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CURRENT APPROACHES TO THE VACCINE DEVELOPMENT FOR AFRICAN SWINE FEVER

(review)

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Abstract

African swine fever (ASF), first described in 1921 by R.E. Montgomery (R.E. Montgomery, 1921), has been a major problem in pig production for over 100 years. The search for effective and universal specific vaccine variants started back in 1933 (J. Walker, 1933). This article presents a literature review on the most important and successful events in the history of ASF vaccine development, presenting the approaches on developing attenuated (C. Mucoz-Pérez et al, 2021), inactivated (E. Cadenas-Fernández et al., 2021), subunit (J.G. Neilan et al., 2004) and live vectored (J.K. Jancovich et al., 2018) vaccines. The widespread use of naturally attenuated non-hemadsorbing isolates as vaccines in the second half of the 20th century in European countries led to a persistent chronic ASF infection in a big number of pigs (J. Manso Ribeiro et al., 1963). Successive passages of field isolates of the ASF virus in many cell cultures did not show the proper result in weakening the virulent properties of the pathogen, despite genetic changes in the virus genome (I. Titov et al., 2017). Only modern technologies (e.g., homologous recombination and CRISPR-Cas9 genome editing) for obtaining genetically modified virus ASFV-G-ΔI177L by deleting specific genes in the genome led to the creation of effective candidate vaccines (M.V. Borca et al., 2020). Inactivated, as well as subunit vaccines based on recombinant proteins, caused the formation of specific humoral immune responses in high titers, but did not confer protective properties (G. Burmakina et al., 2016). Live vectored vaccines have become a new milestone in the fight against infectious animal diseases, in particular ASF; human adenovirus 5 (rAd) and modified vaccinia Ankara (MVA) are among the vectors for the development of such vaccines (L.C. Goatley et al., 2020). Attenuated vaccines based on genetically modified viruses with a deletion of specific genes *I226R* and *18-7GD* require international expertise for further registration and use in veterinary practice.

Keywords: African swine fever, ASF, vaccines, inactivated vaccine, attenuated vaccine, DNA-vaccine, recombinant vaccine, CRISPR-Cas9

African swine fever (ASF) is a contagious, natural-focal, transboundary disease of domestic pigs and wild boars, which can be hyperacute, acute, subacute, chronic and asymptomatic. Its etiological agent is an enveloped virus containing double-stranded DNA (dsDNA) (African swine fever virus — ASFV, genus *Asfivirus*, family *Asfarviridae*) [1] (Fig. 1).

The virus genome consists of 170-193 kb and contains 151-167 open reading frames (ORFs) [2]. The pathogen has 9 seroimmunotypes identified in the

haemadsorption delay test (HAD) and in the immunoassay on susceptible animals, and 24 genotypes based on the variability of the *B646L* gene encoding the vp72 capsid protein [2-4]. Due to the conservatism of the latter, intragenotypic differentiation of ASF virus isolates is also carried out based on the analysis of three highly variable genes, the *B602L* (CVR) gene encoding a non-structural chaperone that is involved in the assembly of the capsid, as well as the *E183L* and *CP204L* genes encoding the structural proteins vp54 and vp30 [5, 6].

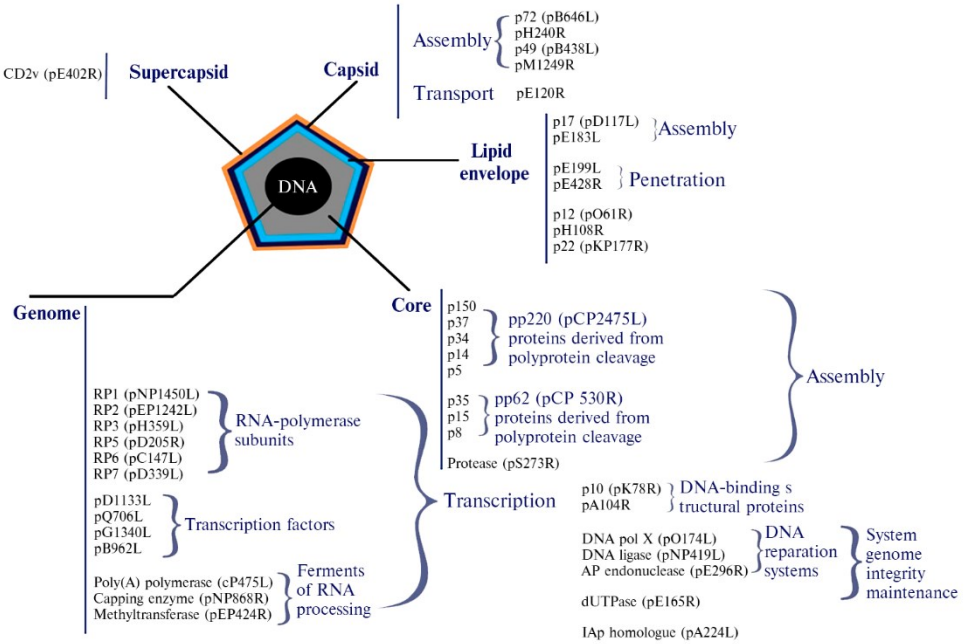
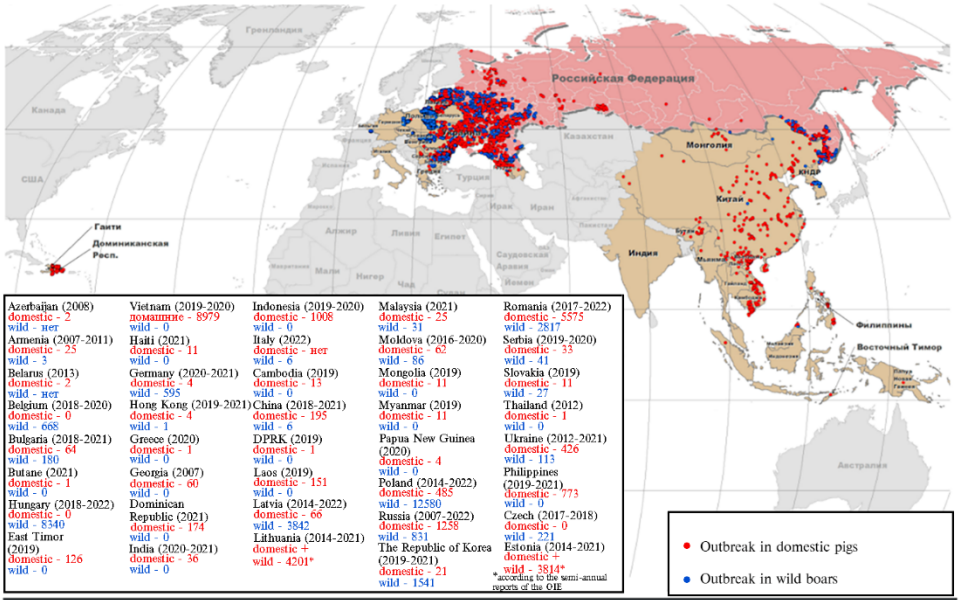


Fig. 1. Morphology of the African swine fever virus virion [1].

Epizootic situation for African swine fever virus in the Russian Federation, Europe, Asia and America, 2007-2022
(OIE emergency report data of 01/17/2022)



Azerbaijan (2008)
domestic - 2
wild - нет

Armenia (2007-2011)
domestic - 25
wild - 3

Belarus (2013)
domestic - 2
wild - нет

Belgium (2018-2020)
domestic - 0
wild - 668

Bulgaria (2018-2021)
domestic - 64
wild - 180

Butane (2021)
domestic - 1
wild - 0

Hungary (2018-2022)
domestic - 0
wild - 8340

East Timor (2019)
domestic - 126
wild - 0

Vietnam (2019-2020)
домашние - 8979
wild - 0

Haiti (2021)
domestic - 11
wild - 0

Germany (2020-2021)
domestic - 4
wild - 595

Hong Kong (2019-2021)
domestic - 4
wild - 1

Greece (2020)
domestic - 1
wild - 0

Georgia (2007)
domestic - 60
wild - 0

Dominican Republic (2021)
domestic - 174
wild - 0

India (2020-2021)
domestic - 36
wild - 0

Indonesia (2019-2020)
domestic - 1008
wild - 0

Italy (2022)
domestic - нет
wild - 6

Cambodia (2019)
domestic - 13
wild - 0

China (2018-2021)
domestic - 195
wild - 6

DPRK (2019)
domestic - 1
wild - 0

Laos (2019)
domestic - 151
wild - 0

Latvia (2014-2022)
domestic - 66
wild - 3842

Lithuania (2014-2021)
domestic +
wild - 4201*

Malaysia (2021)
domestic - 25
wild - 31

Moldova (2016-2020)
domestic - 62
wild - 86

Mongolia (2019)
domestic - 11
wild - 0

Myanmar (2019)
domestic - 11
wild - 0

Papua New Guinea (2020)
domestic - 4
wild - 0

Poland (2014-2022)
domestic - 485
wild - 12580

Russia (2007-2022)
domestic - 1258
wild - 831

The Republic of Korea (2019-2021)
domestic - 21
wild - 1541

Romania (2017-2022)
domestic - 5575
wild - 2817

Serbia (2019-2020)
domestic - 33
wild - 41

Slovakia (2019)
domestic - 11
wild - 27

Thailand (2012)
domestic - 1
wild - 0

Ukraine (2012-2021)
domestic - 426
wild - 113

Philippines (2019-2021)
domestic - 773
wild - 0

Czech (2017-2018)
domestic - 0
wild - 221

Estonia (2014-2021)
domestic +
wild - 3814*

*according to the semi-annual reports of the OIE

Fig. 2. African swine fever (ASF) epizootic situation in the world, 2007-2022 [11].

In the 21st century, in a relatively short period of time, ASF has become a global problem. Over the past 8 years, the rapid spread of infection in Europe (95% of outbreaks in the wild boar population) and Southeast Asia (97% of outbreaks in the domestic pig population), previously free from this disease, has become catastrophic. In the modern ASF panzootic (2007-2022), the first foci of infection officially appeared on the territory of Georgia at the beginning of 2007, when meat products contaminated with the ASF virus, delivered to the port of Poti on ships from the countries of South-East Africa, were sold among the local population . After that, during the spring-summer season around the city of Poti, mass deaths of pigs were recorded, including those that were free-range [7]. In November 2007, the first outbreak of ASF was registered in the Russian Federation in the Chechen Republic among wild boars [8]. In 2012, ASF outbreaks occurred in Ukraine, in 2013 in Belarus, in 2014 in Poland and Estonia, in 2017 in the Czech Republic, in 2018 in Hungary, Belgium and China, in 2020 in Germany , in 2021 in Malaysia, the Republic of Haiti and the Dominican Republic [9, 10]. According to OIE, as of 2021, ASF has been reported in 13 countries in Europe and 11 countries in Asia (Fig. 2) [11]. Between January 2020 and November 2021, the total losses from ASF in Europe and Asia amounted to 1,168,354 and 373,693 domestic pigs, respectively [12].

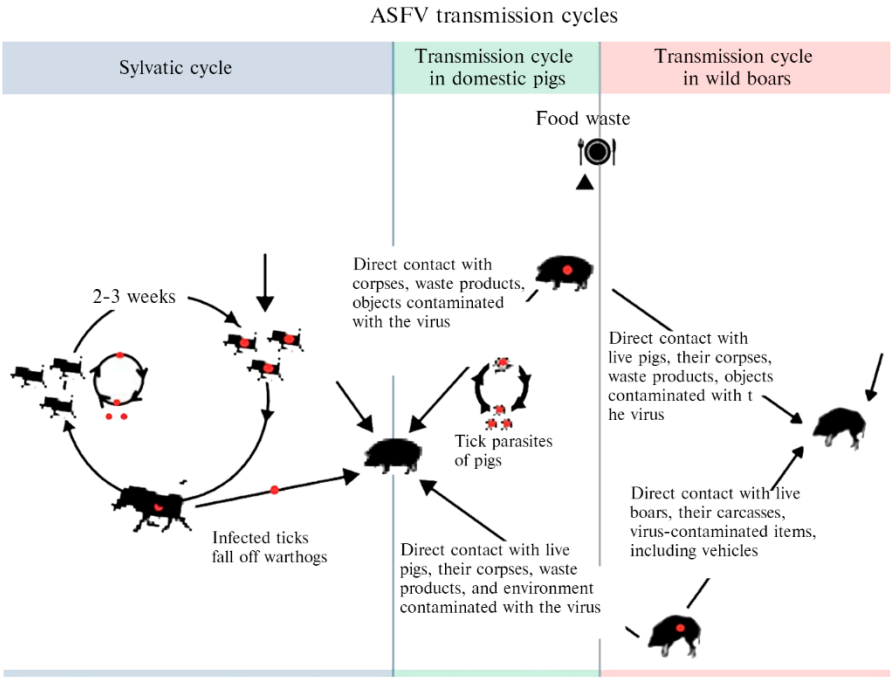


Рис. 3. African swine fever virus (ASFV) transmission [13].

The mechanism of transmission of the ASF virus occurs in three main ways: through soft ticks (sylvatic cycle: countries of South and Southeast Africa, Sardinia), contact transmission and alimentary infection, the last two involving both domestic pigs and wild boars (Fig. 3) [13]. In most regions of Africa, the mutualistic relationship between Ornithodoros moubata (a tick of the family Argasidae) and the warthog Phacochoerus africanus forms a stable sylvatic cycle, thus maintaining the circulation of the virus in nature [14]. In northern hemisphere conditions, wild boars can transmit the virus to domestic pigs by contact [15]. In addition, another mechanism is anthropogenic and associated with active

human activity, including the transportation of pig products and hunting products contaminated with the ASF virus, its entry into the feed of healthy susceptible animals [16].

A distinctive feature of the infection is the formation of a large number of antibodies, but the complete elimination of the virus from the body of a sick animal does not occur [17]. Moreover, due to the presence of the FCγ receptor in a macrophage, an antibody-dependent increase in infection can be observed, leading to excessive synthesis of IL-10, and, as a result, activation of Th2 cells [16, 18-20]. It has been experimentally established that the role of antibodies is to reduce the titers of primary viremia and, as a result, to delay the manifestation of clinical signs [21].

Due to the complexity of studying the mechanism of immune responses in the body of pigs in response to infection with the ASF virus, the search for approaches to the development of effective and safe means of specific prevention continues to this day [22]. In recent years, in the context of the unprecedented spread of ASF in the world, unfortunately, little progress has been made in creating an effective vaccine.

In this regard, in our review, we will consider issues related to the current state of affairs in the development of attenuated, inactivated and recombinant vaccines against ASF.

The role of cellular and humoral immunity in protection against ASF. Most of the available evidence points to the critical role of NK cells in the development of the immune response against ASF. An in vitro study found that virulent ASFV isolates inhibited NK cell activity [23]. A high level of cytotoxic CD8+ T lymphocytes, which destroy infected macrophages in the body, plays an equally significant role in the immune response in pigs [24]. However, hypergammaglobulinemia, as well as increased levels of plasma cells and cytotoxic T-lymphocytes, can cause both specific immunosuppressive and mediated response of the body through IL-4 and IL-10 [25, 26]. To date, attenuated vaccines obtained by deletion of certain genes and recombinant vaccines that induce strong cellular immunity, which is due to the early activity of NK cells and cytotoxic CD8+ T lymphocytes, have shown themselves to be the most effective. The main difficulty associated with the use of DNA vaccines is precisely to ensure the formation of cellular immunity associated with the early activity of NK cells and cytotoxic CD8+ T lymphocytes, which, of course, plays a greater role than short seroconversion to ASF virus proteins. , which are expressed in host cells [27].

Currently, there are works proving the existence of virus-neutralizing antibodies [17]. Convalescent sera obtained after immunization of pigs with the E75CV1-4 variant attenuated in the CV-1 cell culture protected animals from infection with the original E75 isolate, as well as E70, Lisbon 60, Malawi Lil 20/1 isolates of the ASF virus in 86-97% of cases [28]. In pigs passively immunized with purified immunoglobulins against the ASF virus, a delay in primary viremia was observed for 3 days compared with the control group of animals [29]. A study of the virus-neutralizing activity of antibodies in cultures of Vero cells and porcine alveolar macrophages showed 80% neutralization of the radiolabeled ASF virus, while its internalization into cells continued [30]. Antibodies to the cytoplasmic dynein domain DLC8 of the vp54 protein played a leading role in the neutralization of the ASF virus in the Vero cell culture [31, 32]. Despite the inconsistency of the data obtained regarding both the level and the very fact of the formation of humoral immunity against the ASF virus, most researchers agree that specific antibodies are important in the hemadsorption delay reaction upon re-infection with

the ASF virus and in the delay of primary viremia, but they do not play a protective role [17].

Attenuated vaccines. Attenuated vaccine preparations are based on naturally attenuated (or attenuated) viruses obtained in the laboratory through successive passages on sensitive cell cultures, as well as by genetic modification of a virulent virus as a result of the deletion of certain genes.

Naturally attenuated vaccines. The first data on the use of attenuated vaccines were published in 1933 by J. Walker [33]. The percentage of surviving pigs when immunized with such vaccines was low, and the administration of convalescent sera to intact pigs did not protect against infection with a virulent virus. The author then suggested that the low immune protection of pigs against ASF could be due to the high antigenic variability of the isolates due to the large number of strains of the pathogen compared to the classical swine fever virus [33]. Subsequently, it was found that immunization with attenuated vaccines could protect against infection only with a homologous genotype isolate, while infection with a heterologous virus isolate develops the clinical picture of ASF [34, 35].

In the work of F. Boinas et al. [36], pigs were injected with a naturally attenuated ASF virus isolate OURT88/3 (genotype I), isolated from the tick Ornithodoros erraticus of the family Argasidae and showing no haemadsorbing properties, followed by infection with a virulent haemadsorbing virus OURT88/1 (genotype I) as a punch strain. At the same time, neither viremia nor clinical signs of ASF were noted in animals. However, after infection of such pigs with Lisbon 57 isolate (genotype I), the death of the entire population occurred in 10-14 days [36].

When pigs were infected with a non-hemadsorbing NH/P68 isolate (genotype I), isolated in 1968 in Portugal from pigs with a chronic form of ASF, an asymptomatic course of the disease was observed, while early activity of NK cells (from day 7), late viremia (14 days after) and a high level of specific IgM, IgG1, IgG2 and IgA and cytotoxic T lymphocytes detected on days 7-18 after infection. Pigs that showed inapparent infection, early activity, and high NK cell counts were resistant to challenge with the virulent ASF L60 isolate (genotype I). However, in animals with chronic ASF, the level of NK cells was low and approached that of pigs from the control group [37, 38].

K. King et al. [39] immunized pigs with the avirulent OURT88/3 isolate and then with the original naturally attenuated low-virulence strain OURT88/1 of the ASF virus. When such pigs were infected with virulent isolates Benin 97/1 (genotype I) and heterologous Uganda 1965 (genotype X), the level of immune protection was 85.7 and 100%, respectively. Moreover, 78% of immunized pigs challenged with Benin 97/1 isolate and 50% of pigs challenged with Uganda 1965 isolate showed no viremia and no clinical signs of disease [39].

During immunization of pigs with a non-hemadsorbing naturally attenuated isolate of the ASF virus Lv/17/WB/Rie1 (genotype II), isolated from a wild boar in Latvia in 2017, followed by contact with pigs infected with the virulent Arm07 virus (genotype II), in 50% animals of the experimental group noted clinical signs and viremia, and 50% remained clinically healthy, but the viremia persisted. When pigs of the control group were infected with the related hemadsorbing virulent isolate Lv17/WB/Zieme3, death occurred on day 12 [40]. Oral vaccination of wild boars with the Lv/17/WB/Rie1 strain showed 92% immune protection against challenge with Arm07 [41]. A vaccine candidate based on Lv/17/WB/Rie1 is currently being evaluated for safety in the wild boar population. Different survival rates of animals were noted after vaccination and revaccination at doses of

10^3 TCD_{50} and 10^4 TCD_{50}, which shows the importance of further studies of this vaccine prototype [42].

Therefore, naturally attenuated vaccine variants of the ASF virus show wide variability in terms of protective properties even against challenge by homologous genotype isolates [17]. This feature hinders the development of a unified drug with a wide range of protective activity.

Laboratory attenuated vaccines. Cultural vaccines. When attenuating the virulent isolate BA71 of the ASF virus (genotype I), 36 passages were performed on porcine macrophages, followed by 23 passages on a transplanted Vero cell culture. This resulted in the BA71V variant, which proved to be the standard for ASF virus titration by plaque forming units (PFU), but this variant did not reduce the lethality of pigs upon infection [43].

After 50 successive passages of the virulent K49 ASF virus isolate (genotype I) on a transplantable porcine embryonic kidney (SPEV) cell culture and 262 passages on a primary culture of porcine bone marrow cells, the avirulent KK262 strain was obtained. After two injections of KK262 to the pigs of the experimental group (on the 1st and 21st days), viremia was observed in 33% of the animals on day 28, but when infected with the initial virulent K49 isolate, on the 42nd day from the start of the test, all pigs remained alive [44].

When studying the biological properties of the Odintsovo 02/14 isolate (genotype II) isolated from wild boar, three successive passages of the virus were performed on a culture of porcine bone marrow cells (PBC). It is important to note that the initial field isolate isolated from the spleen of a fallen wild boar caused 87.5% lethality in domestic pigs after the isolate was administered to five animals intramuscularly at a dose of 10 HAD_{50} and to five animals intranasally at a dose of 50 HAD_{50} [27, 45]. Adaptation of the Odintsovo 02/14 isolate on a transplanted CV-1 cell culture for 30 consecutive passages (the virus was named ASF/ARRIAH/CV-1/30) resulted in a decrease in hemadsorption from 40-50 to 20-30 erythrocytes attached to an infected cell. Infection of pigs with this variant showed a decrease in mortality to 16.7% and the resistance of surviving animals to control infection with the virulent Arm 07 isolate [46-48].

When adapting the Georgia 2007/1 isolate, 110 passages were carried out in Vero cell culture. At the 80th passage, the ASFV-G/VP80 variant showed a 10-fold reduced ability to replicate in a primary trypsinated porcine macrophage cell culture, and at the 110th passage, the ASFV-G/VP110 variant showed a 10^5-10^6-fold decrease in replication. When pigs were immunized with the ASFV-G/VP110 variant, no clinical signs of the disease were noted during 21 days of observation. However, control infection with the Georgia 2007/1 isolate did not show the effectiveness of such immunization, since all pigs showed clinical signs of ASF and death was recorded on day 9 [49].

The results of the conducted studies on laboratory attenuation of field isolates allow us to conclude that, as a rule, there is no such weakening of the virulent properties of the ASF virus in order to further use it for safe immunization. However, in the development of vaccines, targeted modification of the genes of attenuated variants of the ASF virus using molecular biology methods may be successful.

Vaccines based on genetically modified viruses. One promising new approach to obtaining safe attenuated vaccines involves the deletion of specific ASF virus genes encoding proteins that serve as virulence factors [22, 50]. However, in this case, significant difficulties are associated with the fact that the functions of many proteins have not been studied. For example, deletion of the

KI69R gene encoding thymidine kinase resulted in a loss of virulence in the Malawi Lil-20/1 isolate (genotype VIII), but retained virulence in the Georgia 2007/1 isolate (genotype II), which, apparently, is explained by compensatory mutations in other parts of the genome [51, 52].

Removal of six genes of the *MGF360* and *MGF505* multigene families from Georgia 2007/1 (*MGF505-1R*, *MGF360-12L*, *MGF360-13L*, *MGF360-14L*, *MGF505-2R*, and *MGF505-3R*) completely weakens the virulence properties of the virus. Immunization of animals with this deleted variant at doses of 10^2 and 10^4 HAD_{50} did not cause the development of clinical signs of ASF, and after control infection with the virulent Georgia 2007/1 strain, no signs of the chronic course of the disease were noted, however, moderate viremia was detected for approx. 7.5 days [53]. When immunized with the Georgia 2007/1 variant with a deletion of the *9GL* gene encoding phosphorylase, a higher level of protection was observed when combined with a deletion of the *UK* (*DP96R*) gene [50, 54]. However, simultaneous deletion of the *MGF360* and *MGF505* genes encoding type I interferon inhibitors and *9GL* did not result in a protective effect during immunization [55].

Pigs immunized with the BA71ΔCD2 variant with a deletion of the *EP402R* gene obtained by homologous recombination were resistant to infection with the original virulent BA71 isolate (genotype I), as well as virulent E75 isolates (genotype I), Georgia 2007/1, RSA/11/2017 (genotype XIX), Ken06.Bus (genotype IX) [56, 57].

Deletion of the DP148R gene in the Benin 97/1 ASF virus isolate (genotype I) resulted in a complete loss of virulence. In pigs immunized with BeninΔDP148R, intramuscular infection with the original isolate showed 100% protection, while intranasal infection was 83.3% [58].

When immunizing pigs with NH/P68DA238L-COS7 variants with a deletion of the *A238L* gene, NH/P68DA224L-COS7 with a deletion of the *A224L* gene, NH/P68DEP153R-COS7 with a deletion of the *EP153R* gene at a dose of 10^6 TCD_{50} and NH/P68DA276R-PAM at a dose of 10^2 TCD_{50} followed by a control infection of Arm07 strain at 10 HAD_{50}, the weakening of the virulent properties was achieved only for a deletion of the *A224L* gene. Immune protection in this case reached 100% [59].

In studies on the deletion of the *MGF505-1R*, *MGF505-2R*, *MGF505-3R*, *MGF360-12L*, *MGF360-13L*, *MGF360-14L*, *EP402R*, *9GL*, *DP148R* genes encoding seven different proteins, Chinese scientists [60] obtained a modified version of the ASF virus HLJ/18-7GD, after intramuscular inoculation of which the pigs remained clinically healthy for 3 weeks of observation. When pigs were infected with the virulent HLJ/18 isolate (genotype II) at 200 LD_{50}, animals immunized with 10^3 TCD50 HLJ/18-7GD developed fever for 3-9 days with a maximum rise in temperature up to 42 °C, however, in pigs immunized with 10^5 $TCD5^0$ HLJ/18-7GD, a slight rise in temperature to 40.7 °C occurred only during the first day [60].

In 2020, a group of scientists from the USA published data on 100% protection of the pig population immunized with an attenuated ASFV-G-ΔI177L variant of the ASF virus, obtained from the original virulent isolate of ASFV-G (Georgia 2007/1) as a result of the deletion of the I177L gene, which had previously The study was not typical of such experiments. In the experimental group, pigs were injected with ASFV-G-ΔI177L at 10^2 HAD_{50} after which insignificant titers were recorded during viremia ($10^{1.8}$-10^5 HAD_{50}/cm^3 on day 4, peak at 10^4-$10^{7.5}$ HAD_{50}/cm^3 on day 11 followed with a decrease to $10^{2.3}$-10^4 HAD_{50}/cm^3 up

to day 28) and the absence of any clinical signs of ASF during 28 days of observation. These pigs were subsequently challenged with 10^2 HAD_{50} of the original ASFV-G isolate. Within 21 days, the animals had no clinical signs of ASF, viremia developed, titers did not exceed those in the first observation period, and the virulent virus was not detected in the blood by RT-PCR (qPCR) [61]. The work of M.V. Borca et al. [61] was the first report on the formation of sterile immunity against ASF in the history of the study of this infection. Adaptation of ASFV-G-ΔI177L on PIPEC (Plum Island porcine epithelial cells) cell culture resulted in a stable isolate of ASFV-G-ΔI177L/ΔLVR with a deletion of the *MGF* and *X69R* genes, protecting 100% of pigs from infection with virulent ASFV-G, which can be used in the production of the universal ASF vaccine [62]. Later, the authors showed the effectiveness of an experimental vaccine based on ASFV-G-ΔI177L when administered orally, which is of great importance from the point of view of the prospects for immunization of wild pigs (especially the European boar *Sus scrofa*). At the same dose with intramuscular inoculation, viremia titers for oronasal administration were significantly lower, while IgG1, IgG2 and IgM titers remained at the same level [63]. A recent study showed that an experimental vaccine based on ASFV-G-ΔI177L successfully induced immune protection in Vietnamese pigs against ASF virus (genotype II) field isolates in Vietnam [64].

Similar studies were carried out in the PRC in 2021, where the SY18ΔI226R isolate with a deletion of the previously undescribed functional I226R gene encoding the conserved pI226R protein, localized in the virosome of the cell cytoplasm ("viral factories"), was used as a candidate vaccine. After application of 10^4 TCD_{50} and 10^7 $TCD5_0$ SY18ΔI226R to pigs of two test groups the animals showed no increase in body temperature above 40.1 °C and no clinical signs of the disease. When the first group was infected with the initial isolate SY18 (genotype II) at $10^{2.5}$ TCD_{50}, a 2-day fever was recorded with a maximum temperature increase of up to 41.4 °C. In the second group, when infected with 10^4 TCD_{50} SY18, no fever or other clinical signs of ASF occurred during the observation period with 0% mortality in both groups [65].

1. Genetically modified variants of African swine fever virus (ASFV) used as promising vaccine strains

Experimental ASFV	Delited genes	Control isolate (genotype)	Infected/survived (protection effect, %)	References
BA71ΔCD2, 10^6 HAD_{50}	*EP402R* (CD2v)	BA71 (I)	6/6 (100 %)	[56]
BA71ΔCD2, 10^3 HAD_{50}	*EP402R* (CD2v)	BA71 (I)	6/2 (33 %)	[56]
BA71ΔCD2, $3{,}3\times10^4$ or 10^6 HAD_{50}	*EP402R* (CD2v)	E75 (I)	12/12 (100 %)	[56]
BA71ΔCD2, 10^3 HAD_{50}	*EP402R* (CD2v)	E75 (I)	6/1 (17 %)	[56]
BA71ΔCD2, $3{,}3\times10^4$ or 10^6 HAU_{50}	*EP402R* (CD2v)	Georgia 2007/1 (II)	18/18 (100 %)	[56]
BA71ΔCD2, $3{,}3\times10^4$ or 10^6 HAD_{50}	*EP402R* (CD2v)	RSA/11/2017 (XIX)	6/5 (83.3 %)	[57]
BA71ΔCD2, $3{,}3\times10^4$ and 10^6 HAD_{50}	*EP402R* (CD2v)	Ken06.Bus (IX)	8/4 (50 %)	[57]
HLJ/18-7GD	*MGF505-1R*, *MGF505-2R*, *MGF505-3R*, *MGF360-12L*, *MGF360-13L*, *MGF360-14L*, *EP402R*, *9GL*, *DP148R*	HLJ/18 (II)	4/4 (100 %)	[60]
BeninΔDP148R	*DP148R*	Benin (I), intramuscularly	11/11 (100 %)	[58]

Continued Table 1

BeninΔDP148R	*DP148R*	Benin (I), intranasally	6/5 (83.3 %)	[58]
ASFV-G-ΔI177L, 10^2 HAD_{50}	*I177L*	Georgia 2007/1 (II)	10/10 (100 %)	[61]
ASFV-G-ΔI177L, 10^4 HAD_{50}	*I177L*	Georgia 2007/1 (II)	5/5 (100 %)	[61]
ASFV-G-ΔI177L, 10^6 HAD_{50}	*I177L*	Georgia 2007/1 (II)	5/5 (100 %)	[61]
ASFV-G-ΔI177L (oronasal immunization)	*I177L*	Georgia 2007/1 (II)	10/10 (100 %)	[63]
SY18ΔI226R	*I226R*	SY18 (II)	10/10 (100 %)	[65]
Georgia 2007/1	*9GL* (*B119L*) and *UK* (*DP96R*)	Georgia 2007/1 (II)	5/5 (100 %)	[50]
Pr4Δ9GL	*9GL* (*B119L*)	Pr4 (XX)	4/4 (100 %)	[76]
NH/P68DA238L-COS7, 10^6 TCD_{50}	*A238L*	Arm07 (II)	4/0 (0 %)	[59]
NH/P68DA224L-COS7, 10^6 TCD_{50}	*A224L*	Arm07 (II)	4/4 (100 %)	[59]
NH/P68DEP153R-COS7, 10^6 TCD_{50}	*EP153R*	Arm07 (II)	4/0 (0 %)	[59]
NH/P68DA276R-PAM, 10^2 TCD_{50}	*A276R*	Arm07 (II)	5/0 (0 %)	[59]
ASFV-G-ΔMGF, 10^2 HAD_{50}	*MGF505-1R*, *MGF360-12L*, *MGF360-13L*, *MGF360-14L*, *MGF505-2R*, *MGF505-3R*	Georgia 2007/1 (II)	10/10 (100 %)	[55]
ASFV-G-ΔMGF, 10^4 HAD_{50}	*MGF505-1R*, *MGF360-12L*, *MGF360-13L*, *MGF360-14L*, *MGF505-2R*, *MGF505-3R*	Georgia 2007/1 (II)	10/10 (100 %)	[55]

Published data on ASFV vaccine preparations, including genes to be edited and survival rates after challenge, are presented in Table 1. This summarizes the past decade of ASFV genetic modification studies using the best characterized and studied virulent isolates (in particular, Georgia 2007, Benin, E75).

It can be seen (Table 1) that due to the bioinformatic analysis of the ASF virus genome, the choice of target genes for deletion in order to obtain a candidate vaccine has expanded. When pigs were immunized with experimental viruses, the doses ranged from 10 to 10^6 HAD_{50}. The results obtained so far indicate that deletions in various genes (e.g., *I177L*, *9GL* and I226R) reduce the virulence of the virus and provide sufficient protection against re-infection. The effectiveness of a drug may vary depending on the genotype of the parent isolate, the dose of immunization, and even the route of administration of the virus. Moreover, the deletion of a number of genes can affect the virulence of the virus and the degree of protection against re-infection in different ways, depending solely on the method of immunization. These results demonstrate the difficulty of determining the best vaccination route for ASF control.

Inactivated vaccines. Inactivated vaccines are biological preparations in which the replication activity of the virus is suppressed under the action of a chemical. Attempts to develop an effective inactivated vaccine against ASF, similar to other infectious animal diseases, were made simultaneously with the creation of attenuated variants of the virus. However, already in 1967, when studying the immune response to an inactivated vaccine, it was found that immunized pigs did not develop resistance to infection with a virulent isolate (66). When immunized with a culture vaccine inactivated by glyceralaldehyde, resistance to spleen injury was noted in some animals, but protective immunity was not formed when infected with a virulent ASFV isolate (67). Despite initial setbacks, research continued.

Thus, when using an inactivated ASF virus isolated from an extract of the spleen of diseased pigs and N-octylglucoside as an adjuvant, immunized pigs showed resistance to infection with a homologous isolate, but were sensitive to a heterologous isolate [68]. In 2021, new and extremely active adjuvants (Silicaoil, mGNE, etc,) were used for virus inactivation with binary ethyleneimine at low temperatures. However, infection of immunized pigs with a virulent ASFV isolate did not develop protective immunity [69].

Because of the strong evidence that this type of ASF vaccine is not appropriate, control experiments are being conducted to prove that inactivated ASF vaccines are not viable [70].

As a result, it should be noted that all approaches used to develop an inactivated vaccine were not successful. This, in turn, raises a number of important questions regarding the genetic and antigenic variability of the virus, which require detailed study.

Subunit vaccines. In the late 1990s, the attention of scientists was focused on recombinant proteins obtained in the baculovirus expression system for the immunization of pigs against ASF.

In the experiment, the CD2v protein synthesized in this way was administered to pigs in different doses. After infection of pigs with intact virus, a temporary delay in haemadsorption and a temporary dose-dependent delay in the development of infection were observed, but protective immunity was not formed [71]. Administration of the recombinant vp12, vp30 and vp54 proteins produced specific immunoglobulins in pigs that delayed the entry of the virus into target host cells, but high antibody titers did not result in resistance in pigs when infected with virulent ASFV isolate [72, 73].

The use of the vp54/30 chimeric protein, which was obtained by expression of the *CP204L* gene integrated into the restriction site of the *E183L* gene in the baculovirus system, in porcine macrophage cell culture resulted in 50% neutralization of the ASF virus by specific antibodies. A bioassay with immunization with the vp54/30 chimeric protein and subsequent infection with the E75 isolate showed 100% survival of pigs in chronic ASF [74]. When using chimeric CD2v proteins and type C lectin encoded by the *EP153R* gene, the role of specific antibodies protecting against infection with a homologous ASFV isolate was proven [75].

In 2004, an experiment was conducted on susceptible animals to compare subunit vaccines and vaccines based on genetically modified ASFV, using two groups of pigs: the first was immunized with the Pr4Δ9GL isolate with a deletion of the *B119GL* gene, the second was injected with recombinant proteins vp30, vp54, vp72 and vp22, expressed in the baculovirus system. After infection with the original Pr4 isolate (XX genotype), the animals of the first group showed clinical signs of ASF and viremia with an insignificant titer (2.9±0.6 TCD_{50}/ml), however, death did not occur, while in the second group, viremia developed with a delay on day 2 after which the virus titer was 9.1±0.3 TCD*50*/ml, and on day 8.5±0.5 after infection the animals died [76]. Such data cast doubt on the existence of virus-neutralizing antibodies involved in the formation of immune protection against ASF.

As Table 2 shows, various recombinant proteins can be used as candidate subunit vaccines for immunization of pigs, but almost all protein combinations fail to protect immunized animals from re-infection, and all the results obtained so far are unpromising. The only exception was the vp54/30 chimeric protein,

immunization with which ensured the survival of pigs during the control infection with a virulent isolate, however, ASF took a chronic course and the virus was still isolated from the body of pigs. Similar works related to the use of subunit vaccines as candidates cannot yet be considered promising either. This is largely due to the lack of knowledge of many antigens that can protect pigs from infection with the ASF virus.

2. Used African swine fever (ASFV) **subunit vaccine prototypes**

ASFV proteins	Expression system	Protective effect	References
vp12	Baculovirus	No protection	[73]
CD2v	Baculovirus	No protection	[71]
vp54 and vp30	Baculovirus	No protection	[73]
Chimeric proein vp54/30	Baculovirus	0 % mortality (2/2), chronic ASF	[74]
vp30, vp54, vp72 and vp22	Baculovirus	No protection	[76]
CD2v and lectin type C	Baculovirus	Partial protection	[75]

Recombinant (vector) vaccines. In connection with the development of genetic engineering and molecular biology, the development of recombinant vaccines, including against ASFV, in which plasmids and heterologous viruses can be used as a vector, has become methodically accessible [77-79).

For example, when using the plasmid construct pCMV-UbsHAPQ, encoding the chimeric proteins vp54, vp30 and CD2v coupled with ubiquitin to increase the possibility of expression together with MHC class I molecules on the cytoplasmic membrane of target cells of pigs, as expected, there was a high level of cytotoxic CD8+ T lymphocytes with a peak on the 3rd day after immunization. After infection with the virulent E75 isolate, partial protection against ASF was observed in pigs with a 66.0% mortality [78]. Plasmid BacMam-sHAPQ can be used as a promising vector for the expression of vp54, vp30 and sHA proteins, the combination of which, when immunized pigs, showed high levels of cytokines in four out of six animals [78].

When constructing modified adenoviruses to express ASFV proteins, the *A151R*, *B119L*, *B602L*, *EP402RΔPRR*, *B438L*, *K205R*, and *A104R* genes of the Georgia 2007/1 isolate were incorporated into the adenoviral genome. In cultured porcine bone marrow cells (PMC), this recombinant construct induced a high level of γ-interferon on day 7 after inoculation, but due to the lack of tests on susceptible animals, it cannot be concluded that there is immune protection against virulent ASF isolates [80]. Immunization of wild boars with 35 ASFV antigens expressed using human adenovirus 5 as a vector showed no immune protection when challenged with the virulent Arm 07 isolate [78].

Cloning of 47 antigens of the ASF virus to plasmids and vaccinia virus as vectors did not give notable results in the search for recombinant vaccines, since when pigs immunized with the recombinant variant were infected with the virulent Georgia 2007/1 isolate at 10^4 HAD_{50}, in all animals of the experimental group, tere was an acute form of the disease, despite the high level of production of γ-interferon by cytotoxic CD8+ T lymphocytes in vitro (6×10^5 cells) [81].

In a similar study, the *Escherichia coli* pGEX 4T-1 plasmid served as a vector, in which the genes of the ASF virus were cloned in two variants: in one, encoding proteins CD2v, vp32, vp72, and vp17; in the other, proteins vp15, vp35, vp54, and vp17. The level of γ-interferon synthesized in blood mononuclear cells sensitized with the recombinant variant barely exceeded that in the control sample, and 100% mortality was observed when immunized pigs were infected with the virulent ASF virus isolate Arm 07 [82].

Very encouraging results were obtained from a study in which eight genes

of the ASFV isolate OURT88/3 (*B602L*, *B646L*, *E183L*, *E199L*, *CP204L*, *F317L*, *EP153R*, and *MGF505*-5R) were incorporated into human adenovirus 5 (rAd) and modified vaccinia virus Ankara (MVA) vectors. When pigs were injected with the recombinant rAd virus at a dose of 1.5×10^{10} IU and the recombinant MVA virus at a dose of 2×10^{8} PFU and re-infected with the virulent ASF isolate OURT88/1, viremia was observed for 6 days while neither animal died [83].

An important step in the development of molecular biology was the CRISPR/Cas9 technology, which has already experimentally shown results in the expression of the vp30 protein encoded by the ASFV *CP204L* gene [84]. A recombinant variant of the highly virulent isolate ASFV-Kenya-IX-1033 (genotype IX) with the deletion of the *A238L* gene was synthesized using the CRISPR/Cas9 system [85]. This method is considered as the most promising for editing the genome of viruses, including the causative agent of ASF.

The available generalized information on genetically engineered ASF vaccines is presented in Table 3. From the data in this table, it can be seen that the use of recombination of various genes expressed using plasmid and viral vectors is one of the most interesting and promising approaches in the development of an ASF vaccine. Different genes can be pooled to achieve the best result, which ranges from complete lack of protection to 100% protection (see Table 3). The success of such results depends both on the combination of genes used and on their influence on each other.

3. The use of recombinant constructs as a potential vaccine against African swine fever

ASFV proteins	Expression system, vector	Protective effect	References
Ubiquitin-CD2v, vp54 and vp30	Plasmid pCMV-UbsHAPQ	Partial protection (33 %)	[78]
vp54, vp30, sHA	Plasmid BacMam-sHAPQ	Partial protection	[77]
7 antigenes: vpA151R, vpB119L, vpB602L, vpEP402RΔPRR, vpB438L, vpK205R and vpA104R	Human adenovirus 5	Биопроба не проводилась	[80]
47 antigenes	Plasmid pCMVi-LS and modified vaccinia virus	No protection	[81]
CD2v, vp32, vp72, vp17	Plasmid *Escherichia coli* pGEX 4T-1	No protection	[82]
vp15, vp35, vp54 and vp17	Plasmid *Escherichia coli* pGEX 4T-1	No protection	[82]
35 antigenes	Human adenovirus 5	No protection	[79]
8 antigenes: vpB602L, vpB646L, vpE183L, vpE199L, vpCP204L, vpF317L, vpEP153R, vpMGF505-5R	Human adenovirus 5	Complee protection (100 %)	[83]
8 antigenes: vpB602L, vpB646L, vpE183L, vpE199L, vpCP204L, vpF317L, vpEP153R, vpMGF505-5R	Modified vaccinia virus Ankara (MVA)	Complee protection (100 %)	[83]

Thus, it can be concluded that, despite the considerable accumulated experience in studying the ASF virus genome, as well as the functional and immunological characteristics of viral proteins, today there is not a single certified ASF vaccine in the world. After a relatively long lull, a new ASF epizootic in 2007-2021 resulted in multi-billion dollar losses across all swine and hunting sectors. In China, losses from ASF in 2018-2019 amounted to 0.78% of the country's gross product [86]. In this regard, the issue of the need for effective protection of farms from ASF has again become acute, not only by methods of general prevention, but also by vaccination. An independent evaluation of ASF-specific prophylactic drugs requires further research into potential vaccine candidates in commercial swine production in different countries in order to avoid the chronic course of ASF that persists in a particular country [87]. To implement the strategy for differentiating infected vs. vaccinated animals (DIVA), it is proposed to use a vaccine variant of

the ASF virus with an induced deletion of the conserved E184L gene of the immunogenic protein, which makes it possible to clearly determine the absence of antibodies to this protein in vaccinated individuals [88].

So, the development of effective and safe vaccines against African swine fever (ASF) has been going on for almost 90 years. The widespread use in the middle of the 20th century of attenuated vaccines against homologous isolates of the ASF virus in many European countries (Spain, Portugal) led to large-scale circulation of the pathogenic virus in the population of domestic and wild pigs and to an increase in the number of livestock with a chronic course of the disease. An attempt to weaken the virulent properties of the virus by successive passages on many primary and continuous cell culture lines in order to create a vaccine was not successful. Inactivated and also subunit vaccines based on recombinant proteins had high immunogenicity, but they did not have protective properties, regardless of which adjuvants and inactivants were used. The use of homologous recombination technology to obtain clones of the ASF virus with the deletion of certain genes has led to the creation of successful candidate vaccines. Human adenovirus 5 (rAd) and modified vaccinia Ankara (MVA) virus have proven themselves as vector constructs for transferring ASFV DNA in the development of effective recombinant (vector) vaccines. The most promising, from our point of view, are experimental vaccines based on genetically modified viruses ASFV-G-ΔI177L and SY18ΔI226R, demonstrating 100% protection of pigs, at least against homologous ASFV genotype II isolates. Similar expectations are raised by the HLJ/18-7GD candidate vaccine.

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ANTIOXIDANT STATUS AND QUALITY OF POULTRY AND ANIMAL MEAT UNDER STRESS AND ITS CORRECTION WITH THE USE OF VARIOUS ADAPTOGENS

(review)

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Abstract

Modern animal breeds and poultry crosses do not fully realize their genetic potential for productivity due to the impact of various stresses (V.I. Fisinin et al., 2015). Recently, there has been a marked public concern about the negative impact of intensive rearing on animal health, food safety and quality. Animal health and welfare are prerequisites for both productive performance and obtaining products that are safe for human (K. Proudfoot et al., 2015). Oxidative stress caused by an imbalance between production and accumulation of oxygen reactive species (ROS) and the ability of a biological system to detoxify these reactive products under feed, climatic, technological, and biological stresses negatively affects health, growth rates and product quality. Due to the high level of polyunsaturated fatty acids and non-heme iron Fe^{3+} and Fe^{2+}, chicken meat is most susceptible to lipid peroxidation compared to beef and pork (I.F. Gorlov et al., 2016). The present review paper summarizes the current state of knowledge on the influence of stress factors, including housing conditions (climatic, stocking density), transportation, feeding, veterinary measures on the antioxidant status, meat oxidative properties and quality on the example of chickens and broilers. Climatic and other conditions determine behavioral, physiological and immune responses of birds, affect their antioxidant, biochemical status and productivity. Meat quality deteriorates, as can be seen from changes in pH, muscle protein structure, increased lipid oxidation and the appearance of meat defects (K. Rosenvold et al., 2003; M. Petracci et al., 2015; P.F. Surai et al., 2019). The negative impact on meat quality depends on the type of stresses (chronic or acute), the animal genotype, and the type of muscle fibers (N.A. Mir et al., 2017; P.A. Gonzalez-Rivas et al., 2020; M. Zhang et al., 2020). Transport stress is the result of the simultaneous action of several stress factors (L. Zhang et al., 2014). The intensity of the impact on the body and the change in biochemical markers of stress depends on the conditions of transportation, feeding and keeping, individual characteristics and health status of the bird. Data on the impact of stress on metabolism in animals and birds are rather contradictory. The use of synthetic or natural antioxidants in animal husbandry is currently being discussed due to their ability to influence oxidative stress and meat quality (A. Gouda et al., 2020). This review also provides an analysis of ways to improve the antioxidant protection and meat quality using natural adaptogens (vitamins E and C, taxifolin and quercetin) as feed additives (M. Mazur-Kuśnirek et al., 2019; V.R. Pirgozliev et al., 2020). The study of biomarkers of antioxidant protection is essential for obtaining high quality meat. The use of antioxidants enhances antioxidant protection, increases animal resistance, and improves product quality. This method of preventing the negative effects of stress in animal husbandry and poultry farming is considered the most acceptable and cheapest, especially when natural adaptogens are combined in the diet, which can be more effective than the action of each adaptogen separately.

Keywords: stress, meat quality, antioxidant status, vitamin E. vitamin C, taxifolin, quercetin

In the world animal husbandry, poultry and pig breeding are among the most actively developing sectors that provide the population with high-quality meat, which is associated with high growth energy and the ability of pigs and

poultry to reproduce quickly. Focus on maximum efficiency and profitability leads to major changes in the methods of maintenance, to automation and even greater intensification of production processes. Modern breeds and crosses have a high genetic potential for productivity, but it cannot be fully realized in practice due to the impact of environmental, technological, nutritional and physiological stress factors [1]. In recent years, there has been a marked increase in public concern about the negative impact of intensive production on animal health and food safety [2]. Animal health is an integral part of well-being, which becomes a prerequisite for both high productivity and the production of products that are safe for humans [3].

The response to stress is complex, multidimensional and can be determined by interactions between stressors, leading to unpredictable outcomes. Depending on the source of stress, animals experience fear, dehydration, and hunger. Increased fatigue and physical injuries additionally potentiate disturbances in the energy and ionic intracellular balance, in the protease system, as well as changes in skeletal muscle proteins. All of these factors affect the conversion of muscle to meat [4, 5]. Understanding and controlling the stress response is critical to animal welfare and meat quality.

Research over the past two decades has convincingly shown that most stress, regardless of source, is associated with an imbalance in free radical production and detoxification [6]. Oxidative stress is a major problem for modern livestock production around the world [7]. Accumulating scientific evidence suggests that oxidative stress can impair health, growth performance, and meat quality [8]. It is considered as a key link in the negative consequences of feed, climatic, technological and biological (internal) stresses at the molecular level [9, 10]. Previously, we paid attention to identifying stresses in pig production and leveling their consequences when using flavonoids as feed additives [11].

The purpose of this review is to summarize modern scientific data on the consequences of stresses of various nature and the use of alimentary factors, in particular vitamins E, C and bioflavonoids, to improve the antioxidant status and quality of animal and poultry meat.

Stress affecting the physiological state of animals and poultry and product quality. *Climate factors.* Temperature stress includes both heat and cold stress. Genetic selection for rapid chest growth and mass over the past few decades has reduced the thermoregulatory capacity of birds in modern commercial breeds, making them more vulnerable to heat stress [12]. Heat stress causes altered behavioral and physiological responses and negatively impacts health, productivity and product quality in poultry [13]. High ambient temperature in summer leads to a deterioration in health, reduces the growth rate and quality of the carcass of broiler chickens [14-16].

Numerous studies have been devoted to the impact of climatic stress on the physiological and productive characteristics of birds. For example, chronic heat exposure impairs growth, gut morphology, and appetite, which may be due to increased secretion or expression of appetite-related hormones and genes and higher expression of nutrient-sensing receptors (T1R1 and T1R3) [17]. Heat stress leads to an increase in rectal temperature ($p = 0.001$), respiratory rate ($p = 0.001$) and blood pH ($p = 0.02$), which characterizes the state of respiratory alkalosis in broilers [18]. Broilers kept at an ambient temperature of 32 °C consume 14% less feed than their counterparts kept at normal temperature. K. Sahin et al. [19] believe that reduced feed intake serves as a protective physiological response to reduce heat production. Other authors [20] also note that a decrease in feed intake by broilers is the main reason for a decrease in live weight gain, an increase in mortality, a decrease in fertility and hatchability, changes in the balance of electrolytes and blood pH [21], disorders of secretion and activity of endogenous

enzymes [22], decrease in serum concentrations of thyroid hormones T_3 and T_4, suppression of immune function [23] and decreased intestinal absorption [24]. High ambient temperature negatively affects broiler productivity [25] due to changes in energy, protein, lipid and mineral metabolism, acid-base and electrolyte blood balances, as well as the concentration of hemoglobin. At 32 °C there is a significant decrease in the activity of the digestive enzymes trypsin, chymotrypsin and amylase. Heat stress leads to a decrease in the content of vitamins (C, E and A) and minerals (Fe, Zn, Se and Cr) in the blood serum and liver, and affects the immune response of poultry [26].

V.R. Pirgozliev et al. [27] found that chronic heat stress in poultry production reduced not only feed intake and body weight gain, but also small intestine weight, total weight of the gastrointestinal tract, liver, spleen, heart, villus height, intestinal villus surface area, and negatively affects the activity of blood glutathione peroxidase (GP). The productivity, physiological and immune responses of the body of broiler chickens to the effects of heat stress depend on the composition and nutritional value of the diet and on the genetic characteristics of the bird [28]. High environmental temperatures cause oxidative stress [29], in which there is an increased production of free radicals in the body due to an increase in body temperature [30], as well as due to an increase in oxygen consumption [31]. Increasing oxygen consumption increases the production of reactive oxygen species (ROS) [32, 33].

Climate stress has a negative impact on the quality of poultry products. Exposure to this type of stress increases the incidence of meat defects such as pale, soft, and exudative (pale, soft, exudative, or PSE) and dark, firm, and dry (dark, firm, dry, or DFD) [34]. It was found that both acute and chronic exposure to temperature change meat quality indicators. At elevated ambient temperatures, meat exhibits PSE characteristics, while at lower ambient temperatures, DFD [35]. Acute heat stress affects meat pH limits to a greater extent than chronic stress, while chronic heat stress affects color traits (L* and a*) [34]. According to Y. Hashizawa et al. [35], chronic temperature stress (30 °C for 10 days) can also cause deterioration in broiler meat quality and lead to PSE. The effect of chronic heat stress on meat quality was most significant, causing broiler breast stiffness. Consequently, both chronic and acute heat stress degrade poultry meat quality, with exposure to extreme temperatures shortly before slaughter having an even greater impact [36].

Several reviews have detailed the effect of heat stress on meat quality in poultry, ruminants and pigs [29, 36) and characterize meat quality and defects in poultry [37]. Heat stress reduces the myofibril fragmentation index and increases the reactivity of thiobarbituric acid in broiler muscles [18]. In the skeletal muscles of broilers, thermal stress reduces the rate of protein synthesis and the activity of proteolysis [38]. This is partly due to adaptive endocrine changes: for example, thyroid hormones promote growth and their levels are negatively correlated with elevated temperature. In broiler skeletal muscle, exposure to high temperatures suppresses downstream metabolic pathways for insulin signaling, which is an important regulator of muscle metabolism and protein synthesis [39]. Other effects of heat stress on broiler meat quality include decreased muscle glycogen and muscle pH, paler color [40], increased lipid oxidation [41] and altered muscle fiber structure [42].

As J.H. Feng et al. [43] reported, heat exposure (41 °C) increased the oxidation of muscle proteins, which led to a decrease in the gelling properties of broilers' meat. Other investigators have observed that in meat-cross chickens, heat exposure (34 °C for 18 h) increases oxygen production in skeletal muscle mitochondria, and this correlated with an increase in rectal temperature and weight loss [44].

Chronic heat stress (CTS) has been reported to significantly increase fat deposition in broilers [45]. However, this most likely depends on the genotype of the bird. For example, Q. Lu et al. [46] showed that CTS reduced subcutaneous and intermuscular fat in Arbor Acres broilers while increasing abdominal fat in Beijing You chickens. In addition, the same authors found that L* and meat moisture loss increased in CTS-treated Arbor Acres broilers. At the same time, no significant consequences of such exposure to Peking chickens were observed.

L. Zhang et al. [47] found that the proportion of breast muscle in broilers was reduced by chronic chronic heat stress at 4 to 6 weeks of age, while the effect on thigh muscle was the opposite. Other authors have explained that the decrease in pectoral muscle mass in CTS occurs due to suppression of the signaling pathway of insulin-like growth factors, the mammalian target of rapamycin (mTOR) [48]. CTS does not affect the content of moisture, raw protein and raw fat in breast muscle, but generally impairs breast quality in broilers [49], and ante-mortem transport of broilers under short-term heat stress increases the incidence of PSE meat [50, 51].

Cyclic heat stress, when the birds were at 33±1 °C for 10 h (8.00-18.00) and at 22±1 °C the rest of the time, increased the blood concentration of corticosterone and triacylglycerol, droplet moisture loss and the content of malondialdehyde in muscle, and reduced blood glucose, pH_{24}, total muscle antioxidant capacity (T-AOC), catalase (CAT) and glutathione peroxidase (GSH-PX) activity [52].

Thus, the body response to heat stress (acute and chronic) depends on many factors, i.e., breed, muscle type, specific conditions of detention, but one way or another, this type of stress has multiple negative consequences and brings significant losses to the industry.

Stocking density. High stocking densities have been reported to impair broiler meat quality by causing oxidative stress to develop [29, 53]. At the same time, in a number of other studies [54, 55], stocking density did not affect the quality of broiler meat. D.G. Yu et al. [56] found that high stocking densities impair growth performance, gut barrier function, and enhance stress responses [56].

Growing conditions affect behavioral and physiological responses, muscle composition and meat quality. Thus, with an organic housing system that allows broilers to freely occupy a grass pen, the yield of carcasses increases and the organoleptic qualities of meat improve, but lipid peroxidation (LPO) and the accumulation of their oxidation products (thiobarbituric acid reactive substances, TBARS) in the muscles increase [57].

Transportation stress. Pre-slaughter transportation causes stress and injuries which lead to a noticeable decrease in the quality of poultry meat and significant financial losses. Birds may also be exposed to co-occurring stressors during transport, including thermal changes in the transport microenvironment, acceleration, vibration, movement, shock, starvation, lack of water, social stress, noise [58]. All these factors impair metabolism, especially the secretion of stress hormones, as well as increased muscle anaerobic glycolysis [59].

M.H. Tamzil et al. [60] reported that broilers transported for 3 h before slaughter increased erythrocyte and leukocyte counts, heterophile percentage, higher poultry mortality and meat pH, while decreasing lymphocyte percentage, water-holding capacity (WHC) and cooking loss. A dormancy period after transportation for 12 hours reduced the adverse effect of transport stress on meat quality.

Glucose serves as the body's main source of energy and is stored as glycogen [61]. Transport stress accelerates muscle glycolytic metabolism by affecting muscle, glycolytic enzyme activity and glycolytic potential [62-65]. C. Zhang et al. [66] found that broilers transported within 3 hours before slaughter

had decreased muscle glycogen, increased muscle lactate dehydrogenase (LDH) activity, and increased lactate levels. As the transport time increases, the muscles contract strongly, anaerobic processes increase, which causes the accumulation of lactic acid and reduces the pH of the muscles [36, 68]. The structure of muscle proteins is disturbed, the loss of moisture increases [69]. The latter is due to the fact that lower pH causes actin and myosin to condense and shrink into granules, destroying the spatial structure of the tissue, increasing the amount of free water, reducing WHC, which ultimately affects muscle color [70]. Thus, pre-slaughter transport may increase bird stress by reducing muscle glycogen stores and therefore affect the rate and extent of pH decline, as well as meat quality [59]. Rapid anaerobic glycolysis causes lactate to build up in the muscle and pH to decrease, ultimately resulting in PSE meat [70].

Glucose concentrations, increased levels of lactate and uric acid, and serum LDH activity indicate that the birds are under stress [71]. The deterioration of meat quality caused by transport stress is closely associated with negative changes in muscle energy metabolism and antioxidant status [66, 72]. Transportation of poultry can cause excessive production and accumulation of ROS and ultimately lead to oxidative stress [74], which interferes with collagen metabolism [75] and/or leads to lipid peroxidation and protein oxidation [8, 76].

Poultry transportation within 3 h before slaughter increased the loss of live weight, drip losses; the content of malonic dialdehyde (MDA) in muscles and lactate increased, the activity of the thymus, spleen and Fabritius index, pH_{24}, total antioxidant activity of muscles, catalase and GP activity, glycogen content decreased [72]. In earlier studies of these authors, the same conditions of transportation before slaughter increased the blood concentration of corticosterone, the content of MDA and lactate in the muscles, LDH activity in the muscles, while the content of muscle glycogen, total superoxide dismutase (SOD) activity and GP activity decreased, which worsened breast meat quality (lower pH_{24} and higher drip loss) [66].

When studying the effect of the duration of transportation on the biochemical status and quality of meat, it was shown that 2- and 4-h transportation of broilers before slaughter did not affect the activity of LDH, γ-glutamyl transferase, alanine aminotransferase, creatine kinase and glucose in blood serum, GP in the thigh muscles and mRNA expression heat stress protein in the liver. The concentration of triiodothyronine, thyroxine and insulin in the blood serum decreased after 2 h of transportation and returned to normal after 4 hof transportation. Both variants increased SOD activity in the muscles. In the muscles of the thigh and chest, with an increase in the time of transportation, the amount of MDA and lactic acid increased, fluid losses increased, while the glycogen content decreased. Transportation for 2 h did not affect pH_{24} in the muscles of the chest and thigh, but these parameters decreased with 4 h of transportation [67].

Z. Gou et al. [73] studied the effect of age and duration of transport on stress biomarkers and meat quality in broiler chickens. With an increase in the duration of transportation of medium-sized broiler chickens at the age of 75 days from 0.5 to 3 h, the live weight of the bird linearly decreased, the concentration of adrenocorticotropic hormone, cortisol and corticosterone in plasma, and the activity of glutathione peroxidase increased. At the same time, the content of glucose in the blood did not change. The effect of transporting broiler chickens at this age on meat quality was negligible. Only a decrease in the total antioxidant capacity and drip losses of the pectoral muscle were noted [73].

Thus, transportation stress is the result of the simultaneous action of several stress factors. The intensity of the impact of transport stress on the body of a bird depends on the age, breed, state of health, composition and nutritional value

of the diet, feeding conditions during transport, methods of capture before transport, temperature during transport, and rest time after transport [72, 73]. Data on the impact of transport stress on the metabolism of substances in chickens and broilers are rather contradictory.

Feed stress. Feed stress in poultry occurs when changing feeds, using low-quality ingredients, contamination of feed with xenobiotics, and under the influence of other causes. Thus, fat in the diet significantly affects the growth performance and health of the herd. Poor quality oil reduces the productivity of broiler chickens [77]. Diets rich in polyunsaturated fatty acids (PUFAs) increase lipid peroxidation and reduce antioxidant capacity. Rancid fats undergoing autoxidation processes contain substances that form free radicals. As a result of oxidation reactions, harmful peroxides are formed, which are converted into hydrocarbons, ketones, alcohols, organic acids and aldehydes, including MDA. Oxidation reactions also reduce the content of vitamins A, E and carotenoids [78]. Increased ROS production disturbs the redox balance and leads to oxidative stress with detrimental health consequences [78].

Oxidative stress can be caused by mycotoxins in feed. Ochratoxin A (OTA, a secondary metabolite produced by certain species of *Aspergillus* and *Penicillium*) has an immunosuppressive effect in humans and animals. Ochratoxin A causes oxidative stress, lipid peroxidation and pathological lesions in the tissues of the bursa of Fabricius, spleen and thymus of chickens, as evidenced by a decrease in the amount of catalase and GP and an increase in the content of products that react with thiobarbituric acid (TBA-AP). In addition, the introduction of OTA into the diet leads to apoptosis, which was manifested in an increase in the expression of the *PTEN*, *Bax*, and caspase-3 genes and a decrease in the expression of the *PI3K*, *AKT*, and *Bcl-2* genes [79]. In the review by V. Sorrenti et al. [80] an increase in ROS production and, as a result, oxidative stress and lipid peroxidation are discussed as the causes of OTA toxicity [80]. Repeated exposure of chickens to OTA over a period of time reduces SOD activity, glutathione (GSH), and total antioxidant activity while increasing MDA [81, 82].

Cadmium (Cd) is a heavy metal and one of the most toxic environmental pollutants. Its presence in feed is a serious problem in animal husbandry and agriculture in general. In some cases, the amounts of Cd exceed the maximum allowable. Cd can be ingested by animal feed mineral premixes and can be introduced into plants when cadmium-rich manure is used as organic fertilizer [83]. Cadmium has complex toxicity to mammals ganisms, causes various forms of oxidative damage and damage to animal tissues [84]. Cd induces the formation of free radicals, reduces the activity of antioxidant enzymes [85], and leads to oxidative degradation of lipids [85], proteins and DNA in humans and animals [86]. Hepatotoxicity of cadmium has been described; in chicken liver, it induced oxidative stress and apoptosis [87].

Veterinary manipiulations. Veterinary manipulations also change the redox balance and metabolism, which leads to a deterioration in meat quality. With long-term use of exogenous glucocorticoid dexamethasone, lipid peroxidation products (TBARS) accumulate in plasma and skeletal muscles, which increases the content of saturated fatty acids in broiler skeletal muscles [88]. Exogenous corticosterone causes an imbalance in the skeletal muscle redox system, which affects the oxidative stability of meat during storage [89]. X. Chen et al. [90] found that intraperitoneal administration of 10% H_2O_2 to broilers increased the formation of ROS and decreased the activity of antioxidant enzymes, as a result, increased oxidative stress, decreased the proportion of muscle mass in the carcass, and deteriorated meat quality.

In addition to the considered stresses, other factors can also lead to a

change in the quality of poultry meat. It is well known that the genetic background causes variations in animal responses to stress. The two main genes that induce porcine PSE are known as the *Halothane* gene and the *RN* gene. Their role is reviewed by K. Rosenvold et al. [91]. Genetic selection of broilers for growth rate and increased breast yield is accompanied by myopathy, including deep mammary myopathy and PSE meat, as well as the recently discovered white banding and wood breast [12].

Thus, stresses of various nature negatively affect the state of the immune and antioxidant systems of poultry, which reduces the quality of the products obtained, in particular meat. According to some scientists, chicken meat is more susceptible to LPO processes than beef and pork due to its high content of polyunsaturated fatty acids and non-heme iron (Fe^{3+} and Fe^{2+}). According to the results obtained by I.F. Gorlov et al. [92], the degree of oxidative changes in chilled poultry meat depends on the reactivity of the antioxidant system of the body and the formation of lipid peroxidation products. The weakening of antioxidant activity and the activation of free radical lipid oxidation in the blood plasma of broiler chickens enhance the processes of meat oxidation.

An analysis of stress-induced metabolic changes indicates the importance of reducing the effects of oxidative stress in broiler production and the need for additional protection of the antioxidant system in poultry [6]. It is clear that conditioning avoids critical temperature effects [93], and comfortable transport conditions and balanced diets based on quality ingredients mitigate transport and feed stress. However, often stress cannot be avoided or the technical solution is costly. In this case, it seems appropriate to use natural antioxidants, the main regulators of many physiological processes, as additives to feed or water. The redox balance between anti- and pro-oxidants in feed, gastrointestinal tract, blood and tissues is an important factor in protecting animals from stress and its consequences [94].

We have previously studied the effect of taxifolin on stress in young pigs [95-98]. Particular attention has been paid to the effect of the adaptogen on meat quality [99, 100]. An analysis of publications shows the promise of this approach in poultry farming.

The use of nutritional factors to improve the antioxidant status and quality of animal and poultry meat. Recently, antioxidants have attracted increasing attention in all branches of animal husbandry due to their effect on oxidative stress and meat quality [101-103]. By origin, antioxidants (AO) can be divided into synthetic and natural. Natural AO, as a rule, are molecules present in parts of plants (leaves, bark, seeds, and fruits). The most important are tocopherols (fat-soluble vitamin E) and ascorbic acid (water-soluble vitamin C). The first comes only as part of the diet, and the second is synthesized in the body of poultry [104].

Vitamin E (VE). Natural vitamin E includes four tocopherols and four tocotrienols. RRR-α-tocopherol is the most abundant form in nature and has the highest biological activity [105]. Vitamin E was recognized as an essential nutrient almost a century ago when H.M. Evans and K.S. Bishop (1922) discovered a fat-soluble herbal preparation that restored fertility in rats under dietary restrictions. The compound was named tocopherol (Greek for "bearing offspring"), and to date, its activity has been measured in terms of reproduction in rodents. The main function of α-tocopherol is that it is a lipid-soluble antioxidant that prevents various oxidative damage. α-Tocopherol is necessary for normal permeability of lipid bilayers, cell adhesion, and is involved in the regulation of gene expression. Although the transport of this vitamin and other lipids shares some common steps, some tissues have specific transport mechanisms, including the α-tocopherol transporter protein (αTTP) [106]. VE deficiency is associated with increased

oxidative stress, central and peripheral neuropathies, and impaired immune function. VE is an effective antioxidant that maintains cell integrity during normal cellular metabolism and inflammation [107]. In the poultry industry, the addition of VE is necessary to maintain the fertility and hatchability of the parent flock. It also plays a major role in the prevention of foodborne encephalopathy and myopathy in chickens and turkeys [108].

Z.Y. Niu et al. [109] showed that the use of VE in the diet of broilers increased the total activity of superoxide dismutase (T-SOD) and glutathione peroxidase (GSH-Px) and reduced blood levels of MDA ($p < 0.05$). At the same time, the expression of SOD and GSH-Px mRNA in the liver of broilers increased when additional VE was added to the diet. These results indicate a positive effect of VE in broiler nutrition on meat quality by improving antioxidant status through the regulation of antioxidant enzyme gene expression [109].

Emphasizing the important properties of vitamin E in diets, J.V. Van Vleet et al. [110] described the changes occurring in the cells and organs, skeletal muscles of a bird suffering from exudative diathesis, a disease associated with a lack of VE and selenium and oxidative damage to membranes. According to these authors, blood plasma GP represents the first barrier of antioxidant protection for capillary cells, since it prevents the lipoperoxyl radical from attacking PUFAs in the membrane. Vitamin E present in the membrane acts as a second AO barrier, stopping the spread of the LPO chain. In selenium and VE deficiency, none of these antioxidant mechanisms is activated, leading to lipid peroxidation and its pathological consequences [111].

Ascorbic acid, ascorbate (anion of ascorbic acid), vitamin C (VC). It is a water-soluble antioxidant compound that protects cells from oxidative damage and improves immune system function [112, 113]. Vitamin C is not part of any metabolic pathway, but serves as a necessary cofactor in many enzymatic reactions—collagen, carnitine, and catecholamine synthesis, microsome metabolism, or tyrosine synthesis and catabolism. Vitamin C is a cofactor for dopamine beta-hydroxylase, which is involved in the conversion of dopamine to norepinephrine in nerve tissues [114]. In addition to the biosynthesis of norepinephrine, VC is required for the bioconversion of tyrosine to other catecholamines such as dopamine, norepinephrine, and epinephrine. Feeding tyrosine and VC during stress periiods can reduce stress hormones and reduce body weight loss [115]. Vitamin C also improves hormone stability and activity, regulates body temperature, synthesis of 1,25-dihydroxyvitamin D, and immune system function. It is present in high concentrations in immune cells and is quickly depleted during times of stress. It is not known exactly how VS enhances immune system function, but some evidence points to its effect on phagocytes, cytokine production, lymphocytes, and the number of cell adhesion molecules in monocytes [116].

Vitamin C is a powerful biological antioxidant. Its feeding is effective in reducing oxidative stress in animals reared under various stress conditions [117, 118]. However, VC supplementation has not been widely used in the poultry industry because it is believed that poultry synthesizes sufficient amounts of VC in the body [119]. Although birds produce endogenous vitamin C, vitamin C requirements or the body's synthetic capacity may vary due to individual characteristics, breed, health, environmental conditions [120], which can lead to BC deficiency. Heat stress is one of the most common causes of increased need for additional BC in the diet. F. Rafiee et al. [121] report that VC reduces the adverse effects of heat stress on broiler performance and health [121]. VC can also act as a co-antioxidant in conjunction with other antioxidants, providing a synergistic effect. For example, there is a strong relationship between VC and VE, while both of them have a positive effect on the immune system, increasing antibody production,

macrophage activity and humoral immunity in broilers and laying hens.

The currently known plant-derived antioxidants are mostly flavonoids belonging to the vitamin P group, which has become known as bioflavonoids. Bioflavonoids include antioxidants such as quercetin, rutin, hesperidin, cyanidin, and taxifolin (dihydroquercetin, DHQ).

DHQ is found in small amounts in many plants. In the late 1960s, it was isolated in the USSR by a group of scientists headed by Professor N.A. Tyukavkina from larch wood. Even a small concentration of DHQ with regular use can normalize vascular permeability, reduce the risk of cardiovascular and oncological diseases, prevent blood clots, increase immunity, and improve the general condition of the body [122].

Flavonoids find use as anti-inflammatory, antioxidant compounds, which also have antibacterial effects [123-125]. In vitro studies have shown that quercetin is the most potent antioxidant among its six metabolites and butylated hydroxytoluene [126]. Quercetin introduced into the diet of mice increases the content of glutathione in the blood serum [127]. The use of quercetin in broiler diets increases their immune status [128], the expression of superoxide dismutase (*SOD1*), glutathione peroxidase (*GPx1*), as well as *GLUT2*, peptide transporter 1 (*PEPT1*) and fatty acid synthase (*FAS*) genes [129]. M. Koudoufio et al. [130] consider the use of flavonoids as modulators of genes involved in redox signaling.

Since DHQ is an adaptogen that has a positive effect on the antioxidant status of animals, a number of studies have been conducted on the effect of its feeding alone and in combination with vitamins on the antioxidant and biochemical status of the body, including under stressful conditions. R.V. Nekrasov et al. [95] showed a positive effect of DHQ in pigs under stress (improvement of the oxidative function of the blood, normalization of the number of leukocytes, increase in the number of erythrocytes, hematocrit, metabolic rate and endurance). Methods for improving meat quality using feed antioxidants and adaptogens have been studied [96-98]. It was found that the meat of pigs subjected to simulated stress (regrouping) and fed with a diet of 32 mg DHQ/kg of feed had an increased VSS, contained less fat and more protein than in analogue animals, not treated with adaptogens [99]. The hypothesis was tested that the resistance of pork to the development of hydrolytic and oxidative processes can be increased by introducing DHQ into the feed (confirmed dose of 32 mg/kg). In the experimental group, interrelated trends were noted for an increase in the amount of antioxidants in the blood, an increase in the degree of unsaturation of fat in bacon, and the resistance of muscle and adipose tissue to oxidative processes [100]. Similar effects have been noted in other studies [131-134].

Let us consider in more detail the role of antioxidant adaptogens in the formation of antioxidant protection and meat quality in poultry when such preparations are fed in pure and in combination. The table presents data from different authors over the past 5 years (2017-2022) on the use of vitamins C and E, as well as quercetin and taxifolin, in feeding chickens and broilers, including under various stresses. We searched the available literature in Science Direct, Scopus, PubMED and Google Scholar databases for the following keywords: dihydroquarcetin, taxifolin, quercetin, broilers, stress, meat quality, vitamin C, vitamin E.

Although an adult bird is able to synthesize vitamin C under normal conditions, the need for it increases during times of stress. Feeding ascorbic acid to poultry has been reported to be beneficial [89]. It has been shown that additional feeding of VC significantly reduces the metabolic signs of stress, improves the productivity and immune status of the bird. The optimal feeding dose in terms of the effectiveness of introducing VC into feed and water for broilers and laying hens under conditions of stress of various etiologies, apparently, is 200-250 mg/kg

of feed (the doses up to 1000 mg/kg were studied). Under chronic stressful conditions, such as extreme environmental temperatures, the amount of corticosterone in the body rises, which can ultimately reduce the effectiveness of VC feeding. Under normal conditions, VC controls the release of adrenal corticosterone by decreasing its production and secretion, but during times of stress, endogenous VC is depleted in the adrenal glands, causing systemic secretion of this potent adrenal glucocorticoid. The addition of BC from an exogenous source such as feed can help mitigate the detrimental effects of stress to minimize its negative impact on hen performance [191]. Feeding in combination with other biologically active substances (BAS) enhances the work of vitamin C [112, 113, 153]. A number of studies have shown a positive effect of vitamin C on the amount of ROS [121, 135, 140], productivity [145, 150] and quality of broiler meat [138, 139, 141] under OS caused by TS, high bird density [56], transportation [112, 154], toxicosis [149, 151, 153].

A review of literature sources also showed that the optimal dose of vitamin E feeding to chickens and broilers is 100-400 mg/kg of feed. In a number of studies, the use of BE in diets did not have a positive effect on growth rates [157, 160, 167], but contributed to a decrease in poultry mortality [170]. In other studies, the use of VE alone or in combination with other antioxidants in the diet of broilers contributed to an increase in growth rate and feed conversion [159, 162, 163], AOS parameetetrs [166, 169], and feed consumption [168]. The biological properties of VE in the body are manifested in an increase in the concentration of tocopherol in muscles [165, 166], blood plasma [166], and liver [169]. When feeding the vitamin alone and in combination with other antioxidants, an increase in the antioxidant activity of the blood and muscle tissue was noted [162, 165-167]. Feeding VE to broilers and chickens leads to an increase in the brightness of muscle tissue, in particular breast [155, 157, 169, 170], an increase in muscle pH [157, 170], an increase in the relative mass of the liver [157], stomach [159]. Enrichment of broiler diets with BAS complex (selenium with vitamins E and C) improves the function of vital organs, immune system response and growth performance of broilers under conditions of heat stress [192]. The combination of these supplements alleviates the symptoms of TS more effectively than their individual forms, due to the combination of several mechanisms in a synergistic effect. It has been shown that selenium and vitamins E and C closely interact: protect proteins and lipids from oxidative damage and activate the function of the immune system. The combination of VE and selenium can reduce the negative consequences of OS in the body of birds caused by xenobiotics of a chemical nature [165].

It has also been found that quercetin supplementation leads to a significant increase in the expression of genes associated with oxidative fibers, promoting the switch of skeletal fibers from glycolytic type II to oxidative type I [192]. In vitro studies have shown that quercetin acts as an antioxidant due to its ability to scavenge free radicals through the successive transfer of two electrons and the formation of an oxidized electrophilic product (quinone). In vivo, quercetin tends to generate reactive oxygen species by transferring electrons to oxygen catalyzed by transition metals. The resulting superoxide rapidly dismutes to hydrogen peroxide, which is fairly stable. Hydrogen peroxide or quinone are most likely responsible for the cytoprotective effects by inducing cellular endogenous antioxidant responses [193]. They are controlled by the transcription factor Nrf2 which is activated in response to the presence of hydrogen peroxide and electrophiles and then binds to related antioxidant elements located in the promoter regions of cytoprotective, antioxidant, and detoxification enzyme genes, including those involved in the synthesis and recycling of the widespread endogenous the antioxidant glutathione [194-196].

The effect of vitamins C and E, quercetin and taxifolin on the antioxidant status and parameters of meat in chickens and broilers

Experiment design (genotype, number of animals, age, antioxidant dosage)	Stress type	Antioxydant status	Carcass yield, meat quality, condition of internal organs	Blood biochemil parameters, immunity status	Productivity and other biological effects	References
			Vitamin C			
Broiler chickens Ross 308 (n = 160 heads), from days 25 to 42; 250 mg VC/kg feed	Chronic heat stress (35±2 °C for 8 h dayly, 9 am-5 pm)	A 27.90 % increase in GP	Not studied	Decrease in the amount of LDLP, the ratio of heterophils to lymphocytes in the blood	Increasing BW	[121]
Broiler chickens Ross 308 (n = 162), from days 3 to 35; 200 mg VC/kg feed	Heat stress (32-34/27-29 °C day/nignt)	An increase in AOS in the blood, a decrease in the expression of mRNA of interleukin (IL)-1β, IL-6, interferon (IFN)-γ, Toll-like receptors (TLR)-4 and HSP70 in the liver, a decrease in LPO processes in the blood and liver, mRNA expression of pro-inflammatory cytokines and HSP70	Liver and spleen weight unchanged, statistically significant ($p < 0.05$) increase in relative thymus weight	Not studied	No significant difference in live weight, feed conversion	[135]
Broiler chickens Ross 308 (n = 384), from 0 to 22 weeks; 200 mg BC/kg feed	Absent	Not studied	Not revealed	No effect on the content of alkaline phosphatase in blood serum	No effect on BW, intestinal morphology (villous height, depth of Lieberkün crypts and their ratio), strength and ash content of the tibia. Better feather integrity, reduced number of tail and wing feathers	[136]

Continued Table

Broiler chickens Ross 308 (n = 1368 heads, ♀:♂ = 1:1), from days 21 to 35; 200 mg VC/kg feed	Stocking density (low, 9 birds/m^2 and high, 18 birds/m^2)	Did not affect AOS in the liver (OAS and MDA)	Not revealed	No change in the concentration of H:L (hetero-phil:lymphocyte) in the blood and CORT (cortisol) in feathers, a decrease in the value of TER (transepithelial electrical resistance) in the mucosa of the jejunum as a parameter of intestinal permeability	No impact on growth performance	[56]
Broiler chickens (n = 96, ♀), from day 1 to week 4; 0, 250, 500 or 1000 mg AA/kg feed	Heat stress (дup to 36 °C for 6-10 h)	Not studied	An increase in adrenal weight	Maintaining the concentration of total protein, a slight increase in glucose, cholesterol, a decrease in the concentration of sodium in plasma, an increase in the amount of calcium and phosphorus, potassium	AA, especially at a dose of 250 mg/kg, reduces the negative effects of HS on metabolism and productivity; reduces the ADG of non-heat stressed birds	[137]
Broiler chickens Ross (n = 330), from day 4 to week 6; 0, 10, 50, 100 and 200 mg AA/kg feed	Absent	Not studied	An increase in the yield of steamed and chilled carcasses, the breast muscle weight; the meat is more red, an improved bone strength, increased Ca and P accumulation in the bones	The lymphocyte subpopulation showed more CD4 and T-cell receptor-II (TCR-II) cells	Increased growth, the digestibility of nutrients; AA (200 mg/kg) increases productivity and immunity	[138]
Broiler chickens Ross 308 (n = 270, ♀135, ♂135), day 35; 50 mg VC/l, 100 mg VC/l, 1 g AA/l, 1.5 g AA/l, 50 mg VC/l + 1 g AA/l, 50 mg VC/l + 1.5 g AA/l, 100 mg VC/l + 1 g AA/l and 100 mg VC/l + 1.5g AA/l drinking water	Transporatation stress	Not studied	Not studied	Decreased values of all stress indicators (glucose, albumin, globulin, uric acid, calcium, AlAT, AsAT, creatine kinase and T3). Increasing the concentration of T4	100 mg VC/l + 1.5 g AA/l with drinking water reduces the negative impact of transport stress on the body	[112]

Continued Table

Hens and roosters, line Mandarah (♀288, ♂ 36), from weeks 32 to 48; 1000 mg betaine/kg, 200 mg AA/kg, 150 mg tocopherol acetate/kg feed and their combination	Chronic heat stress (38±1 °C; 55-65 % humidity) for 3 days weekly, 11^{00} am–3^{00} pm)	Not studied	Increased weight of the liver, spleen, thyroid, ovaries, oviduct and length of the oviducts	Decreased values of stress markers (glucose, estrogen, progesterone, T_3, T_4)	Increased productivity	[139]
Broiler chickens Ross 308 (n = 120), from days 25 to 54; 15 g VC/100 l drinking water (equial to 11.25 mg/kg bodyweight)	Oxidative stress (caused by SA on day 35)	Reducing the degree of influence of the OS: a decrease in the concentration of MDA in the blood serum, an increase in OAS	Recovery of histopathological changes	No effect on the concentration of interleukin-6 in the synovial fluid	Not studied	[140]
Broiler chickens Cobb 500 (n = 1680), from days 21 to 38 (final growing); 500 mg VC/kg feed	Heat stress (34±1 °C for 8 h dayly)	Decrease in the concentration of MDA in the pectoral muscle, a decrease in LPO	Not studied	A decrease in the concentration of UA, lactate, no effect on CPK, LDH, T_3, T_4	Not studied	[141]
Laying hens (n = 96), from week 28 for 10 weeks; 0, 50, 100 and 200 mg VC/kg feed	Absent	Not studied	Not studied	Increasing the concentration of vitamins in the blood	Not studied	[142]
Laying hens Isa Brown (n = 13200), from month 13 for 40 days; 1 g VC/kg	Heat stress (+23.84 °C for 20 days followed by +25.54 °C for 20 days with 1 g VC/kg).	Not studied	Not studied	Not studied	Not found	[143]
Broiler chickens (n = 100), from day 22 of feeding to the end of growing (day 42); 2 g VC/l water (200 mg active substance/l)	Heat stress (after 28 days of feeding, the temperature was above the optimal values)	Not studied	Not studied	Increasing the number of erythrocytes, a reduced effect of hemolysis of erythrocytes	Not studied	[144]
Laying hens White Leghorn (n = 96); 100, 200 and 300 mh AA	Heat stress, randomly grouped hens were kept at 26±1,0 °C and under heat stress (40±5.0 °C)	Not studied	Not studied	No change in the concentration of HSP70 (heat shock protein). Decreased corticosterone concentration	Increased feed efficiency ratio, productivity index, egg production (%) in the group fed 300 mg of AA	[145]
Hens Hy-Line W-36, from weeks 65 to 69; 200 mg VC/kg feed	Heat stress, neutral (22 °C) and high (32 °C) temperature	Not studied	Not studied	An increase in the concentration of Na and P in the blood, a decrease in the concentration of Ca and P compared to TN	No significant effect noted	[146]

Continued Table

Hens Bovan (n = 80), from month 4 for 6 weeks); 1000 mg AA/kg feed, 500 mg AA/kg feed, 500 mg AA in water, 1000 mg AA in water	Absent	Not studied	Not studied	No significant change in the ratio of heterophils and lymphocytes	Increase in weight, body temperature, total number of leukocytes	[147]
Broiler chickens Cobb 500 (n = 45), days from 1to 35; 30, 60, 90, 120 mg AA/kg feed	Absent	Not studied	Not studied	Not studied	Increase in body weight, weight gain and feed intake	[148]
Broiler chickens (n = 240), from days 0 to 42; 100, 200 mg VC under various levels of OTA	Toxicosis (OTA)	Not studied	Positive effect on the weight of the liver, kidneys, bursa of Fabricius	Reducing the amount of TP and cholesterol in the blood, increasing the concentration of UA and alkaline phosphatase	Partial reduction in the adverse effects of OTA on performance, relative organ mass and biochemical parameters	[149]
Broiler chickens Shiver (n = 180), from day 1 to week 8; 0, 500 и 1000 mg VC/kg feed	Heat stress, neutral (24 °C) and high (35 °C) temperature	Not studied	Not studied	Not studied	Significant improvement in ADG and FCR	(150]
Broiler chickens Ross (n = 368, ♂, 8 groups of n = 46 each) from day 3 to week 5; 300 mg VC/kg feed and in combination with yeast (SC 3 g + 300 mg/kg feed), and when fed feeds contaminated with OTA (200 mg/kg)	Toxicosis (OTA)	Not studied	Not studied	Not studied	Reducing the toxic effect of OTA when using a combination of VC with yeast	[151]
Broiler chickens Ross 308 (n = 1824, ♂), from days 0 to 35; 200 g VC/1000 l drining water	Heat stress (35 °C during 8^{00} am–2^{00} pm dayly)	Not studied	Not studied	Decrease in the concentration of corticosterone in the blood	Slight increase in productive indicators	[152]
Broiler chickens Ross 308 (n = 160, ♂), from days 25 to 42; lemon verbena (0.5% or 1,0%) and VC (250 mg/kg feed)	Chronic heat stress (35±2 °C for 8 h dayly, 9^{00} амд–5^{00} pm)	An increase in GP by 51.81% with 1.0% lemon verbena and by 27.90% with VC	Higher relative weight of bursa of Fabricius and breast due to 1.0% lemon verbena	Decrease in the ratio of heterophils to lymphocytes, the amount of LDLP decreased by 15.85 and 17.57% when feeding 0.5 and 1.0% lemon verbena	Not studied	[121]

Continued Table

Broiler chickens Cobb 500 (n = 251), from days 1 to 42; 300 mg $CuSO_4$/kg feed separately abd in combination with vitsmin C (250 mg/kg feed), vitsmin E (250 mg/kg feed) and their combination	Toxicosis ($CuSO_4$)	Reduced toxicity due to improved AOS	The addition of vitamins C and E, alone or in combination, had a beneficial effect on microscopic changes in the architecture of the kidneys, impaired OS	Reducing the negative consequences of OS, which manifested itself in a decrease in the number of red blood cells, hemoglobin concentration, hematocrit value, a state of hypoglycemia with an increase in the content of uric acid and creatinine in the blood serum	Preventive effects of dietary antioxidants on hematobiochemical changes, OS and kidney damage caused by $CuSO_4$ toxicity	[153]
Broilers (n = 128); 250 mg VC/l, 500 mg VC/l or 750 mg VC/l	Trransportation stress (24 or 48 km)	Not studied	Not studied	Decreased blood glucose	The addition of VC during transport had a positive effect on weight retention, heart rate and reduced mortality	[154]
			Vitmin E			
Broilers Cobb 500 (n = 750, ♂), from days 42 to 54; 30, 90, 150, 210 and 270 mg VE/kg feed	Absent	Not studied	Increasing the brightness of the breast muscles, increasing the pH of the meat	Not studied	Not studied	[155]
Broiler chickens (n = 96, ♂), from days 1 to 22; 22.00, 220.00 IU VE/kg diet	Veterinary maniplations (birds aged 22 days were injected subcutaneously with *Escherichia coli* 0111:B4 LPS)	Low *IL6* mRNA in the jejunum	Not studied	Not studied	Not studied	[156]
Broiler chickens Ross 308 (n = 420), from day 6 for 26 days; 33, 65 and 100 IU VE/kg feed (vitamin E of various origin)	Absent	Decreased lipid oxidation in the breast, thigh muscles, decreased mRNA expression of pro-inflammatory (IFN-γ, IL-1 and IL-6) and anti-inflammatory cytokines (IL-4, IL-10 and TGF-4) in the jejunum	Reddening of breast meat, slight increase in the relative weight of the liver, spleen, thymus, and bursa of Fabricius	Not studied	No effect on growth rates	[157]

Continued Table

Layinf hens Lohmann (n = 216), from week 50 for 12 weeks; 0, 20, 100 IU VE/kg feed	Absent	It did not affect the content of CF and cholesterol, the expression of acetyl-CoA carboxylase, lipoprotein lipase, fatty acid synthase, or the expression of CMKLR1 mRNA in the liver. An increase in the content of MDA, a decrease in the activity of GP in the blood serum and in the ovaries, a significant increase in the activity of GP in the liver and in the serum, the activity of SOD in serum, the expression of mRNA, SOD in the liver and ovaries	Not studied	Not studied	Not studied	[158]
Broiler chickens Ross 308 (n = 120, ♂), days from c 1 to 42; 100, 200 mg VE/kg feed	Absent	No differences in the activity of OAS or SOD. Increasing the concentration of total tocopherols and vitamin E in the blood serum, liver, and pectoral muscles	An increase in the weight of the stomach, the pH of the stomach contents; pectoral muscles are light with low pH, muscle tissue had a higher yellowness, high concentration of omega-6 PUFAs, low atherogenic index	Not studied	Increased BW	[159]
Parent flock Ross 308 (n = 512 of hens aged 71 weeks and n = 576 of hens aged 75 weeks); 100, 200 or 400 mg VE/kg feed for 12 weeks	Absent	Decreased MDA in ovaries, egg yolks and serum, brain and yolk sac of chickens, AOS in serum and ovaries, increased AOS in egg yolks and yolk sac of chickens, α-tocopherol content in egg yolks	Not studied	Not studied	No effect on egg production and egg hatchability	[160]
Broiler chickens Hubbard-Cobb (n = 960), days from 0 to 42; 200 mg VC and VE added with electrolytes/kg feed	Heat stress (30 °C, 60 % humidity for days from 21 to 42 with various intervals)	Not studied	Not studied	Absent	Adding electrolytes in the diet with the addition of sodium bicarbonate and vitamins C and E reduced the negative effects of heat stress on productivity	[161]

Continued Table

Broiler chickens Arbor Acres (n = 108), days from 7 to 35; 0.1 mg Se nanoparticles/kg feed, 100 mg VE/kg feed and all additives combined	Absent	Increase in AOS when feeding a complex of selenium nanoparticles and VE	Not studied	Increasing the amount of calcium and phosphorus in plasma. Increasing the content of albumin in the feeding Se + VE. Decreased cholesterol, increased triglycerides	Increasing BW and ADG, increasing feed conversion	[162]
Broiler chickens Ross (n = 720) days from 22 to 42; organic Zn (0.0 mg/kg and 120 mg/kg feed) and VE as DL-α-tocopherol acetate (0.0 mg/kg, 300 mg/kg and 600 mg/kg feed)	Heat stress (average temperature and relative humidity of 30.0 °C and 57.7 % for days from 22 to 33, and of 30.7 °C and 58.9 % for days from 34 to 42)	Not studied	No effect on the slaughter yield and the yield of abdominal fat	Not studied	VE supplement improved productivity from day 22 to day 33	[163]
Broiler chickens (n = 100), a subletal dose of 100 mg thiamethoxam/kg bodyweight + 150 mg VE/kg bodyweight or 0.25 мг Se/ml, or vitamin E + Se in drinking water	Toxic stress (thiamethoxam)	Not studied	Not studied	Positive effects of the combination of vitamin E and selenium on hematobiochemical parameters	Reducing the degree of toxic stress	[164]
Broiler chickens Ross 308 (n = 400), days from 21 to 42; VE (200 IU/kg feed), VC (250 mg/kg feed), Se (0,2 mg/kg feed), or VE + VC + Se of the indicated dosage)	Oxidative stress (5 % flaxseed oil in the diet)	An increase in the concentration of α-tocopherol, a decrease in the content of MDA in the pectoral muscle, inhibition of LPO processes in fresh, frozen, freshly cooked meat	No changes in the slaughter yield of breast, drumstick, wings, back and abdominal fat, WHC, pH_{24}, pH_{48}, breaset muscle color	Not studied	Not studied	[165]
Broiler chickens Cobb (n = 150), days from 1 to 21; 200 mg VE/kg feed	Absent	An increase in the concentration of γ-tocopherol in the blood plasma and in the meat of the thigh, the concentration of γ-tocopherol in the meat of the thigh on the 1st day, followed by a decrease. Reducing the concentration of MDA	Not studied	Not studied	Increase in ADG, decrease in feed conversion	[166]

Continued Table

Broiler chickens Ross 708 (n = 210), days from 0 to 58; 10, 200 IU VE/kg feed or omega-3, or VE + omega-3	Absent	Not studied	No difference in meat yield, in muscle mass, in pH, in losses during thawing, cooking; an increase in yellowness in the pectoral muscles, a decrease in the fat content in the pectoral muscles	Not studied	No significant effect of vitamin E, n-3 fatty acids, or a combination of these on growth performance	[167]
Broiler chickens Ross 708 (n = 28), from day 1 to week 5; natural (α-tocopherol acetate — AsAT, 35 mg/kg feed) or synthetic vitamin E (10 and 58 mg/kg feed)	Absent	Not studied	Increasing the concentration of α-tocopherol in the liver and muscles	Increasing the concentration of α-tocopherol in plasma	Increase in feed consumption by 1.72-1.81 times, ADG by 1.58-1.65 times	[168]
Broiler chickens Ross 308 (n = 945), days from 3 to 42; 200 mg VE/kg feed (Kavimix-E-50 α-tocopherol acetate) in the study of bioactive preparations	Absent	Increase in VE concentration in the liver (total VE and α-tocopherol) with the addition of VE	Reduced yellowness of meat and skin	Increased plasma carotenoid concentration in the VE treated group	Decreased feed intake with no negative effect on growth rate throughout the experiment in groups fed 200 mg VE	[169]
Chiks of experimental Polish meat line (n = 420, ♂), days from 1 to 63; 44, 200 mg VE (DL-α-tocopherol acetate)/kg feed	Absent	Decreased content of MDA, oxidative changes in the muscles of chickens 48 h after slaughter	Increasing carcass yield. No effect on the percentage of chest and leg muscles. An increase in the mass of the heart and stomach from the bodyweight, a decrease in the liver. Decreased abdominal fat. Increased pH_{24} and WHC of muscles and reduced losses during cooking. Darker meat, more saturated red and less saturated yellow. Improveв consumer properties of meat. Reducing the diameter and surface area of the fibers, the ratio of fibers to the total area	Not studied	No effect on growth rates, a decrease in mortality	[170]

Continued Table

Quercetins						
Broiler chickens Cobb 500 (n = 150), days from 1 to 42; 0.5, 1 g quercetin/kg feed	Absent	Not studied	Increase the brightness of the breast muscles, increase oxidative stability, decrease in MDA	Not studied	Not found	[171]
Broiler chickens Ross 308 (n = 120), days from 1 to 35; 0.2, 0.4 and 0.8 g quercetin/kg feed	Absent	Increase in the gut expression of mRNA for SOD (*SOD1*), GP (*GSH-Px*)	Not studied	Not studied	Increasing growth intensity and improving feed conversion	[129]
Broiler chickens Ross 308 (n = 80, ♂), days from 7 to 28 and from 28 to 35; extract of *Larix sibirica* (85 % dihydroquercetin) at 0.5 g/kg feed	Absent	Not studied	Change in redness of the breast muscles	Not studied	Not studied	[172]
Broiler chickens meat cross Smena 7 (n = 300), days from 1 to 42; 0.5 mg dihydroquercetin/kg bodyweight	Absent	Not studied	More dry matter and fat, less tryptophan and ash	Not studied	Not studied	[173, 174]
Broiler chickens (n = 300), days from 1 to 42; 0.5 mg dihydroquercetin/kg bodyweight	Absent	Not studied	Not studied	Not studied	Improved feed conversion by 9.2%	[175]
Broiler chickens (n = 300), days from 1 to 42; 0.5 kg dihydroquercetin/t premix	Absent	Not studied	Increased protein concentration in liver tissues and breast muscles	Not studied	Not studied	[176]
Broiler chickens (n = 160), days from 1 to 42; 0.5 mg dihydroquercetin/kg bodyweight	Absent	Not studied	Not studied	Not studied	Increase in productivity by 33.4%, derease in livestock smortality by 5.3%	[176]
Broiler chickens line Vencobb 400 (n = 192), days from 7 to 42; 1g quercetin/kg feed added with oil	Oxidative stress (caused by fat in the diet)	Not studied	Reducing the negative impact on meat quality of additional inclusion of fat in the diet	Not studied	Increased slaughter yield	[177]
Broiler chickens lina Cobb 500 (n = 40), days from 1 to 60; 0.5, 0.75 and 1 g dihydroquercetin/100 kg combned feed	Absent	Not studied	Not studied	Not studied	Increase in bodyweight by 11.91-32.78%	[178]

Continued Table

Broiler chickens line Cobb 500 (n = 40), days from 1 to 60; 0.5, 0.75 and 1 g dihydroquercetin/100 kg combned feed	Absent	Not studied	Not studied	Bringing to normal hematological parameters	Not studied	[179]
Broiler chickens line Cobb 500 (n = 40), days from 1 to 60; 0,5, 0,75 and 1 g dihydroquercetin/100 kg combned feed	Absent	Not studied	Increase in the mass of the butchered carcass by 15-38%, muscle tissue by 3%, pectoral muscles by 0.3-2%, edible part of the carcass - by 2-6%	Not studied	Decrease in the mortality by 20-30%, in bodyweight by 12-33%	[180]
Broiler chickens Ross 308 (n = 320), days from 7 to 35, extract of *Larix sibirica* (85 % dihydroquercetin) at 0.5 g/kg feed and dihydroquercetin with VE at 0.3 g/kg feed	Chronic heat stress (35 °C)	Increased activity of GP in the blood and OAS	Increased heart mass and cecum size (dihydroquercetin); weight gain in the spleen and liver (dihydroquercetin + VE)	Not studied	Not found	[27]
Broiler chickens Ross 308 (n = 100), days from 7 to 21; extract of *Larix sibirica* (85 % dihydroquercetin) at 0.5, 1.5 and 4,5 g/kg feed	Absent	Increased GP activity at maximum dosage	Not studied	Not studied	Slight increase in bodyweight at maximum dosage	[181]
Broiler chickens Arbor Acre (n = 240), days from 1 to 42; 97 % quercetin at a dosage of 0.02, 0.04 and 0.06 % of ration	Absent	Not studied	Not studied	An increase in the index of the spleen and thymus. Increased production of immunoglobulin A (IgA), interleukin-4 (IL-4), immunoglobulin M (IgM) and tumor necrosis factor-α (TNF-α). Increased expression of TNF-α, TNF receptor-associated Factor-2 (TRAF-2), NF-κBp65 and interferon-γ (IFN-γ) mRNA and expression of NF-κB-alpha (IκB-α) inhibitor	Slight effect	[182]

Continued Table

Broiler chickens Arbor Acres (n = 240, ♂), days from 1 to 21, 200 or 500 mg quercetin/kg feed	Oxidative stress (caused by lipopolysaccharides)	Reducing the amount of ROS, MDA. Increased activity of peroxidase, SOD, glutathione content. Reduced damage to jejunal mitochondria and increased expression of genes associated with mitochondrial DNA copy number	Not studied	Alleviation of oxidative damage to the gut through the MAPK/Nrf2 signaling pathway	Not studied	[183]
Broiler chickens Arbor Acre (n = 480), days from 1 to 42; > 95 % quercetin at a dosage of 0.2, 0.4 and 0.8 g/kg feed	Oxidative stress (caused by oxidized oil)	Decreased MDA	Not studied	Activation of Nrf2 and related genes (*CAT, GP 2, SOD1, HO-1*, and thioredoxin) in the ileal mucosa. Strengthening the intestinal barrier by increasing the expression and secretion of mucin 2 (MUC2)	Not studied	[184]
Broiler chickens Arbor Acres (n = 300), days from 1 to 42; 0.2, 0.4 and 0.6 g quercetin/kg feed	Oxidative stress (caused by streptozotocin)	Increasing the activity of antioxidant enzymes, reducing the content of MDA and NO. Activation of expression of genes associated with the PI3K/PKB signaling pathway	Not studied	Activation of expression of genes associated with the PI3K/PKB signaling pathway, regulation of glucose metabolism	Not studied	[185]
Broiler chickens Arbor Acres (n = 640), days from 1 to 35; 97 % quercetin at a dosage of 250, 500 and 1000 mg/kg feed	Chronic heat stress (32 °C for 24 h from day 4)	Increased activity of SOD (T-SOD) and AOS	Decrease in MDA concentration, decrease in the amount of abdominal fat	Increasing the concentration of tumor necrosis factor-α (TNF-α)	Increased bodyweight	[186]
Broiler chickens Cobb (n = 40), days from 1 to 42; 0.5 g quercetin/kg feed	Oxidative stress (caused by OTA)	Normalization of enzyme activity	Not studied	Reducing the immunotoxic effects of OS due to the activation of the PI3K/AKT signaling pathway for its immunomodulatory, antioxidant, and antiapoptotic activities	Not studied	[79]
Broiler chickens Ross 308 (n = 210), days from 1 to 42; 500 and 1000 mg quercetin/kg feed, and 1000 mg quercetin/kg feed + 250 mg VE/kg feed	Absent	Not studied	Not studied	Not studied	Increasing bodyweight of chickens and feed intake	[187]

Continued Table

Broiler chickens Ross 308 (n = 1088), days from 1 to 35; 0.2, 0.4 и 0.6 g quercetin/kg feed	Absent	Not studied	Increase in WHC, decrease in moisture loss of the pectoral muscle	Not studied	Increased ADG, feed intake, nutrient digestibility	[188]
Broiler chickens Arbor Acres (n = 480); 0, 0.2, 0,4 and 0.6 g quercetin/kg feed	Absent	Not studied	The percentage of fat in the abdominal cavity was significantly reduced due to the favorable modulation of the intestinal microbiota	Not studied	Quercetin improved lipid metabolism by modulating gut microbial and AMPK/PPAR signaling pathways	[189]
Broiler chickens Ross 308 (n = 300), 100, 200, 300 mg quercetin/kg feed	Absent	Not studied	Not studied	Not studied	Improved feed conversion rates	[190]

N o t e. HS — heat stress, VC — vitamin C, VE— vitamin E, GP — glutathione peroxidase, LDLP — low density lipoproteins, BW — bodyweight, LPO — lipid peroxidation, AOS — antioxidant status, AP — alkaline phosphatase, OAS — overall antioxidant status, MDA — malonic dialdehyde, AA — ascorbic acid, AlAT — alanine aminotransferase, AsAT — aspartate aminotransferase, OS — oxidative stress, SA — septic arthritis, UA — uric acid, OTA — ochratoxin A, ADG — average daily gain, CF — crude fat, SOD — superoxide dismutase, PUFA — polyunsaturated fatty acids, WHC — moisture holding capacity, DHQ— dihydroquercetin, ROS— reactive oxygen species, TH — thermoneutral, TP — total protein, FCR — feed convertion rate, BAS — bioactive substances.

Thus, quercetin exerts protective functions either directly, by activating antioxidant enzymes, or indirectly, by stimulating transcription factors that enhance the antioxidant defense status, especially under stress. In the sources we considered, quercetin was included in the diets of poultry in the amount of 0.2-1 g/kg of feed. Feeding quercetin at the indicated dosages promotes an increase in the expression of antioxidant defense genes [129], a decrease in the concentration of MDA, ROS, an increase in glutathione activity [184], including under conditions of oxidative [185, 186] and heat stress [79]. The dosage of the introduction of taxifolin (dihydroquercetin) into the diets of broilers and chickens is much less than that of quercetin, due to its increased biological activity - 0.005-0.01 g/kg of feed. The authors noted an improvement in the quality of broiler meat under the influence of DHQ [173, 174, 180].

In conclusion, it should be noted that modern studies of stresses and their adjustment in livestock and poultry farming are quite numerous, but in some part contradictory, so the search for the most effective feed products that counteract the effects of stress remains relevant. Biomarkers are needed to allow for an in vivo assessment of the quality of the products obtained, and the study of correlations between indicators of the biochemical, antioxidant, hormonal status of the animal and the quality of meat. This will allow more targeted use of antioxidants in animal and poultry nutrition. It is necessary to take into account the synergistic effect of antioxidants-adaptogens on the body and slaughter products when used in complex diets, especially under stresses of various nature. In our opinion, the use of natural flavonoids in combination with vitamins is promising, which will enhance antioxidant protection, resistance and, as a result, provide improved quality meat products. It is possible that it is the combination of natural adaptogens as feed additives that will be the most effective method in protecting against the effects of stress. This is the reason for the interest in continuing such studies both in poultry and in monogastric animals.

So, in poultry farming, stresses of various nature (climatic, transport, feed, veterinary, placement density) have a significant impact on the body, primarily on the immune and antioxidant systems. Also, unfavorable conditions lead to significant losses in safety and gains in live weight, feed conversion decreases. The most significant negative impact of stress on product quality. With a decrease in the proportion of muscle mass, lipid peroxidation products accumulate, which lowers the pH of the meat and increases the proportion of meat PSE (pale, soft, exudative). The most effective and simple method of protection against stress and its negative consequences should be recognized as feeding animals with antioxidants. Many studies have established a significant positive role of vitamins C and E, as well as bioflavonoids when fed to laying hens and broilers under various stresses observed in modern industrial poultry farming. Vitamins and bioflavonoids enhance the expression of antioxidant defense genes and reduce lipid peroxidation. They protect proteins and lipids from oxidative damage and increase immune function in general, which leads to an improvement in the quantitative and qualitative indicators of meat productivity.

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FATS AND EMULSIFIERS IN FEEDING BROILER CHICKENS
(review)

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Abstract

The increase in feed prices determines the need to optimize the rationing of high-energy ingredients of the diet, as well as various approaches to improving the efficiency of their use in the digestive process. In industrial poultry farming, fats, having a high energy value, serve as indispensable components of the diet (V.I. Fisinin et al., 2000; V.I. Fisinin et al., 2011). They provide high productivity and economic efficiency (N.C. Baião et al., 2005; M. Nayebpor et al., 2007; H. Fébel et al., 2008), play an important role in the regulation of metabolism, deposit energy, performing a protective function, serve as solvents and carriers of vitamins, hormones, as well as an obligatory component of nervous tissue (A.V. Arkhipov, 2010; M. Poorghasemi et al., 2013; R. Rodriguez-Sanchez et al., 2019). A wide variety of fats and oils and by-products of processing are available for use in diets, for example, animal fats and vegetable oils (soy, corn, sunflower, palm, hemp, mustard, etc.), sunflower fusel (a by-product of the conversion of sunflower seeds into vegetable oil), acidified soapstocks (by-products refining of vegetable oil, mainly containing free fatty acids), hydrogenated fats (A.V. Arkhipov, 2007; V.A. Manukyan et al., 2018; L.N. Skvortsova et al., 2013). The choice of fat for use in feeding farm animals and poultry is largely determined by both its cost and quality characteristics. The main factor that affects the release of energy from fat entering the body with food is its digestibility (V. Ravindran et al., 2016; R. Rodriguez-Sanchez et al., 2019; B. Jimenez-Moya et al., 2021). The digestion of fats is a complex process that requires a sufficient amount of bile acid salts and enzymes (S. Leeson et al., 2009). In addition, the correction of the diet with lipids is effective, but economically impractical. The increase in prices for animal and vegetable fats currently encourages interest in the search and use of alternative energy sources in the feed of farm animals or substances that enhance the processes of digestion and assimilation of lipids, in order to reduce the cost of production (S.A. Miroshnikov et al., 2005; O. Lyutykh, 2020). One of the approaches to increase the amount of available fats can be the use of synthetic and natural emulsifiers. Popular emulsifiers usually consist of hydrophilic and hydrophobic components that can reduce the surface tension of fat and water, reduce chylomicrons of fat, improve emulsification and increase fat absorption, make up for the deficiency of bile acid and lipase in the digestive tract of poultry (M. Rovers et al., 2014; M. Jansen et al., 2015). Natural emulsifiers include bile acids and salts, including cholic and henodeoxycholic, taurocholate, lecithin, casei, phosphatide concentrates, some of which can be produced in the animal's body (M. Soares et al., 2002). Bile acid salts reduce the tension of the oil-water interface, activate pancreatic lipase, and also prevent the denaturation of this enzyme when it throws the surface of emulsified fat droplets (M. Boesjes et al., 2014; Y. Xu, 2016; X.K. Ge et al., 2019). Synthetic emulsifiers (lysolecitin, lysophosphatidylcholine, mono- and polyoxyethylene glycol dioleates) improve liver and bile duct function, accelerate weight gain and improve feed conversion, increase growth rates and nutrient digestibility (B. Zhang et al., 2011; M.M. Gheisar et al., 2015; S.D. Upadhaya et al., 2018). Consequently, the strategy of using emulsifiers and enzymes can be an effective tool for improving the digestion of fats both in young birds with functional immaturity of the digestive system and in adults to further reduce feed losses due to the intensification of the digestive process. The use of this approach will provide increased digestibility and digestibility of nutrients while reducing the introduction of vegetable and animal fats into the diet of broiler chickens.

Keywords: chicken broilers, feeding, diet, fats, oils, lipids, emulsifiers, digestion, microbiome

Currently, industrial poultry farming is an example of an efficient meat production system among other livestock industries [1, 2]. The main condition for the successful development and good productivity of poultry is a full-fledged rationed feeding [3, 4]. Since the formation of the body occurs due to the nutrients of the feed (proteins, fats, carbohydrates, minerals and vitamins), the rate of growth and development, body weight and productivity are directly dependent on the component composition of the diet and its percentage ratio [5-7].

The term "fat" is commonly used as a synonym for lipid. Both terms describe a variety of compounds that are insoluble in water but soluble in organic solvents such as chloroform, acetone, alcohol, and diethyl ether. Lipids play an important role in the nutrition, biochemistry and physiology of animals. From a nutritional perspective, triglycerides, phospholipids, sterols, and fat-soluble vitamins are important [8].

Rising feed costs have led to an increased interest in the use of fat supplements in the diet. The inclusion of fat in diets has become a widespread practice in the poultry industry to meet the high energy requirements of fast growing birds [9-12]. Essential fatty acids and vitamins also enter the body along with fat [8, 13, 14]. However, there are some problems with fat intake, quantity and digestibility in broiler chickens. Fat digestibility depends on the age of the bird, as well as the type and source of fat [15].

Free fatty acids released during the digestion of fats can react with divalent cations to form soluble or insoluble soaps. In case of formation of insoluble soap, fatty acids and minerals can become unavailable to the birds. The divalent Ca^{2+} ions present in the feed bind to fatty acid molecules and result in the formation of soap that is not absorbed or digested, resulting in loss of fat and calcium [16]. Dietary fats also affect the digestibility, absorption, intake, and metabolism of many other substances, such as carbohydrates, proteins, and minerals [17]. There is a relationship between carcass fat content and the type of fat in feed [18]. Some fats used in the diet cause more abdominal fat and lead to the rejection of such a product by consumers in the markets.

An alternative to increasing the amount of fat in diets can be synthetic and natural emulsifiers that intensify its digestion. This approach will provide increased digestibility and absorption of nutrients while reducing the amount of vegetable and animal fats introduced into the diet.

The purpose of this review is to assess the effectiveness of the use of various sources of fat in the diet, to analyze the causes that affect the rate of digestion and absorption of fats, and also to compare ways to control these processes using various substances, including emulsifiers.

The role and properties of fats. The central place in the theory of feeding broiler chickens is occupied by energy nutrition, primarily the content of metabolic energy in the diet. The key condition is a significant increase in the amount of energy used to synthesize products (19). Optimal energy content in the diet ensures high protein conversion. The lack of energy leads to the fact that amino acids begin to be used for energy purposes, which reduces the productivity of broiler chickens. It is important to note that fat synthesis uses the energy of digested protein to a lesser extent than the energy of digested carbohydrates and fats [20, 21].

The biological functions of lipids are diverse: they serve as the main form of energy storage in the body, sources of essential fatty acids, structural components of biological membranes, and the basis for the subsequent synthesis of some biologically active substances [22-25]. Fats can improve the physical properties and palatability of feed, thus increasing feed intake [26, 27].

Along with fats, carbohydrates and proteins serve as energy components of the diet [28]. Fats can be synthesized in the body from carbohydrates (25.2 g of fat is produced from 100 g of starch) and proteins (26 g of fat is synthesized from 100 g of protein) [29]. However, in terms of energy capacity, carbohydrates and proteins differ slightly, while the energy saturation of fats is much higher, approx. 2.25-fold. The energy value of 1 g of fat is approximately equal to 9.3 kcal, or 39 kJ, and when 1 g of carbohydrates are oxidized, 17 kJ is formed, and 1 g of proteins is 24 kJ. In addition to the fact that fat is the main energy store, it also acts as a structural material in the cell and is necessary for the normal functioning of the digestive glands.

Fats are involved in the regulation of metabolism [30-32], perform a protective function (due to deposition in the area of internal organs and in subcutaneous adipose tissue) [33, 34], dissolve and transfer vitamins and hormones, and are also part of the nervous system tissues [35, 36].

A wide variety of fats and oils are available for use in diets, including by-products of processing, such as animal fats and vegetable oils (soybean, corn, sunflower, palm, hemp, rapeseed, etc.), sunflower fuse (a by-product of seed processing into vegetable oil), hydrogenated fats, and acidified soapstocks (free fatty acids are removed from the alkaline refining process and precipitated as alkaline soaps) [23, 25, 29]. These fats and oils vary considerably in composition. The choice of fat for feeding farm animals and poultry is largely determined by both its cost and quality characteristics.

The main factor that affects the release of energy from fat that enters the body with food is its digestibility. The digestibility of fats and oils is affected by many factors. These include the number of double bonds, or the degree of unsaturation of the fatty acid, the amount of free fatty acids and their position in the triglyceride molecule, the structure of the diet, the sex and age of the bird, and the composition of the intestinal microflora [8, 37, 38].

The nutritional value of fats depends on both their energy potential and safety. Oxidation becomes the main reason for the loss of quality of fat. Oxidative rancidity is a process that occurs in unsaturated fatty acids when the double bond of triglycerides is oxidized. It affects smell, color and taste and ultimately reduces the value of fat [39].

Currently, the use of liquid vegetable oils as sources of fats, which differ in the ratio of saturated and unsaturated fatty acids, is justified. This circumstance determines the digestibility and use of fats by poultry. In addition, these foods serve as an additional source of essential fatty acids [40].

Thus, fats, having a high energy value, are indispensable components of the diet. Lipids play an important role in the regulation of metabolism, store energy, perform the protective function of the body, serve as solvents and carriers of vitamins, hormones, prostaglandins, and are also an essential part of the nervous tissue.

Fats in the diet of broiler chickens and their regulation. In Russia, the dietary structure of broiler chickens is predominantly based on a wheat or wheat-barley feed mixture, which makes diets energy deficient [41]. In connection with the increase in the price of grain feed in recent years, the addition of fat to the diets of farm animals has become a necessary measure. Since the energy released during the digestion of fats is higher than the energy of carbohydrates, it makes economic sense to increase the fat content in broiler diets. In case of a lack of fats, metabolic processes, liver functions are disturbed, there is a lack of vitamins A, D, E, K, skin diseases (dermatitis, plumage disorders) occur, as a result, immunity decreases and reproductive function disorders occur [42, 43].

According to the instructions of the All-Russian Research and Technological

Institute of Poultry Farming (2010), the recommended dosage of fats and oils in feed is 4-6%, which positively affects productivity, the use of feed nutrients and metabolism, including lipid (Table 1). However, since the oil content directly affects the structure and granulation of the feed, other authors recommend introducing no more than 4% [5, 43].

1. Metabolized energy, protein, fat, linoleic acid, unsaturated and saturated fatty acids in the diets of broiler chickens [1]

Weeks of growth	Metabolized energy, kJ/100 g	Crude protein, %	Linoleic acid, %	Fats and oils, %	Fatty acids, %	
					unsaturated	saturated
1-3	1297	23.0	1.4	0-6	100	0
4-5	1318	21.0	1.3		75	25
6-7	1339	20.0	1.2	0-8	50	30

In young chicks, fat digestion and absorption is inefficient due to low natural lipase production [24]. Activity and net duodenal lipase secretion increase with age [44]. The problem is exacerbated by the low rate of bile salt synthesis in juveniles [45]. However, these physiological features are leveled with age. In this regard, for broiler chickens, the proportion of fat in the diet in the first 10-14 days is limited to 2.5-3.0% [45-48].

The main factor in the digestibility of fats and oils by the body is the content of saturated and unsaturated fatty acids in them. Animal fats consist mainly of saturated fatty acids, while vegetable fats consist of unsaturated fatty acids. Unsaturated fatty acids ensure normal growth, metabolism, proper skin function, vascular elasticity, and cholesterol metabolism in the body [49-51]. Excess leads to lipid peroxidation, metabolic disturbances, reduced productivity and reproductive function of poultry, as well as to the destruction of fat-soluble vitamins, especially vitamins A and E [52, 53]. From vegetable oils, sunflower and rapeseed oils, less often linseed and palm oils are used as lipid additives [54].

Vegetable oil is an easily accessible source of metabolic energy. Its nutritional value depends on the content of fats and vitamins, in particular polyunsaturated fatty acids (PUFAs) (linoleic 50-60%). Vegetable oils also serve as sources of vitamin E and β-carotene [55]. In Russia, sunflower oil is mainly used as a lipid supplement in feeding broiler chickens. However, given the high content of linoleic acid in it (in the diet no more than 1.8%), which negatively affects productivity, its use is limited.

Linoleic and α-linolenic acids are recognized as metabolically essential fatty acids. Linoleic acid is the only essential fatty acid that has been proven necessary. Linoleic acid deficiency is rare. With a shortage, there is an increased need for water and a decrease in the immune response. Linoleic acid deficiency in bettas can impair spermatogenesis and affect fertility [56]. Insufficient deposition of linoleic acid in the egg will adversely affect embryonic development [57]. The essential fatty acid requirements of growing and adult birds can usually be met by feeding a diet with 1% linoleic acid. Oils of rapeseed, hemp, flax and camelina are rich in linoleic acid [23]. The need for poultry in -linolenic acid has not yet been proven. However, α-inolenic acid plays an important role in the development of specialized membranes in the retina and nervous system [58].

In contrast to Russia, in the United States, corn is the basis of compound feed, a high-energy product that significantly increases the energy of the diet [59]. The energy intake of broiler chickens can vary with age, rearing stage and ambient temperature and is typically between 400 and 450 kcal IU per head per day (or 1640-1845 kJ) [59]. Auxiliary energy components are usually rapeseed oil, animal and palm fat [60].

Thus, the use of vegetable fats in feeding birds, on the one hand, is necessary to ensure the physiological process, on the other hand, it is an effective way

to increase the energy value of the feed.

Digestion of fats. Digestion and absorption of fat occurs in several steps and includes emulsification (fat breakdown into droplets), hydrolysis by pancreatic lipase and formation of mixed micelles, as well as movement of micelles to the intestinal epithelium and absorption [61].

Digestion of fat is greatly accelerated when it enters the duodenum. Bile, formed in hepatocytes, passes into the gallbladder, and then into the intestine in the duodenum [64]. It contains bile pigments, bile salts, phospholipids, cholesterol, electrolytes and some proteins. Bile salts and phospholipids are the major components of bile required for lipid digestion [65]. In poultry, bile salts combine with taurine in the liver, which increases their water solubility and also reduces the cellular toxicity of bile salts. Bile salts are flat amphiphilic molecules, one side of which is a non-polar hydrophobic surface and interacts with water, and the other side is a polar hydrophilic surface that interacts with the oil phase of the emulsion. Due to this unique characteristic, bile salts are at the water-lipid interface and do not penetrate deeply [66].

This step promotes emulsification and activates pancreatic lipase, and prevents denaturation of lipase as it leaves the surface of the emulsified fat droplets [65]. Feed fat enters the intestine in the form of rather large coagulated particles. The presence of bile has a detergent-like effect on dietary lipids, causing this coagulated mass to break up into very fine, stable droplets (i.e., preventing sticking) and increasing the total surface area for lipase action [58].

Lipase is one of the digestive enzymes secreted by the pancreas, including trypsin, chymotrypsin, amylase, and phospholipases. The enzyme acts as a catalyst only when it is on the surface of emulsified fat droplets along with bile salts and co-lipase, a cofactor present in pancreatic juice. By itself, co-lipase has no enzymatic activity, but is required to initiate the activity of pancreatic lipase. Colipase is rich in both hydrophobic and hydrophilic amino acids and interacts with lipase to form a more hydrophobic and less charged complex, which allows the lipase to be maintained in an active configuration at the lipid—water interface. It is believed that the charge characteristics of co-lipase allow it to bind to the surface of fat droplets and act as an "anchor" for lipase, allowing the enzyme to act on triglycerides. Colipase and bile salts are competitive inhibitors of substrate binding sites. The activity of pancreatic lipase is suppressed by high concentrations of bile salts, but is restored by co-lipase [8].

Identification of the functional characteristics of local areas of the intestine during lipid digestion is crucial for understanding the complete picture of digestion. The results of studying this problem are presented in a limited number of works and, moreover, are contradictory. When fats enter the duodenum, their digestion is significantly accelerated. The presence of fat in this segment of the gastrointestinal tract stimulates the secretion of cholecystokinin, which in turn regulates the secretion of pancreatic juice and bile. Cholecystokinin also stimulates the release of bile from the gallbladder [63].

The jejunum is the main site of fat digestion and absorption in poultry [50], with digestion continuing in the upper ileum [67]. There are differences in the qualitative composition of fatty acids depending on the area of the intestine [50]. Linoleic acid is absorbed in the intestinal tract starting from the duodenum, while absorption of palmitic, stearic and oleic acids begins only in the jejunum. The exact reasons for these differences are unclear, but they can partly be explained by the insufficiency of bile due to the anatomical and topographic features of the bile ducts in birds. In addition, the passage of chyme into the duodenum of chickens is very fast, and this time may not be sufficient to emulsify saturated

fatty acids (68). In general, the digestion and absorption of fats is a complex process requiring adequate amounts of bile salts, pancreatic lipase and co-lipase [69]. The absence or decrease in the amount of any of these components will impair the processes of digestion and absorption.

Effect of fats on the gut microbiome of poultry. The gastrointestinal tract (GI) of broiler chickens is inhabited by a complex microbial community including fungi, archaea, protozoa and viruses, but dominated by bacteria [70]. Interactions between the organism and the microbial population have been extensively studied and analyzed by many research groups [71-75], and microorganisms are now thought to play an important role in bird nutrition, gut physiology and development [76-79]. The qualitative and quantitative composition of the microbiota and, consequently, its functionality depend on localization in the gastrointestinal tract. There is a significant difference in the taxonomic composition of the various sections of the digestive tract, so they can be considered as separate ecosystems, despite being interconnected [80)].

The microbiota plays a vital role in digestion and nutrient absorption, immune system development, and pathogen identification [83-85]. The composition and function of the microbial community varies depending on the age of the bird, localization in the gastrointestinal tract, and the ingredients consumed [79, 81, 86]. It should be noted that the taxonomic profiles described for each section of the gastrointestinal tract vary significantly across studies and depend on factors such as breed (cross), sex, genotype, diet, age, section of the intestine, use of antibacterial drugs, which makes it difficult to determine a typical profile for each department [82, 87].

The ingredient composition of the diet has an important influence on the composition of the gut microbiota [88]. In this regard, considerable attention is paid to the role of dietary components in the formation of intestinal microflora [87]. However, high-fat diets have not yet been extensively studied in terms of their effect on the microflora. Eating a high-fat diet typically results in an increase in Firmicutes and induces microbiota changes that are clearly associated with obesity and digestive disorders. In addition, the number of lipophilic bacteria (*Verrucomicrobia*, *Deltaproteobacteria*, *Ruminococcus*, *Lachnospiraceae*, *Bacteroidaceae*) increases [89]. Despite the fact that the bacteria of these groups are mostly not pathogenic or even beneficial to the body under normal nutrition, under conditions of high fat intake, the cumulative products of their metabolism can lead to multiple negative effects. A number of studies [90-93] indicate that high-fat diets increase the number of *Actinobacteria*, *Proteobacteria* and *Deferribacteres* and decrease the abundance of *Spirochaetae*. In addition, the proportion of *Collinsella*, *Streptococcus*, *Gemella* and *Elusimicrobium* is increasing. In a model mouse experiment, analysis of the gut microbiota showed that feeding a high-fat diet significantly altered gut microbiota composition, increasing *Firmicutes* abundance and decreasing *Bacteroidetes* population, resulting in a significant decrease in the *Bacteroidetes/Firmicutes* ratio. Moreover, the populations of *Clostridia* and *Deferribacteres*, *Ruminococcaceae*, *Lachnospiraceae* and *Bacteroidaceae* increased, while the population of *Bacteroidales* decreased. Thus, feeding a high-fat diet altered the qualitative and quantitative composition of the gut microbiota [94, 95]. Another study assessing the effect of fats on the microbiota found an increase in *Firmicutes* and *Proteobacteria*, and an increase in *Clostridia*, *Bacilli* and *Deltaproteobacteria* was also observed [96].

A high dietary fat content has been reported to cause an imbalance in the composition of the avian gut microbiota, resulting in increased intestinal permeability with chronic inflammation and a predisposition to food allergies [97].

Therefore, the intestinal microbiota plays an important role in digestion, and the qualitative and quantitative composition of microbial communities depends on the age of the bird, the gastrointestinal tract physiological conditions, and diet components [92]. It should be noted, however, that for the most part the results obtained so far from these experiments are contradictory or inconclusive. The difficulty in identifying specific populations of bacteria that improve digestion and productivity makes it impossible to change the microbiota to the desired one, given that causal relationships are unclear. The development of innovative tools and technologies will facilitate non-invasive monitoring of the gut microbiota [98].

Alternative sources of fats and emulsifiers in feeding broiler chickens. Rising prices for animal and vegetable fats are currently prompting the search for and use of alternative energy sources in the feeding of farm animals in order to reduce production costs [99, 100]. For this, components with a high exchange of energy can be used, such as soap stock, including soy, phosphatides, calcium salt concentrate of fatty acids, fatty diatomaceous earth and glycerin [101, 102]. The possibility of using glycerin is supported by a number of studies that have confirmed its safety and positive effect when included in the diet in an amount of no more than 5%. However, increasing the glycerol content of the diet above 10% has been shown to adversely affect the growth performance and meat yield of broiler chickens [103-105]. The main problem in the industrial use of these sources of fat is the technological difficulty of introducing them into animal feed and feed mixtures and the lack of large-scale studies on their use.

One of the factors limiting the use of high amounts of fat in the diet of broilers is the difficulty of its transformation, since in young birds the digestive tract is not sufficiently developed for the synthesis and secretion of bile salts and lipase, and the absorption and digestion of large amounts of dietary lipids is inefficient [45, 106]. In order to increase the absorption of lipids in the feed industry, emulsifying agents are used. Emulsifiers popular today usually consist of hydrophilic and hydrophobic components, which can reduce the surface tension of fat and water, reduce fat chylomicrons, improve emulsification and increase absorption of fats, replenish bile acid and lipase deficiency in the digestive tract of birds.

2. Main properties of emulsifiers used in animal feed

Parameter	Emulsifier type	
	hydrophobic (phospholipids)	hydrophilic (special proteins)
Fat binding	1:8	1:10
The amount of stabilized fat per 1 g of emulsifier, g	900	1500
Assimilation of fat in the body, %	Up to 90	95 and more
The composition of the emulsifier, %:		
fat	92	2
protein	2-4	92-95

The main indicator by which hydrophobic and hydrophilic emulsifiers differ is the hydrophilic-lipophilic balance (HLB) (Table 2). It shows the ratio of two opposite groups of molecules - hydrophilic and hydrophobic (lipophilic). Low HLB (lipophilic) emulsifiers are more soluble in fat, while high HLB (hydrophilic) emulsifiers are more water soluble [114]. Since animals and birds consume almost 2 times more water per day than feed, an aqueous environment is formed in their intestines, which means that a hydrophilic emulsifier is preferable both in terms of efficiency and speed of action [115]. In the presence of an emulsifier, oil droplets are distributed in oil-water emulsions, which leads to efficient digestion and absorption of fat (Table 3).

3. Emulsifiers used in the poultry industry

Emulsifiers	Main effect
	Natural
Bile acids and salts (including cholic and chenodeoxycholic acids, taurocholate)	They act as emulsifiers that disperse fat into small droplets in the aquatic environment after fat enters the gastrointestinal tract, and also increase metabolic energy, lower plasma cholesterol, and improve the absorption of dietary fats due to organic endogenous secretion [45, 65, 115, 116]
Lecithin	Reduces cholesterol and low density lipoproteins (LDLP) in blood serum; improves the digestibility of total energy, dry matter; enhancing the antioxidant effect of tocopherols (vitamin E), is able to increase the permeability of cell membranes, which provides better adsorption of fats and fat-soluble biologically active substances [17, 117]
Casein	Reduces the content of cholesterol and LDLP in the blood serum; increases digestibility [118]
	Synthetic
Lysolecithin, lysophosphatidylcholine, polyoxyethylene glycol mono- and dioleates	Conflicting results; improve the function of the liver and bile ducts; accelerate weight gain and improve feed conversion; increase growth performance and nutrient absorption [119-121]

Natural emulsifiers are produced in the animal body and include bile acids, phosphatide concentrates, and casein [122]. Amphiphilic bile salt molecules act as emulsifiers, reducing the tension of the oil-water interface [123-125]. Casein as a natural emulsifier has become an important feed additive. The main sources of casein are skimmed milk powder and soluble caseinates, which are heterogeneous protein aggregates [126]. Soy lecithin, a by-product of soybean oil processing, which serves as an emulsifier of fats, has found wide application in practice. Manufacturers produce lecithin in several forms: defatted supplements in powder form, standard (liquid) forms, and lysolecithins (hydrolyzed lecithins) [122, 127-129].

The main manufacturers of emulsifiers include the Netherlands (FRAmelco, Orffa Additives B.V.), Germany (Berg + Schmidt, Biochem GmbH - Bredol®), USA (Archer-Daniels-Midland Company - ADM), Russia (Kemin, Apex Plus, TEXBET, Cargill, Sodruzhestvo Group).

Studies of synthetic emulsifiers have yielded conflicting results [119]. Only a few publications have reported improvements in growth performance and nutrient absorption in broiler chickens [120, 121]. It has also been found [130, 131] that emulsifiers do not significantly affect the growth performance of broiler chickens. Differences in the effectiveness of exogenous emulsifiers can be explained by many factors., e.g., the type of fat, age of the bird, lipase activity and the state of the hydrophilic-lipophilic balance.

The use of emulsifiers in broiler chickens, consisting of bidistilled vegetable oleic acid and glycerol, polyethylene glycol, ricinoleate, had a positive effect on growth, feed efficiency and lipid metabolism [112].

Thereof, many studies show the positive effect of emulsifiers on growth performance and nutrient absorption [119, 132-134], as well as on the reduction of cholesterol and triglycerides in blood serum. The addition of emulsifiers to the feed improves digestion and fat absorption in birds at an early age and results in improved growth performance [44]. Emulsifiers are widely used in nutrition, increasing lipid digestibility, thereby reducing feed intake and having a positive effect on growth performance.

Thus, fats provide the body with energy and improve the productivity of the bird. When improving the diets of broiler chickens, it is important to use alternative sources of fats. One approach to increase the amount of available fats can be the use of synthetic (lysolecithin, lysophosphatidylcholine, polyoxyethylene glycol mono- and dioleates) and natural (bile acids and salts, including cholic and chenodeoxycholic, taurocholate, lecithin, casein, phosphatide concentrates) emulsifiers. Bile salts reduce the tension of the oil-water interface, activate pancreatic lipase, and prevent its denaturation. Synthetic emulsifiers improve liver and bile

duct function, accelerate weight gain and improve feed conversion, growth performance and nutrient absorption. The use of emulsifiers in the feeding of poultry makes it possible to reduce the cost of compound feed due to a smaller amount of vegetable and animal fats in the diet. This approach provides increased digestibility and absorption of nutrients both in young birds with a functionally immature digestive system and in adults and, as a result, reduces feed losses due to the intensified digestion process.

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MINERAL COMPOSITION OF COW MILK — A MINI REVIEW

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Abstract

Milk is a secretory product of the mammary glands which synthetic capacity is extremely high at the peak of lactation. Cow's milk is a generally recognized source of Ca, K, Mg, Na, P, Se, and Zn for human nutrition. About 50 mineral elements were found in milk (A.V. Skalny, 2019). Given the fact that the deficiency of micro- and macroelements is becoming global (R.L. Bailey, 2015; A.V. Skalny, 2019), interest in milk to solve this problem is increasing (M.L. Astolfi, 2020). Milk is the only source of nutrients for newborn calves. The composition and proportions of milk components are optimal for their gastrointestinal absorption, which ensures the successful survival of the species. The quantity and structural composition of macro- and microelements of milk are complementary to active anabolism and the development of the musculoskeletal system, in particular the skeleton of young animals. The purpose of our review is to summarize relevant data on micro- and macroelements in milk with regard to their biological role in cows. Comparative analysis shows a wide range of mineral content of milk. The content of Zn can vary from 3.09 to 6.48 mg/kg, Cu from 0.83 to 1.73 mg/kg (S.M. Zain, 2016; S. Kinal, 2007). This may be due to i) alimentary factors (A. Costa, 2021) which are closely related to the natural distribution of micro- and macroelements in the Earth's crust (S.M. Zain, 2016) and ii) synergistic and antagonistic interactions of elements in their assimilation (N. Bortey-Sam, 2015; A.V. Skalny, 2019). For example, an excess of potassium and calcium reduces the absorption of magnesium and phosphorus (A.V. Skalny, 2019), and a deficiency of vitamin D disrupts the absorption of Ca (W.P. Weiss, 2017). We also note the variability of the mineral content depending on the lactation period, season of the year (S.M. O'Kane, 2018; E.S. Kandinskaya, 2019), type of housing and feeding (V.S. Kozyr, 2015; I. Orzhales, 2018). Milk iodine and selenium concentration measured by inductively coupled plasma mass spectrometry were higher than indicated in previously created food composition databases (S.M. O'Kane, 2018). Thus, reliance on previously created databases should be partial when choosing milk as a source of mineral components to compensate for the identified deficiency in the human diet. Newly formed databases should be more accessible to the consumer. In addition, molecular tools should help to identify target genes and proteins as markers for assessing the level of macro- and microelements (W.P. Weiss, 2017; A. Costa, 2021), but so far little progress has been made in this research area. Precise elemental analysis of milk is necessary both to confirm its safety in terms of toxic macro- and microelements, and to solve the mineral deficiency problem.

Keywords: cow milk, mineral composition, microelements, macronutrients, fodder, blood

Milk is a complex biological fluid whose nutritional value cannot be overestimated [1]. The dairy diet plays an important role in preventing the development of chronic diseases [2, 3] such as obesity, diabetes, cancer, and some cardiovascular diseases [2, 3]. The fact of the value of milk for human nutrition (1, 3) is of constant interest in different countries [5, 6] among scientists representing different fields of science [2, 4-6].

An important area of modern milk research [1, 7] is the biochemistry of milk and dairy products [8-12]. In the work of M.L. Astolfi et al. [8] clarifies the well-known notion that cow and goat milk are important sources of macro- and

micronutrients (MMEs) [1], in particular Ca, K, Mg, Na, P, Se and Zn [8]. Plant analogues (soybean, rice, oat, almond, coconut milk) lose to animal milk in terms of the content of both MME and other substances [8]. At the same time, soy and coconut milk are good sources of magnesium, while hazelnut milk is rich in sodium ions [8]. In their review, S. Sethi et al. [9], while recognizing the competition of the rapidly developing market for plant-based milk, note that most of the alternatives to cow's milk (type of plant-based milk) lack the nutritional balance of MME required by humans. Vegetable types of milk are inferior to animal milk in a number of parameters, and their use is considered rather as a forced measure (8, 9). At the same time, functionally active components, which the manufacturer focuses on, are the only advantage of vegetable milk types that contribute to the solution of narrow, point problems [9, 10], while about 50 macro- and microelements were found in the composition of cow's milk, which are optimally balanced [1, 11].

Milk feeding is a certain strategy of evolutionary development in mammals, their care for offspring and, as a result, the survival of the species. Milk production involves not only the mammary gland, but also various organs and systems, carrier molecules [12]. As a result of the enzymatic transformation of macro- and microelements of feed [13], a composition [1, 14] is formed with the ratio and balance of milk components [15], contributing to their better intestinal absorption and utilization [13, 14]. This is important, since the nature of all interelement relationships changes from an excess or deficiency of only one micro or macro element [15, 16], which affects and disrupts the work of a number of organs and systems [17-19]. In addition, the interaction of MMEs with each other during digestion and absorption may be accompanied by synergistic and antagonistic effects [15, 16]. So, the presence of mercury and arsenic leads to a deficiency of selenium, cadmium to a deficiency of selenium and zinc, calcium to a deficiency of zinc and phosphorus, iron to a deficiency of copper and zinc, manganese to a deficiency of copper and magnesium, molybdenum to a deficiency of copper, zinc to a deficiency of copper and iron [15]. In addition, soil-forming rocks, the composition of water and air [20] in different parts of the earth's surface are often characterized by an uneven distribution of MMEs [21], which largely determines the composition of microorganisms, plants and animals [21]. Environmental dispersion of MMEs (mainly metals) during mining and use leads to serious anthropogenic pollution [17]. This affects both the health of lactating cows and the quality of milk, the composition of which is directly affected by processes occurring in the environment [17, 19, 22].

In a number of comprehensive studies [12, 13] to elucidate the metabolic mechanisms of milk production [18], for example, in the work of H. Sun et al. [23], simultaneously assessed ruminal fluid, milk, blood serum and urine. It is noted that, compared with a biochemical blood test [15, 24, 25], the analysis of the composition of milk is more indicative in assessing the mineral status of an animal [15, 23]. In addition, information on the composition of milk by MME can be used both to assess the balance of MME in the diet of dairy cows, and to inform the end consumer about the level of MME in the product.

Although many researchers [10, 15, 26] have focused on global micronutrient deficiencies [15, 26] and how to improve the efficacy and safety of providing human MME through food [15, 26, 27], little attention has been paid to informing the final consumer about the composition of food products, in particular milk, according to MME. In Russia, the amount of information provided to the consumer on the packaging of milk is strictly regulated [27] by the Federal Law of the Russian Federation No. 88, the technical regulation for milk and dairy products dated 06/13/2008, as amended on 03/22/2014 and the technical regulation

of the Customs Union 033/2013 “On the safety of milk and dairy products” from 09.05.2014. The documents prescribe the rules for labeling milk and dairy products (the content of fat, proteins, carbohydrates in the finished product as a percentage or in grams per 100 g of the product, energy value in calories or kilocalories is indicated), but it does not regulate the procedure for informing consumers about the content of MME. However, the content of MME and vitamins in the finished product is indicated only in case of enrichment, that is, their additional introduction into the finished product.

The purpose of the presented mini-review is a comprehensive analysis of data on the content of micro- and macroelements (MME) in cow's milk, taking into account the biological role of MME in the animal's body.

Under industrial conditions, the composition of MME milk [28, 29] is influenced by the main diets and feed additives used [16], the characteristics of animal metabolism [30, 31], the technology of their maintenance [32] and milking [33], productivity [34], genetic characteristics [35], season [36], age at first calving [37] and other factors [38, 39]. During the lactation dominant period, the intake of metabolites, in particular MME, into the mammary gland is a priority [12]. In case of MME deficiency, the reserves of these metabolites are mobilized from their depots in the blood, bones, muscles, liver, skin, and adipose tissue [24, 25, 40].

Calcium and phosphorus (Ca, P). These elements are necessary for the formation of calf bone tissue, since the processes of their absorption from the intestine and participation in the process of ossification proceed simultaneously [15]. Calcium is essential for transmission of nerve impulses and muscle contraction, blood clot reaction and many other procssess [24, 25]. E.S. Kandinsky et al. [41] found that the average content of Ca in raw milk of Holstein and Black-and-White cows ranged from 0.7 to 1.1 g/l. In the same study [41] a trend towards an increase in the amount of Ca in milk obtained in the autumn period was noted. It is known [12, 41] that whatever the content of Ca and P in cow's milk, their ratio tends to a ratio of 1:1-1.4:1. It is worth noting the role of phosphorus in maintaining the cellulolytic function of rumen microorganisms [42], as well as in the synthesis of microbial protein, which is extremely important for ruminants [12, 42].

In addition, since Ca and P are important constituents of casein micelles [14, 43], these chemical elements also influence the rennet coagulation ability of milk. M. Malacarne et al. [43] found that milk with the highest content of protein fractions and minerals, low concentration of chlorides and high values of titratable acidity is most suitable for cheese production [1, 11, 43]. Non-curdling milk had high pH values and low titratable acidity [43]. For the formation of casein micelles, the highest values of colloidal Ca associated with P caseins are optimal. However, excessive mineralization can lead to a decrease in the amount of casein phosphate groups available for curd formation [43].

Sodium and potassium (Na, K). Sodium [24, 25] plays an important role in maintaining the buffer capacity of the blood, activating digestive enzymes [12], regulating nerve and muscle conduction, and water-salt metabolism. Potassium maintains osmotic pressure [24, 25], regulates acid-base balance, participates in the transmission of nerve impulses, muscle contraction, transport of oxygen and carbon dioxide, in phosphorylation of creatinine, acts as an activator or cofactor of enzymatic reactions [12, 24, 25]. Using the atomic emission method, it was found that milk is 350.3-427.2 mg/kg sodium and 1159.8-1337.9 mg/kg potassium [44]. The amount of Na in milk increases during lactation, while the amount of K decreases. Subclinical mastitis and intrauterine infection increase Na concentration and decrease K concentration in milk [45].

Magnesium (Mg). This chemical element is part of and affects the activity of more than 300 enzymes [24, 25] that regulate bioenergetic processes in

the body [15], the activity of the cardiovascular system and the level of fats in the blood [25]. Extracellular magnesium is necessary for the functioning of the nervous system, the functioning of muscle tissue, and the formation of bone tissue. Hypomagnesemia is the main cause of herbal tetany.

Copper (Cu). Copper affects the activity of enzymes responsible for the oxidation of metabolites and cellular respiration [12, 15, 25]. Cu stimulates the production of sex hormones, thyroxine and neurotransmitters [24, 25], is necessary for heme synthesis, mobilization and transport of iron from the liver to the bone marrow [46] and maintenance of erythropoiesis [24, 46]. Cu ions facilitate the process of excitation transmission in the brain [15]. The role of copper is extremely important for the formation of connective tissue (cartilage, ligaments, vessel walls), since it is copper that catalyzes the formation of desmosine cross-links in collagen and elastin [12]. Cu is a component of superoxide dismutase (EC 1.15.1.1) [24], which protects cells from oxidative metabolites and is important for maintaining active phagocytosis [15]. It is known [1] that elevated concentrations of Cu in milk lead to the risk of developing oxidative processes and the appearance of a specific taste [11]. Cu deficiency leads to a decrease in the bioavailability of iron deposited in the liver [15, 46]. Over time, this causes changes in hematological parameters, which manifests itself in the form of normocytic normochromic or hypochromic microcytic anemia [46]. Primary copper deficiency in dairy cows is associated with biogeochemical provinces in which copper is less abundant, secondary is caused by excessive intake of antagonists [15], the molybdenum, sulfur, iron and zinc, which reduces the absorption of copper from the gastrointestinal tract [46].

Cobalt (Co). Co is a component of vitamin B_{12} (cobalamin), which is synthesized by rumen microorganisms [12]. The lack of Co and, as a result, vitamin $B1_2$, is especially acute during active cell proliferation, in particular in the hematopoietic system [15]. Young animals are most susceptible to Co deficiency [12] because their liver reserves of vitamin B_{12} are still low, while adults may have enough vitamin B_{12} for several months.

Iron (Fe^{2+}). Ferrous iron is a vital element [15, 24], which is involved in metabolism in the form of Fe^{2+} (in the composition of hemoglobin, myoglobin) or in the form of the Fe^{2+}/Fe^{3+} redox pair (for example, in respiratory chain enzymes). Its main function is associated with the transport of oxygen and carbon dioxide in the composition of hemoglobin [25], participation as a cofactor for the implementation of the function of electron transport chain enzymes and a number of other enzymes [25]. It should be noted that iron deficiency is rare in adult animals [46], which is associated with a significant prevalence of iron in the environment, as well as contamination of roughage with soil [12, 15], but often manifests itself in calves before weaning [46, 47] . The bioavailability of Fe from mammalian milk does not always fully satisfy the needs of offspring [12, 47]. Perhaps this is the exceptional case when, with the problem of Fe deficiency, milk should be excluded from the diet. In our opinion, this is due to the presence in milk of a large number of Fe antagonists - phosphates, manganese, nickel and selenium, since they prevent the absorption of Fe in the proximal small intestine [15, 47]. In turn, J. Joerling and K. Doll [47] attribute the low level of Fe and the development of anemia in calves mainly to their feeding with whole milk without food additives. Of course, this fact should also be taken into account when prescribing a diet for patients with anemia and using milk substitutes prepared from plant materials, as demonstrated in the work of M.L. Astolfi et al. [8]. In this case, soy milk and coconut milk will better provide the need for Fe, in addition, these products do not contain such an amount of Fe antagonists that prevent its absorption.

Iodine (I). Iodine is an essential trace mineral [15, 25] that comes from

the oceans [15]. It is necessary for the synthesis of thyroid hormones that regulate energy metabolism [24], growth and development [25], and to some extent the transmission of nerve stimuli and brain development [48, 49]. During lactation, active synthesis of thyroid hormones occurs [12], which requires a large supply of iodine to the thyroid gland [12, 48]. In addition, most dietary iodine is excreted in milk and urine [12, 15]. Milk can contain between 530.40 and 588.85 micrograms of iodine per liter [50]. It depends on the source of iodine and its content in the feed, the presence of antagonists, the choice in favor of iodine-containing preparations for the disinfection of udder teats [48, 49]. Milk and dairy products are considered important sources of iodine [48, 49], which is especially important for areas with severe iodine deficiency, the Western (Tyumen region, Bashkiria) and Eastern (Krasnoyarsk Territory, Yakutia, Tyva) Siberia, as well as in regions of Russia affected by the accident at the Chernobyl nuclear power plant, which are endemic for goiter [15, 26].

In the work of A. Costa et al. [49] on a sample of 4072 cows found that the iodine content in cow's milk is a trait that is inherited with a low frequency. Thus, breeding strategies are significantly inferior to feeding strategies in terms of the effectiveness of increasing the level of iodine in milk.

Manganese (Mn). The greatest accumulation of manganese is characteristic of the pituitary gland, liver, inorganic matrix of bone tissue, thyroid and pancreas, as well as the mammary gland during lactation [12]. Mn-superoxide dismutase (EC 1.15.1.1) [51] together with other antioxidants limits the accumulation of reactive oxygen species [3, 51]. The lack of manganese intake in the body of calves leads to slow growth, abnormalities in the development of the skeleton, which is associated with a lack of enzymes galactotransferase and glycosyltransferases (EC 2.4) [12]. The content of manganese in milk can vary from 51.24 to 101.84 μg/l [50].

Zinc (Zn). Zinc is a component of many metal-containing enzymes [24, 25] that are directly involved in the metabolism of carbohydrates, proteins, lipids, and nucleic acids [12, 15]. Examples are Zn- and Cu-containing superoxide dismutase (EC 1.15.1.1) [3, 51], carbonic anhydrase (EC 4.2.1.1), alcohol dehydrogenase (EC 1.1.1.1), RNA polymerase (EC 2.7.7) [12, 15]. Zn is involved in the regulation of cell division, reproduction of offspring, production of proteins and digestive enzymes [15, 25]. The content of zinc in milk, according to various sources, varies greatly, from 3.09 to 6.48 mg/kg [22]. The introduction of chelate forms of Zn, Cu, Mn into the diet of highly productive cows increases milk yield by 6.5% for 305 days of lactation, positively affects the level of MME and immunoglobulins in colostrum [52, 53].

Selenium (Se). Se acts as a powerful immunostimulant, participating in the regulation of the formation and activity of T-helpers, natural killers, and the phagocytic activity of neutrophils [54, 55]. Its antioxidant properties are realized through the action of enzymes containing selenocysteine (eg, the glutathione peroxidase family, EC 1.11.1.9) [29, 55]. Se is found in proteins involved in thyroid hormone metabolism, iodothyronine deiodinases (EC 1.21.99.4 and EC 1.21.99.3), and thus affects metabolism [55]. Se directly in milk prevents the accumulation of lipid peroxidation products [3, 12, 29]. Organic forms of selenium in the diet of dairy cows increased the milk content of Se up to 57.25 rg/l on day 90 of the experiment vs. 21.98-25.25 μg/l in the control [29].

Quantitative composition of macro- and microelements and its formation. In a comparative analysis of the results of studies performed in the period from 2005 to 2018 (Table 1), a strong variation of milk samples in terms of the quantitative composition of MME is obvious [22, 29, 44, 50, 52, 53, 55-58].

1. The reported content of mineral elements in cow milk

Element	Concentration
Ca	1.273-2.156 μg/ml [56], 1.06-1.20 g/kg [53], 0.81-0.85 g/kg [52], 942.2-1009.3 mg/l [57]
P	719.93-1216.55 μg/ml [56], 0.95-1.01 g/kg [53], 0.88-0.93 g/kg [52]
Na	350.3-427.2 mg/kg [44], 0.47-0.49 g/kg [52], 321.3-452.9 ml/l [57], 372-495.1 mg/l [22]
K	1159-1337 mg/kg [44], 1474-1550 mg/kg [22], 764.7-1206.5 mg/l [57]
Mg	119.7-130.9 μg/ml [56], 0.11-0.13 g/kg [53], 0.095-0.097 g/kg [52], 62.8-128.3 ml/l [57]
Fe	0.198-0.258 μg/ml [56], 1.33-4.58 mg/kg [22], 0.16-0.63 mg/l [57], 1.01-3.48 mg/kg [22]
Cu	1.49-1.73 mg/kg [53], 65.37-89.85 μg/l [50], 0.83-1.30 mg/kg [22], 0.01471-0.1420 μg/ml [56]
Zn	3.69-4.98 mg/kg [53], 3085.42-3163.68 μg/l [50], 3.09-6.48 mg/kg [22], 2.026-4.800 μg/ml [56]
Mn	0.02 μg/ml [55], 51.24-101.84 μg/l [50], 0.08 mg/kg [22], 0.0116-0.0407 μg/ml [56]
Mo	10.39-10.65 μg/l [50], 0.05-0.098 mg/kg [22]
Co	5.16-8.34 μg/l [50]
I	530.4-588.9 μg/l [50], 423.1-534.3 μg/kg [58]
Se	0.0100-0.0209 μg/ml [56], 22.44-39.20 μg/l [50], 21.98-57.25 μg/l [29]

N o t e. Reference [55] gives data for the Simmental and Holstein-Friesian breeds.

For example, Zn in milk has been reported to range from 3.09 to 6.48 mg/kg [22], Cu from 0.83 mg/kg [22] to 1.73 mg/kg [53], Ca from 0.81 g/kg [52] to 1.20 g/kg [53]. A comparative study of the MME composition in the Simmental and Holstein-Friesian rocks, carried out by R. Pilarczyk et al. [56] showed the best combination of MME in Simmental milk. It contains more Fe, Mg and less Pb and Cd [56]. In the work performed in 2016 by S.M. Zain et al. [22] Ca, Na, Fe, Zn, Mn, K, Ba and Mg have been identified as key mineral elements in a comparative study of Malaysian milk and milk from other regions of the world [22]. Such a difference is obviously related to the composition of the MMEs of soils characteristic of specific geographic zones [17].

In turn, the widespread use of MME additives in the feeding of dairy cattle [29, 52, 57-59] and anthropogenic impact [20, 21] make their own adjustments to milk autoinfection by MME composition [22], which requires regular analysis and timely introduction of the obtained results. results into current databases. S.M. O'Kane et al. [58] showed that in 2018 iodine and selenium concentrations were higher vs. current UK food composition databases.

Undoubtedly, methods of analysis play their role in determining the amount of MME in milk. W.P. Weiss [59] reports this in sufficient detail.. The development of flame spectroscopy has greatly simplified elemental analysis [59] and increased the analytical sensitivity. The late 1960s saw a boom in research on Co, Cu, Fe, Mn, Se, J, Zn, and many other elements [15, 59]. Currently, 22 MMEs are classified as subject to rationing in the preparation of the diet of dairy cattle [59]. Š. Zamberlin et al. [10] identify 20 MMEs as essential in human nutrition, and again, all of these are found in milk. The elemental composition of milk is directly related to the quality of the elemental nutrition of dairy cows. Modern methods of analysis available for monitoring MME in feed, biological fluids and tissues are atomic absorption spectrometry [52, 57], inductively coupled plasma atomic emission spectroscopy [56], inductively coupled plasma mass spectrometry [50, 60], atomic fluorescence spectrometry [29]. Their use makes it possible to multiply the speed and accuracy of studies performed simultaneously on several MMEs when monitoring diets, the elemental status of animals, and the quality of dairy products.

At present, attempts are being made to apply molecular methods to elemental analysis [59]. For example, the possibility of identifying specific genes and proteins as markers for assessing the level of MME is being considered. L.A. Sinclair et al. [60] tested the effect of Cu source on mRNA expression in the liver. However, the authors did not observe an effect ($p > 0.05$) both in the presence of copper antagonists (S and Mo) and without them.

The study of breed characteristics as a factor influencing the quantitative

composition of MME in milk [56, 57] showed that Simmental cows, in comparison with Holstein cows, had more Ca, Mn, Se and significantly more Fe and Mg ($p < 0.05$) [56]. An assessment of the content of mineral elements in the milk of six different breeds (Simmental, Holstein-Friesian, Black-and-White and Red-White, Polish Red, White-tailed) showed that the milk of Simmental cows and native breeds contained more K, Ca, Na, Mg, Zn, Fe [57]. The authors emphasize that aboriginal and Simmental breeds of cows received more green fodder in comparison with Holstein-Friesian, Black-and-White and Red-White cows [57].

Productive longevity of dairy cows and mineral metabolism. Lactation as a physiological process is associated with increased metabolic load [61-63]. Different stages of lactation are accompanied by characteristic hormonal, hematological, biochemical and immunological changes [61, 62]. According to A. Sundrum [64], the functional adaptations that occur in a lactating cow affect the entire body. So, at the peak of lactation, S.B. Kim et al. [61] observed a decrease in hemoglobin and hematocrit, an increase in the concentration of urea nitrogen and total cholesterol, an increase in the activity of alanine aminotransferase (EC 2.6.1.2) [61]. High milk productivity inevitably affects mineral metabolism [61, 62]. The most striking example of this is paresis of smooth and striated muscles caused by active excretion of Ca with milk [59, 61] in the first days and even hours [64] after calving. The early lactation period is characterized by an increased need not only for Ca (6.8-fold), but also for glucose (2.7-fold), amino acids (2-fold), fatty acids (6.8-fold) [64]. Purposeful selection to increase milk production has significantly increased the gap between the consumption of resources in the body and the ability to consume them [64, 65]. This also applies to MME. The energy expended to ensure high milk production, the animal replenishes at the expense of its own tissues [65], which leads to chronic metabolic acidosis [64, 65]. Under such conditions, even increased absorption of Ca from the intestine and mobilization of this element from the bone tissue depot does not maintain a normal level of electrolyte in the blood [64].

Therefore, it is necessary to regularly monitor the condition of lactating animals. In Table 2 we present some data on the blood MME in the of dairy cows [12, 66].

2. The reported content of mineral elements in cow blood

Element	Concentration
Ca	2.50-3.11 mmol/l [25], 1.90-2.33 g/kg [53], 2.53±0.25 mmol/l [61]
P	1.45-2.10 mmol/l [25], 1.94±0.06 mmol/l [61], 1.57-2.42 g/kg [53]
Na	139-148 mmol/l [25]
K	4.1-4.9 mmol/l [25]
Mg	0.5-1.5 mmol/l [25], 1.14-1.30 g/kg [53], 1.08±0.08 mmol/l [61]
Fe	17.9-29.0 mmol/l [25], 29.7-39.7 mmol/l [24], 26.5-29.2 μmol/l [60]
Cu	0.593-0.776 mg/l [63], 5.14-5.54 mmol/l [24], 12.8-13.5 μmol/l [60]
Zn	0.851-0.87 mg/l [63], 16.54-18.83 μmol/l [53], 10.2-10.6 μmol/l [60]
Mn	2.72-4.48 μg/l [63]
Mo	13.1-36.2 μg/l [63], 0.16-0.36 μmol/l [60]
Co	0,.884-1.111 μg/l [63]
I	48.4-83.6 μg/l [63]
Se	33.9-59.8 μg/l [63], 118.19-125.08 μg/l [29], 0.08-0.16 mg/l [55]

Obviously, blood MME composition is less variable due to homeostatic regulation [67, 68]. The determining factor in maintaining productive longevity will be how well the body copes with metabolic stress in both the short and long term [64, 65].

Should not lose sight of the fact that that in large livestock complexes with a strictly regulated type of feeding and composition of the diet, the influence of geographical location (including geochemical provinces) on the mineral status of the animal and the quality of dairy products is either minimal or completely leveled

by the introduction of appropriate mineral additives into the diet. In organic production, both the mineral metabolism of animals and the quality of the products obtained from them depend to a greater extent on local conditions (66). So, with free grazing and feeding with silage, the content of As, Cr, Fe, Pb increased in milk. Undoubtedly, in such cases, it is also possible to normalize MMEs and introduce them additionally into the diet; however, it will be more difficult to exclude and/or take into account the influence of MMEs that are characteristic of the area and supplied with feed. Alternatively, one can take into account the presence, for example, of an excess amount of iron in water, soil, feed, and introduce iron antagonists into the diet or water, preventing its excessive intake into the body. The concentrated type of feeding enriches milk with Co, Cu, I, Se, Zn [66], however, it has its own peculiarities of influencing the health of productive animals (we do not touch on these issues in this publication).

Summing up the discussion of the macro- and microelement composition of cow milk, and the factors determining its formation, we note that, in our opinion, a system of measures is needed to ensure the possibility of using milk as a product that compensates for the deficiency of human elemental nutrition. Firstly, it is necessary to form a sufficient number of healthy livestock with milk productivity corresponding to the physiological capabilities of the animal organism. Secondly, the feeding of dairy cows should be balanced according to MME, taking into account local characteristics (in particular, data on the composition of MME in soil and water, distance from enterprises that are potentially dangerous and pollute the environment with toxic MME, seasonality of conditions, macro - and the microelement composition of feed, especially imported ones). Thirdly, it is important to regularly monitor the MME in milk, indicating reliable and accurate information about the finished product.

It may also be worth paying more attention to the use of local native breeds in dairy cattle breeding, focusing on their adaptive potential for the use of MMEs.

So, cow milk is considered a generally recognized source of macro- and microelements (MME), in particular Ca, K, Mg, Na, P, Se, Zn, in human nutrition. The content of MME in milk is determined by the geochemical conditions of the region (mainly with loose housing) and feeding (taking into account the addition of MME in one form or another). The quantitative composition of milk MME is indicative in assessing the mineral status of dairy cows and the balance of MME in their diet, which is important for normalizing metabolism, ensuring productive longevity of the animal, growth and development of young animals. When choosing milk as a source of mineral elements to compensate for their deficiency in the human diet, it is possible to use previously created databases only in part of the information confirmed by modern methods of qualitative and quantitative analysis. To use cow's milk as a product of functional (elemental) human nutrition, a system of measures is needed, including the formation of an appropriate livestock, control of feeding according to the balance and composition of MME, and monitoring of MME in milk. Reliable and accurate information about the quality of the finished product, in particular, its mineral composition should be available to the consumer.

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DIFFERENTIATION OF QUAIL (*Coturnix japonica*) BREEDS BASED ON THE MORPHOLOGICAL PARAMETERS OF EGGS

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Abstract

For quail breeding in Russia, a variety of populations is characteristic in which the pedigree of individuals is often unknown. Advanced breeding with quails (*Coturnix japonica*) requires identification of breeds, including the common origin. Egg weight is a mandatory descriptive attribute when testing poultry breeds and lines for distinctness, uniformity and stability. This paper is the first to provide data for quail breeds' differentiation by egg morphological parameters and estimates the influence of the quail breed on the parameters under consideration. The work aimed to study if it is possible to discriminate quail breeds by origin based on the morphological characteristics of eggs. Eggs were collected in the breeding herds of Japanese quail (n = 240), Omsk quail (n = 720), Pharaon (n = 720), and Texas white quail (n = 360) of 238-242 days of age (Siberian Research Institute of Poultry — a Branch of the Omsk Agrarian Scientific Center, Omsk). Three successively laid eggs were taken from each laying hen. Morphological parameters of eggs included large diameter (mm), small diameter (mm), egg weight (g), absolute weight of shell (g), yolk (g), and albumen with fractionation (g), albumen height (mm), yolk height (mm), shell thickness (μm) at the sharp pole, at the equator and at the blunt pole. All parameters were assessed according to the "Methodology for anatomical cutting of carcasses and organoleptic assessment of the quality of meat and eggs" (Sergiev Posad, 2013). Statistical analysis was performed using IBM SPSS Statistics v.23 software. Breed affiliation was considered as a factor influencing the egg morphological composition. The strength of the influence of the breed (η^2) was assessed using analysis of variance (ANOVA). As breeds deviated towards meat productivity, the weight of an egg and its components increased. Purposeful selection of Japanese, Omsk, Pharaon and Texas White quails for economically useful traits led to their significant differences in egg weight (η^2 = 0.723; $p < 0.001$). Quail egg weigh depended largely on albumen mass (r = 0.897-0.911; $p < 0.01$). Distinctiveness of egg weight as a breed trait is due to breed differences in a set of morphological characteristics. These are the weight of the outer liquid layer of the albumen (η^2 = 0.642; $p < 0.001$) and outer dense layer of the albumen (η^2 = 0.796; $p < 0.001$); the height of the dense layer albumen (η^2 = 0.627; $p < 0.001$); large and small diameters (η^2 = 0.776 and η^2 = 0.852, respectively; $p < 0.001$). Breed clustering based on the morphological parameters of the eggs, corresponds to the similarity of genotypes. Our findings allow us to suggest the morphological analysis of eggs as a methodology to preliminarily discriminate the relatedness of quail breeds.

Keywords: *Coturnix japonica*, quail, egg morphological characteristics, analysis of variance, breed influence, degree of relationship

The global trend of modern quail breeding is the organization of production based on industrial technologies. As many years of experience in chicken and turkey breeding show, an increase in productivity is achieved through the use of poultry crosses namely, a complex of combined lines [1].

A feature of domestic quail breeding is the diversity of populations, often of unknown origin. This creates a breeding problem, since in order to obtain quail lines, it is necessary to study the productive qualities of the gene pool available in

Russia and to identify promising specialized (egg and meat) breeds. To increase the genetic diversity of the constituent breeds or lines, it is necessary to know the degree of their relationship, that is, to identify existing breeds and populations (2).

Egg weight is one of the obligatory features when testing poultry breeds and lines for distinctness, uniformity and stability [3, 4].

The bird egg is a complex and highly differentiated germ cell containing the nutrients necessary for the development of the embryo. Egg mass and properties are influenced by genotypic and paratypic factors [5). The body of the bird reacts to the conditions of feeding and keeping, which affects the reproductive system. Under the influence of these factors, components of the egg, the albumen, yolk and shell undergo quantitative and qualitative changes [6-8].

In addition, the components of the egg change with the age of the laying hen: the weight of the egg increases, the thickness of the shell and the quantitative ratio of albumen and yolk decrease, and their biological value regresses [9]. Such variability is within the limits of the reaction norm in accordance with the species, breed and lineage of the bird. In particular, significant differences in egg weight, weight and shell thickness between gray and white quails (*Coturnix japonica*) were found when kept at elevated temperatures [10]. As is known, the egg weight heritability coefficient is one of the highest among quantitative traits, on the basis of which it is assumed that this trait is controlled by a smaller number of genes than other economically useful traits [11-13].

From a biological point of view, a purposeful increase in live weight is a deviation from the natural genetic status of a bird species. Such a deviation entails an increase in the mass and morphological qualities of the egg [14, 15]. The tgg weight correlates with other indicators of productivity, including live weight [16, 17].

In chickens (*Gallus gallus*), a positive and significant phenotypic correlation of egg weight with its geometric dimensions (large and small diameters), as well as with the mass of albumen, yolk and shell, and negative with the shell index [18-20] were revealed. Along with the phenotypic correlation, egg weight has statistically significant ($p < 0.01$) positive genetic correlations with shell weight ($r = 0.73$), albumen weight ($r = 0.73$) and yolk weight ($r = 0.68$), with the height of the yolk ($r = 0.51$), the diameter of the yolk ($r = 0.46$) and its index ($r = 0.42$). Most of the internal qualitative characteristics of the egg varied depending on the change in its mass [21]. Comparison of the morphological features of eggs in birds of the same species, the *G. gallus*, but different breeds (Utrerana and Leghorn) showed significant interbreed differences in egg weight, its geometric dimensions, albumen and yolk weight. For both breeds, it was found that the external features of the egg (mass, small and large diameter) are related to its internal morphology [22].

The accumulated scientific data indicate that the mass of the egg is a determinant of most of its quantitative and qualitative characteristics, which determines the species, breed and lineage of the bird, but research in this area is still fragmentary. For example, it is known that egg mass evolves under the influence of selection in the direction of bird productivity. This sign is considered one of the key ones in the identification of a species, breed, and line. There is also evidence that a change in the mass of an egg entails a modification of its internal structure.

The question naturally arises: which components of the egg change to a greater extent under the influence of selection and whether these morphological characteristics can be applied to determine the degree of similarity of the breed genotype. We suggested that the construction of dendrograms according to the degree of similarity of the complex of morphological features of poultry eggs can be used as a basis for the hierarchical classification of breeds and lines and a preliminary assessment of their proximity in origin. The choice of quail (C. *japonica*)

for our study is justified by the fact that it is both an agricultural bird and a model species that is widely used in genetic studies [23-25].

In this paper, for the first time, the results of the analysis of the influence of the breed belonging of quails of different directions of productivity on the morphological characteristics of the egg are presented. It is shown that the selection of quails by live weight to a greater degree affects the geometric dimensions of the egg, its mass as a whole, the mass of its fractions, as well as the thickness of the shell. The possibility of using the morphological characteristics of eggs for preliminary identification of the origin of quail breeds was revealed.

The aim of the work was to study the differentiation of quail breeds and determine the degree of their relationship based on the distinctness of the morphological features of eggs.

Materials and methods. The study was carried out on quail eggs of the Japanese (n = 240), Omsk (n = 720), Pharaon (n = 720), Texas White (n = 360) breeds at the age of 238-242 days (SibNIIP, a branch of the Omsk ARC, Omsk, 2020). The diets and conditions of the birds were in accordance with the recommended ones [26].

Three successively laid eggs were taken from each laying hen. The morphological analysis of eggs was carried out in accordance with the methodology of the Federal Scientific Center All-Russian Research Institute of Typology of the RAS [27] for the large egg diameter (mm), small egg diameter (mm), egg weight (g), the absolute shell weight (g), yolk (g), albumen with fractionation (g), albumen height (mm), yolk height (mm), shell thickness (rm). We used an HL 100 electronic scale (A&D Company, Ltd., Japan), a TOPEX 31C628 digital caliper 150 mm, 0.02 mm (Grupa Topex, Poland), and an altimeter.

Data on albumen mass, mass of outer albumen fractions, albumen height, large and small diameters were used for hierarchical classification of the studied breeds with the construction of a dendrogram with sequential association of clusters according to the degree of similarity of the set of features. The convergence of clusters was judged by the Euclidean distance.

Statistical analysis was performed using IBM SPSS Statistics v.23 software (https://www.ibm.com/support/pages/down-loading-ibm-spss-statistics-23). Means (M) and their standard errors (±SEM) are presented for the measured values. Differences were considered statistically significant at the level of Student's t-test $p < 0.05$. The degree of trait variability was assessed by the coefficient of variation (Cv). Variation of the trait was considered weak at $Cv < 10\%$, moderate at Cv 10-20%, and significant at $Cv > 20\%$. Correlation and regression analyzes were used to measure the strength of the interdependence of indicators. Phenotypic correlation coefficients were established based on paired comparisons of traits. As a factor influencing the indicators of the morphological composition of eggs, the breed was taken into account. Analysis of variance (ANOVA) was used to assess the strength of breed influence (η^2). The influence of the factor was considered significant at the significance level of Fisher's F-test from $p < 0.05$ to $p < 0.001$.

Results. Large samples of eggs from each breed provides representative results. The egg mass within the breed varied slightly ($Cv < 10\%$). This indicates that the study was conducted on consolidated quail breeds. The direction of productivity was reliably manifested in the difference between breeds in terms of egg weight. The Japanese breed (egg direction) was inferior in egg weight to the meat-and-egg Omsk breed by 6.46%, the meat breeds Pharaon and Texas white by 11.22 and 19.74%, respectively. In turn, in the Omsk breed, the egg weight was less than in the Pharaon and Texas White breeds, by 5.09 and 11.04%, respectively. The Texas white breed, which differs from the other breeds in its greater live weight, also had the highest egg weight (Table 1).

1. Morphological parameters of eggs in different quail (*Coturnix japonica*) breeds (Siberian Research Institute of Poultry — a Branch of the Omsk Agrarian Scientific Center, Omsk, 2020)

Parameter	Japanese quail (n = 240)		Omsk quail (n = 720)		Pharaon (n = 720)		Texas White quail (n = 360)	
	M±SEM	Cv, %	M±SEM	Cv, %	M±SEM	Cv, %	M±SEM	Cv, %
Absolute mass, g:								
eggs	12.74±0.111^{bcd}	7,66	13,62±0,075^{acd}	7,07	14,35±0,079^{abd}	4,53	15,31±0,068^{abc}	8,61
shells	1.28±0.013^{bcd}	9,93	1,32±0,007^{acd}	9,40	1,36±0,013^{abd}	7,92	1,47±0,007^{abc}	9,33
albumen	7.41±0.049^{bcd}	8,38	8,03±0,060^{acd}	8,12	8,69±0,060^{abd}	6,05	9,18±0,046^{abc}	8,78
yolk	4.05±0.042^{bcd}	11,39	4,27±0,029^{acd}	10,77	4,30±0,040^{abd}	7,83	4,66±0,028^{abc}	11,76
Absolute mass of albumen layers, g:								
external liquid	2.84±0.038^{bcd}	22,42	3,02±0,031^{ad}	20,75	3,09±0,089^{ad}	26,67	3,33±0,041^{abc}	23,85
outer dense	3.10±0.113^{bcd}	12,08	3,44±0,051^{acd}	15,76	3,60±0,060^{abd}	15,38	3,83±0,042^{abc}	11,43
internal liquid	1.34±0.026^{bcd}	33,44	1,42±0,025^{acd}	34,96	1,84±0,092^{ab}	40,38	1,83±0,019^{ab}	31,30
internal dense	0.13±0.003^{bcd}	49,16	0,15±0,006^{ad}	41,34	0,16±0,009^{ad}	44,03	0,19±0,011^{abc}	47,86
Egg diameter, mm:								
large	34.73±0.093^{bcd}	4,91	35,11±0,046^{acd}	3,51	35,86±0,200^{abd}	4,66	37,19±0,084^{abc}	4,40
small	25.23±0.100^{bcd}	3,44	25,62±0,079^{acd}	4,52	26,51±0,111^{abd}	3,02	27,32±0,079^{abc}	5,60
Shell index, %	74.54±0.531^{b}	6,28	72,97±0,046^{acd}	4,81	73,93±0,420^{b}	4,45	73,46±0,244^{b}	6,45
Height, microns:								
outer dense albumen	510±7.5^{bcd}	21,27	491±5,7^{ad}	29,53	488±4,9^{ad}	24,85	457±5,3^{abc}	22,69
yolk	1111±6.4^{bcd}	5,02	1136±5,3^{acd}	9,55	1162±5,2^{abd}	7,18	1231±4,7^{abc}	7,57
Shell thickness, microns	186±1.1^{bcd}	7,43	192±1,0^{acd}	10,86	198±1,1^{abd}	8,83	202±0,9^{abc}	9,63

N o t e. The age of laying hens is 238-242 days. Latin letters ^{a}, ^{b}, ^{c} and ^{d} indicate significant differences ($p < 0.05$) with the breeds (Japanese, Omsk, Pharaon and Texas white, respectively).

Differences between breeds in egg mass were reflected in the geometric dimensions of the eggs. The heavier breeds of quails significantly exceeded the light breeds in terms of large and small egg diameters. In the Omsk breed, the large and small egg diameters were larger than in the Japanese breed by 1.08 and 1.52%, in the Pharaon breed vs. the Omsk breed by 2.09 and 3.36%, respectively, in the Texas White breed vs. the Pharaon breed by 3.58 and 2.96% (see Table 1).

There was a tendency for a more noticeable difference between breeds in the small diameter of the egg than in the large one. During statistical processing of the entire data array for four breeds, the determination of the regression coefficient for the mass and diameters of eggs showed that with an increase in egg mass by 1 g, the large egg diameter increases by 0.318 mm, the small one - by 0.363 mm ($p < 0.001$). According to the shell index, only a significant difference between the Omsk breed and the other three breeds was established (see Table 1).

The greatest differences between the breeds were found in albumen mass. Thus, the Japanese breed was inferior to the Omsk, Pharaon and Texas White breeds by 7.72; 14.73 and 19.28%, respectively; Omsk breed to the Pharaon and Texas White breeds by 7.59 and 12.53%; Pharaon to Texas White breed by 5.34%. As the egg mass increased, the difference between the breeds in absolute albumen mass decreased (see Table 1).

Differences between breeds in terms of shell and yolk weight were significant and comparable. In terms of yolk mass, the difference between the Japanese and other breeds was 5.15, 5.81 and 13.09%, respectively, between the Omsk breed and the Pharaon and Texas White breeds 0.70 and 8.37%, between the Pharaon and Texas White breeds 7.73%. Between the Omsk and Pharaon breeds, the difference in yolk weight was unreliable. In the Japanese breed, the weight of the egg shell was less than in the Omsk, Pharaon and Texas White breeds by 3.035.88 and 12.93%, respectively, in the Omsk breed vs. the Pharaon and Texas White breeds by 7.59 and 12.53%, in the Pharaon breed vs. the Texas White by 7.48%.

The data obtained are consistent with the results of A. Taskin et al. [28], who showed that quails, divided into groups by live weight, had differences in egg weight and morphological features, while consistently transmitting these differences to offspring.

In our study, there was a trend towards an increase in shell thickness as breeds deviated towards meat productivity (see Table 1). Omsk breed in terms of egg weight, shell and yolk was more different from the Japanese breed than from the Pharaon breed. This can be explained by the fact that the original population of the Omsk breed was created on the basis of the genetic material of the indicated breeds, but had a $^3/_4$ blood ratio for the Pharaon breed.

The variability of egg components in quails of all breeds was low (see Table 1). The coefficients of variation in the mass of the shell and albumen were low while of the yolk, it was medium but close to 10%. Of interest is the coefficient of variation in the mass of egg albumen which is less than the variation coefficients for the mass of the shell and yolk. In our previous studies on egg and meat chicken crosses, it was found that the coefficients of variation in egg albumen mass exceeded those for the shell and yolk [29, 30].

The weight of the egg depended to a greater extent on the weight of the albumen (for the Japanese, Omsk, Pharaon and Texas white breeds, $r_ж = 0.902$, $r_о = 0.903$, $r_р = 0.897$, $r_т = 0.911$; respectively, $p < 0.01$) than on the mass of the shell ($r_ж = 0.699$, $r_о = 0.494$, $r_р = 0.557$, $r_т = 0.535$; $p < 0.01$) and yolk weight ($r_ж = 0.610$, $r_о = 0.580$, $r_р = 0.702$, $r_т = 0.758$; $p < 0.01$). The indicators of albumen, yolk and shell mass positively correlated with each other. The correlation coefficients were moderate or weak, but significant ($p < 0.01$): for albumen and shell mass $r_ж = 0.326$, $r_о = 0.273$, $r_р = 0.405$, $r_т = 0.393$; for the mass of albumen

and yolk $r_ж = 0.213$. $r_o = 0.182$. $r_p = 0.332$. $r_т = 0.436$; for the weight of the yolk and shell $r_ж = 0.300$. $r_o = 0.475$. $r_p = 0.345$. $r_т = 0.379$.

The egg albumen consists of four layers, two outer and two inner. The main share of egg albumen was accounted for by its outer fractions, which accounted for 80% in egg and meat-egg breeds (Japanese and Omsk), and somewhat less in meat breeds (Pharaon and Texas White), 77-78%. Significant differences between all breeds appeared only in the mass of the outer dense albumen. Thus, in the Texas White breed, the albumen content in this layer in the egg was higher than in the Pharaon (by 6.01%), Omsk (by 10.18%), and Japanese (by 19.06%) breeds. In turn, the mass of this albumen layer in the Pharaon breed was greater than in the Omsk (by 4.44%) and Japanese (by 13.89%) breeds. In the Omsk breed, the mass of internal dense albumen was greater than in the Japanese breed (by 9.88%). In terms of the mass of the outer dense layer, the Omsk breed approached the Pharaon breed to a greater extent than the Japanese breed. This trend is consistent with the differences we found between the studied breeds in terms of albumen mass.

In all the breeds studied, we established a positive correlation between egg size and albumen mass and its external fractions. Correlation coefficient values ranged from low to moderate, but were significant ($p < 0.01$). For a large egg diameter, *r* with albumen mass was from 0.345 to 0.613, with the mass of the outer liquid layer from 0.259 to 0.289, with the mass of the outer dense layer from 0.264 to 0.299. For a small egg diameter, *r* with albumen mass was from 0.527 to 0.703, with the mass of the outer liquid layer from 0.304 to 0.571, with the mass of the outer dense layer from 0.285 to 0.315.

The incubation quality of eggs is characterized by albumen index and Howe units. In accordance with the mathematical calculation formulas, these indices are directly related to the height of the dense outer layer of the albumen. A comparative analysis of the eggs made it possible to establish the following regularity: as the breeds deviated towards meat productivity, the height of the outer dense albumen layer decreased. This is consistent with the data of P.P. Tsarenko [23] that the eggs of heavy breeds of chickens, ducks, turkeys and geese have a slightly lower albumen height compared to light breeds.

For the rest of the albumen fractions, we have identified the following trends. The Japanese breed was significantly inferior to the other three in terms of the mass of external and internal liquid and internal dense albumen. The mass of these albumen layers in the Omsk breed was significantly less than in the Texas White breed. In terms of the mass of internal liquid albumen, the Pharaon and Texas White breeds were close to each other, in terms of the mass of the external liquid and internal dense albumen, the Omsk and Pharaon breeds were close. In all breeds, we established a negative significant ($p < 0.01$) correlation dependence of moderate strength between the mass of the liquid and dense outer layers of the albumen ($r_ж = 0.416$, $r_o = 0.300$, $r_p = 0.605$, $r_т = 0.472$,).

The outer layers of the albumen had significantly less variability compared to the inner layers. The smallest variability in all four quail breeds was noted in relation to the outer dense layer of albumen, which was at an average level, approaching the lower limit in the Japanese and Texas White breeds.

The outer albumen fractions, compared to the inner ones, had a closer correlation with both the total albumen mass and the egg mass. The degree of correlation between the mass of albumen and eggs with the mass of the outer liquid albumen was moderate, with the mass of the outer dense - moderate and high. Correlation coefficients for internal albumen fractions turned out to be low and, in most cases, unreliable (Table 2).

2. Correlation coefficients (*r*) between the variables of egg morphological parameters in different quail (*Coturnix japonica*) breeds (Siberian Research Institute of Poultry — a Branch of the Omsk Agrarian Scientific Center, Omsk, 2020)

Albumen layer	Japanese quail (n = 240)		Omsk quail (n = 720)		Pharaon (n = 720)		Texas White quail (n = 360)	
	egg	albumen	egg	albumen	egg	albumen	egg	albumen
Outer:								
liquid	0,219[b]	0,389[b]	0,206[b]	0,400[b]	0,204[b]	0,203[b]	0,309[b]	0,312[b]
dense	0,379[b]	0,659[b]	0,677[b]	0,841[b]	0,244[b]	0,492[b]	0,381[b]	0,439[b]
Internal:								
liquid	0,013	0,153[b]	0,257[b]	0,227[b]	0,152[b]	0,189[b]	0,199[b]	0,145[b]
dense	0,016	0,127[a]	0,007	0,145[b]	0,005	0,101[a]	0,046	0,123[a]

N o t e. The age of laying hens is 238-242 days. Latin letters [a] and [b] indicate statistically significant correlations at $p < 0.05$ and и $p < 0.01$, respectively.

3. The influence of the quail (*Coturnix japonica*) breed on egg morphological parameters (Siberian Research Institute of Poultry — a Branch of the Omsk Agrarian Scientific Center, Omsk, 2020)

Parameter	η^2	F	p
Absolute mass:			
eggs	0.723	10.908	0.000
shells	0.354	8.280	0.000
albumen	0.633	10.958	0.000
yolk	0.496	7.759	
Absolute mass of albumen layers:			
external liquid	0.642	7.682	0.000
outer dense	0.796	12.418	0.000
internal liquid	0.498	7.624	0.000
internal dense	0.058	2.260	0.086
Height of outer dense albumen, microns	0.627	7.774	0.000
Egg diameter:			
large	0.776	11.434	0.000
small	0.852	18.788	0.000
Shell index	0.721	8.704	0.000
Shell index, %	0.291	8.455	0.000

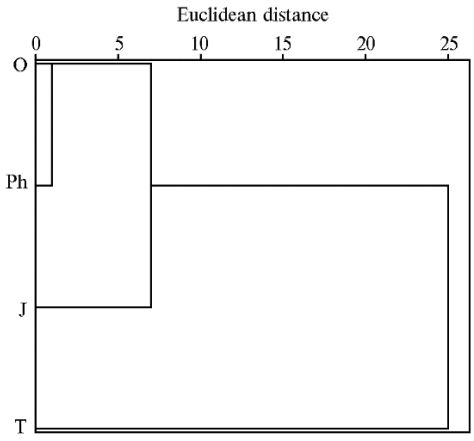
N o t e. The age of laying hens is 238-242 days.

We used one-way analysis of variance to establish the reliability of the distinguishability of quail breeds by the morphological structure of eggs. For all the studied morphological characteristics of the egg (except for the mass of the inner dense albumen layer), the influence of the breed factor was statistically significant ($p < 0.001$) (Table 3).

Species and breeds are one of the main factors limiting the variability of egg mass [31]. In our study, the influence of the breed factor was high. In the constituent parts of the egg, the studied factor determined the mass of the albumen to a greater extent than the mass of the shell and yolk. The genotypic effect on the variability of the masses of the layers turned out to be unequal. The influence of the breed to a greater extent determined the variability of the mass of the outer fractions of the albumen and the height of its outer dense layer. In terms of magnitude, this variability turned out to be comparable to that for the mass of a whole egg. The variability of the inner layers of the albumen, depending on the breed, turned out to be significantly less, although it remained significant. The geometric dimensions of the eggs were also determined by the breed of quails. The effect of the breed factor was stronger for small egg diameter than for large diameter. The shell thickness variability controlled by the breed was low (see Table 3).

In general, it can be stated that the degree of genotypic variability comparable to that for egg weight was characterized by the following morphological features: albumen weight, weight of its outer fractions, large and small diameter of the egg, height of the outer dense albumen layer. Based on the analysis of the

share of influence of the breed on the variability of the morphological parameters of the egg, we chose these features for the hierarchical classification of quail breeds (Fig.).



Hierarchical analysis of egg morphological parameters of quail (*Coturnix japonica*) breeds: J — Japanese quail, O — Omsk quail, Ph — Pharaon, T — Texas White quail (Siberian Research Institute of Poultry — a Branch of the Omsk Agrarian Scientific Center, Omsk, 2020).

The studied breeds have a common origin. The Omsk quails are $^1/_4$ the Japanese breed and $^3/_4$ the Pharaon breed. The Pharaon breed was bred due to many years of selection with Japanese quails. The Japanese breed was also involvrd in the creation of the Texas White breed [1]. The process of data aggregation went in the direction of reducing the degree of breeds' relatedness. At the first stage, the Pharaon and Omsk breeds were grouped into one cluster. At the next cluster of both breeds merged with the Japanese breed, then at a great distance the three breeds merged with the Texas White breed.

Our study shows that the creation of quail breeds specialized in economically useful traits leads to a change in the weight of the egg and its morphological features. In meat breeds, in comparison with egg and meat-egg breeds, the mass of the egg, its geometric dimensions, the mass of the shell, albumen and yolk are greater. This is consistent with reports that bird breeds have egg-specific morphological features [32-35]. A number of authors indicate that there are statistically significant phenotypic and genetic correlations between the mass of an egg and its internal structure [36-38].

N. Vali [39] gives data on the coefficients of heritability in quails, the: 0.32-0.65 for egg weight, 0.35 for albumen weight, and 0.25-0.60 for shell weight. The proportions of influence of breeds on the corresponding morphological characters established by us were close to these values.

Our study complements the known data with a more in-depth analysis of the influence of the quail breed on such morphological features of eggs as the mass of outer and inner albumen fractions, the height of the outer dense albumen layer. Summarizing the available scientific publications and the results of our own research, we put forward a hypothesis about the possibility of using the morphological composition of eggs to identify quail breeds.

The method of hierarchical classification for determining the degree of relationship is often used in biology. B.S. Iolchiev et al. [40] used body conformation indicators for the preliminary identification of hybrids of argali and domestic sheep with different proportions of blood. D. Deeming [41] using cluster analysis revealed a high similarity of the amino acid composition of egg albumen in duck and goose, as well as in quail, turkey and chicken. In our study, in order to identify quail breeds with a common origin, we applied a hierarchical cluster analysis based on the morphological characteristics of eggs. The breeds were grouped into clusters according to the degree of genotype similarity (see Fig.). The clustering step distance increased as the bloodiness decreased.

Thus, targeted selection of Japanese, Omsk, Pharaoh and Texas White quails for economically useful traits led to significant differences between breeds

in egg weight ($\eta^2 = 0.723$; $p < 0.001$). The mass of quail eggs depended to a greater extent on the mass of alnumen (*r* from 0.897 to 0.911; $p < 0.01$). Distinctiveness of egg weight as a breed trait is due to breed differences in such morphological features as the mass of the outer liquid layer of the albumen ($\eta^2 = 0.642$; $p < 0.001$), the outer dense layer of the albumen ($\eta^2 = 0.796$, $p < 0.001$), the height of the dense layer albumen ($\eta^2 = 0.627$, $p < 0.001$), large and small egg diameter ($\eta^2 = 0.776$ and $\eta^2 = 0.852$, respectively; $p < 0.001$). The breeds were grouped into clusters according to the degree of genotype similarity. Our findings allow us to propose the morphological analysis of eggs for the differentiation of quail breeds.

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PROSPECTS FOR THE APPLICATION OF EXTRACELLULAR PROTEINASES OF MICROMYCETE *Aspergillus ochraceus* IN THE TREATMENT OF MASTITIS IN COWS

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Abstract

Cow mastitis, the etiological agents of which are pathogenic and opportunistic microorganisms, is considered one of the diseases that cause significant economic damage to dairy farming with a risk to the health of dairy consumers. With bacterial infections of the mammary gland in cows, the formation of protein exudates occurs. Proteinases that reduce the severity of the inflammatory response are included in the treatment regimens for various pathologies in medicine, but this practice is limited in veterinary medicine. In this work, we proved that a drug based on *Aspergillus ochraceus* proteinase increases the effectiveness of antibiotic therapy for cow mastitis. The aim of the study was to experimentally evaluate the possibility of using *A. ochraceus* BKM F-4104D micromycete proteinase in veterinary medicine. The studies were performed on lactating black-and-white cows (*Bos taurus*) with milk productivity for the previous lactation of 6900-7110 kg, which were divided into two groups (n = 16 each). All experimental animals were intracisternally injected with an anti-mastitis drug based on the beta-lactam antibiotic amoxicillin, clavulonic acid and prednisolone (ACP) at a dose of 3.0 g (one syringe dispenser) once per day for 3-4 days until the disappearance of clinical signs of mastitis. In the second group, 12 hours before the use of the ACP preparation, animals were additionally intracisternally administered a drug with the working name PAO-1. PAO-1 is an oil suspension containing 4.0 g (syringe-dosing device) extracellular proteinase of the micromycete *A. ochraceus* BKM F-4104D as an active ingredient. This proteinase is able to degrade heterogeneous protein substrates in a wide range of environmental conditions, which can increase the effectiveness of the etiotropic therapy of bovine mastitis. The condition of the breast, morpho-biochemical status were evaluated before treatment, after treatment-and 7-10 days after the end of the administration of the drugs. It was found that the combined use of an antimicrobial agent and a preparation based on the proteinase of the micromycete *A. ochraceus* BKM F-4104D was accompanied by the recovery of 93.8 % of cows with clinical mastitis, which is 12.5 % higher ($p < 0.05$) than when using only the antimastitis drug ACP. Recovery of animals was characterized by normalization of morpho-biochemical status. The amount of β-globulins increased by 14.2 % ($p < 0.05$), triglycerides by 31.4 % ($p < 0.05$), creatinine decreased by 24.2 % ($p < 0.05$) compared to animals treated with ACP therapy. Endogenous intoxication and lipid peroxidation decreased, e.g., the concentration of malonic dialdehyde decreased by 40.4 % ($p < 0.00005$), medium-weight molecules by 46.3 % ($p < 0.00005$), NOx 3.6-fold ($p < 0.00005$), endogenous intoxication index was 33.7 % lower ($p < 0.005$) compared to sick animals. The activity of the enzymatic and non-enzymatic components of the antioxidant defense increased, the concentration of vitamin A increased by 36.8 % ($p < 0.005$), vitamin E by 32.8 % ($p < 0.05$), vitamin C by 39.2 % ($p < 0.005$), catalase activity increased by 39.4 % ($p < 0.005$), glutathione peroxidase activity increased by 30.6 % ($p < 0.005$) compared to sick animals. Optimization of protein, lipid and mineral metabolism occurred. After the end of the therapeutic course, the number of somatic cells and their

composition in the secret of the udder was normalized and there was no pathogenic microflora, which confirmes the complete clinical recovery of the cows. Our findings indicate that *Aspergillus ochraceus* BKM F-4104D micromycete proteinase which has high anticoagulant and fibrinolytic activity, can be very promising for the creation of domestically produced enzymatic veterinary drugs competitive in the world market.

Keywords: proteinases, *Aspergillus ochraceus* BKM F-4104D, PAO-1 drug, mastitis, cattle, enzyme preparations, combination therapy

In many countries, cow mastitis is considered a common pathology that leads to significant economic losses in dairy farming [1-5]. Among the main causes of mastitis are the effects of pathogenic and opportunistic microorganisms [6-9].

Most drugs for the treatment of mastitis contain antibiotics as an active ingredient. However, their use has led to the emergence of drug-resistant strains of microorganisms [5], the persistence of biofilm resistance of *Staphylococcus aureus* to antibiotics [1], and the isolation of multidrug-resistant microbial isolates from cows with clinical mastitis [9, 10]. In addition, antibiotics can have a toxic effect on the fetus during pregnancy, cause allergic reactions, dysbacteriosis and immunodeficiency in young animals [11]. Therefore, reducing the frequency and/or frequency of antibiotic use is a task that requires more and more attention. It can be solved by increasing the effectiveness of antibiotic therapy [12, 13].

The consequences of bacterial infections of the reproductive organs and mammary gland in cows include the formation of blood clots and protein exudates, which plays a significant role in the development of infertility and a decrease in milk production [14, 15]. To solve the problem in therapeutic practice, drugs containing proteinases are successfully used, which reduce the severity of the inflammatory response, which is manifested in the normalization of microcirculation and the reduction of edema, and the improvement of tissue trophism [16]. This is due to the proven presence of fibrinolytic, immunomodulatory and other effects in proteinases [16, 17].

The fibrillar proteins fibrin and collagen are difficult to hydrolyze substrates that require proteolytic enzymes with specific activity for their cleavage [18]. In clinical practice, preparations based on such proteinases are used to eliminate blood clots, purulent masses, and necrotic tissues in the affected area [18]. According to a number of studies, among such enzymes, proteinases of micromycetes, especially representatives of the genus Aspergillus, which produce several types of enzymes with a directed action of limited proteolysis, stand out with high activity and efficiency [18-20]. In this regard, the proteases of filamentous fungi, which can effectively cleave fibrin, collagen, elastin, keratin, and other fibrillar proteins, can be of great practical importance for veterinary medicine.

It is also known that many processes of homeostasis are regulated by various forms of proteases, the activity of which, in turn, is in a complex interdependence with the action of the oxidant and antioxidant systems [17].

Proteolytic enzymes, having a range of biological effects, can affect the condition of animals during antibiotic therapy of mastitis, in particular, their hematological status and sanitary characteristics of the udder secretion. However, such studies are not well represented in the scientific literature.

In our work, we compared the results of a morpho-biochemical blood test, determination of markers of the activity of the lipid peroxidation-antioxidant protection system, microbiological and morphobiochemical analysis of mammary gland secretion in two treatment regimens and proved that a drug based on *Aspergillus ochraceus* proteinase, which is able to cleave heterogeneous protein substrates in a wide range of environmental conditions, increases the effectiveness of antibiotic therapy for mastitis in cows.

The purpose of our study is an experimental and clinical evaluation of the

possibility of using Aspergillus ochraceus BKM F-4104D micromycete proteinase to increase the effectiveness of treatment of cow mastitis in combination with etiotropic agents.

Materials and methods. All procedures performed in the study were previously reviewed and approved at a meeting of the bioethical commission of the ARVRIPP&T RAS and corresponded to type A (manipulations with animals that do not cause pain or cause minimal pain and discomfort). The personnel participating in the experiment were trained in the correct and humane treatment of animals in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (ETS 123, Strasbourg, 1986); Directive 2010/63/EU of the European Parliament and of the Council of the European Union of 22 September 2010 on the protection of animals used for scientific purposes; Guide for the Care and Use of Laboratory Animals, Washington (DC) (1996); Code of Ethics of a Veterinarian of the Russian Federation, recommended at the XIII Moscow International Veterinary Congress of the Association of Practitioners of Veterinary Doctors of Russia (2005).

In the experiment, we used a drug with the provisional name PAO-1 (developed at the ARVRIPP&T), which contains recombinant *Aspergillus ochraceus* BKM F-4104D micromycete proteinase with anticoagulant and fibrinolytic properties. Proteinase was expressed in *Escherichia coli* BL 21 strain (DE3) in soluble form and inclusion bodies and subsequently in high yield was obtained using refolding and affinity chromatography on Ni-NTA-agarose [21].

Lactating cows (*Bos taurus*) of black-motley breed of tie-down housing (n = 32; Agrotech-Garant LLC Rostoshinsky, Ertilsky District, Voronezh Province, February-March 2021) with milk production for the previous lactation of 6900-7110 kg divided into two groups of 16 cows each to determine the therapeutic efficacy of drugs. All cows (groups I and II) were intracisternally injected with an anti-mastitis drug based on the β-lactam antibiotic amoxicillin, clavulonic acid and prednisolone (ACP) at a dose of 3.0 g (one syringe dispenser) 1 time per day for 3-4 days until disappearance of clinical signs of mastitis. Animals from group II, 12 hours before the use of the ACP preparation, were additionally injected once intracisternally with the drug PAO-1 (oil suspension, 4.0 g, one dosing syringe).

The clinical state of the mammary gland was assessed before treatment, during treatment (3-4 days) and 7-10 days after the administration of the last therapeutic dose of ACP. In addition, blood was taken from 5 cows from each group from the jugular vein into Green Vac-Tube vacuum tubes (Green Cross, South Korea) for laboratory studies of morpho-biochemical parameters (the amount of total protein and its fractions, urea, creatinine, glucose, total lipids, triglycerides, cholesterol, activity of alkaline phosphatase ALP, aspartate aminotransferase AsAT, alanine aminotransferase AlAT, γ-glutamyl transferase GGT, total calcium, inorganic phosphorus, copper, zinc, manganese, magnesium, selenium, protein-bound iodine PBI) and activity markers of the lipid peroxidation, i.e., the antioxidant defense system (LPO-AOD) (concentrations of malonic dialdehyde MDA, activity of glutathione peroxidase GPO and catalase Cat, content of vitamins A, E, C, medium-weight molecules MWM, stable metabolites of nitric oxide NOx) [22, 23]. Morphological studies were performed on an ABX Micros 60 hematological analyzer (HORIBA ABX SAS, France). Biochemical studies were performed on a Hitachi-902 analyzer (Hitachi, Japan).

The secret of the udder from cows with catarrhal mastitis was collected according to the instructions [24, 25]. Bacteriological studies of the udder secretion (in 5 animals in the group), the study of the cultural, morphological and biochemical properties of the isolated microorganisms were carried out in accordance with the recommendations [25]; the number of somatic cells was determined

according to GOST 23453-2014 using a somatic cell analyzer in milk DCC (DeLaval, Sweden) in accordance with the instructions for the device [26], the composition of the leukocyte population was determined by microscopy of udder secretion preparations stained according to Romanovsky-Giemsa (microscope Bioscope-1, LOMO, Russia). The content of circulating immune complexes (CIC) in udder secretion was determined by PEG precipitation using a UV 1800 spectrophotometer (Shimadzu, Japan) [27].

Statistical data processing was performed using the MedCalc 15.8 program (MedCalc Software, Ltd., Belgium). The mean values (*M*) and standard errors of the means (±SEM) were determined. Statistical significance was assessed using the nonparametric Mann-Whitney U-test, differences were considered statistically significant at $p < 0.05$.

Results. In group I of cows with mastitis, when using only the antimicrobial drug ACP, 81.3% of animals recovered (when taken into account by udder shares, this figure was 83.3%). In group II, with the combined use of antimicrobial and enzyme preparations, the therapeutic efficacy was 93.8 and 94.7%, respectively. Therefore, the additional use of the enzyme preparation significantly ($p < 0.05$) increases the therapeutic effect by 12.5%.

The positive effect of the complex use of antimicrobial and enzyme preparations was confirmed by our results of a study of the morphological and biochemical characteristics of the blood of animals. The recovery of cows treated with the antimicrobial agent ACP (Table 1) was accompanied by a decrease in the content of -globulins in the blood by 31.4% ($p < 0.005$), creatinine by 9.9%, ALP activity by 37.7% ($p \leq 0.00001$), AsAT by 17.6% ($p < 0.05$), AlAT by 47.2% ($p \leq 0.00002$), GGT by 8.9% with an increase in albumin content by 24.1% ($p < 0.05$), triglycerides by 1.5 times ($p \leq 0.00001$), the amount of total calcium by 48.4% ($p < 0.005$) compared to the state before the start of treatment. The combined use of ACP and PAO-1 in the treatment of clinical mastitis led to a more pronounced increase in the albumin fraction of the protein (by 25.5%, $p < 0.05$), β-globulins by 19.8% ($p < 0.05$), the amount of triglycerides by 2 times ($p < 0.00005$), cholesterol by 15.1%, total calcium by 54.3% ($p < 0.005$), copper by 12.8% ($p < 0.05$), zinc by 13.2% ($p < 0.05$) with a decrease in the α-globulin fraction of the protein by 38.0% ($p < 0.005$), creatinine by 31.7% ($p < 0.005$), ALP activity by 38.7% ($p \leq 0.00005$), AsAT by 27.3% ($p < 0.005$), AlAT by 53.1% ($p < 0.00005$), GGT by 19.8% ($p < 0.05$). In the group of animals that received the antimicrobial drug in combination with PAO-1, an increase in the amount of β-globulins by 14.2% ($p < 0.05$), triglycerides by 31.4% ($p < 0.05$) occurred with a decrease in the content of creatinine by 24.2% ($p < 0.05$) relative to the indicators in the group of animals that were administered only an antibacterial drug.

An analysis of the prooxidant-antioxidant status of cows showed that mastitis occurs against the background of intensified lipid peroxidation, which indicates the presence of oxidative stress, endogenous intoxication also increases and AOD decreases. This was indicated by high concentrations of MDA, MWM, NOx and the value of the endogenous intoxication index (EII) at low values for the enzymatic and nonenzymatic AOD units (Table 2).

After a course of antibiotic therapy with the use of ACP, the recovery of animals was accompanied by a decrease in the blood index of endogenous intoxication by 16.0%, the content of medium-weight molecules by 23.7% ($p < 0.00005$), malondialdehyde by 25.4% ($p < 0.05$), NOx by 57.7% ($p < 0.00005$) with an increase in the content of vitamin E by 14.6%, vitamin C by 11.0%, vitamin A by 23.7% ($p < 0.05$), GPO activity by 14.9%, Cat by 18.2%.

1. Morphobiochemical blood parameters in black-motley cows (*Bos taurus)* with mastitis before and after treatment with antibiotic and enzyme preparation (*M*±SEM, Agrotech-Garant LLC Rostoshinsky, Ertilsky District, Voronezh Province, February-March 2021)

Parameter	Reference values	Before treatment (a basal level)	After treatment	
			group I (n = 5)	group II (n = 5)
Erythrocytes, $\times 10^{12}$/l	4.8-7.0	5.72±0.37	5.81±0.41	5.79±0.45
Hemoglobin, g/l	90-140	116.3±6.1	115.6±7.2	120.4±7.9
Total protein, g/l	72-86	78.4±4.00	80.3±4.80	82.4±4.11
Albumins, %	38-50	37.7±2.30	46.8±3.12*	47.3±2.84*
α-Globulins, %	12-20	24.5±1.92	16.8±0.81**	15.2±1.30**
β-Globulins, %	10-16	10.1±0.41	10.6±0.31	12.1±0.38**▲
γ-Globulins, %	25-40	27.7±1.11	25.8±0.90	25.4±1.13
Urea, mmol/l	3.0-6.7	3.82±0.21	3.94±0.19	3.99±0.22
Creatinine, μmol/l	40-180	119.4±7.7	107.6±5.0	81.6±4.60**▲
Glucose, mmol/l	2.1-3.8	3.12±0.12	3.45±0.22	3.51±0.13
Total lipids, g/l	1.4-5.6	4.24±0.21	4.31±0.19	4.61±0.18
Triglycerides, mmol/l	0.25-0.70	0.34±0.01	0.51±0.01***	0.67±0.02***▲
Cholesterol, mmol/l	1.3-5.5	3.45±0.22	3.56±0.19	3.97±0.22
ALP, E/l	100-200	289.6±5.5	180.3±4.1***	177.5±3.9***
AsAT, U/l	10-50	85.4±4.11	70.4±3.90*	62.1±3.6**
AlAT, U/l	10-30	38.6±1.64	20.4±1.53***	18.1±1.42***
GGT, U/l	7-15	19.2±1.1	17.5±1.34	15.4±1.09*
Calcium total, mmol/l	2.25-3.15	1.88±0.12	2.79±0.15**	2.90±0.19**
Phosphorus inorganic, mmol/l	1.45-2.3	1.72±0.11	1.80±0.17	1.74±0.11
Copper, μmol/l	12.6-30.0	14.8±0.58	15.1±0.71	16.7±0.44*
Zinc, μmol/l	46.2-77.0	44.7±3.1	48.6±3.9	50.6±1.15*
Manganese, μmol/l	2.7-4.6	2.82±0.21	2.99±0.17	3.19±0.16
Magnesium, mmol/l	0.8-1.25	0.87±0.09	0.91±0.08	0.95±0.07
PBI, μg%	4-8	5.36±0.31	5.41±0.37	5.65±0.29

N o t e. For a description of the groups, see the Material and methods section. AP, AsAT, AlAT, GGT stand for alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transferase, respectively, PBI — protein-bound iodine.

*, **, *** Differences from the basal level are statistically significant at $p < 0.05$, $p < 0.005$, $p \le 0.00005$, respectively.

▲ Differences from group I are statistically significant at $p < 0.05$.

2. Parameters of the lipid peroxidation-antioxidant protection system and endogenous intoxication in black-motley cows (*Bos taurus*) with mastitis before and after treatment with an antibiotic and an enzyme preparation (*M*±SEM, Agrotech-Garant LLC Rostoshinsky, Ertilsky District, Voronezh Province, February-March 2021)

Parameter	Reference values	Before treatment (a basal level)	After treatment	
			group I (n = 5)	group II (n = 5)
MDA, μmol/l	0.8-1.2	3.32±0.24	2.32±0.18*	1.98±0.07***
MSM, U OD_{254}	< 0.3	0.41±0.01	0.29±0.01***	0.22±0.01***
EII, CU		16.9±1.24	13.7±0.91	11.2±0.63**
GPO, μmol G-SH/(l · min · 10^3)	20.0-35.0	14.3±1.24	16.2±1.41	18.8±1.23*
Cat, мμmol H_2O_2/(l · min · 10^3)	30.0-40.0	41.1±3.37	48.1±3.84	57.7±4.21**▲
Vitamin A, μmol/l	0.84-2.78	1.33±0.12	1.67±0.11*	1.82±0.09**
Vitamin E, μmol/l	15.0-30.0	12.3±1.12	14.1±1.33	16.2±1.23*
Vitamin C, μmol/l	34.1-85.2	20.0±1.92	22.3±1.80	27.7±2.31*
Sw, μmol/l	1.0-1.5	1.02±0.05	1.24±0.07	1.35±0.06**
NOx, μmol/l	40-120	128.6±6.66	52.8±2.56***	36.3±2.23***▲

N o t e. For a description of the groups, see the Material and methods section. MDA, MWM, EII, GPO, Cat stand for malondialdehyde, medium-weight molecules, endogenous intoxication index, glutathione peroxidase, and catalase, respectively.

*, **, *** Differences from the basal level are statistically significant at $p < 0.05$, $p < 0.005$, $p \le 0.00005$, respectively.

▲ Differences from group I are statistically significant at $p < 0.05$.

In the blood of animals that additionally received PAO-1, there were more pronounced changes vs. those before treatment (a basal level). Thus, the blood content of NOx decreased by 3.6 times ($p < 0.00005$), malondialdehyde by 40.4% ($p < 0.00005$), medium-weight molecules by 46.3% ($p < 0$, 0005), the index of endogenous intoxication decreased by 33.7% ($p < 0.005$) with an increase in the content of vitamin C by 39.2% ($p < 0.05$), vitamin A by 36.8% ($p < 0.005$),

vitamin E by 32.8% ($p < 0.05$), selenium by 32.4% ($p < 0.005$), glutathione peroxidase activity by 30.6% ($p < 0.05$), catalaseby 39.4 % ($p < 0.005$), which indicates a decrease in the rate of lipid peroxidation and activation of enzymatic and non-enzymatic components of antioxidant protection (see Table 2). In group II, compared to group I, catalase activity increased by 20.2% ($p < 0.05$), and the NOx decreased by 31.3% ($p < 0.05$).

The complex use of ACP and PAO-1 preparations favorably affected the cytological composition of milk cells (Table 3).

3. Immune and cytomorphological parameters of the udder discharge in black-motley cows (*Bos taurus*) before and after treatment with an antibiotic and an enzyme preparation (M±SEM, Agrotech-Garant LLC Rostoshinsky, Ertilsky District, Voronezh Province, February-March 2021)

Parameter	Reference values	Before treatment (a basal level)	After treatment	
			group I ($n = 5$)	group II ($n = 5$)
Somatic cells, $\times 10^3$/ml	< 200	4620.2±718.1	351.4±56.8***	189.7±22.1***▲
Lymphocytes, %	20-30	5.1±0.35	25.1±4.9**	25.8±3.2**
Neutrophils, %	12-20	91.1±7.71	41.2±3.7**	19.1±2.7***▲
Macrophages, %	55-65	3.8±0.25	33.7±3.1***	55.1±5.2***▲
Lysozyme, μg/ml	0.5-1.8	2.01±0.03	0.71±0.07***	0.55±0.04***
Circulating immune complexes, g/l	0.05-1.0	0.253±0.04	0.099±0.01**	0.061±0.01**▲

N o t e. For a description of the groups, see the Material and methods section.
*, **, *** Differences from the basal level are statistically significant at $p < 0.05$, $p < 0.005$, $p \le 0.00005$, respectively.
▲ Differences from group I are statistically significant at $p < 0.05$.

The content of somatic cells in the secretion of the mammary gland in animals of group I (ACP) at the end of the experiment decreased by 13.0, in group II (ACP + PAO-1) by 24.7 times, and this decrease was almost 1.9 times higher compared to group I.

Cytomorphological analysis of somatic cells of the secret from the affected lobes of the mammary gland of cows with mastitis found that neutrophils were predominant, the content of which was 89.7 and 92.5% in animals of groups I and II, respectively. At the end of treatment in the milk of cows from group II, the number of lymphocytes was close to normal (25.8%) while the number of neutrophils (19.1%) and macrophages (55.1%) approached the optimal value. In the milk of cows treated only with ACP, an increased content of neutrophils (41.2%) and a decreased content of macrophages (33.7%) were noted, the content of lymphocytes was close to optimal (25.1%). After treatment, the observed changes in parameters relative to the background in both groups, as well as in group II vs. group I (except for the number of lymphocytes and the lysozyme level) were statistically significant (see Table 3). A 1.62-fold excess of the concentration of CIC in animals of group I compared to cows that received additional PAO-1 may indicate a violation of the permeability of the vascular wall, an increase in the inflammatory response, the release of lysosomal enzymes and suppression of T lymphocytes [27].

Bacteriological studies of the secret of the mammary gland of cows showed that at the end of treatment with ACP, the microflora was not isolated in 60.0% of cases, *Staph. aureus* was detected in 20.0% of cases and E. coli in 20.0%. In the milk of cows from group II, subjected to complex treatment (ACP + PAO-1), no microflora was found.

It is known that some proteolytic enzymes realize a therapeutic effect through the influence on the inflammatory process, vascular-platelet hemostasis and immune responses [17]. Proteolytic enzymes improve tissue trophism by destroying protein formations and fibrin clots in the area of inflammation, as well as reducing platelet aggregation, thereby preventing the transition of a chronic inflammatory process to a recurrent stage [28, 29]. Enzymes can act as natural highly

active inflammation modulators to speed up the healing process [28].

A number of studies have shown the ability of extracellular proteinases of micromycetes of the genus Aspergillus to exhibit hydrolytic properties and activate protein C and factor X in blood plasma [30]. Proteinase, an activator of blood plasma protein C isolated from the culture liquid of *A. ochraceus* VKM F4104D, which is a serine proteinase [31] with high biological activity and anti-inflammatory action, was obtained [32].

Our clinical trials have shown the effectiveness of an experimental preparation based on *A. ochraceus* micromycete proteinase in the complex treatment of mastitis in cows. Additional use of PAO-1 increased the therapeutic effect by 12.5% which was confirmed by the data of morpho-biochemical studies. Changes in homeostasis indicators in the process of recovery of cows with the combination of the antimicrobial drug ACP with PAO-1 indicate, on the one hand, a decrease in the inflammatory response and functional load on the liver and kidneys due to a decrease in endogenous intoxication, on the other hand, the normalization of protein, lipid and mineral metabolism.

Lipid peroxidation is an important metabolic factor in both normal and pathological conditions [33]. The pathogenesis of many animal diseases is based on the intensification of lipid peroxidation processes, which leads to disruption of cellular energy exchange, protein synthesis, inhibition of membrane-dependent enzymes due to the accumulation of a number of toxic products (MDA, conjugated dienes, ketodienes) [34, 35]. Mastitis proceeded with oxidative stress and an increase in endogenous intoxication, as indicated by the increase in the concentration of MDA and MWM, high EII, low values characterizing the activity of the enzymatic (Cat, GPO), non-enzymatic (vitamins A, E and C) AOD and high NOx content. When analyzing the prooxidant-antioxidant status of recovered animals, it was found that in cows that additionally received PAO-1, positive changes were more pronounced. So, in these animals, the concentration of MDA, MWM decreased, the EII decreased with an increase in the content of vitamins A, E, C and the activity of Cat and GPO, which indicates a decrease in the intensity of lipid peroxidation and activation of the enzymatic and non-enzymatic AOD.

The recovery of animals after a course of ACP in combination with PAO-1 was accompanied by a more significant decrease in the blood NOx content (the difference was 31.3%), which also indicates a weakening of oxidative stress, since NO is able to act both as a powerful pro-oxidant and participate in endogenous antioxidant protection [36, 37].

The detected changes may be due to the fact that the enzymatic activity of the proteinase improves microcirculation in inflammatory foci, reduces vascular porosity, provides more complete removal of damaged tissues and pus clots from the milk ducts and, consequently, faster elimination of pathogenic microorganisms and bacterial toxins with milk, while antibiotics reached the inflammatory focus more quickly [28, 38].

The clinical recovery of cows after the end of the therapeutic course is confirmed by the normalization of the number and composition of somatic cells, as well as the absence of pathogenic microflora in the udder secretion with the additional use of PAO-1 in combination with etiotropic treatment.

So, the results of experimental and clinical studies allow us to draw the following conclusions. The use of the micromycete *Aspergillus ochraceus* BKM F-4104D proteinase in combination with etiotropic treatment for mastitis of cows provides a significant increase in the effectiveness of antibiotic therapy (by 12.5%, $p < 0.05$) and the complete release of the mammary gland from mastitis pathogens *Staphylococcus aureus* and *Escherichia coli*. Changes in the blood

morphobiochemical parameters during treatment indicates the normalization of metabolic processes, a decrease in the toxic effect of endogenous metabolites (the content of malondialdehyde decreased by 40.4% at $p < 0.00005$, medium-weight molecules by 46.3% at $p < 0, 00005$) and stimulation of the enzymatic and non-enzymatic components of the antioxidant system (an increase in the content of vitamin A by 36.8% at $p < 0.005$, vitamin E by 32.8% $p < 0.05$, vitamin C by 39.2% at $p < 0.005$, catalase activity by 39.4% at $p < 0.005$, glutathione peroxidase activity by 30.6% at $p < 0.005$). This reduces the metabolic load on the liver and kidneys of animals. Combined therapy induces a more pronounced modulation of pro- and antioxidant status compared to etiotropic treatment, i.e., a 3.6-fold decrease in NOx content ($p < 0.00005$). This is probably due to a decrease in inflammation at the site of infection, since in the secret of the udder there was a significant decrease in the number of somatic cells (neutrophils to the greatest extent, up to 19.1% at $p < 0.05$) and the content of circulating immune complexes (up to 0.061 g/l at $p < 0.05$). The absence of pathogenic bacteria in the udder secretion with the proteinase preparation means that the decrease in the inflammatory response is also associated with a decrease in the action of endotoxins produced by bacteria and pathogen-associated molecular patterns. We believe that the reduction of endo- and exogenous intoxication, as well as the activation of reparative processes in the focus of infection due to fibrinolytic remodeling of the extracellular matrix under the action of proteinase, can contribute to the normalization of morphobiochemical parameters. Thus, the proteolytic functions of the studied enzyme preparation are likely to underlie the increase in the effectiveness of therapy. The *A. ochraceus* BKM F-4104D micromycete proteinase with anticoagulant and fibrinolytic properties may be very promising in the development of drugs for veterinary medicine, since its use enhances the antibiotic effect and, possibly, will reduce the amount of the drug itself.

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IRON DEFICIENCY ANEMIA IN LABORATORY RATS TO BE USED AS AN EXPERIMENTAL MODEL FOR FARMED FUR-BEARING ANIMALS

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Abstract

Iron is an essential trace element necessary for the implementation of many processes in the body (metabolism regulation, DNA and ATP synthesis, oxygen transfer, tissue respiration, erythropoiesis, immune response). In caged fur animals, iron deficiency anemia leads to significant economic losses due to a decrease in viability and fertility, and a deterioration in the fur quality. Therefore, the study of the causes of this microelementosis, the development of pharmacological agents and techniques for its prevention and treatment remain a topical issue. In our report, we present data confirming the modeling of this pathology using an atraumatic approach, i.e., the diet we proposed, which is low in cost and simple in its ingredients. As a model object, white rats were used, which, in terms of physiological parameters, are more similar to fur-bearing animals than other laboratory animals. The aim of the study was experimental modeling of iron deficiency anemia in laboratory rats in order to extrapolate the results obtained on this model to fur animals in the future. From 4-month-old white outbred laboratory rats weighing 200 g, two groups of 10 individuals were formed. Control animals received a generally accepted balanced diet which corresponded to the consumption norms for laboratory rats and was 4 g proteins, 2 g fats, 25 g carbohydrates, and 0.5-1.0 g fiber. In the experimental group a specially developed diet was applied which was 4 times less in the iron content, but corresponded to the feeding norms in terms of the nutrients, vitamins and minerals (excluding iron). After 45 days, the rats in the experimental group developed iron deficiency anemia. As compared to the control rats, receiving a diet not deficient in iron, there was a significant ($p \leq 0.05$) decrease in hemoglobin (by 37.5 g/l), hematocrit (by 18.35 %), the number of erythrocytes (by 3.57×10^{12}/l), the concentration of serum iron (by 18.44 μmol/l), the average volume of erythrocyte (by 14.02 fl), the average content of hemoglobin per erythrocyte (by 6.26 pg) and per erythrocyte mass (by 73.29 g/l). The anemia of the animals was hypochromi and macrocytic. From day 17 of the experiment, shortness of breath and increased heart rate occurred, from day 24, the body temperature decreased which indicates the development of an anemic syndrome in the rats. Up to day 14, the color of the skin and mucous membranes, as well as the general condition of the rats in both groups corresponded to the norm. After day 14, anemic skin and mucous membranes of the oral cavity were observed in rats receiving an experimental diet with a limited iron content. In addition, lethargy and general depression occurred. Our results demonstrated the ability to effectively simulate iron deficiency anemia in laboratory rats, minimizing stress and eliminating physical and mental traumatization of animals, the risk of their death, and side effects. The model has been successfully applied in evaluating the effectiveness of a complex microelement preparation based on a polymaltose complex of Fe^{3+} hydroxide. In the future, we plan to use the model of iron deficiency anemia in rats to develop methods for the prevention and correction of this pathology in farmed fur-bearing animals.

Keywords: fur farming, iron deficiency anemia, iron preparations, anemia modeling, laboratory rats

Iron is an essential micronutrient with a complex metabolism, recycling

system, and content control, which allows us to speak about Fe homeostasis in the body [1-4]. Inorganic iron supplied with food [5] is mainly in the trivalent form Fe^{3+}. It is absorbed by the duodenal mucosa with the participation of beta-3-integrin and mobilferrin, a 56 kDa protein. In the cytosol of the absorbing cell, iron binds to a complex known as paraferritin, which contains integrin, mobilferrin, and flavin monooxygenase. This complex serves as a ferrireductase and reduces iron to the divalent state Fe^{2+}, in which it is available for the formation of heme proteins [6-8]. In the cells of the intestinal mucosa, iron in the form of Fe^{3+} combines with the protein apoferritin to form ferritin, the main form of deposition of this trace element [4,] (in the bone marrow, liver, and spleen). It is believed that the amount of iron entering the blood depends on the content of apoferritin in the intestinal walls [10]. The transport of iron from the intestine to the hematopoietic organs is carried out by a complex with the blood plasma protein transferrin [8]. In the form of fumarate, the bioavailability of iron is increased [11].

In the body, iron compounds are involved in oxidative reactions. Hemoglobin serves as a carrier of oxygen, myoglobin (protein of skeletal muscles and heart muscle) binds oxygen and creates a reserve to make up for its deficiency. Parenteral and enteral administration of iron salts increases the content of hemoglobin in the blood and iron in the blood serum [12-16]. Iron-containing enzymes cytochromes, cytochrome oxidase, catalase, peroxidase provide tissue respiration, iron is in the prosthetic group of ferroflavoproteins - xanthine oxidase, succinate dehydrogenase [17].

Thus, iron is necessary for the implementation of basic processes in the body (metabolism regulation, DNA and ATP synthesis, oxygen transport, tissue respiration, erythropoiesis) [17-19], it affects immunoresistance [20]. Iron deficiency can cause impaired conversion of protoporphyrin IX to heme. As a result, the content of porphyrins in erythrocytes increases (21). With iron deficiency, a hematological syndrome develops, characterized by impaired hemoglobin synthesis and manifested by erythrocytopenia and sideropenia (low iron and iron-containing enzymes) [22-24].

The variety of Fe functions determines the significant physiological abnormalities caused by iron deficiency anemia [25-27]. In animal husbandry, its etiology in most cases is associated with improper feeding and inadequate care of animals [26]. Iron deficiency anemia accounts for the majority of all diagnosed anemias [16, 28]. With this pathology, the growth and development of animals slows down [29-31], in fur-bearing animals under conditions of industrial breeding, the condition of the skin and hair integuments worsens, the quality of furs decreases [2, 32-34], including its physical and mechanical characteristics [35].

The prevalence of iron deficiency anemia in mammals, its dangerous consequences and the damage it causes determines the volume of fundamental physiological, biochemical [36-40] and genetic studies in this pathology [18, 25, 41], as well as practical developments to compensate for the iron deficiency state in humans [12, 42] and animals [13, 29-31, 43]. For these purposes, iron-deficient diets are widely used in world practice [18, 44-46], which are proposed for various animal species - rodents, dogs, cats, rabbits, guinea pigs, ferrets, pigs, sheep, goats, cows, primates and are produced in commercial scale, which allows standardization of the design of the experiments. Examples include Teklad from Envigo, USA (https://www.envigo.com/) and AIN (American Institute of Nutrition Approved Diets) [46-48], which continue to be improved. In such experiments, an adequate choice of a biological model is also important, which makes it possible to apply the results obtained on a laboratory animal to solve practical problems [49]. The most common animal models used in biomedical research are rats, usually males [18], which makes it possible to exclude the effect of hormonal changes on the

results.

In fur farms, iron deficiency anemia is a common pathology [34, 50, 51] and leads to significant economic losses due to a decrease in the viability and fertility of animals, and a deterioration in the quality of the resulting furs [26, 34, 52]. Iron deficiency anemia is most common in minks [2], in part because their diet includes marine fish [34, 53], which are rich in trimethylamine oxide (TMAO, or triox), which binds iron and converts it from divalent to non-ferrous. digestible trivalent. With regular consumption of such fish, animals, especially growing young animals, develop iron deficiency [34, 53].

Until now, in Russia, there was no publicly available information about the method of obtaining experimental models of iron deficiency anemia which would be similar to the known ones, but low-cost, accessible, atraumatic and at the same time sufficient for the selection of pharmacological prevention and treatment of this microelementosis and schemes for their use in the practice of fur farming. In our report, we present data confirming the possibility of such modeling in rats using the original diet developed by us presented in the work.

The aim of our study was to confirm the development of experimental iron deficiency anemia in laboratory rats when using a diet with a limited iron content.

Materials and methods. Physiologically healthy female outbred laboratory rats (n = 20, aged 4 months with body weight of 200 g) were used in the experiment. According to the principle of pair-analogues, two groups of 10 rats each were formed. The conditions of the animals were in accordance with the International Guidelines for Biomedical Research Involving Animals [54]. Rats of both groups were kept in standard cages in compliance with veterinary and zootechnical requirements according to the recommendations for biological models [55]. As the main (control group I) we used a balanced diet (Delta Feeds, a feed for laboratory rats and mice P-22, AO BioPro, Russia), corresponding to the consumption norms for laboratory rats (4 g proteins, 2 g fats, 25 g carbohydrates, 0.5-1.0 g fiber), water in plenty. In group II (test group), rats received a specially designed diet (26 g individually for each animal 1 time per day), which corresponded to the age norms for animals of this species in terms of the content of nutrients, minerals and vitamins with a decrease in the content of Fe in the feed (9.12 mg/kg vs. 35.00 mg/kg in control). In both groups, access to water was not restricted. The experiment continued for 45 days.

During the entire observation period, body temperature, heart rate (HR), and respiration rate (RR) were measured every week in animals. We used a PowerLab® 8/30 recorder (ADInstruments Pty Ltd., Australia) and a piezoceramic sensor for recording respiration rate with a BNC for connecting to the recorder, an elastic cuff for fixing the sensor (different sizes depending on the type of animal). The general condition of the rats was also assessed.

At the end of the experiment (on day 45), a hematological analysis of peripheral blood of each laboratory rat collected from the tail vein with a needle into a test tube with an anticoagulant was performed. The number of erythrocytes and the amount of hemoglobin [56], hematocrit [57], the average volume of erythrocytes, the average content of hemoglobin in the erythrocyte and in the erythrocyte mass, as well as the content of iron in the blood serum [58] were determined.

From rats with iron deficiency anemia, modeled according to the method we proposed by (n = 10), two groups were formed (n = 5 each) to study the effectiveness of a new complex trace element preparation based on the Fe^{3+} hydroxide polymaltose complex (OOO A-BIO, Moscow). Animals of group I served as control (rats continued to receive the diet developed by us), in group II, it was supplemented with a complex microelement preparation at a dose of 0.1 ml for

each animal. After 30 days, hematological examination of peripheral blood was performed as described above.

Statistical analysis of the obtained data was performed using the Microsoft Excel program. The arithmetic mean of the measured parameters (*M*) and the standard error of the mean (±SEM) were calculated. To assess the significance of differences between the compared means, Student's *t*-test was used ($p \leq 0.05$).

Results. The study of the efficacy and safety of pharmacologically active compounds in experimental models is a necessary preliminary step in the search for drugs for the treatment and prevention of microelementoses in fur animals.

Various schemes for modeling iron deficiency anemia in animals are known. For example, in pigs [59] and rats [60], this microelementosis was induced by bloodletting (blood-removing induced anemia). In Russian researchers, it was proposed to administer the drug Deferoxamine subcutaneously (0.5 g/kg twice with an interval of 3 days, Patent RU 2553344 C1, publ. 10.06.2015, Bull. No. 16) [61]. A significant drawback of the proposed method is the multiple negative side effects. Another technique involves the administration of Desferal® (also a complexing compound, the dosage and frequency of administration are not indicated by the authors) [60, 61]. However, these techniques are traumatic (up to the risk of death of animals) and cause severe stress in test animals [61], which distorts the results of the experiment. In world practice, standardized iron-deficient diets are used [18, 44-46]. In Russia, the use of a diet with an iron content of 27 mg/kg for the induction of experimental iron deficiency anemia in rats was reported, but the composition of the diet was not specified by the author [61].

1. The diet for modeling iron deficiency anemia in white laboratory rats

Ingredient	Daily dose per animal, g
Sodium chloride	0.3
Magnesium sulfate · 7H20	0.01
Sodium phosphate	0.4
Calcium gluconate	0.55
Potassium chloride	0.02
Microcrystalline cellulose	0.25
Tea powder	0.08
Vitamin blend	0.003
Sunflower oil	2.0
Abiopeptide dry	2.0
Semolina	20.0
Corn starch	0.67

N o t e. The composition of the vitamin blend is specially selected and contains the following components (one daily dose): α-tocopherol acetate (0.24 mg), ascorbic acid (1.8 mg), calcium pantothenate (0.072 mg), nicotinamide (0.48 mg), pyridoxine hydrochloride (0.072 mg), retinol palmitate (0.044 mg), riboflavin (0.048 mg), rutoside (0.24 mg), thiamine hydrochloride (0.048 mg), folic acid (1.68 mcg), cyanocobalamin (0.048 μg). Each rat received individually 26 g of the mixture per day.

For the induction of iron deficiency anemia, we have proposed and tested a diet, the composition of which is presented in Table 1.

When developing an experimental model, an adequate choice of animal is important [44, 49]. The most common biological models are mice [43, 62] and rats [15, 18, 25, 39, 44]. In our experiment, rats were model animals which are more similar to fur-bearing animals, and in particular to minks, in terms of physiological and biochemical features. An important advantage of rats as laboratory animals is that they are quite resistant to infectious diseases. Males are usually used [18], as well as females at the age of 3-5 months (up to 6 months of age, only about 1% of individuals start reproduction) [54, 55]. We used 4-month-old female white outbred laboratory rats based on the fact that, in particular, in minks, it is the iron deficiency anemia of females that poses a serious problem. Anemia-prone adult male breeding males are mostly sterile, while females have reduced body weight, a high percentage of infertility, cannibalism, and loss of maternal

instinct [52]. Their puppies are underweight at birth, often suffer from maldigestion, grow poorly, often die at an early age, and the survivors remain small and even dwarf [52].

In our experiment, when rats received the experimental diet, the blood hemoglobin significantly ($p \leq 0.05$) decreased, hematocrit, erythrocytes and serum iron concentration also decreased compared to control by 37.5 g/l, 21.35%, 3.57×10^{12}/l and 18.44 μmol/l), respectively, hypochromia and microcytosis were recorded (Table 2) which are characteristic signs of iron deficiency anemia.

2. Hematological parameters in white laboratory rats characterizing the state of iron deficiency anemia ($M \pm$SEM)

Parameter	Group I (control) ($n = 10$)	Group II ($n = 10$)
Erythrocytes, $\times 10^{12}$/l	7.82±0.43	4.25±0.37*
Hemoglobin, g/l	121.1±6.2	83.6±0.4*
Hematocrit, %	44.58±3.55	23.23±2.18*
Average erythrocyte volume, fl	62.26±6.17	51.24±3.16*
Average hemoglobin content per erythrocyte, pg	22.64±2.32	16.38±1.13*
Average hemoglobin content in erythrocyte mass, g/l	348.45±20.11	275.16±28.12*
Serum iron concentration, μmol/l	45.68±2.74	27.24±3.82*

N o t e. The groups formed from 4-month-old rats were used in a 45-day test.
* Differences from control are statistically significant at $p \leq 0.05$.

The significant hematological changes we identified are similar to those described in animals of other species where these indicators, i.e., a decrease in hemoglobin [40], a decrease in hematocrit [63], erythrocytopenia, a decrease in blood iron concentration, and hypochromia and microcytosis [64] which are characteristic of iron deficiency anemia.

3. Dynamics of physiological parameters of white laboratory rats with experimental iron deficiency anemia ($M \pm$SEM)

Days	Group ($n = 10$)	Parameter		
		RR	HR	T
One day prior to the experiment	I	99±7	398±20	37.3±0.2
	II	98±10	402±15	37.4±0.3
Day 3	I	101±11	411±21	37.5±0.3
	II	106±9	409±19	37.3±0.2
Day 10	I	102±8	387±18	37.4±0.3
	II	112±11	406±21	37.5±0.4
Day 17	I	109±8	395±12	37.6±0.4
	II	129±9*	428±10*	37.4±0.4
Day 24	I	112±11	403±16	37.4±0.3
	II	132±8*	429±17	36.4±0.4*
Day 31	I	99±7	409±15	37.3±0.3
	II	133±8*	439±14*	36.5±0.4*
Day 38	I	110±9	413±16	37.5±0.2
	II	138±10*	448±15*	36.8±0.4*
Day 45	I	104±6	404±18	37.4±0.3
	II	137±8*	453±21*	36.9±0.4

N o t e. The groups formed from 4-month-old rats. RR — respiration rate, HR — heart rate, T— body temperature.
* Differences from control are statistically significant at $p \leq 0.05$.

Iron deficiency anemia induced in rats from group II affected the physiological state of the animals, the respiration rate, heart rate, and body temperature (Table 3). Consequently, the body of laboratory rats from the experimental group as a whole reacted to the development of the disease, while from day 17 significant changes began, when shortness of breath and palpitations were noted in the animals. From day 24, body temperature decreased which indicates the development of anemic syndrome.

During the experiment, we also assessed the color of the skin and mucous membranes and the general condition of the animals. Until day 14, these indicators in laboratory rats from the test and control groups corresponded to the norm. After 2 weeks, in rats fed an experimental diet with a limited iron content, we

observed anemia of the skin and mucous membranes of the oral cavity, as well as lethargy and depression of the general condition.

Laboratory rats with iron deficiency anemia, modeled by our method, were further used to study the effectiveness of a new complex trace element preparation based on the Fe3+ hydroxide polymaltose complex (Table 4). The studied drug in 1 ml contains 50 mg Fe(III) as active substances, Cu 0.1 mg, Co 0.2 mg, Se 0.07 mg, Mn 0.6-0.7 mg, Zn 0.6-0.7 mg. As excipients, the preparation is 1.5 g methylhydroxybenzoate, 0.15 g propylhydroxybenzoate, 100 g sucrose, 140 g sorbitol (drinking water up to 1.0 l). In appearance, the drug is an odorless, opaque, reddish-brown liquid.

4. Hematological parameters in white laboratory rats with experimental iron deficiency anemia in an experiment to study the effect of a complex trace element preparation based on a polymaltose hydroxide Fe^{3+} complex ($M \pm$SEM)

Parameter	Group I (control) (n = 5)	Group II (n = 5)
At the beginning of the test		
Erythrocytes, $\times 10^{12}$/l	4,42±1,63	4,35±0,31
Hemoglobin, g/l	81,8±0,4	82,3±0,8
Hematocrit, %	23,34±1,82	22,27±2,32
Average erythrocyte volume, fl	61,21±1,19	58,45±3,63*
Average hemoglobin content per erythrocyte, pg	16,45±3,56	15,91±2,17
Average hemoglobin content in erythrocyte mass, g/l	278,65±15,41	271,92±21,78
Serum iron concentration, μmol/l	24,91±3,93	26,85±3,14*
At the end of the test		
Erythrocytes, $\times 10^{12}$/l	5,14±0,24*	7,71±0,39*
Hemoglobin, g/l	85,3±5,2*	118,7±4,8*
Hematocrit, %	36,41±3,82*	44,12±2,74*
Average erythrocyte volume, fl	58,92±7,46	57,44±5,31
Average hemoglobin content per erythrocyte, pg	15,35±3,19*	23,54±2,71*
Average hemoglobin content in erythrocyte mass, g/l	288,56±19,53*	353,27±16,25*
Serum iron concentration, μmol/l	27,65±2,71*	46,32±1,68*

Note. A 30-day experiment was performed. The manufacturer of the drug is A-BIO LLC, Moscow.
* Differences from control are statistically significant at $p \leq 0.05$.

At the end of the experiment (after 30 days), in rats from group I (control), hematological parameters (erythrocytes, hemoglobin, hematocrit, average hemoglobin content in erythrocytes, average hemoglobin content in erythrocyte mass and serum iron concentration) were still reduced - by 2.57×10^{12}/l, 33.4 g/l, 7.71%, 8.19 pg, 64.71 g/l and 14.67 μmol/l, respectively. In rats from group II (test), hematological parameters returned to normal, which indicated the elimination of iron deficiency anemia. Thus, a positive experience has been obtained in using the developed model of experimental iron deficiency anemia in assessing the effectiveness of its correction schemes. It was shown that the use of a complex microelement preparation of a microelement preparation based on the Fe^{3+} hydroxide polymaltose complex allowed normalization of hematological parameters (erythrocytes, hemoglobin, hematocrit, average hemoglobin content in an erythrocyte, average hemoglobin content in an erythrocyte mass and serum iron concentration) in laboratory rats after modeling iron deficiency anemia.

Based on the results of the experiments, it can be argued that we have proposed a model of iron deficiency anemia in laboratory rats. The model is effective, atraumatic, low-cost, accessible and sufficient for solving the problems of practical fur farming in assessing the effectiveness of the developed techniques for correcting this pathology [13, 15, 32, 42] and the safety of the agents used, given the pathophysiological effects of excess iron [65-68].

R.M. Kaufman and P. Simeon [44] studied the effect of an iron-deficient diet on iron absorption in rats weighing 200-350 g. The total iron content of the diet used in the study was 3.90 μg/g (daily feed intake 10 g); in a standard commercial diet, the iron content was 186 μg/g (daily feed intake of 10-15 g). The authors concluded that iron absorption is controlled by the depletion of its reserves

from a different pool than in the liver and erythrocytes. The basis was the data that with a lack of iron in the diet, its absorption in rats increased (after at least 5 days of limited iron intake), the lack of iron in the diet did not affect erythropoiesis, and up to 14 days the content of serum iron in animals in the experimental group did not differ from normal [44]. The observation time, depending on the variant of the experiment, ranged from 2 to 30 days [44]. These and other available data indicate that body adaptive responses and iron homeostasis [4, 36, 44, 67] need to be considered when developing a model of iron deficiency anemia. In our model, the daily feed intake was 26 g, the iron content in the proposed diet was 9.12 mg/kg vs. 35.00 mg/kg in the control, that is, the difference was approximately 4-fold vs. 48-fold in the report by R.M. Kaufman and P. Simeon [44]. Our experiment lasted 45 days and resulted in the significantly decreased amount of hemoglobin, hematocrit, erythrocyte count, serum iron concentration, average erythrocyte volume, average hemoglobin content in erythrocytes and average hemoglobin content in erythrocyte mass in the laboratory rats. Additional observations confirmed that from day 17 the general condition and physiological parameters of the animals changed and anemic syndrome developed.

Thus, our results demonstrate that the provided simple and available diet can effectively simulate iron deficiency anemia in laboratory rats, minimizing stress and eliminating the physical and mental traumatization of animals, the risk of their death and the unreliability of the results. The iron deficiency of the animals was characterized by a decrease ($p \leq 0.05$) in hemoglobin concentration by 37.5 g/l, in hematocrit by 21.35%, in erythrocytes by 3.57×10^{12}/l, in the blood iron concentration by 18.44 μmol/l, in mean erythrocyte volume by 14.02 fl, in mean hemoglobin content per erythrocyte by 6.26 pg, and in erythrocyte mass by 73.29 g/l compared to the control (iron-free diet). There were shortness of breath and palpitations from day 17 of the experiment and a decrease in body temperature from day 24, which indicates the development of an anemic syndrome in animals. Up to day 14, the skin and mucous membrane color, as well as the general condition of the animals in both groups corresponded to the norm, but after day 14, in rats fed feed with a limited iron content, we observed anemia of the skin and mucous membranes of the oral cavity, lethargy and oppression of the general state. The proposed model was successfully applied to study the effect of a complex microelement preparation based on the Fe^{3+} hydroxide polymaltose complex. Further, we plan to use the model of iron deficiency anemia in rats to develop methods for the prevention and correction of iron deficiency anemia in other animals, including fur animals, which, by their biological features and physiology are similar to rats.

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EFFICACY OF A COMPLEX PREPARATION TO CORRECT DIGESTION IN BROILER CHICKENS (*Gallus gallus* L.) IN EXPERIMENTAL MYCOTOXICOSIS

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Abstract

Mycotoxins have a negative effect on the health and productivity of farm animals. The main criterion for the diagnosis of mycotoxicoses in the absence of a pronounced clinical picture of the disease is the presence of toxins in the feed. Different preparations are used to prevent mycotoxicoses, but their effect on the bird organism has not been fully studied. In the present work, the peculiarities of digestive function, metabolism and haematological values in broiler chickens of Smena 8 cross from 34- to 48-days old when using protease combined with sorbent in the case of experimental mycotoxicosis caused by T-2 toxin were shown first. The investigation was aimed at determining the effect of sorbent Zaslon 2+ and enzyme preparation Axtra Pro on duodenal enzymes activity, protein metabolism and morphobiochemical blood parameters in broiler chickens of Smena 8 cross with chronic intestinal fistula in experimental mycotoxicosis caused by T-2 toxin. The experiments were performed according to requirements of the European Convention on protection of vertebrate animals used for experiments or other scientific purposes (ETS № 123, Strasburg, 1986). The broiler chickens were kept in the vivarium from 1- to 48-day-old chickens (All-Russian Research Institute for Scientific and Technical Studying of Poultry Farming, 2021) respecting the regime of feeding and keeping according to the requirements for the definite age group and cross of poultry. Surgical operations of fistula implantation into the duodenum were carried out on 25 birds at the age of 20-25 days. A cannula was implanted opposite the place where the pancreatic and bile ducts ran into the intestine. Five groups of 5 birds were formed of clinically healthy birds. each group was formed according to the principle of analogues: Group I (control) was kept on the basic diet (OR) without the addition of mycotoxins, experimental group II received OR + T-2 toxin (0,1 mg/kg) + sorbent Zaslon 2+ (BIOTROF, Ltd., Russia) (2 g/kg food), group III — OR + T-2 toxin (0.4 mg/kg) + Zaslon 2+ (2 g/kg food), group IV — OR + T-2 toxin (0.1 mg/kg) + Zaslon 2+ (2 g/kg food) + Axtra Pro enzyme (DuPont de Nemours, Inc., USA) (0,1 g/kg feed), group V — OR + T-2 toxin (0.4 mg/kg) + Zaslon2+ (2 g/kg feed) + Axtra Pro (0.1 g/kg feed). The feed was contaminated with T-2 toxin to MAC levels (groups II and IV) and 4 MAC levels (groups III and V) by mechanical means in compliance with personnel safety requirements. Standard T-2 toxin (powder with mass fraction of main substance 99.7±0.3 %; Romer Labs, Austria, LOT No. S17052T) was used. The preparation period lasted from 26- to 33-day-old birds, the experiment period lasted 14 days (from 34- to 48-day-old birds). Chyme (1.0-2.0 ml) and litter (5.0 g)

samples were collected daily from each bird in the morning. Blood (2 ml) was taken 1 day before slaughter (at the age of birds 47 days) from the sub wing vein. When using Zaslon 2+ sorbent in combination with Axtra Pro protease to prevent mycotoxicosis, the enzymatic activity of duodenal chyme was increased compared with that of the basic preparation (sorbent): protease activity in duodenal contents by 15.5 % ($p < 0.05$), trypsin by 12.8 % ($p < 0.05$), alkaline phosphatase by 46.1 % ($p < 0.05$), total phosphorus content by 25.6 % ($p < 0.05$), at a toxin dose of 0.1mg/kg feed. amylase activity increased by 9.6 and 14.7 % at the dose of T-2 toxin 0.1 and 0.4 mg/kg, respectively, compared with using a single sorbent. There was a statistically significant increase in total proteolytic activity, trypsin (at the dose of 0.1 mg/kg toxin), and lipase activity (at the dose of T-2 toxin 0.4 mg/kg). The activity of enzymes in the litter of birds of experimental groups did not increase compared to the control group, indicating the positive role of the preparations in normalizing digestion when using the toxic feed in the bird's diet. The use of contaminated feed with T-2 toxin for 14 days adversely affected the state of protein metabolism, which was manifested in the reduction of nitrogen use by poultry in all experimental groups; there was also a negative trend in the availability of amino acids, especially when the toxin dose was 0.4 mg/kg. Biochemical blood parameters of broiler chickens in experimental mycotoxicosis showed impairment of protein, fat and carbohydrate metabolism, as well as signs of stress response due to the action of the toxin on digestive organs (pancreas, liver).

Keywords: T-2 toxin, HT-2 toxin, T-2 toxicosis, broilers, chyme, droppings, digestive enzymes, feed additive Zaslon 2+, enzyme Axtra Pro

Mycotoxins adversely affect the health and productivity of farm animals [1-3]. Studying the biochemistry and properties of mycotoxins, developing methods for their detection, identifying symptoms of diseases and complying with regulatory guidelines established by the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) are the main areas of research and practice aimed at preventing or minimizing mycotoxin contamination of food and feed, reducing toxicity, and reducing economic losses [4-6].

It has been shown that activation in pancreatic tissues of genes of regulatory molecules for the development of an inflammatory response (*IL6* and *PTGS2*), genes associated with cell death (*Casp6*), as well as genes of antimicrobial factors (primarily *AvBD10*) can serve as an early prognostic marker of T-2 toxicosis in broilers [7]. Using principal component analysis (PCA), it was demonstrated that the expression of *PTGS2* genes in the pancreas, *IL6*, *PTGS2*, *IL8*, *IRF7*, *AvBD9*, *AvBD10* and *Casp6* in the caecum of the intestines of Smena 8 cross broilers, as well as the content in the blood total protein, glucose, triglycerides, activity of alkaline phosphatase and trypsin and the ratio of the activity of these enzymes were in close relationship [7].

A large number of different preparations have been proposed for the prevention of mycotoxicoses in animals [8-10]. The Russian Federation has patented a number of feed additives designed to reduce the negative impact of mycotoxins on animals. Methods for obtaining these drugs and their composition are very diverse. Mineral substances such as clay, zeolites, silicates are used [11)]. Enrichment of adsorbents with humic acids, yeast cell walls is possible (12). It is proposed to carry out the biological neutralization of mycotoxins using enzyme preparations of hydrolases, polypeptide esterases, multicomponent mixtures of enzymes and bacteria [13]. The effectiveness of the proposed drugs in relation to the sorption of mycotoxins in in vitro experiments and the effect on the productivity of animals and poultry in in vivo experiments has been proven, however, the mechanism of the observed positive effect on the digestion and body of the bird has not yet been sufficiently studied.

In the present work, it was shown for the first time that the mechanism of the positive effect of a complex preparation containing a sorbent and a protease on broiler chickens of the Smena 8 cross aged from 34 to 48 days with experimental T-2 toxicosis is associated with modulation of the activity of duodenal content proteases, trypsin in blood plasma. and the number of lymphocytes in the blood.

The aim of the work was to determine the effect of the Zaslon 2+ sorbent and the Axtra Pro enzyme preparation on the activity of duodenal enzymes, protein metabolism, and morphobiochemical blood parameters in broiler chickens of the Smena 8 cross with chronic intestinal fistula in experimental T-2 toxicosis.

Materials and methods. Physiological experiments were carried out in 2021 on broiler chickens (*Gallus gallus* L.) of the Smena 8 cross in accordance with the requirements of the European Convention for the Protection of Vertebrate Animals used for experiments or for other scientific purposes (ETS No. 123, Strasbourg, 1986) [14]. Chickens from 1 to 48 days of age were kept in a vivarium (FSC All-Russian Research and Technological Institute of Poultry Farming RAS); feeding and maintenance regimes met the requirements for the age group and cross [15].

Surgical operations for implantation of fistulas into the duodenum were performed on 25 birds at 20-25 days of age, the cannula was implanted opposite the place where the pancreatic and bile ducts enter the intestine according to the author's method [16]. During the first day after the operation, the bird was limited in food, and then the feeding was normalized, following the passage of chyme in the intestine. The sutures were removed on day 5 after the operation. From clinically healthy birds, five groups were formed (5 broilers each) according to the principle of analogues. Group I (control) was kept on the basal diet (BD) without the addition of mycotoxins. Group II received BD + T-2 toxin (0.1 mg/kg) + sorbent Barrier 2+ (2 g/kg feed). Group III received BD + T-2 toxin (0.4 mg/kg) + Barrier 2+ (2 g/kg feed). Group IV received BD + T-2 toxin (0.1 mg/kg) + Zaslon 2+ (2 g/kg feed) + Axtra Pro enzyme (0.1 g/kg feed). Group V received BD + T-2 toxin (0.4 mg/kg feed) kg) + Barrier 2+ (2 g/kg feed) + Axtra Pro (0.1 g/kg feed). Feed additive Zaslon 2+ (OOO BIOTROF, Russia) consisted of a sorbent material of diatomite, bacteria *Bacillus* sp., a mixture of natural essential oils of eucalyptus, thyme, garlic and lemon. The proteolytic activity of the enzyme preparation Axtra Pro (DuPont de Nemours, Inc., USA) was 897.0±47.5 $mg \cdot g^{-1} \cdot min^{-1}$. Feed was contaminated with T-2 toxin up to 1 MPC (groups II and IV) and 4 MPC (groups III and V) mechanically in compliance with personnel safety requirements. We used standard T-2 toxin (powder with a mass fraction of the main substance 99.7±0.3%; Romer Labs, Austria, LOT No. S17052T). Fresh feed was given to the birds daily, access to water was not limited.

The preparatory period lasted from 26 to 33 days of age of the birds, the period of the experiment lasted 14 days (from 34 to 48 days of age). Samples of chyme (1.0-2.0 ml) and droppings (5.0 g) were collected daily during the experiment from each bird in the morning, placed in a refrigerator at –20 °C, samples (5 g each) were dried in freeze dryer of the TFD series (ilShinBioBase Co., Ltd., South Korea) for 34 h at –77,8 °C and a pressure of 5 mTorr (removal of 97% moisture from the substrate with the preservation of biologically active substances). In duodenal chyme and litter, the activities of digestive enzymes, alkaline phosphatase, mineral content were determined, the balance of nutrients, nitrogen absorption, and the availability of amino acids were assessed.

Blood (2 ml) was taken 1 day before the slaughter of a bird (at the age of 47 days) from the axillary vein (cutanea ulnaris) on the inner side of the wing above the elbow joint. The puncture site was clamped with a sterile swab for several minutes. Samples for biochemical studies were taken into sterile vacuum tubes with lithium heparin (4.0 ml; Shandong Weigao Group Medical Polymer Co., Ltd., China), for morphological studies with anticoagulant K3-EDTA (2.0 ml; SOYAGREENTEC Co., Ltd., South Korea). To separate plasma from formed elements, the samples were centrifuged in a Hettich EBA 200 centrifuge (Andreas Hettich GmbH & Co. KG, Germany) at 5000 rpm for 5 min.

Feed samples for analysis were taken according to GOST 13496.0-2016 [18] from bags. The arbitration sample was prepared from an average sample weighing 1 kg. Each food sample was analyzed 3 times. Sample preparation was carried out according to GOST 34140-2017 [17]. The quantitative content of T-2 and HT-2 toxins in the original compound feed was measured twice in each analyzed sample by tandem high-performance liquid chromatography-mass spectrometry (HPLC-MS/MS) (Agilent 1260 Infinity chromatographic system, Agilent Technologies, Germany; a mass spectrometer AB SCIEX Triple Quad™ 5500, Applied Biosystems, USA; Gemini® C18 chromatographic column with a reverse-phase sorbent based on silica gel with an organic polymer, particle size 5 μm, 150×4.6 mm, Phenomenex, USA) [18].

Amylase in duodenal contents and litter was determined according to Smith-Roy in the modification for high enzyme activity [19], protease activity was determined by hydrolysis of casein purified according to Hammersten (colorimetric control at $\lambda = 450$ nm), lipase, alkaline phosphatase, calcium and phosphorus content were measured (a semi-automatic biochemical analyzer SINNOWA BS-3000P, SINNOWA Medical Science & Technology Co., Ltd, China) with a set of veterinary diagnostic reagents (DIAKON-VET, Russia). Biochemical blood tests were performed on a Sinnowa BS-3000P semi-automatic biochemical analyzer (SINNOWA Medical Science & Technology Co., Ltd., China) with a kit for determining total protein, alkaline phosphatase, glucose, cholesterol, triglycerides, lipase (DIAKON-VET, Russia). Trypsin activity in blood plasma was measured on a semiautomatic biochemical analyzer BS-3000P by the kinetic method [20] using Na-benzoyl-DL-arginine-p-nitroanilide (BAPNA, Acros Organics, Switzerland) as a substrate. Morphological blood tests were performed on a DF-50 automatic hematological analyzer for veterinary medicine (Dymind Biotech, China) using branded reagents.

The JMP Trial 14.1.0 software (https://www.jmp.com/en_us/software/data-analysis-software.html) was used for statistical processing of the results. The results are presented as arithmetic means (*M*) and standard deviations (±SD). Significance of differences was determined by Student's t-test, differences were considered statistically significant at $p < 0.05$.

Results. Preparations for the neutralization of mycotoxins affected the enzymatic activity in the duodenal contents (Table 1). Amylolytic activity increased in group II by 34.5% ($p < 0.05$), in group III by 40.9%, in group IV by 44.1%, in group V by 55.6% compared to the control. Proteolytic activity increased statistically significantly ($p < 0.05$) in groups IV and V by 38.5 and 22.9%, respectively. Trypsin activity increased only in group IV (by 22.8%, $p < 0.05$). The lipolytic activity of the duodenal contents tended to increase in all experimental groups, but the indicator changed statistically significantly ($p < 0.05$) only in group V by 19.3% compared to group I. It should be noted that the increase in protease activity in the duodenal chyme in group IV was more pronounced than in group II, where the enzyme preparation was not used (indicators differed by 15.5%, $p < 0.05$).

Alkaline phosphatase produced during the destruction of cells of bone tissue, liver, intestines, performs the function of hydrolysis of monoester compounds of phosphoric acid with the formation of alcohol [21]. In groups treated with T-2 toxin, the activity of the enzyme significantly increased compared to the control, in group II by 13.9%, in group III by 22.3% ($p < 0.05$), in group IV by 66.5% ($p < 0.05$), in group V by 68.7% ($p < 0.05$). This indicates degenerative processes in the intestinal tissue, aimed at adaptation to the action of the toxin [22]. The total phosphorus in the duodenal contents increased in group IV by 62.1% ($p < 0.05$). Therefore, the studied preparations have the ability to neutralize the negative effect of mycotoxin on the intestines.

1. Activity of duodenal enzymes in broiler chickens (*Gallus gallus* L.) Smena 8 cross fed additive Zaslon 2+ and the Axtra Pro enzyme preparation under experimental T-2 toxicosis (absolutely dry matter, $n = 5$, $M \pm SD$; vivarium, FSC ARRTPI RAS, 2021)

Parameter	Group				
	I (control)	II	III	IV	V
Amylase, mg · ml · min^{-1}	2722±278.4	3662±244.9*	3837±234.7*	3922±131.0*	4237±240.3*
Proteases, mg · ml · min^{-1}	161±15.3	193±6.4	203±19.2	223±2.9*	198±2.7*
Trypsin, U/l	6770±339.2	7371±201.4	7609±408.5	8313±206.5*	7202±215.4
Lipase, U/l	17482±1225.2	18784±1731	18289±911.2	21941±2172.7	20863±312.4*
Alkaline phosphatase, U/l	130085±6466.6	148260±7699.4	159106±6269.9*	216656±14191.5*	219429±8676.0*
Calcium, mmol/l	243±5.5	258±14.4	248±9.9	245±3.5	224±4.5*
Phosphorus, mmol/l	161±29.5	172±22.6	180±20.3	261±6.5*	226±12.6

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

Determination of the activity of digestive enzymes in poultry litter serves as a diagnostic test for assessing intestinal health [31]. The activity of total proteases and alkaline phosphatase in the litter of broiler chickens of the Smena 8 cross did not change significantly when the feed sorbent Zaslon 2+ and the enzyme preparation Axtra Pro were added to the diet against the background of experimental mycotoxicosis (Table 2). The exception was the activity of alkaline phosphatase, which decreased by 28.1% ($p < 0.05$) in group IV vs. control. In the litter, ther was a statistically significant ($p < 0.05$) decrease of amylolytic activity (by 46.7% in group II, by 66.7% in group III, by 73.3% in group IV, and by 40.0% in group V), the activity of lipase (by 48.9, 73.1, 49.4, 80.4%, respectively) and trypsin (respectively by 34.7, 18.8, 30.2, 26.8%) compared to group I.

2. Activity of digestive enzymes in the litter of broiler chickens (*Gallus gallus* L.) Smena 8 cross fed additive Zaslon 2+ and the Axtra Pro enzyme preparation under experimental T-2 toxicosis (absolutely dry matter, $n = 5$, $M \pm SD$; vivarium, FSC ARRTPI RAS, 2021)

Parameter	Group				
	I (control)	II	III	IV	V
Amylase, mg · ml · min^{-1}	900±69.4	480±0.1*	300±23.1*	240±0.1*	540±3.1*
Proteases, mg · ml · min^{-1}	45±5.8	36±6.3	35±1.9	49±5.3	35±5.8
Lipase, U/l	9586.5±678.03	4902.0±135.64*	2585.0±193.44*	4852.0±343.35*	1881.5±301.54*
Alkaline phosphatase, U/l	72977±3803.5	60833±6603.1	67559±3075.7	52479±3840.8*	62528±3410.4
Trypsin, U/l	3883.5±146.60	2537.0±94.02*	3154.5±90.75*	2712.0±112.90*	2842.0±29.28*

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

The decrease in the activity of digestive enzymes in the feces could be due to several reasons: the return of enzymes from the small intestine to the blood [23], the degradation of enzymes by serine proteinases [24], the inactivation of enzymes by specific inhibitors, or their absorption by the intestinal microflora [25].

In general, the results of our experiment allow us to conclude that the drugs used to neutralize toxins correct the cycles of digestive enzymes [26] and alkaline phosphatase in the intestine and have a positive effect on enteral homeostasis.

How efficient the process of protein digestion is and what is the availability of nitrogen in experimental T-2 toxicosis can be judged by the balance of the protein components of the feed and amino acids [27]. With a high feed contamination with T-2 toxin (0.4 mg/kg), the digestibility of crude protein in group III was lower by 2.8% (with the addition of a sorbent to the feed), in group V by 2.7% (with the addition of sorbent and protease to the feed) vs. control (Table 3). Nitrogen uptake decreased in the groups receiving the sorbent by 14.1% for 0.1 mg/kg T-2 toxin and 20.1% for 0.4 mg/kg, when using the sorbent and the enzyme preparation by 14.7 and 22.3% ($p < 0.05$), respectively. The greatest decrease in

crude protein digestibility and nitrogen availability occurred in groups where for feed contamination 0.4 mg/kg T-2 toxin was used.

3. Crude protein digestibility and nitrogen assimilation in broiler chickens (*Gallus gallus* L.) Smena 8 cross fed additive Zaslon 2+ and the Axtra Pro enzyme preparation under experimental T-2 toxicosis (absolutely dry matter, $n = 5$, M±SD; vivarium, FSC ARRTPI RAS, 2021)

Parameter	Group				
	I (control)	II	III	IV	V
Crude protein, %	90.75±0.35	90.57±1.26	88.20±1.34	90.33±1.02	88.31±1.32
Nitrogen assimilation, %	62.96±1.43	54.08±3.75	50.34±5.68	53.69±4.88	48.90±3.98*

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

4. Availability of amino acids in the intestines of broiler chickens (*Gallus gallus* L.) Smena 8 cross fed additive Zaslon 2+ and the Axtra Pro enzyme preparation under experimental T-2 toxicosis (absolutely dry matter, $n = 5$, M±SD; vivarium, FSC ARRTPI RAS, 2021)

Amino acid	Group				
	I (control)	II	III	IV	V
Aspartic	78.56±0.82	78.78±2.84	74.52±2.91	77.79±2.34	75.79±2.73
Threonine	80.35±0.75	80.90±2.55	75.36±2.81	79.81±2.13	77.70±2.51
Serene	80.02±0.77	80.59±2.60	74.95±2.86	79.89±2.12	77.36±2.55
Glutamine	89.74±0.39	89.39±1.42	88.26±1.34	89.66±1.09	88.43±1.30
Proline	87.39±0.48	87.03±1.73	84.19±1.80	86.54±1.42	84.81±1.71
Glycine	54.56±1.75	51.95±6.43	41.92±6.64	39.50±6.39	32.33±7.63*
Alanine	80.54±0.75	80.02±2.67	78.68±2.43	80.20±2.09	78.39±2.43
Valine	81.29±0.72	80.77±2.57	77.76±2.54	70.02±2.11	78.65±2.41
Isoleucine	83.82±0.62	83.65±2.18	80.95±2.17	82.72±1.82	81.71±2.06
Leucine	84.36±0.60	84.20±2.11	81.74±2.08	83.54±1.74	82.19±2.00
Tyrosine	81.53±0.71	81.89±2.42	77.78±2.54	80.27±2.08	79.33±2.33
Phenylalanine	84.43±0.60	84.27±2.10	81.99±2.05	84.16±1.67	82.71±1.95
Histidine	77.08±0.88	79.16±2.79	70.71±3.34	76.14±2.52	72.58±3.09
Lysine	84.81±0.58	84.65±2.05	80.58±2.21	83.47±1.74	82.04±2.02
Arginine	86.20±0.53	86.32±1.83	84.35±1.78	86.37±1.44	84.98±1.69
Cystine	77.15±0.88	77.72±2.98	72.82±3.10	76.44±2.48	74.86±2.83
Methionine	91.24±0.33	89.98±1.34	89.56±1.19	89.40±1.11	89.97±1.13

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

The pattern was similar to the assimilation of amino acids in the intestines of broilers (Table 4). Despite the trend towards a decrease in the availability of amino acids in groups III and V, only the assimilation of the amino acid glycine in group V decreased statistically significantly (by 40.8%, $p < 0.05$). This may affect the processes of inhibition in the central nervous system, since glycine serves as a mediator in the transmission of nerve impulses [28]. Consequently, contamination of feed with T-2 toxin for 14 days had a negative effect on protein metabolism, reducing the digestibility of crude protein and the availability of glycine in group V.

We did not observe deviations in the general condition of broilers during the experiment, but we performed a morphobiochemical blood test in order to identify changes in the body during experimental mycotoxicosis (Tables 5, 6).

In groups IV and V, there was an increase in trypsin activity in blood plasma, respectively, by 75.7 and 58.4% ($p < 0.05$) compared with the control (see Table 5). These indicators exceeded the physiological norm and could indicate an inflammatory process in the intestine and pancreatic tissues [29], which is associated with the presence of PARs (proteinase-activated receptors) receptors that regulate cellular signaling and can cause an immune inflammatory response [30]. With mycotoxicoses in the digestive organs of broilers, apoptosis phenomena may occur when caspase is activated in the pancreatic tissue [7]. Trypsin activity increased in

groups IV and V which received a protease supplement along with the sorbent, by 110.3% ($p < 0.05$) and 103.2% ($p < 0.05$), respectively, compared to groups II and III. The activity of alkaline phosphatase decreased ($p < 0.05$) when using dietary sorbent, in group II by 39.5% and in group III by 60.8%. The phosphatase-protease index decreased 2.0-fold compared to control. Total protein in group III increased by 10.3% ($p < 0.05$) while in group IV it decreased by 19.3% ($p < 0.05$), that is, it depended on the blood trypsin activity. The change in protein metabolism occurred in group V with an increase in the amount of uric acid by 175.9% ($p < 0.05$), which indicates the inefficient use of nitrogen in birds when the feed was contaminated with T-2 toxin at maximum dosage (0.4 mg/kg). In other experimental groups, there was a negative trend in the use of amino acids. This indicates a violation of protein metabolism confirmed by a decrease in the assimilation of nitrogen by birds in the experimental groups (see Table 3).

5. Blood biochemical parameters in broiler chickens (*Gallus gallus* L.) Smena 8 cross fed additive Zaslon 2+ and the Axtra Pro enzyme preparation under experimental T-2 toxicosis (absolutely dry matter, $n = 5$, $M \pm SD$; vivarium, FSC ARRTPI RAS, 2021)

Parameter	Group				
	I (control)	II	III	IV	V
Trypsin, U/l	244.4±15.72	204.2±1.38	190.5±3.20	429.4±3.67*	387.2±29.90*
Alkaline phosphatase, U/l	3525±564.3	2133±167.3*	1382±34.3*	2384±28.9	4710±416.9
Phosphate-protease index	14.4	10.4	7.2	5.5	12.1
Total protein, g/l	44.5±1.18	46.1±1.68	49.1±0.67*	35.9±1.72*	46.2±1.71
Uric acid, rmol/l	177.1±18.52	223.4±15.25	217.0±8.12	202.7±10.70	488.4±25.68*
Glucose, mmol/l	8.2±0.58	10.8±0.05*	9.6±0.09*	11.6±0.17*	12.4±0.24*
Cholesterol, mmol/l	2.8±0.14	1.8±0.04*	2.5±0.16	2.6±0.20	3.7±0.05*
Triglycerides, mmol/l	0.28±0.02	0.57±0.06*	0.33±0.01*	0.43±0.01*	0.35±0.04
Calcium, mmol/l	2.6±0.07	3.6±0.06*	2.8±0.05	1.9±0.01*	2.4±0.13
Phosphorus, mmol/l	2.0±0.07	1.6±0.07*	2.2±0.03*	1.6±0.04*	1.8±0.09

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

6. Hematological parameters in broiler chickens (*Gallus gallus* L.) Smena 8 cross fed additive Zaslon 2+ and the Axtra Pro enzyme preparation under experimental T-2 toxicosis ($n = 5$, $M \pm SD$; vivarium, FSC ARRTPI RAS, 2021)

Parameter	Group				
	I (control)	II	III	IV	V
Leukocytes, 10^9/l	41.2±4.36	37.2±1.95	62.4±7.79*	58.0±2.09*	38.5±4.87
Heterophiles, %	51.7±3.58	65.1±1.79*	54.3±0.61	58.2±4.29	64.3±4.92*
Eosinophils, %	8.4±0.97	8.8±2.29	8.5±1.04	7.3±0.37	7.2±1.68
Basophils, %	0.1±0.01	0.1±0.02	0.2±0.05	0.1±0.01	0.1±0.02
Lymphocytes, %	37.6±1.37	22.6±2.29*	35.6±1.35	34.3±1.79	27.8±2.70*
Monocytes, %	2.2±0.30	3.4±0.52	1.4±0.13	0.2±0.02	0.1±0.04
Erythrocytes, $\times 10^{12}$/l	3.5±0.15	3.3±0.11	4.4±0.34*	4.1±0.05*	3.3±0.24
Hemoglobin, g/l	165.3±6.15	154.3±4.71	200.5±12.34	200.8±4.56	164.5±14.44
Hematocrit, %	44.3±2.20	42.2±1.30	52.6±3.58	54.7±1.31	43.5±4.65

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

A clear parameter to characterize the state of carbohydrate metabolism in broiler chickens was the blood glucose concentration which increased statistically significantly ($p < 0.05$) in all test broilers, in group II by 31.7%, in group III by 17.1%, in group IV by 41.5%, and in group V by 51.2%. This can be explained by the stress developed under an experimental mycotoxicosis [31].

The state of lipid metabolism is determined by the amount of cholesterol and triglycerides in the blood. The greatest increase in cholesterol levels in our experience was observed in group V by 32.1% compared to the control ($p < 0.05$). All experimental groups showed an increase in the amount of lipids in blood plasma but the most significant changes occurred in groups II (by 103.6%, $p < 0.05$)

and IV (by 53.6%, $p < 0.05$).

Hematological parameters of broilers reflected the metabolic and immune status of the bird (see Table 6). The number of leukocytes in test group III increased by 51.4% ($p < 0.05$) and in group IV by 40.8% ($p < 0.05$) compared to the control. Analysis of the leukoformula showed that in groups II and V the number of heterophils increased by 25.9 and 24.4%, respectively, which indicated an increase in resistance under the action of T-2 toxin [32]. The decrease in the number of lymphocytes in the experimental groups can be explained by changes in the lymphoid tissue of the intestines of chickens under the influence of T-2 toxin [33]. The most noticeable changes we observed in groups II (by 39.9%, $p < 0.05$) and V (by 26.1%, $p < 0.05$). In chickens treated with a protease sorbent as an additive, the number of monocytes was significantly lower (by 94.1 and 92.9%, $p < 0.05$) than in those receiving only the sorbent, which was apparently associated with a decrease in inflammatory response. The number of erythrocytes in the blood of a bird is closely related to oxidative processes in the body. It can be assumed that in groups III and IV, metabolism was activated due to an increase in the number of erythrocytes in the blood, hemoglobin and hematocrit. With an increase in these indicators, blood oxygen saturation increased markedly.

The mechanisms of action of mycotoxins on the digestive system of animals remain poorly understood, although the relevance of this problem is associated with the health of people who consume meat, including poultry meat [34]. Earlier, in a chronic experiment in vivo on broilers, we first studied the physiological and biochemical processes during the development of T-2 toxicosis and proposed methods for diagnosing the disease [24]. In this work, we considered the effect of the sorbent as a means of preventing mycotoxicoses in various ways of its application. An increase in the efficiency of the sorbent in combination with the protease indicates the promise of these studies and the expediency of their continuation [35].

It is known that cellular enzymes play an important role in detoxification in birds. S.Yu. Gulyushin and V.O. Kovalev [36] note that the generation of free radicals (free forms of oxygen) and antiradical (antioxidant) protection are in dynamic equilibrium. Under the equilibrium disturbance in poultry fed mixed feed contaminated with mycotoxins, an oxidative stress occurred together with the inhibition of the main enzymes of antiradical protection (catalase, peroxidase, superoxide dismutase, glutathione peroxidase, glutathione-S-transferase, glutathione reductase) and the accumulation of a larger amount of hydroperoxide products. As a result, there was a destabilization of biological membranes, a violation of homeostasis, oxygenation and tissue trophism, and a potentiation of various cytopathogenic effects. The authors suggest selenium preparations to increase the activity of serum and cellular antiradical protection factors, to normalize physiological and biochemical processes and, as a result, to provide a prolonged effect of productivity correction in the combined chronic mycotoxicoses. The results of studies [36] showed that Selexen and DAFS 25 had the most effective prophylactic properties in the experiments while the mineral salt Na_2SeO_3 was slightly inferior. The authors also came to the conclusion that monotherapy with the use of selenium preparations is not effective enough. It seems appropriate to combine it with other available methods of nonspecific prophylaxis that stimulate protein synthesis and/or excretion of xenobiotics.

The results of our study of a complex preparation for broiler chickens showed for the first time that the activity of digestive enzymes in the duodenal chyme and blood can serve as a criterion for evaluating the effectiveness of this preparation. Taking into account the observed decrease in the phosphatase-protease index in the test groups, it can be argued that the transition of the T-2 toxin

to the HT-2 metabolite [19] is slowed down due to the sorption capacity of the drug. The negative effect of T-2 toxin on protein metabolism follows from a decrease in nitrogen utilization in all test groups and a trend towards a decrease in the availability of amino acids, especially at 0.4 mg/kg T-2 toxin. The blood biochemical parameters indicate a violation of protein, fat and carbohydrate metabolism of broiler chickens, as well as signs of stress caused by the influence of the toxin on digestive organs, the pancreas and liver. The immune status of birds in experimental T-2 toxicosis changed mainly due to an increase in the number of heterophils capable of phagocytosis [37]. The number of lymphocytes, immunocompetent cells that counteract pathogenic biological agents, decreased, which makes the bird less protected from infectious diseases [38, 39].

Thus, in the experimental T-2 toxicosis of Smena 8 cross broiler chickens, the Zaslon 2+ sorbent in combination with the Axtra Pro enzyme preparation containing protease lead to more pronounced changes in the activity of duodenal enzymes than the sorbent alone. There was a statistically significant increase in total proteolytic activity (by 15.5%, $p < 0.05$), trypsin activity (by 12.8%, $p < 0.05$), alkaline phosphatase activity (by 46.1%, $p < 0.05$), total phosphorus (by 25.6%, $p < 0.05$). Amylase activity in the litter decreases 2.0-fold. The activity of blood trypsin in the groups fed the protease supplement along with the sorbent increased by 110.3% ($p < 0.05$) and 103.2% ($p < 0.05$) compared to the birds that received only Zaslon 2+. Also, the combined use of the sorbent and protease provides a higher number of blood lymphocytes (by 51.8%, $p < 0.05$). The obtained results suggest that in mycotoxicoses the correction of protein metabolism by the toxin sorbent in combination with proteases is associated with the stimulation of digestive enzyme activity and metabolism. We plan to continue these studies to gain a more complete understanding the described processes and to develop a new drug for the prevention and complex therapy of mycotoxicosis.

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COMPOSITION AND METABOLIC POTENTIAL OF THE INTESTINAL MICROBIOME OF *Gallus gallus* L. BROILERS UNDER EXPERIMENTAL T-2 TOXICOSIS AS INFLUENCED BY FEED ADDITIVES

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Abstract

Mycotoxins can adversely affect the composition and function of the poultry gut microbiota, with implications for host health. The introduction of feed additives into contaminated feed is a strategy for restoring the intestinal microbiome under mycotoxicoses. This paper shows for the first time that the feed additive Zaslon 2+ effectively improves the structure and metabolic potential of the intestinal microbiome in broiler chickens with experimental T-2 mycotoxicosis. Our goal was to identify changes in the chyme microbiota and its functional annotation after 14-day exposure to T-2 toxin, artificially introduced with feed, and under the influence of the feed additive Zaslon 2+, fed alone and in combination with the proteolytic drug Axtra Pro. The experiments were carried out in the vivarium of the Federal Scientific Center ARRTPI RAS in 2021. Broiler chickens of the Smena 8 cross aged 33 days were assigned into four groups of 5 birds each. Control group I received a basal diet (BD) without T-2 toxin, group II was fed with BD added with T-2 toxin (200 μg/kg) (BD + T-2), group III — BD + T-2 + additive Zaslon 2+ (1 g/kg feed) (BIOTROF Ltd, Russia), group IV — BD + T-2 + additive Zaslon 2+ added with proteolytic preparation Axtra Pro (DuPont de Nemours, Inc., USA) (100 mg/kg feed). Zaslon 2+ contains diatomite, two *Bacillus* strains, and a mixture of natural essential oils (eucalyptus, thyme, garlic, and lemon). Feed intake averaged 150 g/day, i.e. the birds of the experimental groups received 30 μg T-2 toxin daily. At the end of the experiment, the caecum content was sampled from three broilers of each group. Total DNA was isolated from the samples using the Genomic DNA Purification Kit (Fermentas, Inc., Lithuania). The caecal bacterial community was assessed by NGS sequencing on the MiSeq platform (Illumina, Inc., USA) using primers for the V3-V4 region of the 16S rRNA gene. Bioinformatic data analysis was performed using QIIME2 ver. 2020.8 (https://docs.qiime2.org/2020.8/). The reconstruction and prediction of the functional content of the metagenome, gene families, and enzymes was carried out using the PICRUSt2 (v.2.3.0) software package (https://github.com/picrust/picrust2). MetaCyc base data (https://metacyc.org/) was used to analyze metabolic pathways and enzymes. NGS-sequencing revealed changes in biodiversity and composition of the gut microbiota at the level of phyla. I.e., in group II, the population of superphylum *Actinobacteriota* and phylum *Proteobacteria* increased 1.8 and 3.5 times, respectively ($p \le 0.05$) while the superphylum *Desulfobacterota*, on the contrary, decreased 2.2 times ($p \le 0.05$). In group IV (BD + T-2 supplemented with Zaslon 2+ and Axtra Pro), the abundance of superphylum *Actinobacteriota* and phylum *Proteobacteria* also increased compared to group I ($p \le 0.05$), while in group III (BD + T-2

supplemented with Zaslon 2+) no change occured. The members of superphylum *Verrucomicrobiota* completely disappeared in groups II and IV, while in group I they accounted for 14.1±0.8 %. In group III compared to group I, bacteria of the genus *Lactobacillus* increased ($p \leq 0.01$) from 15.9±1.32 to 30.7±1.84 %. The genus *Akkermansia* represented by the only species *A. muciniphila* sharply decreased in all groups fed T-2 toxin (groups II, III, and IV) as compared to group I ($p \leq 0.001$), up to a complete absence in groups II and IV. Pathogenic microorganisms which were absent in group I (*Enterococcus cecorum*, *Campylobacter concisus*, *Campylobacter gracilis*, *Streptococcus gordonii*, *Flavonifractor* spp.) appeared in group II. In groups III and IV, these pathogens were either absent or were present in a significantly smaller amount than in group II ($p \leq 0.05$). Gut microbial community showed differences between groups ($p \leq 0.05$) in 163 predicted metabolic pathways. When exposed to T-2 toxin (group II compared to group I, $p \leq 0.05$), there was an increase in the predicted metabolic pathways for the degradation of aromatic compounds, including xenobiotics, and amino acids and for the synthesis of coenzymes, cofactors and formation of biofilms, cell walls, spores and protective substances in cells. The feed additive Zaslon 2+ contributed to the adjustment of metabolic pathways to the level of group I. The combined use of the feed additive Zaslon 2+ and protease (group IV) had no positive effect on the potential of metabolic pathways. Thus, feed contamination with T-2 toxin has a negative impact on the composition and predicted metabolic potential of the gut microbiome of Smena 8 cross broiler chickens. In general, the effect of the feed additive Zaslon 2+ and its complex with protease was positive though the additive without the enzyme showed greater efficiency.

Keywords: mycotoxins, T-2 toxin, broilers, gut microbiome, gene expression, poultry

Mycotoxins, the compounds of the secondary metabolism in molds, are highly toxic to animals, birds and humans [1)]. Micromycetes of the genera *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria*, *Phomopsis*, *Emericella*, *Cephalosporium*, *Myrothecium*, *Trichoderma*, *Trichothecium*, *Neopetromyces*, *Byssochlamys*, *Neotyphodium* and *Claviceps* are responsible for their production. On average, 25% of the world's agricultural products and feeds are contaminated to some extent with mycotoxins [2], which causes mass poisoning of both people and farm animals in many countries [1].

T-2 toxin is one of the most dangerous mycotoxins [3]. It belongs to trichothecenes [4] produced by micromycetes of the genera *Fusarium*, *Myrothecium* and *Stachybotrys*. T-2 toxin is commonly found in grains of wheat, corn, barley, rice, soybeans, oats [5], its derivatives and compound feeds, including compound feed for poultry [6].

The main symptoms of T-2 toxicoses in poultry include hemorrhagic necrotizing ulcerative inflammation of the digestive tract with thickening of the mucous membrane, staggering gait and refusal to feed [7]. It has been noted [8] that acute intoxication in broiler chickens is manifested by symptoms of internal hemorrhage, lesions of the oral cavity and skin (necrohemorrhagic dermatitis), deterioration of the quality of feathers and disorders of the nervous system. At low doses, a significant decrease in the amount of hemoglobin in the blood of broiler chickens was observed [8]. In addition, when exposed to low doses of T-2, a decrease in total protein and cholesterol and an increase in serum uric acid and lactate dehydrogenase were noted [9, 10], which convincingly proves the toxic effect of T-2 toxin even at low doses. Pathological examinations usually reveal fatty degeneration and severe granular degeneration in the liver, kidneys, and rarely in the heart [11].

Acting as a selective permeable barrier places the intestinal mucosal epithelium at the center of interactions between the mucosal immune system and chyme, which includes normal flora, pathogens, and food toxicants [12]. It is known that mycotoxins can somehow modulate the composition of the gut microbiota, which has a detrimental effect on the health of the host [13]. When exposed to toxins, the integrity of the intestinal epithelium is disrupted, as a result of which pathogenic microorganisms penetrate into the macroorganism [14]. At the same time, the gut microbiota can metabolize mycotoxins, thereby converting them into different chemical structures with greater or lesser toxicity than the

original compounds [13]. It appears that the gut microbiome not only has a direct metabolic potential for xenobiotics, but also influences gene expression and host enzyme activity. W. Meinl et al. [15] demonstrated that the gut microbiota affects the expression of xenobiotic detoxification-associated *GSTs*, *GPX2*, *EPHXs*, and *NNAT 1* genes in colon and liver tissues of the host. Studies on the composition of the microbiome of animals and birds under the influence of mycotoxins are extremely limited [16, 17]. An analysis of changes in the predicted functional potential of the microbiome of animals, birds and humans using bioinformatic software systems such as PICRUSt2 and the like has not been previously carried out. The effect of T-2 toxin on the composition and functional potential of the gut microbiome in birds has also not been studied.

For the prevention of mycotoxicosis, sorbents are used that selectively bind toxins during digestion, preventing their absorption from the gastrointestinal tract and, consequently, reducing the toxic effect. It is known that enrichment of feed with probiotics, amino acids, lipids, enzymes has a positive effect and reduces the symptoms of T-2 toxicosis [3]. For example, the inclusion of bacterial cultures of *Lactobacillus* spp. in the diet of broiler chickens reduced the toxic effects of aflatoxin B_1, zearalenone [18] and DON [19]. H. Tozaki et al. [20] demonstrated that a promising approach for the degradation of xenobiotics could be the use of enzymes, in particular proteases.

In this work, for the first time, we established changes in the composition of the broiler microbiome under the influence of the feed additive Zaslon 2+ and the proteolytic enzyme Axtra Pro against the background of artificial contamination of feed with T-2 toxin.

Our goal was a comprehensive analysis of the effect of the 14-day exposure to T-2 toxin added to the feed, as well as the feed additive Zaslon 2+ and the proteolytic enzyme Axtra Pro, on the composition and functional potential of the chyme microbiome of the Smena 8 cross broiler chickens.

Materials and methods. The 1-day experiment was carried out in 2021 in the vivarium of the All-Russian Research and Technological Institute of Poultry Farming on broiler chickens (*Gallus gallus* L.) of the Smena 8 cross from 33 to 47 days of age in accordance with the requirements of the European Convention for the Protection of Vertebrate Animals used for experiments or for other scientific purposes (ETS No. 123, Strasbourg, 1986) [21]. Feeding and housing conditions met the requirements for broiler crosses [22]. For feeding, PK-6 compound feed (Russia) was used for broilers in the form of a scattering. Feeding and watering of the birds were not limited.

An experimental contamination of feed with T-2 toxin (2-fold excess of MPC) was performed mechanically in compliance with personnel safety requirements. We used a certified calibration standard T-2 toxin in the form of a powder with a mass fraction of the main substance of 99.7±0.3% (Romer Labs, Austria; cat. No. 10000310, LOT #S17052T). Before and after contamination, the compound feed was examined for the presence of mycotoxins by high-performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS, Agilent 1260 Infinity LC chromatographic system, Agilent Technologies, USA; mass spectrometer AB SCIEX Triple Quad™ 5500, AB Sciex, USA; Gemini® C18 110A 5 μm 150×4.6 mm reverse-phase separation column, Phenomenex, USA). A standard solution of T-2 toxin was used to build calibration graphs and as internal standards. In addition to the above, the diet of broilers practically did not contain background amounts of mycotoxins. The HPLC-MS/MS system was used to determine the background content of mycotoxins in feed. Standard solutions of mycotoxins (Romer Labs, Austria) were used in the construction of calibration graphs

and as internal standards. No aflatoxins (B_1, G_1), fumonisins (B_1, B_2, B_3), deoxynivalenol, T-2 toxin, zearalenone and ochratoxin A were found in the feed used for infection.

The birds were divided into four groups of 5 broilers each. In group I (control) broilers received the basal diet (BD) without T-2 toxin, in group II broilers received BD added with T-2 toxin (200 μg/kg), in group III BD added with T-2 toxin (200 μg/kg) and feed additive Zaslon 2+ which is a sorbent material of diatomite + two *Bacillus* spp. cultures, a mixture of of eucalyptus, thyme, garlic and lemon natural essential oils (OOO BIOTROF, Russia) (1 g/kg of feed), group IV received BD added with T-2 toxin (200 μg/kg), feed additive Zaslon 2+ (1 g/kg of feed) and enzyme preparation with proteolytic activity Axtra Pro (DuPont de Nemours, Inc., USA) (100 mg/kg of feed). Feed intake by broilers averaged 150 g/day, that is, the birds of the est groups received T-2 toxin daily with feed in the amount of 30 μg.

At the end of the experiment, the bird was decapitated and an autopsy was performed. Chyme samples were manually taken from the blind processes of the intestine from three birds from each group (30-50 g) with the maximum possible observance of aseptic conditions. The selected samples were immediately placed in sterile plastic centrifuge tubes. All samples were frozen at –20 °C and transported in dry ice for subsequent DNA isolation.

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

The caecal bacterial community was assessed by NGS sequencing on the MiSeq platform (Illumina, Inc., USA) with primers for the V3-V4 region of the 16S rRNA gene. Forward primer is 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3'; reverse primer is 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'.

PCR was performed as follows: 3 min at 95 °C; 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C (25 cycles); 5 min at 72 °C (final elongation). Sequencing was performed with Nextera® XT IndexKit library preparation reagents (Illumina, Inc., USA), Agencourt AMPure XP PCR product purification (Beckman Coulter Inc., USA), and MiSeq® ReagentKit v2 (500 cycle) (Illumina, Inc., USA). The maximum length of the resulting sequences was 2×250 bp.

Bioinformatic data analysis was performed using QIIME2 ver. 2020.8 (https://docs.qiime2.org/2020.8/). After importing sequences in the .fastq format from the sequencing instrument and creating the necessary mapping files containing the metadata of the studied files, paired read lines were aligned. The sequences were filtered for quality using the default settings. Noise sequences were filtered using the DADA2 method built into the QIIME2 package, which includes quality information in its error model, which makes the algorithm robust to lower quality sequences, while using a maximum trimming sequence length of 250 bp. (https://benjjneb.github.io/dada2/tutorial.html). To build a de novo phylogeny, multiple sequence alignment was performed using the MAFFT software package (https://mafft.cbrc.jp/al-ignment/software/), followed by masked sequence alignment to remove positions that differed significantly. The reference database Silva 138.1 (https://www.arb-silva.de/documentation/release-138.1/) was used for taxonomy analysis.

Based on the obtained table of operational taxonomic units (OTU), using the plugins of the QIIME2 software package, biodiversity indices were calculated,

and a graph of the dependence of the number of OTUs on the number of reads was plotted. In the statistical analysis of diversity indices, their additional transformation was not carried out.

The reconstruction and prediction of the functional content of the metagenome, gene families, and enzymes was carried out using the PICRUSt2 (v.2.3.0) software package (https://github.com/picrust/picrust2) (23). We worked with the program according to the recommended scenario of actions, all settings were used by default. The OTUs of each sample were arranged according to their content, from largest to smallest, and the values were converted using the logarithmic transformation of Log2. The MetaCyc database (https://metacyc.org/) was used to analyze metabolic pathways and enzymes. The predicted profiles of MetaCyc metabolic pathways were assessed by the abundance of ASV (Amplicon Sequence Variants) [24]. Data visualization and calculation of statistical indicators were performed using the Phantasus v1.11.0 web application (https://artyomovlab.wustl.edu/phantasus/), which, in addition to the main visualization and filtering methods, supports R-based methods such as like k-means clustering, principal component analysis, or differential expression analysis with the limma package.

Mathematical and statistical data processing was carried out by the method of multifactor analysis of variance (ANOVA) in Microsoft Excel XP/2003, R-Studio (Version 1.1.453) (https://rstudio.com). Results are presented as means (*M*) and standard errors of the means (±SEM). Significance of differences was established by Student's *t*-test, differences were considered statistically significant at p 0.05. Means were compared using Tuke's significant difference (HSD) test and the TukeyHSD function in the R Stats Package.

Results. When performing NGS-sequencing of the microbiome, a total of 85,121 sequenced sequences of the 16S rRNA gene were generated (with a median of reads of 7.253, min = 3.330, max = 10.859). The number of OTUs did not differ significantly ($p > 0.05$) between the experimental groups (Fig. 1).

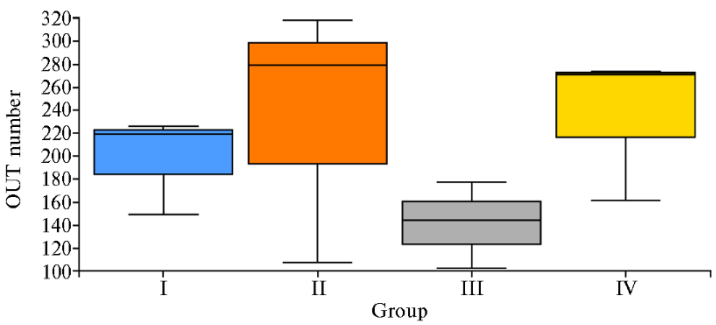

Fig. 1. The number of operational taxonomic units (OTU) **according to the results of NGS sequencing of the intestinal microbiome of broiler chickens (*Gallus gallus* L.) Smena 8 cross fed T-2 toxin, additive Zaslon 2+** (BIOTROF LLC, Russia) **and enzyme drug with proteolytic activity Axtra Pro** (DuPont de Nemours, Inc., USA) (vivarium, FSC ARRTPI RAS, 2021). For a description of the groups, see the Materials and methods section.

When compared by the Simpson index, it turned out that in group III, biodiversity decreased ($p \leq 0.05$) vs. control (group I, Fig. 2). We assume that the decrease in biodiversity is associated with the stabilization of the microbiome under the influence of the feed additive Zaslon 2+. According to J. Knol et al. [25], this can have a beneficial effect, as a large number of interacting species often tend to have a destabilizing effect on the microbiome. It has been previously shown that prebiotics, by inhibiting the growth of potentially pathogenic *Clostridium* spp. and *Salmonella* spp., contributed to the formation of more stable gut microbial communities with low biodiversity [26]. In group IV, there was an increase

($p \leq 0.05$) in biodiversity in the intestine compared to group III (see Fig. 2) which is probably associated with the introduction of a proteolytic enzyme. J.M. Lourenco et al. [27] reported that the addition of protease to the diet of broilers had an effect on both the richness and diversity of microbial populations: with the introduction of the enzyme, the number of OTUs ($p = 0.04$) and the value of the Chao1 index ($p = 0.09$) increased.

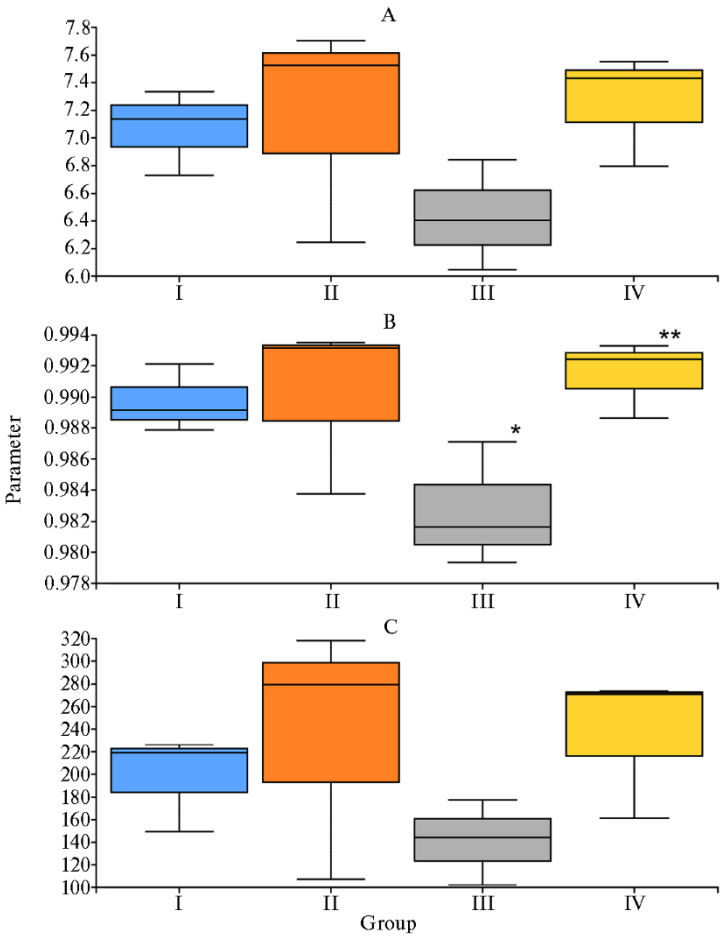

Fig. 2. Absolute values of the Shannon (A)**, Simpson** (B)**, and Chao1 (C) biodiversity indices for the gut microbiome of broiler chickens (*Gallus gallus* L.) Smena 8 cross fed T-2 toxin, additive Zaslon 2+** (BIOTROF LLC, Russia) **and enzyme drug with proteolytic activity Axtra Pro** (DuPont de Nemours, Inc., USA) (vivarium, FSC ARRTPI RAS, 2021). Calculated using plugins of QIIME2 ver. 2020.8. For a description of the groups, see the Materials and methods section.
*, ** Differences are statistically significant at $p \leq 0.05$ when compared to groups I and III.

The intestinal microbiome of birds from all groups contained 15 bacterial phyla and superphyla (Fig. 3). The phyla *Firmicutes*, *Verrucomicrobiota*, *Proteobacteria*, *Actinobacteriota*, *Bacteroidota* dominated of which the phylum *Firmicutes* was the most abundant (from 71.1±3.9 to 94.8±5.7%). The predominance of bacteria of the phylum *Firmicutes* in the poultry intestinal microbiome has been shown previously [28, 29]. An important function of *Firmicutes* is the ability to degrade complex polysaccharides with subsequent formation of short-chain fatty acids [30]. Short-chain fatty acids play an important role in host energy metabolism by promoting the growth and normal functioning of intestinal cells [31].

In the intestines of birds from group II, when T-2 toxin was introduced into the feed, bacteria of the superphylum *Actinobacteriota* and phylum *Proteobacteria*

became 1.8-fold and 3.5-fold more abundant ($p \leq 0.05$), while the bacteria of the superphylum *Desulfobacterota* decreased 2.2-fold ($p \leq 0.05$). In the intestines of broilers from group IV, the number of bacteria of the superphylum *Actinobacteriota* and the phylum *Proteobacteria* also increased compared to group I ($p \leq 0.05$), while in group III (with Zaslon 2+ feed additive), no such changes occurred.

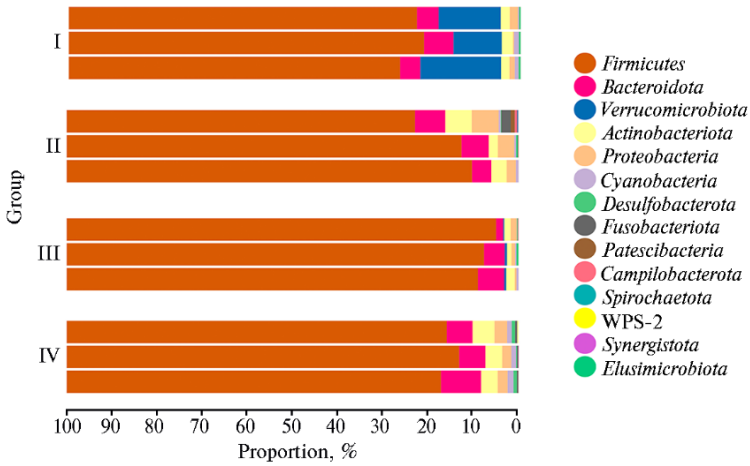

Fig. 3. The gut microbiome composition at the phyla levels (based on data of NGS sequencing of 16S rRNA gene amplicons) in broiler chickens (*Gallus gallus* L.) Smena 8 cross fed T-2 toxin, additive Zaslon 2+ (BIOTROF LLC, Russia) **and enzyme drug with proteolytic activity Axtra Pro** (DuPont de Nemours, Inc., USA) (vivarium, FSC ARRTPI RAS, 2021). For a description of the groups, see the Materials and methods section.

Interestingly, new superphyla, *Fusobacteriota* and *Spirochaetota*, appeared in the microbiome in groups II and IV (pathogens are often found among representatives of these taxa) [32, 33]. In group III, which received the feed additive Zaslon 2+, we did not identify these phyla. Particularly noteworthy is the fact that representatives of the superphylum *Verrucomicrobiota* completely disappeared in groups II and IV, while in group I, the members this superphylum was present in significant numbers (14.1±0.8%). In group III fed T-2 toxin with the Zaslon 2+ additive, their number was very low (0.3±0.03%) compared to group I ($p \leq 0.001$). It is likely that the bacteria of the superphylum *Verrucomicrobiota* are most sensitive to the T-2 toxin compared to other intestinal microbiota. It is known [34] that *Verrucomicrobiota* bacteria synthesize many glycoside hydrolases for degradation of stable polysaccharides. Therefore, a sharp decrease in their content in the intestines of birds under the influence of T-2 toxin could be associated with inhibition of fiber digestion processes.

The most abundant microorganisms among the phylum *Firmicutes* were bacteria of the genus *Lactobacillus* spp. of the *Lactobacillaceae* family (from 11.1±0.72 to 30.7±1.84% depending on the experimental group) and Clostridia_UCG-014 of the Clostridia_UCG-014 family (from 17.6±0.91 to 25.5±1.34%. Microorganisms of the phylum *Verrucomicrobiota*, bacteria of the genus *Akkermansia* of the *Akkermansiaceae* family, were also widely represented in group I (14.1±0.82%) (Fig. 4, 5). For bacteria *Lactobacillus* spp. they have also previously been shown to constitute one of the predominant groups in the gastrointestinal tract of farm birds [35, 36].

An increase in the bacteria of the genus *Lactobacillus* from 15.9±1.32 to 30.7±1.84% ($p \leq 0.01$) in the intestines of birds from group III fed feed additive Zaslon 2+ compared to group I, probably was considered positive. These microorganisms are able to produce significant amounts of lactic and acetic acids, which lower the pH values in the gastrointestinal tract [37], compete with pathogens for

nutrients and epithelial sites for adhesion [38].

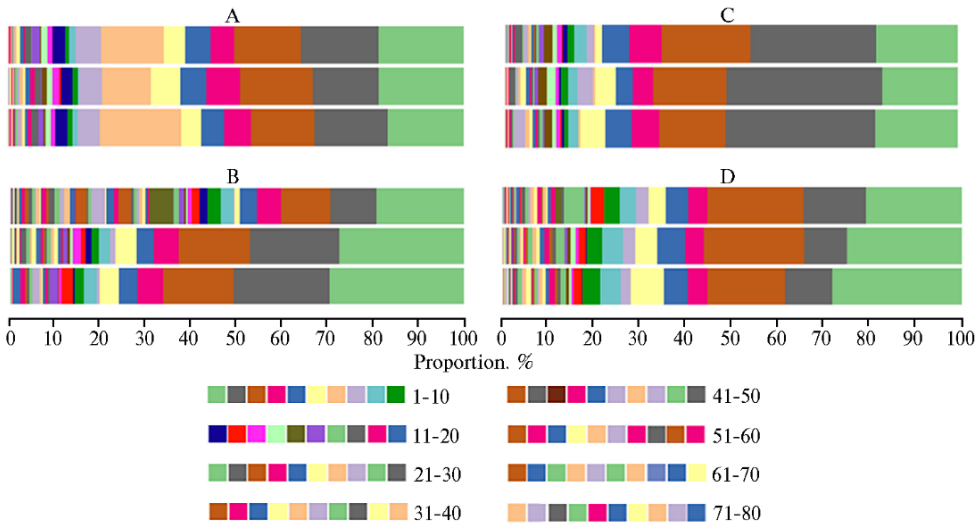

Fig. 4. The gut microbiome composition at the families levels (based on data of NGS sequencing of 16S rRNA gene amplicons) in broiler chickens (*Gallus gallus* L.) Smena 8 cross fed T-2 toxin, additive Zaslon 2+ (BIOTROF LLC, Russia) **and enzyme drug with proteolytic activity Axtra Pro** (DuPont de Nemours, Inc., USA)**:** 1-10 — Clostridia_UCG-014, *Lactobacillaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Oscillospiraceae*, *Rikenellaceae*, *Akkermansiaceae*, *Eubacterium coprostanoligenes* group, *Christensenellaceae*, RF39; 11-20 — *Erysipelatoclostridiaceae, Bifidobacteriaceae, Anaerovoracaceae, Butyricicoccaceae,* Clostridia vadinBB60 group, *Enterobacteriaceae*, *Clostridiaceae*, *Coriobacteriales*, *Gastranaerophilales*, *Peptococcaceae*; 21-30 — *Monoglobaceae*, *Erysipelotrichaceae*, *Veillonellaceae*, *Peptostreptococcaceae*, *Enterococcaceae*, *Chitinophagaceae*, *Desulfovibrionaceae*, *Prevotellaceae*, *Xanthomonadaceae*, *Pseudomonadaceae*; 31-40 — *Neisseriaceae*, *Anaerofustaceae*, *Corynebacteriaceae*, *Sphingomonadaceae*, *Actinomycetaceae*, *Leptotrichiaceae*, *Fusobacteriaceae*, *Carnobacteriaceae*, UCG-010, *Eggerthellaceae*; 41-50 — *Streptococcaceae*, *Selenomonadaceae*, *Flavobacteriaceae*, *Micrococcaceae*, *Campylobacteraceae*, *Bacillaceae*, *Saccharimonadales*, *Defluviitaleaceae*, *Xanthobacteraceae*, *Saccharimonadaceae*; 51-60 — *Izemoplasmatales*, *Oxalobacteraceae*, *Peptostreptococcales-Tissierellales*, *Spirochaetaceae*, *Propionibacteriaceae*, *Tannerellaceae*, *Gemellaceae*, *Lentimicrobiaceae*, *Atopobiaceae*, *Bacteroidaceae*; 61-70 — *Burkholderiaceae*, *Weeksellaceae*, *Pasteurellaceae*, *Porphyromonadaceae*, *Staphylococcaceae*, *Acholeplasmataceae*, *Alcaligenaceae*, JCI 0000069-P22, *Synergistaceae*, *Chloroplast*; 71-80 — *Sphingobacteriaceae*, *Cardiobacteriaceae*, *Caulobacteraceae*, *Coriobacteriaceae*, *Oscillospirales*, *Hungateiclostridiaceae*, *Mycoplasmataceae*, *Elusimicrobiaceae*, *Dysgonomonadaceae*, *Rhizobiaceae* (vivarium, FSC ARRTPI RAS, 2021). For a description of the groups, see the Materials and methods section.

The exact role of members of the genus Clostridia_UCG-014 is not completely clear due to the lack of ability to grow on laboratory media. According to C. Yang et al. [39], Clostridia_UCG-014 play a positive role for the macroorganism. The authors showed that in the human intestine against the background of ulcerative colitis and the use of sodium dextran sulfate, there was a violation of the composition of the microbiome, in particular, a decrease in the number of Clostridia_UCG-014. The use of turmeric polysaccharide led to the restoration of the microbiota composition and an increase in the content of these microorganisms. In our experiment, with introduction of T-2 toxin into the diet (group II), the abundance of Clostridia_UCG-014 increased compared to the control, which may indicate participation of Clostridia_UCG-014 in the initiation of dysbiotic disorders when the toxin was fed. Moreover, Clostridia_UCG-014 may be more resistant to T-2 toxin than other members of gut microbiota due to the ability to sporulate, high tolerance to acids and other aggressive substances [40, 41] and involvement into toxin detoxification.

Microorganisms of the genus *Akkermansia* represented by the only species *A. muciniphila* attract special attention. Their presence sharply decreased in all

groups with the introduction of T-2 toxin into the diet compared with group I ($p \leq 0.001$), up to a complete absence in groups II and IV (see Fig. 5). Decreased numbers of *A. muciniphila* in the gut can have negative consequences for poultry. The presence of *A. muciniphila* in the digestive system has been shown to be associated with a decrease in the abundance of pathogens, including *Salmonella pullorum* [42]. An increase in the proportion of *A. muciniphila* is associated with an increase in the processes of regeneration of the intestinal mucosa in chickens, which is accompanied by an increase in the number of goblet cells and an increase in mucin synthesis [43].

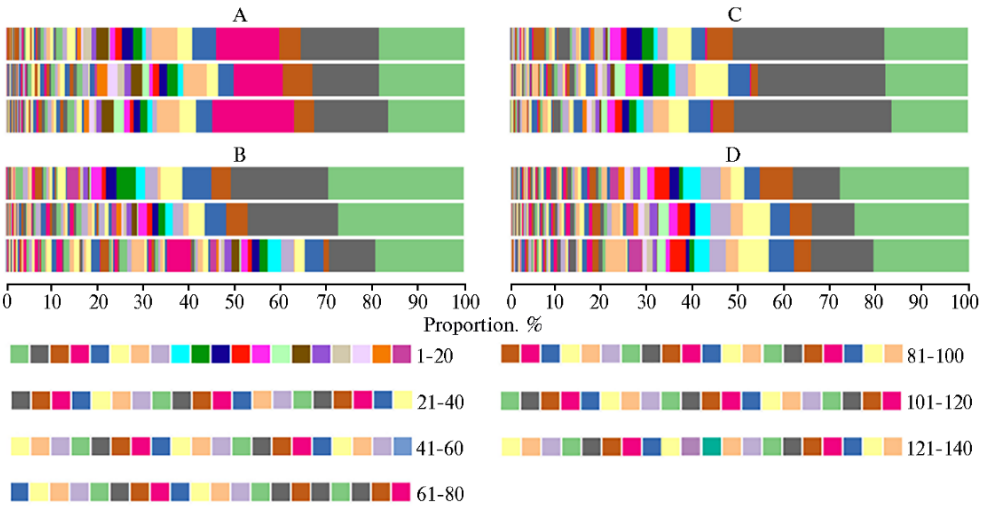

Fig. 5. The gut microbiome composition at the genera levels (based on data of NGS sequencing of 16S rRNA gene amplicons) in broiler chickens (*Gallus gallus* L.) Smena 8 cross fed T-2 toxin, additive Zaslon 2+ (BIOTROF LLC, Russia) **and enzyme drug with proteolytic activity Axtra Pro** (DuPont de Nemours, Inc., USA)**:** 1-20 — Clostridia UCG-014, *Lactobacillus*, *Alistipes*, *Akkermansia*, *Subdoligranulum*, *Faecalibacterium*, *Eubacterium coprostanoligenes* group, *Christensenellaceae* R-7 group, RF39, *Ruminococcus torques* group, UCG-005, *Fournierella*, *Erysipelatoclostridium*, *Bifidobacterium*, Clostridia vadinBB60 group, *Escherichia-Shigella, Clostridium* sensu stricto 1, *Blautia*, *Ruminococcus, Veillonella*; 21-40 — *Gastranaerophilales*, UCG-010, *Lachnoclostridium*, f. *Anaerovoracaceae* XIII AD3011 group, *Negativibacillus, Monoglobus*, *Butyricicoccus*, *Streptococcus*, *Romboutsia*, *Enterococcus,* f. *Anaerovoracaceae* XIII UCG-001, *Stenotrophomonas*, *Pseudomonas*, f. *Oscillospiraceae* V9D2013 group, *Erysipelotrichaceae*, *Eisenbergiella*, *Bilophila*, *Prevotella*, *Anaerofustis*, *Neisseria*; 41-60 — *Corynebacterium*, f. *Oscillospiraceae* NK4A214 group, *Sellimonas*, UCG-009, *Flavonifractor*, *Paludicola*, *Caproiciproducens*, *Actinomyces*, *Leptotrichia*, *Fusobacterium*, *Holdemania*, UCG-008, *Granulicatella*, *Gemella*, *Alloprevotella*, *Sphingomonas*, *Intestinimonas*, *Porphyromonas*, UC5-1-2E3, *Chryseobacterium*; 61-80 — *Ralstonia*, *Anaerotruncus*, *Gordonibacter*, *Staphylococcus*, *Anaerofilum*, *Capnocytophaga*, *Marvinbryantia*, *Haemophilus*, *Tyzzerella*, *Rothia*, *Campylobacter*, CHKCI001, *Kingella*, *Eubacterium hallii* group, *Oscillibacter*, *Saccharimonadaceae*, *Enterorhabdus*, *Anaerostipes*, *Defluviitaleaceae* UCG-011, *Gardnerella*; 81-100 — *Saccharimonadales*, *Selenomonas*, *Izemoplasmatales*, *Massilia*, *Candidatus* Soleaferrea, *Eubacterium nodatum* group, *Lautropia, Centipeda, Shuttleworthia, Treponema, Cutibacterium,* GCA-900066575, *Tannerella*, WPS-2, *Merdibacter*, *Peptococcus*, *Peptoniphilus*, *Lentimicrobium*, *Bergeyella*, *Atopobium*; 101-120 — *Aggregatibacter*, *Oscillospira*, *Frisingicoccus*, *Erysipelotrichaceae* UCG-003, *Catenibacillus*, *Vibrionimonas*, *Alcaligenes*, JGI 0000069-P22, *Johnsonella*, *Rikenellaceae* RC9 gut group, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Megasphaera*, DTU014, *Papillibacter*, *Auricoccus-Abyssicoccus*, *Anaeroplasma*, *Bacteroides*, *Fretibacterium*, *Anaerococcus*, *Angelakisella*; 121-140 — *Tuzzerella*, *Stomatobaculum*, *Solobacterium*, *Chloroplast*, *Finegoldia*, *Lachnospira*, *Nubsella*, *Prevotellaceae* UCG-001, *Prevotellaceae* Ga6A1 group, WCHB1-41, *Cardiobacterium*, *Ezakiella*, *Filifactor*, *Hydrogenoanaerobacterium*, *Ureaplasma*, *Elusimicrobium*, *Dysgonomonas*, *Collinsella*, *Kocuria*, f. *Lachnospiraceae Incertae Sedis* (vivarium, FSC ARRTPI RAS, 2021). For a description of the groups, see the Materials and methods section.

Interestingly, in group II fed feed contaminated with T-2 toxin, pathogenic microorganisms appeared which were absent in group I. These are the *Enterococcus*

cecorum (causing gastroenteritis and diseases of the joints of birds) [44], *Campylobacter concisus* (the causative agent of gastroenteritis) [45], *Campylobacter gracili*s (gastrointestinal infections, including bacteremia which may occur primarily due to immunosuppression) [46], *Streptococcus gordonii* (infective endocarditis) [47], *Flavonifactor* spp. (bloodstream infections) [48]. This seems to be natural, since earlier Y. Li et al. [49] found that low concentrations of T-2 toxin alter the activation of Toll-like receptors, thereby reducing pathogen recognition and preventing the initiation of inflammatory immune responses against bacteria and viruses [49]. In groups III and IV, these pathogens were either absent or present in a significantly smaller amount than in group II ($p \leq 0.05$), which indicates a positive effect of the feed additive Zaslon 2+ and its complex with the enzyme on the quantitative composition of intestinal microbiota.

Interestingly, some taxa of intestinal microorganisms, mainly of the phylum *Firmicutes* which were present in birds from group I, completely disappeared in group II when food was contaminated with T-2 toxin. These were representatives of the genera *Marvinbryantia* (family *Lachnospiraceae*), UCG-008 (family *Butyricicoccaceae*), and V9D2013_group (family *Oscillospiraceae*). This probably could have a negative effect on the non-starch polysaccharides digestion and the synthesis of butyrate, since representatives of *Lachnospiraceae* and *Oscillospiraceae* belong to an important group of cellulolytic microorganisms [50, 51], while bacteria of the *Butyricicoccaceae* family are active producers of valuable butyric acid [52]. The abundance of *Eubacterium coprostanoligenes*, a representative of the *Firmicutes* phylum, also sharply decreased in group II vs. control (from 5.2 ± 0.28 to 0.68 ± 0.042 at $p \leq 0.001$) and increased vs. group I in group III ($p \leq 0.05$) when Zaslon 2+ was fed and in group IV when the enzyme + Zaslon 2+ were fed. *Eubacterium coprostanoligenes* belongs to the normoflora and produces valuable organic acids such as acetic, formic, and succinic [53].

Based on the results of reconstruction and functional annotation, we found 756 predicted metabolic pathways in the broiler gut microbial community, 163 of which showed differences ($p \leq 0.05$) between the experimental groups. These pathways were involved into protein metabolism (biosynthesis of amino acids, conversion of nitrogenous compounds), lipid metabolism (biosynthesis of lipids, oleate, palmitoleate, palmitate, stearate), carbohydrate metabolism (breakdown of complex polysaccharides such as chitin, degradation of glucose), energy metabolism (for example, Krebs cycle), into the synthesis of volatile fatty acids (in particular, propionic and butyric), nucleic acids, nucleotides and nucleosides, cofactors and coenzymes (tetrahydrofolate, acetyl-CoA, ubiquinols 7-10, heme), vitamins (biotin, thiamine diphosphate, menaquinols 6-13, dimethylmenaquinols 6, 8, 9, adenosylcobalamin), xenobiotic biodegradation, cell wall formation and spore formation (synthesis of peptidoglycan, teichoic acids), pathogenesis (synthesis of O-antigens, siderophores), biofilm formation. Of particular note is the fact that a significant number of predicted metabolic pathways that ensure the degradation of various organic substances, primarily amino acids and aromatic compounds (in particular, xenobiotics), differed between the experimental groups ($p \leq 0.05$). A similar trend was also found for metabolic pathways associated with the synthesis of cofactors and coenzymes (25 pathways), as well as vitamins (19 pathways) ($p \leq 0.05$).

When exposed to T-2 toxin (group II vs. group I), there was an increase in the following predicted metabolic pathways for the degradation of aromatic compounds, including xenobiotics: for PWY-5182 (toluene IV degradation) by 6.8 times ($p \leq 0, 05$), for PWY-5415 (degradation of catechol I) by 5.7 times ($p \leq 0.05$), for 3-HYDROXYPHENYLACETATEDEGRADATION-PWY (4-hydroxyphenylacetate degradation) by3.0 times ($p \leq 0.05$), and for PWY0-321 (degradation

of phenylacetate I) by 5.0 times ($p \leq 0.05$) (Fig. 6). The effects of probiotics [54] and bird age [55] on the metabolic potential of the gut microbiome have been previously studied. We did not find any reports on the effect of xenobiotics on the predicted metabolic pathways in the intestines of birds, animals, and humans in the available scientific literature. Nevertheless, the ability of many microorganisms to degrade various xenobiotics has long been known. Thus, the possibility of decomposition of phenylacetate, which is the main intermediate in the bacterial degradation of many aromatic compounds, was shown [56]. Microorganisms can oxidize phenylacetate under both aerobic and anaerobic conditions with the participation of the enzymes phenylacetate-CoA ligase, phenylacetyl-CoA 1,2-epoxidase, and oxygenase [57, 58]. It has also been demonstrated [59] that active processes of xenobiotic metabolism take place in the intestine with the participation of β-glucuronidase, nitroreductase, and sulfoxide reductase.

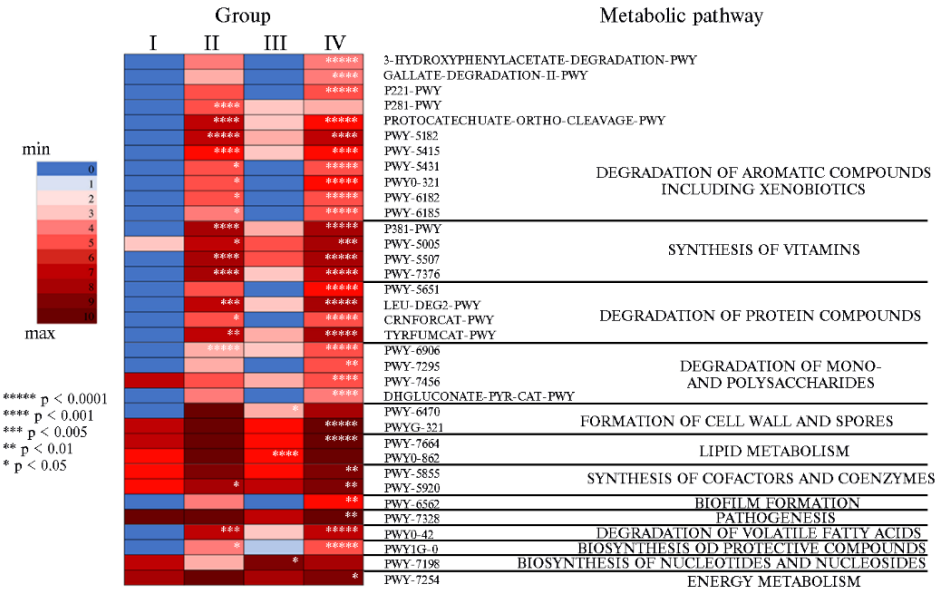

Fig. 6. Functional annotation of predicted metabolic pathways of caecum microbiome in broiler chickens (*Gallus gallus* L.) Smena 8 cross fed T-2 toxin, additive Zaslon 2+ (BIOTROF LLC, Russia) **and enzyme drug with proteolytic activity Axtra Pro** (DuPont de Nemours, Inc., USA) (vivarium, FSC ARRTPI RAS, 2021). The data were obtained using the PICRUSt2 software package (v.2.3.0). The MetaCyc database (https://metacyc.org/) was used to analyze metabolic pathways and enzymes. The scale reflects the intensity of potential metabolic pathways of the microbiome, blue is the lowest (minimum) intensity, red is the highest (maximum). For a description of the groups, see the Materials and methods section.

In our study, the Zaslon 2+ added to feed (group III) of broilers fed T-2 toxin led to a decrease in the potential of metabolic pathways for the degradation of aromatic compounds, including xenobiotics, compared with group II ($p \leq 0.05$). The activity of the PWY-5182 pathway decreased by 2.9 times, PWY-5415 by 4.1 times, 3-HYDROXYPHENYLACETATEDEGRADATION-PWY by 3.0 times, PWY0-321 by 5.0 times ($p \leq 0.05$). This effect may be associated with a decrease in the toxic load when the feed additive Zaslon 2+ is introduced into the diet. It is interesting that in group IV, when the enzyme preparation was added to the diet, there were no differences ($p > 0.05$) from group II in these metabolic pathways. That is, the introduction of protease reduced the effectiveness of the feed additive Zaslon 2+. When feed was contaminated with T-2 toxin, an increase in the potential of metabolic pathways for the degradation of such compounds important for birds as amino acids was observed ($p \leq 0.05$). In particular, in group

II vs. group I, the potential for LEU-DEG2-PWY (degradation of L-leucine I) increased 6.9-fold ($p \leq 0.05$), for PWY-5651 (degradation of L-tryptophan to semialdehyde 2-amino-3-carboxymuconate) 4.6-fold, for TYRFUMCAT-PWY (degradation of L-tyrosine I) 6.5-fold, and for CRNFORCAT-PWY (degradation of creatinine I) 6.9-fold. The enhancement of amino acid degradation pathways may be associated with the appearance of bacteria of the phylum *Fusobacteriota* in birds from groups II and IV. Members of this taxon are known to use amino acids as an energy source [60]. The Zaslon 2+ added to the diet of broilers which were fed T-2 toxin (group III) reduced ($p \leq 0.05$) the metabolic potential for amino acid degradation to the level of group I (without feed contamination with T-2 toxin). This is a positive effect, since amino acids serve as an important plastic material for protein synthesis and bird growth [61].

In addition, feed contamination with T-2 toxin (group II vs. group I) resulted in activation of the predicted metabolic pathway PWY0-42 (2-methylcitrate cycle I) leading to propionic acid (volatile fatty acid) degradation ($p \leq 0.05$). Organic acids, in particular propionic acid, are produced by representatives of the normal flora present in the intestine [62]. These substances may increase the performance of poultry by altering the pH of the digestive system and therefore altering the composition of the microbiome [63]. In particular, propionate causes acidification of the cytosol in pathogenic bacteria, dispersion of the proton-motive force, disruption of CoA homeostasis, and, in some cases, inhibition of the key enzymes of the tricarboxylic acid cycle aconitase and citrate synthase due to the formation of (2S,3S)-2-methylcitrate. In addition, organic acids are able to improve the morphology and physiology of the gastrointestinal tract [64]. However, some intestinal bacteria can degrade propionate and even use it as their sole carbon source. Of all the propionate degradation pathways, the 2-methylcitric acid cycle is the most widely used. In this pathway, the methylene group of calcium propionate is oxidized to the keto group with the formation of pyruvate, a common precursor for biosynthesis and energy generation. This pathway is well known in *Salmonella enterica enterica* serovar *Typhimurium* [65] and *E. coli* [66]. In these microorganisms, the pathway begins with the activation of propanoate to propanoyl-CoA by propionate-CoA ligase, followed by the synthesis of (2S,3S)-2-methylcitrate from propanoyl-CoA and oxaloacetate catalyzed by 2-methylcitrate synthase. Further, (2S,3S)-2-methylcitrate is dehydrated to cis-2-methylaconitate using 2-methylcitrate dehydratase, followed by rehydration to (2P,3S)-2-methylisocitrate and cleavage of the latter into pyruvate and succinate.

It also seems logical that contamination of feed with T-2 toxin led to the activation ($p \leq 0.05$) of the predicted metabolic pathways PWY-6562 (norspermidine biosynthesis) and PWY1G-0 (mycothiol biosynthesis) by 4.0 and 3.8 times, respectively. The polyamine norspermidine is known to be involved in the regulation of biofilm formation in microorganisms [67], in particular in *Vibrio cholera*, the causative agent of cholera with symptoms of diarrhea [68, 69]. Bacteria form biofilms as a survival mechanism, since such structures contribute to protection against pH extremes, osmotic stress, ultraviolet radiation, antimicrobials [70] and, in our study, probably T-2 toxin. An increase in the production of mycothiol against the background of food contamination with T-2 toxin in groups II and III compared with group I ($p \leq 0.05$) is also natural. Mycothiol is the main thiol contained in the cells of actinomycetes [71]. Probably, the activation of mycothiol production was associated with an increase ($p \leq 0.05$) in the abundance of *Actinobacteriota* superphylum bacteria in the intestines of birds from groups II and IV compared to control. In terms of functions, mycothiol is in many respects similar to glutathione, the dominant thiol in other bacteria, which is absent in actinobacteria.

This substance is involved in the detoxification of alkyl reducing agents, reactive oxygen and nitrogen species, antibiotics [72]. It also acts as a thiol buffer that has reducing properties and protects against disulfide stress [73].

Previously, it was noted [74, 75] that the reaction of birds to the introduction of proteases into diets is not always favorable. It has been shown [75] that despite an increase in amino acid digestibility in the ileum, growth and feed intake rates declined and feed conversion deteriorated. The authors [75) explained this by the difficulties in selecting the optimal dosages of proteases in each specific case. The introduction of protease at a dose of 160 mg/kg into the diet of broilers significantly reduced the activity of pancreatic trypsin. M. Mahagna et al. [76] reported that the introduction of amylase and protease preparations into the diet of sorghum and soybean meal significantly reduced the activity of its own amylase, chymotrypsin and trypsin in the intestinal contents of broilers. According to L. Liu et al. [31], proteolytic systems and selective proteolysis are considered as key regulators of tumor progression processes; proteolysis processes are directly related to the reactions of inflammation and tissue destruction.

Thus, with experimental contamination of feed with T-2 toxin (2-fold excess of MPC), in the intestines of broiler chickens, the biodiversity and composition of the microbiome changed already at the level of phyla. The abundance of bacteria of the superphylum *Actinobacteriota* and phylum *Proteobacteria* increased (by 1.8 and 3.5 times, respectively, $p \leq 0.05$), while the abundance of the superphylum *Desulfobacterota*, on the contrary, decreased (by 2.2 times, $p \leq 0.05$). With the introduction of the feed additive Zaslon 2+ into the diet in combination with the proteolytic enzyme preparation Axtra Pro, the abundance of the superphylum *Actinobacteriota* and the phylum *Proteobacteria* also increased compared to the control ($p \leq 0.05$), while the feed additive Zaslon 2+ used separately did not give such an effect. Representatives of the superphylum *Verrucomicrobiota* completely disappeared in the groups that received T-2 toxin without additives, as well as when using a complex of feed additive and enzyme, while in the control these microorganisms were present in a significant amount, 14.1±0.8%. The use of Zaslon 2+ alone increased the abundance of bacteria of the genus *Lactobacillus* vs. control from 15.9±1.32 to 30.7±1.84% ($p \leq 0.01$). With the introduction of T-2 toxin into the diet, the number of *Akkermansia muciniphila* sharply decreased ($p \leq 0.001$), and pathogenic microorganisms that were absent in the control appeared (*Enterococcus cecorum*, *Campylobacter concisus*, *Campylobacter gracilis*, *Streptococcus gordonii*, *Flavonifractor* spp.). When using a feed additive or its complex with an enzyme, these pathogens were either absent or present in a significantly smaller amount ($p \leq 0.05$). The test groups differed ($p \leq 0.05$) in 163 predicted metabolic pathways. Under the influence of T-2 toxin, metabolic pathways for the degradation of aromatic compounds (including xenobiotics), amino acids, the synthesis of coenzymes, cofactors, the formation of biofilms, cell walls, spores and protective substances increased 3.0-6.9-fold ($p \leq 0.05$) in cells. The feed additive Zaslon 2+ reduced the potential for metabolic degradation of aromatic compounds, including xenobiotics, and amino acids by 2.9-5.0 times. In general, the feed additive Zaslon 2+, as well as a complex of this additive with a proteolytic enzyme have a positive effect on the composition and potential functional activity of the intestinal microbiome in broiler chickens, and the feed additive without the enzyme is more effective.

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BIOINFORMATIC ANALYSIS OF THE *Bacillus velezensis* KR-2 GENOME TO REVEAL BIOTECHNOLOGICALLY IMPORTANT PROPERTIES

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Abstract

Members of the genus *Bacillus* are actively used to create biological preparations for agriculture due to their ability to produce a wide range of biologically active molecules with antimicrobial activity, stimulating plant growth and restoring the balance of microorganisms in the digestive system of animals. This paper presents for the first time research data on the identification of a unique pathway for intracellular synthesis of the osmoprotectant glycine betaine encoded by the *BetA*, *BetB*, *BetT*, and *BetC* genes, which has not previously been found in the genus *Bacillus*. The peculiarity of the *B. velezensis* KR-2 genome associated with the synthesis of the siderophore myxochelin A, which we have identified, is probably also unique among other strains of *B. velezensis*, since it has not been previously described. The aim of the study was whole genome sequencing (WGS) and bioinformatic annotation of the *Bacillus velezensis* KR-2 genome to identify genetic determinants capable of encoding biosynthesis of various bioactive substances for agriculture. The *B. velezensis* KR-2 strain isolated from the rumen of a dairy cow (the collection of OOO BIOTROF+) was examined. Its antimicrobial activity was investigated using the differed antagonism assay. DNA was isolated according to standard procedures with the Genomic DNA Purification Kit (Thermo Fisher Scientific Inc., USA). A DNA library for WGS was prepared using the Nextera XT kit (Illumina, Inc., USA). Nucleotide sequences were determined using a MiSeq instrument (Illumina, Inc., USA). Paired-end reads filtered by length not less than 50 to 150 bp were assembled de novo using the SPAdes-3.11.1 genomic assembler with appropriate keys. Comparative analysis of the *B. velezensis* KR-2 genome with other microorganisms was performed using the NCBI databases (https://www.ncbi.nlm.nih.gov/). For phylogenetic analysis, the 16S rRNA gene sequence was transferred to the Nucleotide BLAST web service (https://blast.ncbi.nlm.nih.gov). PROKKA 1.12 (https://bioweb.pasteur.fr/packages/pack@prokka@1.12) was used to convert contig sequences into an amino acid sequences. Functional annotation of the genome was performed using the RAST 2.0 web service (https://rast.nmpdr.org). The KEGG Pathway database (http://www.genome.jp/kegg/) was used to evaluate the pool of genes associated with biotechnologically valuable properties and build metabolic maps. WGS resulted in 16 contigs with a total length of 3936398 bp, containing 46.6 % of GC; the N50 and N75 conting size was 2109194 and N75 844068 bp, respectively. The chromosome contained 3854 coding sequences (CDS) associated with the synthesis of polypeptides. Among the 464 identified metabolic subsystems, the subsystems of amino acids and their derivatives and carbohydrates

were the most numerous, 431 and 416, respectively. The *B. velezensis* KR-2 strain has a whole range of potential properties, including the production of antimicrobial peptides, fatty acids, vitamins, siderophores, auxins, the ability to adhere, to resist toxic compounds and stress factors, to stimulate plant growth and phosphate metabolism, the motility and chemotaxis. In particular, the *B. velezensis* KR-2 genome contains genes (*BacA*, *BacB*, *BacG*, *BacF*, *BacD*) involved in the production of the dipeptide bacilysine, a non-ribosomal bacteriocin. This is consistent with the phenomenon of antagonism against *Staphylococcus aureus*, *Escherichia coli*, *Fusarium oxysporum*, and *Clostridium butyricum*. In the *B. velezensis* KR-2 genome, we have found a unique pathway for intracellular synthesis of the osmoprotectant glycine betaine (the *BetA*, *BetB*, *BetT*, *BetC* genes) not previously detected in the genus *Bacillus*. *B. velezensis* KR-2 is a putative producer of auxins, such as indole-3 ethanol (*IAR*, *TO*), indole-3-acetaldehyde (*IAD*, *AAD*, *AO*) and indole-3-acetonitrile (*N3*). Several gene clusters associated with siderophore synthesis (*DhbA*, *DhbB*, *DhbC*, *FeuA*, *FeuB*, *FeuC*, *FeuD*) were also identified in the genome of *B. velezensis* KP-2. Our findings indicate that *B. velezensis* KR-2 is a bacterial resource for agriculture. The unique biosynthesis pathway for glycine betaine (*BetA*, *BetB*, *BetT*, and *BetC*) that we have discovered are important for the *B. velezensis* KP-2 adaptation to high-osmolar stress under fluctuations in water content, for example, in dried silage and upper layers of soil.

Keywords: whole genome sequencing, *Bacillus velezensis*, bioactive substances, antimicrobial activity, bacilysine, glycine betaine, probiotics, PGPR, starter cultures

Bacteria of the genus *Bacillus* are widely used in agriculture as the basis of probiotics, plant growth stimulants, biopesticides and insecticides, starter crops for ensiling, because, having wide metabolic capabilities, they serve as an important source for the synthesis of biologically active molecules with useful properties [1, 2]. This demand is primarily associated with the presence in the genome of microorganisms of this taxonomic group of a large number of chromosomal loci that determine the synthesis of antimicrobial compounds [3]. This is one of the most important functional properties taken into account in the selection of potential biologics producers [4-6]. P. Piewngam et al. [4)] demonstrated that one of the fengycin-producing strains of the *Bacillus* genus was active against *Staphylococcus aureus* in mice. E. Lara et al. [2)] observed a decrease in the number of mold micromycetes and yeasts when introduced into the silage ecosystem of a starter culture based on the *B. subtilis* strain.

In addition, the possibility of *Bacillus* spp. to carry out metabolism along the pentose phosphate pathway makes them effective producers of vitamins, among which the most significant are cobalamin, riboflavin, folic acid and biotin [7-9]. The spectrum of valuable metabolites also includes substances with anti-inflammatory activity [10]. T.-Y. Lee et al. [10] showed that a strain of *B. subtilis* bacteria capable of synthesizing poly-γ-glutamic acid was effective in treating dermatitis in mice by suppressing the Th2-biased immune response and synthesis of IL-17A. The ability of bacillus strains to synthesize surface-associated proteins, including S-layer proteins, aminopeptidases, flagellin, and metalloproteases, provides the ability to specifically bind to mucin and fibronectin, which may play an important role in adhesion in the gastrointestinal tract and provide a probiotic effect [11]. The high capacity of the secretory systems of bacteria of the genus *Bacillus* predetermines the potential for the production of many hydrolytic extracellular substances. Among the enzymes of interest for animal husbandry are amylases (α- and β-amylases), β-glucanases [1], cellulases and xylanases [12], which are important for enhancing the degradation of complex polysaccharides during the introduction of strains into the digestive system. Another example of *Bacillus* spp. enzymes of biotechnological importance is the insecticidal chitinase metabolites produced by *B. thuringiensis* [1, 13], which act in conjunction with δ-endotoxins (Cry or Cyt) [14].

A number of bacteria of the genus *Bacillus* belong to the so-called PGPR group (plant growth promoting rhizobacteria) [15]. These microorganisms, by synthesizing phytohormones such as auxins (indole-3-acetic acid), contribute to the intensification of plant growth and beneficially affect their nutrition by solubilizing phosphates and chelating iron with siderophores [16].

Modern advances in the sequencing of the genomes of various microorganisms make it possible to discover many new gene clusters that encode new or alternative variants of already described pathways for the synthesis of bioactive molecules [4, 17]. *Escherichia coli* is traditionally used as an experimental model in molecular biology, in particular, in genomic studies [18, 19]. However, a significant body of data has now been accumulated on the genomics of many other microorganisms, including *B. velezensis*, which is recognized as an effective antibacterial agent and an important biological control agent in agricultural lands as an alternative to chemical antibiotics [20]. In many studies on the genomes of *B. velezensis* strains were carried out to establish the viability, antimicrobial and probiotic potential of this microorganism for its use in medicine [21], to identify industrially significant characteristics for the production of valuable raw materials [22], to assess the ability to synthesize antibacterial and antifungal metabolites [23] active against phytopathogens.

In the present work, in the *B. velezensis* KR-2 strain, we for the first time revealed a unique pathway of intracellular synthesis of the osmoprotectant glycine-betaine with the participation of the *BetA*, *BetB*, *BetT*, and *BetC* genes, which was not previously known in bacteria of the genus *Bacillus*. The peculiarity of the *B. velezensis* KR-2 genome associated with the synthesis of the siderophore myxochelin A, which we discovered, is probably also unique for this strain, in contrast to other studied representatives of the *B. velezensis* species, since it has not been described in the literature.

The aim of the work was a molecular analysis and bioinformatic annotation of the genome of the *Bacillus velezensis* KR-2 strain to identify genetic determinants that determine the possibility of biosynthesis of various biologically active substances important for the creation of biological products for agriculture.

Materials and methods. The *B. velezensis* KR-2 strain from the collection of OOO BIOTROF+ was isolated from the rumen of a dairy cow.

The antimicrobial activity of the strain was studied by the method of delayed antagonism (method of perpendicular strokes) according to the recommendations [24]. To do this, a suspension of the test culture (10^7 CFU/ml) was streaked along the diameter of a Petri dish on a GRM agar medium dried for 24-48 h (SRC PMB Obolensk, Russia) added with glucose (7 g/l). After 24 h of incubation at 37±1.0 °C, test strains of *Staphylococcus aureus*, *E. coli*, *Fusarium oxysporum* and *Clostridium butyricum* were added to the grown culture perpendicularly to the direction of growth. After 24 h of incubation at 37±1.0 °C, the growth inhibition of test strains was assessed by the distance to the stroke of the test culture.

DNA was isolated by standard procedures using the Genomic DNA Purification Kit (Thermo Fisher Scientific, Inc., USA) according to the attached instructions [25]. The method is based on selective detergent-mediated precipitation of DNA from a substrate using solutions for cell wall lysis and DNA precipitation, 1.2 M sodium chloride, chloroform.

A DNA library for whole genome sequencing was prepared using the Nextera XT kit (Illumina, Inc., USA). Nucleotide sequences were determined using a MiSeq NGS system (Illumina, Inc., USA) with a MiSeq Reagent Kit v3 (300-cycle) (Illumina, Inc., USA). Invalid sequences and adapters were removed using the Trimmomatic-0.38 program (https://www.osc.edu/book/ex-port/html/4385) [26]. Filtered by length not less than 50 to 150 bp pair-end sequences were assembled de novo using the SPAdes-3.11.1 genomic assembler (http://cab.spbu.ru/software/spades/) [27] with appropriate keys. Chromosomal and plasmid contigs were distinguished according to the information in the description (contigs assembled as plasmid had the corresponding mark "-plasmid"). Quality of ansavbling was

assessed using QUAST Version: 5.0.2 (A. Gurevich, 2017; http://quast.sourceforge.net/download.html).

The NCBI database (https://www.ncbi.nlm.nih.gov/genome/microbes/) was used to compare the genome of the *B. velezensis* KR-2 strain with the nucleotide sequences of other microorganisms. For phylogenetic analysis, the 16S rRNA gene sequence was transferred to the Nucleotide BLAST web service (https://blast.ncbi.nlm.nih.gov). Search settings have been set by default. The nucleotide sequences of the contigs were translated into amino acids using the PROKKA 1.12 program (https://github.com/kbaseapps/ProkkaAnnotation) [28]. Functional annotation of the genome was performed using the RAST 2.0 web service (https://rast.nmpdr.org) (29). The KEGG Pathway database (http://www.genome.jp/kegg/) was used to evaluate the pool of genes associated with biotechnologically valuable properties and build metabolic maps [30, 31]. For this, the resulting translated protein sequence was transferred to the KEGG-KAAS database server (https://www.genome.jp/kegg/kaas/). The GHOSTX and bi-directional best hit (BBH) algorithms were set as search criteria. Additionally, the UniProt database (https://www.uniprot.org/) was used.

Mathematical and statistical processing of the results was carried out using Microsoft Office Excel 2003 software packages.

Results. The strain *B. velezensis* KP-2 had a pronounced antagonistic effect (Fig. 1) against the studied test cultures of *S. aureus*, *E. coli*, *F. oxysporum*, and *C. butyricum*. The width of the zone of growth inhibition ($n = 5$) was 13.0±0.75, 5.0±0.30, 19.0±0.75, and 14.0±0.70 mm, respectively. This suggests the presence of antimicrobial substances diffusing into the agar in the culture liquid of *B. velezensis* KP-2.

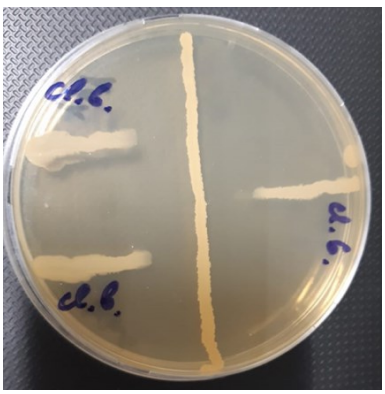

Fig. 1. Antagonistic effect of *Bacillus velezensis* KR-2 on the test culture *Clostridium butyricum*. Suspension of *B. velezensis* KR-2 was plated along the diameter of a Petri dish. The test strain *C. butyricum* was plated by streaking in the perpendicular direction. Closer to the central part, a zone of no growth of *C. butyricum* is visualized.

The data obtained are of great practical importance, since *S. aureus* is dangerous for farm animals, as it can cause diseases in cattle, primarily mastitis [32]. *C. butyricum* is able to act as the main initiator of clostridial fermentation in silage fermentation, resulting in loss of feed quality [33]. Therefore, *B. velezensis* KP-2 is promising in the development of biocontrol agents to suppress pathogenic microbiota, in particular, when introduced into the silage and into the digestive system of farm animals.

In this regard, we performed whole genome sequencing and genome annotation of the *B. velezensis* KP-2 strain using the RAST 2.0 web service. When performing BLAST analysis in the NCBI database, contig NZ_JAILSD010000003.1 was determined as the 16S C region of *Bacillus velezensis* (strain FZB42). The match was 99.81% (1547/1550 bp, 3 mismatches). The genomic sequence was deposited with the BioProjects Collection (NCBI, https://www.ncbi.nlm.nih.gov/bioproject/) under accession number PRJNA756418.

For uploading to the server, 16 contigs were used with a total length of 3936398 bp, a share of GC pairs of 46.6%, and N50 assembly quality indicators of 2109194 bp and N75 844068 bp. We did not find any inconsistencies and inconsistencies in the resulting assembly.

The chromosome included 3854 coding sequences (CDS) associated with

the synthesis of polypeptides, with 93 genes for tRNA and 9 genes for rRNA. Plasmid DNA (8162 bp) contained 52.1% GC pairs. The largest of the annotated proteins in length consisted of 5434 amino acid residues, the smallest of 37 amino acid residues (Fig. 2). The dominant number of proteins had a length of 37 to 500 amino acid residues.

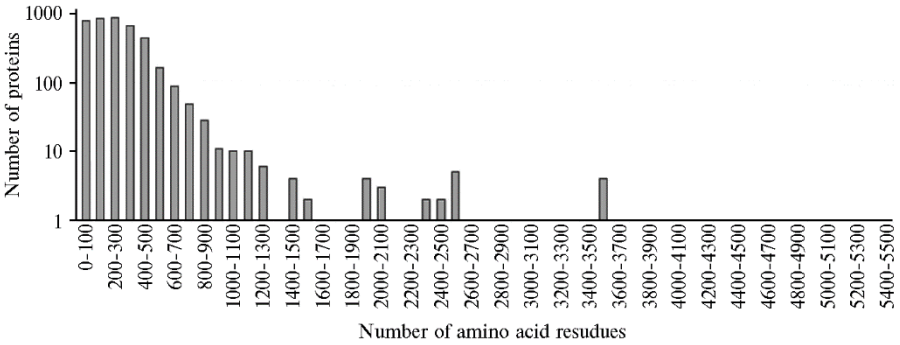

Fig. 2. The length profile of the annotated proteins of the *Bacillus velezensis* KR-2 strain based on whole genome sequencing data and genome annotation (the RAST 2.0 web tool, https://rast.nmpdr.org).

Comparison of the *B. velezensis* KP-2 genome with the nucleotide sequences of other microorganisms in the NCBI Microbial Genomes database revealed a high degree of similarity with the genome of *Bacillus velezensis* (strain FZB42), as well as with the genomes of other members of the *Bacillus* genus (*B. subtilis* QB928, *B. subtilis* subsp. *subtilis* str. AUSI98, *B. subtilis* subsp. *subtilis* str. 168). In addition, the *B. velezensis* KR-2 strain was closely related to the *B. amyloliquefaciens* and *B. atrophaeus* clusters.

B. amyloliquefaciens strains are of interest for their ability to stimulate the growth of host plants through the production of auxins, suppress soil pathogens by synthesizing antibacterial and antifungal metabolites, and induce plant resistance to adverse environmental factors [34]. Among *B. atrophaeus* strains, industrially important ones are also often found, including active producers of antimicrobial substances used as biological protection agents [35]. Previously, A. Niazi et al. [34] reported about high genetic identity of *B. amyloliquefaciens* UCMB5033 to *B. atrophaeus* and *B. subtilis* revealed by whole genome sequencing.

Based on the analysis of the *B. velezensis* KP-2 genome using the RAST web service, we identified 464 metabolic subsystems for the groups of proteins that together ensure the implementation of a certain biological process (Fig. 3). The most represented subsystems were those for the metabolism of amino acids and their derivatives (431 subsystems) and the metabolism of carbohydrates (416 subsystems).

In the *B. velezensis* KR-2 strain, we found a set of potential properties, including the synthesis of antimicrobial peptides, fatty acids, vitamins, siderophores, auxins, the ability to adhere, motility, and chemotaxis, resistance to toxic compounds, the ability to withstand stress factors, stimulate plant growth, and participate in the metabolism of phosphates (see Fig. 3).

In the genome of *B. velezensis* KR-2, we have identified genes (*BacA*, *BacB*, *BacG*, *BacF*, *BacD*) involved in the synthesis of bacilizin. Bacilizin is a nonribosomal synthesized antimicrobial dipeptide that was discovered in one of the strains of *B. subtilis* as early as 1946 as a substance that causes partial lysis of growing cultures of *Staphylococcus aureus* [36]. Later, M. Kenig and E. Abraham [37] noted a high activity of bacilizin (10^{-3} mg · ml^{-1}) against *E. coli*. The data on the possibility of synthesizing this peptide are consistent with the observed

phenomenon of *B. velezensis* KR-2 antagonism against *S. aureus* and *E. coli*. It is known [38] that bacilizin (L-alanine-[2,3-epoxycyclohexano-4]-L-alanine) consists of an L-alanine residue at the N-end and a non-protein amino acid L-anticapsin at the C-end. Despite its relatively simple chemical structure, bacilizin is active against a wide range of bacteria, yeasts, and micromycetes [37].

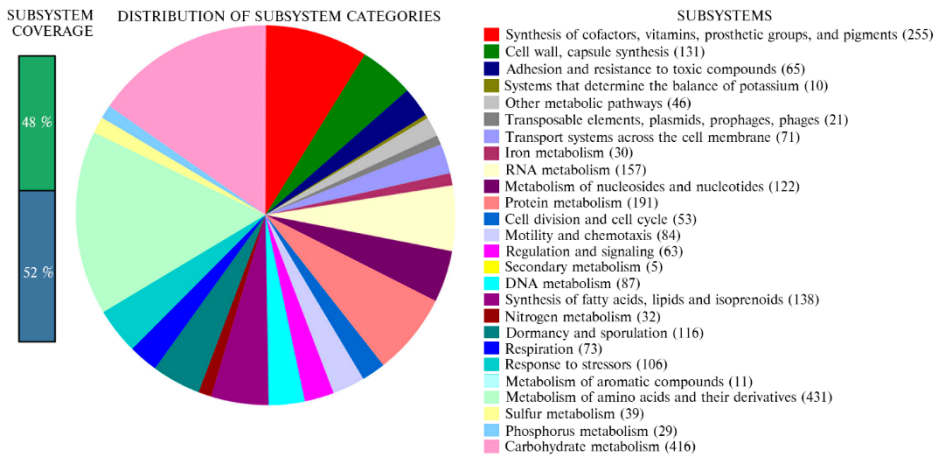

Fig. 3. Distribution of cell metabolism subsystems in the *Bacillus velezensis* KR-2 strain based on functional annotation (RAST, https://rast.nmpdr.org). The pie chart represents the percentage of proteins for each subsystem category. The categories of subsystems are listed in the legend from top to bottom according to the direction of movement along the pie chart in a clockwise direction. The numbers in parentheses are the number of metabolic pathways in the corresponding category of the subsystem. Subsystem coverage is the ratio of known proteins that can be placed into existing subsystems (green) and unknown proteins that cannot be placed into any existing subsystem (blue).

It is interesting that by the annotation in the PROKKA 1.12 program and the UniProt database, in *B. velezensis* KP-2, the *ComA* and *ComP* genes were identified which are responsible for the "quorum sensing" in bacterial populations, that is, the ability to coordinate individual behavior for secretion of molecular signals [38]. It is suggested that a quorum-sensitive pathway involving these genes regulates the bacilizin production in the genus *Bacillus* [38].

The described systems for the synthesis of antimicrobial peptides are not new for bacteria of the genus *Bacillus* and are quite widespread among them [39]. Thus, earlier, using the example of *B. subtilis* BAB-1, it was found that about 5.2% of the strain genome is associated with the synthesis of antimicrobial products, including antibiotics produced by nonribosomal peptide synthetases and polyketide synthases, lantibiotics, and bacillibactin. C. Luo et al. [17] performed whole genome sequencing of the *B. subtilis* 916 strain and found four clusters of genes (*srf*, *bmy*, *fen*, and *loc*) associated with the synthesis of lipopeptides surfactins, bacillomycin, fengycin, and lokillomycins which are active against moulds. Previously [21], whole genome sequencing of the *B. velezensis* KMU01 strain showed that its genome contained the lantibiotic mersacidin operon, including the genes for premersacidin (IM712_RS05205), modification protein (IM712_RS05195), and bacteriocin export protein (IM712_RS05185).

In addition, we found that the *B. velezensis* KR-2 strain is capable of synthesizing and accumulating osmoprotectors (see Fig. 3). One of the most important osmoprotectants is glycine-betaine which is present in the environment, for example, is synthesized by plants [40]. *B. velezensis* KP-2 has the potential to accumulate glycine-betaine directly from the environment through three osmotically regulated uptake systems controlled by the *OpuD*, *OpuAA*, and *OpuAB* genes. The relationship of these genes with the accumulation of glycine-betaine, as well

as their presence in the genome of *Bacillus* spp. has been described in other studies [41]. Earlier, C. von Blohn et al. [42] sequenced a 2781 bp DNA fragment of the *B. subtilis* pORT4 plasmid. This region was associated with the synthesis of the OpuE protein which is important for proline uptake in high osmolarity media. Proline serves as an osmoprotectant in *B. subtilis*. The proline uptake system controlled by *OpuE* works independently of the known transport systems for the osmoprotectant glycine-betaine. S. Heo et al. [21] showed that the genome of the strain *B. velezensis* KMU01 contained two osmoprotective uptake systems for glycine betaine and proline betaine (the *OpuA* and *OpuD* genes, respectively).

It turned out that, in addition to the direct intake of glycine betaine from the environment, *B. velezensis* KR-2 has the potential to accumulate this osmoprotectant through its intracellular synthesis, which requires the presence of precursors (choline or glycine betaine aldehyde) in the environment [43]. The *BetA* gene identified in the genome of *B. velezensis* KP-2 is associated with the synthesis of flavin adenine dinucleotide-dependent choline dehydrogenase (EC 1.1.99.1) which oxidizes choline to glycine betaine aldehyde. The *BetB* gene is associated with the production of betaine aldehyde dehydrogenase (EC 1.2.1.8) which converts glycine betaine aldehyde into osmoprotective glycine betaine, while having a high substrate specificity. The use of choline (the precursor molecule) is due to the high affinity of the BetT transporter for it [43].The *betIBA* operon was under the transcriptional control of the *AnoR* quorum regulator. Previously, a similar pathway for the synthesis of glycine-betaine involving the genes *BetA*, *BetB*, *BetT*, *BetC* was found in *E. coli* [43] and *Acinetabacter nosocomialis* [44], but was not demonstrated for bacteria of the genus *Bacillus*, therefore, it is a unique characteristic of the strain studied by us.

Traditionally, the conditions of increased osmotic pressure in the media are considered as limiting for the development of microorganisms, since high salinity is associated with a decrease in water activity [45]. The pathways we discovered not only for absorption but also for the synthesis of glycine-betaine are important from the point of view of cellular adaptation of the *B. velezensis* strain to high-osmolar stress [45] which is created in environments subject to frequent fluctuations in water content, for example, in dried plant silage and upper layers of the soil [46]. It is believed that the synthesis and accumulation of osmoprotectants is the most flexible response of microorganisms to limited water availability [47].

In addition, *B. velezensis* KR-2 showed a potential for the synthesis of indole derivatives, such as indole-3-ethanol, with the production of which the *IAR*, *TO* genes, indole-3-acetaldehyde (*IAD*, *AAD*, *AO* genes) and indole-3-acetonitrile (*N3* gene) are associated. The possibility of synthesizing tryptophan, an important precursor of auxin, the indolyl-3-acetic acid, has also been found [48], which is determined by the *PRAI*, *IGS*, *TSa*, *TSb*, and *APRT* genes. It is known that auxins have a positive effect on the growth rate, the time of flowering and fruiting of plants, the photosynthesis and production of various metabolites, the resistance to outer stressors, and regulate gene expression [49]. Therefore, the synthesis of auxins is considered as an important advantage for the associative interaction of PGPR bacteria with plants [50]. Biochemical studies have shown that strains of the genus *Bacillus* can produce auxins, in particular indole-3-acetic acid [51]. Whole genome sequencing of *B. subtilis* EA-CB0575 predicted the potential for the synthesis of some auxins (metabolism of indole via tryptophan, as well as the possibility of production of indole acetate and indoacetamide) [52]. Analysis of the genome of *B. velezensis* BS89 revealed the presence of gene clusters responsible for the synthesis of indole-3-acetic acid [53].

With the functional annotation of the *B. velezensis* KR-2 genome, we also

identified potential pathways for the synthesis of vitamins, including biotin, thiamine, riboflavin, and menaquinone. In particular, we showed the presence of genes associated with the synthesis of biotin, the *BPL*, *BR*, BioF, *BioA*, *BioD*, *BioB*, *BioW*, *BioC*, *BioN*, *BioG*, *BioK*, *BioZ*. The ability to synthesize vitamins was previously discovered in many strains of the genus *Bacillus* [41, 54]. Thus, whole genome sequencing of *B. subtilis* UBBS-14 [55] isolated from fermented food products revealed genes associated with the biosynthesis of biotin, riboflavin, vitamin K, cobalamin, vitamin B_6, and folic acid. Vitamins play an important role in many metabolic processes in animals, affecting productivity [56]. It has been reported that these substances can increase plant stress tolerance as well as disease resistance when infected with plant pathogens [57].

In the *B. velezensis* KR-2 genome, we identified several gene clusters (*DhbA*, *DhbB*, *DhbC*, *DhbE*, *DhbF*, *YuiI*, *FeuABCD*) associated with siderophore synthesis and phosphorus assimilation (*PstS*, *PstC*, *PstA*, *PstS hal*, P*stS C*, *PhoP*, *PhoR*, *PhoB*). Almost all genes necessary for the process of iron binding with the participation of bacillibactin (*DhbA*, *DhbB*, *DhbC*, *DhbE*, *DhbF*) were found. A cluster of the *DhbA*, *DhbB*, *DhbC* genes associated with the production of 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase (EC 1.3.1.28), isochorismatase (EC 3.3.2.1), and isochorismate synthase (EC 5.4.4.2) enzymes, which are responsible for synthesis of the bacillibactin precursor. The *FeuA*, *FeuB*, *FeuC*, and *FeuD* gene cluster is associated with the synthesis of substrate-binding proteins of the iron transport system. In the genome of *B. velezensis* KP-2, genes involved in the synthesis of other siderophores, the enterochelin (*EntE*, *EntB*) and myxochelin A (*MxcE*, *MxcF*), were also found. Probably, bacteria of the genus *Bacillus* can synthesize several siderophores acting in synergy to increase their competitiveness, which was previously shown for other microorganisms [58].

Bacterial siderophores are of great interest for agricultural applications [59]. The fact is that many bacteria, primarily pathogenic ones, require metal ions, in particular iron cations, which are involved in electron transfer and serve as cofactors for enzymes that control DNA and RNA synthesis to ensure the vital activity of many bacteria [60, 61]. Therefore, during evolution, microorganisms have formed siderophores, the specific molecular structures (low molecular weight chelating agents) that provide assimilation of iron ions in a bound state [62]. The use of probiotic preparations based on beneficial microorganisms that produce siderophores, which reduce the concentration of iron ions available to pathogens, have a positive effect on animal health [63]. Through the production of siderophores and successful competition for iron ions present in the soil, PGPR rhizobacteria can inhibit pathogenic microorganisms [64]. Previously, whole genome sequencing of *B. subtilis* EA-CB0575 [52] revealed the potential for the production of siderophores such as bacillibactin, enterochelins, and vibriobacins.

Summing up, it should be noted that the potentially useful properties of *B. velezensis* KP-2 revealed via whole genome sequencing make it possible to recognize this strain as a bacterial resource promising for use in agriculture. Due to the potential for synthesis of a complex of metabolites, the strain can adapt to a specific environment in the host's digestive system, in the rhizosphere or in feed during fermentation, successfully compete with other members of the autochthonous microbiota. Additionally, *B. velezensis* KP-2 exhibit functions that mediate the positive effect of the strain on microbiological processes during introduction into various environments.

Thus, as a result of whole genome sequencing of the *Bacillus velezensis* KR-2 strain isolated by us from the rumen of a dairy cow, 16 contigs are obtained with a total length of 3936398 bp, containing 46.6% GC pairs, with quality N50 2109194 bp and N75 844068 bp. The chromosome includes 3854 coding sequences

(CDS) associated with the synthesis of polypeptides. We have found 464 metabolic subsystems the most represented of which are the subsystems of the metabolism of amino acids and their derivatives (431 subsystems) and the metabolism of carbohydrates (416 subsystems). The functional annotation revealed a complex of potential properties, including the synthesis of antimicrobial peptides, fatty acids, vitamins, siderophores, auxins, the ability to adhere, motility and chemotaxis, resistance to toxic compounds, the ability to withstand stress factors, stimulate plant growth and participate in phosphate metabolism. In particular, in the genome of the *B. velezensis* KR-2 strain, genes involved in the production of bacillicin bacillisin (*BacA*, *BacB*, *BacG*, *BacF*, *BacD*) are found. A unique pathway for intracellular synthesis of the osmoprotectant glycine-betaine with the participation of the *BetA*, *BetB*, *BetT*, and *BetC* genes is also identified. The potential for the synthesis of auxins such as indole-3-ethanol (*IAR*, *TO* genes), indole-3-acetaldehyde (*IAD*, *AAD*, *AO*) and indole-3-acetonitrile (*N3*) is shown. In addition, in the *B. velezensis* KP-2 genome, several clusters of genes associated with siderophore synthesis (*DhbA*, *DhbB*, *DhbC*, *FeuA*, *FeuB*, *FeuC*, *FeuD*) are identified. The peculiarity of the *B. velezensis* KP-2 genome associated with the synthesis of the siderophore myxochelin A which we have identified, is probably unique, since this was not found in other strains of the species *B. velezensis*. Glycine-betaine synthesis pathways involving the *BetA*, *BetB*, *BetT*, and *BetC* genes identified by us are important for the cellular adaptation of the *B. velezensis* KP-2 strain to high-osmolar stress occurred in environments subject to frequent fluctuations in water content, for example, in dried plant mass of silage and in the upper layers of the soil. The ability to synthesize bacteriocins are annotated in the *B. velezensis* KR-2 strain, which is consistent with the antagonism against *Staphylococcus aureus*, *Escherichia coli*, *Fusarium oxysporum*, and *Clostridium butyricum* observed in vitro. In the future, using various methods (liquid chromatography, mass spectrometry, nuclear magnetic resonance spectroscopy), we plan to compare the obtained molecular characteristics with the empirically observed biochemical and physiological patterns of beneficial metabolite production in *B. velezensis* KR-2. This will provide new fundamental knowledge about the genetic control and regulation of the synthesis of bioactive substances in bacilli and the use of *B. velezensis* KR-2 in agriculture.

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CATTLE COLIBACILLOSIS IN PERM KRAI: PREVALENCE, SOURCES OF THE CAUSATIVE AGENT AND ITS BIOLOGICAL CHARACTERIZATION

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Abstract

Infectious diseases in agricultural enterprises of the Russian Federation are annually recorded in 50 % of the livestock, while the death of young calves during the first weeks of life ranges from 14 to 60 %. Colibacillosis remains the main infectious pathology in terms of morbidity and mortality, despite the widespread use of modern antibiotics and vaccines. To assess the prevalence of colibacillosis among cattle and identify the source of the infectious agent, for the first time an integrated approach was used covering a triad of sick animals—healthy animals—environment, which is of theoretical significance because it contributes to a better understanding the patterns of the epizootic process. An important practical aspect of the work was the analysis of data from long-term sanitary and zoo-hygienic records, which indicates that the control of the risk of *Escherichia coli* infections should be aimed not only at the natural reservoirs but also at the environment factors. The purpose of the study was to assess the prevalence of colibacillosis in cattle in various agricultural enterprises of the Perm Territory and to study the biological properties of the pathogen. To assess the *Escherichia coli* contamination of inventory, feeding and watering systems, data from sanitary and zoo-hygienic studies were analyzed. The incidence of the infections in cattle was summarized based on the reports from the Perm Veterinary Diagnostic Center, the livestock department of the Ministry of Agriculture of the Perm Krai for 2010-2020, statistical data from the Department of Veterinary Medicine of the Perm Krai and form laboratory records. The reports also covered bacteriological data for 22,480 samples from beef and dairy farms (n = 146). Sanitary and zoo-hygienic analyses of swabs from dairy equipment, inventory of slaughterhouses, feed of plant and animal origin, mixed feed were carried out for 29,207 samples from the same farms. The antigenic structure of the *E. coli* isolates was determined. The sensitivity of strains to antibacterial drugs was assayed by disk diffusion method (ampicillin 10 μg, cefoxitin 30 μg, ceftriaxone 30 μg, cefepime 30 μg, meropenem 10 μg, imipenem 10 μg, aztreonam 30 μg, amikacin 30 μg, gentamicin 10 μg, ciprofloxacin 5 μg, levofloxacin 5 μg, moxifloxacin 5 μg, tetracycline 30 μg, chloramphenicol 30 μg). In a prospective study (2020-2021), the prevalence of Shiga toxin-producing *E. coli* (STEC) strains in a population of healthy animals was assessed. Cultures (n = 61) were isolated from cattle feces. Genes encoding Shiga toxins 1 and 2 (*stx1* and *stx2*) were detected by polymerase chain reaction at the end point. The research results indicate that in recent years the prevalence of colibacillosis in the Perm Krai does not exceed 20 %. An increased number of dead animals and sick animals with diarrheal syndrome corresponded to a decreased immunization of pregnant cows. There were sporadic cases of the disease, but the risk of horizontal transmission of the pathogen, including through environmental objects, was high. *E. coli* was mostly isolated from swabs from dairy equipment and inventory of slaughterhouses, as well as from animal feed. Five most epizootically significant serogroups were identified, the *E. coli* O8, O15, O20, O101, and O115; in rare cases, *E. coli* O157 was isolated. The antibiotic resistance profiles of *E. coli* strains isolated from sick, dead and healthy animals did not differ significantly, except for tetracycline and chloramphenicol, resistance to which was significantly higher in *E. coli* strains of the first group. Of note is the higher proportion of cultures resistant to ampicillin and ciprofloxacin in the *E. coli* subpopulation from healthy cattle. In addition, the *stx1*

(2.0 %) and *stx2* (6.1 %) genes were found in *E. coli* from healthy cattle. That is, our data confirm that pathogens can persist in the gastrointestinal tract of both sick and healthy farm animals, which become the source of STEC. Additional sources of *E. coli* infection, including STEC strains, are drinking water, feed, and other abiotic components.

Keywords: cattle, colibacillosis, *Escherichia coli*, Shiga-like toxins, on-farm contamination

One of the priorities of the agro-industrial complex of the Russian Federation is to provide the population with environmentally friendly meat and dairy products [1, 2]. For this purpose, modern industrial technologies are actively used in livestock farms, which, however, have a number of disadvantages. Year-round stall keeping, high concentration of livestock in limited areas, lack of walking and insolation lead to an increase in the functional load on the animal's body and, as a result, to an increase in morbidity, especially of an infectious nature [3-5]. As a result, the number of stillborn and non-viable calves increases, which causes enormous economic damage, which is associated not only with the death of offspring, but also with a decrease in productivity, overspending of feed and significant treatment costs [6, 7]. Diseases of the gastrointestinal tract are the most significant in terms of mass and economic damage.

Infections of the gastrointestinal tract in livestock farms in Russia are annually recorded in 50% of the livestock, while the death of young animals in the first weeks of life ranges from 14 to 60% [8]. In newborn calves, diseases of the digestive system, accompanied by a diarrheal symptom complex, account for 60-70% of pathologies. Colibacillosis in terms of the number of cases and mortality, despite the widespread use of modern antibiotics and vaccines, remains the main infection in animals. This is an acute disease that occurs with diarrhea, signs of dehydration, intoxication, and dysfunction of the cardiovascular and central nervous system [9-11]. According to numerous studies, the prevalence of colibacillosis varies widely, from 5.4 to 90%, and the associated mortality of calves is estimated at approx. 20% [12]. Such variability in incidence rates is due to environmental problems, the conditions of keeping and raising animals, as well as the quality of sanitary and hygienic measures at agricultural enterprises in different countries [13]. The spread of colibacillosis in livestock complexes depends on the presence of a source of the infectious agent, the susceptibility of calves, and on the effectiveness of preventive measures [14].

In the etiology of colibacillosis in cattle (cattle), the most significant are enterotoxigenic (enterotoxigenic *Escherichia coli*, ETEC), enteropathogenic (enteropathogenic *E. coli*, EPEC) and enterohemorrhagic (enterohemorrhagic *E. coli*, EHEC) pathotypes of diarrheal *E. coli*, which cause enterocolitis in people, which makes this problem relevant not only for veterinary medicine, but also for medicine [12, 15, 16]. Representatives of enterotoxigenic serovars produce thermostable (STa or STb) and/or thermolabile (LT1 or LT2) enterotoxins that cause dehydration and are often found in calves in the first three days of life. Enterohemorrhagic and Shiga-toxin-producing (EHEC, STEC) strains of *E. coli* produce a toxin that, by damaging the intestinal microvilli, causes hemorrhagic diarrhea in calves at the age of 2-5 months [17, 18]. Infection of animals with less virulent cultures of extraintestinal *E. coli* leads to the occurrence of diseases of extraintestinal localization, in particular, polyarthritis, meningitis, less often uveitis and nephritis, which can become chronic.

It is known that representatives of diarrheagenic *E. coli* pathotypes are able to circulate for a long time in livestock enterprises among the livestock [19]. According to most researchers, the main source of the infectious agent is excreta (faeces) of apparently healthy animals, since *Escherichia* strains of enteropathogenic serogroups can persist in the intestines of adults [12, 18]. The udder of cows with a lack of bedding and irregular cleaning of manure can be contaminated with

bacteria, which leads to infection of calves through the digestive tract during sucking, as well as when drinking contaminated milk. Among calves, transmission of the pathogen occurs through direct contact ("nose-to-nose") or through the respiratory tract [17]. Consequently, adult animals and young animals serve as a reservoir and/or source of the infectious agent, which allows the bacteria to persist in the herd, circulating among animals of all ages. In agricultural enterprises, colibacillosis pathogens can also be transmitted through drinking water and feed. The data of the microbiological study of feed on the territory of Russia for 2014-2018 indicate that representatives of enteropathogenic *E. coli* were most often isolated from feed of plant origin and compound feed [20].

Monitoring of pathogens of bacterial infections is an essential component of the system of epizootic surveillance of farm animals. Because of the increased cases of food poisoning when using animal products contaminated with Shiga toxin-producing *Escherichia*, control over the circulation of these pathogens in a population of healthy individuals is of particular importance.

This report presents for the first time data on the prevalence of colibacillosis and the biological properties of pathogens circulating in agricultural enterprises in the Perm region. For the first time, an integrated approach was applied to assessing the prevalence of colibacillosis among cattle (sick animals-healthy animals-environment), which is of theoretical importance, deepening the understanding of the patterns of the epizootic process in this disease. An important practical aspect was the analysis of data from long-term sanitary and zoohygienic studies, the results of which indicate that on-farm control of the risk of infection of animals and personnel of enterprises with diarrheal *Escherichia* should extend not only to the natural reservoir, but also to the environment.

The purpose of the work is to assess the prevalence of colibacillosis in cattle in various farms of the Perm Territory and to characterize the pathogen by biological properties. In order to control the contamination of equipment, feed and watering system with *Escherichia* in livestock farms, an analysis of the data of sanitary and zoohygienic studies was carried out.

Materials and methods. The dynamics of the incidence of bacterial infections in cattle was analyzed based on the reporting data of the Perm Veterinary Diagnostic Center, the Department of Animal Husbandry of the Ministry of Agriculture of the Perm Territory for 2010-2020, the statistical data of the Department of Veterinary Medicine of the Perm Territory (form No. 1-vet, 2-vet, form No. 3, 4), as well as laboratory journals for 2016-2020. The reporting included results for 146 beef and dairy enterprises of all categories, including 92 (63.0%) agricultural enterprises, 46 (31.5%) farms and 8 (5.5%) individual farms. The livestock for the entire observation period averaged 76754.2±850.1 animals. Sanitary and zoohygienic studies were carried out at the same enterprises. Biological fluids, excreta and pathological material obtained from sick and dead animals were studied, i.e., feces, blood, urine, nasal mucus, vaginal mucus, preputial mucus, liver with gallbladder, a segment of the affected small intestine (22480 samples were analyzed in total). In addition, in dynamics, swabs from dairy equipment, inventory of slaughterhouses, feed of plant and animal origin, compound feeds were analyzed (29207 samples in total).

Bacteriological examination was carried out according to the "Guidelines for bacteriological diagnosis of colibacillosis (escherichiosis) in animals" (Moscow., 2000). The antigenic structure of the isolated *E. coli* strains was studied using the O-coli agglutinating sera kits (FKP Armavir Biofactory, Russia) and test sera for typing adhesive antigens of *Escherichia coli* F4 (K88), F5 (K99), F6 (987P), F41, A20 (Att25) (Vyshelessky Institute of Experimental Veterinary Medicine, Russia) according to the manufacturer's instructions.

The sensitivity of strains to antibacterial drugs was determined according to MUK 4.2.1890-04. The strains were tested by disk-diffusion method using Muller-Hinton agar (SRC PMB, Russia) and disks (OOO NITsF, Russia) for sensitivity to penicillins (ampicillin, 10 μg), cephalosporins (cefoxitin, 30 μg; ceftriaxone, 30 μg; cefepime, 30 μg), carbapenems (meropenem, 10 μg; imipenem, 10 μg), monobactams (aztreonam, 30 μg), aminoglycosides (amikacin, 30 μg; gentamicin, 10 μg), fluoroquinolones (ciprofloxacin, 5 μg; levofloxacin, 5 μg; moxifloxacin, 5 μg), tetracyclines (tetracycline, 30 μg), and fenicol (chloramphenicol, 30 μg). Insensitivity of strains to at least one drug from three or more groups of antibiotics was considered multidrug resistance (MDR).

In a prospective study (2020-2021), the prevalence of Shiga toxin-producing *E. coli* strains was assessed in a population of healthy animals (calves and cows). Cultures ($n = 61$) were isolated from cattle feces at animal husbandry enterprises in the Perm Territory.

DNA was isolated by the following method. A separate colony of each strain was resuspended in 0.5 ml of ultrapure water in Eppendorf test tubes, incubated in a Termit solid-state thermostat (NPO DNA-Technology LLC, Russia) for 10 min at 98 °C, cooled and centrifuged for 5 min at 13000 rpm. The supernatant was used for genetic studies immediately or after storage at –18 °C. Genetic typing of cultures was carried out in a double control system by rep-PCR (repetitive element sequence-based PCR) with primers M13 (5′-GAGGGTGGCGGTTCT-3′) and ERIC1R/ERIC2 (5′-CACTTAGGGGTCCTCGAATGTA-3′/5′-AAGTAAGTGACTGGGGTGAGCG-3′) using appropriate reaction modes [21, 22]. Detection of the *stx1* and *stx2* genes was performed using primers stx1-F/stx1-R (5′-ATAAATCGCCTATCGTTGACTAC-3′/5′-AGAACGCCCACTGAGATCATC-3′; 180 bp fragment) and stx2-F/stx2-R (5′-GGCACTGTCTGAAACTGCTCC-3′/5′-TCGCCAGTTATCTGACATTCTG-3′; 255 bp fragment). For both pairs, the general amplification regimen was used as recommended [23]: 3 min at 95 °C (initial denaturation); 1 min at 95 °C, 2 min at 60 °C, 1.5 min at 72 °C (25 cycles); 5 min at 72 °C (final elongation). Oligonucleotide primers were synthesized at OOO Sintol (Russia). DNA amplification was carried out using reagents manufactured by OOO Sintol (Russia) on a DNA Engine Dyad thermal cycler (Bio-Rad, USA) in 25 μl of the reaction mixture. PCR products were detected uding a horizontal electrophoresis in 1.2% agarose gel at an electric field strength of 6 V/cm at room temperature. A buffer containing 0.25% bromophenol blue and 30% glycerol in ultrapure water was used to apply the samples. Agarose gels were stained with ethidium bromide solution (1-2 μg/ml) for 10-15 min. The bands were visualized and data were recorded using the Gel-Doc XR gel documentation system (Bio-Rad, USA).

Statistical data processing was carried out using Microsoft Office XP Excel 2013 and Statistica v.6.0 (StatSoft, Inc., USA). To compare qualitative features, the $\chi 2$ test (with Yates correction) was used. At $p < 0.05$, the difference between the compared samples was considered statistically significant.

Results. According to statistical reports for the period from 2010 to 2020, 1361 cases of infectious diseases of bacterial etiology were registered among cattle at livestock enterprises in the Perm Territory. The share of colibacillosis in the infectious pathology of animals varied from 0.2 to 61.5% and averaged 14.4±11.9% over 11 years (Fig. 1).

It should be noted the high incidence in 2011. The decrease in the proportion of colibacillosis among animal infections in 2012-2014 was apparently due to an increase in the prevalence of leptospirosis.

In 2020-2021, 39 cultures of diarrheal *E. coli* were isolated from sick and dead animals (calves and adult cattle). Among them, we identified 5 epizootically

most significant serogroups (O8, O15, O20, O101, O115), *E. coli* O157 was detected sporadically. The frequency of occurrence of A20 adhesin among pathogenic strains of *E. coli* was 6%. In most cases, *E. coli* cultures showed resistance to ampicillin and cefazolin (61.5% each). We found high resistance of cultures to other cephalosporin antibiotics, the cefoxitin (30.7%) and ceftriaxone (23.1%). Imipenem, meropenem, amikacin, tobramycin and moxifloxacin had the highest activity against *Escherichia*. The proportion of sensitive strains was 100, 97.4, 92.3, 100 and 92.3%m respectively. A high frequency of resistant strains of *E. coli* has been reported to tetracycline (79.5%) and chloramphenicol (61.5%).

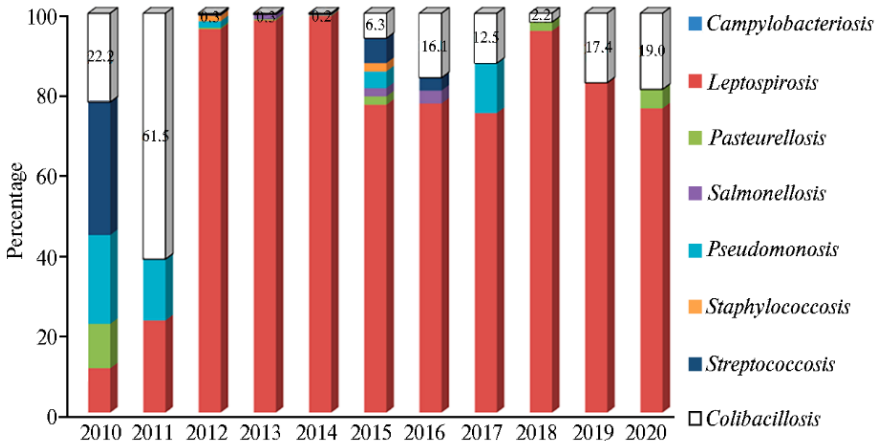

Fig. 1. Proportion of colibacillosis among cattle at livestock enterprises in the Perm Territory (statistical reporting, 22480 samples).

According to the results of ERIC typing of the primary collection of isolates (n = 61) isolated from the faeces of healthy calves and cows at industrial animal husbandry enterprises, we identified 49 representatives of individual genomogroups, which were used in further work. The *stx1* gene was detected in only one culture (2.0%), *stx2* was present in the genome of three (6.1%) cultures. That is, among these 49 *E. coli* strains, we found four STEC strains (8.1%).

1. The prevalence of antibiotic resistance among strains of *Escherichia coli* isolated from cattle at livestock enterprises in the Perm Territory (2020-2021)

Group of antibiotics	Antimicrobial agent	Resistant strains, %	
		sick/dead animals (n = 39)	healthy animals (n = 49)
Penicillins	Ampicillin	61.5	77,6
Cephalosporins	Cefoxitin (II)	30.7	20,4
	Ceftriaxone (III)	23.1	16,3
	Cefepime (IV)	23.1	14,3
Monobactams	Aztreonam	25.6	16,3
Carbapenems	Meropenem	2.6	0
	Imipenem	0	0
Aminoglycosides	Gentamicin (II)	7.7	2,0
	Amikacin (III)	7.7	0
Fluoroquinolone	Ciprofloxacin (II)	8.2	10,2
	Levofloxacin (III)	nd	6,1
	Moxifloxacin (IV)	7.7	nd
Tetracyclines	Tetracycline	79.5	49,0* (p = 0,0066)
Phenicols	Chloramphenicol	61.5	20,4* (p = 0,0002)

N o t e. In parentheses the generation of the antimicrobial agent is indicated, nd means no data.
* Differences from the group of sick/dead animals are statistically significant.

The formed collection of strains was tested for sensitivity to antimicrobial agents. Comparison of the antibiotic susceptibility profiles of E. coli strains isolated from sick and dead (group 1) and healthy (group 2) animals showed that for

most of the drugs the proportion of resistant strains was lower among cultures of the second group, but the difference was statistically significant only for tetracycline and chloramphenicol (Table 1). It should be noted a higher proportion of cultures resistant to ampicillin and ciprofloxacin in the subpopulation of Escherichia isolated from healthy cattle.

The results of microbiological studies of swabs from inventory and equipment, water samples from the animal watering system, feed and feed raw materials showed that the analyzed objects were contaminated to one degree or another by various microorganisms, but on average, the number of positive samples was a little more than 6%. Most often, the surfaces of equipment and inventory, as well as water sources, were contaminated. Representatives of *Salmonella* and *Escherichia*, *Proteus*, *Enterococcus*, *Staphylococcus*, *Streptococcus*, anaerobic bacteria and mold fungi were found in the studied samples. A total of 1806 bacterial cultures were isolated, of which almost 80% were *E. coli* isolates (Table 2).

2. The assessment of the sanitary and zoohygienic condition at livestock enterprises in the Perm Territory (2010-2020, statistical reporting)

Materials	*n*	Positive samples (%)	*Escherichia coli* (% of the number of positive samples)
Swabs:			
from dairy equipment[a]	6928	613 (8.8)	559 (91.2) p^{cdef}
from the inventory of slaughterhouses[b]	7486	721 (9.6)	594 (82.4) p^{cdef}
Feed:			
vegetable origin[c]	4414	71 (1.6)	50 (70.4) p^{def}
compound feed[d]	3060	106 (3.5)	99 (93.4) p^{ef}
animal origin[e]	5445	25 (0.5)	7 (28.0) p^{f}
Water for use[f]	1874	270 (14.4)	123 (45.5)
Total	29207	1806 (6.2)	1439 (79.7)

[a], [b], [c], [d], [e], [f] Differences between the indicated material and materials marked with the corresponding Latin letters are statistically significant at $p < 0.01$.

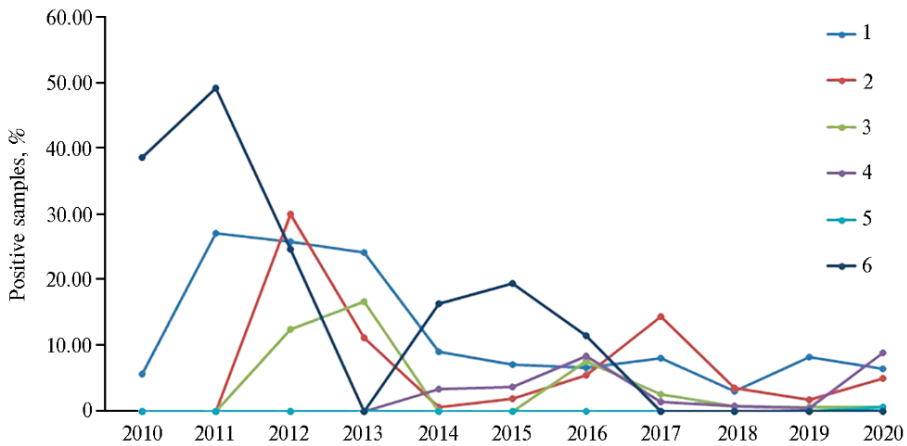

Fig. 2. Isolation of *Escherichia coli* from samples collected at livestock enterprises in the Perm Territory: 1 — swabs from dairy equipment, 2 — swabs from inventory of slaughterhouses, 3 — feed of plant origin, 4 — compound feed, 5 — feed of animal origin, 6 — water for use (statistical reporting, 29207 samples).

Most often, *Escherichia* was isolated from samples containing swabs from dairy equipment and inventory of slaughterhouses, as well as in animal feed. The proportion of samples containing *E. coli*, in most cases, did not exceed 20% of the number of all studies carried out in a year. In 2011, the highest percentage of

Escherichia-contaminated samples (49.1%) taken from drinking systems was revealed (Fig. 2). Apparently, these results indirectly reflect the situation of colibacillosis at the livestock enterprises of the region in that period (see Fig. 1).

It is known that gastrointestinal diseases of bacterial etiology occupy a special place among cattle infections, while enteropathogenic strains of *E. coli* play a leading role in the microbial profile of infectious pathology [6, 13, 24]. The high prevalence of colibacillosis of farm animals is recorded in the countries of South Asia and Africa. So, according to a number of studies, in animals with a diarrheal symptom complex EPEC occurs in 75% of cases in India, in 54%in Pakistan, in 86%in Iran, in 63.6-82.0% in Egypt. In European countries, this figure is significantly lower, for example, in Sweden, the frequency of isolation of *Escherichia* in intestinal diseases of cattle was 11.5%, in Germany 42.0%, in France 20.3%, in Spain 35.9% [12]. The variation in the prevalence of colibacillosis in different geographical regions may be associated with climate, the environmental situation, the form of keeping animals (free or stall), the practice of using antibiotics and carrying out sanitary and preventive measures.

In most regions of Russia, constant microbiological monitoring is carried out, and data on the incidence of colibacillosis differ significantly in different farms. An analysis of long-term (1996-2015) statistics of infections of farm animals in the Krasnodar Territory showed that the incidence of colibacillosis in calves remained high (33.5-53.6%) throughout the entire study period and averaged 40.5% of all registered infections [25]. Colibacillosis is widespread in the farms of the Amur region, the share in the infectious pathology of calves in some areas reached 60% [26]. The incidence of calves with escherichiosis on dairy farms of the GUSP MTS Tsentralnaya (2014-2016) in the Republic of Bashkortostan was more than 30% [9]. On the territory of the Irkutsk region in 2001-2010, the incidence of colibacillosis in cattle ranged from 0.2 to 20.5%, however, the authors state that the epizootological situation, both in general for gastrointestinal diseases, and for colibacillosis during the period under review was tense. This pathology remains one of the main problems in farms in almost all regions of the Irkutsk region, especially during the stall period of keeping animals [27]. Similar patterns in relation to colibacillosis were revealed by us in the Perm Territory: over an 11-year period, the proportion of the disease in cattle fluctuated widely, while it should be noted that the situation in this nosology in the region has been relatively stable in recent years.

E. coli bacteria are extremely heterogeneous in antigenic structure and toxigenic properties, which determine the pathogenetic features of their persistence in the macroorganism. More than 700 *Escherichia* serotypes are known, but only some of them have the ability to cause a diarrheal symptom complex in animals, therefore, simultaneously with the identification of bacteria, serotyping of cultures is carried out in an agglutination reaction with specific O-coli agglutinating sera. The main role in the development of diarrhea in newborn calves is played by enterotoxigenic *Escherichia* strains with adhesion antigens K88, K99, 987P, F41, F18, A20, which are more common among bacteria of serogroups O8, O9, O15, O41, O78, O86, O101, O115 , O119, O137 [9, 12, 27]. Of particular importance is adhesin A20, which, according to the literature, is common among *Escherichia* that cause colibacillosis [9, 28]. STEC representatives do not usually cause disease in recovered or vaccinated animals with post-amnestic or post-vaccination immunity, but can cause mortality among young animals, hemorrhagic colitis and hemolytic uremic syndrome in humans [29]. Zoonotic strains of STEC include cultures of EHEC O157:H7, as well as representatives of other serogroups (non-O157 EHEC O26, O111, to a lesser extent O17, O56, O87, O108, and O109),

which have recently begun to circulate in agricultural enterprises with increasing frequency [19]. In the Perm Territory, the most significant bacteria in the etiology of colibacillosis in cows and calves turned out to be bacteria of the traditionally common serogroups O8, O15, O20, O101, and O115. It should be noted that strains of *E. coli* O157 were also recorded at agricultural enterprises, this serotype predominates in outbreaks of escherichiosis caused by STEC in Russia and other industrialized countries [30, 31].

Active use of antibiotics for the prevention and treatment of infectious animal diseases, as well as for fattening in many countries, leads to the emergence and spread of antibiotic resistance among commensal and pathogenic microbiota. Thus, studies of the antibiotic susceptibility of pathogenic isolates of Escherichia isolated from colibacillosis of calves at enterprises in the Altai Territory showed that most strains were resistant to seven to nine antibacterial drugs [32]. In Iran, of 63 *E. coli* isolates isolated from diarrheal calves, 76% were resistant to at least one of the drugs tested, and 62% of the cultures were multidrug resistant [33]. According to N.M. Sobhy et al. [34], 54.5% of isolates from calves with diarrheal syndrome in livestock farms in Egypt were multidrug resistant. The percent resistance to tetracycline, streptomycin, ampicillin, and trimethoprim/sulfamethoxazole (a combination known as co-trimoxazole) was 79.5, respectively; 67.0; 54.5 and 43.0%. Ceftazidime (14.8%), amoxicillin-clavulanate (13.6%), and aztreonam (11.3%) were the most effective, and none of the isolates was resistant to imipenem.

In our studies, the resistance of escherichiosis pathogens to beta-lactam antibiotics also varied widely, the proportion of those resistant to ampicillin was 61.5%, while all cultures were sensitive to imipenem. Interestingly, in *E. coli* strains isolated from sick/dead and healthy animals, the profiles of resistance to antibiotics did not differ significantly (with the exception of tetracycline and chloramphenicol, resistance to which was significantly higher in *Escherichia* of the first group). The MDR phenotype was observed in 14 (35.9%) and 16 (32.7%) cultures of the studied subpopulations, respectively, although, according to the literature, among the *E. coli* isolates circulating in livestock enterprises, more than half have multiple resistance [33, 35]. However, it should be noted that the percentage of beta-lactam-resistant *E. coli* strains exceeded those obtained by other researchers in relation to *Escherichia* isolated from both diseased and healthy animals [34, 36-38]. Significant differences in the prevalence of antibiotic-resistant microorganisms circulating in livestock farms in different countries may be due to the peculiarities of the conditions in which animals are kept and approaches to the use of antimicrobial drugs.

Cattle are the most important reservoir of zoonotic strains of STEC, which are transmitted to humans through dietary or faecal-contaminated water, and through direct contact with infected animals. The causative agents of infectious diseases can be isolated from the gastrointestinal tract not only of sick and dead, but also of healthy farm animals. Our studies confirm that the latter are the source of Shiga-like toxin-producing *E. coli* isolates, which occurred in 8.1% of cases. It should be noted that the published materials provide different data on the prevalence of *E. coli* producing Shiga toxin in herds of healthy animals, from 2.9 to 27.3% [39, 40]. At the same time, the frequency of detection of STEC in cattle with symptoms of intestinal infection, according to foreign studies, ranges from 10 to 40% [19, 34, 37]. The results of observations by V.I. Terekhova et al. [25] showed that 21.5% of *E. coli* strains isolated from calves with colibacillosis carried genes for Shiga-like toxins. Data on the prevalence of STEC are of great importance for the development of preventive measures in livestock enterprises and

the assessment of food safety, so monitoring the spread of STEC among livestock, including among healthy individuals, is one of the elements of infection control. The identification of factors that influence the carriage and shedding of STEC in cattle and lead to the development of the disease in humans is of both veterinary and medical importance [41].

On farms, the main sources of infection of cattle with *Escherichia*, including STEC strains, are drinking water, feed, and other abiotic components of the ecological system [19]. To a large extent, the health of farm animals, their reproductive functions and the biological value of final food products depend on the sanitary quality of feed. An analysis of data from microbiological monitoring of feed in the Russian Federation for 2014-2018, conducted in 2020, showed that pathogenic E. coli serotypes were most often isolated from feed of plant origin and compound feed [20]. In our studies, similar data were obtained: samples contaminated with Escherichia decreased in the series feed > feed of plant origin > feed of animal origin (see Table 2).

Vaccination of livestock with drugs containing various escherichial antigens of adhesion factors and enterotoxins is extremely important for the prevention of colibacillosis in livestock enterprises. Currently in Russia, including on the Perm Territory, commercial preparations used for active immunization against colibacillosis of farm animals are OKZ (*E. coli* O9: K99, *E. coli* O138: K88) (OOO Agrovet, Russia), Kombovak-K (*E. coli* O9, O78, O115; capsular polysaccharides K80, K30; adhesive antigens K99, F41) (OOO Vetbiokhim, Russia), Rotagal which contains *E. coli* EC/17 with adhesive antigen F5 (K99) (OOO Vetbiokhim, Russia), Skaugard 4KS (ScourGuard 4KS, enterotoxigenic strains of *E. coli* with adhesion factor K99) (Zoetis, Inc., USA). At the same time, it can be assumed that for the prevention of colibacillosis in different regions, it is more appropriate to use local strains of microorganisms for the preparation of vaccines and hyperimmune sera [26]. In this regard, interesting studies were carried out at the Vyshelessky Belarusian Research Institute of Experimental Veterinary Medicine in 2002, the purpose of which was to determine the correspondence between the O-serogroup affiliation of isolated epizootic strains and vaccine cultures [28]. The authors showed that O-serotypes of the applied *E. coli* vaccine strains are found in field isolates with a frequency of 30.3 to 55.2%, while in diseased and dead calves there was an increase in the number of isolates (up to 20%) carrying antigens that are absent in commercial vaccines.

In the Perm Territory, *E. coli* of non-vaccine serogroups were also encountered as etiological agents of colibacillosis in cows and calves, which confirms the need for specific prevention of acute intestinal diseases in calves, taking into account the spectrum of circulating enteropathogenic strains. Its basis is the immunization of pregnant cows, which allows you to protect young animals in the first days after birth. This approach is supported by data on the increase in the number of diseases of the gastrointestinal tract, recorded against the background of a decrease in the proportion of pregnant cows immunized against colibacillosis. Thus, according to the reports of the livestock department of the regional Ministry of Agriculture, in 2016, 57.6% of cows were vaccinated in the region, in 2020 - no more than 40%, while the number of sick calves increased from 21.9 to 24.8%, and the number fallen/forcibly killed, from 2.3 to 2.4% (data not fully presented). Nevertheless, it should be noted that over the past 5 years, the total number of cattle in the Perm Territory has remained stable in terms of numbers with an upward trend (172,029 heads in 2016, 173,944 heads in 2020).

The development of the epizootic process is mainly due to the peculiarities of the relationship of the infectious agent with the populations of the obligate and potential hosts, which, in turn, is determined by the biological properties of the

etiopathogen, the immune status of the macroorganism, as well as natural and economic factors [42]. Our comprehensive study, which analyzed the proportion of colibacillosis in the infectious pathology of animals, the prevalence of *Escherichia* in the on-farm environment, and also described some of the biological properties of the pathogen, allows us to better understand the epizootic process in colibacillosis and optimize preventive measures.

Thus, our sanitary and zoohygienic analysis indicates the need for more thorough bacteriological control of feed, dairy equipment, water and water supply sources, and inventory of slaughterhouses. In addition, on-farm control of the risk of infection of employees with diarrheal *Escherichia* is mandatory, which should extend not only to the natural reservoir of infection, but also to the environment. Vaccination, currently used as the main method of preventing colibacillosis, is not always effective enough. To reduce the colonization of the intestines of cattle by pathogenic *E. coli*, it is necessary to use probiotics, bacteriophages, as well as modification of the nutrition of young and adult animals.

So, the epizootic situation for colibacillosis in cattle in the Perm region in 2010-2020 was quite favorable: its share among all bacterial infections did not exceed 20%. The epizootic process was characterized by sporadic cases of the disease, while there was a high risk of transmission of the infectious agent in a horizontal way, including through environmental objects. *Escherichia* was mainly isolated from swabs from dairy equipment and inventory of slaughterhouses, as well as from animal feed. Among the isolates, five most epizootically significant serogroups were identified, the O8, O15, O20, O101, O115, in several cases *Escherichia coli* O157 were isolated. Antibiotic resistance profiles in *E. coli* isolates from diseased/deceased and healthy animals did not differ significantly (except for tetracycline and chloramphenicol, resistance to which was significantly higher in *Escherichia* of the first group). It should be noted a higher proportion of cultures resistant to ampicillin and ciprofloxacin in *Escherichia* from healthy cattle. In addition, we detected the *stx1* (2.0%) and *stx2* (6.1%) genes in the latter, i.e., our findings have confirmed that pathogens of infectious diseases can be present in the gastrointestinal tract of not only sick, but also healthy farm animals, which become a source of Shiga toxin-producing *E. coli*.

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KAZAKHSTAN STARTER COMPOUND FEED FOR AFRICAN CATFISH (*Clarias gariepinus*): FORMULATION, QUALITY CHARACTERIZATION, AND EFFICIENCY IN AQUACULTURE

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Abstract

Currently, the breeding of *Clarias gariepinus* is a promising area of aquaculture in Kazakhstan. African catfish is a fast-growing, rapid-maturating, hardy and disease-resistant species. In the Almaty region, breeding of African catfish is most relevant due to a large number of geothermal sources which can significantly reduce the cost of fish growing. However, fish farmers use imported starter feeds. Here, we submit research data on approbation of the Kazakhstan starter feed for larvae of *C. gariepinus*. A technology has been developed for the production of mixed feed based on grain raw materials using the extrusion method (LLP Kazakh Research Institute of Processing and Food Industry, Almaty). The experimental feed is a 50.7 % protein, 11.53 % fat, and 17.31 MJ/kg of metabolic energy. Experimental rearing (LLP Kapshagai spawning and rearing farm-1973 and a peasant farm MG, Almaty region, 2020). Trout feed Aller Futura EX 0.5-0.2 mm (Aller Aqua, Denmark), the most common feed for growing *C. gariepinus* in Kazakhstan was a control. The experiment was arranged in duplicate, two pools for the control and two pools for the experiment. The fish was fed by hand 12 times during daylight hours in equal portions. The growth rate of juvenile *C. gariepinus* was assessed by control catches at 10-day intervals and a final catch. Volumetric method was used for the *C. gariepinus* larvae counting. Fish-breeding and biological parameters of the juvenile *C. gariepinus* were obtained by expert assessments. Absolute bodyweight gain, the feed conversion ratio and the survival rate of juveniles were 1313.7 g, 0.93 vs. 0.82 in the control and 75 % vs. 77 % in control, respectively, (the LLP KSRF-1973) and 1588.5 g, 0.93 vs. 0.88, 72 % vs. 74 % (the MG farm). Thus, the developed starter feed is not inferior to the standard in terms of nutritional value, it is physiologically complete, balanced in the main nutrients, including essential amino acids, and easier to digest. The compound feed is a well-flowing crumbs from dark to light brown in color, has high water resistance, swells quite well (swelling time is 40 minutes), and retains its shape during swelling. It was well eaten by fish. The granules practically did not break, did not crumble, and were of a size convenient for fish. Compound feed complies with sanitary standards in terms of microbiological indicators and can be stored for 10 months. The cost of the developed starter compound feed is 3 times lower than that of the imported one, and the cost of African catfish juveniles was less by 0.39-0.73 tenge per individual while fish breeding and biological parameters remained quite high. That is, in terms of the combination of price-quality, the Kazakhstani starter mixed fodder corresponded to the used standard sample. The results obtained allow us to conclude that the proposed feed is competitive, and its use will improve the efficiency of rearing juveniles of African catfish and the capabilities of fish farms in Kazakhstan

Keywords: aquaculture, starting compound feed, *Clarias gariepinus*, juveniles, fodder coefficient, survival, extruding

Aquaculture in Kazakhstan is currently experiencing rapid development and may well provide local population with fresh processed fish products. This requires technologies for breeding fast-growing fish of commercially valuable species. One of these species is the African sharp-toothed catfish *Clarias gariepinus*, since when growing this species, products can be obtained in a short time and with minimal labor. [1-4].

The meat of *C. gariepinus* (is high in nutritional value [5, 6], contains an optimal ratio of proteins, fats, amino acids, and polyunsaturated (omega-3) fatty acids, the amount of which is greater than in the meat of salmon fish. In addition, the clariid catfish as an object of aquaculture has a number of advantages compared to the fish species traditionally grown in Kazakhstan, such as precocity, endurance, rapid growth (the commercial weight of 1000-1500 g is reached at the age of 6-7 months, puberty at 11-13 months), resistance to water turbidity and disease [1, 2, 7]. *C. gariepinus* is omnivorous, but in nature, it is mainly a predatory fish. On day 5, *C. gariepinus* larvae begin to be fattened with natural feed, on day 10 day with artificial starter feeds of fine grinding (0.1-0.5 mm) [8]. It is especially important that *C. gariepinus* can be reared at very high stocking densities, since clariid catfish do not require high oxygen content in water due to the ability to breathe atmospheric air. Thus, this fish is omnivorous, unpretentious and undemanding in feed, with feed consumption of 0.8-1.2 kg per 1 kg of product, which significantly affects its cost and production costs [9, 10].

In Kazakhstan, technologies for the production of feed for heat-loving fish species (tilapia) began to be developed earlier with good results obtained in experiments [11]. However, starter feeds are still imported from abroad (in particular, when growing catfish, the most common feed for trout is Aller Futura EX 0.5-0.2 mm, Aller Aqua, Denmark), which significantly increases the cost of fish products. Therefore, at present, it is especially important to create formulas for starting compound feeds and their production use for the wide distribution and cultivation of *C. gariepinus* in the warm-water farms of the republic. Such compound feeds were created jointly by NPC SPC of fisheries (Almaty) and TOO KazNIIPPP (Almaty) and tested under production conditions. Based on the results of these tests, work continued to improve the feed composition and properties [8, 12].

In this report, for the first time, it was confirmed that the improved formula of the starter feed from local ingredients fully meets the physiological needs of juvenile catfish. Production tests of compound feed and economic calculations of rearing juveniles show that the cost of sales when using this feed turned out to be lower than for imported feed by 1.3% in TOO Kapshagai Spawning and Nursing Farm-1973 (TOO KNVKh-1973) and by 2.3% in the Farm MG (KH MG). The starter feed of Kazakhstan production is balanced in terms of the main nutrients, has an increased digestibility, and is cost-effective and quite competitive.

The purpose of our study is to evaluate the physiological and economic efficiency of the composition-optimized starter feed for *Clarias gariepinus* juveniles developed in Kazakhstan.

Materials and methods. The composition of the starter feed and the technology of its production (TOO KazNII processing and food industry, Almaty) were developed and optimized taking into account the physiological needs of *Clarias gariepinus* larvae in the main nutrients [12, 13]. The nutritional value of raw materials and feed, its organoleptic and physico-chemical parameters, technological properties, microbiological parameters were evaluated according to the standards, including GOST 13496.0-2016, GOST ISO 6498-2014, GOST 13496.13-2018, GOST 28254-2014, GOST 13496.3-92 (ISO 6496-83), GOST 13496.4-2019, GOST 32905-2014, GOST 32933-2014, GOST 13496.2-91, GOST 26657-97, GOST 28497-2014, GOST 28758-97, GOST ISO 7218-2015, GOST

10444.15-94, GOST 10444.12-2013, GOST 31878-2012, GOST 31659-2012 (ISO 6579:2002), GOST 32011-2013 (ISO 16654:2001), GOST 10444.7-86, GOST 8.207-76, and the sanitary requirements for commodities subject to veterinary inspection (supervision) (approved by the Decision of the Commission of the Eurasian Economic Union dated June 18, 2010 No. 317).

The granulometric composition of crushed and not crushed farinaceous raw materials was determined by laboratory sieving with the installation of sieves with a hole diameter of 2, 1 and 0.5 mm. Based on the sieve analysis data, the grinding size modulus was calculated. The mixing efficiency was evaluated by the coefficient of variation V_c as a qualitative indicator of the component distribution in the mixture:

$$V_C = \frac{100}{\bar{x}} \sqrt{\frac{\sum (x_i - \bar{x})^2}{n-1}},$$

where $\bar{x}$ is the arithmetic mean of the content of the studied component in the mixture, %; x_i is the value of the studied indicator in the i-th sample, %; n is the number of analyzed samples.

When calculating the coefficient of heterogeneity, the formula of Lastovtsev and Khvaltsov was used:

$$V_C = \frac{100}{C_0} \sqrt{\frac{\sum (C_i - C_0) n_i}{n-1}},$$

where V_c is the heterogeneity coefficient, %; n_i is the number of samples with C_i; concentration; C_0 is the concentration of the same ingredient in its ideal distribution in the mixture; Ci is the concentration of the same ingredient in its single distribution in the mixture.

During the production of pilot batches of compound feed and development of the technology for its production by extrusion (TOO Pet Food KZ, Almaty region), the ingredients of plant and animal origin were crushed to 0.2-0.5 mm grains, dosed according to the recipe, mixed, the moisture content of the mixture was adjusted to 30% including the initial level and subjected to extrusion at a 110 to 140 °C and a pressure of 4 MPa. Fat heated to 48 °C was added by spraying in a drum machine. The resulting starter feed for *C. gariepinus* had the form of grits with a particle size of 2 mm.

To establish the shelf life under production conditions (TOO Pet Food KZ), 100 kg (5 bags) of each compound feed were stored for 2, 4 and 10 months at 10-25 °C and relative humidity from 60 to 75% in dry, clean, pest-free grain stocks, well-ventilated closed warehouses in packaged form, away from direct sunlight, sources of heat and moisture. The quality of feed during storage was assessed in terms of peroxide value and acid value of fat according to GOST 13496.18-85, GOST 13496.2-91, and GOST R 51850-2001.

Before the start of production tests of the developed compound feed, a general hydrochemical analysis of water in fish reservoirs was performed; during the experiments, the water temperature and the content of dissolved oxygen in the pools were determined 3 times a day, the pH of the aquatic environment once day. For measurements, a Consort 932 thermooximeter (Consort, Belgium) was used.

Production tests of the effectiveness of compound feed were carried out on juveniles of clariid catfish in fish farms of TOO KNVKh-1973 and KH MG (Almaty region). The duration of the production experiment was 30 days. Danish feed for trout Aller Futura EX 0.5-0.2 mm (Aller Aqua, Denmark) was used as a control. The experiment was carried out in duplicate (two pools for control and two pools for experiment), the fish were fed in equal portions by hand 12 times during daylight hours. Based on the results of control every 10 days and the final

catch, the growth rate of *C. gariepinus* juveniles was estimated. The number of larvae was measured by volume accounting. Fish breeding and biological indicators (initial and final weight of fish, absolute gain, relative gain, average daily gain, survival) and the feed coefficient were determined by the method of expert assessments; food intake and feeding behavior of fish were recorded [14-17].

The cost of fish seed material of clariid catfish when rearing on starter feeds of Kazakhstani and imported production was calculated according to the recommendations [18-20].

Statistical processing of the results of assessing the physicochemical and technological characteristics of the feed was performed in accordance with GOST 8.207-76 and GOST 11.004-74 (ST SEV 876-78). Statistical analysis of fish breeding and biological indicators was performed according to G.F. Lakin [21]. Data are presented as means (*M*) and their standard errors (±SEM). Differences between experiment and control were considered statistically significant at $p \leq 0.05$.

Results. Given the fact that artificial fish foods are made from raw materials such as fishmeal and fish oil, there are environmental and economic (high cost) restrictions on their use. Therefore, in the development of effective feed formulations, alternative sources of protein of plant and animal origin are of interest [22-24].

Currently, there are no domestic artificial starter foods for larvae and fry of *Clarias gariepinus* in Kazakhstan. As a result, the biological characteristics of fish in these age periods have not yet been of practical importance, and its nutritional requirements for feed in aquaculture have not been fully studied.

Based on the analysis of publications [12, 13], we determined the amount and the main sources of protein necessary and corresponding to the physiological needs of *C. gariepinus* juveniles, and the nutritional and energy value of starter mixed feeds used in the industrial cultivation of catfish:

Indicator	Value
Exchange energy	18.0 MJ/kg
Mass fraction (MF) of crude protein	Not less than 50.0 %
MF crude fat	Not less than 8.0 %
MF crude fiber	No more than 2.0 %
MF Raw ash	No more than 10 %
MF Lysine	Not less than 2.4 %
MF methionine + cystine	Not less than 1.1 %
MF Phosphorus	Not less than 1.2 %
Acid number of fat	No more than 30.0 mg KOH/g
Peroxide value of fat	No more than 0.2 %J/г
Moisture in compound feed:	
in granular	No more than 13.5 %
in extruded	No more than 10.0 %
Crumbly:	
granules	No more than 3.0 %
extrudate	No more than 2.0 %
Water resistance of granules	Not less than 30.0 мин

Raw materials for the production of starter feed (average samples) were evaluated by organoleptic properties and quality indicators (Table 1).

1. Physicochemical properties of ingredients for the development of starter feeds for *Clarias gariepinus* in aquaculture (average samples)

Ingredient	Metabolic energy, MJ/kg	Percentage							
		linoleic acid	crude				NFE	P	Ca
			protein	fat	cellulose	ash			
Flour from:									
fish	11,17	0,52	54,82	15,80		18,80	6,90	3,30	4,92
blood	11,74	0,10	78,20	1,10		5,60	9,43	0,38	0,42
meat and bone	9,04	0,78	48,20	25,20		22,20	11,7	4,89	9,23
Corn gluten	14,81	1,12	49,60	5,08	5,20	2,00	16,88	0,52	0,32
Yeasts	9,20	0,05	36,50	1,44	1,67	4,90	46,22	1,40	0,73
Soybean meal	9,63	0,54	42,02	1,20	10,6	7,00	32,2	0,65	0,37

Continued Table 1

Embryos:									
corn	21,67	20,70	16,20	46,50	3,20	5,80	23,7	0,32	0,11
wheat	13,85	3,41	29,90	10,90	3,00	5,60	37,6	0,39	0,29
Wheat gluten	15,80		75,10	1,80	0,60		13,8	0,30	0,43
Soy isolate	11,90		89,52	4,04		5,00		0,29	0,39
Wheat bran	7,20	1,77	14,40	4,10	9,88	4,97	54,87	1,00	0,24
Wheat	12,34	0,99	11,50	1,60	2,77	1,84	70,8	0,30	0,08
Oats	12,00	1,58	12,25	4,72	2,25	1,62	67,34	0,32	0,11
Corn	14,90	1,80	8,56	4,00	2,60	1,53	74,2	0,23	0,02
Fish oil	34,31	7,10		98,10					
Soybean oil	35,88	49,30		99,90					
Bentonite								2,34	

N o t e. NES — nitrogen-free extractive sudstances.

On this basis, we calculated the variation rates for including ingredients in starter feeds for *C. gariepinus* larvae in aquaculture:

Component	Percentage
Wheat	0-20
Peas	0-15
Wheat bran	0-5
Meal, cake:	
soy	0-20
sunflower	0-10
Corn gluten	0-15
Flour:	
meat and bone	0-15
meat	0-20
bloody	0-27
fish	0-70
Yeasts	0-15
Skimmed milk powder	0-10
Bentonite	0-1
Soy isolate	0-15
Wheat gluten	0-4
Oil:	
soy	0-3
sunflower	0-3
linen	0-4
Fish oil	0-8

There is an active search in the world for innovative ways to grow predatory fish seed stock, such as *C. gariepinus*, using artificial starter foods [25, 26]. The physiological usefulness of starter feeds for juvenile fish lies in the ability to satisfy the body's needs for basic nutrients and biologically active substances, to ensure high growth rate and survival at an early stage of development.

The starter feeds developed by us are physiologically complete in terms of nutritional value (protein content is not less than 50.0%, fat content is not less than 8.0%). The composition of the starter feeds being developed included components with a high content of crude protein and a low fat content, since the formulation was developed for extruded feeds. The rest of the fat (soybean oil, fish oil) was injected by spraying (Table 2).

2. Nutritional value of optimized starter feed for *Clarias gariepinus* in aquaculture

Parameter	Concentration
Moisture content, %	9,04
Crude protein, %	50,7
Crude fat, %	11,53
Crude fiber, %	0,83
Ash, %	10,0
Linoleic acid, %	1,58
Nitrogen-free extractive substances (NES), %	15,68
Lysine, %	3,46
Methionine, %	1, 02
Methionine + cystine, %	1,6
Tryptophan, %	0,6

Continued Table 2

Sugar, %	0,5
Starch, %	6,22
Phosphorus, %	1,85
Calcium, %	2,85
Gross energy, kcal/100 g (MJ/kg)	492,78 (20,61)
Metabolic energy, kcal/100 g (MJ/kg)	413,93 (17,31)
Composite energy value of feed, %	Protein — 58.4; fat — 22.1; carbohydrates — 19.5

As a control, the starting feed for trout Aller Futura EX 0.5-0.2 mm (Aller Aqua, Denmark) was chosen, which is 60% proteins, 15%fat, 5.7% carbohydrates, 12.6% ash, 0.7% fiber, 1.4% phosphorus, with energy value of 472.3 kcal/100 and digestible energy of 396.7 kcal/100 g.

Thus, the improved Kazakh starter feed for larvae and fry of *C. gariepinus*, manufactured by TOO Pet Food KZ is a well-flowing grist from dark to light brown. The ratio of components in the recipe creates a complete biological complex that provides balancing the feed in terms of exchange energy, protein, limiting amino acids, vitamins and minerals, and physical and chemical parameters. This compound feed fully meets the physiological needs of *C. gariepinus* juveniles.

One of the important indicators of produced feed pellets is their water resistance, which reduces feed losses, increases feeding efficiency and improves the ecological situation in water bodies, which is currently being given special attention [27]. Properly formulated feed can contribute to minimal water pollution [27]. According to the requirements of GOST 28758-97, the crumbling of extruded feed should not exceed 3%. In the experimental batch of starter compound feed developed by us, crumbling did not exceed 2.35%. The compound feed retained its shape during swelling, the swelling time was 40 min, that is, the developed starter compound feed has high water resistance, while swelling quite well.

The quality of compound feed made by extrusion is significantly improved due to thermodynamic methods of processing raw materials (pressure, temperature). During extrusion, gelatinization of starch occurs, that is, amylopectin is formed, as a result, the absorption of carbohydrates is significantly improved. Extrusion processing significantly improves the palatability of the feed by inactivating certain enzymes, making it more palatable; when exposed to high temperatures, toxins and pathogenic microflora are neutralized [26, 28, 29]. Protein under the influence of high temperature (1300 °C) and pressure breaks down into amino acids, which are much easier to digest. Losses of amino acids occurring during extrusion are not critical. The temperature of decomposition is 224 °C for lysine, 282 °C for tryptophan, 284 °C for phenylalanine, 283 °C for methionine, 337 °C for leucine, 284 °C for isoleucine, 315 °C for valine, 258 °C for threonine; the total proportion of essential amino acids in the diet remains almost unchanged compared to the original [28-30].

3. Fat oxidation in the optimized starter feed for *Clarias gariepinus* during commercial storage (TOO Pet Food KZ, Almaty region)

Parameter	Shelf life	Value
Acid number of fat, mg KOH/g	0	8.34
	2	16.93
	4	20.09
	10	26.36
Fat peroxide value, %J/g	0	0.10
	2	0.12
	4	0.17
	10	0.19

It is known that in stored raw materials and finished products, fat is oxidized to the formation of peroxides and acids, under the influence of which fat-soluble vitamins are destroyed and not absorbed by fish, which leads to diseases

and even death. In the feed for *C. gariepinus*, oxidation was assessed by the acid and peroxide values of fat as indicators of fat hydrolysis and oxidation (Table 3).

When stored for 10 months in all samples, an increase in acid number (up to 26.36 mg KOH) and fat peroxide number (up to 0.19%) was observed. The maximum permissible values according to the Unified veterinary (veterinary and sanitary) requirements for commodities subject to veterinary control (surveillance) are 30 mg KOH/g and 0.2 J%/g, respectively.

The absence of growth of pathogenic microflora during 10-month storage means that the sanitary indicators of the feed did not decrease during this period. Microbiological studies of compound feed did not reveal spore-forming bacteria *Bacillus subtilis*, *B. mesentericu*s, as well as lactic acid bacteria, yeast and filamentous fungi in samples with different shelf life.

The results of hydrochemical analysis of water in TOO KNVKh-1973 showed that it is suitable for fish breeding purposes. The oxidizability of water is low (1.12 mg/l in the well, 4.51 mg/l in the pond when heated), the phosphorus content did not exceed the standard (0.02 mg/l in the well, 0.09 mg/l in the pond). The mineralization of water from the well and the pond as a whole corresponded to the standard values (371 mg/l for the well, 665 mg/l for the pond). The increased content of nitrates in artesian water was corrected. Water from deep wells first entered the degasser tank, and then into the aerator tank, where it was saturated with oxygen. In general, in terms of hydrochemical parameters, water from an artesian well after carrying out the described measures becomes suitable for growing valuable fish species [31, 32]. The content of oxygen dissolved in water in the pools ranged from 6.0-8.2 mg/l, the pH was stable (average pH 7.9 for the pool), the temperature ranged from 19.2 to 29.3 °C (on average, 24.3 °C), the content of the main biogenic elements corresponded to generally accepted indicators [33]. The water samples from the water supply canal of KH MG were slightly alkaline (pH 7.96), the amount of organic matter was low (according to permanganate oxidation 5.9 mg O/dm^3), the content of biogenic elements was sufficient for the development of aquatic vegetation. The concentration of ammonium nitrogen accounted for 0.06 mg/dm^3, nitrites and mineral dissolved phosphorus for 0.001-0.003 mg/dm^3, nitrates for 1.3 mg/dm^3, iron for 0.03 mg/dm^3, siliconfor 3.5 mg/dm^3. In terms of technical properties, this water sample corresponds to the category of hard (with a total hardness of 6.1 mg-eq/dm^3), in terms of the total content of dissolved salts it belongs to fresh water with a mineralization of 715 mg/dm^3, in terms of dominant ions it belongs to the hydrocarbonate class, magnesium group. The quality of water from the water supply canal of KH MG according to the main indicators met the requirements for fish farms [31-33].

4. Fish breeding and biological parameters of *Clarias gariepinus* larvae of 5-week age when fed a standard and novel feed (N = 2, n = 25, TOO KNVKh-1973, Almaty region, 2020)

Parameter	Feed	
	novel	standard Aller Futura EX 0.5-0.2 mm
Growing time, days	30	30
Planting density, pcs/m^3	10000	10000
Initial weight, mg (M±SEM)	1.3±0.1	1.3±0.1
Final weight, mg (M±SEM)	1315±58.1	1380±62.3
Absolute weight gain, g	1313.7	1378.7
Average daily weight gain, mg	43.79	45.9
Feed ratio	0.93	0.82
Survival, %	75	77

In production tests, the proposed starter feed was well eaten by fish, the particles practically did not break, did not crumble, corresponded to the size required for grown fish, and had a high feed coefficient of 0.93. This is 0.11 higher

than for the most common in Kazakhstan feed company Aller Agua served as a control and meets the physiological requirements of catfish in terms of nutrition (Tables 4, 5). The survival of fish when using the two compared feeds practically did not differ and varied from 75 to 77% (see Tables 4, 5).

5. Fish breeding and biological parameters of *Clarias gariepinus* larvae of 5-week age when fed a standard and novel feed (N = 2, n = 25, KH MG, Almaty region,, 2020)

Parameter	Feed	
	novel	standard Aller Futura EX 0.5-0.2 mm
Growing time, days	30	30
Planting density, pcs/m^3	10000	10000
Initial weight, mg (M±SEM)	1.5±0.1	1.6±0.1
Final weight, mg (M±SEM)	1590±57.1	1680±61.2
Absolute weight gain, g	1588.5	1678.4
Average daily weight gain, mg	52.95	55.9
Feed ratio	0.93	0.88
Survival, %	72	74

At TOO KNVKh-1973, the average weight of *C. gariepinus* fed the developed feed was only 65 mg lower ($p > 0.05$) than that of fish fed standard feed, the average daily gain differed by 2.11 mg, the survival rate by 2% (see Table 4).

Satisfactory results were also obtained in both variants under the conditions of KH MG. The values of absolute and average daily gains in both variants differed slightly, by 89.9 and 2.95 mg, respectively; survival rates were within the standard values and with a difference of 2%; the value of the feed coefficient was 0.93 vs. 0.88; the mean weight of *C. gariepinus* was 90 mg lower ($p > 0.05$).

It should be noted that in Kazakhstan there are no starter feeds for larvae and juveniles of *C. gariepinus* and there are no regulatory documents for such feeds. Our production tests showed good results in both trials.

6. Estimated economical indicators of juvenile *Clarias gariepinus* aquaculture with standard and novel feed (TOO KNVKh-1973, Almaty region, 2020)

Parameter	Feed	
	novel	standard Aller Futura EX 0.5-0.2 mm
Planting material (for 10,000 fry), tenge	200000.00	200000.00
Payroll fund for employees, including taxes, tenge	6400.00	6400.00
Amount of feed, kg	9.16	8.70
Feed price, tenge	505.58	1500.00
Total cost of feed, tenge	4630.14	13051.04
Overhead costs, tenge	10551.51	10972.55
General production costs, tenge	221581.65	230423.59
Product output, pcs.	7500.00	7700.00
The cost of products sold, tenge/pcs.	29.54	29.93
Final weight, mg	1315.00	1380.00
Price-quality ratio, tenge/mg	0.022	0.022

7. Estimated economical indicators of juvenile *Clarias gariepinus* aquaculture on standard and novel feed (KH MG, Almaty region, 2020)

Parameter	Feed	
	novel	standard Aller Futura EX 0.5-0.2 mm
Planting material (for 10,000 fry), tenge	200000.00	200000.00
Payroll fund for employees, including taxes, tenge	6400.00	6400.00
Amount of feed, kg	10.63	10.93
Feed price, tenge	505.58	1500.00
Total cost of feed, tenge	5375.90	16390.70
Overhead costs, tenge	10551.51	10972.55
General production costs, tenge	222364.70	233930.24
Product output, pcs.	7200.00	7400.00
The cost of products sold, tenge/pcs.	30.88	31.61
Final weight, mg	1590.00	1680.00
Price-quality ratio, tenge/mg	0.019	0.019

The calculated parameters for rearing catfish juveniles when fed with different starter artificial feeds in different fish farms are presented in tables 6 and 7.

The largest part of the cost in rearing juvenile catfish in TOO KNVKh-1973 was the cost of fish stock as an intermediate fish product, the 90.3% of the total production costs when using the feed developed by us and 86.8% for the feed of the company Aller Aqua. This is followed by other expenses (utilities, feed costs, wage fund, etc.), 9.7 and 13.2%, respectively.

In the farm KH MG, the main part of the cost for reared fry was the cost of fish seed, the 89.9% of the total production costs for the developed compound feed vs. 85.5% for the feed of the company Aller Aqua. Other expenses accounted for 10.1% and 14.5%, respectively.

Despite the fact that the cost of the starter mixed fodder of Kazakhstan production is 3 times less than that of the imported one, the cost of reared juveniles of catfish using the proposed mixed fodder turned out to be lower by 0.73 tenge per individuum at KH MG and by 0.39 tenge per individuum at TOO "KNVKh-1973 while fish rearing and biological indicators remain at the same lavel.

So, the starter feed for *Clarias gariepinus* developed in Kazakhstan, is a well-flowing grits from dark to light brown. In terms of nutritional value (protein content of 50.70%, fat content of 11.53%, metabolic energy 17.31 MJ/kg), physico-chemical properties and attractiveness, the starter compound feed fully meets the physiological needs of *C. gariepinus* juveniles. When tested in two fish farms, the absolute increase in live weight was 1313.7-1588.5 g (vs. 1378.7-1678.4 g in control), the feed coefficient was 0.93 (vs.0.82-0.88 in control), survival rate was 75 and 72% (vs. 77 and 74% in control). The average weight of *C. gariepinus* fed the developed feed was 65-90 mg lower ($p > 0.05$). However, the cost of the developed feed is significantly (3-fold) lower than that of the standard, and in terms of nutritional value it is not inferior to the standard. The cost of reared juveniles of catfish in farm trials was less by 0.39-0.73 tenge per individuum while fish breeding and biological indicators of juveniles remain the same. The developed feed complies with microbiological sanitary standards and is well stored for 10 months. The use of this starter feed makes it possible to increase the profitability of rearing *C. gariepinus* juveniles in pools.

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REARING OF *Cherax quadricarinatus* (Von Martens, 1868) JUVENILES USING FEED FOR STURGEONS

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Abstract

At present, the Australian red-clawed crayfish *Cherax quadricarinatus* (Von Martens, 1868) farming is not well-developed and is mainly limited to temperate and subtropical regions where the initial stages of growth occur under well controlled conditions. In Russia, *C. quadricarinatus* farming technologies are under development and the values of bioproducts are much more lower. Juveniles of crayfish are more demanding on feed and require at least a 30-50 % protein-based diet for rapid growth. Starter feed for sturgeon fish species has the appropriate indicators, which opens up prospects for its use in crayfish farming. In this work, for the first time, we submit data on feeding norms, growth rate, survival and hematological indicators of juvenile crayfish fed sturgeon feeds with a protein content of 46 %. Scientific research was aimed at studying the parameters of hemolymph and growth of juvenile Australian red-clawed crayfish that received mixed feed for sturgeons. The experiments were conducted at the Kuban State University. Equal-aged 480 juveniles of Australian red-clawed crayfish with an average weight of 150 mg were selected from three females, mixed, and distributed in three rearing tanks of 160 individuals each, according to feeding rates (group I — 9 %, group II — 6 %, group III — 3 %). The tnaks were mounted in a multi-tiered closed aquaculture system. The crayfish juveniles were fed twice a day (in the morning at 9^{00} and in the evening at 1800) with granulated feed Coppens vital (0.8-1.2 mm) (Alltech Coppens B.V., the Netherlands), a protein content of 46.0 %. Survival rates and growth rates were recorded every 8 days (July 21, July 29, August 6, August 14, and August 22). A dayly feeding rate was adjusted with respect to changes in survival and biomass of the groups. On day 32, 13 individuals weighing from 0.67 to 1.39 g were selected, boiled and weighed separately. The meat was separated from the carapace and other inedible parts and weighed. To determine the total hemocyte counting (THC) and the proportion of granulocytes in the hemolymph of crayfish, the cuticle was pierced at the base of the first pair of pleopods from the ventral side of the first segment of the abdomen and a small amount of hemolymph was removed with a micropipette. At the end of the experiment, the median length was 3.50 cm for groups I and II, 3.40 cm for group III; the median weight was 0.94 g, 0.98 g, and 0.89 g for groups I, II, and III, respectively. The differences between the groups were statistically insignificant. The final mortality was 57 individuals (35.6 %) for group I, 62 individuals (38.7 %) for group II, and 58 individuals (36.2 %) for group III. Differences between the groups in total hemocyte counts (THC) and percentage of granulocytes were statistically insignificant. The THC average values ranged from 1005 to 1073 cells/μl, granulocytes accounted for 20.1 to 21.1 %. The median THC was 965 cells/μl for group I, 840 cells/μl for roup II, and 1101 cells/μl for group III; the median percentage of granulocytes was 21.1 %, 20.1 %, 20.6 %for groups I, II, and III. The THC values at different daily feeding rates does not depend on the percentage of granulocytes (the correlation coefficients ranged from –0.02 to –0.08). The relative weight gain decreased from 99.8 to 17.6 % (group I), from 102.6 to 19.1 % (group II), and from 105.4 to 16.9 % (group III). The specific growth rate was from 8.6 to 2.0 % (group I), from 8.8 to 2.2 % (group II), and from 9.0 to 2.0 % (group III). The meat yield index of crayfish juveniles did not differ significantly between the groups. Average indicators ranged from 31.1 to 32.5 %. In group I, the feed cost was the highest, 2.00 vs. 1.47 in group II and 0.72 in group III. Low feed consumption (daily feeding rate 3 %) with similar values

of growth rates, survival rates, average weight and length, and their medians indicate efficient assimilation of feed by *C. quadricarinatus* juveniles in group III and excessive feeding rate in other groups.

Keywords: *Cherax quadricarinatus*, Australian redclaw crayfish, juveniles, feeding, hemolymph, hemocytes, granulocytes, recirculating aquaculture system

The Australian red claw crayfish (*Cherax quadricarinatus*) belongs to the thermophilic aquaculture objects and has valuable consumer and economic qualities [1, 2]. The technology of industrial cultivation of Australian crayfish has not yet been sufficiently developed. Cultivation of this species is increasingly spreading in countries with a temperate and subtropical climate, where cultivation begins under controlled conditions and then continues in open water. In Russia, cultivation technologies for *C. quadricarinatus* are at the development stage and bioproduction indicators are much more modest [1, 3].

Cold climatic conditions require the obligatory use of closed systems for keeping spawners in winter, spawning, keeping females with eggs and juveniles, rearing juveniles [2, 4-6]. The most important element of this technology is the development of feeding. There are lines of specialized aquarium food on the market, which are disproportionately expensive and inapplicable for industrial breeding. Research and development of new methods and formulations of feeding met the physiological characteristics of crustaceans [7] is underway. These are the search for alternatives to traditional feed components [8, 9], the use of various additives [10-13] and various feeding regimes [14-17], but, as practice shows, it is long and expensive. The possibility of using already proven high-quality fish feeds in crayfish breeding has been poorly studied.

Young crayfish are more demanding on feed, and for its effective rapid growth, they must contain at least 30-50% protein [1, 18-20]. Starter feeds for sturgeon species meet the same requirements, which opens up prospects for their use in crayfish breeding. There have been attempts to explore the possibility of introducing these types of feeds in comparison with analogues [21], but different feeding regimens have not been sufficiently considered.

Malnutrition can lead to an inadequate balance of energy and material intake with macro- and micronutrient deficiencies, which is the most common cause of immunodeficiencies and is a risk factor in productive animals. In animal husbandry practice, violations of energy protein nutrition most often occur, which is accompanied by an increase in the sensitivity of individuals to infections due to suppression of innate and adaptive immunity [22, 23]. In this regard, when developing a diet, it is required to analyze indicators that characterize the state of the immune system. For crustaceans, these may be indicators of hemolymph. i.e., the total number of hemocytes (THN) and the proportion of granulocytes [24-28]. There are few studies that take these indicators into account when developing a diet for Australian red claw crayfish [15, 29].

In this work, for the first time, the feeding norms, growth rate, survival rate and hematological parameters of juvenile Australian red claw crayfish were established using specialized feed for sturgeon species with a protein content of 46%.

The purpose of the study was to study the effectiveness of the use of compound feed for sturgeon species and their effect on hemolymph parameters when growing juvenile Australian red claw crayfish.

Materials and methods. The experiment was carried out on the basis of the business incubator of the Kuban State University from July 21 to August 22, 2019. A multi-tiered recirculating water supply installation was 0.97 m in length, 0.70 m in width, 1.92 m in height. It consists of four nursery plastic containers with dimensions 78×56×18 cm (bottom 65.5×51 cm, area 0.334 m^2). Water treatment unit was divided into five compartments, the first for rough mechanical cleaning

from sponge layers of different porosity, the second-fourth for floating polypropylene loading BioElements (RK Plast, Denmark) with a density of 0.93 g/cm3 and a specific surface area of 750 m^2/m^3. The fifth is a storage tank with a pump and a thermostat with a volume of 0.11 m^3; pump 14HF HyperFlow (RIO, Taiwan) 3.4 m^3/h; piston compressor Hailea Electrical Magnetic AC ACO-208 (Hailea, China) with atomizers for air supply to the biological treatment compartment; connecting polypropylene pipes and taps. The containers were installed on a metal frame. Three growing tanks were used for the experiment. Each was covered with a Styrofoam lid, which reduced water consumption by minimizing evaporation and reducing the likelihood of cancer coming out of the containers.

For experiments, coeval juveniles of the Australian red claw crayfish with an average weight of 150 mg (480 ind.) were selected from three females, then mixed and distributed over nursery tanks. A total of 160 individuals were stocked in nursery tanks.

To reduce the damage from cannibalism, we used multi-story shelters made of polycarbonate and plastic mesh that we designed. As the juveniles grew, the shelters were changed in accordance with the size characteristics. Shelters were designed to provide juvenile crayfish both with individual cells (with a margin), and to create an area on the floors necessary for safe molts, as well as served as an additional substrate for the fixation of nitrifying bacteria.

For the experiment, the juveniles were divided into three experimental groups with different daily feeding rate (as a percentage of biomass), 9% in group I, 6% in group II, and 3% in group III. Juveniles were fed twice a day, in the morning at 9.00 and in the evening at 18.00, with granulated feed for sturgeon species Coppens vital (0.8-1.2 mm) (Alltech Coppens B.V., Netherlands). The Coppens vital is 46.0% protein, 10.0% fat, 1.5% phosphorus, 14000 IU/kg vitamin A, 2140 IU/kg vitamin D, 280 mg/kg vitamin E, 500 mg/kg stable vitamin C: total energy is 19.4 MJ/kg.

During the experiment, the main hydrochemical indicators of water (NO_3, NO_2, NH_4/NH_3, pH) were within the limits of fish breeding standards, they were checked with colorimetric tests of the Sera brand (Germany) and Api (USA). Cleaning the bottom with a siphon and partial replacement of about 30% of water was carried out once every 4 days. The water temperature during the experiment varied from 27.1 to 29.5 °C, the average was 28.3 °C.

Every 8 days (July 21, July 29, August 6, August 14, August 22) the survival rate, individual weight, biomass growth were assessed and the actual daily feeding rate was corrected taking into account changes in the number and biomass of the experimental groups. The measurement was carried out with a ruler with a 1 mm accuracy. For weighing, an electronic balance MEM-EBS (Mercury, South Korea) with an accuracy of 0.01 g was used. The sample size was 50 ind. At the end of the experiment, all individuals were measured.

To determine the yield of meat at the end of the experiment (on day 32), 13 individuals weighing from 0.67 to 1.39 g were selected and boiled, and thes individually weighed. The meat was separated from the carapace and other inedible parts of the body and weighed.

Relative growth rate (ΔM, %) and specific growth rate (C_w, %) of biomass were calculated [30] as $\Delta M = (M_t - M_0)/M_0 \cdot 100$ %; $Cw = (\ln M_t - \ln M_0)/t \cdot 100\%$ where M_0, M_t is the biomass at the beginning and end of the period, g. Feed consuption (FC, g) per unit of weight gain was calculated as $FC = M_f/WG$, where M_f is the amount of feed consumed, g, WG is the weight gain, g.

The THN and the proportion of granulocytes in the cancer hemolymph were determined at the end of the experiment (on day 32). The hemolymph was collected as follows: the cuticle was pierced at the base of the first pair of pleopods

on the ventral side of the first segment of the abdomen [31] and a small amount of hemolymph was removed with a micropipette.

Hemolymph samples were examined under a light microscope Mikromed-1 (Micromed, Russia) using the Goryaev chamber. The following formula was used to calculate the THN in 1 μl: THN = $N \times 10$ where N is the number of hemocytes in 25 large squares of the camera grid [32].

Mathematical data processing was carried out by standard methods of variation statistics. Mean values (M), standard deviations ($\pm\sigma$), coefficients of variation (C_V), medians (Me), 25th and 75th percentiles (Q1 and Q3) were calculated. Statistically significant differences between groups were identified using the Mann-Whitney U test and the Kruskal-Wallis test for non-parametric and independent groups. Calculations and graphic presentation of the obtained data were performed using Microsoft Excel and Statistica 12 software (StatSoft, Inc., USA).

Results. Figure 1 shows a multi-tiered closed water supply system which we used in the work.

Fig. 1. A multi-tier recirculating water supply facility used to rear juvenile Australian red-clawed crayfish (*Cherax quadricarinatus*).

As a result of the studies, the indicators of the dynamics of the average weights and biomass of juvenile Australian red claw crayfish during 32 days were obtained (Table 1). At the beginning of the experiment, the average weight of juveniles was 0.15 g. The average weight of juveniles at the end of the experiment was 1.06 g in group I, 1.01 g in group II, and 1.02 g group III. Differences between groups were not statistically significant.

The average weight in our experiment was comparable with that obtained by S.V. Sevasteev et al. [33]. In the authors' experiment, when feeding crayfish at the rate of 10% of the biomass with decapsulated brine shrimp, granules from brine shrimp, and brine shrimp "amber", juveniles weighed 1.11-1.54 g in 30 days vs. initial weight of 0.08-0.12 g.

The coefficient of body weight variation in juveniles in our experiment changed over time from 11.5-11.8 to 45.7-51.9%, and the differences between the groups were small. Such dynamics of variation in size and mass characteristics can be considered quite large for the Australian red claw crayfish, when compared with the few data of other works [2, 4, 5]. In our studies, this was most likely due to the greater variability of initial indicators and high stocking densities, which led to growth inhibition of initially lagging individuals. In the work of A.V. Zhigin et al. [6], the coefficient of variation in body weight changed from 8.51 to 14.17% when the weight stocking juveniles ranged within 0.44-0.57 g and an initial stocking density of 44.4/m^2. In our case, juveniles had such a mass 16 days after the start of the experiment, and the stocking density was in the range of 385.1-388.1/m^2.

Medians (Fig. 2) as averages of samples are considered more objective for the analysis of results. The medians for length were 3.50 cm in groups I and II, 3.40 cm in group III; the medians for weight were 0.94 g in group I, 0.98 g in group II, and 0.89 g in group III. Differences in medians according to the Kruskal-Wallis test were not statistically significant. The minimum weight and length of

juvenile Australian red claw crayfish at the end of the experiment in all groups were approximately the same and amounted to 0.33-0.40 g and 2.3-2.4 cm, respectively (see Fig. 2). The maximum indicators differed. In groups I and II, the values were greater, at 5.3 cm length of juveniles, the weight was 2.60 and 2.53 g, respectively, in group III, at 4.8 cm length, the weight averaged 2.34 g.

1. Weight of juvenile Australian red claw crayfish (*Cherax quadricarinatus*) fed Coppens vital (Alltech Coppens B.V., Netherlands) **granular feed for sturgeon species** (lab test, 2019)

Date	Weight (n = 50)	Group I	Group II	Group III
06/21 (the befininng of the test)	$M\pm\sigma$, g	0.15±0.017	0.15±0.017	0.15±0.018
	Cv, %	11.5	11.5	11.8
07/29	$M\pm\sigma$, g	0.34±0.059	0.34±0.062	0.34±0.060
	Cv, %	17.3	18.2	17.7
08/06	$M\pm\sigma$, g	0.56±0.161	0.57±0.173	0.60±0.169
	Cv, %	28.7	30.3	28.2
08/14	$M\pm\sigma$, g	0.81±0.432	0.72±0.310	0.76±0.361
	Cv, %	37.4	38.5	36.6
08/22 (the enf of the test)	$M\pm\sigma$, g	1.06±0.551	1.01±0.461	1.02±0.506
	Cv, %	51.9	45.7	49.7

N o t e. See the description of the experiments in the Materials anad methods section.

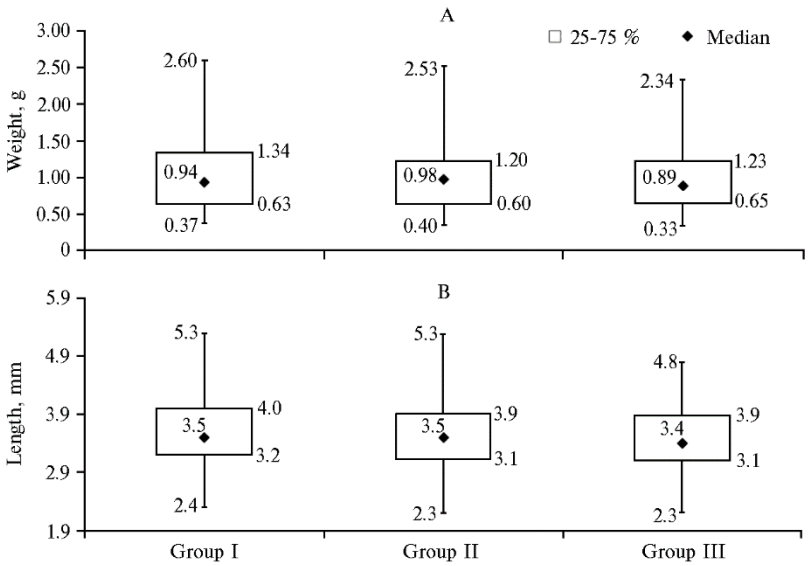

Fig. 2. Final weight (A) **and length** (B) **of juvenile Australian red claw crayfish (*Cherax quadricarinatus*) fed granular feed for sturgeon Coppens vital** (Alltech Coppens B.V., Netherlands) **on day 32 of the experiment** (lab test, 2019). See the description of the experiments in the Materials anad methods section.

Variation series were compiled to estimate the size structure of juveniles obtained at the end of the experiment (Fig. 3, 4). Weight was estimated according to 13 size classes with an interval of 0.2 g, length according to 10 classes with an interval of 0.3 cm. In all groups, the most representative individuals for weight and length were those of 0.40-1.39 g and 3.10-3.99 cm (see Fig. 3, 4). As the mass increases, one can notice a systematic decrease in the number of individuals in size groups.

The Australian red claw crayfish is characterized by high cannibalism, so the survival rate during the experiment (Table 2) was in the range of 61.3-64.4%. Mortality eventually amounted to 57 ind. (35.6%) in group I, 62 ind. (38.7%) in group II, and 58 ind. (36.2%) in group III.

The change in biomass during the experiment is indicative (see Table 2).

Initially, it was 24 g for each group. By the end of the experiment, group I was characterized by the largest actual increase in biomass of 85.5 g vs. 75.0 g in group II and 79.9 g in group III. This difference are not significant, since, given close average masses, it is due to greater survival in the group.

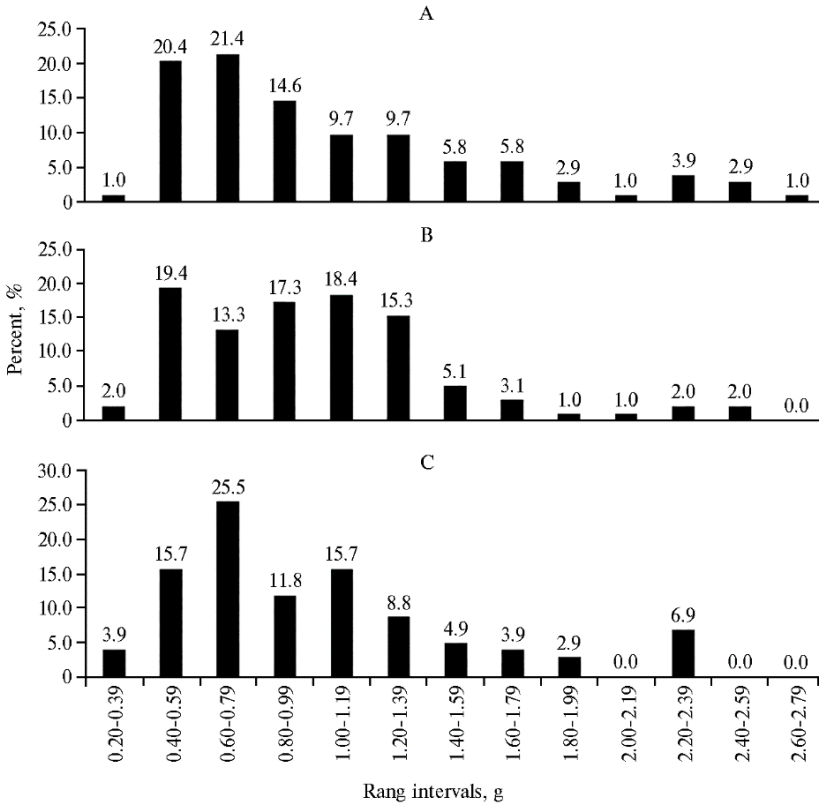

Fig. 3. Variation series of weight parameters in juvenile Australian red claw crayfish (*Cherax quadricarinatus*) fed granular feed for sturgeon Coppens vital (Alltech Coppens B.V., Netherlands) **on day 32 of the experiment:** A — group I, B — group II, C — group III (lab test, 2019). See the description of the experiments in the Materials anad methods section.

Since the daily feeding rate expressed as a percentage, increased in proportion to the growing biomass at each stage, it is incorrect to focus only on the above actual values. The description of the relative growth and specific growth rate can be considered decisive here, which decreased as they grew older in all groups. The relative increase in 32 days decreased from 99.8 to 17.6% in group I, from 102.6 to 19.1% in group II, and from 105.4 to 16.9 in group III. This is due to a natural decrease in metabolism as individuals mature and an increase in molt spacing in crustaceans, which is observed in many decapods. The specific growth rate ranged from 8.6 to 2.0% in group I, from 8.8 to 2.2% in group II, and from 9.0 to 2.0% in group III.

Malnutrition can lead to an inadequate ratio of energy and substance intake with a number of macro- and micronutrient deficiencies. It becomes the most common cause of immunodeficiencies and is a risk factor in productive animals. The red swamp crayfish (*Procambarus clarkii*) has been studied as an alternative to fish oil for feed [34]. Its complete replacement with beef showed potential harm to the health of crayfish, while partial replacement did not suppress growth and did not impair antioxidant capacity and innate immunity. An increase in hemolymph triglycerides and free fatty acids was observed compared to crayfish fed a complete traditional diet ($p < 0.05$) [34].

To some diets, various components (e.g., immunopotentiators, such as glycyrrhizic acid as an antiviral agent) are periodically or regularly added. F. Liu et al. [35] showed that crayfish in groups receiving the optimal dose of 50-150 mg/kg feed had increased final body weight, weight gain, specific growth rate, and reduced feed conversion rate compared to control ($p < 0.05$). The total number of hemocytes and the content of phenol oxidase in the hemolymph were increased, immune responses and expression of immunity-related genes were improved. The use of *Codonopsis pilosula* polysaccharides in the diet [36] gave similar results. The influence of the probiotic *Pediococcus acidilactici* [37], *Lactobacillus acidophilus*, *L. plantarum* [38] on the immunological parameters of hemolymph and the enzymatic system of juvenile *Astacus leptodactylus* and *Cherax cainii*, as well as chitosan on *Procambarus clarkii* [39] was studied. These approaches to varying degrees affected the hemogram of crustaceans and the general physiological state.

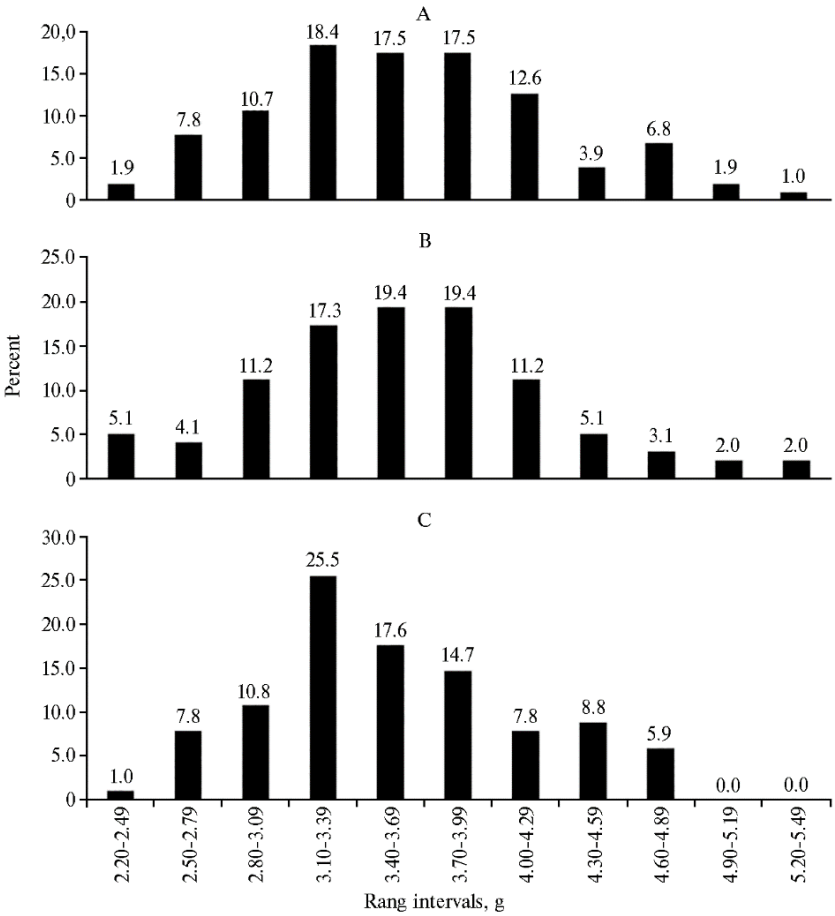

Fig. 4. Variation series of length parameters in juvenile Australian red claw crayfish (*Cherax quadricarinatus*) fed granular feed for sturgeon Coppens vital (Alltech Coppens B.V., Netherlands) **on day 32 of the experiment:** A — group I, B — group II, C — group III (lab test, 2019). See the description of the experiments in the Materials anad methods section.

2. Weight gain and survival of juvenile Australian red claw crayfish (*Cherax quadricarinatus*) fed Coppens vital (Alltech Coppens B.V., Netherlands) **granular feed for sturgeon species** (lab test, 2019)

Date	Group I	Group II	Group III
	W e i g h, g		
07/21 (the befininng of the test)	24.0	24.0	24.0
08/22 (the enf of the test)	109.5	99.0	103.9

Continued Table 2

	Weigh gain, %		
07/29			
ΔM, %	99.8	102.6	105.4
Cw, %	8.6	8.8	9.0
08/06			
ΔM, %	50.7	52.4	57.0
Cw, %	5.1	5.3	5.6
08/14			
ΔM, %	28.9	12.1	14.8
Cw, %	3.2	1.4	1.7
08/22			
ΔM, %	17.6	19.1	16.9
Cw, %	2.0	2.2	2.0
	Survival, %		
07/21	100	100	100
07/29	88.1	89.4	90.6
08/06	80.6	81.3	80.6
08/14	71.9	72.5	73.1
08/22	64.4	61.3	63.8

Note. See the description of the experiments in the Materials anad methods section. ΔM — relative weigh gain, Cw — specific biomass growth rate.

In our experiments, there were no statistically significant differences in THN and the proportion of granulocytes between groups ($p > 0.05$). The average values of the THN in the groups ranged from 1005 to 1073 per 1 μl, the proportion of granulocytes was from 20.1 to 21.1% (Table 3).

3. Hematological parameters of juvenile Australian red claw crayfish (*Cherax quadricarinatus*) fed Coppens vital (Alltech Coppens B.V., Netherlands) **granular feed for sturgeon species** (lab test, 2019)

Parameter	Group I	Group II	Group III
Total hemocyres number/μl			
M±σ	1030±499	1005±493	1073±507
Cv, %	48.5	49.1	47.2
Granulocyte , %			
M±σ	21.1±5.97	20.2±5.50	20.1±5.48
Cv, %	28.3	27.3	27.2

Note. See the description of the experiments in the Materials anad methods section.

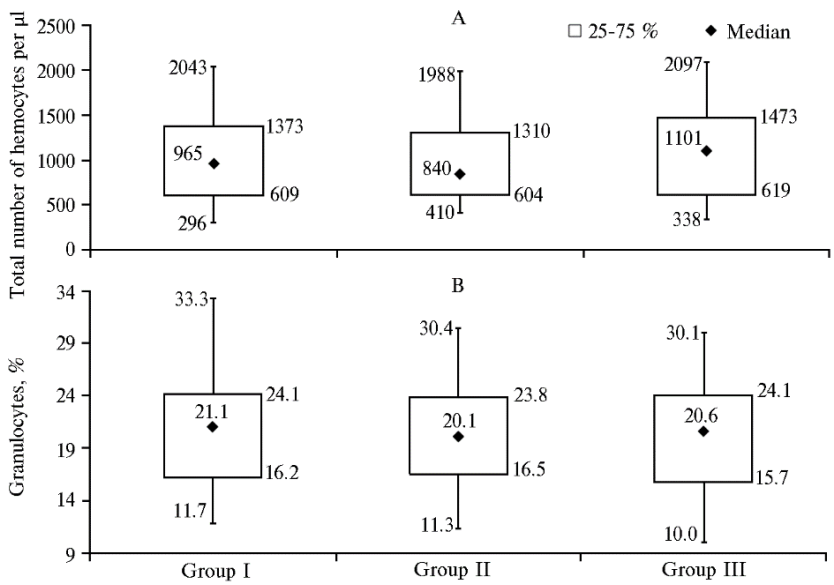

Fig. 5. Total hemocyres number (THN, A) **and granulocytes** (B) **in juvenile Australian red claw crayfish (*Cherax quadricarinatus*) fed granular feed for sturgeon Coppens vital** (Alltech Coppens B.V., Netherlands) **on day 32 of the experiment** (lab test, 2019). See the description of the experiments in the Materials anad methods section.

The medians (Fig. 5) for THN were 965 per 1 μl in group I, 840 per 1 μl in group II, and 1101 per 1 μl in group III. Granulocytes amounted to 21.1% in group I, 20.1% in group II, and 20.6% in group III.

The total number of hemocytes in crayfish at different daily feeding rates did not depend on the proportion of granulocytes ($p > 0.05$), the correlation coefficients ranged from -0.02 to -0.08 (Fig. 6).

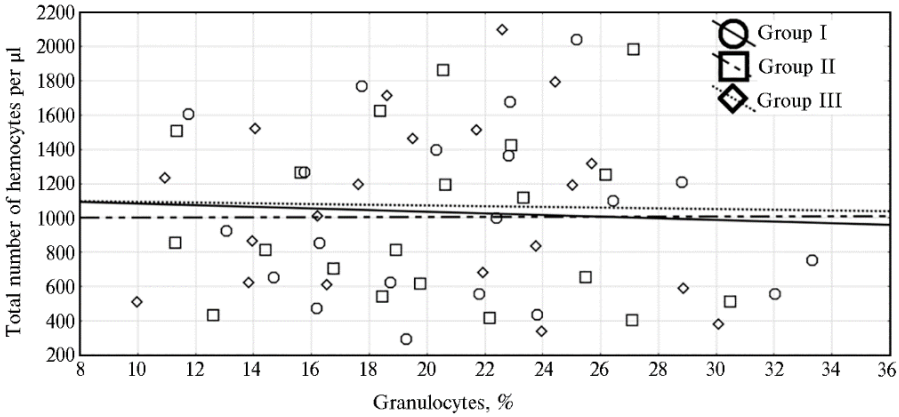

Fig. 6. The ratio of total hemocyres number (THN) **to granulocytes in juvenile Australian red claw crayfish (*Cherax quadricarinatus*) fed granular feed for sturgeon Coppens vital** (Alltech Coppens B.V., Netherlands) **on day 32 of the experiment** (lab test, 2019). The lines on the graph are straight lines of zero correlations of the experimental groups. See the description of the experiments in the Materials anad methods section.

4. Economic indicators of rearing juvenile Australian red claw crayfish (*Cherax quadricarinatus*) fed granular feed for sturgeon Coppens vital (Alltech Coppens B.V., Netherlands) (lab test, 2019)

Parameter	Group I	Group II	Group III
Initial stocking density, ind/m2	479	479	479
Feed consumption for the period, g:			
July 21-July 28	17.3	11.5	5.8
July 29-August 5	34.5	23.3	11.8
August 6-August 13	52.0	35.6	18.6
August 14-August 22	67.1	39.9	21.3
Total, g	170.9	110.3	57.5
Total weight gain, g	85.5	75.0	79.9
Feed consumption, units	2.00	1.47	0.72
Productivity, ind/m^2	308	293	305
Productivity, g/m^2	326.9	295.4	310.1
Cost (feed costs), rub/kg	421.4	309.7	151.7
Meat output			
Bodyweight before treatment, g			
$M\pm\sigma$	1.06±0.167	1.10±0.156	1.08±0.172
min-max	0.76-1.34	0.67-1.30	0.79-1.39
Bodyweight after treatment, g			
$M\pm\sigma$	0.95±0.146	0.92±0.136	0.97±0.149
min-max	0.68-1.16	0.66-1.15	0.72-1.21
Abdominal muscles, g			
$M\pm\sigma$	0.31±0.065	0.30±0.067	0.32±0.073
min-max	0.15-0.40	0.14-0.32	0.17-0.46
Output of meat from abdomen, %			
$M\pm\sigma$	32.5±3.85	31.1±2.99	32.0±3.90
min-max	22.1-37.6	21.9-37.0	22.9-36.9

N o t e. See the description of the experiments in the Materials anad methods section.

An important indicator of the nutrient assimilation in aquaculture is the yield of edible parts (in our case, muscle fiber, meat) from the total bodyweight. Since the mass of juvenile crayfish is small and it is not possible to remove the entire body meat, the amount contained only in the tail part was estimated. The remaining parts of the body were considered as inedible (Table 4).

The meat yield in the experimental groups in juvenile crayfish did not differ statistically significantly. The mean values ranged from 31.11% (group II) to 32.54% (group I). Close dvalues, the 30-32% of body weight which is characteristic of older individuals weighing from 22 to 86 g, were noted in *C. quadricarinatus* when grown in a recirculating water supply installation [2, 40], in other crayfish species these values amounted to 15-20% [40]. It should be noted that our work presents estimats for juveniles that other authors have not studied.

One of the indicators of growing efficiency in general is feed costs (see Table 4). For the entire experiment, the most feed was eaten in group I (170.9 g) where the highest feed costs (2.00 units) were also noted.

Low feed coefficients are shown in the work of S.V. Sevasteev et al. [33]. They reported thet, when feeding Artemia-based products, juveniles of close final sizes were obtained at similar times with a feed consumption of 0.5 to 1.6 units. Based on the OOO Pioneer Trade (Russia) price (1500 rubles/kg, 2018) of decapsulated Artemia offered by S.V. Sevasteev et al. [33] as a feeding option, the calculated cost of feed will be from 750 to 2400 rubles/kg, which is several times more expensive compared to feed for sturgeon species.

According to the price list dated February 21, 2019, at OOO AlpheusFeeds (the official Russian distributor of Coppens International B.V.—Alltech Coppens, the Netherlands), the cost of Coppens vital (0.8-1.2 mm) is 2.82 euros/kg, or 210.7 rubles/kg (at the exchange rate of 74.7 rubles prt euro in February 2019). With such cost of feed, the cost of juveniles in group III is minimal and amounts to 151.7 rubles/kg vs. 309.7 and 421.4 rubles/kg in groups I and II, respectively.

Thus, the low feed cost per unit of weigh gain of juvenile Australian red claw crayfish when fed Coppens vital granulated feed for sturgeon species at a daily feeding rate of 3% resulted in similar growth rates, survival, average weights, lengths, length medians and hematological status indicating effective feed assimilation and an excessive feeding rate in other groups. The daily feeding rate of more than 3% did not lead to an increase in the efficiency of rearing. When growing juveniles for 32 days from an average weight of 0.15 g to 0.89 g, the feed cost was 0.72 units. With an initial stocking density of 479 ind/m^2, 61-64% of crayfish survived with a productivity of 293-308 ind./m^2, or 295-327 g/m^2. For the first time, the yield of abdominal meat in young crayfish was estimate which ranges within 31-33%. An increase in the feeding rate did not affect the total number of hemocytes (1005-1073 per 1 μl) and the number of granulocytes (20-21%). Our trials confirmed the effectiveness of the feeding regimen for juvenile Australian red claw crayfish. Nevertgeless, more experimentation is needed to determine the minimum threshold for daily feeding both for the indicated age group of juvenile Australian red claws and for older ages, as metabolism, and with it the need for food, change with adulthood.

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