrum 145, 3±1.4 and 142.4±1.7 cm, respectively. Moreover, the difference between individuals in height at the withers and rump (respectively 2.1 cm and 2.9 cm) was significant (td = 2.46 and td = 2.20). The width of the chest, the width in the hook bones and the width in the ischial tuberosities in the first heifers from group 1 were significantly higher, by 3.1 cm (td = 4.07), 3.3 cm (td = 2.13) and 2.0 cm, respectively (td = 2.95). It should be noted that, according to other measurements (except for the girth of the metacarpus), first-calf heifers from group 1 were characterized by higher rates. Despite somewhat lower body measurements, the animals of group 2 also had a fairly good physique.

In contrast to the comparative characteristics of measurements expressed in absolute values, the use of physique indices makes it possible to obtain relative numerical indicators characterizing the exterior type of dairy cattle in the relative harmony of all articles [29]. Dairy cattle have a lower stretch index. In our studies, in cows in group 1, it was within 119.6%, in group 2, it was 2.7% higher (differences are not significant, Table 5). The pelvic-thoracic and thoracic indices testify to the pronounced milk type of the evaluated animals. Thus, the chest index in cows from group 1 was 3.7% higher than in individuals in group 2 (differences are not significant). The overrun index (compactness, index of general development and, in particular, live weight) was higher in first-calf heifers from group 1. The difference (3.2%) tended to be significant (td = 1.75, P > 0.90). Heifers from group 2 were characterized by a higher overgrowth index. According to the bone index, the differences between the groups were insignificant, 14.0% in group 1 vs. 13.9% in group 2.

5. Body indexes (%) in black-and-white Holstein replacement heifers of different origin (Molochnik LLC, Kursk Province, 2020)

Index	Group 1 (n = 15)		Group 2 (n = 15)	
	M±SEM	Cv, %	M±SEM	Cv, %
Leggy	46.30±0.19	4.5	45.20±0.88	6.7
Lengthiness	119.60±0.24	6.6	122.30±2.97	8.6
pelvic-thoracic	85.30±0.33	9.9	84.70±1.05	7.5
Thoracic	62.90±0.31	5.4	59.20±0.96	7.1
Downed	120.10±1.18	8.0	116.90±1.04	8.3
Overgrowth	105.70±1.41	4.7	106.70±2.11	4.4
Bonyness	14.00±0.07	5.3	13.90±0.06	6.2

N o t e. Imported animals of European selection (Denmark) were group 1, heifers derived from mothers born at the farm and subjected to adaptation were group 2.

Commercial dairy farming provides for the intensive exploitation of animals, as a result of which their body is constantly exposed to a wide variety of stressors. Stress negatively affects many physiological functions, the intensity of metabolic processes, which affects the health and productivity of cows. Metabolic disorders are one of the main factors hindering the realization of the genetic potential of animals [30, 31].

The results of a biochemical analysis of the blood of first-calf heifers of both groups indicated a properly organized, complete and balanced feeding. At the 6th month of lactation, all the studied biochemical parameters of the blood of animals varied within the physiological norm. The concentration of total protein in the blood serum in group 1 averaged 83.11±5.62 g/l, in group 2 it was higher by 0.67 g/l (differences are not significant) (Table 6), which indicates the compliance of protein nutrition with current standards.

6. Biochemical parameters of blood serum in black-and-white Holstein replacement heifers of different origin (M±SEM, Molochnik LLC, Kursk Province, 2020)

Parameter	Group 1 (n = 5)	Group 2 (n = 5)
Total protein, g/l	83.11±5.62	83.78±6.04
Albumins (A), g/l	27.80±1.73	27.17±1.82
Globulins (G), g/l	54.02±3.94	55.75±5.02
A/G	0.51±0.06	0.49±0.07
Glucose, mmol/l	3.74±0.11	3.71±0.16
Total cholesterol, mmol/l	3.63±0.28	3.76±0.22
Alanine aminotransferase, IU/l	27.02±2.06	25.19±1.93
Aspartate aminotransferase, IU/l	80.86±5.72	81.95±6.64
Alkaline phosphatase, IU/l	128.02±8.47	127.16±8.18
Ca, mmol/l	2.16±0.09	2.20±0.12
P, mmol/l	2.28±0.17	2.34±0.19
Ca/R	0.95±0.13	0.94±0.16
Mg, mmol/l	0.98±0.06	0.94±0.08

N o t e. Imported animals of European selection (Denmark) were group 1, heifers derived from mothers born at the farm and subjected to adaptation were group 2.

Aminotransferases belong to a group of enzymes indicative of the functional state of the liver, an organ involved in all metabolic processes [32], including the balance of protein nutrition. We found out that in the examined animals the activity of AlAT, AsAT and alkaline phosphatase was within the physiological norm, the differences in the indices between the groups were insignificant. The same can be said about the content of calcium and phosphorus in the blood serum (2.16-2.20 mmol/l and 2.28-2.34 mmol/l, respectively) and the ratio of these elements characterizing the state of calcium-phosphorus metabolism (0, 95±0.13 and 0.94±0.16).

Blood is the connective tissue of the internal environment of the body, participating in all processes occurring in it, while changing both qualitatively and quantitatively. Morphological parameters of blood vary and take values that are optimal for the adaptation of an individual to changing environmental conditions [33]. We noted a slight increase in the number of leukocytes in the blood of cows

of European selection, which may be due to the protective and adaptive reactions of their body (Table 7), while the number of erythrocytes and the level of hemoglobin were slightly lower (see Table 7).

7. Hematological parameters in black-and-white Holstein replacement heifers of different origin (M±SEM, Molochnik LLC, Kursk Province, 2020)

Parameter	Group 1 (n = 5)	Group 2 (n = 5)
Leukocytes, $\times 10^9$/l	8.37±1.99	8.19±1.43
Erythrocytes, $\times 10^{12}$/l	7.33±0.88	7.54±1.05
Hemoglobin, g/l	99.86±3.27	101.00±3.44
Hematocrit, %	32.60±1.11*	26.60±1.16

N o t e. Imported animals of European selection (Denmark) were group 1, heifers derived from mothers born at the farm and subjected to adaptation were group 2.
* Differences between groups are statistically significant at P > 0.95.

The number of red blood cells in the blood of cattle is determined by sex, age, productivity, feeding and housing conditions. The ratio of the volume of formed elements to the total volume of blood characterizes the hematocrit. The sizes of erythrocytes, as a rule, are inversely related to their number per unit volume of blood and the metabolic activity characteristic of the body [34, 35].

Our data on the acclimatization features and productive qualities of dairy cattle are consistent with the results of other researchers. Thus, it is reported about the successful adaptation of Holstein cattle of domestic and American selection in the conditions of the Kabardino-Balkarian Republic [36, 37]. It is important to assess the state of cattle of various selections in conditions of ecological trouble. Realization of the genetic potential of black-and-white Holstein first-calf heifers of Hungarian selection of different lines is shown (from 78.9 to 91.42%, an average 305-day milk productivity of 6957 kg, a fat content of 3.75% and protein content of 3.06%) [38].

The study of blood parameters is an objective method for assessing the functional state of an animal's body in conditions of adaptation to technology and environmental factors [2, 39]. For example, it was noted that in Holstein animals brought to the Samara region from Holland, each new generation improved blood morphology and biochemical parameters, and elevated the level of cellular and humoral factors of natural resistance. As a result, adaptation to new natural-ecological, fodder and technological conditions took place [40].

Large-scale studies of economically useful features of Holstein heifers imported from the USA, Denmark, Germany and Australia to the Lower Volga region showed that animals of American and German origin had a higher level of natural resistance and adaptability to the natural and climatic conditions of the region. They also had higher productivity and improved reproductive function compared to their peers of Danish and Australian selection. A comparative study of economically useful traits of black-and-white heifers of the Leningrad and Danish selection in the conditions of the Rostov region showed that Danish heifers had a higher resistance to changing environmental conditions compared to their peers of the Leningrad selection. In the conditions of the Central Non-Black Earth Region, imported black-and-white Holstein animals realize their high genetic potential of milk productivity, significantly exceeding domestic breeds in terms of milk yield [41-44].

So, in our studies, we compared animals of European selection (Denmark) (group 1) and those derived from mothers born at the farm and undergone adaptation (group 2), according to the age of the 1st calving, milk productivity, milk fat and protein yield, body measurements, exterior signs, biochemical parameters and blood morphology. Significant (P > 0.95) differences occurred only in milk yield normalized to standard fat content (by 134 kg in favor of animals born at the farm), in exterior, i.e., in the strength of the physique, milking characteristics,

the condition of the legs in favor of imported animals. Hematocrit was also higher in imported animals. Animals of group 1 and group 2 realized their genetic potential for milk yield by 93.1 and 93.0%, for mass fraction of fat by 99.5 and 100.7%, of protein by 100.0 and 99.3%. When comparing the type of physique according to five classification criteria, the scores for the groups were 83.0 and 81.7 points. A slight increase in the number of leukocytes occurred in first-calf heifers of European selection. It can be assumed that in this group, the ongoing adaptation led to more intense metabolic processes. Thus, under conditions that meet the biological needs of animals, the acclimatization of cows of European selection is quite successful.

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ADAPTIVE RESPONSES OF CATTLE DIGESTIVE SYSTEM AS INFLUENCED BY DIETARY ULTRAFINE IRON PARTICLES COMBINED WITH FAT DIETS

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Abstract

Fats are a concentrated source of energy; the fatty components in the diets of farm animals are economically feasible and efficient. The fatty supplements in the diet of cattle improves the palatability of the diets and reduces the rate of feed passage through the gastrointestinal tract, which increases the availability of nutrients and increases livestock productivity. However, some papers indicate a decrease in the digestibility of nutrients in the presence of dietary fat. To increase the availability of nutrients in rations, it is necessary to use additional components in the feed, in particular, ultrafine particles. They, unlike their counterparts in micro- and macro-form, have higher physical activity, chemical neutrality, and high bioavailability ensured by an increased surface area. The limited practical use of ultrafine particles (UFP) in animal husbandry is due to insufficient knowledge about their biological effects on metabolism. Here, for the first time, we evaluated the effect of an ultrafine iron preparation on pancreatic secretion, enzymatic activity of pancreatic juice, morphological and biochemical parameters of blood, and digestibility of feed enriched with sunflower and soybean oils. The aim of our research was to characterize ultrafine iron particles as modulators of metabolic activity when using vegetable fats in the diet of ruminants. The experiments were carried out on Kazakh white-headed calves aged 8 months with an average weight of 120-130 kg (a vivarium of Federal Research Centre of Biological Systems and Agrotechnologies RAS, October 2019-October 2020). A Latin square 4×4 design was applied in five replicates. Control group were fed a standard balanced basal diet (BD), group I — BD supplemented with UFP Fe, group II — BD added with sunflower oil, group III — BD added with sunflower oil + UFP Fe, group IV — BD added with soybean oil, and group V — BD added with soybean oil + UFP Fe. Oils replaced 3 % dry matter of feed concentrates. To produce UDP Fe, we used electric explosion of a conductor in an argon atmosphere (Advanced Powder Technologies, Tomsk). UFPs Fe (d = 90 nm, Z-potential 7.7±0.5 mV) are 99.8 % Fe. Before use, ultrafine iron particles were dispersed in a physiological solution using UZDN-2T (NPP Akadempribor, Russia) (35 kHz, 300 W, 10 μA, 30 min) and added at a dosage of 2.2 mg per animal. To study the exocrine function of the pancreas, a duodenal anastomosis surgery technique was performed. Pancreatic juice and chyme samples were collected over 8 hours with a 60 min interval. The activity of amylase, proteases, and lipase was measured. The blood NO metabolites and trypsin activity were measured. Feed digestibility was assessed on day 7 in balance experiments based on the amount of the consumed feed, uneaten feed and excreted feces. The digestibility coefficient (DC) was calculated as the ratio of the digested nutrients to those entered the body. Dry matter, crude protein, fat and ash contents were measured. Blood for quantitative analysis of the morphological and biochemical parameters was sampled in the morning on an empty stomach on day 7 of the experiment. The research data indicate that dietary UFP Fe with the fat diets contributed to a significant ($p \leq 0.05$) increase in the digestibility of crude fat, organic matter and nitrogen-free extractive substances, while the digestibility of crude fiber and crude protein decreased. Enrichment with UFP Fe and fatty ingredients had a stimulating effect on pancreatic secretion, leading to an increased amount of pancreatic juice. The

UFP Fe selectively changed the activity of the digestive pancreatic enzymes. UFP Fe added to BD, increased the activity of lipase by 35.7 %, intestinal proteases by 43.1 % while the amylolytic activity decreased by 28.8 %. Dietary UFP Fe combined with sunflower and soybean oils reduced the enzymatic activity of the pancreas compared to the control: in group III, the activity of lipase and intestinal proteases increased by 12.1 and 16.7%, respectively ($p \leq 0, 05$), in group V — by 133.2 and 38.4 %, respectively ($p \leq 0.05$). The BD supplementation with fatty ingredients, alone and in combination with UFP Fe, increased the level of NO-metabolites in all experimental groups compared to the control. When replacing BD with fat diets, the trypsin activity increased in group II by 106.6% ($p \leq 0.05$), in group IV by 130.9 % ($p \leq 0.05$). Added UFP Fe reduced the trypsin activity. Morphological analysis revealed a statistically significant ($p \leq 0.05$) increase in the hemoglobin content in calves of the experimental groups, in group I by 9.7 %, in group II by 31.2%, in group III by 41.9 %, in IV by 28.0 %, in V by 30.1 %. A biochemical blood test showed that all the studied parameters were within physiological norms, however, it should be noted that UFP Fe had a stimulating effect on protein, fat and carbohydrate metabolism in calves. A significant increase in the de Ritis ratio occurred in the groups that fed UFP Fe with fat diets, up to 3.98 in group III and 4.1 in group V ($p \leq 0.05$). As compared to the control, the bilirubin index (BI) increased by 17.8 % ($p \leq 0.05$) in group I and by 5.5% ($p \leq 0.05$) in group IV, in all other groups the BI values were lower than in the control.

Keywords: ultrafine particles, iron, blood morphology, blood biochemical test, pancreas, enzymes, pancreatic juice, chyme, cattle, fats, sunflower oil, soybean oil

Today, the production of high-quality beef has transformed fat from a simple additive to a valuable high-energy cereal substitute, energy source, and cellular metabolism modifier [1]. Fats as a feed agent serve as concentrated sources of energy, contain and transport fat-soluble vitamins, provide the body with essential fatty acids, and also give the feed certain aromatic, taste and structure. The addition of fat components to complete diets for farm animals is cost effective and efficient [2, 3].

The inclusion of vegetable oils (coconut, palm, soybean, sunflower, flaxseed and canola) in ruminant diets reduced in vitro intestinal methane production by 40.55-48.58%. It did not affect the pH of the rumen, the amount of microbial protein, the digestibility of dry and organic matter [4]. The addition of sunflower oil to the diet of cattle (cattle) led to a decrease in the number of protozoa, a decrease in methanogenesis and the concentration of ammonia nitrogen, and an improvement in the production of microbial biomass and propionic acid in the rumen [5]. However, some researchers point to a decrease in nutrient digestibility in the presence of fat [6, 7]. There was a decrease in the digestibility of neutral detergent fibers in the rumen due to the addition of fat, while the efficiency of microbial protein synthesis increased, and the abundance of protozoa tended to decrease [8]. Fat supplements led to a statistically significant ($p \leq 0.01$) decrease in the digestibility of organic matter and neutral detergent fiber in the intestines of young fattening cattle [9].

To increase the effectiveness of feed products, such components of diets as mineral supplements, in particular metal nanopowders, are considered and studied [10-12]. Currently, the influence of ultrafine metal particles as independent additives, as well as in combination with other feed components on metabolic processes in the gastrointestinal tract, the enzymatic activity of the digestive glands, and the composition of the microbiome is being actively studied. These supplements have been shown to be effective in reducing side effects, improving nutrient bioavailability, and increasing performance [13-15].

In the presented work, we for the first time established the effect of an ultrafine iron preparation on pancreatic secretion with the additional inclusion of sunflower and soybean oils in the diet of calves. An increase in the enzymatic activity of pancreatic juice, as well as the digestibility of the nutritional components of the feed, was noted.

The purpose of the study was to evaluate the possibility of using ultrafine iron particles as modulators of the activity of metabolic processes when vegetable fats (sunflower and soybean oils) are added to the diet of calves.

Materials and methods. In vivo experiments were carried out from October 2019 to October 2020 at the Federal Scientific Center for Biological Systems and Agrotechnologies of the Russian Academy of Sciences on calves (*Bos taurus taurus*) of the Kazakh white-headed breed. Groups of four animals aged 8 months (average live weight 120-130 kg) were formed. The experiment was performed according to the scheme of the Latin square 4×4 in five repetitions.

Animal care and research were carried out in accordance with the instructions and recommendations of Russian Regulations, 1987 (Order No. 755 on 12.08.1977 the USSR Ministry of Health) and The Guide for Care and Use of Laboratory Animals (National Academy Press Washington, D.C. 1996). Every effort has been made to minimize animal suffering and reduce the number of samples taken. Animals were kept in separate metabolic cages (1.0×2.2 m) in a room with optimal temperature and humidity (during the experiment, the ambient temperature was maintained between 23 and 25 °C), with free access to water.

The calves of the control group received a basal diet (BD) which included mixed grass hay (2 kg), a mixture of concentrates (1.5 kg), corn silage (5 kg), wheat straw (1 kg), fodder molasses (0.1 kg), table salt (0.04 kg), vitamin and mineral premix. To the BD, ultrafine particles (UFP) of Fe were added in group I, sunflower oil in group II, sunflower oil + UFP Fe in group III, soybean oil in group IV, soybean oil + UFP Fe in group V. Oils were added at the rate of 3% of the dietary dry matter by replacing the concentrated part of the diet. Animals were fed twice a day, in the morning and in the evening in equal proportions. The diets met the need for nutrients and energy but differed in the fatty acid composition of the vegetable fats [16, 17].

Ultrafine iron particles obtained by electrical explosion of a conductor in an argon atmosphere (Advanced Powder Technologies, Russia). UFP (d = 90 nm, Z-potential 7.7±0.5 mV) were 99.8% Fe. Before adding to the diet, they were dispersed in physiological saline using UZDN-2T (NPP Akadempribor, Russia) (35 kHz, 300 W, 10 μA, 30 min). Animals were fed UFP Fe after mixing with a concentrated feed mixture at a dose of 2.2 mg per snimal.

To study the exocrine function of the pancreas, duodenal anastomosis was performed [18].

The tests were carried out after 16 hours of empty stomach. Pancreatic juice and chyme were collected over 8 hours at 60 min intervals. After taking the first sample, the animals were fed, and the juice and chyme collection continued. The amount of juice and the enzymatic activity of juice and chyme were determined in cito.

Amylase activity was measured by the Smith-Roe method modified for high values of the parameter [19]. Protease activity was evaluated by Hammerstenpurified casein hydrolysis under calorimetric control (λ = 450 nm) [20], lipase and α-amylase activity, concentration of total protein, phosphorus and calcium were measured using an automatic biochemical analyzer CS-T240 (DIRUI Industrial Co., Ltd, China) with commercial veterinary biochemical kits (ZAO DIACON-DS, Russia) [21].

The concentration of blood NO metabolites was determined spectrophotometrically with the Griess reagent (an Infinite PRO F200 microplate analyzer, Tecan Austria GmbH, Austria; λ = 540 nm) [22].

Blood trypsin activity was determined using a CS-T240 automated biochemical analyzer (DIRUI Industrial Co., Ltd, China), sodium benzoyl-DL-arginine-4(p)-nitroanilide hydrochloride (BAPN) was used as a substrate.

Feed digestibility was assessed for 7 days in balance experiments, based on the amount of feed consumed by animals, uneaten leftovers, and the amount of excreted feces. The digestibility coefficient (DC) was calculated as the ratio of

digested nutrients to those taken. After freezing, drying, and homogenization, the content of dry matter, organic matter, crude protein, crude fat, nitrogen-free extractives (NFE), and ash in feces and feed was analyzed according to the recommendations of the Association of Official Agricultural Chemists [23]. Digestibility was assessed as described by S. Hashemi et al. [24].

Blood for morphological and biochemical tests was taken from the jugular vein into vacuum tubes with a coagulation activator (thrombin) in the morning on an empty stomach on day 7 of the experiment. A CS-T240 automatic analyzer (DIRUI Industrial Co., Ltd, China) and commercial kits for veterinary medicine (DiaVetTest, Russia) were used.

Statistical analysis was performed using ANOVA methods (Statistica 10.0 software package, StatSoft, Inc., USA) and Microsoft Excel. Means (*M*) and standard errors of the means (±SEM) are sunmitted. The significance of differences between the compared indicators was determined by Student's *t*-test. Differences were considered statistically significant at $p < 0.05$.

Results. Table 1 shows the composition and quality indicators of the diets fed to the experimental calves. The difference in the content of crude fat, crude protein and metabolic energy was quite significant, 45.5, 8.3 and 12.9%, respectively.

1. Composition and quality parameters of the diets fed to Kazakh white-headed calves (*Bos taurus taurus*) in the experiment (FSC of biological systems and agricultural technologies RAS, 2019-2020)

Parameter	Diet		
	basal	added with sunflower oil with soybean oil	added with soybean oil
Composition of the diet			
Mixed grass hay, kg	7.0	7.0	7.0
Concentrates, kg	2.0	2.0	2.0
Sunflower oil, kg		0.3	
Soybean oil, kg			0.3
Molasses fodder, kg	0.6	0.6	0.6
Premix PK-60, kg	0.06	0.06	0.06
Salt, kg	0.02	0.02	0.2
UFP Fe, mg		2.2	2.2
Nutritional value of the diet			
Dry matter, kg	8.42	8.42	8.42
Crude fiber, kg	2.56	2.56	2.56
Crude fat, kg	0.244	0.355	0.355
Crude protein, kg	0.72	0.66	0.66
NFES, kg	5.4	5.0	5.0
Calcium, g	42.2	42.6	43.2
Phosphorus, g	30.0	29.8	30.4
ME, MJ	63.0	71.1	71.1

N o t e. NFES — nitrogen-free extractive substances, ME — metabolic energy. The vitamin-mineral premix contains Mn — 48 mg, Zn — 36 mg, Fe — 60 mg, Cu — 10 mg, — - 0.24 mg, Co — 0.12 mg; vitamin A — 2640 IU, vitamin D — 302 IU; vitamin E — 17 mg (per 1 kg of concentrate).

The main challenge in the production of high quality beef is to provide animals with the necessary nutrients to meet metabolic needs and increase productivity. However, traditional grains in cattle diets adversely affect dry matter content and inhibit fiber digestion [25, 26]. The use of fats in diets is essential in the feeding of farm animals. Lack of fat leads to growth retardation, disruption of reproductive function, reduced productivity and poor product quality. The presence of a large amount of fat in the diet creates a load on the digestive system as a whole, especially in cattle. When diets are saturated with fats, the activity of digestive enzymes changes, as a result, complex food components are not broken down well enough and are poorly absorbed [27]. Dietary fat that is not biolyzed and biohydrogenated by rumen microorganisms but is digested in the lower digestive tract is known as bypass fat or rumen protected fat (inert fat) [28]. The introduction of fats into the diet of cattle grazing on pastures increases the production of meat and dairy products. However, increasing the amount of fats and fatty acids

inhibits the digestion of fiber in the rumen and reduces the digestion of organic matter in the anterior part of the stomach [29].

In our work, with the introduction of sunflower and soybean oil into the diets, the digestibility of crude fat decreased by 38.2 and 10.9% ($p \leq 0.05$) vs. control, respectively (Table 2). UFP Fe added to the basal diet increased the digestibility of organic matter by 9.6% ($p \leq 0.05$), crude fat by 2.2% ($p \leq 0.05$), and nitrogen-free extractive substances by 9% ($p \leq 0.05$). Z. Khan et al. [30] revealed that feeding calves with a diet high in iron reduced average daily body weight gain, dry matter intake, and feed nutrient digestibility.

2. Nutrient digestibility coefficients (%) **in Kazakh white-headed calves (*Bos taurus taurus*) fed diets supplemented with vegetable oils and ultrafine Fe particles** (n = 4, M±SEM, FSC of biological systems and agricultural technologies RAS, 2019-2020)

Parameter	Group					
	control;	I	II	III	IV	V
Dry matter	74.3±0.04	70.8±0.03*	76.1±0.01*	72.1±0.02*	60.9±1.05	68.4±0.03*
Organic matter	87.8±0.30	96.2±0.40*	83.83±0.21*	98.7±0.42	59.4±0.03*	95.8±0.05*
Crude protein	76.3±3.60	71.0±4.10	81.4±2.30*	74.5±2.82	72.8±1.02	70.4±2.4
Crude fat	72.7±1.23	74.3±1.40*	44.9±1.88*	62.7±1.54*	64.8±0.75*	76.4±1.23*
Crude fiber	37.4±0.18	36.6±0.20	45.5±0.08*	37.5±0.12	43.7±0.45*	36.2±1.12
NFES	80.3±0.90	88.2±0.70*	82.3±1.10	91.8±0.93*	75.6±0.38*	84.3±0.24*

N o t e. NFES — nitrogen-free extractive substances. For a description of the groups, see the Materials and methods section.

* Differences from control are statistically significant at $p \leq 0.05$.

UFP Fe in the diets statistically significant increased the digestibility of crude fat, in group III vs. group II by 39.6% ($p \leq 0.05$), in group V vs. group IV by 17.9% ($p \leq 0.05$). A similar trend occurred in the digestibility of organic matter and NFES. It should also be noted that dietary UFP Fe reduced the digestibility of crude fiber and crude protein in groups I, III and V.

Additional enrichment of diets with mineral components and changes in the quantitative and qualitative composition of the feed significantly affect pancreatic secretion and the activity of digestive enzymes [31-34]. The structure and composition of the diet, as well as the volume and frequency of feeding, have a regulatory effect on digestive functions due to reflexive and humoral mechanisms.

In the first hour before feeding the animals, pancreatic secretion in all groups was significantly lower than after feeding. Secretion increased in the reflex and gastric phases, and then decreased in the intestinal phase, in the period 360-480 min after the start of measurements.

3. The amount of pancreatic juice (ml) **excreted in Kazakh white-headed calves (*Bos taurus taurus*) fed diets with vegetable fats and ultrafine Fe particles** (n = 4, M±SEM, FSC of biological systems and agricultural technologies RAS, 2019-2020)

Time, min	Group					
	control	I	II	III	IV	V
0-60	32.0±2.81	48.0±3.24	28.0±1.72	109.0±19.02	18.0±2.43	88.0±12.23
60-120	66.0±3.42	76.0±3.61*	48.0±3.51	138.0±6.52	78.0±6.72*	141.0±5.64*
120-180	67.0±5.32	80.0±5.12	58.0±4.62*	228.0±4.11*	72.0±7.12	218.5±14.64
180-240	59.0±4.71	84.0±6.04	50.0±5.03	156.5±1.93*	62.0±5.83	184.5±3.83
240-300	55.5±5.20	88.0±4.22	48.0±3.72	142.5±9.92	5.0±4.31	168.5±11.31*
300-360	59.5±4.11	91.0±2.81	47.0±3.30*	77.0±5.02	48.0±5.31	81.5±18.04
360-420	67.0±6.31	90.0±4.52	38.0±5.43	23.0±6.23	46.0±3.82	39.0±6.40
420-480	51.5±4.70	84.0±3.02*	50.0±5.22	40.0±2.90	42.0±3.22	28.0±3.91
0-480	457.5±37.83	641.0±32.40*	367.0±55.42*	914.0±55.53*	417.0±45.22*	949.0±75.81*

N o t e. During the first hour of the experiment, the indicators were recorded on an empty stomach. For a description of the groups, see the Materials and methods section.

* Differences from control are statistically significant at $p \leq 0.05$.* Differences from control are statistically significant at $p \leq 0.05$.

When replacing the control compound feed with experimental samples with UFP Fe, the amount of pancreatic juice produced increased, which indicates an increase in the load on the pancreas with such diets (Table 3). Thus, when using the basal diet with UFP Fe, the amount of pancreatic juice increased by 40.1% over the entire time of the experiment ($p \leq 0.05$). With sunflower oil added to the diet, the production of pancreatic juice decreased by 19.8% ($p \leq 0.05$), with soybean oil by 8.8% ($p \leq 0.05$) vs. control. Dietary UFP Fe stimulated the secretion of pancreatic juice by 149.0% in group III vs. group II, and by 127.6% in group V vs. group IV ($p \leq 0.05$).

4. Activity of pancreatic juice enzymes in Kazakh white-headed calves (*Bos taurus taurus*) fed diets with vegetable fats and ultrafine Fe particles ($n = 4$, $M \pm$SEM, FSC of biological systems and agricultural technologies RAS, 2019-2020)

Parameter	Group					
	control	I	II	III	IV	V
Lipase, U/l	90.9±18.2	123.4±19.4*	773.0±14.8*	101.9±12.7*	667.0±37.0*	212.0±11.3*
Amylase, $mg \cdot ml^{-1} \cdot min^{-1}$	5137.5±450.0	3337.5±330.0	2537.0±400.0	1698.4±330.0	1931.0±69.0	1456.0±34.0
Proteases, $mg \cdot ml^{-1} \cdot min^{-1}$	133.5±24.3	191.0±22.6	249.0±21.1*	155.8±14.6*	200.0±12.6*	184.8±13.5*
Total protein, g/l	0.46±0.12	0.48±0.16	0.18±0.01	0.38±0.01*	0.33±0.01	0.41±0.020
Phosphorus, mol/l	0.14±0.02	0.12±0.03*	0.03±0.00	0.10±0.00	0.08±0.01	0.10±0.010*
Calcium, mol/l	2.33±0.12	2.46±0.15*	2.43±0.22*	2.26±0.18	2.39±0.10*	2.41±0.12
α-Amylase, U/l	416.0±4.8	536.0±6.2	578.0±11.5	559.1±6.7	767.0±13.8	758.0±16.8

N o t e. For a description of the groups, see the Materials and methods section.
* Differences from control are statistically significant at $p \leq 0.05$.

The introduction of additional ingredients and a change in the qualitative composition of the diet lead to a selective change in the activity of digestive enzymes [35-38]. UHF Fe added to the BD led to a significant increase in the activity of lipase (by 35.7%, $p \leq 0.05$), intestinal proteases (by 43.1%) vs. a decrease in amylolytic activity (by 35.0%, $p \leq 0.05$). In group I, the content of phosphorus in the pancreatic juice decreased by 14.3% ($p \leq 0.05$) with an increase in the amount of Ca by 5.6% ($p \leq 0.05$) vs. control (Table 4).

Fatty diets stimulated lipase and intestinal proteases, in group II 8.5-fold ($p \leq 0.05$) and 1.9-fold ($p \leq 0.05$), respectively, in group IV 7.3-fold and 1.5-fold ($p \leq 0.05$) vs. control. Due to fat components in diets, the activity of the amylase decreased.

UFP Fe addede to the diets containing sunflower and soybean oils reduced the enzymatic activity of the pancreas, that is, the load on the pancreas decreased. In group III compared to control, the activity of lipase and proteases significantly increased, by 12.1 and 16.7%, respectively ($p \leq 0.05$), in group V by 133.2 and 38.4% ($p \leq 0.05$). Fat components in the diet increased the secretion of lipase, but not amylase. Obviously, an increase in the amount of any nutrient leads to an increase in the production of digestive enzymes in the pancreas.

A downward trend also occurred in the acticity of intestinal proteases. Proteolytic activity decreased by 52.0% for BD + UFP Fe vs. BD, but the differences were not significant. A statistically significant decrease in protease activity occurred, by 28.8% ($p \leq 0.05$) in group II, by 62.9% ($p \leq 0.05$) in group III, by 50.0% ($p \leq 0.05$) in group V, by 3.4% in group IV. The activity of lipase in the duodenal chyme increased in animals receiving fat diets, however, the introduction of UFP Fe led to a decrease in the parameter value. Thus, , lipolytic activity decreased 4.3-fold ($p \leq 0.05$) in group I, 4.6-fold ($p \leq 0.05$) in group III, and 4.5-fold ($p \leq 0$ .05) in group V.

Dietary UFP Fe significantly reduced the activity of pancreatic amylase in the duodenal chime, by 16.0% with dietary sunflower oil and by 66.0% with

dietary soybean oil ($p \leq 0.05$) (Fig. 1).

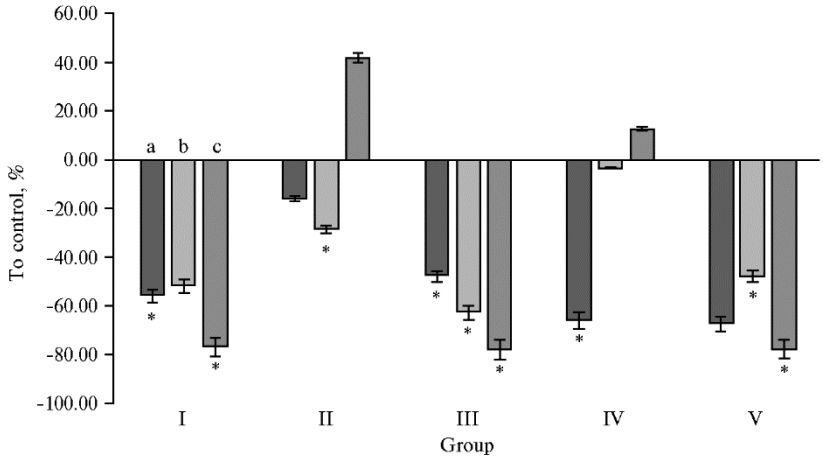

Fig. 1. Changes in the activity of digestive enzymes of pancreatic juice in duodenal chyme of Kazakh white-headed calves (*Bos taurus taurus*) fed diets with vegetable fats and ultrafine Fe particles vs. control: a — amylase, b — protease, c — lipase ($n = 4$, $M \pm$SEM, FSC of biological systems and agricultural technologies RAS, 2019-2020). For a description of the groups, see the Materials and methods section.

The activity of nitric oxide metabolites in the blood serum mediates a whole cascade of physiological processes, including the regulation of vascular tone, plasma and platelet hemostasis, neurotransmission and the formation of an immune response, inhibition of the proliferation of smooth muscle cells and has a significant effect on metabolic processes in the digestive tract [39]. In our study, an increase in the content of NO metabolites was observed in all experimental groups relative to the control values (Fig. 2).

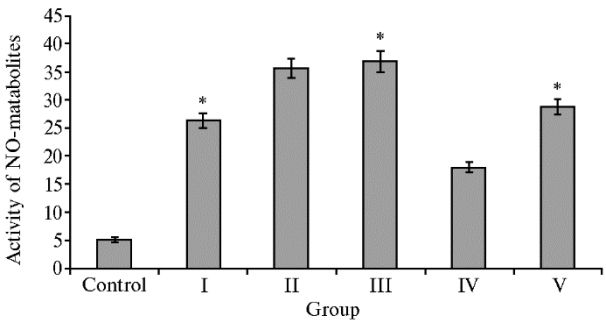

Fig. 2. The content of NO metabolites in the blood serum of Kazakh white-headed calves (*Bos taurus taurus*) fed diets with vegetable fats and ultrafine Fe particles ($n = 4$, $M \pm$SEM, FSC of biological systems and agricultural technologies RAS, 2019-2020). For a description of the groups, see the Materials and methods section.

* Differences from control are statistically significant at $p \leq 0.05$.

Additional administration of UFP Fe statistically significantly increased the content of nitric oxide metabolites 5.1-fold ($p \leq 0.05$) in group I, 7.2-fold ($p \leq 0.05$) in group III, 5.6-fold ($p \leq 0.05$) in group V. Insufficient production of NO in animals from the control group is associated with the development of disorders in the cardiovascular and other body systems. S.V. Rama Rao et al. [40] found that excessive production of NO, which provides an antimicrobial effect in inflammation, could turn from an adaptation link into a link in pathogenesis and become no less dangerous damaging factor for the body than NO deficiency. An increase for NO-metabolites in calves in group III fed UFP Fe indicated a compensatory reaction of the body to the changing lipid profile of the diet.

Indicators of amylase and lipase activity in blood serum may not always indicate the physiological stress of pancreatic function when changing diets, since there is an extrapancreatic production of these enzymes. Trypsin is the optimal marker for detecting changes in the physiological state of the pancreas, since it is

specific to this organ. It has been established that the entry of trypsin into the blood reduces the release of enzymes with pancreatic juice, while the administration of a trypsin inhibitor, on the contrary, is accompanied by an increase in the secretion of enzymes [41].

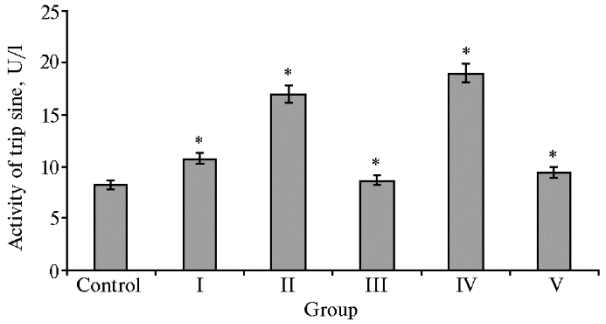

Fig. 3. Trypsin activity in the blood serum of Kazakh white-headed calves (*Bos taurus taurus*) fed diets with vegetable fats and ultrafine Fe particles (n = 4, M±SEM, FSC of biological systems and agricultural technologies RAS, 2019-2020). For a description of the groups, see the Materials and methods section.

* Differences from control are statistically significant at $p \leq 0.05$.

In our experiments, the introduction of UFP Fe into the diet in all experimental groups increased the activity of trypsin in the blood serum, which plays a critical role in initiating the cascade of activation of digestive enzymes in the intestine (Fig. 3). With the introduction of UFP Fe, trypsin activity increased by 31.2% ($p \leq 0.05$) vs. control. When the basal diet was replaced with fat diet, trypsin activity increased in group II by 106.6% ($p \leq 0.05$), in group IV by 130.9% ($p \leq 0.05$) vs. control. When UFP Fe was addaed to fat diets, trypsin activity decreased by 48.8% ($p \leq 0.05$) in group III vs. group II and by 50.5% ($p \leq 0.05$) in group V vs. group IV.

Trypsin activation by proteolytic breakdown of trypsinogen in the pancreas can lead to a series of events that induce pancreatic self-perception. One of the consequences of the autosomal recessive disease cystic fibrosis is insufficient transport of trypsin and other digestive enzymes from the pancreas [42].

5. Blood morphology in Kazakh white-headed calves (*Bos taurus taurus*) fed diets with vegetable fats and ultrafine Fe particles (n = 4, M±SEM, FSC of biological systems and agricultural technologies RAS, 2019-2020)

Parameter	Group					
	control	I	II	III	IV	V
Leukocytes, $\times 10^9$/l	7.5±1.32	7.1±1.20	7.2±1.63	6.9±1.32	11.8±2.22*	8.2±1.72*
Lymphocytes, $\times 10^9$/l	2.9±0.83	2.6±0.63	4.1±1.21	3.2±1.13	5.5±1.21	4.3±1.32
Monocytes, $\times 10^9$/l	1.3±0.32	1.2±0.25	1.5±0.31	1.2±0.22	2.5±0.42	1.6±0.33
LMR	2.21	2.13	2.72	2.61	2.22	2.50
Granulocytes, $\times 10^9$/l	1.90±0.63	2.10±0.52	5.30±0.82*	4.30±0.62*	4.80±0.61	4.36±0.71
Erythrocytes, $\times 10^{12}$/l	5.08±2.91	4.98±2.22	5.37±3.13	4.88±2.50	7.62±1.81*	4.86±1.61*
Hemoglobin, g/l	93.0±11.12	102.0±9.81*	122.0±13.80*	132.0±11.51*	119.0±14.70*	121.0±12.22*
Hematocrit, %	20.1±4.34	22.6±3.63	21.6±4.91	22.0±2.82	24.2±3.94	20.8±2.61
MCH, g/l	349±26.92	324±28.63	440±26.42	388±36.53	424±27.72	368±34.23
Platelets, $\times 10^9$/l	201±19.81	212±14.32	224±17.71	216±18.22	220±21.70	206±14.63

N o t e. LMR is the lymphocytes-to-monocytes ratio, MCH quantifies the amount of hemoglobin per red blood cell. For a description of the groups, see the Materials and methods section.

* Differences with the control group are statistically significant at $p \leq 0.05$.

Blood morphology in calves changed when the basal diet was replaced with the experimental diet supplemented with soybean oil. The number of leukocytes increased statistically significantly by 57.3% ($p \leq 0.05$) vs. control, but the added UFP Fe led to a 30.5% decrease in leukocytes vs. group IV ($p \leq 0.05$) (Table 5).

The addition of UFP Fe led to increased absorption of iron from the gastrointestinal tract, improved synthesis of iron-containing metabolites (including hemoglobin), and stimulation of erythropoiesis [32]. In experiments on 4-month-old heifers treated with iron nanopowder, the number of erythrocytes increased by 19.6%, and the amount of hemoglobin by 17.1% compared to control.

We found that the UHF Fe added to both control and fat diets reduced the number of lymphocytes and monocytes. The number of erythrocytes decreased in group I by 2.0% vs. control, in group III by 9.0% vs. group II, in group V by 36.2% ($p \leq 0.05$) vs. group IV.

The direct participation of iron in the hemoglobin synthesis contributed to an increase in the content of hemoglobin in the experimental groups treated with UFP Fe. The blood hemoglobin level in animals of the experimental groups increased statistically significantly ($p \leq 0.05$) in group I by 9.7%, in group II by 31.2%, in group III by 41.9%, in group IV by 28.0%, in group V by 30.1% vs. control. The high iron content of heme makes hemoglobin an ideal molecule for targeted iron extraction during endogenous exposure to UFP. Significant levels of blood erythrocytes and hemoglobin indicates more intense redox processes in the animsl body and corresponds to higher productivity parameters [43].

The highest number of platelets was noted in the experimental group II (224×10^9/l), which was 11.4% higher than the control value ($p \leq 0.05$). Competition for heme iron between blood cells and a pool of exogenous bacteria together with an intense erythropoiesis, low absorption of iron by the endothelium and increased motility of the gastrointestinal tract could cause some increase in the parameter.

The number of formed elements of the blood leukocytes in control and test groups was within the physiological norm. In the leukogram which reflects the percentage ratio of different populations of leukocytes, no deviations from the norm were recorded. The lymphocytes-tomonocytes ratio (LMR) which characterizes the relationship between the affector and effector parts of the immune response, showed that LMR prevailed in group II and then decreased in the series group III > group V > group IV > control > group I (see Table 5).

The revealed blood morphological parameters in calves from the test groups corresponded to a higher metabolic activity.

6. Blood biochemical parameters in Kazakh white-headed calves (*Bos taurus taurus*) fed diets with vegetable fats and ultrafine Fe particles (n = 4, M±SEM, Federal Scientific Center for Biological Systems and Agrotechnologies of the Russian Academy of Sciences, 2019-2020)

Parameter	Group					
	control	I	II	III	IV	V
Total protein, g/l	72.05±3.98	76.05±2.82*	86.85±4.51*	144.70±4.81*	99.43±6.98	154.50±4.54*
Albumin, g/l	29.00±6.12	38.00±5.41	36.00±5.80*	38.00±4.72	42.00±5.33*	44.20±3.82
Glucose, mmol/l	3.41±0.87	4.94±0.55*	3.52±0.63	4.94±0.46*	4.21±0.74*	5.22±0.41*
Triglycerides, mmol/l	0.29±0.07	0.34±0.06*	0.37±0.03	0.09±0.01*	0.45±0.09*	0.07±0.01*
Cholesterol, mmol/l	2.67±0.19	1.08±0.05*	3.63±0.31*	0.92±0.03	4.99±0.81*	1.06±0.07
AlAT, U/l	23.80±4.31	22.50±3.81*	31.80±5.12	26.60±2.71*	28.80±5.11	26.30±2.91
AsAT, U/l	44.20±5.93	42.20±2.92*	52.30±6.33	105.80±6.12*	54.90±5.82	108.60±4.82
De Ritis coefficient (AsAT/AlAT ratio)	1,86	1,87*	1,64	3,98*	1,91*	4,12*
Bilirubin total, rmol/l	2.43±0.07	2.61±0.08*	3.25±0.08*	2.16±0.12	3.67±0.09*	1.94±0.22
Bilirubin direct, rmol/l	1.11±0.13	1.01±0.11	1.72±0.18	1.05±0.02	1.59±0.16	1.84±0.06**
Billyrubin index	2.19	2.58*	1.91*	2.05*	2.31*	1.05
LDH, U/l	3049±56.05	3856±62.21*	5272±64.31	3659±51.12*	4098±63.70*	3426±42.01*
α-Amylase, U/l	415.00±23.11	712.00±30.22	471.00±63.12*	358.00±16.21	423.00±21.91	346.00±11.62
Lipase, U/l	17.30±3.42	18.00±2.20	16.80±1.21	8.00±0.63	28.40±3.91	8.60±0.52
Urea, mmol/l	3.20±0.72	4.20±0.63	5.10±0.91*	5.00±0.91	4.60±0.92	4.40±0.55
Creatinine, rmol/l	74.50±6.31	81.20±5.11	88.70±7.23*	93.10±5.31	89.60±7.21	92.80±4.83
γ-GT, U/l	18.30±2.60	23.20±3.21	32.40±4.11	24.00±2.12	23.60±3.13	21.00±2.32
Uric acid, rmol/l	15.50±3.22	16.00±2.82	18.90±4.32	21.20±3.82	16.10±3.92	19.80±3.61
Iron, rmol/l	19.20±3.81	22.70±4.61*	33.40±5.12*	49.30±4.41*	34.60±4.31	36.80±3.62*
Magnesium, mmol/l	1.22±0.21	1.08±0.08	1.73±0.31	0.84±0.02	1.68±0.91	0.78±0.06
Calcium, mmol/l	2.45±1.12	2.68±1.21	3.01±1.22	2.60±0.58	2.71±0.83	2.32±0.12
Phosphorus, mmol/l	1.54±0.04	2.04±0.08	2.03±0.06	1.97±0.09	1.68±0.62	1.44±0.23

N o t e. AlAT — alanine aminotransferase, AsAT — aspartate aminotransferase, LDH — lactate dehydrogenase, γ-GT — glutamyl transpeptidase. For a description of the groups, see the "Materials and methods" section.
*, ** Differences with the control group are statistically significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.

Changes in biochemical parameters reflect the adaptation of all body systems, including digestion and general homeostasis, to changing feeding conditions [43]. In our experiments, the introduction of UFP Fe into the diet stimulated protein metabolism in calves (Table 6). An increase in the amount of total blood protein indicated a better assimilation of feed nitrogen, which was also facilitated by an increase in enzymatic activity [29]. When the control diet was replaced with fat, this indicator increased by 20.6% in group II ($p \leq 0.05$), and by 38.0% in group IV. Additional introduction of UFP Fe increased the content of total protein in group I by 5.6%, in group III by 100.8%, and in group V by 114.6% vs. control.

An upward trend was also observed for albumin (see Table 6). Its amount was statistically significant ($p \leq 0.05$) increased by 24.1% in group II and by 44.8% in group IV. In the test groups, the exceeding of the urea content vs. control ranged from 31.2% in group I up to 59.4% ($p \leq 0.05$) in group II.

Unbound iron also is an inducer of lipid peroxidation and protein peroxidation. Metals in microparticles have a low degree of release and assimilation rate, thereby eliminating toxic effects on the body and intestinal microflora [12]. The change in fat metabolism was assessed by the content of triglycerides and cholesterol in the blood serum. When UFP Fe was added, the amount of triglycerides increased by 17.2% ($p \leq 0.05$) (see Table 6).

Vegetable oils in animal diets have a significant effect on blood lipid profile [45]. In our experiment, when replacing the control diet with fat, the content of triglycerides increased in group II by 27.6%, in group IV by 55.2% ($p \leq 0.05$). UFP Fe added to fat diets contributed to a significant decrease in the amount of triglycerides in the blood serum of calves, in group III by 68.9% ($p \leq 0.05$), in group V by 75.9% ($p \leq 0.05$) vs. control. A similar trend occurred for the content of cholesterol (see Table 6).

The impact of UFP Fe on carbohydrate metabolism in animals was assessed by the glucose content in blood serum. The parameter value increased statistically significantly ($p \leq 0.05$), by 44.9% in groups I and III, by 53.1% in group V vs. control (see Table 6).

Dietary UFP Fe contributed to an increase in the content of iron ($p \leq 0.05$), however, the amount of magnesium, calcium and phosphorus decreased insignificantly, with the exception of group III. When the control diet was replaced with fat diet, the content of P increased in group II by 31.8%, in group IV by 9.1%, the content of Ca increased in group II by 22.9%, in group IV by 10.6%, the content of Mg increased in group II by 41.8%, in group IV by 37.7% vs. control.

UFP Fe added to the control and fat diets contributed to both stimulation and inhibition of certain processes. An increase in the AsAT/AlAT ratio (de Ritis coefficient) could indicate chronic processes associated with parenchymal liver damage due to heavy metal intoxication. AsAT in the de Ritis coefficient reflects the activity of a central metabolic link. Its regulate the use of substrates in the Krebs cycle with their subsequent aerobic oxidation, performs ammonia detoxification, involving ammonia into the urea synthesis cycle, and also provides recovery of the aspartate level in tissues which decreases with an imbalance of amino acids and hypoxia [43, 45]. The higher the AlAT level, the lower the de Ritis coefficient.

The content of AlAT increased in the groups which received fat diets. i.e., in group II by 33.6%, in group IV by 21.0%, however, these changes were not statistically significant. Additional administration of UFP Fe led to a decrease for AlAT by 5.5% ($p \leq 0.05$) in group I vs. control and by 16.4% ($p \leq 0.05$) in group III vs. group II that received the same diet except the addition of iron. The de

Ritis coefficient was significantly ($p \leq 0.05$) higher in the groups receiving UFP Fe with fat diets (see Table 6).

The blood bilirubin index (BI) was also calculated, which characterizes the excretory function of the liver and shows the degree of toxicity of UFP Fe. BI increased by 17.8% ($p \leq 0.05$) in group I and by 5.5% ($p \leq 0.05$) in group IV vs. control, in all other test groups, the BI values were lower than in the control.

Thus, the ultrafine iron (UFP Fe) when added to the fat diets of Kazakh white-headed calves contributes to a significant increase in the digestibility of crude fat, organic matter, and nitrogen-free extractive feed substances, while the digestibility of crude fiber and crude protein decreased. Enrichment of rations with UFP Fe and fatty components has a stimulating effect on pancreatic secretion, leading to an increase for pancreatic juice production. UFP Fe selectively changes the activity of the digestive enzymes of the pancreas. Due to UFP Fe added to the control (basal) diet, the lipase activity increases by 35.7%, intestinal proteases by 43.1% while the amylolytic activity decreases by 28.8%. The use of UFP Fe with fat diets reduced the enzymatic activity of the pancreas vs. the control group. With sunflower oil, the activity of lipase and intestinal proteases increased by 12.1 and 16.7%, respectively ($p \leq 0.05$), with soybean oil by 133.2 and by 38.4% ($p \leq 0.05$). Supplementation of the diet with fatty components, separately and in combination with UFP Fe, led to an increase in the of NO-metabolite content in all test groups compared to control. With sunflower and soybean oil, an increase occurres in the trypsin content by 106.6% ($p \leq 0.05$) and 130.9%, respectively. UFP Fe, when aadded, decreases the trypsin content. Morphological analysis revealed a statistically significantly higher blood hemoglobin in animals of the test groups, by 9.7-41.9% ($p \leq 0.05$). All the studied biochemical parameters were within acceptable physiological norms. However, UFP Fe had a stimulating effect on protein, fat and carbohydrate metabolism. An increase in the de Ritis coefficient occurred in the groups receiving UFP Fe with sunflower and soybean oil in the diets. In all other test groups, the values of the bilirubin index were lower than in the control, which indicates a rather low toxicity of the preparation of ultrafine iron particles.

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FEATURES OF NITRIC OXIDE METABOLISM IN EMBRYOS OF DIFFERENT BIRD SPECIES AS GENETICALLY DETERMINED SIGN ASSOCIATED WITH MEAT PRODUCTIVITY

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Abstract

At present, the role of nitric oxide (NO) in embryogenesis, in particular in myogenesis, is widely discussed. Earlier we noted that the main part of nitric oxide synthesized in the avian embryo can accumulate in tissues as part of the so-called NO donor compounds or be oxidized to nitrate. The degree of this oxidation correlates with the meat productivity of adults. This report shows that in broiler embryos NO is oxidized to nitrate by 90% or more, while in embryos of egg poultry NO oxidation is negligible. That is, the degree of NO oxidation is determined by some features of the embryo tissues rather than NO itself determines these features. Consequently, the degree of NO oxidation in bird embryogenesis is an indicator associated with tissue properties correlating with meat productivity. Since this sign is inherited, it is assumed to be genetically determined. The purpose of this work is to characterize the manifestation and inheritance of the intensity of nitric oxide oxidation and the associated physiological characteristics of embryos in birds of different species. The experiments were carried out in a vivarium (Zagorskoye, Sergiev Posad, Moscow Province, 2015-2021). It was shown that in poultry of different breeds characterized by the same degree of NO oxidation the live weight can vary significantly. This is especially evident in hens. The proportion of oxidized NO in the embryo was higher in lines, breeds and crosses obtained as a result of breeding to increase meat productivity. Thus, in the embryos of broilers and meat quails, by the day 7th, more than 90% of embryonic NO is oxidized, in egg forms oxidation was insignificant (several percent), most meat-egg forms occupied an intermediate position according to this index. The analysis of inheritance of the index in the F_1 generation in several bird species suggests that this trait is formed due to the expression of various genes that can both promote and counteract its manifestation. Oxidation of NO to nitrate in embryos of both meat and egg forms can be induced by light at the beginning of incubation. In embryos of egg forms, the proportion of oxidized NO can increase up to 60 % under the action of light. Consequently, there is a possibility of oxidation of NO in embryos of both meat and egg forms. Apparently, the mechanism of activation of this process is inherited, which can also be partially induced by light. Further analysis of the inheritance of the intensity of oxidation of embryonic NO in a number of generations will show which genes are associated with the intensity of oxidation of NO. This will allow using this indicator as a highly sensitive marker for the corresponding genes.

Keywords: nitric oxide, NO, NO donors, NO oxidation, trait inheritance, nitrate, myogenesis, *Gallus gallus domesticus* L., chickens, *Coturnix coturnix* L., quail, *Numida meleagris* L., guinea fowl, *Struthio camelus* L., ostriches

At present, the role of nitric oxide (NO) in embryogenesis, in particular in myogenesis, is widely discussed. It is believed that NO mediates proliferation of myocytes [1-5], formation of muscle fibers [3, 4], and proliferation of satellite

cells [6]. According to modern concepts, the physiological effect of NO is due to the nitrosation of enzymes guanylate cyclase [7, 8], caspase]9-11] and the structures that determine gene expression [12, 13].

It is believed that the synthesized nitric oxide is incorporated in the NO donor molecules — S-nitrosothiols (RSNO), dinitrosyl iron complexes (DNIC), and high molecular weight nitro compounds (RNO_2). These compounds play the role of a nitric oxide depot, prolonging its physiological lifetime [7, 14, 15]. Their concentration in cells can reach tens of micromoles [16, 17], being comparable with the concentration of nitrate, the end product of NO metabolism. Therefore, to determine the role of NO in a particular process, it is necessary to control the content of deposited NO and the products of its metabolism.

But until now, the quantifiction of all NO metabolites in living tissues is difficult due to the lack of methods that allow one to quickly analyze the entire spectrum of nitro and nitroso compounds and its changes during physiological processes.

The enzyme biosensor we have developed jointly with the Federal Research Center for Chemical Physics RAS is based on the reversible inhibition of catalase by all nitroso compounds that initially have an NO^+ group or acquire it when influenced by sertain factors. Halide ions increase the inhibition efficiency by two orders of magnitude. Nitroso compounds lose their inhibitory properties when interact with substances specific to each of their groups. This allows the concentration of S-nitrosothiols (RSNO), DNIC, nitrite, and nitrosamines to be measured with a 50 nM accuracy [17].

Using the developed sensor, it was shown that embryogenesis in birds, as in other animals, is associated with increased production of NO which either accumulates in the embryo as part of donor compounds or is oxidized to nitrate. Within a species, the NO synthesis is approximately of the same intensity while the intensity of nitric oxide oxidation to nitrate varies. The latter indicator in meat poultry is many times higher than in egg poultry [17, 18].

Earlier we evaluated the embryonic NO oxidation level in 42 breeds, lines and crosses of 5 poultry species [18-20]. All 25 egg breeds, lines and crosses were low in intensity of embryonic NO oxidation, no more than 5% of the total synthesized NO on day 14. In 19 breeds, crosses and lines designated as meat poultry, the level of NO oxidation was high (90% or more), and in meat-and-egg breeds, the indicator showed intermediate values [18-20]. Such data suggest that the intensity of NO oxidation is somehow related to meat productivity, especially since NO oxidation occurs predominantly in muscle tissues [20]. Since the intensity of NO oxidation is a hereditary trait with a deviation of less than 10% within the line and cross [18-20], it is reasonable to assume that the property is genetically determined.

The hypothesis of using the NO oxidation intensity as a selection marker could be confirmed or refuted by data on this trait manifestation in the embryos of birds of different species and different types of productivity. We did not find such data in the available scientific publications.

In this work, we have studied for the first time the relationship between the intensity of NO oxidation to nitrate in the embryo and the rate of postembryonic growth in quails, chickens, and ostriches. Also, the mechanism of heritability of this trait was analyzed for the first time in F_1 from crosses of different lines and breeds were crossed. The proposed methodology allowed us to estimate a highly sensitive parameter associated with meat productivity. Its successful use in breeding programs requires an in-depth study of the mechanism of such a relationship.

Our goal was to reveal the features of the inheritance of nitric oxide oxidation intensity and related physiological traits in the embryos of birds of different species.

Materials and methods. Experiments were carried out in a vivarium of the Genetic and Breeding Center Zagorskoye (VNITIP, Sergiev Posad, Moscow Province, 2015-2021) using fertile eggs of chickens (*Gallus gallus domrticus* L., the White Cochinchina, Fawn Brama, Andalusian Blue, Cornish lines B5, B6, B56, Plymouth Rock lines B7, B9, B79, Kulangi breeds; cross Hisex White and its lines X1, X2, X12, X3, X34; crosses Hisex Brown, Smena 8, Cobb 500, Ross 308, mini-hens of lines B77 and A77 groups No. 1 and No. 2), quails (*Coturnix coturnix* L., Estonian meat-egg, Japanese Gray, Manchurian Golden, Pharaoh, White Heavy breeds), and guinea fowl (*Numida meleagris* L., lines ZB1 and ZB2 of the Zagorskaya white-breasted breed, obtained from Genofond LLC, Russia). The eggs of ostriche (*Struthio camelus* L.) black-necked and blue-necked subspecies were obtained from the Vorob'i bird park (Kaluga Province). F_1 hybrids of the Japanese Grey, Estonian Meat-Egg, and Manchurian Golden breeds derived from various crossing combinations.

The temperature during incubation was 37.6 °C, during the hatching period 37.2 °C in accordance with the recommendations of VNITIP (Moscow, 2014) (incubators Stimul Ink-1000, Russia),.

With the use of green light during incubation, the control group was kept in the dark. The experimental group was kept under around-the-clock lighting (15 W Navigator NCL-SH10 energy-saving lamp with a green filter, light flux of 975 lm). The illumination period continued in course of 4 or 14 days from the beginning of incubation. The experiment was repeated 4 times, repetitions were performed with a change of incubators at the incubation temperature of 37.6 °C and hatching temperature of 37.2 °C as described (Recommendations of VNITIP. Moscow, 2014).

To determine the proportion of oxidized NO on days 2, 3 (formation of the amniotic membrane, the embryo is separated from the egg contents), 7, 8, 10 and 15 (after the complete formation of allantois), for ostriches on day 24, 20-40 eggs of each breed, line and cross were selected from the incubated batches. The concetration of NO metabolites in the samples (egg content homogenate, amnion content, allantois content) was measured no later than 30 min after sampling.

Shell-free eggs were homogenized (8 min, 6 °C, 40 frictions/min, a glass homogenizer, DWK Life Sciences GmbH, Germany); after 11-day incubation, a chopper (Oster, Mexico) was used.

The concentration of nitro and nitroso compounds was measured by using the highly sensitive sensor based on Dithermanal calorimeter (Hungary) [21]. The total concentration of NO donor compounds (depot), the S-nitrosothiols (RSNO), dinitrosyl iron complexes (DNIC), high-molecular-weight nitro compounds capable of transforming into DNIC (RNO_2), as well as the concentration of nitrite and nitrosamines, nitrate (NO_3^-) were assessed {17]. The nitrate content was evaluated after reduction with vanadium trichloride to nitrite, followed by quantitative determination [21, 22]. The proportion of NO oxidized to nitrate was determined from the concentration ratio nitrate/(NO donors + nitrate) × 100%.

Meat productivity was assessed by the live weight of birds in samples of 20-40 individuals after hatching and at the age of 28 days [23]. Young birds were kept in cages, feeding and rearing conditions, sex ratio during rearing were as commonly used (Recommendations of VNITIP. Moscow, 2014).

The BioStat software package (https://www.softsalad.ru/software/znaniya/matematika-i-nauka/biostat-2008) was used for statistical processing. Mean values (M) and standard errors of the mean (±SEM) were calculated with a 95%

confidence interval ($t_{0.05}$ × SEM). Differences between the variants were assessed by parametric statistics (Student's *t*-test) and were considered statistically significant at $p < 0.05$.

Results. To assess the amount of deposited NO and its oxidation product, nitrate, we used an enzymatic sensor developed by us based on the property of nitrite, nitrosamines (RNNO), nitrosothiols (RSNO), dinitrosyl iron complexes (DNIC) and nitro derivatives of macromolecular compounds (RNO_2) to inhibit catalase in the presence of halide ions and the loss of this ability under the influence of factors different for each group of compounds. Since the catalase reaction is highly exothermic (47.2 kcal/mol of released oxygen), its kinetics can be analyzed based on the kinetics of heat production accompanying the process [17, 21]. The method makes it possible to estimate the content of NO derivatives without preliminary sample preparation, since there is no need to remove colored impurities and turbidity, and is characterized by a sensitivity of 50 nM [17, 21].

1. The concentration of NO donors and nitrate (μmol/l) **in the egg content homogenate, in the amnion and allantois of embryos of different breeds, lines and crosses of chickens** (*Gallus gallus domesticus* L.) **during incubation** ($n = 40$, vivarium, Genetic and Breeding Center Zagorskoye, VNITIP, Sergiev Posad, Moscow Province)

Sample	Day 2		Day 3		Day 10		Day 15	
	NO donors	nitrate	NO donors	nitrate	NO donors	nitrate	NO donors	nitrate
Cornish line B56, $M \pm (t_{0.05} \times SEM)$								
Homogenate	37.1±2.9	< 0.1	134.5±8.9	8.1±3.2	5.7±1.6	146.6±8.8	18.7±2.5	465.5±19.9
Amnion			19500.3±45.5	< 0.1	23.7±7.6	< 0.1	19.8±7.4	< 0.1
Allantois			6.1±3.5	12.3±3.4	5.9±1.4	138.4±8.8	4.2±1.7	448.7±20.5
Plymouth Rock line B79, $M \pm (t_{0.05} \times SEM)$								
Homogenate	45.8±3.1	< 0.1	148.4±9.9	< 0.1	158.5±10.1	< 0.1	437.3±19.4	16.4±3.5
Amnion			12125.0±169.5	< 0.1	5500.0±150.0	< 0.1	5340.0±180.0	< 0.1
Allantois			10.5±3.3	< 0.1	10.2±3.9	< 0.1	8.9±2.9	13.9±4.1
Cross Smena 8, $M \pm (t_{0.05} \times SEM)$								
Homogenate	25.8±4.4	4.1±3.2	7.6±1.8	133.6±10.1	5.8±1.8	153.6±8.2	12.8±4.8	569.8±16.4
Cross Hisex White, $M \pm (t_{0.05} \times SEM)$								
Homogenate	40.4±6.2	< 0.1	142.4±8.1	< 0.1	160.4±8.7	< 0.1	466.4±15.4	12.5±2.8

Note. On day 3, given the small size of the amnion, homogenates of three embryos covered with an amniotic membrane were used in 1.5 ml of 40 mM K-phosphate buffer, pH 7.4. Up to day 15 (until the allantois was completely closed on days 12-13), the concentration in the liquid medium outside the amnion was measured. The concentration of nitrite and nitrosamines in all samples was < 0.1 μmol/l.

Table 1 shows the data on the content of deposited NO and its oxidation product, nitrate, in the homogenates of avian embryos of the Hisex White egg cross, Smena 8 broiler, its paternal form Cornish B56, and maternal Plymouth Rock B79. NO donor compounds are accumulated from day 1 to day 3 in the amniotic fluid. At the age of 2-3 days, their concentration decreases in the embryos of the Smena 8 cross and the Cornish B56 line. On the example of the Cornish B56 line, we see that there is a decrease in the concentration of NO donors in the amniotic fluid and a simultaneous increase in the concentration of nitrate outside the amnion. In the amnion, nitrate and nitrite are present in trace concentrations. The total content of nitrate and NO donors in the homogenates of all embryos differed insignificantly (see Table 1), 140-160 μmol/l on day 10 and 450-570 μmol/l on day 15. However, the proportion of oxidized NO deposited in donors to nitrate varied tremendously: from more than 90% in embryos of the Cornish B56 line and the Smena 8 cross to less than 5% in the embryos of the Highsex White cross and the Plymouth Rock B79 line (see Table 1). Consequently, the intensity of embryonal NO oxidation is due to definite features of the embryo tissues. It is not NO donors that spontaneously dissociate with the release of NO, but physiological targets in tissues are responsible for degradation of donor compounds and binding NO which is rapidly oxidized to nitrate and moved out of the amnion. This is confirmed by data on the oxidation of exogenous NO

donors introduced into the egg. They were completely oxidized in broiler embryos and almost not oxidized in egg poultry embryos [18, 20].

NO oxidation occurs during the entire embryonic period. Before hetching, the total concentration of nitrate and NO donors can reach several hundred micromoles (see Table 1).

2. Oxidation of NO to nitrate in egg content homogenates on day 7 in chickens, quails and guinea fowls vs the live weight gain after hatching (vivarium, Genetic and Breeding Center Zagorskoye, VNITIP, Sergiev Posad, Moscow Province)

Breed, line, cross	Purpose of use	Egg weight, g	Chick weight, g			NO oxidized to nitrate, %
			day 1	day 28		
				♂	♀	
Chickens (*Gallus gallus domesticus* L.), $M \pm (t_{0.05} \times SEM)$						
Highsex White (n = 40)	Eggs	63.8±0.8	41.8±2.1	230.1±4.5		2.4±1.3
Oryol calico (n = 20)	Eggs	51.7±0.5	35.5±0.9	167.8±8.7		2.1±1.3
Yurlovskaya golosistaya (n = 20)	Meat-and-egg	63.8±0.7	40.6±0.9	253.9±7.7		3.8±1.8
Plymouth Rock B79 (n = 40)	Meat (bred for egg production)	63.9±0.8	47.1±0.7	1044.6±34.3		2.6±1.5
Andalusian Blue (n = 30)	Eggs	48.5±0.6	38.2±1.0	218.6±5.6		2.1±1.3
Blue Meat-Egg (n = 30)	Meat-and-egg	49.9±0.6	40.1±1.0	231.8±5.5		61.8±2.9
Brama Fawn (n = 20)	Meat-and-egg	52.1±0.5	38.2±0.3	254.8±4.4		82.2±3.1
Smena 8 (n = 40)	Meat	64.9±0.6	48.1±0.7	1188.4±53.2		98.1 ±2.5
Cornish B56 (n = 40)	Meat	65.4±0.6	47.9±0.7	1299.7±54.6		96.9±3.1
Kulangi (n = 20)	Fighting	57.1±0.6	39.9±0.9	234.4±9.1		96.6±2.9
Cobb 500 (n=40)	Meat	63.1±0.7	48.3±0.7	1214.2±44.2		97.8±2.6
Ross 308 (n = 40)	Meat	63.8±0.7	45.8±0.5	1177.5±31.6		97.4±2.5
Guinea fowl (*Numida meleagris* L.), $M \pm (t_{0.05} \times SEM)$						
ZB2 (n = 20)	Eggs	46.8±0.5	31.4±0.6	376.4±5.4		2.2±1.4
ZB1 (n = 20)	Meat	6.7±0.5	31.3±0.5	395.5±8.5		97.8±2.6
Quail (*Coturnix coturnix* L.), $M \pm (t_{0.05} \times SEM)$						
Manchurian Golden (n = 40)	Eggs	12.9±0.2	10.0±0.2	181.1±3.6	163.4±1.9	2.3±1.3
Japanese Gray (n = 40)	Eggs	12.7±0.2	10.8±0.2	173.4±3.0	161.2±3.1	2.4±1.4
Estonian Meat-and-Egg (n = 40)	Meat-and-egg	13.1±0.2	11.2±0.2	198.4±3.9	182.1±2.4	95.5±3.6
♂Japanese Gray × ♀Estonian Meat-and-Egg (n = 20)		12.2±0.2	10.3±0.2	178.3±2.5	171.6±2.3	98.8±3.4
♂Manchurian Golden × ♀Japanese Gray (n = 20)		12.4±0.2	10.2±0.2	176.9±2.9	163.7±2.5	2.2±1.4
Pharaoh (n = 30)	Meat	13.3±0.2	11.0±0.2	208.8±3.5	196.5±4.1	98.4±3.6
White Heavy (n = 20)	Meat	13.9±0.2	11.5±0.2	289.1±3.5	274.0±3.9	97.1±3.3

Poultry with a high intensity of NO oxidation (Table 2) turned out to be meat or fighting birds. In these breeds, lines, and crosses, chicks on day 28 mostly exceeded in weight the birds with low NO oxidation, which are considered egg or initial forms.

But, as follows from the data of Table 2, in birds of different breeds, despite the same intensity of NO oxidation, the live weight differs significantly, especially in chickens. Therefore, it makes sense to determine not the correlation between the proportion of oxidized embryonic NO and body weight, but to assess how the proportion of oxidized embryonic NO changes within the same breed during selection for meat productivity.

The available data suggest that selection for increasing meat productivity within the same breed results in an increase in the intensity of oxidation of NO synthesized during embryogenesis. The Blue Meat-Egg breed is a product of the Andalusian Blue chicken breeding for meat productivity. In the embryos of Blue Meat-Egg chickens, the level of oxidized NO is appr. 60%, while in the Andalusian Blue breed, as in all egg-type chickens, this figure is insignificant (see Tables 2, 3). The growth rate of the Blue Meat-Egg breed is significantly ($p < 0.05$) higher than that of the Andalusian Blue, so, the difference in live weight reaches 10% on day 14 and 7% on day 21 (see Table 2).

3. The proportion of NO oxidized to nitrate in egg content homogenates in different breeds, lines and crosses of chickens (*Gallus gallus domesticus* L.) on day 10 of incubation and its inheritance in F_1 (vivarium, Genetic and Breeding Center Zagorskoye, VNITIP, Sergiev Posad, Moscow Province)

Breed, line, cross	Description	Purpose of use	NO oxidized to nitrate, %, $M \pm (t_{0,05} \times \text{SEM})$
X_1 ($n = 20$)	Paternal line of the paternal form of the Hisex White cross		44.2±3.9
X_2 ($n = 20$)	Maternal line of the paternal form of the Hisex White cross		1.9±1.3
X_{12} ($n = 20$)	The paternal form of the Hisex White cross ♂X_1 × ♀X_2		2.1±1.4
X_3 ($n = 20$)	The paternal line of the maternal form of the Hisex White cross		2.2±1.4
X_{34} ($n = 20$)	Maternal form ♂X_3 × ♀X_4		2.4±1.4
Highsex White ($n = 30$)	Final hybrid ♂X_{12} × ♀X_{34}	Egg	2.3±1.4
Andalusian Blue ($n = 30$)		Egg	2.3±1.6
Blue Meat-Egg ($n = 30$)	Derived from selection of Andalusian Blue breed for live weight gain	Meat-and-egg	59.9±2.7
Cornish B5 ($n = 30$)	Line of paternal form of Smena 8 cross		98.2±2.7
Cornish B6 ($n = 30$)	Line of paternal form of Smena 8 cross		97.9±2.8
Cornish B56 ($n = 30$)	Parenal form of cross Smena 8 ♂B5 × ♀B6		96.9±3.1
Plymouth Rock B7 ($n = 30$)	Line of maternal form of cross Smena 8		3.3±1.4
Plymouth Rock B9 ($n = 30$)	Line of maternal form of cross Smena 8		2.9±1.5
Plymouth Rock B79 ($n = 30$)	Maternal form of cross Smena 8 ♂B7 × ♀B9		2.6±1.5
Smena 8 ($n = 30$)	Final cross, ♂B56 × ♀B78	Meat	98.4±2.4
B77(1) ($n = 20$)	Mini-hen line B77 of group 1	Mini-egg	81.4±3.1
B77(2) ($n = 20$)	Mini-hen line B77 of group 2	Mini-egg	90.7±3.5
A77(1) ($n = 20$)	Mini-hen line A77 of group 1	Mini-meat	98.8±3.4
A77(2) ($n = 20$)	Mini-hen line A77 of group 2	Mini-meat	99.3±3.5

N o t e. The proportion of NO oxidized to nitrate is calculated as the concentration ratio nitrate/(NO donors + nitrate) × 100%. Groups 1 and 2 are derived from selection of the corresponding lines for an increase in live weight.

Mini-hens are characterized by an increased NO oxidation compared to ordunary chickens. However, for mini-hens, selection for higer growth rate leads to intensification of nitric oxide oxidation (Table 3). Thus, 8 weeks after hatching, the live weight in line B77 group 2 is 17% higher compared to group 1 [23] with 81.4 and 90.7% ($p < 0.05$) NO oxidation on day 10 of incubation (see Table 3). A77 line group 2 on week 8 also exceeded group 1 in live weight 17% [23]. Since the eviscerated carcass yield of broilers and egg hens differs by 5%, we considered live weight as an indicator of meat productivity. It is a known that the higher the live weight, the higher the eviscerated carcass yield [23].

Lines ZB1 and ZB2 derived from Zagorskaya white-breasted (ZB) guinea fowl after beedng for meat (ZB1) and egg (ZB2) productivity. In ZB1, almost complete oxidation of NO synthesized in the embryo occurs, while in ZB2, only a few percent are oxidized, although the growth rate of ZB1 slightly exceeds that of ZB2 (see Table 2). Therefore, the growth rate is determined by many factors, and not all of them are associated with the activation of NO oxidation. Nevertheless, selection for higher growth rate leads to intensification of nitric oxide oxidation.

It can be assumed that there is a gene that is either not present in the original forms (and the selection process captures some mutant forms), or this gene or genes are present everywhere, but its (their) expression can be suppressed by other genes. In this regard, it was of interest to assess the inheritance of this trait when crossing different breeds. The Smena 8 cross broilers were obtained by crossing a maternal and paternal forms, which, in turn, also result from crossing certain lines of the Cornish and Plymouth Rock breeds. The data of Table 3 indicate that the Smena 8 cross and its paternal lines and forms (Cornish B5, B6, and B56) show almost complete oxidation of NO in the embryo. On the contrary, in the maternal lines and forms (Plymouth Rock B7, B9, and B79), only a few

percent of NO is oxidized. The growth rate of the Smena 8 cross is somewhat lower than that of the Cornish B56 line, but higher than that of the Plymouthrock B79 line (see Table 3).

4. The proportion of NO oxidized to nitrate in egg content homogenates in different breeds of quails (*Coturnix coturnix* L.) on day 8 of incubation and its inheritance in F_1 (vivarium, Genetic and Breeding Center Zagorskoye, VNITIP, Sergiev Posad, Moscow Province)

Breed	Purpose of use	NO oxidized to nitrate, %, $M\pm(t_{0.05} \times \text{SEM})$
Manchurian Golden (M) ($n = 40$)	Egg	2.6±1.4
Japanese Gray (J) ($n = 40$)	Egg	2.5±1.5
Estonian Meat-and-Egg (E) ($n = 40$)	Meat-and-egg	93.8±4.1
Hybrid (♂J × ♀E) ($n = 20$)		97.9±3.5
Hybrid (♂E × ♀J) ($n = 20$)		99.1±3.1
Hybrid (♂J × ♀M) ($n = 20$)		2.4±1.5

N o t e. The proportion of NO oxidized to nitrate is calculated as the concentration ratio nitrate/(NO donors + nitrate) × 100%.

In quails, heavy breeds (Pharaoh, White Heavy, and Estonian) also show almost complete oxidation of embryonic NO, while it is insignificant in egg breeds Golden Manchurian and Japanese Gray. In hybrids of the Estonian Meat-and-Egg and Japanese Gray breeds, embryonic NO is almost completely oxidized (Table 4). The growth rate of the hybrids turned out to be intermediate between that of the Estonian Meat-Egg and Japanese Gray breeds, regardless of which breed were males and which were females (see Table 2). In the embryos of hybrids of the Japanese Gray and Manchurian Golden breeds, NO oxidation is insignificant, as it is in the embryos of the parent breeds (see Table 4). In terms of growth rate, these hybrids also do not differ from their parents (see Table 2).

In addition to cases when it is possible to assume the presence of a gene or its dominant allele associated with intense NO oxidation, insignificant ($\sim$ 2%) oxidation of embryonic NO can occur in the offspring from crossing a line with significant (44.2±3.9%) NO oxidation (e.g., X_1, the paternal line of the Hisex White paternal form) with a line in which this oxidation is insignificant ($\sim$ 2%, $p < 0.05$) (e.g., X_2, the maternal line of the Hisex White paternal form (see Table 3, X_1, X_2, X_{12} lines and the final hybrid). The X_1 line used as the paternal line of the paternal cross is the heaviest with the strongest skeleton. On day 28, it exceeds the final hybrid and all other lines in live weight by 11-12% [23]. X_1 is also the only line of the Hisex White cross with a significant (44.2%) oxidation of embryonic NO (see Table 3). However, this property was not inherited in further crosses. Therefore, our hypothesis about the dominant allele of the gene that determines the intense oxidation of NO is not confirmed.

5. The proportion of NO oxidized to nitrate in egg content homogenates in different subspecies of ostriches (*Struthio camelus* L.) on day 24 of incubation and its inheritance in F_1 ($n = 20$, vivarium, Genetic and Breeding Center Zagorskoye, VNITIP, Sergiev Posad, Moscow Province)

Breed, subspecies	Описание Description	NO oxidized to nitrate, %, $M\pm(t_{0.05} \times \text{SEM})$
Black-necked (B)		1.6±0.8
Blue-necked (Bl)		1.5±0.8
Hybrid (♂B × ♀ Bl)	A significantly higher rate of postembryonic growth than parental forms.	90.7±3.8
Hybrid (♂Bl × ♀ B)	A significantly higher rate of postembryonic growth than parental forms.	91.2±3.8

N o t e. The proportion of NO oxidized to nitrate is calculated as the concentration ratio nitrate/(NO donors + nitrate) × 100%.

Crossing the black-necked and blue-necked subspecies of ostriches gives us another example that refutes the hypothesis about the dominant allele of the gene for NO oxidation intensity. The offspring of ostriches is characterized by 90%

oxidation of NO in the embryo to nitrate (Table 5) and a significantly higher growth rate than parental forms [19, 24]. NO oxidation in embryos of parental forms is negligible (see Table 5).

All the data obtained, on the one hand, indicate that the intensity of embryonic NO oxidation is genetically determined and inherited within the line, cross, and breed with a variation of the indicator of no more than 10% [18-20]. On the other hand, the considered examples allow us to assume that the intensity of NO oxidation is determined not by single specific gene, but, apparently, by the combined action of different genes. Perhaps they can both provide and reduce the manifestation of the trait in question.

Previously, we showed that the intensity of embryonic NO oxidation does not depend on the age of laying hens, keeping conditions, and the sex of the embryo [18, 19]. However, the use of green light during incubation is known to stimulate post-embryonic growth [25, 26]. We have shown that green light promotes the intensification of embryonic NO oxidation. In this, no activation of NO synthesis occurs, since the total concentration of nitrate and NO donors did not change, remaining within 150-160 μmol/l on day 10 and 470-490 μmol/l on the day 15, whereas NO oxidation intensifies, since the concentration of nitrate increased, and the concentration of NO donors decreased (Table 6). The following regularities turned out to be characteristic. According to our data for 7, 10 and 15 days, illumination of the Hisex White cross eggs from day 1 to day 6 of incubation and from day 1 to day 15 of incubation induces oxidation of up to 60% of the synthesized NO to nitrate (see Table 6). A further increase in light intensity did not increase the proportion of oxidized NO. This percentage is maintained throughout the embryogenesis, even when green illumination is canceled from day 6. Moreover, nitrate was accumulated outside the amnion, as in embryos with an initially high NO oxidation (see Tables 1, 6). Resumed illumination on day 6 no longer led to the intensification of nitric oxide oxidation, although donor compounds were also present in the embryo. Thereofore, the light acts on the embryonic tissues that cause oxidation but does not affect the NO donor compounds itsef (see Table 6).

6. Influence of green light regimes on the composition of nitro- and nitroso compounds in the egg content homogenate, amnion and allantois of Hisex White (*Gallus gallus domesticus* L.) embryos on day 15 of incubation ($N = 4$, $n = 20$, vivarium, Genetic and Breeding Center Zagorskoye, VNITIP, Sergiev Posad, Moscow Province)

Sample	Day 5		Day 7		Day 10		Day 15	
	NO donors	nitrate	NO donors	nitrate	NO donors	nitrate	NO donors	nitrate
Control (darkness), $M \pm (t_{0.05} \times SEM)$								
Homogenate	139.8±7.7	< 0.1	148.5±8.2	< 0,1	155,8±8,9	< 0,1	465,5±19,9	18,6±1,9
Amnion							5230.0±170.0	< 0.1
Allantois							11.2±2.2	12.7±2.9
Lighting from day 1 to day 15, $M \pm (t_{0.05} \times SEM)$								
Homogenate	141.1±7.5	< 0.1	59.8±3.6	90,3±4,4	64,1±10,1	96,1±4,5	164,3±10,2	316,4±15,5
Amnion							1870.0±90.0	< 0.1
Allantois							12.6±2.4	278.6±12.1
Lighting from day 1 to day 4, $M \pm (t_{0.05} \times SEM)$								
Homogenate	147.8±7.4	< 0.1	151.4±7.8	< 0.1	158.8±8.1	< 0.1	478.3±17.9	22.3±1.9
Lighting from day 1 to day 6, $M \pm (t_{0.05} \times SEM)$								
Homogenate	146.6±7.9	< 0.1	79.4±8.6	74.6±7.9	82.7±8.2	81.9±7.9	217.4±10.4	261.5±12.2
Lighting from day 6 to day 14, $M \pm (t_{0.05} \times SEM)$								
Homogenate	145.5±7.2	< 0.1	150.2±7.6	< 0.1	154.9±7.8	< 0.1	469.7±18.1	20.8±2.0

Note. Until day 15, the concentration was measured in a liquid medium outside the amnion. The concentration of nitrite and nitrosamines in all samples was < 0.1 μmol/L.

Based on the data obtained, we can conclude that nitric oxide is involved in a specific process in avian embryogenesis, in which it does not participate (or participates, but not so actively) after hatching. The fact that most of the deposited

NO (90%) in broiler embryos is oxidized to nitrate indicates that the high concentrations of deposited NO (over 90%) that we observe in the amnion of egg forms (see Table 1) are not vital necessary (at least in order to provide vital NO-dependent processes). As shown earlier, the oxidation of NO to nitrate occurs in the tissues of the embryo, and mainly in the muscle tissue. There is virtually no oxidation in the liver and intestines [18, 20]. Therefore, it is reasonable to believe that this oxidation is somehow related to the development of muscle tissue.

To assess the role of NO in increasing the rate of body weight growth, two approaches are possible. The first is to try to artificially minimize NO oxidation in the embryo, for example, by using NO synthase blockers. The second is to use exogenous NO donors. According to our data, a decrease in the intensity of NO synthesis even by 80% from the initial one at the beginning of incubation did not significantly affect the rate of postembryonic growth [18, 20]. In addition, NO donor compounds in an amount equal to that occurred on day 3 had no reliable effect when introduced into the egg [18, 20].

It is possible that NO is synthesized in excess in the embryo to ensure any physiological processes. That is, it is not the amount of oxidized NO that is of interest, but the features of the tissues of the embryo that induce this oxidation. From the data presented in Table 1, it follows that NO donor compounds are initially accumulated in the embryo. Starting from a certain time (from days 2-3), these compounds begin to oxidize to nitrate. In embryos of egg poultry, oxidation is practically absent (see Tables 1-5). What is this period and what process is it associated with? Myotome formation is intiated in the embryo at the age of 2-3 days. Proliferation of myoblasts lasts up to 14 days. But NO oxidation in embryos of meat forms occurs throughout the embryogenesis. Histological studies did not reveal any qualitative differences in the development of muscle tissue in embryos of broilers and egg forms of chickens, as well as in embryos of egg and meat forms of quails, characterized by high and low intensity of embryonic NO oxidation [18]. It can be assumed that some structures appear on days 2-5, the further development of which is associated with NO oxidation. The formation of these structures, apparently, is genetically determined, since the oxidation intensity index is inherited within lines, crosses [18]. Perhaps, under the influence of genetically determined factors, a population of some cells is formed, the growth of which is associated with oxidation processes.

But what is the nature of these genetically determined factors and what genes are associated with their appearance? D. Cazzato et al. [27] studied the expression of seven genes that determine the course of myogenesis at the earliest stages of embryogenesis. The expression was influenced by the NO synthase inhibitor and NO donors. Without denying these data, we note that they refer to the beginning of embryogenesis. The studied factors did not affect the features of postembryonic development [18, 20].

Embryonic oxidation of NO can also be partly due to external factors such as light (see Table 6). This effect was also shown by us in a number of other works [20, 28]. It has been shown that light induced NO oxidation in embryos of both meat and egg chickens, followed with a slight (by a few percent) increase in the rate of postembryonic growth [20]. The latter is consistent with other reportes [26, 29].

Thus, the analysis of the embryonic NO oxidation intensity inheritance in F_1 of several bird species suggests that this trait is the result of the expression of various genes that can both promote and suppress the NO oxidation. The oxidation of NO to nitrate in all avian embryos can be induced by light at the beginning of incubation, after which the process proceeds even in the dark. In embryos of egg forms, under the action of light, the proportion of oxidized NO can increase up to 60%. Therefore, embryos of both meat and egg forms have mechanisms that

ensure the oxidation of NO. Apparently, the ability to activate this process is inherited, which can also be partially induced by external factors (light). To find out for which genes the embryonic intensity of NO oxidation can serve as a marker and how it can be used in the theory and practice of breeding, we will study the inheritance of this trait in several generations. The mechanism of NO oxidation in the embryo and the specific physiological role of this process are still not clear.

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THE CAUSATIVE AGENTS OF COLIBACILLOSIS IN POULTRY: CARRIERS OF GENES ASSOCIATED WITH EXTRAINTESTINAL AND INTESTINAL PATHOGENIC *Escherichia coli*

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Abstract

The expansion and intensification of poultry farming increases the risk of spreading colibacillosis among poultry, so there is an urgent need to monitor avian pathogenic *Escherichia coli* (APEC), study their genetic diversity and identify strains that pose a threat to human health. Determination of virulence-associated genes and the degree of specific adhesion may be useful for a comprehensive assessment of the epidemic and epizootic significance of *E. coli* strains isolated from livestock. In this study, an extended molecular analysis of *E. coli* strains isolated from poultry during outbreaks of colibacillosis was performed with the objective to genotypically characterize the isolated *E. coli* strains and to evaluate the relationship between genes encoding adhesins and specific adhesion to erythrocytes. It was shown for the first time that the strains were characterized by a high potential for pathogenicity and could be carriers of genes for several pathotypes at once, while the genes of intestinal pathogenic *E. coli* (IPEC) were detected often than others. A positive adhesive profile for a number of genes correlated positively with the activity of strain adhesion to chicken (*Gallus gallus* L.) and human erythrocytes. In the study 28 non-clonal *E. coli* strains, as determined by ERIC-PCR, isolated from various organs (except the intestine) of Ross 308 cross broilers (*Gallus gallus* L.) with generalized colibacillosis in 2016-2018 were characterized. Polymerase chain reaction (PCR) was used to detect virulence-associated genes characteristic of four different *E. coli* pathotypes, the APEC, extraintestinal pathogenic (ExPEC), intestinal pathogenic *E. coli* (IPEC: Enteropathogenic *E. coli* EPEC, Enterotoxigenic *E. coli* ETEC, Enterohemorrhagic *E. coli* EHEC, Enteroaggregative *E. coli* EaggEC), and uropathogenic *E. coli* (UPEC). Previously published protocols were used for all types of PCRs and amplifications were performed in the DNA Engine Dyad Thermal Cycler (Bio-Rad, USA). Band visualization and data documentation were performed using the Gel-Doc XR gel documentation system (Bio-Rad, USA). Formalinized human erythrocytes of the type 0(I) Rh(+) and avian erythrocytes were used as cell substrates for the determination of bacterial adhesion to erythrocytes. To evaluate the bacterial adhesion properties the adhesion index was calculated as the average number of bacteria bound to an erythrocyte in the adhesion assay. The obtained results showed that the characterized strains possessed a high pathogenic potential, as they carried genes associated with APEC, ExPEC as well as IPEC. The presence of APEC-specific marker genes identified most of the strains as APEC. However, potential for human pathogenicity was also found among the analyzed strains. As the IPEC-associated genes were found more frequently than ExPEC-associated genes, the *E. coli* strains studied were more similar to strains causing acute intestinal infections in humans, particularly due to the fact that they carried genes encoding toxins characteristic of IPEC (with the exception of genes for Shiga-like toxins and enterohemolysins). Based on cluster analysis of genetic profiles, the strains studied could be classified into three groups: (i) pathogenic to birds and humans, characterized by the presence of 2-6 genes associated with APEC and 2-6 genes associated with ExPEC or IPEC (24 strains), (ii) pathogenic to birds and nonpathogenic to humans, characterized by the presence of 2-6 genes associated with APEC and 0-1 gene associated with ExPEC or IPEC (2 strains), and (iii) nonpathogenic, characterized by the possession of none or one gene from each pathotype, APEC, ExPEC, IPEC (2

strains). It was found that 75 % of the first group, pathogenic to birds and humans, carried not only a high number of virulence-associated genes, but also pathogenicity island SHI-2, as well as genes for extended-spectrum beta-lactamases and class 1 integrons. Specific adhesion of *E. coli* strains was more pronounced on chicken erythrocytes than on human ones. Statistical analysis revealed several positive correlations between the chicken and human erythrocytes adhesion profiles and a number of genes encoding adhesins. The high adhesion activity of the bacteria, regardless of the type of erythrocyte, also correlated with longer survival in host blood serum (genotype *iss*+) and the possibility of erythrocyte lysis (genotype *hlyF*+). The obtained data on the molecular and adhesive properties of causative agents of colibacillosis in birds allow us to assess their zoonotic potential and epizootic significance and can also serve as the basis for improving the monitoring system for colibacillosis in poultry farms.

Keywords: avian pathogenic *Escherichia coli*, APEC, ExPEC, IPEC, virulence-associated genes, zoonotic potential

Aavian pathogenic *Escherichia coli* (APEC) is an animal pathotype of *Escherichia* found in the gut microbiota of some bird species that causes extraintestinal infections in immunocompromised individuals [1]. APEC strains become the main cause of colibacillosis, a syndrome associated with aerosacculitis, pericarditis, and often sepsis in poultry [2, 3]. Outbreaks of the disease in poultry enterprises lead to a reduction in egg production by 2-3% and mortality of livestock up to 30% which entails economic losses [4]. Experts estimate that at any given time, at least 30% of the individuals of all commercial herds in the United States have colibacillosis [5)]. In Russia, colibacillosis accounts for 60 to 88% of all poultry infections [6].

According to the current classification, the APEC population is included in the group of extraintestinal pathogenic *E. coli* (ExPEC), which also includes uropathogenic *E. coli* (UPEC) associated with neonatal meningitis-causing *E. coli*, (NMEC), sepsis-associated *E. coli (*SEPEC) and other pathotypes [2, 5]. The use of molecular approaches to identify *Escherichia* virulence factors has significantly expanded knowledge of the pathogenetic mechanisms of APEC infection [7]. According to the hypothesis proposed by L.K. Nolan et al. [3], the APEC pathotype is due to the presence of specific marker genes. The presence of a minimal set of genetic determinants allows defining a strain in this group, i.e., *ompT* (outer membrane protease), *iutA* (aerobactin receptor), *iss* (serum survival factor), *iroN* (enterochelin receptor) [3, 5]. In addition, various virulence genes encoding adhesins, toxins, defense factors, iron production systems, as well as autotransporters and the IbeA protein are involved in the development of colibacillosis, which determines many manifestations (forms) of avian infections resulting from the expression of various combinations of virulence determinants [6].

V.G. Maturana et al. [8] concluded that APEC strains do not represent a homogeneous group, but, depending on the combination of pathogenicity determinants, are divided into subgroups-subpathotypes, each of which is associated with a specific infectious syndrome. Studies by L. Mageiros et al. [9] showed that strains of the APEC pathotype arise from ubiquitous commensal intestinal bacteria, including through horizontal transfer of genes expressing pathogenicity factors, allowing divergent clones to infect poultry. Most APECs have been found to contain a highly conserved cluster of plasmid-linked virulence genes found in relatively few *E. coli* faecal isolates from healthy birds (AFEC) [10].

Recent studies have suggested that APEC strains may pose a risk to human health [11, 12]. On the one hand, the possibility of APEC transmission through food products, including poultry meat, has been described [13], on the other hand, the revealed DNA sequence homology between APEC and other ExPEC pathotypes shows that they are closely related phylogenetically [10]. For example, in extraintestinal strains of *E. coli* pathogenic for humans, the iss gene was detected in the genome, which expresses a factor responsible for the survival of bacteria in blood serum. The gene is located on the large virulence plasmid ColV, typical of

avian *E. coli* strains, indicating possible plasmid transfer and hence virulence gene exchange between human and avian *E. coli* strains [10, 14]. The results obtained by K.E. Rodriguez-Siek et al. [15] and T.J. Johnson et al. [16, 17] confirm the close relationship between APEC and UPEC/MNEC cultures. The emergence and spread of hybrid and heteropathogenic strains of *E. coli* carrying, respectively, patterns of ExPEC genes and representatives of intestinal pathogenic *E. coli* (intestinal pathogenic *E. coli*, IPEC) or two or more IPEC pathotypes was noted [18, 19]. The presence of similar virulence-associated genes found in IPEC/ExPEC and APEC strains confirms that the latter can either act as zooanthroponotic pathogens themselves or serve as a reservoir of virulence determinants for *E. coli* that cause infections in humans [12, 20].

Various biological systems, including experimental infection, are used to establish a relationship between the presence of certain APEC pathogenicity factors and their manifestation in host biotopes [21, 22]. The role of pColV plasmid in avian and possibly human virulence has been confirmed: transconjugants (commensal strain with pAPEC-O2-ColV) caused death of chick embryos and urinary tract infection in mice, and also grew well in human urine [23], nevertheless APEC's ability to cause disease in humans has not been conclusively proven.

In this work, based on the results of a comprehensive molecular screening of *E. coli* strains isolated from birds during outbreaks of colibacillosis for the presence of genes of three *Escherichia* pathotypes (APEC, UPEC, IPEC), it was shown for the first time that the strains were characterized by a high pathogenic potential and could be carriers of genes all pathotypes at once, while IPEC determinants were more common than others. It has been established for the first time that a positive adhesive profile for a number of genes positively correlates with the level of strain adhesion to chicken and human erythrocytes.

The aim of the work is to give a genotypic characterization of *Escherichia coli* strains isolated from poultry with colibacillosis, as well as to evaluate the relationship between the adhesive genotype and specific adhesion to erythrocytes.

Materials and methods. We used 28 *E. coli* strains with a unique genotype according to ERIC-PCR, isolated in 2016-2018 from different organs (excluding the intestines) of broiler chickens (*Gallus gallus* L.) of the Ross 308 cross with generalized colibacillosis [24]. The strains were deposited in the Ex culture collection of the Department of Biology, Faculty of Biotechnology, University of Ljubljana (Univerza v Ljubljani, Slovenia).

Determining whether a strain belongs to a phylogenetic group, sensitivity to antibiotics, and the presence of genes encoding the most common extended-spectrum beta-lactamases (ESBLs) was described by us previously [24]. In the sample ($n = 28$), polymerase chain reaction (PCR) at the end point was used to detect virulence genes characteristic of four conditional groups: genes that ensure the pathogenicity of bacteria of the *E. coli* species occurring in various pathotypes, genes that are pathogenic for birds *E. coli* (APEC), enteric pathogenic *E. coli* (IPEC: EPEC/ETEC/EHEC/EaggEC) and uropathogenic *E. coli* (UPEC).

For all types of PCR, primers and protocols of the authors who proposed them were used. Amplification was performed on a DNA Engine Dyad Thermal Cycler (Bio-Rad, USA). Band visualization and data documentation were performed using the Gel-Doc XR gel documentation system (Bio-Rad, USA).

The adhesion of bacteria to erythrocytes (specific adhesion) was determined by the method of V.I. Brilis et al. [38]. Formalized human erythrocytes of the 0(I) Rh(+) group and avian erythrocytes served as the cell substrate. The cells were preliminarily washed twice in 0.01 M phosphate-buffered medium (PBS) and standardized to a density of 100 million/ml. A suspension of microbial

cells, standardized in PBS to 2.0 according to McFarland, and erythrocytes were mixed in equal amounts (0.1 ml) in Eppendorf tubes, shaken for 20 min at 37 °C, after which smears were prepared on a glass slide. The smears were dried at room temperature, fixed with methanol for 10 min, and stained with 2% methylene blue.

The number of microbial cells attached to one erythrocyte was counted for at least 25 erythrocytes. The adhesive properties of cells were assessed using the microorganisms' adhesion index (MAI): the average number of bacteria attached to one erythrocyte involved in the adhesion process. Microorganisms were considered non-adhesive at MAI ≤ 1.75, low-adhesive at 1.76-2.5, medium-adhesive at 2.51-4.0, highly adhesive at MAI ≥ 4.0.

Statistical data processing was carried out using Microsoft Excel 2013 and Statistica v. 6.0 (StatSoft, Inc., USA). To assess quantitative indicators, the median *Me* and quartiles, Q1-Q3 were calculated. Relationships between traits were identified using Spearman's nonparametric rank correlation coefficient (Rs). Significance of differences between two dependent samples was assessed using the Wilcoxon signed-rank *W*-test, independent samples were compared using the Mann-Whitney *U*-test. The classification of strains was carried out by the method of hierarchical clustering (tree cluster analyses; the measure of distance is the Euclidean distance). Qualitative features were compared using χ^2 (with Yates correction) or Fisher's exact *F*-test. At $p < 0.05$, it was concluded that there was a statistically significant difference between the compared samples.

Results. Among 28 individual *E. coli* strains, carriers of both virulence genes common to all pathotypes and genes characteristic of representatives of the APEC, UPEC, and IPEC groups were found (Table 1).

1. Virulence genes detected in APEC (avian pathogenic *Escherichia coli*) strains isolente from cross Ross 308 broiler chickens (*Gallus gallus* L.) with generalized colibacteriosis (n = 28, 2016-2018)

Gene	Pathotype	Virulence factor and its function	References
fimH	UPEC, NMEC,	Universal fimbrial adhesive	[25]
ompT	SEPEC, APEC	Surface protein with protease activity	[26]
kpsMTII		Capsule formation type 2 gene	[27]
iroN		Receptor protein of the iron uptake and transport system	
traJ		Positive regulator of plasmid conjugative transfer	[28]
hlyF	APEC	Specific avian hemolysin F	[29]
Iss		Factor that increases cell survival in blood serum	[10]
iutA		Adhesin homologue	[30]
yqi		Specific avian adhesin	[21]
beA	EPEC, ETEC,	Invasive protein	[27]
Iha	EHEC, EaggEC	Adhesin	[31]
eaeA		Intimin	
*stx*1		Shigatoxin	
stx2			
estI		Thermostable enterotoxin	
estII			
ehxA		Enterohemolysin	
eltA		Heat-labile enterotoxin	
east1		Enteroaggregative thermostable enterotoxin	
subAB		Cytotoxin subtilase	[32]
hlyA		Alpha hemolysin	[33]
papGII	UPEC	Fimbrial adhesin, binds the Gal-alpha1-4Gal receptor found on	[30]
papGIII		epithelial cells lining the upper urinary tract	
papC		Outer membrane carrier protein involved in the export and assembly of pili subunits across the outer membrane	
sfaDE		Non-fimbrial adhesin	
afa/		Hemagglutinins of uropathogenic Escherichia coli mediate adherence	[27]
draBC		to the upper urinary tract	
upaG		Mediates aggregation, biofilm formation and adhesion for a number of extracellular matrix proteins; mediates adhesion to human T24 bladder epithelial cells	[34]
usp		Uropathogenic specific protein, colicin	[35]

N o t e. UPEC — uropathogenic *E. coli*, NMEC — neonatal meningitis-causing *E. coli*, SEPEC — sepsis-associated *E. coli*, APEC — avian pathogenic *E. coli*, EPEC — enteropathogenic *E. coli*, ETEC — enterotoxigenic *E. coli,* EHEC — enterohemorrhagic *E. coli*, EaggEC — enteroaggregative *E. coli*, ExPEC — extraintestinal pathogenic *E. coli.*

The studied *Escherichia* strains had a high overall virulence potential. The *fimH* gene encoding fimbrial adhesin was carried by 92.8% of the strains, the capsular formation gene *kpmsT* by 82.1%, the ompT gene for outer membrane protein with protease activity by 71.4%, and the iron uptake and transport system gene *iroN* by 67.8%. In almost half of the strains (46.4%), we identified all of the listed genes, in 32.1% three genes, in 14.3% two genes, one strain carried one gene and none of the listed genes. Separately, it should be noted that 53.6% of the strains had the gene for the positive conjugation regulator *traJ*.

Among the genes that most often characterize the APEC pathotype, the most common gene was the specific avian hemolysin gene *hlyF* (82.1%), the next was the avian adhesin Yqi (the *yqi* gene, 60.7%), *iss* was carried by 57.1% of strains, *iutA* by 42.8%. A quarter of the strains had all four genes, 21.4% had three genes, 25.0% had two genes, 21.4% had one gene, and only two strains had none of the genes from this group. The presence of specific marker genes made it possible to identify most *Escherichia* strains as APEC.

The present sample of strains lacked genes for Shiga-like toxin (*stx1/2*), genes for the main adhesion factor EHEC intimin (*eaeA*) and enterohemolysin (*exhA*). At the same time, 75% of the cultures were carriers of the *subAB* gene encoding the cytotoxin subtilase which is characteristic of Shiga toxin-producing *E. coli* strains. Carriers of other enterotoxin genes from the ETEC group were widely represented. The genes for thermostable enterotoxins *estI/II* were detected in 42.8 and 82.1% of strains, respectively, more than half of the strains (60.7%) carried the gene for enteroaggregative thermostable enterotoxin (*eastI*), thermolabile enterotoxin (*eltI*) was found in 14.3% cultures. In addition, 75% of APECs had the iha adhesin gene, which is one of the pathogenicity factors of diarrheagenic *E. coli*. Nineteen strains (67.8%) carried four or more of the listed genes, six strains (24.1%) carried from one to three genes, three strains did not have the genes of this group.

Of the group of genes most characteristic of uropathogenic *E. coli* strains, only *upaG* (67.8%) and *usp* (7.1%) were found.

When comparing the prevalence of the analyzed marker genes in avian strains, it turned out that the pathogenicity genes common to all *E. coli* pathotypes were more common in the sample than the APEC (*W*-test: $p = 0.029$) or UPEC (*W*-test: $p < 0.01$) genes. .01); APEC were more common than UPEC (*W*-test: $p < 0.01$); IPEC genes had a similar frequency of occurrence compared to genes common to all groups, but significantly exceeded the frequency of APEC and UPEC (*W*-test: $p < 0.01$) (Fig. 1).

According to the results of a cluster analysis of the presence of pathogenicity genes, three conditional groups of strains were identified (Fig. 2): pathogenic for birds and humans (the presence of 2-6 genes associated with APEC and 2-6 genes associated with ExPEC or IPEC) (24 strain); pathogenic for birds and not pathogenic for humans (presence of 2-6 genes associated with APEC, and 0-1 gene associated with ExPEC or IPEC) (2 strains); non-pathogenic (0-1 gene from any group, APEC, ExPEC, IPEC) (2 strains). Strains with phylogroup B1 in 85.7% were assigned to the first group. Based on the data obtained earlier [24], the group of strains isolated as pathogenic for birds and humans was characterized by a high frequency of occurrence not only of virulence genes, but also of ESBL genes, such as CTX (57.1% of strains) and TEM (71.4 % strains). In addition, 42.8% of the representatives of this group had the *traJ* gene (60% of the total frequency of occurrence of the gene in the sample) and 28.5% had segments of class 1 integrons (75% of the total frequency of occurrence in the sample).

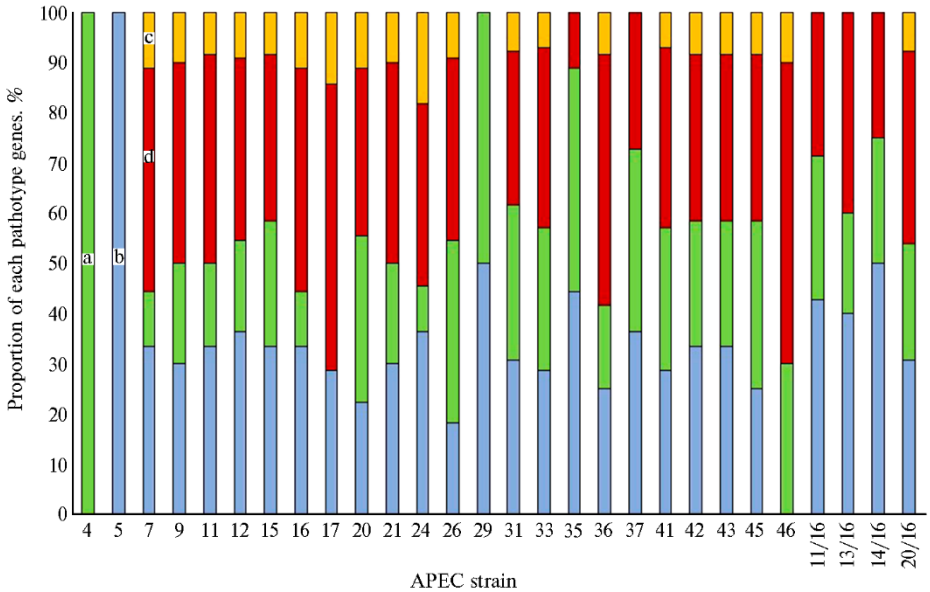

Fig. 1. Ratio of genes common to all pathotypes (b)**, APEC (avian pathogenic *Escherichia coli*)** (a)**, UPEC (uropathogenic *E. coli*)** (c) **and IPEC (intestinal pathogenic *E. coli*)** (d) **in APEC (avian pathogenic *E. coli*) strains isolated from the Ross 308 cross broiler chickens (*Gallus gallus* L.) with generalized colibacillosis** (*n* = 28, 2016-2018)**.**

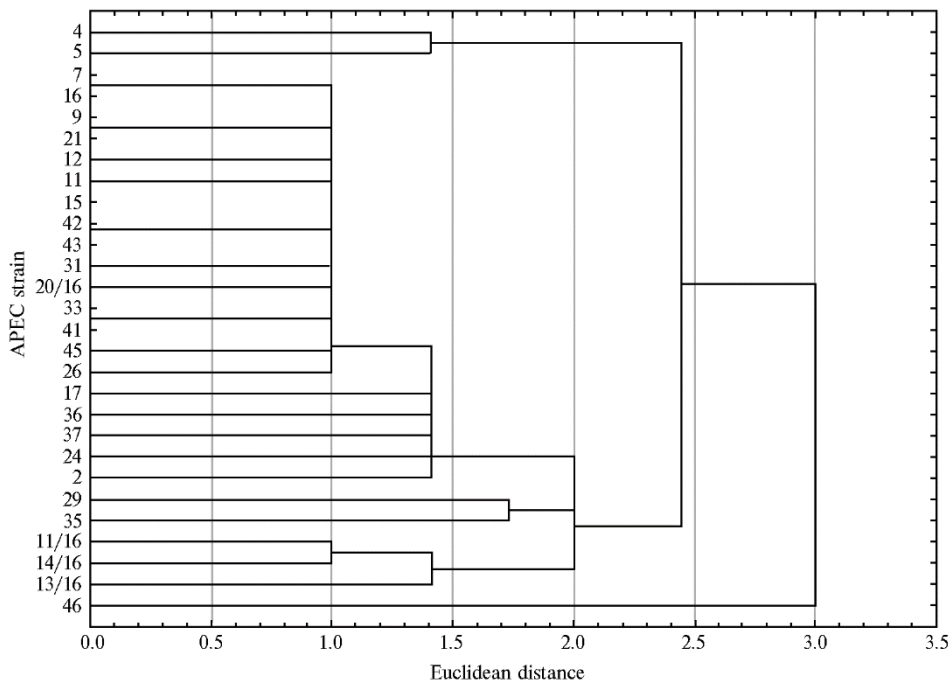

Fig. 2. Results of cluster analysis (Cluster analysis, Statistica v.6.0) **of the distribution of pathogenicity genes among APEC (avian pathogenic *Escherichia coli*) strains isolated from broiler chickens (*Gallus gallus* L.) of the Ross 308 cross with generalized colibacillosis** (*n* = 28, 2016-2018).

To determine the relationship between the presence of genes associated with virulence and antibiotic resistance, cultures were divided into groups with 0-2 and 3-4 genes (for genes of general virulence and APEC markers) and 0-3 and 4-6 genes (for IPEC markers). APEC with 0-2 genes from the group of common virulence were resistant to five or more antibiotics in 42.85% of cases, while strains

with 3-4 genes in 57.14% of cases. For genes characterizing the APEC group, the difference was even more significant: 42.85% resistant to five or more antibiotics among carriers of 0-2 genes and 64.28% among carriers of 3-4 genes. Interestingly, for the genes of the IPEC group, the ratio was reversed. The strains with 0-3 genes were resistant to five or more antibiotics in 66.66% of cases while the strains with a large number of genes only in 47.36%. This trend continued for any combination of the number of genes present and the antibiotics to which the strain developed resistance. It should also be noted that the correlation between these traits was significant only for the genes of the APEC group (Rs = 0.426), while for the IPEC genes it was completely absent (Rs = 0.041). The predominant part of the strains studied by us, according to MAI, was assigned to a low-adhesive group, regardless of the type of erythrocytes used (60.71% of cultures in the test with chicken erythrocytes and 85.71% of cultures with human erythrocytes), while the no correlation between MAI indicators was detected (Rs = 0.046) (Fig. 3).

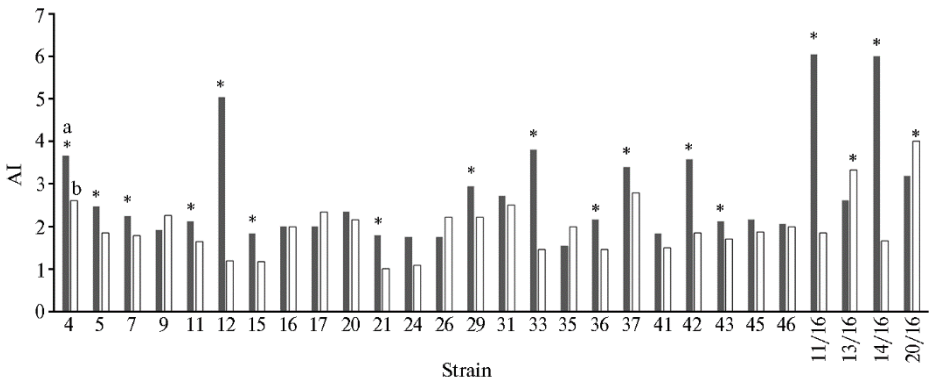

Fig. 3. Microbial adhesion index (MAI) **for APEC (avian pathogenic *Escherichia coli*) strains isolated from the Ross 308 cross broiler chickens (*Gallus gallus* L.) with generalized colibacillosis in the tests with chicken** (a) **and human** (b) **erythrocytes** (*n* = 28, 2016-2018).
* Differences between variants with different types of erythrocytes are statistically significant at p ≤ 0,05 (*W*-test).

For most of the APEC strains, the adhesive activity in relation to chicken and human erythrocytes was significantly different. Cells of 15 strains (53.57%) adhered better to chicken erythrocytes, two strains (7.14%) to human erythrocytes, and in 11 cultures (39.29%) adhesion indices did not differ. The average MAI index Me(Q1-Q3) for avian and human erythrocytes was 2.21(1.96-3.25) and 1.87(1.61-2.24), respectively, and was lower in the latter case (p = 0.0057). In the group of strains pathogenic for birds and humans according to the genotype, the degree of adhesion to avian erythrocytes, as in the general sample, was still significantly higher than to human erythrocytes (*W*-test: p = 0.007), which was determined by the greater tropism of APEC to bird erythrocytes.

When analyzing the relationship between the number of virulence genes and the degree of adhesion to two types of erythrocytes, dependences similar to those for antibiotic resistance were obtained. If in the groups of virulence genes common to all pathotypes and the APEC genes, the adhesion indices were approximately the same in strains with different numbers of detected genes, then within the IPEC gene group, the degree of adhesion to both types of erythrocytes was higher in strains with a smaller number of genetic determinants (Table 2). Separately, indicators of the adhesive activity of bacterial cells with *iss*$^-$ and *iss*$^+$ as well as *hlyF*$^-$ and *hlyF*$^+$ genotypes, which give strains with a positive genotype an advantage of survival during systemic coli infection and damage to erythrocytes, were analyzed. A tendency to an increase in MAI indices in *iss*$^+$ strains was revealed, 2.88(1.85-3.40) vs 2.14(2.00-2.47) and 2.07(1.67-2.23) vs 1 .83(1.47-

2.27) upon adhesion to human and avian erythrocytes, respectively. Similarly, the MAI indices for *hlyF*+ strains were higher in both models, while this difference turned out to be statistically significant with chicken erythrocytes, 2.35(1.93-2.27) vs 2.00(2.00-2.07) at $p \leq 0.01$.

2. The degree of specific adhesion of APEC (avian pathogenic *Escherichia coli*) strains isolated from the Ross 308 cross broiler chickens (*Gallus gallus* L.) of with generalized colibacillosis, depending on the genotype (n = 28, 2016-2018)

Group	AI	
	0-2 genes	3-4 genes
Genes found in various *E. coli* pathotypes	2.35 (2.00-2.54)	2.16 (2.00-3.410)
	2.23 (2.07-2.47)*	1.79 (1.47-2.00)
Genes of the APEC group (avian pathogenic E. coli)	2.20 (2.00-3.40)	2.24 (2.00-3.13)
	1.82 (1.51-2.20)	2.00 (1.75-2.23)
	0-3 genes	4-6 re genes
Genes of the IPEC (intestinal pathogenic E. coli) group	2.94 (2.47-3.66)*	2.00 (1.46-2.98)
	2.15 (1.86-2.60)*	1.79 (1.47-2.11)

N o t e. AI — adhesion index. - AIs for chicken erythrocytes are above the line, AIs for human erythrocytes.are below the line.
* Differences between the strains with different numbers of genes are statistically significant at $p \leq 0.05$ (*U*-test).

The APEC pathotype is considered relatively new in the classification of *E. coli*, and despite the active study of its representatives throughout the world, questions about the autonomy of this ecological group and its zoonotic potential remain open. Each of the described extra- and intra-intestinal pathotypes is a group of serotypes united by certain virulence factors. However, it should be noted that due to the plasticity of the *E. coli* genome, *Escherichia* subpathotypes cannot be conclusively identified, as some strains combine the main virulence characteristics of different groups and are considered potentially more virulent hybrid variants [18]. Phylogenetic analysis has shown that APECs have significant genetic similarity to dominant human ExPEC pathogens and, in addition, can be a source of ColV-localized genes or even whole plasmids for other ExPEC strains [10]. L. Zhao et al. [37] reported that the various UPEC and APEC genes had a similar tendency to be expressed in a mouse-avian cross-infection experiment. Through modeling of neonatal meningitis in rats, it has been shown that some strains of APEC are capable of causing meningitis in mammals, and possibly humans, and strains of NMEC cause colisepticemia in birds. These data support the hypothesis that APECs have zoonotic potential [38]. Currently, studies of *Escherichia* biodiversity are aimed, on the one hand, at searching for phylogenetic relationships between APEC representatives and strains of other pathotypes, and on the other hand, at assessing their potential pathogenicity (including epidemic danger) for humans. Considering that in our study all *E. coli* strains were isolated from parenchymal organs (spleen, liver, and kidneys), lungs, and internal part of the bones of a dead bird, they were regarded by us as the APEC pathotype and further genotyped.

It is known that there is not a single ExPEC pathogenicity factor associated exclusively with a specific disease or macroorganism, while the ability of opportunistic Escherichia to cause an infectious process in various biotopes of immunocompetent hosts is mediated by the presence of certain virulence determinants. According to J.R. Johnson et al. [39], a strain can be considered ExPEC if it contains two or more of the following virulence genes, the pap (P-pilus), *sfa/foc* (S/F1C-pilus), *afa/dra* (Dr, binding adhesins), *iutA* (aerobactin receptor) and *kpsM* II (group 2 capsule synthesis). The distribution structure of the ExPEC genes (see Table 2) made it possible to associate *E. coli* strains isolated during poultry colibacillosis with this group, except for two cultures. To classify a strain as belonging to the APEC group according to T.J. Johnson et al. [5], the presence of at least two marker genes in its genome is necessary. Twenty-five (89.3%) cultures

were assigned strictly to the APEC pathotype. Likewise R.R. Spurbeck et al. [40] proposed a set of four genes to identify ExPEC strains with uropathogenic potential. In our study, from the group of genes most characteristic of uropathogenic *E. coli* strains, only *upaG* and *usp* were revealed which are often found in the APEC genome [41, 42].

Comparison of the genetic and phenotypic characteristics of APEC and IPEC remains an area of extensive study in terms of identifying heteropathogenic and hybrid *Escherichia* strains [18]. In Russia, a new strain of *E. coli* serotype O101:H33 has been identified that exhibits properties and carries genes that are simultaneously characteristic of enterohemorrhagic and enterotoxigenic strains of *E. coli*, i,e., *stx2a*, eae_{γ}, *ehxA* and *est1* [19]. Hybrid strains phylogenetically located between Shiga toxin-producing *E. coli* and UPEC have also been described. They have been shown to possess pathogenicity factors characteristic of both pathogroups and are capable of causing both diarrhea and urinary tract infection [43]. Few works are devoted to comparison of the genotypes of avian pathogens and causative agents of acute intestinal infections in humans [44]. In our study, the presented sample of strains lacked the Shiga-like toxin (*stx1/2*) genes; nevertheless, 75% of the cultures were carriers of the *subAB* gene encoding the cytotoxin subtilase, which is characteristic of Shiga-toxin-producing *E. coli* strains — STEC [45]. STECs are known to synthesize two different types of cytotoxins, the StxI/II itself and cytotoxin subtylase (SubAB) which are structurally similar and consist of a single A subunit and a B subunit pentamer. Cytotoxin subtilase causes various cellular effects, including inhibition of protein synthesis, suppression of nuclear factor-kappa B activation, apoptotic cell death, and stress granule formation [46]. Intraperitoneal administration of purified subtylase cytotoxin to mice resulted in extensive microvascular thrombosis, as well as necrosis of the brain, kidneys, and liver, and was fatal for animals. Oral infection of animals with *E. coli* K-12 strain with cloned *subA* and *subB* genes caused dramatic weight loss in mice. These data suggest that subtilase cytotoxin may contribute to the pathogenesis of human diseases and become a novel toxic virulence marker in animal-derived *E. coli*.

Horizontal gene transfer is an important evolutionary mechanism in bacteria, which determines the complexity and plasticity of their genomes. It is known that opportunistic and pathogenic *E. coli* can originate from commensal strains after the acquisition of virulence-associated genes, which are usually located on the chromosome in certain regions called pathogenicity islands (PAI) [9, 47]. PAI may include genes for type III secretion system proteins, toxins, invasion factors, and iron uptake systems by which PAI can be identified [48]. In our study, we tested two PAI-determining genes, the *eaeA*, encoding the main adhesion factor intimin in the pathogenicity island LEE, locus enterocyte effacement in EPEC and EHEC strains, and *iutA*, encoding the aerobactin receptor in the pathogenicity island SHI-2 (Shigella pathogenicity island 2). The *eaeA* gene, which marks the enterocyte smoothing locus, was not found, while *iutA*, indicating the presence of one of the Shigella pathogenicity islands and designated as SHI-2 PAI, was detected in 42.8% of cultures, which also indicates their potential pathogenicity for humans. For example, 75% of $iutA^{+}$ isolates carried 10 or more virulence genes and were resistant to 5 or more antibiotics, while $iutA^{-}$ met these criteria only in 50.0 and 33.3% of cases. In addition, more than half of all strains had regions of conjugative plasmids, 60% of which were classified as pathogenic for birds and humans, which indicates the possibility of effective spread of pathogenicity determinants through horizontal transfer.

Plasmids pColV have long been associated with *E. coli* virulence, despite the fact that their eponymous trait, ColV bacteriocin production, is not considered a virulence trait [10]. Considering that the *iss*, *ompT*, *hlyF*, *repA* (RepFIB replication

protein) and *traJ* genes, encoding the sequences of putative pAPEC-O2-ColV virulence and transfer regions, were found in our APEC collection at a high frequency, it can be assumed that they were carriers of this plasmid. However, it should be noted that the pathogenicity of APEC does not directly correlate with the presence of other plasmids, as in some *E. coli* pathotypes [49]. For example, putative plasmid genes were widely distributed among both APEC and commensal chicken *E. coli* strains, and in the latter case the average number of plasmid genes per isolate was even higher than among APEC [9].

To identify the pathogenicity of a strain for humans, a quantitative method is used to determine the adhesion of bacteria on erythrocytes [36]. The ability of *E. coli* to attach to and agglutinate RBCs may be determined by mannose-sensitive fimbriae type 1. They are the most common type of bacterial adhesins and are expressed by both commensal and pathogenic strains of *Enterobacteriaceae*. Despite the common primary specificity of these pili for mannose, there is variation in the degree of adhesion between different species, as well as between different isolates of the same species. Thus, FimH of most fecal *E. coli* strains does not provide strong binding to receptors that contain terminal monomannose residues (Man1), however, some FimH variants of uropathogenic *E. coli* have a relatively high ability to bind Man1 due to the presence of functional point mutations at various positions in the FimH molecule [50]. In addition, mannose-resistant adhesins, designated Afa/Dr adhesins, recognizing the Cromer blood group system antigen, complement decay-accelerating factor, or CD55 (complement decay-accelerating factor; DAF) as a receptor, were found in IPEC and UPEC isolates. Interestingly, the AfaE adhesin expressed in E. coli isolates from various animals does not recognize human DAF, while the *afaE8* subtype, first identified in animal *E. coli* isolates, has subsequently been associated with human uropathogenic *E. coli* [51].

Apparently, the natural appearance of various variants of adhesins may reflect the ongoing adaptive molecular evolution of *E. coli* to improve the mechanisms of fixation in biotopes of various hosts. In this regard, we evaluated the adhesive phenotype of APEC using various erythrocytes to prove a possible selective advantage of target cells. The strains we studied are mostky classified as a low-adhesion group, regardless of the type of erythrocytes, while the average MAI when using avian erythrocytes turned out to be higher than that of human ones, which is directly related to the origin of the strains. Nevertheless, in 11 strains, the degree of adhesion did not depend on the type of erythrocytes, and in two cultures it was significantly higher on human than on avian cells. We should also emphasize that the indicators of adhesive activity of bacteria with the iss^+ and $hlyF^+$ genotypes were higher than in the group of strains that do not carry these genes, which gives them an advantage during systemic coli infection.

Thus, the vast majority of *Escherichia coli* strains isolated from the organs of broiler chickens with generalized colibacillosis were characterized as pathogenic for birds and humans, which indicates the potential of APEC as a reservoir of virulence factors for human infectious agents. Their genome simultaneously contained virulence genes characteristic of several pathotypes (with a predominance of APEC/IPEC hybrid pathotypes) while many APEC strains had an affinity with a group of diarrheagenic *Escherichia* in their genetic profile. Being epidemiologically dangerous for humans, they can realize their pathogenic potential to a greater extent due to toxin production genes and genetic determinants associated with general virulence than due to adhesion factors, and without connection with the antibiotic sensitivity profile. The specific adhesion of *E. coli* strains was more pronounced for chicken erythrocytes than for human ones. At the same time, regardless of the type of erythrocytes, high adhesive activity of bacteria correlated with

greater survival in the host's blood serum (*iss*$^{+}$ genotype) and the possibility of erythrocyte lysis (*hlyF*$^{+}$ genotype). The data we obtained on the molecular and adhesive properties of avian colibacillosis pathogens make it possible to assess their zoonotic potential and epizootic significance, and can also serve as the basis for improving the colibacillosis monitoring system in poultry farms.

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EXPERIMENTAL COMBINED MYCOTOXICOSIS IN PIGS AS AFFECTED BY INFECTION LOAD

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Abstract

Animal and human mycotoxicoses occur due to the ingestion of metabolites of toxicogenic microfungi. The effect increases in case of the co-ingestion of several mycotoxins, their mix with another ecotoxicants and biological agents. However, published research data only partially cover the nature of mixed mycotoxicoses in infectious diseases. This work shows for the first time the effect on pigs of the infection load of *Clostridium perfringes* and the combined effect of T-2 toxin, zearalenone, and deoxynivalenol in low doses. Our goal was to study the chronic form of combined mycotoxicosis in weaned pigs with a persistent infection in herd on the animal productivity, blood morpho-biochemical and immunological parameters, pathological changes in organs and tissues. Combined experimental mycotoxicosis with infectious load was modeled under the conditions of the vivarium complex (the Federal Center for Toxicological, Radiation and Biological Safety, 2018) on the weaning Large White piglets (*Sus scrofa domesticus*) divided into three groups 3 pigs each. Group I received no mycotoxins, group II received dietary T-2 toxin (70 μg/kg feed), group III received mixed dietary mycotoxins (DON 1000 μg/kg, ZEN 50 μg/kg and T-2 70 μg/kg). All animals were orally administered a suspension of *Clostridium perfringes* No. 392 type C (1×10^6 CFU/ml, 2 ml). On day 15, the animals were vaccinated intramuscularly in the posterior thigh with 1 ml of the associated vaccine against rota-, coronavirus and colorectal diarrhea of newborn piglets (FCTRB-VNIVI). Group I (control) was considered clinically healthy. Signs of intoxication, blood biochemical parameters (total protein, total bilirubin, glucose, malondialdehyde, alkaline phosphatase, aspartate aminotransferase and alanine aminotransferase activity), blood morphology (counts of erythrocytes, leukocytes, the hemoglobin level) and immunological parameters (T- and B-lymphocytes, titer of antibodies to vaccine antigens) on day 10, 20 and 30. The antibody titers to the *Escherichia coli* vaccine strain were determined by the agglutination reaction, to the coronavirus vaccine antigen by the ELISA test using a Multiscan FC photometer (Thermo Scientific, USA), and to the rotavirus antigen by an indirect hemagglutination test. At the end of the experiment, pieces of organs were fixed in 10 % neutral formalin, followed by generally accepted pathomorphological processing for histological studies. Histopreparations were stained with hematoxylin and eosin. Feed contamination with mycotoxins combined with clostridiosis had an adverse effect on the clinical and immune status, blood morpho-biochemical parameters, and pathoanatomical patterns. The changes were more apparent in co-contamination with ecotoxicants. Average daily bodyweight gain in piglets of group II was lower by 20.5 % compared to the control ($p \geq 0.05$), of group III by 39.2 % ($p \leq 0.05$). In group III, by the end of the experiment, there was a decrease in the erythrocyte counts by 40 % ($p \leq 0.001$), in the level of hemoglobin by 20 % ($p \leq 0.01$), glucose by 57 % ($p \leq 0.001$), and total protein by 13 % ($p \leq 0.05$). The concentration of bilirubin increased 5.1-fold ($p \leq 0.001$), the activity of alanine aminotransferase and aspartate aminotransferase 2.2- and 1.8-fold ($p \leq 0.001$), respectively, the concentration of malondialdehyde 2.8-fold ($p \leq 0.001$), the activity of alkaline phosphatase decrease by 41.5 % ($p \leq 0.001$). Co-mycotoxicosis combined with an infectious load led to immunological changes. Titers of specific antibodies to rotavirus were 8 times lower, to

coronavirus 6.4 times lower ($p \leq 0.05$), to *Escherichia* 5 times lower ($p \leq 0.05$) compared to the control. Marked pathological changes in the internal organs also occurred. Therefore, the co-mycotoxicosis due to T-2 toxin-, deoxynivalenol- and zearalenone-contaminated feed combined with the persistence of *Clostridium perfringens*, the causative agent of intestinal infection lead to suppression of immunological parameters (a decrease in the titer of specific protective antibodies, the number of T- and B-lymphocytes), activation of lipid peroxidation, and pathological changes in tissues and organs of the piglets.

Keywords: mycotoxins, pigs, blood, morpho-biochemical parameters, immune suppression, histological study

Mycotoxins, the secondary metabolites of microscopic fungi represent a serious problem for animal husbandry [1, 2]. Trichothecene mycotoxins [3] with cytotoxic and cytostatic properties [4] interfere with protein synthesis [5], and damage parenchymal organs, nervous and immune systems [6], are a health hazard for both humans and animals.

T-2 toxin is the most toxic of the secondary metabolites produced by *Fusarium* micromycetes [7]. As early as 1973, the World Health Organization (WHO) included T-2 toxin among the inevitable contaminants that contaminate feed and agricultural products [8]. Zearalenone (ZEN) is a mycotoxin that contributes to the development of estrogen syndrome [9]. Deoxynivalenol (DON) can cause a variety of symptoms, including necrosis of the intestinal tract, liver, and bone marrow with leukopenia, vomiting, and diarrhea [10, 11]. The issue of the allowed and toxic content of DON still remains an unaddressed problem [12].

To better assess animal and human health risks, it is important to study the toxicological effects of mycotoxin combinations. The vast majority of studies on this topic were carried out on cell cultures in vitro and focused on the study of the toxic effect of two mycotoxins [13-15], a number of authors note an increase in the toxic effect of several mycotoxins [16-19], as well as in combination with other ecotoxicants [20, 21].

Under production conditions, there are frequent cases of the presence in feed of such mycotoxins as T-2 toxin, DON and ZEN [21-22]. Pigs are thought to be sensitive to mycotoxins [23]. Studies on the combined effect of three mycotoxins at once on these animals are few [24-26]. There are few data on the study of combined mycotoxicoses under infectious diseases [27, 28]. Mycotoxins adversely affect immune parameters, increase the susceptibility of animals to pathogenic agents of a bacterial and viral nature [29]. We have not seen any works devoted to the combined effects of mycotoxins against the background of an infectious load of *Clostridium perfringes* in pig models.

This paper shows for the first time the effect on pigs of infection load of *Clostridium perfringes* and the combined effect of mycotoxins T-2 toxin, zearalenone and deoxynivalenol at low dosage. Consumption of feed containing T-2 toxin, deoxynivalenol and zearalenone under persistence of C. perfringens, a causative agent of intestinal infection in piglets, is accompanied by inhibition of the immune system function (decrease in the titer of specific protective antibodies, the number of T- and B-lymphocytes), activation of lipid peroxidation (LPO), the development of pathological processes in tissues and organs.

Our goal was to study the effect of the chronic form of combined mycotoxicosis on the background of infection persisting in the herd in weaned piglets on the productivity of animals, morpho-biochemical, immunological blood parameters, and the pathoanatomical picture of organs and tissues.

Materials and methods. Experimental combined mycotoxicosis under an infectious load was modeled in the conditions of the vivarium complex of the Federal Center for Toxicological, Radiation and Biological Safety (FCTRBS-VNIVI) in 2018 on Large White piglets (*Sus scrofa domesticus*) of weaning age (35 days) divided into three groups of 3 pigs each. Feeding and maintenance was carried out in a

group way. We used complete feed for feeding piglets (GOST 34109-2017. M., 2017).

Animals received a diet that did not contain mycotoxins (group I), that contained T-2 toxin (70 rg/kg of feed, group II), three mycotoxins (DON at 1000 μg/kg, ZEN at 50 μg/kg and T-2 at 70 μg/kg, group III). Access to water was not restricted. Mycotoxins (purity 96.7-99.8%, obtained at the FCTRBS-VNIVI) were introduced into the feed using sequential and stepwise mixing. The experiment lasted 30 days. Pathogenic isolate *Clostridium perfringes* No. 392 type C (collection of FCTRBS-VNIVI) was used as an infectious agent. All animals were orally administered a suspension of *Clostridium perfringes* (1×10^6 CFU/ml) in 2 ml. The amount of *C. perfringens* No. 392 type C microbial cells taken by us was previously estimated as a dose that does not cause clinical manifestations but ensures the carriage of clostridium. On day 15 of the experiment, the animals were vaccinated intramuscularly (in the back of the thigh in a 1 ml volume) with the associated vaccine against rota-, coronavirus and escherichiosis diarrhea of newborn piglets (FCTRBS-VNIVI).

When forming groups of animals, the principles of humanity and sufficiency were taken into account, while trying to bring the experiment closer to production conditions. Animals of group I were considered as clinically healthy, and the parameters of the remaining piglets were compared with their performance.

The signs of intoxication in pigs, biochemical parameters (total protein, total bilirubin, concentration of glucose, malondialdehyde MDA, activity of alkaline phosphatase AP, aspartate aminotransferase AsAT, alanine amine transferase AlAT) in blood serum, hematological parameters (number of erythrocytes, leukocytes, hemoglobin content) and immunological indicators (the number of T-, B-lymphocytes in the blood, the titer of antibodies to vaccine antigens) were assessed. Blood for research was taken from the tail vein on days 10, 20, and 30 of the experiment.

Hematological studies were carried out on a Mythic 18 Vet analyzer (Orphee Geneva, Switzerland). Biochemical parameters were measured on an ARD-200 analyzer (OOO VITAKO, Russia) using special reagent kits (Chronolab Systems S.L., Spain). The number of T- and B-lymphocytes was determined by the method of spontaneous rosette formation with goat erythrocytes [30]. The accumulation of MDA in the reaction with 2-thiobarbituric acid was used to assess the intensity of lipid peroxidation [31].

The titers of antibodies to the vaccine strain of *Escherichia coli* were determined in the agglutination test (AT). The titer was the last dilution with clear agglutination observed. The result was assessed by the amount of sediment and the transparency of the supernatant after 18-20 h (OD at λ = 490 nm). A positive result (titer) was taken as the ratio ≥2.0 of OD in the well with the studied serum to OD in the well with negative serum. The titers of antibodies to the rotavirus antigen of the vaccine were determined in the indirect hemagglutination test (IHA). The result of IHA was considered positive when the erythrocytes evenly covered the bottom of the well of the plate in the form of an umbrella for 2-4+. With a negative reaction, the accumulation of erythrocytes looked like a small disk ("buttons").

At the end of the experiment, animals of all groups were slaughtered in accordance with ethical standards. The samples for histological examination were fixed in 10% neutral formalin followed by processing by conventional pathomorphological methods. Histopreparations were stained with hematoxylin and eosin. Microphotography of histological preparations was carried out in transmitted light (Leica DM 1000 microscope, Leica DFC 320 camera, Leica Microsystems,

Germany) 200× magnification (10× objective, 20× eyepiece) and 600× (15× objective, 40× eyepiece).

During statistical processing, mean values (*M*) and standard errors of the means (±SEM) were calculated. Significance of differences was assessed by Student's *t*-test. Differences were considered statistically significant at $p \leq 0.05$; $p \leq 0.01$ and $p \leq 0.001$.

Results. The first signs of intoxication began to appear on days 6-8 of the experiment in animals treated with T-2 toxin, DON and ZEN. For several days there was a partial refusal of food. Subsequently, the animals consumed food in smaller quantities. The development of feed refusal syndrome is probably because trichothecenes disrupt the synthesis of liver proteins, cause hyperaminoacidemia, and increase the concentration of tryptophan and serotonin in the brain, and this affects the perception of satiety. Also, in piglets from group III, diarrhea and vomiting periodically occurred which are characteristic DON poisoning syndrome in pigs. Other researchers reported similar results [2, 32].

Clinical manifestations intensified in the second part of the experiment: we registered an increase in body temperature, the piglets huddled together, they were depressed, pursed their stomach, signs of gastrointestinal upset were noted. On day 23, one pig fell in group III. A *C. perfringes* No. 392 type C strain was isolated from organs of the piglets.

By the end of the experiment, pigs from group III had minor bleeding from the vagina. The bleeding was associated with the content of dietary zearalenone, an estrogenic mycotoxin. This is surprising given the low concentrations of ZEN. Zearalenone is a mycotoxin that contributes to the development of estrogen syndrome, including infertility, abortion, and resorption of fetuses [9]. Exposure of pregnant sows to ZEN reduces the number of follicles in piglets, leading to premature depletion of oocytes in adulthood [33]. Probably, the presence of trichothecene mycotoxins in the feed creates prerequisites for greater sensitivity of gilts to the action of zearalenone. Other researchers have also found that the effects of simultaneous exposure to mycotoxins are poorly recognized and difficult to predict [34]. Animals that received only T-2 toxin were more active and ate feed well.

1. Live weight, feed conversion rate and survival of Large White weaned piglets (*Sus scrofa domesticus*) with experimental combined mycotoxicosis under infectious load (*M*±SEM, Federal Center for Toxicological, Radiation and Biological Safety, 2018)

Parameter	Group		
	I ($n = 3$)	II ($n = 3$)	III ($n = 3$)
Weight at the beginning of the experiment, kg	14.9±0.2	15.2±0.2	14.6±0.2
Weight at the end of the experiment, kg	22.2±0.3	21.0±0.4	19.1±0.6*
Average daily increase in live body weight, g	244.0	194.0	148.3
feed conversion	3.55	4.06	5.07
Survival rate of piglets, %	100	100	66.6

N o t e. For a description of the groups, see the "Material and methods" section.

* Differences from control are statistically significant at $p \leq 0.05$.

The average daily live weight gain vs. control in piglets of group II was lower by 20.5% ($p \leq 0.05$) and from group III by 39.2% ($p \leq 0.05$). The deterioration in feed conversion occurred in group III (it was 1.4 times higher than in the group of biological control, $p \leq 0.05$) (Table 1).

Long-term consumption of toxic feed adversely affects morphological, biochemical, and immunological blood parameters (Table 2). Hematological parameters and blood biochemistry in the piglets of group I who received orally a suspension of *C. perfringes* No. 392 type C and did not receive mycotoxins, were

mostly within the normal physiological values for healthy animals. Thus, the number of erythrocytes was $5.45\pm0.15\times10^{12}$/l vs. physiologically normal values of $5.1\text{-}6.8\times10^{12}$/l. Leukocytes accounted for $16.47\pm0.51\times10^{9}$/l vs. $11\text{-}22\times10^{9}$/l, protein for 63.2±0.87 g/l vs. 58-83 g/l. We observed similar patterns in other parameters, including more labile traits, such as the activity of serum enzymes AlAT (compared to normal values of 22-47 U/l), AsAT (compared to normal values of 15-55 U/l) (see Table 2). The data obtained indicate that the animals of group I were clinically healthy.

2. Morphological, biochemical and immunological blood parameters of Large White weaned piglets (*Sus scrofa domesticus*) with experimental combined mycotoxicosis under infectious load, depending on the time from the beginning of the experiment (*M*±SEM, Federal Center for Toxicological, Radiation and Biological Safety, 2018)

Parameter	Group		
	I (n = 3)	II (n = 3)	III (n = 3)
	Day 10		
Erythrocytes, $\times10^{12}$/l	5.4±0.1	5.3±0.1	6.1±0.1
Leukocytes, $\times10^{9}$/l	16.4±0.5	17.5±0.4	19.3±0.5*
Hemoglobin, g/l	93.0±1.5	92.0±1.9	95.0±1.3
Total protein, g/l	63.2±0.8	62.2±0.9	62.6±0.8
Bilirubin total, μmol/l	1.5±0.2	4.4±0.5***	4.2±0.1***
Glucose, mmol/l	3.5±0.1	3.5±0.1	2.9±0.1*
AlAT, U/l	33.2±1.6	28.8±1.7	46.3±1.9***
AsAT, U/l	36.9±2.4	35.2±2.6	60.2±2.2***
ALP, U/l	137.9±14.9	76.8±15.9	312.2±18.7***
MDA, μmol/l	1.9±0.2	3.3±0.1***	5.2±0.1***
T-lymphocytes, %	53.2±2.4	51.6±2.2	53.7±2.8
B-lymphocytes, %	26.5±1.4	26.0±1.8	28.6±1.5
	Day 20		
Erythrocytes, $\times10^{12}$/l	5.6±0.1	5.2±0.1	5.8±0.1
Leukocytes, $\times10^{9}$/l	15.9±0.5	19.4±0.5**	26.9±0.2***
Hemoglobin, g/l	95.0±1.7	90.0±1.3	91.0±1.3
Total protein, g/l	63.3±0.7	60.6±0.7	58.1±0.8
Bilirubin total, μmol/l	3.2±0.2	6.3±0.3***	4.9±0.2***
Glucose, mmol/l	4.2±0.1	3.4±0.1*	2.5±0.1***
AlAT, U/l	29.2±1.9	39.4±1.3***	80.6±1.5***
AsAT, U/l	35.2±2.3	43.4±1.9**	75.2±2.2***
ALP, U/l	143.4±13.9	144.4±13.7	267.7±17.2***
MDA, rmol/l	2.8±0.1	5.6±0.1***	6.9±0.1***
T-lymphocytes, %	51.4±2.8	55.0±2.8	48.3±2.1
B-lymphocytes, %	25.3±0.9	28.6±0.4	26.1±0.4
	Day 30		
Erythrocytes, $\times10^{12}$/l	6.4±0.1	5.0±0.1**	4.0±0.2***
Leukocytes, $\times10^{9}$/l	17.9±0.4	17.4±0.5	19.1±0.5
Hemoglobin, g/l	99.0±1.8	85.0±1.6*	79.0±2.1**
Total protein, g/l	63.9±0.9	57.3±0.9	55.5±0.9*
Bilirubin total, μmol/l	2.5±0.1	7.3±0.1***	12.7±0.5***
Glucose, mmol/l	4.4±0.1	4.3±0.1	1.9±0.1***
AlAT, U/l	51.9±1.8	38.4±1.9***	114.8±1.9***
AsAT, U/l	55.3±2.5	48.8±2.6	99.5±2.5***
ALP, U/l	175.3±15.1	143.0±14.8*	102.6±14.0***
MDA, μmol/l	3.0±0.1	7.3±0.2***	8.7±0.2***
T-lymphocytes, %	46.7±3.0	52.0±1.8	32.1±3.0***
B-lymphocytes, %	28.5±1.3	27.2±0.4	23.2±1.8*

Note. ALT — alanine aminotransferase, AST — aspartate aminotransferase, ALP — alkaline phosphatase, MDA — malondialdehyde. For a description of the groups, see the "Materials and methods" section.

*, **, *** Differences from control are statistically significant at $p \le 0.05$, $p \le 0.01$, and $p \le 0.001$.

When compared to control, more pronounced deviations occurred in animals from group III. Thus, there was a 17.7% ($p \le 0.05$) increase in the number of leukocytes by day 10 of the experiment and a 69.2% ($p \le 0.001$) increase by day 20. In piglets of group II, the number of leukocytes by day 20 was 21.8% ($p \le 0.01$) higher than in the control group. Statistically significant changes in the number of erythrocytes and the hemoglobin level in animals from the experimental

groups occurred on day 30. In group III, on day 30, the erythrocyte number and hemoglobin level were 36.9 and 20.2% ($p \leq 0.01$) lower compared to control.

Progressive erythropenia and leukopenia and anemia indicated destructive changes in immunocompetent and hematopoietic organs known as a target for T-2 toxin [35]. This also occurred in chronic toxicosis caused by zearalenone and deoxynivalenol [36].

A pronounced decrease in the content of protein and glucose in animals treated with mycotoxins was recorded on day 30. In group III, the total protein decreased by 13.1% ($p \leq 0.05$) and the glucose level by 55.8% ($p \leq 0.001$). In groups II and III, by the end of the experiment, the level of bilirubin was 2.9 and 5.1 times higher vs. control ($p \leq 0.001$), respectively. In group III on day 20, the activity of blood ALP, AlAT, and AsAT was 1.9, 2.8, and 1.7 times higher ($p \leq 0.001$) vs. control. In group III vs. group I on day 30, the activity of ALP was 41.5% lower ($p \leq 0.001$), the activity of AlAT and AsAT was 2.2 and 1.8 times higher ($p \leq 0.001$). The data obtained indicate the destructive effect of the combination of mycotoxins on hepatocytes.

Activation of lipid peroxidation plays an important role in the pathogenesis of mycotoxicoses [37]. In groups II and III on day 30, the content of MDA, a quantitative marker for assessing the degree of lipid peroxidation increased 2.4-fold and 2.8-fold ($p \leq 0.001$), respectively, compared to control. Data of interest are changes in immunological parameters under the infectious load. On day 30, the number of T-lymphocytes in animals of group III was 31.3% lower ($p \leq 0.001$) than in the control.

The immunosuppressive effect of mycotoxins has long been known [38-40]. The decrease in the body's resistance to infectious diseases in mycotoxicoses is due to immunosuppression and the direct damaging effect of mycotoxins on the gastrointestinal tract [41].

In the gut, mycotoxins are metabolized and their toxicity decreases until a concentration is accumulated that can affect the intestinal mucosa [42]. Mycotoxins damage mucosal tissues, increase the permeability of the intestinal epithelial barrier, and cause a syndrome of poor absorption (or malabsorption). DON and T-2 toxin can directly damage mucosal tissues. Intestinal epithelial cells are the main targets for DON and T-2 toxin [43]. Deoxynivalenol has also previously been reported to reduce nutrient absorption [44]. Some studies indicate that the mechanisms of action of ZEN on intestinal function are unknown [12], while others indicate an increase in apoptosis and a decrease in the proliferation of Peyre's patches lymphocytes [45].

When the intestinal epithelial barrier is disrupted, pathogens are translocated under the action of mycotoxins, as, for example, in salmonellosis [46], pathologies caused by *C. perfringens* [47] and *Helicobacter* sp. [48]. DON-induced toxicosis leads to leak of plasma amino acids into the intestinal lumen, providing the necessary growth substrate for *C. perfringens* [49], which is a risk factor for intestinal disease and increases vaccination costs [50].

Determination of titers of specific antibodies in experimental animals confirmed the immunosuppressive effect of the combination of mycotoxins in the studied doses in the presence of a pathogenic bacterial pathogen in the herd (Table 3).

Severe intestinal infections (clostridium, rota-, coronavirus infections, escherichiosis) can be triggered by exposure to mycotoxins. Mycotoxins entering the body with food prevent the formation of specific antibodies in the required amount, which reduces the intensity of post-vaccination immunity. In our experiments, this was especially true for animals from group III which consumed a diet artificially contaminated with T-2 toxin, ZEN and DON. Also, in these piglets,

the titers of specific antibodies compared to group I (biological control) were lower, 8-fold ($p \leq 0.05$) to rotavirus, 6.4-fold ($p \leq 0.05$) to coronavirus, and 5-fold ($p \leq 0.05$) to *Escherichia*. Antibody titers in group I were typical for healthy animals. In group II, there was a tendency to decrease in titers, but the indicators remained within those for healthy animals.

3. Serum specific antibody titers in vaccinated Large White weaned piglets (*Sus scrofa domesticus*) with experimental combined mycotoxicosis under infectious load ($M \pm$SEM, Federal Center for Toxicological, Radiation and Biological Safety, 2018)

Group	Titer (dilution) of antibodies		
	to rotavirus	to coronavirusy	to *Escherichia coli*
I ($n = 3$)	8533.0±2090.0	170.6±52.0	213.3±65.0
II ($n = 3$)	7680.0±3135.0	128.0±0.0	160.0±0.0
III ($n = 3$)	1066.6±261.0*	26.7±6.5*	46.6±21.6*

N o t e. For a description of the groups, see the "Material and methods" section.

* Differences from control are statistically significant at $p \leq 0.05$.

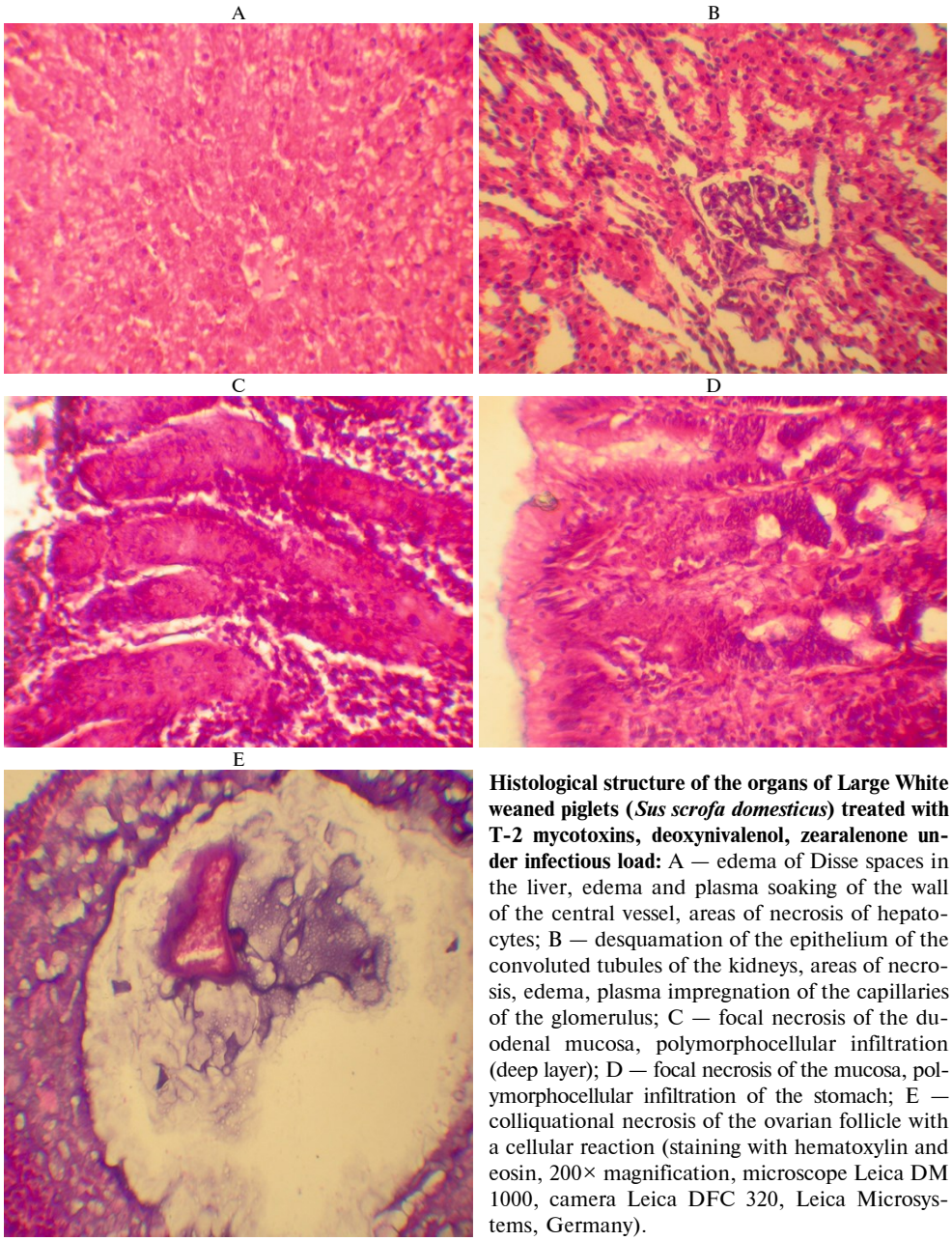
Our results indicate that mycotoxins, in particular, combination of T-2 toxin, ZEN and DON, can block the functions of the immune system of animals, preventing the synthesis of specific antibodies after vaccination. This increases the risk of reducing the protective effect of vaccination, which must be taken into account when organizing anti-epizootic measures.

After exposure to mycotoxins, severe intestinal illness caused by *C. perfringens* is more likely. At autopsy, gilts that received a diet with only T-2 toxin showed hyperemia of the mucous membrane in the stomach and in the small intestine. Pathological changes in internal organs with combined exposure to mycotoxins were more pronounced. Registered catarrhal and catarrhal-hemorrhagic lesions of the gastrointestinal tract, catarrhal inflammation of the lungs. The liver was enlarged, flabby, yellow in color. The muscles of the heart are flabby, the vessels of the brain are filled with blood. The kidneys are flabby, with a smoothed border. The spleen is enlarged, with blunt edges, cherry-red color. No such changes occurred in animals from groups I and II. The revealed histological changes confirm studies of long-term consumption of feed contaminated with DON and ZEN [51]. We also recorded polymorphocellular infiltration and focal necrosis in the mucosa of the wall of the stomach and duodenum (Fig.).

In animals exposed to three mycotoxins, protein dystrophy was noted in the kidneys, the epithelium of the tubules was with areas of necrosis. Protein dystrophy and foci of necrosis occurred in the liver, as well as the response of Kupffer cells in the form of an expansion of the Disse space, their enlargement and deformation. There was a depletion of the white pulp of the spleen. Serous edema of the interalveolar septa developed in the lungs. It is interesting to identify colliquatative necrosis of the ovarian follicle with a cellular reaction (see Fig.).

The intake of low levels of mycotoxins with feed is a serious problem for animal husbandry. In our studies, we tested the maximum allowable concentrations of mycotoxins. In the standard feed assessment scheme, their presence at the MPC level does not mean that the feed is potentially dangerous for pigs. It was of interest to us to methodically correctly reproduce this combination using a benign basic diet and the introduction of toxins against the background of persistent infection in the herd with the maximum exclusion of the influence of other significant toxins and xenobiotics.

Based on information about the individual toxicity of xenobiotics, it is not always possible to predict the effect of their combinations, as well as other influencing factors [52]. The relationship between mycotoxin exposure and infectious disease requires further study, as was noted previously [53].



Histological structure of the organs of Large White weaned piglets (*Sus scrofa domesticus*) treated with T-2 mycotoxins, deoxynivalenol, zearalenone under infectious load: A — edema of Disse spaces in the liver, edema and plasma soaking of the wall of the central vessel, areas of necrosis of hepatocytes; B — desquamation of the epithelium of the convoluted tubules of the kidneys, areas of necrosis, edema, plasma impregnation of the capillaries of the glomerulus; C — focal necrosis of the duodenal mucosa, polymorphocellular infiltration (deep layer); D — focal necrosis of the mucosa, polymorphocellular infiltration of the stomach; E — colliquational necrosis of the ovarian follicle with a cellular reaction (staining with hematoxylin and eosin, 200× magnification, microscope Leica DM 1000, camera Leica DFC 320, Leica Microsystems, Germany).

Thus, combined mycotoxicosis under an intestinal infection was accompanied by suppression of immunological parameters. The titers of specific antibodies to rotavirus decreased by 8 times ($p \leq 0.05$), to coronavirus by 6.4 times ($p \leq 0.05$), to *Escherichia* by 5 times ($p \leq 0.05$) compared to control. There was a decrease in the number of T-lymphocytes by 31.3% ($p \leq 0.001$), B-lymphocytes by 18.6% ($p \leq 0.05$). Due to activated lipid peroxidation, the concentration of malonic dialdehyde increased by 2.4-2.8 times ($p \leq 0.001$) in animals fed feed contaminated with T-2 toxin and three mycotoxins (T-2 toxin, deoxynivalenol and zearalenone) compared to control. Protein dystrophy and foci of necrosis were found in the liver and kidneys of piglets. Our findings indicate the negative impact of T-2 toxin, DON and ZEN combined intake at allowed concentrations on the clinical condition, morphobiochemical, and immunological parameters of piglets.

The data obtained can be used to diagnose animal diseases. In addition, these results should be accounted when carrying out anti-epizootic measures.

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ALEUTIAN MINK DISEASE: THE EFFECTIVENESS OF IMMUNOCORRECTIVE THERAPY

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Abstract

Aleutian mink disease is one of the main problems of fur farming, since it causes colossal losses to the industry due to the mass death of animals and due to the lack of effective means of treatment and prevention. In the presented work the results of using an antiviral agent based on alloferon Allokin-alpha in Aleutian mink disease are reported for the first time, an effective scheme of drug use has been developed, differences in clinical signs, blood biochemical parameters, morphological changes in internal organs have been established for animals with and without the peptide. The objective of our work was to evaluate the effectiveness of the use of the drug Allokin-alpha in Aleut mink disease and to investigate the effects of the drug as a means of immune correction, ensuring the maintenance of the body condition of sick minks. The experiments were performed in compliance with the requirements of the European Convention for the Protection of Vertebrate Animals used for experiments or for other scientific purposes. The research was conducted from May to December 2019 in one of the fur farms of the Northwestern region of the Russian Federation on the mink (*Neovison vison* Schreber, 1777) of the sapphire breed of 30-day age. The control and experimental groups included 5 males and 25 female mink each. All individuals in the experiment were intraperitoneally injected with Aleutian Mink disease virus (Sapphire isolate) at a dose of 2 cm^3. The experimental minks were twice injected subcutaneously with the drug Allokin-alpha (0.5 mg per animal) with a 6-day interval. The control animals were injected subcutaneously with sterile saline solution (0.9 % NaCl) in the same dosage regimen. Clinical, laboratory and economic indicators were assessed in both groups over 6 months of the experiment. Minks' mobility, coordination, appetite, color of the mucous membrane, fur condition and response to external stimuli were recorded. Animal mortality in both groups was checked daily, and all minks were weighed at the beginning of each month. Biochemical blood parameters were determined after 1, 3 and 6 months. Macroscopic, microscopic and chemical studies of feces were performed 3 days after repeated use of the drug Allokin-alpha. At the end of the experiment, animals were subjected to diagnostic slaughter, the kidneys, spleen, ovaries of females and testes of males were collected. The biomaterial was fixed, dried, and poured into paraffin. Sections (5-7 microns thick) were stained with hematoxylin and eosin and examined under a microscope (LOMO Micromed-5, JSC LOMO, Russia). Our research data show that in the test group the minks were strong and had a proportional physique, the animals were very mobile and responded vividly to external stimuli compared to the control group, in which individuals exhibited reduced reactions, lethargy and drowsiness. The experimental minks had their digestion normalized. After receiving Allokin-alpha, the average bodyweight was 19.7 % higher in females and 15.6 % higher in males compared to control. The mortality in the test group was 0 % vs. 20 % in the control. The control animals had a high level of urea (84.05±4.22 mmol/l), creatinine (142.06±2.62 mmol/l), and transaminase activity (73.60±5.84 IU/l for AlAT and 286.60±3.36 IU/l for AsAT). In contrast, in the experimental minks, the indicators were significantly lower, the 9.88±3.88 mmol/l for urea, 97.71±1.47 mmol/l for creatinine, 130.73±4.43 IU/l for AlAT and 184.88±3.22 IU/l for AsAT. The use of Allokin-alpha caused a decrease in pH from 8.7±0.25 to 6.8±0.18 in feces. Intestinal epithelial cells, blood pigments, and soluble protein were not

found in the feces of the experimental minks, but they appeared in the control minks, which indicated the normalization of digestion. Internal organs' morphology showed the signs of glomerulonephritis and foci of lymphoplasmacytic infiltration of kidneys, spleen, liver and ovaries of female and testes of males. Nevertheless, these changes was much less pronounced in the test group than in the control group. Our findings indicate that the use of Allokin-alpha according to the developed scheme has a positive effect on the Aleutian mink disease symptoms, leads to a decrease in animals' death, an increase in bodyweight gain and significantly reduces economic in losses.

Keywords: Aleutian mink disease, viral plasmocytosis, Allokin-alpha, alloferon, immunocorrector

Aleutian mink disease is still a serious problem for commercial fur farming [1, 2]. As an epizooty, the disease causes significant economic damage due to the high mortality of minks, the deterioration of the quality of furs and the death of young animals [3]. An important factor is the lack of specific means of treating animals with this viral pathology, and, as a result, the disease has become widespread throughout the world [4, 5], including the Russian Federation [6]. Thus, from 1990 to 2000, approximately 50% of fur farms ceased to exist in our country, some of which due to 100% damage to the main mink population by Aleutian disease [7]. Now, the disease occurs in many areas in Russia, affecting up to 70% of the livestock in some fur farms [8].

The causative agent of the disease (*Aleutian disease virus*, *Amdoparvovirus*, *Parvoviridae* family, *Parvovirinae* subfamily*)* [9, 10], can persist asymptomatically in the body, causing chronic course of the disease due to antibody-dependent enhancement [11, 12]. This is explained by the fact that during phagocytosis of the antigen-antibody complex, the pathogen does not degrade. Phagocytic cells migrate from the entry site of infection to the liver, where it is destroyed. Intact virus infects hepatocytes and generalization of infection occurs. In addition, the virus inhibits the activity of dendritic cells and cytotoxic T-lymphocytes. B-lymphocytes, on the contrary, are highly active and during antigen-dependent blast transformation develop into plasma cells which produce antibodies in excess [13]. This leads to hypergammaglobulinemia, plasmacytosis and the lifelong presence of the virus in the infectious immune complexes [14-16].

Minks of all color variants at any age get sick, but the Aleutian blue and sapphire are the most vulnerable [17]. The source of infection is animals that have been ill and excrete the virus with urine, feces and saliva [18, 19]. Infection, as a rule, occurs during mating, less often by aerogenic or alimentary ways. At first, the disease is asymptomatic. With the accumulation of sick animals and the development of pathological changes, as well as the action of stress factors, the infection acquires an epizootic character. In this case, significant death of animals can occur, 70-80% of those diseased.

Pathoanatomic signs of the disease are pronounced. The kidneys significantly increase in size, acquire a light orange color, with multiple whitish areas, the capsule is easily separated, and there are a large number of stellate hemorrhages in the parenchyma [20]. Hemorrhages appear in the mucous membrane of the stomach and intestines, the liver becomes red, edematous, enlarged, splenomegaly is observed. Histologically, periarteritis is found in all organs, and the tissues of the organs are infiltrated with a large number of plasma cells [21].

Given the above features of pathogenesis, it can be assumed that successful treatment of viral plasmacytosis requires correction of the immune status in order to increase the efficiency of phagocytosis, cytotoxicity of T-lymphocytes, and activity of dendritic cells.

In recent years, many studies have been published showing that insect

and animal oligopeptides play an important role in the regulation of host innate immunity during invasion of pathogenic microorganisms, since they contribute to the production of cytokines that stimulate the action of T-cytotoxic cells and NK cells [22]. Expression of molecules of the major histocompatibility complex also occurs in infected cells to provide the presentation of viral peptides to other cells of the immune system [22]. It is for this reason that one of the options for solving the problem can be the use of the antiviral agent Allokin-alpha (RU N002829/01-210610) which is a linear oligopeptide histidyl-glycyl-valyl-seryl-glycyl-histidyl-glycyl-glutaminyl-histidyl-glycyl-valyl-histidyl-glycine (alloferon), originally isolated by Professor S.I. Chernysh from insects [23].

Alloferon induces the synthesis of endogenous interferons, mainly IFNG (interferon gamma) and activates cytotoxic CD3+HLA-DR+ T cells even under a decrease in the absolute number of CD3+CD8+ cells, which is important for the antiviral and antitumor response [24, 25].

After the administration of alloferon to mammals and humans, IFNG synthesis increases with an increase in its concentration in the cervical mucus by 37 times compared to the initial level and by 32 times compared to control [26]. IFNG activates the effector functions of neutrophils, macrophages, cytotoxic T-lymphocytes and natural killers, since these cells have receptors for this interferon. They have increased cytotoxicity, microbicidal activity, increased production of cytokines, nitrooxide radicals, superoxide radicals, which leads to the death of intracellular parasites, including viruses [27]. Along with this IFNG oppresses anti-inflammatory IL4 and B-cell response, but enhances the production of pro-inflammatory IL2 which stimulates the proliferation of killer T-cells [28]. IFNG increases the expression of major histocompatibility complex antigens of both classes, I and II in different cells and induces the expression of molecules even in those cells that do not constitutively express them. This leads to an increase in the efficiency of antigen presentation and antigen recognition by T-lymphocytes and natural killer cells.

When using alloferon, the concentration of IL1B increased by 24 times compared to the original [26]. This cytokine can induce NO synthases, thereby increasing the production of nitric oxide by phagocytes [29] which is directly involved in phagocytosis, in particular, in antigen degradation. There was also an increase in the concentration of nonspecific esterase by 3.4 times compared to the initial level and 2.7 times compared to control [26]. An increase in the activity of macrophages also illustrates an increase in the concentration of myeloperoxidase. Nonspecific esterase serves as a cytoplasmic enzyme of dendritic cells [30] and T-killers, so it can be concluded that their activity increases proportionally.

In the complex treatment of severe dysplasia of the cervical epithelium and cervical cancer, the use of alloferon leads to a significant decrease in the immunosuppressive proteins TGFB and FOXP3 which block the activation of lymphocytes and macrophages, and also enhance angiogenesis in the tumor [24]. It should be emphasized that these changes develop in the localization of the infectious agent or tumor tissue, and not systemically. The most important general immune effect from the use of Allokin-alpha is an increase in CD4 content from 32.8 to 50.54% by days 12-18 from the start of treatment and correction of the CD4/CD8 immunoregulatory index from 1.16 to 2.00 [30].

In Russia, Allokin-alpha was studied as an antiviral agent by modeling an experimental viral infection on the example of avian herpes virus where the drug showed high efficiency against the pathogen [32]. However, data on the use of synthetic oligopeptides, in particular the Allokin-alpha as a therapeutic or prophylactic

antiviral agent in fur farming, in particular, under Aleutian mink disease, could not be found.

Here, for the first time, we show the antiviral effectiveness of the alloferon Allokin-alpha-based agent against Aleutian mink disease. An effective scheme for the drug use was developed. When comparing groups that received and did not receive the peptide, differences in clinical signs, biochemical blood parameteres and morphological changes in the internal organs of animals were established.

The work aimed to evaluate the Allokina-alpha effectiveness under the Aleutian disease of minks and to reveal applicability of the drug as an immune correction agent that ensures the maintenance of the condition of diseased minks.

Materials and methods. For the experiment carried out from May to December 2019 at one of the fur farms of the North-West region of the Russian Federation, 60 sapphire minks (*Neovison vison* Schreber, 1777), including 50 females and 10 males at the age of 30 days were selected. Tests were performed in compliance with the requirements of the European Convention for the Protection of Vertebrate Animals used for experiments or for other scientific purposes [33]. All individuals were intraperitoneally injected with 2 cm^3 of the culture isolate Sapphire of the Aleutian Mink Disease virus. To confirm the development of viral plasmacytosis, animals were analyzed for specific antibodies in blood serum by immunoelectroosmophoresis (IEOF) test {34]. Glass plates with 0.7% agar gel and barbital acetate buffer solution (pH 8.6) were used. A virus-containing diagnosticum suspension (TOO IMGEN, Russia) was the antigen. Electrophoresis was carried out for 30 min at a voltage of 120 V in a device for immunoelectrophoresis PEF-3 (JSC Medlabortechnika, Russia). The results were evaluated visually by the appearance of precipitation bands.

Diagnostics were additionally performed using polymerase chain reaction (PCR) to exclude false positive results. DNA was isolated from faecal samples with a commercial kit AmpliPrime DNA-sorb-B reagents (OOO Next-Bio, Russia) according to the manufacturer's instructions. PCR was performed using a commercial kit of amplification reagents (ABN Test System, Central Research Institute of Epidemiology of Rospotrebnadzor, Russia). Amplification reaction (a Tertsik cycler, OOO NPO DNA Technology, Russia) was carried out in a 25 μl mix according to the following protocol: a hot start at 95 °C: 5 min at 95 °C (initial denaturation); 10 s at 95° °C (denaturation), 10 s at 63 °C (primer annealing), 10 s at 72 °C (polymerization) (42 cycles); 1 min at 72 °C (final polymerization). Amplification products were detected by electrophoresis in a 2% TopVision Agarose agarose gel (Thermo Fisher Scientific, USA) with the addition of GelRed (Biotium, USA) in 1× TAE buffer (ZAO Evrogen, Russia) for 35 min at a voltage of 85 B. DNA Ladder 100 bp (Thermo Fisher Scientific, USA) was a molecular weight marker. Electropherograms were visualized using a Cellmager transilluminator with Quantity One version 4.6.3 (Basic) software (Bio-Rad Laboratories, Inc., USA). The results of the PCR diagnostics revealed the presence of the virus in infected animals.

The minks selected for the study were divided into two groups (control and test) of 30 individuals (25 females and 5 males) each. All animals were kept in separate cages, in separate two-row sheds, equipped with running water and electric lighting, and were fed standard rations; the minks received feed once a day.

The minks of the experimental group were subcutaneously injected with the drug Allokin-alpha (RKNPK of the Ministry of Health of Russia, Moscow) 2 times with a 6-day interval at a dose of 0.5 mg per animal. To prepare the

solution, the contents of the drug ampoule (1 mg of alloferon) were diluted in 1 ml of sterile saline (0.9% NaCl). After dilution, the drug was injected into the skin fold at the withers. Minks of the control group were injected subcutaneously with sterile saline (0.9% NaCl) in the same dosing regimen. The condition of both groups of minks was assessed by clinical, laboratory and economic indicators for 6 months.

The functional state of the animals was assessed by clinical methods, including mobility, coordination of movements, appetite, color of mucous membranes, coat condition, response to external stimuli, body weight and mortality. The mucous membranes were examined once a week selectively in 3-5 minks from each group. For examination, animals were fixed by conventional methods with traps and thick gloves, the mouth cavity was opened with ribbons. The fur quality was also assessed, the position of the body in space and behavior were recorded, and after slaughter, the pelt size was calculated based on the body length and chest girth. In both groups, the mortality was checked daily, all minks were weighed at the beginning of each month.

Blood biochemical parameters were determined after 1, 3 and 6 months. To collect the material, the minks were fixed in traps, the hair was cut off at the tip of the tail, the skin was treated with an alcohol solution, and 2-3 mm from the tip of the tail were cut with scissors. Blood was collected drop by drop into Improvacuter plastic tubes (China) with a blood coagulation activator (SiO_2) sprayed onto the inner walls. The samples were delivered to the laboratory, observing the storage temperature regime (+4 °C). Biochemical studies were carried out using an automatic biochemical analyzer Idexx Catalist One (IDEXX Drive, USA). Total protein, albumin, globulin, urea, creatinine, alanine aminotransferase (AlAT) and aspartate aminotransferase (AsAT) were measured in the blood serum.

Macroscopic, microscopic and chemical examination of mink feces were carried out according to S.V. Written [35]. Fecal samples were taken 3 days after the repeated administration of Allokin-alpha. Freshly isolated feces were placed in clean disposable containers and delivered to the laboratory no later than in 8 hours. The color, texture, odor, presence of visible impurities were determined macroscopically; pH was determined with litmus indicator test strips. Microscopic examination of feces was carried out in wet native and stained preparations using Lugol's solution, Sudan-III, 0.5% methylene blue solution. Chemical studies included benzedine and sublimate tests.

Morphological study was carried out at the end of the experiment during the slaughter of the main stock. All animals were subjected to diagnostic slaughter, kidneys, spleen, ovaries from females and testes from males were taken. The selected organs were placed in glassware and fixed in a buffered 10% formalin solution. The fixative volume was 10-20 times the sample volume. The fixed material was dried and embedded in paraffin. Sections 5-7 μm thick were placed on glass slides and stained with hematoxylin and eosin. The resulting preparations were examined under a microscope (LOMO Mikromed-5, JSC LOMO, Russia). Microphotographs were made with an MS-3 camera (OOO LOMO-MA, Russia), image analysis was performed using the MCview program (https://www.lomo-microsystems.ru/doc/po-ru-ms.pdf).

The results were processed using Statistica 10.0 software (StatSoft, Inc., USA) and Microsoft Excel 2016. Data are presented as arithmetic means (M) and standard errors of the mean (±SEM). Groups were compared using Student's t-test. Differences were considered statistically significant at $p < 0.05$.

Results. The mucous membranes of the oral cavity in minks had a pale pink color. The color of the mucous membranes remained unchanged during

the experiment. All the minks of the experimental group were very mobile and responded vividly to external stimuli (the appearance of people near the cage, shouting, knocking, distributing food) compared to the animals of the control group, where there were individuals with a reduced response. The physique of the minks from the experimental group was strong and proportional in contrast to the animals from the control group. In general, individuals from the control group by the end of the study showed signs of malaise, the lethargy and drowsiness.

1. Bodyweigh of sapphire minks (*Neovison vison* Schreber, 1777) infected with Aleutian mink disease virus upon administration of Allokin-alpha injected subcutaneously (*M*±SEM, a fur farm of the North-West region of the Russian Federation, 2019)

Bodyweight, g	Age, days	Control		Test	
		females	males	females	males
At the beginning of the experiment	30	192±5.9 (n = 25)	207±5.7 (n =5)	190±6.1* (n = 25)	209±5.6* (n = 5)
6 months from the beginning of the experiment	210	1320±6.1 (n = 21)	2230±5.6 (n = 3)	1580±6.4* (n = 25)	2580±6.2* (n = 5)

N o t e. For a description of the groups, see the "Materials and methods" section.
* Differences with the control group are statistically significant at $p < 0.05$.

Animals in both groups ate feed completely. The average weight of females treated with the drug was 1580±6.4 g and was 19.7% higher ($p < 0.05$) than in the control group. In males, the average weight in the experimental group was 2580±6.2 g which was 15.6% higher than the control ($p < 0.05$) (Table 1).

By the end of the experiment, all animals of the test group were alive, while 20% of the control minks died. The pelt area in the experimental group from females averaged 1120.1±3.8 cm^2, from males 1586.2±4.1 cm^2, in the control group the values were 1069.2±4.4 and 1524.3±4.1 cm^2, respectively. The increase in live weight in minks treated with alloferon led to an increase in the pelt size by an average of 51 cm^2 in females and by 62 cm^2 in males. In animals that were injected with Allokin-alpha, an increase in fur density was visually recorded. When blowing through the hair, no free skin areas were found. The hairs were strong and elastic. The silkiness of the fur was tactilely noted. The color was uniform throughout the body with a pronounced brilliance.

Biochemical blood tests revealed changes characteristic of the Aleutian disease. In the minks of the control group, the concentration of globulins, urea, and creatinine was significantly higher than in the animals treated with Allokin-alpha (Table 2). In the test group, these changes also occurred, but their severity was significantly less.

2. Blood biochemical parameters of sapphire minks (*Neovison vison* Schreber, 1777) infected with the Aleutian disease of minks upon administration of Allokin-alpha injected subcutaneously (6 months after the injection *M*±SEM, a fur farm of the North-West region of the Russian Federation, 2019**)**

Parameter	Norm	Control (n = 24)	Test (n = 30)
Total protein, g/l	72.8	97.50±1.53	88.30±1.49*
Albumin, g/l	36.9	33.00±0.82	31.60±1.16
Globulins, g/l	31.0	65.00±0.94	55.43±0.87*
Urea, mmol/l	3.52	84.05±4.22	9.88±3.88*
Creatinine, rmol/l	50.9	142.06±2.62	97.71±1.47*
Alanine aminotransferase, IU/l	80.1	273.60±5.84	130.73±4.43*
Aspartate aminotransferase, IU/l	125.6	286.60±3.36	184.88±3.22*

N o t e. For a description of the groups, see the "Materials and methods" section. Normative indicators are given according to O.Yu. Bespyatykh et al. [37], C.Zh. Batoeva et al. [38], N.V. Mantatova et al. [39].
* Differences with the control group are statistically significant at $p < 0.05$.

Analysis of digestion products showed that the use of Allokin-alpha caused a decrease in pH from 8.7±0.25 to an almost neutral value (pH 6.8±0.18). In addition, in the feces of minks from the experimental group, we did not find intestinal epithelial cells, blood pigments, soluble protein while in the control these were detected which indicates the normalization of the overall digestion process when the drug was injected. Consistency, color, smell, presence of neutral fat, pus, plant cells, detritus were the same in both groups (Table 3).

3. Coprogram of sapphire minks (*Neovison vison* Schreber, 1777) infected with the Aleutian disease of minks upon administration of Allokin-alpha injected subcutaneously (*M*±SEM, a fur farm of the North-West region of the Russian Federation, 2019)

Parameter	Control (n = 30)	Test (n = 30)
Consistency	Solid	Solid
Color	Dark green with a brownish tint	Dark green with a brownish tint
Smell	Specific	Specific
pH	8.7±0.25	6.8±0.18*
Neutral fat	+	+
Fatty acid	–	–
Soaps	–	–
Starch	Minor amount	A very small amount
Bilirubin	Doubtful	Absent
Blood	–	–
Leftover undigested food	Present	Present
Plant cells	+	+
Detritus	+	+
Pus	+	+
Intestinal epithelial cells	+	–
Blood pigments	+	–
Soluble protein	+	–

N o t e. For a description of the groups, see the "Materials and methods" section. Normative indicators are given according to O.Yu. Bespyatykh et al. [37], C.Zh. Batoeva et al. [38], N.V. Mantatova et al. [39].
* Differences with the control group are statistically significant at $p < 0.05$.

When examining tissue morphology of kidneys in the control group, we found proliferation of mesangial cells, an increase in the thickness of the mesangial matrix of the renal bodies, narrowing of the lumen of the capillaries, edema of the glomeruli, glomerulonephritis and lymphoplasmacytic infiltration characteristic of interstitial nephritis (Fig. 1, A). In the medulla there were focal, in some places confluent hemorrhages. Microscopic areas of calcification were also observed. In the experimental group, similar signs were found in the kidneys, however, an increase in the mesangial matrix and obliteration of the capillary lumen were 2.5-3 times less, there was a plethora of blood vessels, while there were no foci of hemorrhage, and lymphoplasmic infiltration was 2 times less pronounced (see Fig. 1, B).

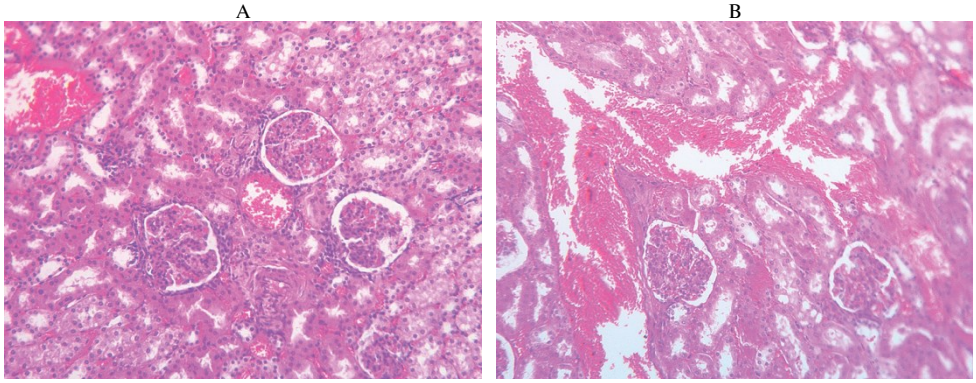

Fig. 1. The kidneys (histological sections) of sapphire minks (*Neovison vison* Schreber, 1777) infected with the Aleutian disease in the control (A) **and upon administration of Allokin-alpha injected subcu-**

taneously (B)**:** A — an increase in mesangial matrix of the renal glomeruli, capillary loops are poorly distinguishable, B — an increase in the mesangial matrix and a lower degree of obliteration of the capillaries of the renal bodies, less pronounced lymphoplasmacytic infiltration compared to the control (staining with hematoxylin and eosin, a microscope LOMO Micromed-5, JSC LOMO, Russia, magnification 10×). For a description of the groups, see the "Materials and methods" section.

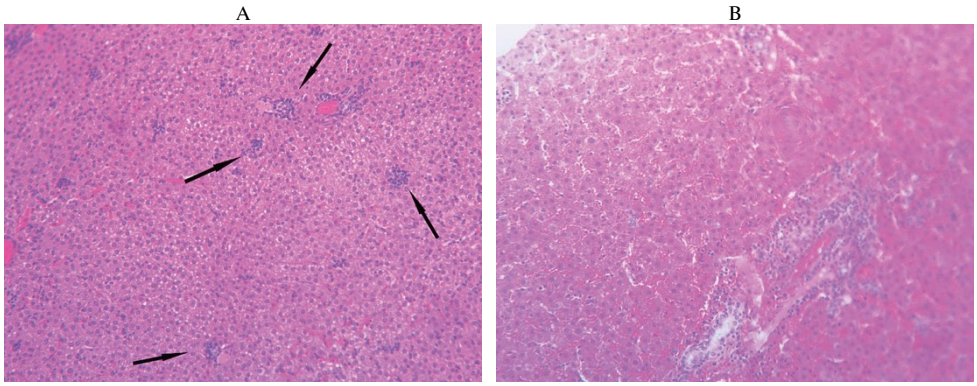

Fig. 2. The liver (histological sections) of sapphire minks (*Neovison vison* Schreber, 1777) infected with the Aleutian disease in the control (A) **and upon administration of Allokin-alpha injected subcutaneously** (B)**:** A — foci of lymphocytic plasma infiltration (indicated by arrows), B — lymphocytic plasma infiltration, no local foci (staining with hematoxylin and eosin, microscope LOMO Mikromed-5, JSC LOMO, Russia, magnification 10×). For a description of the groups, see the "Materials and methods" section.

Total hydropic dystrophy of hepatocytes occurred in the liver of control animals. Foci of lymphoplasmacytic infiltration of the stroma of the liver and bile ducts were revealed (Fig. 2, A). In the minks from the experimental group, the lymphoplasmic infiltration of the liver stroma was 40-50% less, and there were no local foci (see Fig. 2, B).

In the spleen of minks of the control group, we revealed hyperplasia (Fig. 3). Focal accumulations of a large number of plasma cells were observed around the blood vessels. Similar changes in the experimental group were 1.5 times less pronounced.

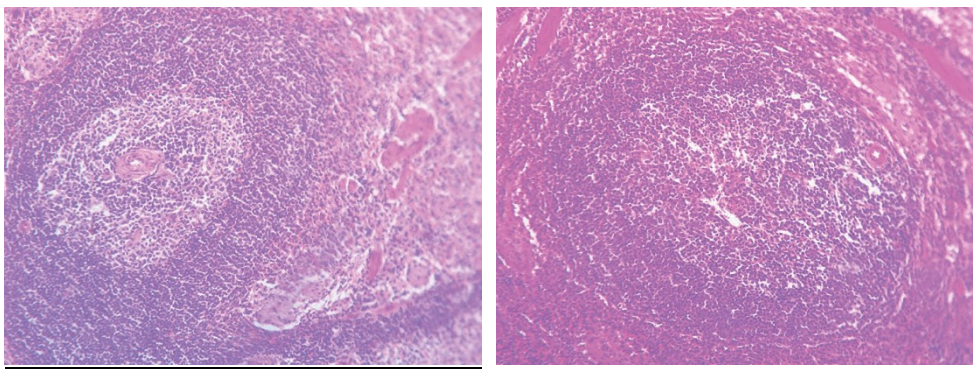

Fig. 3. Hyperplasia of the follicle in the spleen of sapphire minks (*Neovison vison* Schreber, 1777) infected with the Aleutian disease in the control (A) **and upon administration of Allokin-alpha injected subcutaneously** (B) (staining with hematoxylin and eosin, microscope LOMO Micromed-5, JSC LOMO, Russia, magnification 10×). For a description of the groups, see the "Materials and methods" section.

In the ovaries of control females, a large number of atretic bodies (dead oocytes), primordial, primary, secondary and tertiary follicles were found. In the experimental group, the number of primordial follicles in females increased while the number of atretic follicles decreased. Lymphocyte-plasmatic infiltration was expressed 2.5-3 times weaker (Fig. 4). In males, foci of lymphoplasmacytic infil-

tration also appeared in the convoluted tubules of the testes, however, these changes were 2 times less pronounced in individuals from the experimental group (Fig. 5).

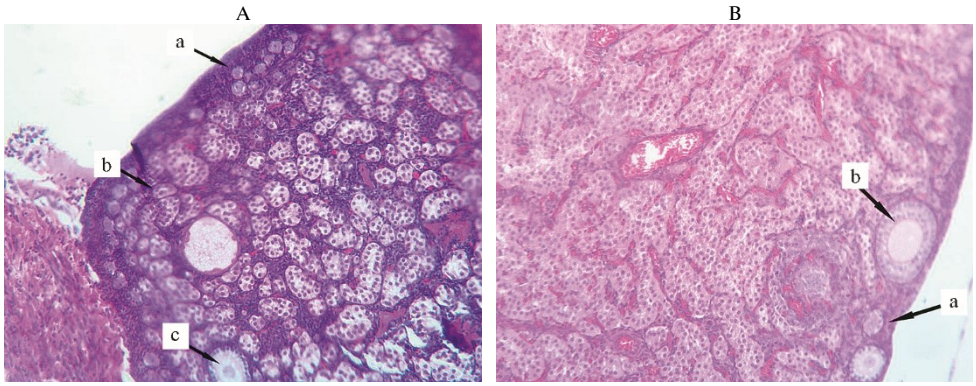

Fig. 4. Lymphoplasmacytic infiltration of the ovaries in female of sapphire minks (Neovison vison Schreber, 1777) infected with the Aleutian disease in the control (A) **and upon administration of Allokin-alpha injected subcutaneously** (B)**:** a — primordial, b — primary, c — secondary follicles (staining with hematoxylin and eosin, microscope LOMO Micromed-5, JSC LOMO, Russia, magnification 10×). For a description of the groups, see the "Materials and methods" section.

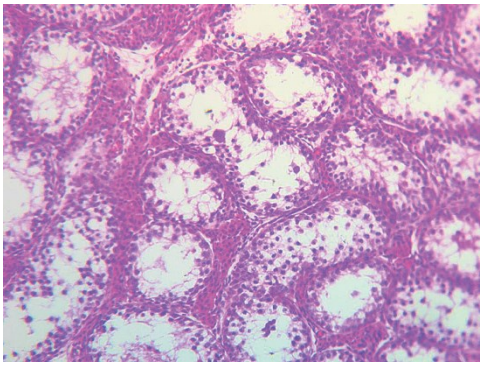

Fig. 5. Convoluted tubules of the testis in male of sapphire minks (Neovison vison Schreber, 1777) infected with the Aleutian disease in the control (A) **and upon administration of Allokin-alpha injected subcutaneously** (B) (staining with hematoxylin and eosin, microscope LOMO Micromed-5, JSC LOMO, Russia, magnification 10×). For a description of the groups, see the "Materials and methods" section.

A unified approach to the treatment and prevention of Aleutian disease of minks has not yet been developed [4, 5]. It is known that viral plasmacytosis can give a severe clinical signs characterized by anemia, cachexia, deterioration of fur quality, kidney failure and high mortality, which leads to enormous losses in mink farming [12, 36]. The use of the drug Allokina-alpha in our study prevented the development of such signs in the experimental animals, in contrast to the control group. The drug not only facilitated the course of the disease and mitigated its negative consequences, but also contributed to the restoration of body functions, as evidenced by increased activity and good quality of fur in experimental animals. In addition, the digestion processes normalized, the live weight indicators of females increased by 19.7%, of males by 15.6%, the physique of the minks treated with the drug remained stronger and more proportional. In addition, the use of Allokin-alpha ensure 0% mortality of the test animals vs. 20% of the control minks.

Many researches distinguish two stages in the pathogenesis of Aleutian mink disease: infectious and autoimmune. The infectious stage is characterized by stimulation of the proliferation of plasma cells that invade the spleen, liver, kidneys and other organs [18, 36, while the autoimmune phase is associated with the development of hypergammaglobulinemia [21]. The results of blood biochemical study obtained by us are consistent with the data of the authors who assert that at the autoimmune stage, the total protein in the blood of sick animals increases [36].

Thus, the total blood protein in the minks of the control group increased due to a sharp increase in the amount of globulins. This suggests that a significant amount of protein compounds in the blood are antibodies that form immune complexes which are subsequently fixed in the glomeruli of the kidneys, causing the development of glomerulonephritis. This is also confirmed by A. Prieto et al. [2], A.H. Farid et al. [10], O.Yu. Bespyatykh et al. [37]. In turn, the development of renal failure leads to an increase in the content of metabolic end products (urea and creatinine) which we observed in individuals of the control group. The morphological signs characteristic of glomerulonephritis and interstitial nephritis, i.e., proliferation of mesangial cells, an increased thickness of the mesangial matrix, narrowing of the capillary lumen, swelling of the glomeruli, hemorrhages, caused a significant decrease in the quality of the filtration capacity of the kidneys. All these changes, combined with a high-protein diet of minks, led to a higher urea content in the control animals and could indicate the transition of renal dysfunction to a chronic form. N.V. Mantatova et al. [39] also associated high urea content in minks (50.0 ± 0.58 mmol/l at $p \leq 0.05$) with kidney pathologies and a high-protein diet.

An increased amount of AsAT and AlAT indicated that during the phagocytosis of immune complexes by the reticuloendothelial system, the virus was released in the nuclei of liver macrophages and caused the destruction of hepatocytes. In the experimental group, on the contrary, the indicators for urea, creatinine and transaminase activity were much lower, which indirectly indicates a smaller scale of destructive changes in the kidneys and liver. Our data on organ morphology in the control animals are consistent with the reports of other researchers who claim that the Aleutian disease of minks is characterized by lymphoplasmacytic infiltration of internal organs, signs characteristic of the histological picture of glomerulonephritis, splenomegaly, and hydropic dystrophy of hepatocytes [17, 20]. It should be noted that in the test group, such changes were on average 2.5-3 times less pronounced. There were signs of intensive regeneration and functional restoration of damaged organs (e.g., plethora of blood vessels, absence of foci of hemorrhages, low the degree of oocyte apoptosis, low intensity of plasmacytic infiltration), which indicates an improvement in the general physiological state of sick animals treated with Allokin-alpha.

Thus, in mink infected with Aleutian disease, 2-fold subcutaneous injections of Allokina-alpha at a dose of 0.5 mg per animal with a 6-day interval had a positive effect on animal health, productive parameters and pelt quality. In individuals of the test group, the clinical condition improved markedly, activity and response to external stimuli increased. A decrease in the pH of feces from 8.7 ± 0.25 to 6.8 ± 0.18 indicates the restoration of digestion. The minks treated with the drug showed a noticeable increase in weight gain, their bodyweight was on average 19.7% higher in females and 15.6% in males. The pelts from test animals were larger, on average by 51 cm^2 for females and 62 cm^2 for males. Subcutaneous injections of Allokin-alpha significantly improved blood biochemical parameters. In the test animals, the urea content was 8.5 times less than in the control, which indicates a decrease in the development of renal failure. A significant decrease in the amount of alanine aminotransferase (by 2 times) and aspartate aminotransferase (by 1.5 times) indicates a low degree of destructive-inflammatory processes in the liver in animals from the test group. Morphological changes in the internal organs of the minks treated with the drug were 2.5-3 times less pronounced, while signs of restoration of damaged organs were observed. The positive effect of the antiviral agent Allokin-alpha on the biochemical mechanism of the disease development and the low degree of histo-

logical changes, contributed to avoiding mortality in the test group and an increase in the amount of pelts obtained, which significantly reduces economic damage. We can recommend Allokin-alpha for use at fur farms as an immunocorrector under viral plasmacytosis of minks, which improves the general physiological state of sick animals and fur maturation. This will minimize losses from the Aleutian disease of minks, and can also be considered as a means of nonspecific prevention and treatment. However, additional experiments are needed to clarify this possibility.

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