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Address: build. 16/1, office 36, pr. Polesskii, Moscow, 125367 Russia
Tel: + 7 (916) 027-09-12
E-mail: felami@mail.ru, elein-k@yandex.ru **Internet:** http://www.agrobiology.ru

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A NEW GENUS OF INFLUENZA VIRUS — *Influenza D virus*
(review)

A.V. LYAPUNOV ✉, G.A. DANCHINOVA

Scientific Centre for Family Health and Human Reproduction Problems, 16, ul. Timiryazeva, Irkutsk, 664003 Russia, e-mail liapunov.asp@mail.ru (✉ corresponding author), dan-chin@yandex.ru
ORCID:
Lyapunov A.V. orcid.org/0000-0002-6947-5771 Danchinova G.A. orcid.org/0000-0002-6705-3070
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Abstract

Influenza D virus (IDV) discovered in swine in 2011 and then in cattle and other animals was subsequently classified as a separate genus *Influenza D virus* (*Orthomyxoviridae*, *Deltainfluenzavirus*) (B.M. Hause et al., 2014). It is assumed that influenza D virus descended from human influenza C virus (ICV) from 300 to 1,500 years ago (Z. Sheng et al., 2014; S. Su et al., 2017). Its virion contains seven segments of RNA. The IDV genome sequence is 50 % different from ICV, no recombinants are formed between IDV and ICV, and no cross-reactivity of the antibodies occurs as well (B.M. Hause et al., 2011). Retrospective analysis showed that the virus has been circulating in North America since 2002 at the latest (M. Quast et al., 2015). Cattle is the main reservoir of the pathogen (L. Ferguson et al., 2015) but it also infects small ruminants, horses (H. Nedland et al., 2018), camels (E. Salem et al., 2017), and pigs (Z. Yan et al., 2018), including in wildlife (L. Ferguson et al., 2018). The virus provokes bacterial infections which affect the lung parenchyma, slowing growth, decreasing milk yields, and causing reproductive delay. In severe acute disease, IDV can move into the bloodstream in cattle and goats via penetration through capillaries lining respiratory tract. Calves possess passive immunity due to natural feeding that weakens in the 6-8-month-old animals, making them susceptible to the infection. Small ruminants serve as a reservoir for IDV and can transmit infection to other livestock. Wild boars can also be dangerous as IDV vectors between wild and domestic animals. IDV has not yet been found in poultry. At present, three types of influenza D virus are circulating simultaneously. The experiments have shown that the virus infects polecats (B.M. Hause et al., 2011) and guinea pigs (C. Sreenivasan et al., 2015). IDV successfully replicates in human's respiratory epithelial cell culture at 33 to 37 °C (M. Holwerda et al., 2019). The selection pressure for IDV is higher in pigs than in cattle and goats, so IDV, if successfully adapted, can spread widely among pigs. Therefore, a new public health risk could arise given the similarity in receptors between pig and human. The accumulated data on the ability of IDV to infect humans are ambiguous and require further in-deep study. Particular attention should be paid to persons involved in the management of farm animals susceptible to IDV. The pathogen is widespread across the planet and poses a potential threat to agriculture in countries where the breeding of cattle, small ruminants and pigs is of great importance to the economy. The fact that the virus is capable of infecting a wide range of hosts makes it potentially harmful to humans too.

Keywords: influenza D virus, influenza C virus, large cattle, small ruminants, pigs

Influenza viruses comprise four genera of *Ortomyxoviridae* family, the *Influenza A* (*Alphainfluenzavirus*), *Influenza B* (*Betainfluenzavirus*), *Influenza C* (*Gammainfluenzavirus*) isolated in 1930-1940, and *Influenza D* (*Deltainfluenzavirus*) discovered in 2011 in a pig and then in cattle and other animals [1-3]. The formal assignment to the genera is based on no serological cross-reactivity determined by antigenic properties of the internal proteins (ribonucleoproteins) of the virion.

Influenza viruses can infect and cause disease in humans, mammals, birds, and possibly other members of the animal kingdom. This pathogen is able

to overcome the interspecies barrier [4, 5]. Its variability is due to two mechanisms, the genetic drift (point mutations) and reassortment. The reassortment occurs when multiple viruses replicate in a host cell during co-infection. When the progeny viruses are assembled, the original viral RNA segments are shuffling and a pathogen with new properties appears [6]. The body's immune system fails to provide proper protection against an altered virus, which results in re-infection of the host macro-organism [7]. This virus spreads over a population, causing severe pandemics [8-10].

This review summarizes the latest data on *Influenza D virus*, its global spread and the range of host species susceptible to this pathogen.

Currently, influenza A viruses (IAVs) are the most widespread, diverse, and epidemiologically significant. IAVs differ in two surface proteins, the hemagglutinin and neuraminidase (18 and 11 subtypes, respectively). Waterfowl is the IAV reservoir, with the exception of H17N10 [11] and H18N11 [12] viruses detected only in bats [13, 14]. IAVs are isolated from a large number of wild and domestic animals. However, reports of IAV infection in cattle are rather rare compared to other agricultural mammals [15]. Over a long period of observations, only few works have addressed the problem of IAV infection in ruminant ungulates. One of the first was the report on the detection of antibodies to H3N2 in yaks in Nepal in 1974 [16]. In 1977 IAV was isolated from a calf [17]. Later, IAV was shown to infect various domestic ruminants without causing massive serious diseases [18, 19] but with a decrease in milk yield [20, 21]. In a number of studies, antibodies to the seasonal human influenza viruses H1N1 and H3N2 were detected in cattle [22-24]. It was serologically evidenced that cultured bovine respiratory epithelium is permissive for the growth of equine H3N8 influenza virus in vitro [25] and that calves, when infected experimentally, can support replication of the H5N1 virus isolated from cats [26].

In the human population, two antigenically distinct influenza B virus (IBV) strains, Victoria and Yamagata [27] capable of reassorting [28] circulate. Their primary host is humans, but since 1999, the virus has been isolated from seals (*Phoca vitulina*) [29, 30], and in the last decade, data on susceptibility of domestic animals to the pathogen have appeared [31]. Influenza C viruses (ICVs) are widespread in humans. As a rule, the disease is asymptomatic or a mild respiratory distress occurs in children under 6 years of age [32, 33]. Six genetically and antigenically different lines have been described [34] that form reassortants during co-circulation [35]. The main reservoir is humans, although ICVs are also detected in domestic pigs [36, 37] and dogs [38, 39]. In pigs, the transmission of the virus from one animal to another has been experimentally shown [40, 41]. Interspecies transmission of influenza C virus between humans and pigs is possible in vivo [42]. It was recently found that camels have antibodies to ICV [43], and in 2016, ICV which was 95 % similar to human influenza viruses was isolated from a sick calf in the United States [44, 45].

A novel genus of influenza virus. D/swine/Oklahoma/1334/2011 (D/OK) influenza virus isolated in April 2011 in Oklahoma from a 15-week-old pig exhibiting influenza-like illness, was only 50 % similar to human ICVs. The isolate was initially designated as C/OK. Like ICV, the isolate has a segmented RNA genome (seven segments). Phylogenetic analysis found that the divergence between human ICV and C/OK is similar to that between influenza A and B viruses. Hemagglutination inhibition (HI) assay showed no cross-reactivity between human ICV and C/OK. Serological screening revealed prevalence of C/OK antibody diagnostic titers in 9.5 and 1.3 % sera from pigs and humans, respectively. In cell culture, the C/OK virus exhibits a broader cellular tropism than ICV. In ST cells (swine testis cell line) on day 3, this virus, like the influ-

enza virus, caused cytopathic effects. In addition, the new virus was easier to culture than ICV [1]. It was found that ICV and C/OK failed to form reassortants. On this basis, the authors proposed to classify the new group as influenza D virus (IDV), a separate genus of the *Orthomyxoviridae* family [46].

Further investigations showed the widespread distribution of IDV on other continents and the ability of the pathogen to infect other mammalian species (cattle, goats, sheep, etc.). This pose a potential threat of IDV to the agriculture and public health around the world, including in the Russian Federation, the countries of the former USSR, and Mongolia, where the breeding of cattle, small ruminants and pigs is economically important.

Prevalence and hosts of influenza D virus. The IDV was first isolated from pigs, but further studies have shown that cattle play a major role in IDV circulation [47, 48]. Currently, IDV has been found in cattle in China [49], France [3], USA [50], Italy [51], Mexico [52], Japan [53], Ireland [54], Luxembourg [55], Great Britain [56]. IDV plays an important role in the Bovine Respiratory Disease Complex (BRDC) and can significantly reduce animal performance indicators in animals. It is most likely that IDV provokes the development of bacterial infections [57] with a damage to the pulmonary parenchyma, slowing growth rate, and delayed puberty onset. The death of animals, a decrease in milk and meat production, and the cost of treatment cause huge losses to the economy of farms.

Using cell cultures, Hause et al. [46] detected another five isolates that were more than 96 % identical to the D/swine/Oklahoma/334/2011 virus, with 18 % of animals being infected. Later Ferguson et al. [50] showed that 6-8-month-old calves at pre-sale points (Mississippi, USA) are massively infected. The virus was detected in 2.4 % of healthy animals and in 23.6 % of animals with BRDC symptoms [50]. By this age, passive immunity due to natural feeding weakens and the calves become susceptible to infection.

IDV is detected in USA, France, Italy, Ireland, and Great Britain, when studying outbreaks of respiratory diseases in cattle, including those associated with the death of animals. Collin et al. [2] identified IDV in 4.8 % of sick animals from Kansas, Texas and Nebraska and established co-circulation of two lines, the D/swine/Oklahoma/1334/2011 (D/OK) and D/bovine/Oklahoma/660/2013 (D/660). The reassortment between viruses of these two lines has also been found [2]. Ducatez et al. [3] examined archival cattle samples (2010-2014) and proved that IDV has been circulating in France since at least 2011. In the Po Valley (Northern Italy), IDVs have also been isolated during outbreaks of respiratory disease [51]. In 12 provinces of Italy, 6.5 % of 895 dead animals died from influenza D virus [58].

In 2014-2016, IDV was detected in Ireland in nasal swabs in cattle with clinically diagnosed respiratory diseases [54]. In the UK, in 8.7 % of dead animals with respiratory infection syndromes, influenza D virus was usually detected as the only viral agent, always combined with bacterial infection [56]. To date, all viral sequences described in European countries are grouped in clade D/swine/Oklahoma/1334/2011.

In China, IDV has been circulating since 2014. In a screening surveillance, the RNA of the virus was found in 0.7-2 % of animals that had no signs of the disease cases, which indicates an asymptomatic infection [49, 59]. In the study of nasal washings in cattle with various clinical sings of diseases in 2016, Zhai et al. [59] found the virus in 12.8 % of cases in the Holstein breed and in 7.3 % in the local yellow cattle and established the penetration of the virus into the circulatory system through the capillaries lining the respiratory tract. The homology of isolates from China ranged from 95.35 to 99.22 % as compared to

the American isolates [49].

Seroprevalence of animals is an important indicator of the IDV prevalence. In 2014 Hause et al. [46], using D/OK and D/660 as antigens, found geometric mean titers for both viruses at a dilution of 1:40 and higher in cattle from different states. Interestingly, in a retrospective study of the blood sera of adult animals from the state of Nebraska (USA) in 2003-2004, Luo et al. [60] identified 81.9 % of animals with antibodies to IDV at all 40 randomly selected farms. Therefore, IDV emerged in this territory at least in 2003. In 2014, testing of paired serum samples from 242 calves revealed that 98% and 76% of animals were seropositive for IDV at 1 week and 3 months, respectively, while antibody titers in most calves decreased. These results prove that newborn calves have high levels of maternal antibodies against IDV. Similar data were reported for eight prefectures of Japan in 2010-2016 (1267 samples from 166 farms): the IDV seropositive samples averaged 30.5 %, increased with the age of the animals and did not depend on the breed. This means that IDV has been circulating in Japan since at least 2010. Currently, the virus is widespread in cattle populations throughout the country [61]. Antibodies to IDV were detected in 92.4 % of animals from 42 farms in Northern Italy (the Province of Mantova) [58], in 80.2 % of adult animals without respiratory symptoms in Luxembourg [55]. From 31 to 70 % (47.2 % on average) of animals were seropositive in the regions of France [62]. IDV prevalence has also been shown in North (Morocco, 2012-2015) and West Africa since 2012 (Togo and Benin, 2014) [43]. The authors associate the emergence of this new pathogen with the import of animals from Europe to Morocco.

Reports on the isolation of the virus in different countries over the past years and retrospective studies of blood sera for anti-IDV antibodies indicate that IDV began circulating in cattle no later than in 2003 and is now common on different continents. The high percentage of seropositive animals suggests that the virus spreads rapidly and leads to a decrease in milk yield, weight gain in young animals, and even death.

Interspecies transmission of IDV can occur when keeping different species of farm animals together. A survey of 648 animals from 141 farms in the United States and Canada showed the presence of anti-IDV antibodies in 5.2 % of sheep and 8.8 % of goats [63]. In a retrospective study of the sera of 64 goats and 85 sheep (2001-2007), one goat sample contained antibodies to IDV (Massachusetts, 2002). This allowed the authors to believe that IDV has been circulating in the United States since at least 2002, and has become most widespread since 2011. It was found that on the North American continent, small ruminants have antibodies to two lines, the D/bovine/Oklahoma/660/2013 (D/660) and D/swine/Oklahoma/1334/2011 (D/OK) isolated from a cow and a pig. It can be concluded that these species are susceptible to the new virus and have been in contact with it in different states of the United States and provinces of Canada. Salem et al. [43] found antibodies to IDV in small ruminants in North Africa (Morocco) and West Africa (Togo and Benin) in 2013 and found the virus to be similar to D/Bovine/Nebraska/9-5/2012 strain. In 2016 in China (the Guangdong Province), RT-PCR detected IDV infection in 33.8 % of goats with various symptoms of diseases and in one of eight rectal smears from animals with severe diarrhea [59]. In France, antibodies were found in 1.5 % of small ruminants [62]. Consequently, small ruminants serve as an IDV reservoir and can transmit infection to other animal species, change the biological characteristics of the virus, and contribute to its evolution.

The pig is the animal from which IDV was first isolated [1]. Wild pigs are mobile and contacting with sources of infection, primarily cattle, and can

spread the virus. In 2012-2013, in the states of Gawai, North Carolina, Oklahoma and Texas (USA), from 7.8 to 28.6 % of wild boar populations had antibodies to IDV [64]. Also, 42.7 % of 96 IAV seropositive samples (2010-2013) contained antibodies to IDV. This study showed that wild boars pose a danger with regard to IDVs and also other viruses as vectors of infection between wild and domestic animals.

IDV is rapidly spreading among domestic pigs in the USA, Italy, China, and Luxembourg. During outbreaks of respiratory diseases in the Po Valley (Northern Italy), IDV RNA was detected in a nasal swab of a sow, and, furthermore, D/swine/Italy/199723-3/2015 was isolated on cell cultures [51]. During the same outbreak, other authors examined 845 clinical samples from 448 pig farms, identified IDV RNA in 2.3 % of the specimens, and isolated three strains closely associated with D/swine/Oklahoma/1334/2011 cluster [65]. The serological screening that they also performed for 3698 pig blood sera from the archived collections of 2009 and 2015 found 0.6 and 11.7 % seropositive samples, respectively. In Guangdong Province (PRC) in 2016, RT-PCR detected 36.8 % of nasal washes positive for IDV in pigs with respiratory symptoms and 28.9 % of lung samples positive for IDV from dead pigs [59]. In Luxembourg, a study of nasal lavages from healthy domestic pigs showed a low spread of the virus, 0% in 2009 and 0.7% in 2014-2015, and the low concentration of viral RNA in the samples did not allow nucleotide sequencing [55]. Low seroprevalence in pigs indicates that they are less involved in IDV circulation than cattle, but as a result of the evolution of the virus, it may become more dangerous for this species. It has been shown that the selection pressure on the virus in pigs is higher than in cattle and goats [66]. It cannot be ruled out that successful adaptation of the virus will lead to its wide spreading among domestic pigs and even among humans, given the similarity of pig's and human's receptors.

Influenza D virus infection in horses was first reported in 2018. Two IDV lines were shown to co-circulate in populations in the western United States [67]. Antibodies were detected in 15.7 % of the sera of adult horses. Out of 57 positive samples, 23 were positive for both lines.

Currently, IDV infection has arisen in one-humped camels in Kenya [43] and Ethiopia [68]. In some territories of Ethiopia, up to half of the camels have antibodies to IDV, with both detection rates and titers being the highest for the strain from Japan, the D/bovine/Yamagata/10710/2016 as compared to D/swine/Oklahoma/1334/2011 and D/bovine/Nebraska/9-5/2013. Nevertheless, in Mongolia, no antibodies were detected when testing the blood sera of two-humped camels [68].

When animals of various species have the same grazing area and watering points, frequent contacts between them enable the virus to adapt to a new host. Possibly, this is happening with IDV which was initially capable of infecting cattle, and now its various strains can infect other mammalian species as well. Given the features of the evolution of other genera of the influenza virus, especially IAV, IDV adaptation to humans is quite possible. Therefore, IDV which is spread worldwide among domestic animals and can affect a wide range of hosts, is potentially dangerous to humans and birds. Although Quast et al. [63] in a study of 150 blood sera of turkeys and 100 chickens from 25 poultry farms in the states of Minnesota and Iowa in 2014 did not reveal antibodies to IDV, it is impossible to reject the ability of IDV to infect birds.

Experimental infection of mammals with IDV. The experimental infection with IDV was studied in domestic and wild pigs, ferrets, guinea pigs, and cattle. In the first experiment, pigs aged 2.5 months had D/swine/Oklahoma/1334/2011 virus replicating in the turbinates and found in nasal swabs, but

not in the trachea and lungs, that is, its replication could be limited to the upper respiratory tract. No clinical symptoms or lesions characteristic of influenza were observed. Virus was transmitted to "clean" animals through direct contacts with infected animals [1]. An experiment with 4-month-old healthy calves showed the likelihood of D/bovine Mississippi/C00046N/2014 infection with moderate signs of the disease (dry cough, nasal flow, apathy). The virus was detected in the turbinates, trachea, bronchi and lungs and was also transmitted by contact when infected and healthy calves were kept together [48]. A similar experiment with wild boars captured in nature also showed the transmission of the virus between animals [64].

The ferret is one of the best experimental models for respiratory infections in mammals, as it develops signs of disease similar to humans, and these animals are also able to become infected through aerosols. In experimental infection, D/swine/Oklahoma/1334/2011 was detected in the turbinates and absent in the lower respiratory tract of the ferret. Infection occurred as a result of direct contact of animals, but no airborne transmission of the pathogen was observed. Clinical symptoms and lesions characteristic of IAV were absent [1]. No transmission of the virus from calves to ferrets was observed when toys soaked with nasal secretions from a calf infected with IDV were used as fomite [48] to infect 30-day-old guinea pigs. In guinea pigs, unlike ferrets, replication of D/bovine/Oklahoma/660/2013 virus occurred in both upper and lower respiratory tracts and the lungs, but no clinical symptoms appeared. The transmission of the virus from infected to "clean" animals occurred only through direct contact [69]. The Table summarizes information on susceptibility to IDV.

Mammals susceptible to *Influenza D virus* (*Orthomyxoviridae*, *Deltainfluenzavirus*), the pathogen detection and spread of

Species	Detection	Country	References
Cattle (*Bos taurus*)	RT-PCR, isolation, ELISA, experimental infection	USA, China, France, Italy, Ireland, Luxembourg, Morocco, Togo, Benin	[2, 3, 43, 46-56, 58-62]
Buffalo (*Bubalus arnee*)	RT-PCR	China	[59]
Sheep (*Ovis aries*)	ELISA	USA, Canada, Togo, Benin	[43, 62, 63]
Goat (*Capra hircus*)	RT-PCR, ELISA	China, USA, Canada, Togo, Benin	[43, 59, 61, 62]
Pig (*Sus scrofa*)	RT-PCR, isolation, ELISA, experimental infection	USA, China, Italy, Luxembourg	[1, 51, 55, 59, 64, 65, 79]
Horse (*Equus ferus caballus*)	ELISA	USA	[67]
Dromedary (*Camelus dromedarius*)	ELISA	Morocco	[43, 68]
Ferret (*Mustela putorius*)	Experimental infection		[1, 50]
Guinea pig (*Cavia porcellus*)	Experimental infection		[69]
Human (*Homo sapiens*)	ELISA, in vitro	USA	[1, 77, 78]

Note. RT-PCR — reverse transcription PCR, ELISA — enzyme-linked immunosorbent assay.

Based on the study of hemagglutinin-esterase-fusion (HEF) glycoprotein which drives the fusion between viral and host cell membranes, Song et al. [70] hypothesized the mechanisms that provide influenza D virus with a broader cell tropism to different hosts compared to influenza C virus.

IDV origins, differences, and diversity. Although research on IAV, IBV, and ICV began in the 1930s-1940s, IDV was not discovered until 2011. Probably, it previously circulated in another unknown animal [1], and after adaptation to cattle it quickly spread across the globe. IDV is identical to ICV in structure, has a negative sense RNA genome represented by seven segments of single-stranded RNA. The IDV and ICV genome sequences are shown to be only half similar [1] while the six known lines of ICV are 95 % identical [34].

The IDV virion is composed of four structural proteins M2, M1, NP and a hemagglutinin esterase (HEF) fusion protein, three subunits of the RNA polymerase complex P3, PB1 and PB2, and two non-structural proteins NS1 and

NS2. IDV is an enveloped virus. The outer layer of the virion envelope is a lipid membrane with the matrix protein M2 which forms ion channels. The lipid membrane also contains an envelope glycoprotein HEF that penetrates the wall of a host cell and plays the role of hemagglutinin (HA) and neuraminidase (NA) of IAV and IBV. The matrix protein M1 is located under the lipid membrane, forms the inner layer of the virus envelope, and gives stability and rigidity to the outer lipid envelope. Ribonucleoprotein complex vRNP (a nucleoprotein in a complex with the genomic RNA) contains RNA fragments attached to a nucleoprotein (NP) and three proteins of the polymerase complex (PB1, PB2, and PA) [1, 71, 73].

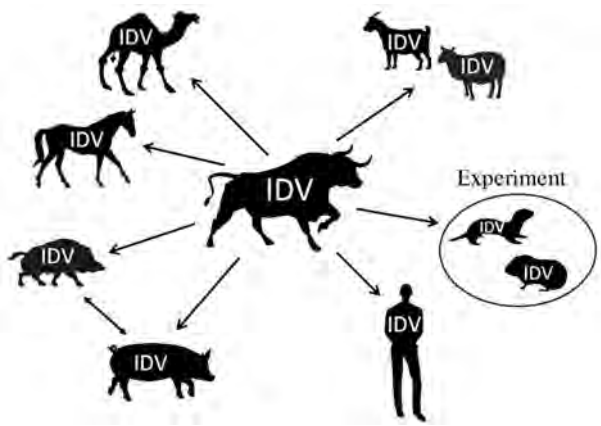
In vitro experiments found no convincing evidences of the ICV and IDV reassortment. Using two strains of human ICV C/Taylor/1947 (C/Tay) and C/Johannesburg/66 (C/JHB), a pig strain D/OK, and a cow strain D/660, Hause et al. [46] found that recombination only occurred between human viruses C/Tay and C/JHB or between animal viruses D/OK and D/660.

It was also found that IDV exhibits a different mechanism in the production of the M1 protein compared to ICV. Another important distinguishing feature of the strains is based on antibody cross-reactivity. It was shown that there is no such reactivity between IDV and antiserum to ICV. The polyclonal antibody to C/Tay does not recognize the D/OK and D/660 antigens, while reacting with the C/JHB antigen. Conversely, the anti-D/OK polyclonal antibody did not recognize human viruses C/JHB and C/Taylor [46]. Using a rabbit antiserum, a cross-reactivity was shown to two IDV lines, D/swine/OK/1334/2011 (D/OK) and D/bovine/660/2013 (D/660) but not to human ICV Victoria/2/2012 (C/Vic) strain [67]. In this case, the D/660 antiserum showed equal reactivity to both D/660 and D/OK, the anti-D/OK antibodies also cross-reacted with D/660 but was more specific for D/OK with titers 4 times higher. Based on these differences, the International Committee on Taxonomy of Viruses (ICTV) assigned the virus to a separate genus *Influenza virus D* of the *Orthomyxoviridae* family and recommended the D/swine/Oklahoma/1334/2011 as the prototype virus.

Analysis of five gene segments showed that IDV could have separated from ICV from 300 to 1200 years ago [73]. Other researchers [74] suggest that IDV originated from human ICV about 1,500 years ago. It can be argued that there are several lines within the genus which demonstrate cross-reactivity, however, it is reduced (up to 10-fold) when using a heterologous serum compared to a homologous. According to Su et al. [74], the two main lines circulating in domestic and wild ungulates (D/swine/Oklahoma/1334/2011 and D/bovine/Oklahoma/660/2013) diverged about 50 years ago. In nasal swabs of a cow with clinical signs of illness, Murakami et al. [53] discovered the influenza D virus (D/bovine/Ibaraki/7768/2016) which had significant differences from the IDVs previously identified in America, Europe and China. In Japan, during the outbreak of the disease in a herd of cows, antibody titers to the strain isolated in Yamagata Prefecture (D/bovine/Yamagata/10710/2016) were 4 times higher compared to strains from Europe and America [75]. That is, there are at least three IDV lines circulating simultaneously. Zhai et al. [59] believe that the variability within the D/660 line indicates its ongoing evolution. However, the low level of nucleotide substitutions characteristic of the known IDVs shows the slow rate of its evolution. The surface glycoprotein is antigenically stable and, therefore, new variants will be infrequent and infection with these viruses can induce long-term immunity. The reason for the slow evolutionary change in IDV proteins is unknown. It can be associated with a low frequency of polymerase errors or with the loss of protein functionality as a result of amino acid

substitutions [74].

Potential risk of the new virus to humans. The ability of IDV to infect ferrets, guinea pigs and other mammals indicates a wide range of hosts for this pathogen and a potential threat to human health. To date, there is little information about the ability of the new virus to infect humans. In 316 sera from patients in Vancouver (British Columbia, Canada) and Hartford (Connecticut, USA) collected during the 2007-2008 and 2008-2009 influenza seasons, antibodies to IDV were detected in four samples of which three samples also had high titers (1:160, 1:320, and 1:1280) to the C/Yamagata/10/1981 strain. One sample had a titer of 1:40 to D/OK and was negative for the tested human ICV strains [1]. Similarly, in 2011, titers to D/OK were detected in 9.5 % of blood sera from 220 pigs aged 3 to 20 weeks, however, only 2.8 % of pig sera had measurable antibody titers to human ICV C/Taylor/1233/47 used to assess the specificity. Therefore, it can be argued that the D OK virus circulates in swine populations, but is not typical for humans [1]. Only ICV was detected by RT-PCR in the study of Scotland archival samples from the human respiratory tract [76]. However, in Florida (USA), antibodies to the new virus (strain D/Bovine/Kansas/1-35/2010) were detected in almost 95 % blood sera of 35 people working with cattle. Also, out of 11 adults who had no contact with animals, two were found to have anti-bodies [77]. Using in vitro human respiratory tract epithelium cells (HAEC), it was found that IDV is able to effectively replicate and be released from cells at temperatures from 33 to 37 °C. The replication proceeded without long-term adaptation to the cell culture used in the work and even more intensively than in ICV [78].



Host range of *Influenza D virus* (IDV). Arrows indicate the probable routes of infection transmission.

Thus, the new influenza virus IDV is involved in the complex of respiratory diseases of cattle, is widespread in the Europe, Asia, North America and Africa, and has a significant impact on the livestock economy. This virus plays the role of a primary infection, provoking further pathologies caused by pathogenic bacteria which colonize the lower respiratory tract [47, 52, 56, 62]. In addition to cattle, IDV infects a wide range of domestic animals, including goats, sheep, pigs, camels, and horses (Fig.). Experiments on ferrets and guinea pigs have proved the susceptibility to IDV and IDV transmission [1, 69]; IDV also successfully replicates in human cell lines.

The global cattle population amounts to approximately 1.5 billion, small ruminants to more than 2.2 billion, pigs to approximately 1 billion, horses to 61 million, and camels to 35 million, so viral monitoring is necessary not only among cattle, but among other domestic animals. These species play a significant role in the agricultural economy of many countries, including Russia and the bordering countries of the former USSR. Cattle, small ruminants and pigs are the most abundant in the Russian Federation. According to the Federal Customs Service of the Russian Federation, from 2014 to 2018, the import of cattle (primarily of the pedigree black and white Holstein breed from the EU countries) also increased by

54 % due to several projects within the framework of import substitution and a zero VAT rate (until 2019) for the import of bull sires and young animals. Currently, there is no data on the spread of IDV in the Russian Federation and the role of the pathogen in the complex of respiratory diseases of cattle, but detection of the infection in a large number of countries, including those bordering on the Russian Federation, suggests that the virus is circulating in Russia as well. Therefore, surveillance studies in different regions of the country are relevant to obtain data reflecting the Russian specifics of the evolution and pathobiology of IDV.

Since a significant number of people are in daily contact with these animals, the potential threat of IDV to public health cannot be ruled out. Retrospective studies on pigs conducted in different countries have shown that there has been an increase in the number of immune animals since 2009 [55, 65]. Despite the fact that at present there are no confirmed cases of human disease due to IDV infection, attention should be paid to animal farm workers, since there are cases of detection of the virus in pigs and cattle without signs of the disease [79].

In populations of domestic animals, at least three IDV lines co-circulate [73]. Different animal species are often kept together, and these contacts increase the likelihood of interspecies transitions of the virus, its adaptation to new hosts, acceleration of evolution and molecular changes. In 2017, a RT-PCR method was developed to detect IDV [80], in 2018, a test was suggested for detecting antibodies in different animal species by enzyme immunoassay [81]. Epidemiological, biological and immunological research on IDV should continue and, if necessary, a vaccine should be developed to protect humans against this new pathogen. Since IDV reassortants can arise at any time, there is a high probability of the emergence of viruses capable of infecting people and transmitting from person to person. Information on the ability of IDV to infect humans is little and controversial, therefore, further serological and molecular studies of the population professionally associated with animal care is required for timely preventive measures.

Thus, the influenza D virus (IDV) derived from the human influenza C virus and originally infecting mainly cattle is now widespread among the main ungulate farm animals on different continents. The pathogen also circulates in ungulate populations in the wild. Infection with this virus can lead to both asymptomatic and severe pathology, up to the animal death. There is no conclusive evidence yet of infection with this virus in poultry. Nevertheless, information about genetic diversity of the pathogen and an increase in the world population of chickens and other poultry suggests the possibility of interspecific transfer of the virus when conditions are suitable, as it was with other influenza viruses. The fact that the pathogen is actively spreading among domestic pigs makes IDV potentially dangerous for humans.

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ENZOOTIC BOVINE LEUKOSIS — DIAGNOSTICS, ERADICATION, AND ANTHROPOZOONOTIC POTENTIAL (BACKGROUND)

(review)

I.M. DONNIK[1] ✉, M.I. GULYUKIN[2], V.A. BUSOL[3], L.V. KOVALENKO[3], A.M. KOVALENKO[4]

[1]*Russian Academy of Sciences*, 14, Leninskii prosp., Moscow, 119991 Russia, e-mail ktqrjp7@yandex.ru (✉ corresponding author);
[2]*Federal Scientific Center — Skryabin and Kovalenko All-Russian Research Institute of Experimental Veterinary Medicine,* 24/1, Ryazanskii prosp., Moscow, 109428 Russia, e-mail admin@viev.ru;
[3]*National Scientific Center Institute of Experimental and Clinical Veterinary Medicine of the National Academy of Agricultural Sciences of Ukraine*, 83, Pushkinskaya Street, Kharkov, 61023 Ukraine, e-mail inform@vet.kharkov.ua;
[4]*Gorin Belgorod State Agricultural University*, 1, ul. Vavilova, Belgorod District, Belgorod Province, pos. Maiskii, 308503 Russia, e-mail: mycobacteria@rambler.ru
ORCID:
Donnik I.M. orcid.org/0000-0002-8593-7470
Gulyukin M.I. orcid.org/0000-0002-7489-6175
Busol V.A. orcid.org/0000-0002-9618-0493
Kovalenko A.M. orcid.org/0000-0002-7219-4049
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Abstract

Of tumor diseases in the global animal husbandry industry, the greatest danger is enzootic bovine leukosis. Since the last century, this neoplastic disease has remained relevant for veterinary medicine and, in addition, more and more questions arise concerning the potential threat of the Bovine leukemia virus (BLV) for humans. The problem is being discussed (G.Yu. Kosovskiy et al., 2016) which additionally stimulates both fundamental research of the pathogen and the pathology, as well as the methods for detection to improve dairy cattle breeding and veterinary safety. A number of publications are devoted to BLV prevalence, peculiarities, the prospects for improving animal health and welfare based on selection and vaccines developed in the world (S.G. Hopkins et al., 1997; M.A. Juliarena et al., 2017). In our report, we retrospectively compare the experience of BLV eradication in the USSR, Russia and abroad, additionally focusing on the possible role of this pathogen in the occurrence of cancer in humans. In veterinary practice, serological tests, i.e., agar gel immunodiffusion assay (AGID) and enzyme-linked immunosorbent assay (ELISA) are mostly used to detect the BLV infection. G.C. Buehring et al. (2019) reported the detection of BLV proviral DNA in blood leukocytes in 38% of patients examined by PCR and DNA sequencing. IgG antibodies to BLV were detected in 32%, IgM — in 58%, and IgA — in 32% of the samples tested. Accumulated data indicate metabolic changes in the BLV-positive animals, e.g., disorder in the metabolism of tryptophan, a critical essential acid, leading to the accumulation of dangerous endogenous metabolites in the body. Particularly, free tryptophan, indole and anthranilic acid increased in level 4-8-fold are deposited in the organs of the immune and hematopoietic system (lymph nodes, spleen, liver), in the mammary gland, lungs, and kidneys. It has been established that the milk of leukemic animals differs from the milk of healthy cows in terms of physicochemical, bacteriological (lysozyme), technological parameters and mineral composition. In C57 mice fed with pasteurized milk and heat-treated meat from ID-positive cows, the blood cell profile changed. Available data drive to the unambiguous conclusion that the products derived from BLV-infected cattle pose an increased risk to humans. Despite the lack of convincing evidence of the BLV pathogenicity for humans, concerns about the etiological role of this virus in the occurance of cancer in humans additionally necessitates continuing research on the control and eradication of this common oncogenic retro-virus in livestock farms.

Keywords: leukemia, Bovine leukemia virus, proviral DNA, PCR indication, IgA, IgM, IgG, blood cells, endogenous metabolites, milk, vaccination, eradication

Enzootic bovine leukosis (EBL) is a dangerous chronic infectious disease caused by oncogenic Bovine leukemia virus (BLV) [1, 2]. Cattle and other domestic ruminants, as well as wild ruminants are BLV susceptible [3, 4]. Treatment of cattle enzootic leukemia is ineffective. By the mid-1960s, the incidence

of the disease was reported on several continents in most countries with developed cattle breeding, which, in turn, provokes even higher incidence and wider spread of the diseases which poses a threat to the industry [5, 6]. Bovine leukemia causes huge economic damage to pedigree and commercial livestock raising, and also via restrictions in live animal markets and animal product markets [7, 8]. The improvement of BLV sanitary situation is a prerequisite for increasing dairy cattle performance and ensuring the veterinary and sanitary safety of animal products [9, 10].

B-lymphotropic bovine leukemia virus (a C-type retrovirus with single-stranded RNA) is a member of the *Retroviradae* family [11, 12]. Like hepatitis virus-derived transcripts [13], BLV transcripts are detected in malignant human tissues. BLV is phylogenetically close to human T-cell leukemia virus type 1 (HTLV-1) [12] infecting CD4+ T cells

Common patterns of neoplastic processes in the hematopoietic organs of humans and cattle arouse interest in veterinary, medical, and biological aspects of the pathology caused by BLV [14, 15]. A possible role of this pathogen in the etiology of oncological diseases in humans is being discussed [16, 17] which additionally stimulates fundamental studies of the BLV and the pathology it causes, as well as the improvement of methods for BLV detection and eradication.

This review gives a retrospect view on and prospects of bovine leukemia eradication in Russia and abroad and debates the zoonotic and anthropozoonotic potential of BLV.

Enzootic bovine leukosis — sings, diagnostics and indication aspects. CEL was first described in 1871 [18, as cited in 19], although its causative agent was first identified only in 1969 which made it possible to further study the pathogen and to develop eradication measures [1, 12, 20, 21]. It is known that BLV-induced multistep leukemogenesis is characterized by several steps. Many BLV-infected animals do not develop any clinical symptoms of viral infection while some cows develop humoral immune response to virus antigens. The next progressive stages are persistent lymphocytosis (hematological stage of the disease) and lymphosarcoma with a malignant transformation [22]. Hematological changes develop in 30 % of dairy cattle older than 3 years while lymphosarcomas of internal organs occur in 0.1-10 % of infected animals [23-25]. Transition from one stage to another, especially at the beginning of the disease progression, explains the disappearance and reappearance of clinical signs in some animals, as well as remissions in animals with persistent lymphocytosis [26].

BLV in naturally transmitted mainly horizontally (with infected lymphocytes, including through the biological secrets) [26]. Transplacental transmission occurs in 5-8 % of cows with asymptomatic infection and in 10-20 % of cows with clinical manifestations of leukemia. Close contacts between animals and improper therapeutic and prophylactic measures enhance the BLV contagiousness. Animals become infected when lymphocytes containing BLV enter the body enterally or parenterally. It was experimentally found that for a cow to become infected with the leukemia virus, it is enough to inject 2500 infected leukocytes intradermally into the skin. It has been established that 0.5 μl of blood infected with the leukemia virus is sufficient to infect an animal [27]. The transmission by flies and through mucosal surfaces or broken skin cannot be ruled out either [28, 29].

The strategy for bovine leukemia eradication is specific to a country conditions and based on data about the pathogenesis of the disease, the characteristics of the immune response in susceptible animals, and the transmission routs of the pathogen. In Europe, in the 1950s, the first successfully applied method for detecting infected animals was based on clinical, pathological and hematological

studies followed by isolation and elimination of sick cows from the herd [23].

In 1967, the World Organization for Animal Health (OIE) recommended diagnosing the disease by a hematological study using a hematological key to determine persistent lymphocytosis. Counting the ratio of lymphocytes has become the main diagnostic tool for many years in all countries, including the USSR [30, 31].

Miller and Olson [32], considering the absence of EBL clinical signs at the initial stages of the developing infection while the level of virus-specific antibodies is rather high, have proposed the anti-BLV antibody test (agar gel immunodiffusion assay, AGID) to diagnose EBL. AGID assay was advanced for its time and, being approved as a basic diagnostic approach in veterinary practice, significantly accelerated both preventive measures and the eradication of bovine leukemia [33]. Currently, the AGID assay [34] and enzyme-linked immunosorbent assay (ELISA) [35] are basic tests for the detection of antibodies to BLV in blood and milk. In Europe, in cattle farms, serological tests have been used since 1970 to detect virus-specific antibodies. By 1979, hematological studies for leukemia were completely abolished in Danish livestock herds and the mandatory use of the immunodiffusion reaction was recommended [36, 37]. Since 1988, EU legislative documents have also provided for the use of ELISA test for the diagnosis of leukemia in addition to ID test [38]. ELISA detects virus-specific antibodies with low titers in both blood and milk, which cannot be detected by AGID test [39].

The next advance in CEL diagnostics was due to polymerase chain reaction (PCR) analysis and its modifications allowing detection of BLV proviral DNA in the genomic sequence of infected lymphocytes [40-42]. PCR analysis is often used for emergency detection in clinical material as early as 7 days after infecting of a macroorganism (especially calves) with a pathogen in cases of uncertain serological tests [43]. PCR analysis, due to its high accuracy, is used to detect BLV in semen samples [44] and in milk [45].

Eradication of EBL. Practical approaches to EBL eradication are represented by several strategies [46]. The first strategy called “check and remove” aims to identify infected animals hematological, serological and molecular methods, to immediately remove such animals from the herd for rapid slaughter [23, 47]. This strategy has played an important role in eradication of the disease in several European countries (in particular, in Belgium and Finland). However, the strategy's limitations are the mandatory low initial spread of infection and financial losses due to government compensation for culled animals. Countries such as the United States, Canada, Argentina and Japan where no financial compensation was applied were unable to implement this strategy [46].

The second strategy, or “check and separate”, is less costly because the infected animals are not culled but separated from the BLV-free animals. Only clinically sick animals are subject to culling [48]. This strategy has proven to be effective to significantly reduce the prevalence or even eradicate the disease in most countries [49, 50].

As for the third strategy of “check and manage”, no animals should be added to the herd instead of those BLV-infected and culled. In fact, this strategy is limited to biosafety measures, minimizing the exposure of animals to an infectious agent, thus requiring minimal financial investment [46].

Prevalence, diagnostics and measures to combat bovine leukemia in the USSR and the Russian Federation. It is believed that the spread of leukemia in our country is associated with the importation of pedigree cattle from Germany to Western Siberia, Kaliningrad, Moscow, Leningrad regions in 1940 and 1945-1947 [51]. In the USSR, the study of CEL and its causative agent began at the Kovalenko All-Union (now All-Russian) Institute of Experimental Veterinary Medicine (VIEV,

Moscow) in 1961 and at the NSC Institute of Experimental and Clinical Veterinary Medicine and Belotserkovsky State Agrarian University (the Ukrainian SSR) in the 1960s. CEL incidence was first officially reported in the Soviet Union in 1965-1966 [52]. The researchers of VIEV have developed instructive and normative materials (1965, 1969, 1984, 1989), regulating measures to combat this infectious disease.

For the last 30 years in Russia, ID assay and ELISA test have been used to detect antibodies to BLV antigens in blood and milk or colostrum to diagnose CEL. Many researchers have shown the possibility of using PCR for early diagnosis of bovine leukemia [53, 54].

In the Soviet Union, since the early 1990s, a check-and-separate strategy was used. A systemic approach was developed based on biological properties of BLV, infectious and epizootic processes. BLV is inactivated by heating to 56 °C for 15 min, to 70-74 °C for 15-17 s. In milk, BLV remains intact at 9-15 °C for in 24-48 hours. The virus remains active at a pH below 6.0, in 0.5 % sodium hydroxide solution, in 0.5 % formaldehyde and phenol solutions, and in 2 % ethyl alcohol. A 4-hour exposure to direct sunlight kills the bovine leukemia virus, ultraviolet radiation kills BLV in 30 min [10]. Importantly, there are four stages of the CEL development, namely, i) incubation period (from the inoculation until the appearance of antibodies to the pathogen); ii) serological stage (asymptomatic infection, from the appearance of antibodies until the detection of hematological changes); iii) hematological stage which corresponds to the development of persistent lymphocytosis; iv) malignant stage characterized by development of malignant tumors in the tissues of blood-forming and other organs [25, 55]. One of the main immunogenetic factors conferring cattle resistance or susceptibility to bovine leukemia are histocompatibility antigens. In particular, the resistance of animals to leukemia is determined by the alleles of the BoLA-DRB3 gene encoding class II antigens of the main cattle histocompatibility complex [56].

Since 1997, bovine leukemia has ranked first among infectious diseases in the Russian Federation [5, 11]. As of January 1, 2014, up to 10 % of herd infection was observed in 53 constituent entities of the Russian Federation, and in the Nizhny Novgorod region this figure exceeded 30 % [57]. In 2016, new outbreaks of bovine leukemia were registered in more than 27 regions of Russia. In 2016, bovine leukemia was registered in 68 constituent entities of the Russian Federation (58 thousand cows in total). Only a few constituent entities of the Russian Federation are free from CEL [10]. A decrease in average annual milk production in BLV-infected herds is 218 kg per cow as compared to BLV-free herds [57, 58].

A retrospective analysis of the incidence of leukemia for 2005-2015 in the Ural region (statistical reporting data of the Department of Veterinary Medicine of the Sverdlovsk Region) revealed a high epizootic intensity for cows in most dairy farms located in the zone with combined technogenic pollution characteristic of this region. It was also found that in farms located in the most contaminated areas of the East-Ural Radioactive Trace (EURT), the seropositive cows accounted for 56-76 % and 8-14 % had hematological pathology, with the average seropositivity across farms of 20 % and 5.7 % of characteristic hematological abnormalities [59]. In areas not exposed to technogenic pollution, BLV infection was about 1 %, and animals with hematological disorders were practically absent. Hematological studies revealed that 12 % of cows were positive in ID test throughout the region with combined radioactive contamination. At dairy farms located along the EURT axis, the number of animals with persistent lymphocytosis was 1.5-2.0 times more as in the region [5]. Disorder for bovine leukemia

in areas of technogenic and radioactive contamination is associated with immune disorders, leading to the rapid clinical development of leukemia [60].

The histomorphological analysis of malignant tumors in cows, carried out for the first time in the Soviet Union, showed that 85.0 % of the 5243 intravitally diagnosed CEL are leukemia (of which 82.70 % are lymphocytic leukemia, 2.07 % are hemocytoblastosis, 0.23 % are myeloid leukemia); reticulosis accounted for 14.7 % (7.20 % for reticulosarcoma, 3.40 % for lymphosarcoma, 2.10 % for systemic reticulosis, 1.52 % for lymphoreticulosis, and 0.48 % for lymphogranulomatosis)/ The remaining types of tumors accounted for 0.3 % [61].

Foreign experience in the CEL eradication. Ukraine. After 1990, in Ukraine, measures to detect and control bovine leukemia are regulated by the relevant legislation. Since 2007, the use of serological and genomic methods in research and diagnostics has been regulated. In general, since 1990, there has been a significant decrease in the incidence of leukemia in livestock [62, 63].

The annual detection of new sites of bovine leukemia indicated the presence of factors contributing to the maintenance of a tense epizootic situation. These were ignoring the incubation period of leukemic infection; errors and untimely diagnostics; the presence in healed farms of few BLV-infected cows which, when overexposed in the herd, become the main sources of the pathogen; favorable conditions for the transmission of the pathogen; human factor (improper execution of veterinary, zootechnical and organizational and economic measures, in particular, untimely isolation and delivery of animals infected with leukemia virus for slaughter). Based on the analysis of the epizootic situation, by the beginning of the 2000s, a clear set of anti-leukemic measures was proposed, including constant monitoring of each dairy farm; identification of sources of the causative agent using highly specific diagnostic tests to detect early infection; immediate (just once detected) isolation of sources of the causative agent and its elimination within 10 days; reliable zootechnical recording of animals; adaptation of technological systems for livestock moving, keeping, feeding, and raising calves in accordance with the requirements of health-improving veterinary and sanitary measures; veterinary and zootechnical measures; large-scale step-be-step program for CEL eradication in herds, which ultimately led to positive results [20, 63, 64].

In 1988-1990, within the framework of a large-scale veterinary and zootechnical prophylaxis and rehabilitation, a diagnostic kit for the early detection of bovine leukemia was produced by NPS Orion (Kharkov, Ukraine) [65]. In 1991-1997, an integral assessment of the CEL incidence was performed using ID test to determine the active BLV sources. A through zootechnical survey was carried out and conditions were created for the immediate isolation of identified sick animals and their slaughter no later than in 15 days. To maintain herd size, the reproduction was intensified. Since 1998, using relevant methods of intravital diagnostics, preventive and recreational measures have been actually implemented with regards to epizootology, etiopathogenesis, and epizootic aspects [33, 63, 66, 67].

In bovine leukemia, the effectiveness of a zootechnical and veterinary system is assessed by the epizootic indicators, prevalence of the virus, incidence and the number of sites unfavorable for CEL. According to veterinary statistics, over a 17-year period (1997-2014), the prevalence of CEL in Ukraine decreased 369-fold, the incidence rate 376-fold, and the frequency of detection of unfavorable site 745-fold. As a result, by 2013-2014, the transition of the epizootic to the state of sporadic cases was recorded [63]. The developed zootechnical and veterinary system of anti-CEL measures is generally recognized as effective [63, 68]. Some researchers believe that the complete eradication of cattle is possible only through

identifying infected animals without specific prophylaxis. However, this is rather difficult, since to date the developed diagnostic tests fail to identify all animals which just began to replicate the virus [15].

Republic of Belarus. In the Republic of Belarus, in the 1990s, epizootic BLV infection was registered in 97.8 % of farms [68]. Active anti-CEL measures [69, 70] reduced the proportion of ID-positive animals from 19.6 to 2.4 % in a dairy herd and from 7.4 to 3.2 % among young animals and made it possible to improve the health of herds at 397 dairy farms. By 2011, the proportion of seropositive animals decreased to 0.1 % [68].

The Republic of Kazakhstan. In Kazakhstan in 2002-2011, from 2.3 to 43.7 % of the livestock were immunologically surveyed for CEL using ID and ELISA tests, and 3.3 % were found to be infected, ranging from 2.2 in 2009 to 11.0 % in 2005 [71]. Measures to combat this disease are given constant attention [72-74], taking into account the importance of the problem for cattle breeding in Kazakhstan [71].

Countries of Western and Eastern Europe, Asia, Africa, and America. UK is an example of successful national programs for control and eradication of CEL based on serological testing of blood and milk samples. The first outbreak of the disease was reported here in 1978, the last case in 1996, and since 1999 the UK has been officially recognized as BLV-free [68, 75-77]. The disease control strategy adopted in the UK presupposes maintaining the existing status on the basis of continuous serological monitoring of blood and milk samples and the import of livestock exclusively from Nordic countries free of bovine leukemia [68].

As per the OIE reports, the countries free of BLV are also Andorra (since 1994), Cyprus (since 1995), Czech Republic (since 2010), Denmark (since 1990), Egypt (since 1997), Estonia (since 2013), Finland (since 2008), Georgia (since 1996), Ireland (since 1999), Kyrgyzstan (since 2008), New Zealand (since 2008), Norway (since 2002), Slovenia (since 2006), South Africa (since 2012), Spain (since 1994), Sweden (since 2007), Switzerland (since 2005), Tunisia (since 2005), and Poland (since 2017 years) (the years of eradication of the disease are indicated). In Italy, Portugal, Latvia, Greece, Romania, and Bulgaria, BLV either persists with minor manifestations or the disease occurs sporadically [68]. In the countries of North and South America, Africa and Asia, as well as in Australia, in spite of the anti-CEL measures carried out there, the level of the BLV infection in dairy cattle remains high [68].

Note, however, that each country has its own plan to combat CEL. For instance, in the United States, there are no federal lows to limit the BLV spreading in herds, and there is no mandatory recording of BLV infection which causes great difficulties in assessing the problem [6]. In 2007, seropositivity for BLV was detected by ELISA test on average in 83.9 % of the surveyed herds where pooled samples were collected, and in large herds of more than 500 cows the infection rate reached 100 % [68]. Seroprevalence for BLV was also found at 38.0 % of meat farms [68]. According to data for 2018, when examining milk samples by ELISA test, BLV-specific antibodies were detected on average in 46.5 % of samples [78]. The figures increased from 29. 7% in cows of the 1st lactation to 58.9 % in cows of ≥ the 4th lactations [78].

Since 2000 in Canada, up to 37.2 % of cows and 89.0 % of herds have been reported to be BLV-positive [79, 80-82]. Leukemia infection is also widespread in China and Japan with seroprevalence, reaching in some herds of dairy cattle 49.1 and 40.9 %, respectively [82]. Less than 6 % of cattle were infected in Mongolia (3.9 %) and Cambodia (5.3 %) [82].

It should be noted that the status of zones (states) and herds free of BLV

is determined by the sanitary requirements for international animal and livestock product trade set forth in the "Sanitary Code of Terrestrial Animals" approved by the OIE [83]. These are territories where, within 3 years, 99.8 % of herds are BLV-free, and control of pathological material from animals with suspicion for lymphosarcoma is carried out. To maintain the status of a BLV-free territory, annual serological monitoring is performed, covering up to 99 % of the livestock/ All requirements for imported livestock and genetic resources are also mandatory.

Zoonotic and possible anthropozoonotic potential of BLV. Functional and structural similarity of BLV with causative agents of human T-cell leukemia (HTLV-I, HTLV-II) and T-lymphotropic viruses of monkeys, which tend to overcome the species barrier and, under experimental conditions, provoke an infectious process in sheep [84, 85], rabbits, pigs, and monkeys [29, 86, 87], is the reason for social significance of CEL [88]. One of the earliest and most cited works of McClure et al. [87] described the development of erythroleukemia and pneumonia (*Pneumocystis carinii*) in two out of six chimpanzee infants who, from birth, ate unpasteurized milk from cows naturally infected by BLV. Chimpanzees died at 34 and 45 weeks of age after an illness that lasted 5 to 6 weeks and was characterized by lethargy, anorexia, leukocytosis, anemia, and progressive pneumonia. Blast and immature myeloid cells were found in the bone marrow and peripheral blood of the dead animals [87].

Noteworthy are the findings of Soviet scientists reported in the early 1970s on the development of a neoplastic process in calves experimentally infected with the blood of a person with leukemia. The presence of common antigenic determinants in the organs of people and animals with leukemia has been proven [26].

In 1981, summarizing the accumulated data, Burridge [38] concludes that there are no epidemiological or serological findings that indicate that BLV can infect humans.

The studies were continued, as the presence of BLV-infected cells in the milk of most naturally infected cows indicates that humans are often exposed to this pathogen when ingested orally. The interspecies BLV transmission to rabbits by direct injection into the bloodstream or through the gastrointestinal tract has been proven [29], which confirms the infectious properties of milk from cows with CEL and indicates the potential danger of deinking row milk consumption for humans. Molecular methods revealed the BLV *gag* gene in 49 % of the samples of raw milk and beef [15]. This was the first study to highlight the presence of *gag* gene in food and confirm the presence of viral DNA in raw milk. Since milk pasteurization completely inactivates BLV, the possibility of human infection through milk should be studied, first of all, among livestock breeders working with BLV-infected cows and consuming unpasteurized milk [38, 89, 90].

Extensive epidemiological studies carried out in the late 20th and early 21st centuries in the USA, Denmark and Sweden failed to demonstrate a link between human leukemia and bovine leukemia. Serological studies also did not detect antibodies to BLV in people with various possible exposure to the pathogen [38].

The absence of BLV-specific sequences in 157 cases of acute lymphoblastic leukemia in children with lymphoma (USA) [91] and in 517 cases of human leukemia and 162 patients with lung cancer (Korea) [92] provided additional evidence that BLV was not an etiological factor in human hemoblastosis.

Concerns about human infection with BLV have re-emerged from the findings of Buehring et al. [93] who found reactivity to the BLV p24 protein in 74 % of the examined human sera. The authors argued that the serological methods used in the original studies were not sensitive enough to detect BLV-specific antibodies in humans. Although less than 10 % of people with specific antibodies

report direct contact with cows or their biological products, the authors conclude that antibodies in humans could be a response to oral exposure to heat-denatured virus in food or resulted from direct human infection with this pathogen [93]. It was also found that sera from people infected with HTLV-1 and HTLV-2 cross-react with p24 BLV due to a common epitope [37].

Another evidence of the possible transmission of the BLV from cattle to humans was the PCR detection of virus sequences in 44 % of breast tissue samples. The most striking discovery was the immunohistochemical detection of BLV protein p24 expression in the secretory epithelium of the mammary gland [89]. The analysis showed that the number of BLV sequences in samples from patients with breast cancer is twice as in sections of normal breast tissue. The authors concluded that the presence of BLV in breast tissue was associated with breast cancer [89]. Retro-transcribed BLV DNA was found in 40 out of 50 women (80 %) with breast cancer. When comparing paired breast tissue samples collected with an interval of 3-10 years from patients in whom the first sample was diagnosed as benign and the second as malignant, BLV was found at the first examination in 74 % of women in benign breast tissue [89]. This is consistent with the assumption of a causal relationship between BLV infection and cancer development. It is also noted that the women with BLV proviral DNA in the mammary gland and no history of cancer accounted for 41 % [89].

Buehring and colleagues reported on the detection of BLV proviral DNA in 38 % of human leukocytes detected by PCR analysis and sequencing of genomic proviral DNA. IgG antibodies to BLV were detected in 32 %, IgM in 58 %, and IgA in 32 % of the examined persons [90]. However, there are also reports that antibodies to BLV and its genomic sequences are not detected in healthy women and women with breast cancer [16, 94]. Studies of more than 3700 human malignant tumors, including 810 breast adenocarcinomas, using RNA sequences did not confirm previous data on BLV expression in breast tissue [13].

Products from BLV-infected animals. A large body of data has been accumulated on metabolic changes in animals infected with BLV. During infection, the metabolism of tryptophan, the critical essential acid is disrupted, which leads to the accumulation of dangerous endogenous metabolites. E.g., free tryptophan, indole and anthranilic acid increase 4-8-fold in amount and are deposited in the organs of the immune and hematopoietic system (lymph nodes, spleen, liver), mammary gland, lungs, and kidneys [68, 95]. It has been established that milk from leukemic animals differs from the milk of healthy animals in terms of physicochemical, bacteriological (lysozyme), technological parameters and mineral composition. In C57 mice, drinking pasteurized milk and heat-treated meat from ID-positive cows led to changes in the blood cell composition [96]. Veterinary and sanitary examinations classify meat and slaughter products from BLV-infected animals as conditionally suitable raw materials, which requires special processing and veterinary control to ensure the biosafety of livestock products [27, 68, 97, 98].

Thus, bovine leukemia (cattle enzootic leukemia), a dangerous chronic oncogenic infectious disease, is caused by the bovine leukemia virus (BLV), a member of the *Retroviradae* family. Many countries are officially recognized as free of BLV, nevertheless, its eradication is still a problem. Analysis of the available data allows us to make an unambiguous conclusion about the increased risk of products from BLV-infected cattle for human health. Despite the lack of convincing evidence of BLV pathogenicity for humans, concerns about the etiological role of this virus in the onset of human cancer requires further in-depth research and improved control over this common infectious retroviral proliferative disease

common in livestock farms.

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Mycoplasma bovis, *M. bovigenitalium* AND *M. dispar* AS BOVINE PATHOGENS: BRIEF CHARACTERIZATION

(review)

M. ABED ALHUSSEN[1], V.V. KIRPICHENKO[2], S.P. YATSENTYUK[3], A.A. NESTEROV[2], O.P. BYADOVSKAYA[2], T.V. ZHBANOVA[2], A.V. SPRYGIN[2] ✉

[1]*People's Friendship University of Russia (RUDN University)*, 6, ul. Miklukho-Maklaya, Moscow, 117198 Russia, e-mail alhussenmohammed85@hotmail.com;
[2]*Federal Center for Animal Health Control*, FGBU VNIIZZh, mkr. Yurievets, Vladimir, 600901 Russia, e-mail kirpichenko@arriah.ru, nesterov@arriah.ru, bjadovskaya@arriah.ru, zhbanova@arriah.ru, sprygin@arriah.ru (✉ corresponding author);
[3]*Russian State Center for Animal Feed and Drug Standardization and Quality*, 5, Zvenigorodskoe sh., Moscow, 123022 Russia, e-mail pcr-lab@vgnki.ru
ORCID:
Abed Alhussen M. orcid.org/0000-0002-1210-0303
Kirpichenko V.V. orcid.org/0000-0002-2494-3826
Yatsentyuk S.P. orcid.org/0000-0002-4819-2131
Nesterov A.A. orcid.org/0000-0002-4288-1964
Byadovskaya O.P. orcid.org/0000-0002-8326-7151
Zhbanova T.V. orcid.org/0000-0002-9857-5915
Sprygin A.V. orcid.org/0000-0001-5982-3675
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Abstract

The cattle mycoplasmas are widespread throughout the world (A.M. Parker et al., 2018; M. Abed Alhussen et al., 2020). This review presents data on the epidemiology and diagnosis of mycoplasmosis in cattle caused by *M. bovis, M. bovigenitalium*, and *M. dispar*. Mycoplasmas can cause economically important diseases in cattle, including mastitis, arthritis, keratoconjunctivitis, otitis media, pneumonia, and reproductive disorders (R.A.J. Nicholas et al., 2008; F.P. Maunsell et al., 2011). Mycoplasmas are characterized by a size of up to 150 μm, small genome (0.58-1.38 million base pairs) a low G-C composition (23-40 %) and the absence of a cell wall which determines their polymorphism and resistance to antibiotics, influencing the synthesis of the bacterial cell wall (R.A.J. Nicholas et al., 2008; P. Vos et al., 2011). Mycoplasma surface antigens are highly variable both in vitro and in vivo, which leads to significant variability of isolates (M.A. Rasheed et al., 2017). They also play an important role in overcoming the host's immune system. In addition, some of these antigens are involved in the adhesion of mycoplasmas to host cells (Y. Guo et al., 2017). After adhesion, many mycoplasmas produce a variety of products that damage host cells and enhance pathogenesis (L.A. Khan et al., 2005). They can also form biofilms that increase resistance to drying out and heat stress (L. McAuliffe et al., 2006; F. Gomes et al., 2016). Moreover, the invasion and intracellular survival of mycoplasmas in cattle cells contributes to the preservation and spread in the host organism (J. Van der Merwe et al., 2010). The incubation period for mycoplasma infection in cattle depends on many factors, i.e., the infectious dose, the presence of associated infections, the conditions of keeping the animals in the herd and the stress state of the animals (M.J. Calcutt et al., 2018). Sick animals are a source of infection, because they can shed the pathogen with nasal discharge and sperm for several months and sometimes for several years (K.A. Clothier et al., 2010; V. Punyapornwithaya et al., 2010). It should be noted that at low temperatures, mycoplasmas remain viable for a long time: in deeply frozen cattle semen, the pathogen can remain infectious for many years (A. Kumar et al., 2011). The high contagiousness of some species of *Mycoplasma* spp., their low sensitivity to treatment and the associated consequences of culling for the affected population make timely and accurate diagnosis important for disease control and prevention (A.M. Parker et al., 2018). The cultural methods can be applied for isolation and identification of the pathogen. However, these methods have limitations. Cultivation of mycoplasmas requires a complex medium, special equipment and technical skills (R.A.J. Nicholas et al., 2008; M.J. Calcutt et al., 2018; A.M. Andersson et al., 2019). Mycoplasmas require 7-10-day cultivation at a temperature of 37 °C and 5-10 % CO_2. The colony has the "Fried-egg" appearance characteristic of most mycoplasmas (P.J. Quinn et al., 2011). By contrast, PCR provides a rapid and accurate diagnosis of the disease by detecting mycoplasmal DNA (A.M. Andersson et al., 2019). Furthermore, many other methods of diagnostics of bovine mycoplasma are used, such as MALDI-TOF MS (Matrix assisted laser desorption ionization time-of-light mass spectrometry), latex agglutination,

immunochromatographic assays etc., however, each method has its advantages and disadvantages, which should be considered before application (M.J. Calcutt et al., 2018; B. Pardon et al., 2020).

Keywords: *Mycoplasma bovis, Mycoplasma bovigenitalium, Mycoplasma dispar,* cattle, pathogens, epidemiology

Genus *Mycoplasma* (class *Mollicutes*) comprises small microorganisms characterized by the absence of cell wall, a low GC content (23-40 %) and a small genome size (0.58-1.38 million bp) [1]. The first representative of the genus *Mycoplasma*, the *Mycoplasma mycoides* subsp. *mycoides* was isolated from sick cows in 1898 and characterized as the causative agent of contagious bovine pleuropneumonia [2]. This dangerous disease, having reached a global spread in the 19th century, was successfully eradicated in most countries, with the exception of Africa, where it still remains a serious problem [3]. Other mycoplasma species that pose a threat to cattle include *M. bovis*, *M. bovigenitalium*, *M. dispar*, *M. californicum*, *M. bovirhinis*, *M. bovoculi*, *M. leachii* (formerly *Mycoplasma* sp. *bovine* group 7) [4], *M. canis*, *M. canadense*, *M. alkalescens*, *M. arginini*, and *M. wenyonii* [5-7].

Mycoplasmas are both a secondary microflora, aggravating pathological processes caused by other microorganisms, and primary etiological agents in a host organism with a weak general resistance. Mycoplasmas cause numerous diseases in cattle, including mastitis, arthritis, keratoconjunctivitis, pneumonia, and reproductive pathologies [8]. Currently, te most common pathogenic and clinically significant species are *M. bovis*, *M. bovigenitalium*, and *M. dispar*.

This review focuses on three pathogenic mycoplasmas of cattle, the *Mycoplasma bovis*, *M. bovigenitalium*, and *M. dispar*, their distribution, biological properties, and laboratory methods of identification.

Clinical signs of the infections caused by *M. bovis*, *M. bovigenitalium*, and *M. dispar*. *M. bovis* is the most common species affecting cattle, however, there have been cases of its isolation from buffaloes, small ruminants, and chickens [9]. *M. bovis* is the main cause of pneumonia in young cattle, however, adult cattle are also susceptible to this mycoplasma [10]. In calves, mortality rate due to *M. bovis* is 5-10 %, and morbidity reaches 35 % [6]. The pathogen does not cause characteristic clinical signs, and the infected animals have a sharp dry cough, fever, apathy, and discharge from the eyes (6).

When pneumonia develops, *M. bovis* is always detected in association with other pathogenic microorganisms. Lung lesions may not occur or they are limited to reddish areas of multilobular consolidation in the apical lobes [10]. However, a severe course is associated with extensive lung lesions which appear as an area of coagulation necrosis and abscesses [6]. Chronic *M. bovis* infections are characterized by lymphocytic pneumonia with hyperplasia of the peribronchial lymphoid tissue, causing stenosis of the airway lumen followed by compression and collapse of the adjacent pulmonary parenchyma [11]. The *M. bovis* antigen is found on the periphery of areas of coagulation necrosis, in necrotic exudates and is closely associated with infiltrating macrophages and neutrophils [12]. Pneumonia can occur as a single manifestation of infection or in combination with other clinical signs, including polyarthritis in adult animals and otitis media in young calves [13]. *M. bovis* can also cause subclinical, clinical or chronic mastitis, being a serious problem for milk producers [14]. *M. bovis* was also detected in aborted fetuses [14]. Both the respiratory and mastitis forms of the disease can induce arthritis [6, 16] with impaired motor function and, in severe cases, a decreased feed intake and exhaustion [6]. In addition, *M. bovis* can cause epididymitis, orchitis, urethritis, and bovine seminal vesiculitis [17].

M. bovigenitalium was isolated from the lungs and reproductive tract of dead cows and from aborted fetuses of cattle and buffaloes with arthritis, mastitis

or both [18]. *M. bovigenitalium* is known to be a pathological factor associated with bovine necrotic vulvovaginitis, resulting in significant losses to breeders of pedigree livestock [19]. Acute mycoplasmosis causes severe damage to the udder, affecting one to four quarters [8]. Udder hardness and a decrease in milk yield occur. In mastitis, the antibiotic therapy has no treatment effect. In recent years, *M. bovigenitalium* has often been isolated from samples of vaginal swabs and respiratory tract of cows with reduced fertility, endometritis, granular vulvitis, or all of these conditions. Also, *M. bovigenitalium* is often found in sperm samples and on the mucous membranes of the genital organs in association with *M. bovis* [20].

M. dispar is found in the airways of clinically healthy animals. In a study of calves in the Netherlands, *M. dispar* was detected in 92 % of sick and 40 % of healthy animals [21, 22]. In Denmark, *M. dispar* was equally detected in lung samples from calves with signs of fibrinous necrotic purulent bronchopneumonia and embolic pneumonia [23]. Studies of respiratory diseases in dairy calves have shown a possible initiating role of *M. dispar* in the *P. multocida* invasion [24].

M. dispar colonizes the mucous membrane epithelium of the respiratory tract, exerting a cytostatic and even cytopathic effect on the cells of the distal bronchi and bronchioles, which impaired tracheobronchial clearance [25]. *M. dispar* causes purple to red consolidations, mainly in the cranioventral areas of the lung [17]. In the UK, *M. dispar* is frequently detected in calves showing respiratory signs. It is believed that the pathogen becomes the main cause of severe pleuropneumonia in adult animals, sometimes with a fatal outcome [26].

Epizootology. *M. bovis* was first isolated in the United States in 1961 from cows with severe mastitis [29]. The pathogen has spread to many countries, including Israel (1964), Spain (1967), Australia (1970), France (1974), Great Britain (1974), Czechoslovakia (1975), Germany (1977), Denmark (1981), Switzerland (1983), Morocco (1988), South Korea (1989), Brazil (1989), Republic of Ireland (1994), as well as Northern Ireland (1993) and South Africa (2005) [6].

M. bovis is the most economically significant causative agent of bovine mycoplasmosis. Its study was a part of the EU funded DISCONTOOLS project [30]. In 34 regions of the Russian Federation during 2015 to 2018, *M. bovis* genome was detected in 10.1 %, *M. bovigenitalium* genome in 8.6 %, and *M. dispar* genome in 37.15 % of 1186 samples from cows with clinical signs of respiratory, reproductive pathology or both [31].

An outbreak of mastitis due to *M. bovigenitalium* was first reported in England in 1960 [18]. Numerous works confirm the spread of this mycoplasma throughout the world. Single cases of *M. bovigenitalium* isolation from an aborted horse fetus and boar semen have also been described [32]. *M. bovigenitalium* in cattle was detected in the UK, USA, Brazil, Egypt, India, Germany, Austria, Croatia, Denmark, Nigeria, Italy, Japan, Turkey, the Netherlands, Switzerland, South Africa, France, Canada, and Morocco [6, 33].

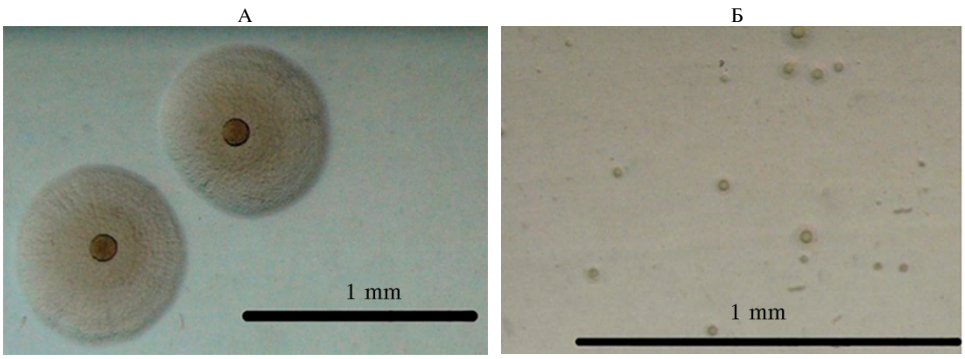
M. dispar is one of the pathogens causing respiratory diseases in cattle [34, 35]. *M. dispar* was first isolated in England in 1969 from affected lungs of calves [36]. Then *M. dispar* was found in Denmark, Belgium, Holland, France, Australia, the US, Canada, Korea, and Japan. In Europe, reports of this causative agent came from Great Britain [26]. *M. dispar* infection has been also reported in Brazil [37] and Italy [38].

Microbiological features. The identification and detection of mycoplasmas infecting cattle is carried out primarily by microbiological methods. Due to the reduced genome, mycoplasmas cannot synthesize a number of amino acids and, depending on the species, are completely or partially unable to synthesize fatty acids. Mycoplasma receives these nutrients from the host cell.

To culture mycoplasmas, the growing media with bovine heart broth,

serum, yeast extract, peptone, and other additives is necessary with buffering to a final pH of 7.3-7.8 [39]. Examples of nutrient media for mycoplasmas are Edwards nutrient medium (based on bovine heart muscle extract and peptone with horse blood serum and yeast extract), UNIIEV medium (tryptic hydrolyzate), Morton's medium (bovine heart extract and bactopeptone), Hayflick medium (modified Morton's medium added with horse serum and yeast extract).

To suppress growth of other bacteria when isolating the causative agents of mycoplasmosis, the nutrient media are added with antibacterial drugs to which mycoplasmas are insensitive [5]. The growth conditions are 37 °C and 5-10 % CO_2 for 7-10 days. The "fried eggs" appearance is characteristic of most *Mycoplasma* colonies (Fig.), as the central part of a colonies grows into agar while a zone of surface growth is located on the periphery. A number of mycoplasmas have their own characteristic colonies, which makes it possible to differentiate them [39].



Colonies of *Mycoplasma bovis* (3-day culture) **(A) and *M. bovigenitalium*** (5-day culture) (B) **on solid medium** (https://www.mycoplasma-exp.com/speccultured.html).

M. bovis is not glucose fermenting and does not hydrolyze arginine [40], instead, *M. bovis* uses organic acids such as lactate and pyruvate as energy sources. One of the metabolic products is hydrogen peroxide, an important virulence factor of *M. bovis* [40, 41]. *M. bovigenitalium* does not hydrolyze arginine and ferment glucose, but has phosphatase activity and reduces tetrazolium salts under anaerobic conditions [42]. *M. bovigenitalium* also produces hydrogen peroxide [43, 44]. *M. dispar* does not form typical "fried eggs" shaped colonies, especially in the early passages. Growth on nutrient media is slow and requires 7-14 days. *M. dispar* ferments glucose and reduces tetrazolium salts under aerobic and anaerobic conditions, but does not hydrolyze arginine, does not catabolize serum, and does not exhibit phosphatase activity [6, 22, 44].

Antimicrobial resistance. Mycoplasmas lack cell walls and thus are resistant to antibiotics which suppress the synthesis of the bacterial cell wall. Mycoplasmas are also resistant to polymyxins, sulfonamides, trimethoprim, nalidixic acid, and rifampicin [45-47]. The antibiotics most commonly used to control mycoplasma infections in cattle are macrolides and tetracyclines. Lincosamides, fluoroquinolones, pleuromutilins, fenicols, and aminoglycosides may also be active against mycoplasmas [48, 49]. Most strains of mycoplasmas remain sensitive to fluoroquinolones, but pleuromutilins are the most effective [50].

In mycoplasmas, target genes have been identified in which point mutations confer antibiotic resistance, namely, those encoding DNA-gyrase and topoisomerase IV for fluoroquinolones, 23S rRNA for macrolides, lincosamides, pleuromutilins, and amphenicols, and 16S rRNAs for tetracyclines and aminoglycosides [49, 51, 52].

Routs of transmission. The main transmission mechanism is the

aerogenic pathway. Mycoplasmas enter the body through inhalation of contaminated micro-droplets and dust particles. With hematogenous transmission, joint damage is characteristic.

Calves can be infected both horizontally (via aerosols infecting respiratory tract) and vertically from an infected dam. Milk is also among the main sources of infection for calves, especially if the cow has mastitis [53]. With artificial infection of various materials, the survival time of mycoplasmas outside the host is quite long at low temperatures, for example, at 4 °C, the *M. bovis* remains stable in foam for 57 days, in milk for 54 days, on straw for 20 days, on wood and in water for 17 days. The survival period reduced to 1-2 weeks at 20 °C and to 1 week at 37 °C [54].

In a 6-week study of the dairy herd, no evidence of transmission of the pathogen with contaminated sand was found. However, in 12 studied calves, mycoplasmas were detected in samples taken from the upper respiratory tract though the autopsy revealed no signs of pathology [55]. In contrast, another study showed that in a clinical outbreak of *M. bovis*, sand from animal litter without proper treatment may pose a risk of udder infections [56].

In Denmark, studies based on four rounds of milk screening have shown that proximity to farms with confirmed mycoplasmosis increases the risk of introducing the pathogen into a healthy farm. Moving cattle between these farms also poses the risk of infection [57, 58]. Analysis of mycoplasmosis incidence at closely located farms indicates that a rather rapid spread of mycoplasmas if sanitary control measures are not duly observed [59].

Mycoplasma contamination of sire semen. *M. bovis* can remain infectious for many years in deep frozen bovine semen. Antimicrobial treatment of semen is ineffective [60].

In in vitro microinjections of *M. bovis* into embryos, the pathogen remained virulent even when thoroughly washed and treated with trypsin and anibiotics (a combination of penicillin, streptomycin, lincomycin and spectinomycin or a combination gentamycin, tylosin, lincomycin and spectinomycin) [61]. In Russia, PCR analysis revealed *M. bovis* DNA in 1.2 %, *M. bovigenitalium* in 43.4 % of 410 sperm specimens from Russian and foreign breeding centers, and the genetic material of *M. californicum* and *Ureaplasma diversum* [62]. The sperm infection by several types of mycoplasmas at once was frequent [62]. In the study of 483 sperm samples from 13 constituent entities of the Russian Federation in 2015-2018, the *M. bovigenitalium* genome was found in 29 %, *M. bovis* in 11.6 % of the specimens [31]. The release of mycoplasmas by infected animals into the environment is not constant, therefore, isolated studies on the detection of a pathogen may be unreliable (63).

The mucous membrane of the upper respiratory tract and mammary glands seem to be the most important site of persistence and shedding of *M. bovis* [64]. Transportation, rearrangement, transfer to the feedlot, and cold stresses cause more aggressive shedding of *M. bovis* from mucous membranes [65]. Chronic asymptomatic infection with intermittent shedding of *M. bovis* is epidemiologically critical [8].

Incubation period. Incubation periods of mycoplasma infection largely depend on the age and condition of the animal. In experimental infections, the incubation period is 2-4 days prior to the onset of mastitis and 7 days for pneumonia. In outbreaks of mastitis caused by mycoplasmas, the incubation period was 14 days [66], while an earlier publication reported from 2 to 6 days for similar outbreaks [67]. The incubation period may also depend on the infectious dose, the presence of associated infections, the conditions of the herd, and the

stresses, especially after transportation [68].

Pathogenesis and its molecular mechanisms. Mycoplasmas adhere onto host cells to survive as cell surface-associated parasites or further invade into the host cells. Adhesion is an important mechanism of mycoplasma virulence, which is confirmed by the study of avirulent strains unable to adhere [69]. Several adhesive proteins have been identified in *M. bovis*, including the membrane-localized P26 protein [70], α-enolase, a plasminogen-binding enzyme [71], NADH oxidase [41], and several highly variable membrane proteins (VspA, VspB, VspE, VspF, and VpmaX) [72, 73]. Proteins of the Vsp family spontaneously undergo a random switching between "on" and "off" states of gene expression. The high rate of spontaneous phenotypic Vsp switching is due to frequent rearrangements in the corresponding genes [68].

Such genetic changes affect the virulence of mycoplasmas, biofilm properties [74], susceptibility to phagocytosis and complement-mediated lysis [75], and molecular shielding of antigens [76]. Comparative genomic analysis of different isolates of *M. bovis* revealed many antigenic variations in the surface proteins of the pathogen. The identification and characterization of adhesins contributes to a better understanding of the interactions between *M. bovis* and host cells. It is assumed that fibronectin-binding proteins and a multifunctional glycoprotein of the extracellular matrix can stimulate adhesion of mycoplasmas to host cells [77]. Several of these proteins have been identified in mycoplasmas. For example, the *M. bovis* TrmFO protein plays an important role in the adhesion of a pathogen to cells, and also performs the function of tRNA methyltransferase [77]. Another distinctive feature of mycoplasmas is their ability to penetrate and multiply in various types of cattle mononuclear cells in the peripheral blood and in erythrocytes, which allows the pathogen to evade host immune immune system and antimicrobial drugs, and also contributes to rapid spreading in the body of an infected animal [78]. *M. bovis* antigens were visualized in hepatocytes and epithelial cells [79], and invasion accompanied by intracellular replication was demonstrated in epithelial cells of the respiratory tract of cattle embryos [80].

The ability of many mycoplasma species to form biofilms is noteworthy [74]. Despite the fact that the biofilms were mainly demonstrated in vitro [74, 81, 82], some researchers report that the mycoplasma biofilms can affect the course of the disease or the pathogenicity of microorganisms in cattle [83, 84]. Nevertheless, the genes encoding the ability to form biofilms characteristic of other bacterial species were not found in mycoplasmas.

In *M. mycoides* subsp. *mycoides*, key proteins associated with biofilm formation have been identified, such as elongation factor Tu, glucose-specific transporter IIB of the PTS system, phosphoenolpyruvate protein phosphotransferase, fructose bisphosphate aldolase II, and pyruvate dehydrogenase [85]. A comparative study of different isolates of *M. bovis* revealed correlation between biofilm formation and the corresponding Vsp expression profile. The ability to develop biofilms can explain the *M. bovis* survival in the litter [56, 86].

To date, only one study has reported putative role of specific genes in pathogenicity of *M. bovis*. When comparing the reduced pathogenicity in the 115th, 150th and 180th passages of the *M. bovis* isolate as compared to its parent wild-type isolate *M. bovis* HB0801, 11 genes were identified that affect the attenuation process. Of these, 10 are associated with metabolism and one encodes a variable surface protein [87]. However, in vivo, no gene has been proven to influence the virulence profile of *M. bovis* [68].

Laboratory diagnostics. Lab tests are critical for clinical diagnosis, since bovine mycoplasma-induced clinical signs are not pathognomonic. The

etiology of *M. bovis* is often overlooked until other pathogens have been ruled out or animals stop responding to antibiotic therapy [68, 88]. Culture, molecular, and serological methods are used to identify *M. bovis* and other types of cattle mycoplasmas in milk, articular fluid, bronchoalveolar lavage fluid, smears from mucous membranes or sera [89]. Identification of mycoplasma isolates to species is extremely important, since species such as *M. bovigenitalium*, *M. bovis*, and *M. dispar* are classified as primary pathogens while others are deemed part of a resident microbiome that does not play a significant role in the development of diseases [6].

Culture, as a conventional microbiology method, is the standard for the detection of mycoplasmas, but it takes a long time [18] and requires enriched media and antibiotics [68]. There is also a risk of false positives due to the similarity of *M. bovis* colonies to colonies of other bacteria of the same class. Also, this method is applicable for viable microorganisms [20]. The sensitivity and specificity of the microbiology testing have not been determined for the majority of bacteria involved in the development of respiratory diseases in cattle [88]. A 70.7 % sensitivity and 93.9 % specificity have been shown for growing *M. bovis* on a solid medium with Tween 80 [90].

Serological methods are used to assess the effectiveness of vaccination, to determine the immune status of the herd and the spread of infection on a wider scale [91[. The enzyme-linked immunosorbent assay (ELISA) is a tool only for indirect confirmation of infection, since it detects antibodies to *M. bovis* but not the pathogen itself [20]. ELISA is recommended in combination with other methods to minimize false positives due to the presence of antibodies to *M. bovis* in healthy animals [20, 88].

Over the past two decades, the polymerase chain reaction (PCR) has been recognized as the main test for diagnosing mycoplasmosis [88, 92]. However, PCR requires specific oligonucleotide primers designed based on sequencing genomes of the pathogens [88]. PCR analysis, as a rule, is expensive, but pooled samples allow a group of animals to be tested [93-95].

In general, molecular methods can be divided into those that allow the detection of a specific type of mycoplasma (using classical PCR or real-time PCR) [96, 97] or mycoplasmas without determining their species. The methods enabling differentiation of mycoplasma species are multiplex PCR [98, 99], PCR with denaturing gradient gel electrophoresis [100], DNA microarrays, and multilocus sequence typing [38, 101].

In the last decade, the matrix assisted laser desorption ionization time-of-light mass spectrometry (MALDI-TOF MS) which enables identification of bacteria by their unique protein profiles is recognized a revolutionary milestone in diagnostics. The method is mainly used for identification of cultured bacteria and mycoplasmas [91, 102], but it is less effective in polymicrobial samples and in samples with mixed infection.

Recently, next generation sequencing (NGS) technique has been widely used to study the bacterial genome. NGS data are used for clinical diagnostics, in the study of outbreaks of diseases, and in the control of the microbial resistance to antimicrobial drugs [104]. To date, complete genome sequences are available for five isolates of *M. bovis* [105-107], two isolates of *M. californicum* [108, 109], and for *M. arginini* [110], *M. bovigenitalium* [111], *M. canadense* [112], *M. bovoculi* [113], *M. leachii* [114], one isolate each.

In addition to the described methods, latex agglutination test and immunochromatographic analysis are under development [68]. A number of studies have recently reported application of a loop-mediated isothermal amplification assay for the detection of *M. bovis* [115, 116].

Thus, *Mycoplasma bovis*, *M. bovigenitalium* and *M. dispar* are the most important members of the *Mollicutes* class which affect cattle. They cause many diseases, the most significant of which are mastitis, pneumonia, reproductive disorders and arthritis in calves and adult animals. These diseases can be chronic, reducing the resistance of cattle to other viral and bacterial pathogens. Mortality and morbidity can reach 10 and 35 %, respectively. Mycoplasma infections has a negative economic impact on animal farming. The herd mostly becomes infected from animals with a subclinical course of the disease. However, there are many other routes to transmit mycoplasmas. The chronic character of mycoplasma diseases and subclinical forms hamper the identification of the infection. The resistance of mycoplasmas to a large number of antimicrobial drugs complicates antibiotic therapy so much that for some diseases, such as mastitis, it is now recommended to slaughter all affected animals. The complexity of the treatment of mycoplasmosis determines the relevance of prevention measures. Unfortunately, the development of a mycoplasma vaccine is a difficult task, and so far, such vaccines cannot be considered as a tool for the control of mycoplasma infections in cattle. Epidemiological studies have identified the main factors of biosecurity risk for the spread of mycoplasmosis, e.g., the introduction of new cattle and poor milking hygiene. Timely and accurate diagnosis contributes to the prevention of the disease. However, additional research and effective control programs for pathogenic mycoplasmas are required to fully understand the mechanism of mycoplasma spread.

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RISK OF FISH MYCOTOXICOSIS IN AQUACULTURE
(review)

G.P. KONONENKO ✉, D.A. ONISCHENKO, M.I. USTYUZHANINA

All-Russian Research Institute of Sanitary, Hygiene and Ecology — Branch of FSC ARRIEV RAS, 5, Zvenigorodskoe sh., Moscow, 123022 Russia, e-mail kononenkogp@mail.ru (✉ corresponding author), mitya_anest@mail.ru, ustpuma@list.ru
ORCID:
Kononenko G.P. orcid.org/0000-0002-9144-615X
Ustyuzhanina M.I. orcid.org/0000-0001-7405-7577
Onishchenko D.A. orcid.org/0000-0003-2534-9455
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Abstract

Modern fish aquaculture is a large-scale and rapidly developing industry of global production (FAO, 2018). In order to improve the quality of the products produced, an active search is underway for effective ways to control the safety of artificial feeds (J. Bostock et al., 2010). Based on the results of monitoring projects carried out in Argentina, Brazil, the United States, China, Korea and Central European countries (C. Pietsch et al., 2013; B.T.C. Barbosa et al., 2013; M. Greco et al., 2015; L. Pinotti et al., 2016), the situation of contamination of fish feed with mycotoxins is recognized as extremely serious both in terms of prevalence and content, and in terms of combined occurrence (I. Matejova et al., 2017; C. Pietsch, 2019). For the Russian fishery, which in recent years has become a multi-destination, specialists of academic and university science, as well as industry research institutes proposed feed rations that account for age and species characteristics of fish (J.A. Zheltov, 2006; Y.V. Sklyarov, 2008), and discussed in detail the problem of microbial contamination (I.V. Burlachenko, 2008). In the Russian Federation, mandatory requirements for compliance with quality and safety indicators have been introduced for raw materials and finished feed products (GOST 10385-2014) and a modern methodological base for mycotoxicological control has been created (GOST 31653-2012, GOST 31691-2012, GOST 32587-2013, GOST 34108-2017, GOST R 51116-2017).The purpose of this review is to update information on mycotoxin contamination of domestic raw materials for the production of aquafeeds, to generalize world data on the nature of acute action of the most occurring contaminants, as well as to analyze clinical signs, pathologic-anatomical and biochemical changes accompanying chronic fish mycotoxicosis. In recent years, we have received convincing evidence that the group of the most likely contaminants of raw ingredients — wheat, barley and corn flour, bran, sunflower cake and meal — includes T-2 toxin, deoxynivalenol, fumonisins of group B and zearalenone, related to fusariotoxins, as well as alternariol, ochratoxin A, citrinin, cyclopiazonic acid, mycophenolic acid and emodin (G.P. Kononenko et al., 2018, 2019). Analysis of world data on experimental mycotoxicoses of different age groups of common carp (*Cyprinus carpio*), channel catfish (*Ictalurus punctatus*), grass carp (*Ctenopharyngodon idellus*), Nile tilapia (*Oreochromis niloticus*), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salma salar*), shows that fusariotoxins should be considered as key risk factors and efforts should continue to refine their safe thresholds. Intoxications caused by ochratoxin A remain insufficiently studied, and the situation with regard to other possible feed contaminants is unclear. Reasonable proposals for regulation in fish feed were reported only for T-2 toxin for common carp (V.T. Galash, 1988), for deoxynivalenol — for grass carp (C. Huang et al., 2018, 2019, 2020) and Atlantic salmon (A. Bernhoft et al., 2018), for fumonisin B_1 — for channel catfish (M.N. Li et al., 1994, S. Lumlertdacha et al., 1995). Data on the degree of retention of these mycotoxins in fish tissues is limited (C. Pietsch et al., 2014, 2015; A. Ananter et al., 2016), and therefore regulations on product residues have not yet been adopted. However, the search for new approaches to correctly assess the consequences of their negative effects and transmission to fish products continues, and this leaves no doubt that a solution will be found.

Keywords: aquaculture, fish mycotoxicoses, feed raw materials, combined feeds, mycotoxins

Nowadays, aquaculture is a rapidly developing global industry. The total market value of its products at initial sales prices in 2016 reached 232 billion US dollars [1]. The search for new forms, methods, techniques, technologies and novel

approaches aimed at increasing the efficiency of artificial fish farming and expanding its assortment is especially active [2]. The importance of studies of mycotoxins in fish feed became apparent for the first time in the early 1960s, when massive outbreaks of rainbow trout aflatoxicosis, accompanied by the development of hepatocellular carcinomas, were recorded in the United States with the use of cotton seed meal [3]. Surveys and experiments showed that other mycotoxins also negatively affect non-commercial fish, causing varying degrees of intoxication and specific signs [4-6].

In each country, fish feed safety is mainly related to the of mycotoxins naturally contaminating ingredients of plant origin which are the main sources of protein for fish of low trophic levels. Monitoring studies carried out in Argentina, Brazil, the United States, China, Korea and Central European countries [7-10] have assessed the real situation in the national fish farming and drown to a holistic idea of the overall scale of the threat. Feed contamination with mycotoxins is recognized as extremely high in prevalence, content, combined occurrence and leading, according to world experts, to significant economic losses [11, 12].

In recent years, due to government support [13], Russian fish farming has become a developed industry for rearing mainly carp, herbivorous fish and salmon. Russian scientists have developed balanced species-specific and age-specific recipes of compound feeds with recommendations for fish feeding procedure and feed quality control [14-17] and considered in detail the risks posed by microbial contamination [18]. However, there are few studies on the effect of mycotoxins on fish and attempts to systematize the information necessary for specialists are very schematic [19, 20].

This review aims to update information on mycotoxin contamination of raw materials used for the production of aquafeeds, to generalize world data on the acute action of the most likely contaminants, ana to analyze clinical, pathological and biochemical changes accompanying acute and chronic mycotoxicosis of fish.

Raw materials of vegetable origin in the recipes of domestic mixed feeds are mainly represented by wheat, barley and corn flour, bran, sunflower cake and meal [14, 16, 17]. For grain intended for fodder purposes, contamination with fusaryotoxins with an extensive occurrence of T-2 toxin (T-2) and deoxynivalenol (DON), sometimes together with zearalenone (ZEN), is characteristic and fumonisin B_1 (FUM B_1) is often found in corn kernels [21-25]. Focal contamination of grain with ochratoxin A (OA) was also established [26], and frequent cases of joint detection of OA and citrinin were revealed in grain of wheat, maize and in fodder products from processed sunflower seeds [27, 28]. Typical contaminants of sunflower cake and meal are OA and alternariol, as well as citrinin, cyclopiazonic acid, mycophenolic acid, and emodin [29]. Aflatoxins associated with the main threat to the aquaculture sector in most countries of the African and Asia-Pacific regions [30] are extremely rare in Russian feed raw materials, therefore, they are not considered in this work.

1. Lethal doses of mycotoxins for various fish species after a single oral (po) **and intraperitoneal** (ip) **administration**

Species	Mycotoxin	Lethal doses, mg/kg body weight	Ссылка
Cyprinus carpio	T-2 toxin	ip LD_{50} = 0.21±0.01 po LD_{50} = 0.46±0.04	[31]
Labeo rohita	Citrinin	ip LD_{100} = 12.5	[32]
Ictalurus punctatus	Cyclopiazonic acid	ip LD_{50} = 2.82	[33]
Oncorhynchus mykiss	T-2 toxin	po LD_{50} = 5.37±0.40	[31]
	Ochratoxin A	ip LD_{50} = 4.7	[34]

Mycotoxins which are relevant for fish compound feed in our country

(Table 1) became the subject of research in the 1970-1990s when their lethal doses were established and interspecific differences in fish susceptibility to these fungal metabolites were found (see Table 1).

These early works first reported the characteristic lesions from acute intoxication after injections and oral administration of mycotoxins. Under the influence of T-2 in common carp (*Cyprinus carpio*), necrosis appeared in the hepatopancreas, kidneys, anterior and middle intestine, being especially strongly manifested in the walls of blood vessels, gills, and posterior intestine. The lethal effect on fish of different age groups was the same [31]. In adult juveniles of roho labeo (*Labeo rohita*), dietary exposure to citrinin at 12.5 and 25.0 mg/kg led to a death-causing damage to the kidneys, liver, stomach and depigmentation and hyperemia of the caudal fin [32]. According to the effect on the 19 g-weighted channel catfish (*Ictalurus punctatus*), cyclopiazonic acid is a neurotoxin: 30 minutes after its injections at ≥ 2.4 mg/kg, the fish showed severe convulsions and tetanic seizure [33]. In rainbow trout (*Oncorhynchus mykiss*), OA caused necrosis of kidney and liver tubule cells [34].

The study of the impact of long-term intake of mycotoxins on fish which were fed diets added with naturally contaminated ingredients or the biomass of mycotoxin producing fungi, has disclosed disturbances occurred under close-to-real conditions. It was found at what content of mycotoxins in the feed of common carp and channel catfish there is a deterioration in morphological and fish-breeding parameters, i.e., a decrease in condition, an increase in feed costs, a decrease in vitality (Table 2).

2. Mycotoxin concentrations in feed causing a negative effect on morphological and breeding parameters in two fish species

Mycotoxin, its source (concomitant mycotoxins)	Age, initial body weight, g	Feeding period	Toxin concentration, mg/kg feed	References
Common carp *Cyprinus carpio*				
T-2 toxin, *Fusarium sporotrichioides* biomass (HT-2 toxin, neosolaniol)	Not indicated	67 days	0.14; 1.02	[31]
T-2 toxin, *F. sporotrichioides* biomass (HT-2 toxin, neosolaniol	Not indicated	122 days	0.45; 0.92	[31]
T-2 toxin, *F. sporotrichioides* biomass (HT-2 toxin, 0.45 mg/kg)	1 year of age, 23 g	4 weeks	4.11	[35]
T-2 toxin, preparation	Not indicated	4 weeks	5.3	[36]
Deoxynivalenol, contaminated feed ingredients	Young of-the-year	30 days	1.25	[37]
Deoxynivalenol, contaminated feed ingredients	2 years of age	2.5 months	1.25	[37]
Deoxynivalenol, *F. graminearum* biomass (15-acetyl deoxynivalenol, 0.33 mg/kg)	1 year of age, 23 g	4 weeks	5.96	[35]
Fumonisin B_1, prepraration	1 year of age	42 days	0.5; 5.0	[38]
Fumonisin B_1, prepraration	1 year of age	42 days	10; 100	[39]
Channel catfish (*Ictalurus punctatus*)				
T-2 toxin, preparation	Juvenile fish, 8.9 g	8 weeks	0,625; 1,25; 2,5	[40]
Deoxynivalenol, contaminated feed ingredients	Juvenile fish, 5 g	6 weeks	15; 17,5	[41]
Fumonisin B_1, *F. moniliforme* biomass	1 year of age, 1.2 g	10 weeks	20; 80; 320; 720	[42]
Fumonisin B_1, *F. moniliforme* biomass	Juvenile fish, 6.1 g	12 weeks	20; 40; 80; 240	[43]
Fumonisin B_1, *F. moniliforme* biomass	2 years of age, 31 g	14 weeks	80; 320; 720	[42, 44]
Ochratoxin A, *Aspergillus ochraceus* biomass	Juvenile fish, 6.1 g	8 weeks	1; 4; 8	[45]
Cyclopiazonic acid, preparation	Juvenile fish, 7.5 g	10 weeks	0.1	[33]

In ponds, common carp fed T-2 (0.14 and 1.02 mg/kg) for 67 days exhibited a 30 % decrease in body weight, with a 2-fold increase in feed costs per season per unit gain. The basins fish fed diets with T-2 (0.45 mg/kg and 0.92 mg/kg) for 122 days almost completely stopped growing, with a 40-50 % less average body weight by the end of the experiment compared to control [31]. In channel catfish, a decrease in gain was noted at ≤ 1 mg/kg of T-2 or OA [39, 44] and at 0.1 mg/kg

of cyclopiazonic acid [33].

The growth rate of Nile tilapia (*Oreochromis niloticus*) juveniles with a 2.7 g initial body weight decreased after 8-week when fed FUM B_1-containing feed (40, 70, and 150 mg/kg fungal biomass) [46]. In adults fed 0.5 μg/kg of OA, the fish-breeding parameters significantly ($p < 0.05$) decreased by the end of the 2-month experiment [47]. In 16-week long observation of juvenile rainbow trout with initial body weight of 1.0 g, there was a clear slowdown in growth and consumption of feed containing T-2 at ≥ 1.0 mg/kg [48]. The feed contaminated with 1.0 to 12.9 mg/kg DON slowed down both growth and feeding efficiency within 8 weeks without clinical signs of intoxication and with the preserved survival rate, whereas at 20 mg/kg DON, fish exhibited refusing to eat [49]. Later, the effect of reducing food consumption for rainbow trout with an initial body weight of 24 g was confirmed after 8-week feeding a diet with 0.3 to 2.6 mg/kg DON [50, 51]. Other researchers indicate a negative effect as early as on day 23 and day 32 of feeding this fish with 2 mg/kg DON [52, 53].

The productivity of Atlantic salmon (*Salmo salar*) decreased after 15-week consuming 3.7 mg/kg DON [54]. In an 8-week experiment on one-year-old fidh (58 g initial body weight), 5.5 mg/kg DON in the feed led to a decrease in the average body weight to 80.2 g compared to 123.2 g in the control [55]. In an 8-week experiment on smolts fed a diet added with 0.5-6.0 mg/kg of pure DON preparation, the feeding efficiency, body weight, length, and the condition factor correlated inversely with the toxin dose [56].

In all these species (common carp, channel catfish, Nile tilapia, rainbow trout, Atlantic salmon), long exposure to toxins in feeds had a negative impact on blood biochemical parameters, the activity of digestive, antioxidant and transforming enzymes, caused organ pathologies and functional disorders of body systems and decreased resistance to infectious diseases.

In common carp fed a diet supplemented with 5.3 mg/kg T-2 for 4 weeks, changes in the hemoglobin level with anemia and leukopenia were reported, the plasma glucose concentration and the alanine aminotransferase activity sharply increased while the concentration of triglycerides and ceruloplasmin activity significantly decreased [36]. T-2 at 4.11 mg/kg along with HT-2 in a smaller amount fed to one-year-old carp for 4 weeks did not cause significant differences in markers of lipid oxidation compared to control, but decreased the activity of the glutathione redox system and glutathione-S-transferase [35]. The authors hypothesized the differences in the activity of transforming and antioxidant enzymes to be explained by the long exposure to the toxin. Earlier, in a 3-day experiment on common carp exposed to T-2, a slight increase in the activity of glutathione-S-transferases in the hepatopancreas occurred [57]. In using 0.52 and 2.45 mg/kg T-2 for 7 and 28 days, the activity of both glutathione-S-transferases and glutathione peroxidases increased and the expression of *gpx4* paralogs changed but in different ways. The *gpx4a* expression significantly ($p < 0.05$) increased on day 21 and decreased on day 28, while for *gpx4b*, it remained increased both on days 21 and 28 [58].

In young of-the-year carp fish grown in aquariums and fed naturally contaminated feeds with 1.25 mg/kg DON for 30 days, the blood protein level, triacylglycerols, lipoproteins, cholesterol, the activity of aspartate aminotransferase and alkaline phosphatase decreased. The amount of β-lipoproteins decreased sharply, the glucose concentration increased, and a dysfunction of hepatopancreas occurred. When fed the same contaminated feed for 2.5 months, the two-year-old carp grown in cages exhibited an increase in activity of transferases, alkaline phosphatase, trypsin, amylase, a decrease in blood protein (due to β- and γ-globulin

fractions) [37]. Functional disorders occurred in hepatocytes, pancreatic cells and regulation of secretion, kidney tissues were damaged, vitality was reduced, and intestinal mucosa was atrophied [37]. In one-year-old carp fed DON with admixture of its natural analogue 15-acetyl-DON (a total contamination of 5.96 mg/kg) for 4 weeks, lipid oxidation and glutathione transferase activity in hepatopancreas corresponded to the control, and the concentration of the reduced form of glutathione significantly ($p < 0.05$) increased by the end of the experiment on day 28. For DON, dietary exposure over an extended period of time significantly affects the expression of *gpx4* genes which was above control on days 21 and 28 for *gpx4a* and throughout the observation period for *gpx4b* [58]. The results obtained allowed the authors to draw a general conclusion that the expression of *gpx4* paralogs depends on how long the fish were exposed to T-2 and DON though the activity of glutathione peroxidases remained unchanged [58].

In one-year-old carp regularly fed 0.5 and 5.0 mg/kg FUM B_1 for 42 days, along with a decrease in fish body weight, there were an increase in the number of erythrocytes and platelets, in the concentrations of blood creatinine and total bilirubin, and in the activity of aspartate aminotransferase and alanine aminotransferase. The changes allowed the authors to suggest that the kidneys and liver are the key organs affected by this toxin, and that the erythrocyte membrane may be disrupted or the respiratory process may be affected [38]. In carp fed 5.0 mg/kg FUM B_1, the incidence of superficial erythrodermatitis caused by *Aeromonas salmonicida* subsp. *nova* was higher [38]. In fish of the same age, receiving feed containing 10 and 100 mg/kg of added FUM B1 for 42 days, there were histological changes in blood vessels, liver, kidneys, heart and brain, as well as scattered damage to the exocrine and endocrine parts of the hepatopancreas and renal tissue, probably due to ischemia, increased endothelial permeability, or both [39]. Examination of the brain revealed deep neuronal damage [59].

After 6-week dietary exposure of young channel catfish to 1.0 and 2.0 mg/kg T-2, the mortality 21 days after infection with the virulent isolate *Edwardsiella ictaluri* was significantly higher than in the control [60]. DON-contaminated corn diets also increased susceptibility to this pathogen [61]. After 5-week experimental feeding of *Fusarium* culture material containing 35, 62, 170 and 313 mg/kg FUM B_1 to adult channel catfish, no significant lesions were seen in the brain, heart, liver, spleen, gills, head and trunk kidneys, stomach, intestines, skin, or gonads of the control or treatment groups. These findings suggest that adult channel catfish can tolerate feed contaminated with FUM B_1 up to 313 mg/kg [62]. Nevertheless, in one-year-old channel catfish, a 10-week feeding FUM B_1 at ≥ 80 mg/kg decreased the hematocrit level, the number of leukocytes and erythrocytes, and in 2-year-old fish, a 14-week exposure to 320 mg/kg FUM B_1 led to a decreased hematocrit and erythropenia with leukocytosis [42]. In the liver of 1- and 2-year-old catfish fed ≥ 20 mg/kg FUM B_1 for 10 and 14 weeks, foci of hyperplasia of subcapsular fat cells, swollen hepatocytes with vacuoles filled with lipids, infiltration of lymphocytes and scattered necrotic hepatocytes were seen [42]. In juvenile fish (initial weight 6.1 g), receiving 80 and 240 mg kg FUM B_1 for 12 weeks, the hematocrit significantly decreased, and at 40, 80, and 120 mg/kg FUM B_1, glycogen accumulation in the liver, vacuolization of nerve fibers and an increase in the perivascular lymphohistiocytic layer in the brain were seen [43]. A significant increase in the ratio of free sphingamine and free sphingosine in blood, liver, kidneys, and muscles, but not in the brain occurred in 2-year-old catfish fed a 12-week diet with *Fusarium* culture material containing ≥ 10 mg/kg FUM B_1 [63]. In 2-year-old fish, fed a 14-week diet with *Fusarium* culture material (20 and 80 mg/kg FUM B_1), resistance to *Edwardsiella ictaluri* infection was lower with

poorly formed antibodies as compared to the control group [44].

In juveniles of Nile tilapia (initial weight 2.7 g), after a 8-week feeding a diet with containing 150 mg/kg FUM B_1 of *Fusarium* culture material, the hematocrit was significantly lower, and the ratio of free sphingamine to free sphingosine in the liver increased [46].

In 8-week experimental feeding 2.6 mg/kg DON to rainbow trout (initial weight 24 g), morphological abnormalities seen in the liver were subcapsular hemorrhages, edema, changes in hepatocytes, and fatty infiltration [51]. In a one-year-old rainbow trout fed 2.0 mg/kg DON for 23 days, the mean hemoglobin concentration in the erythrocyte and biochemical parameters significantly ($p < 0.05$) decreased [64). Histological studies revealed degeneration of epithelial cells of the convoluted tubules of the kidneys in 9 out of 10 fish [64], and in several individuals, there were hemorrhages in the liver described earlier by Hooft et al. [50]. When using a feed containing 2.0 mg/kg DON for 23 and 32 days, changes were observed in the activity of glutathione peroxidase in the kidneys, glutathione reductase in the gills and kidneys, catalase in the kidneys and liver, glutathione transferase in the gills and liver. This indicates that DON induced oxidative stress but practically did not affect lipid oxidation [65]. Later studies gave confirmed the regulatory effect of DON on the expression of key genes and the main metabolic processes in rainbow trout [52, 53].

In Atlantic salmon smolts fed a diet with 0.5 to 6.0 mg/kg DON for 8 weeks, the clinical biochemical parameters and protective immune response to *Aeromonas salmonicida* vaccine correlated inversely with the toxin dose [56].

All these findings provided important information about the effect of T-2, DON and FUM B_1 on performance and the state of internal organs based on changes in biochemical parameters, the activity of enzymes in the liver, kidneys, gills, and the susceptibility to diseases upon artificial infections. The next step was estimation of the thresholds for safe exposure to mycotoxins and elucidation mechanisms of their toxic action. To that end, a full-value, nutrient-balanced diet that does not contain plant ingredients, and, therefore, contaminants of a mycogenic nature, were added with individual preparations of mycotoxins to correctly assess the effect of the selected toxicant. For two fusariotoxins, DON and ZEN, these studies provide new insights into effects of mycotoxins at molecular level and the innate immunity responses in cyprinids, involving factors that provoke or prevent the development of inflammatory processes.

The effect of DON at the doses not affecting productivity (0.352, 0.619 and 0.953 mg/kg) was studied on young carp 12-16 cm in length in a series of 6-week experiments. At the lowest dose, the number of blood leukocytes was reduced, and the activity of antioxidant enzymes superoxide dismutase and catalase in erythrocytes was increased, which indicated the immunosuppressive effect of DON [66]. At the highest concentration, lipid oxidation in the liver, head kidney and spleen increased and fat was accumulated in the body. The changes in the activity of lactate dehydrogenase in kidneys and muscles and blood lactate accumulation indicated the effect of DON on anaerobic metabolism, while a decrease in blood albumin at medium and high doses of the toxin was characteristic of their ribotoxic effect [67]. Further, the dynamics of liver damage, changes in the activity of liver enzymes, and the immune response were studied on fish (9-12 cm long) fed 0.953 mg/kg DON for 7, 14, 26 and 56 days. During the first 2 weeks, there was an inhibition of biotransforming enzymes followed by activation of alanine aminotransferase, which indicates damage to liver tissue: after 14 and 26 days, lipid aggregation, vacuolization and hyperemia were seen in histological sections together with inhibition of enzymes involved in glutathione cycle and reduction of oxidative stress [68]. In the first 2 weeks, the intake of DON led to the activation

of enzymes and cytokines, both inhibiting and promoting the development of the inflammatory process, and after a 26-day exposure, the activation of arginases to the highest levels was detected in the leukocytes of the head kidney [69]. Despite some immunomodulatory effects at the beginning of the experiment, the authors concluded that DON has a systemic immunosuppressive effect on carp [69].

In young carp (12-16 cm in length), 0.332, 0.621, and 0.797 mg/kg ZEN fed for 4 weeks led to no decrease in growth and no estrogenic effects characteristic of other fish species, as measured by the concentration of blood vitellogenin. Nevertheless, hematological parameters underwent significant changes. The effect of medium and high doses of the mycotoxin on the number of leukocytes, granulocytes and monocytes was shown, and micronuclei seen in the erythrocytes confirmed ZEN genotoxicity [70]. In addition, the effect of ZEN on carbohydrate metabolism, lipid oxidation in organs, and oxygen metabolism was established, which indicates its ability to increase the overall metabolic load [71]. In a comparative study of nitric oxide accumulation in leukocytes from the head and trunk kidneys, a bioassay for respiratory activity, chemiluminescence test and arginase activity showed that the higher concentrations of ZEN have a distinct suppressive effect while the lower concentrations enhance immune responses [72]. In the liver, all concentrations of ZEN led to a decrease in the expression of genes regulating the immune response, antioxidant system, and sensitivity to estrogens. Also, there was a significant increase in the expression of vacuolar-type H+-adenosine triphosphatase, which was consistent with the previously established association between ZEN and lysosomal functions [73]. These studies confirmed the effect of ZEN on many key processes in carp and evidenced that the permissible concentrations of ZEN previously approved for compound feed are too high and do not prevent its damaging effect.

Chinese researchers studied the effect of DON, using another representative of the cyprinids, the grass carp (*Ctenopharyngodon idella*) [74-76]. On juveniles (initial weight approximately 12 g) fed mixed fodders with 0.318, 0.636, 0.922, 1.243 and 1.515 mg/kg DON for 60 days, it has been shown for the first time that this toxin can cause malformations in fish and lead to histopathological changes, oxidative damage, decreased antioxidant capacity, cell apoptosis and destruction of tight junctions in the intestine through signaling systems of Nuclear factor-erythroid 2-related factor 2 (Nrf2), c-Jun N-terminal kinases (JNK) and myosin light-chain kinase (MLCK). Based on the totality of the measured parameters, the DON dose of 0.318 mg/kg was called safe [74]. Further studies have shown that DON disrupts intestinal immune function by mechanisms partially associated with two signaling pathways, the nuclear factor kappa B (NF-kB) and target of rapamicin (TOR). Given the incidence of enteritis caused by *Aeromonas hydrophila*, the activity of lysozyme and acid phosphatase, and the IgM level in the proximal intestine, the recommended doses of DON were 0.252-0.310 mg/kg [75]. In the gills of these fish under the same experimental conditions, DON added to feed at > 0.318 mg/kg also led to histopathological changes, oxidative damage, decreased antioxidant capacity, cell apoptosis, and destruction of tight junctions, which was presumably due to Nrf2, JNK, and MLCK signal systems without affecting the expression of *Keap1b*, *claudin-b*, *claudin-3c*, and *claudin-15b* genes. The allowable amounts of DON calculated based on the activity of malondialdehyde and the pool of antioxidants were 0.376 and 0.413 mg/kg [76].

In grass carp, dietary ZEN (0.535, 1.041, 1.548, 2.002 and 2.507 mg/kg) caused oxidative damage, apoptosis and disruption of the intestine integrity [77]. The authors assumed these effects to be associated with signaling pathways of Nrf2, p38 mitogen activated protein kinases (p38MAPK), and myosin

light chain kinase (MLCK). There were no changes in the antioxidant genes *Keap 1b*, *GSTP1* and *GSTP2* encoding glutathione S-transferases, and *occludin*, *claudin-c*, and *claudin-3c* responsible for the intestinal integrity [77].

Aquarium fish are good models for investigating chronic effects of mycotoxins on cyprinids. In *Danio rerio*, dietary DON at 0.1-3 mg/kg generated higher levels of liver gene biomarkers and adverse effects on the reproductive system [78]. In *D. rerio* adults, based on vitellogenin (Vtg) protein levels and relative abundance of molecular biomarker *vitellogenin-1* mRNA (*vtg-1*), ZEN, in addition to its direct estrogenic effects, was established to be capable of influencing other pathways during ontogenesis [79, 80].

Significant progress has also been achieved in the study of toxicosis of fish of a high trophic level. Despite the absence of histological changes in juveniles of the rainbow trout fed a diet with T-2 (1.0 and 1.8 mg/kg) for 24 days, a distinct oxidative stress occurred, which affects the detoxifying system and may lead to an increase in the sensitivity to other stress factors [81]. At 2 mg/kg of dietary DON for 23 days, higher levels of cytokines TNF-α and IL-8 provoking inflammatory processes were detected in the head kidney of one-year-old fish [82].

In rainbow trout, ZEN has been confirmed being capable of binding estrogen receptors and inducing expression of the corresponding genes [83]. In juveniles (initial weight 55 g), no signs of liver damage occurred 24, 72, and 168 hours after a single intraperitoneal injection of ZEN at 10 mg/kg, since the activity of alanine aminotransferase and aspartate aminotransferase and the blood glucose level did not change. However, iron accumulation in the liver and ovaries significantly decreased which, according to the authors, could be both a consequence and a cause of oxidative stress [84]. In an 8-week experiment on 12-month-old Atlantic salmon (initial weight 58 g), DON (5.5 mg/kg) disrupted integrity of distal and mid-intestine, namely, the expression of barrier protein markers (*claudin 25b*, *occludin*, and *tricellulin*) decreased vs. an increase in the expression of nuclear antigen marker of proliferating cells. Importantly, in the distal intestine, the relative expression of *SOCS1* and *SOCS2* encoding two suppressors of cytokines signaling increased. However, according to the authors, though the damaging effect was mitigated by suppressors of cytokine signaling, this dysfunction of the intestinal barrier should not be underestimated [55]. In 8-week experiment, smolts of Atlantic salmon fed dietary DON (0.5 to 6 mg/kg) exhibited a relative increase in weight of organs as the dose of toxin increased, and no-observed adverse effect level (NOAEL) of the toxin was 1 mg/kg [56].

Unfortunately, so far, the problem of OA intoxication in fish has been poorly studied. In juvenile canal catfish fed a diet added with *Aspergillus ochraceus* culture material (8 mg/kg OA), the hematocrit decreased though the number of blood leukocytes did not change [45]. In the experimental fish which ate 4 mg/kg OA for 6 weeks and then was infected with the virulent isolate of *Edwardsiella ictaluri*, mortality on day 21 was significantly higher than in the control [60]. In catfish, necrosis of the renal tubules was not seen but extensive multifocal melanomacrophage centers appeared in the loose connective tissue of the kidneys at OA doses of 4 and 8 mg/kg. For OA at ≥ 1 mg/kg, the most apparent histopathological lesion was necrosis of hepatopancreatic tissues, especially exocrine cells of the pancreas surrounding the portal veins, and this pathology ultimately led to obliteration of normal pancreatic tissue [41]. In smolts of Atlantic salmon fed five diets supplemented with OA pure preparation (0.2-2.4 mg/kg), after 8 weeks, the performance indicators remained unchanged although after the first 3 weeks there was a tendency to an increase in some clinical biochemical parameters and increased expression of two immune markers in the spleen; however, it was not

possible to calculate NOAEL from the available concentration range [56].

Data on toxic effect of cyclopiazonic acid are very limited. In juvenile canal catfish (initial weight 7.5 g) fed for 10 weeks with dietary toxin (10 mg/kg), histological lesions were seen in the kidneys and stomach as protein granules in the epithelium of the renal tubules and necrosis of the gastric glands, however, liver damage and effects on hematocrit, hemoglobin concentration, leukocyte and erythrocyte counts were not revealed [33]. As to citrinin, mycophenolic acid, alternariol, and emodin, the situation remains unexplored.

As to the effect of the considered mycotoxins on silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Hypophthalmichthys nobilis*), tench (*Tinca tinca*), peled (*Coregonus peled*), paddlefish (*Polyodon spathula*), goldfish (*Carassius auratus*), crucian carp (*C. carassius*), eels (*Anquila* spp.), Siberian sturgeon (*Acipenser baerii*), bester (*Huso huso* × *Acipenser ruthenus*) which form the basis of Russian fish farming no publications are available. Therefore, it is advisable to gradually involve the most commercially demanded species in research to assess their tolerance to the entire set of expected toxicants in a timely manner. In addition, the expanding range of farmed fish necessitates careful generalization and analysis of newly emerging information. It should be noted the works which showed a high sensitivity to OA in common sea bass (*Dicentrarchus labrax* L.) from the *Moronidae* family (LD_{50} 0.277 mg/kg body weight) [85], a chronic intoxication with feed-born FUM B_1 in juvenile African sharptooth catfish (*Clarias gariepinus*) [86, 87], and the effects of polycontaminated feed containing, along with DON, other fusariotoxins and alternariol, on red tilapia (*Oreochromis niloticus* × *O. mossambicus*) [88]. The effect of moniliformin, often concomitant with FUM B_1 in affected corn grain, deserves separate consideration, as it was shown that feeds supplemented with culture material of *F. moniliforme* or *F. proliferatum* which produce moniliformin can cause distinct shifts in hematological and histological parameters in channel catfish [89] and Nile tilapia [46, 90].

There is a general consensus that the identification of mycotoxins in aquatic feed and assessing their adverse effects should remain a major focus, especially due to the general trend of replacing fishmeal as a source of expensive animal proteins with cheaper vegetable proteins. In addition to the aforementioned ingredients, new recipes contain glutens (by-products of grain processing into starch and molasses) and dry grain stillage with hydrolysates [91] for which multiple mycotoxin contamination is known [92]. Expanded use of sorghum grain for compound feed is recommended [9] although its toxicological risk has not been investigated. Flaxseed, pumpkin meal [94, 95], cottonseed meal, seaweed, and grass meal should be mentioned among the minor additives which can also be sources of mycotoxins. Special examinations of the meal are still coming, but for grasses and algae intended for processing into feed meal, the possibility of multiple contamination with mycotoxins has already been established [96, 97].

Fishmeal and its substitutes which are the main source of protein in diets for salmon fish [98] can easily become infected with microscopic fungi during transportation, storage, and use. Recently, 11 potentially toxigenic species of *Penicillium* fungi have been identified in the mycobiota of fish, meat and bone meals [99], however, the contamination of commercial batches with mycotoxins was not monitored. Given prospects for transfer of aquaculture industry to the domestic feed base [100], it is necessary to coordinate regular surveys of flour lots of animal origin.

All efforts to assess the actual contamination of feed and accumulate data on the damaging effect of mycotoxins on fish aim at introducing norms for their permissible concentration. However, reasonable proposals for the regulation of

mycotoxins are still very few, i.e., for T-2 in common carp [31], for DON in grass carp [74-76] and Atlantic salmon [56], and for FUM B_1 in channel catfish [42, 43]. Another key point in the prevention of mycotoxicosis in aquaculture is to ensure the safety of fish products for consumers. In general, data on the transformation of mycotoxins in fish and their preservation in organs and muscle tissue indicate a weakly expressed or moderate accumulation and slow excretion, which is explained by the physiological peculiarities of poikilothermic organisms [54, 101-103]. This information is still limited and, probably, that is why regulations on residual amounts of mycotoxins in products have not yet been adopted. However, the search for new informative approaches to the correct assessment of the risks that mycotoxins pose to fish and, via fish products, to human health continues, and a solution, despite the complexity of the task, will most likely be found.

In Russia, the Technical Regulation of the Eurasian Economic Union (EAEU) "On the safety of fish and fish products" [104], which entered into force in September 2017, defines the maximum allowable concentrations of residues of veterinary therapeutic drugs and growth stimulants permitted for use in aquaculture. The introduction of mycotoxins as congeners in the near future is unlikely, since the block of necessary information has not yet been formed. The first step should be systematic regular monitoring surveys of compound feeds produced in all federal districts of Russia. Generalization of these data will give a reasonable methodology to study metabolism, accumulation and circulation of significant mycotoxins for species intended for fish farming, and then to determine the priority criteria for the regulation of residual contents in feed and fish products. In Russia, mandatory requirements have been introduced for the quality and safety indicators of raw materials and finished feed products (GOST 10385-2014 "Combined feeding stuffs for fishes. General specifications". Moscow, 2014) and a modern methodological base has been created for mycotoxicological control (GOST 31653-2012 "Feedstuffs. Method of immunoenzyme mycotoxin determination". Moscow, 2012; GOST 31691-2012 "Grain and products of its treatment, mixed feeds. Determination of zearalenone content using high-performance liquid chromatography". Moscow, 2012; GOST 32587-2013 "Grain and products of its processing, mixed feeds. Determination of ochratoxin A by high performance liquid chromatography". Moscow, 2013; GOST 34108-2017 "Feeds, mixed feeds and raw material. Determination of mycotoxins content by direct solid-phase competitive immunoenzymatic method". Moscow, 2017; GOST R 51116-2017 "Compound feed, grain and products of its processing. Deoxynivalenol content determination method ohm of high-performance liquid chromatography". Moscow, 2017).

Thus, to date, science has convincing evidence of the deep damaging effect of mycotoxins on non-commercial fish when fed with contaminated feed. From a practical point of view, large datasets on threshold levels at which the risk of developing alimentary toxicosis can be significantly reduced are of particular value. Data on blood biochemical parameters, the activity of digestive, antioxidant and transforming enzymes, as well as on changes in the susceptibility of fish to infections are also of significant interest. In recent years, the first studies of molecular mechanisms leading to impaired detoxifying, immune and reproductive functions in fish have been carried out, and original methodological techniques have been proposed for assessing the permissible doses of these toxicants. However, research should be consistent with the real landscape of mycotoxin contamination, in particular, combinations of two or more mycotoxins at different concentrations should be investigated. This approach and the timely systematization of the

accumulated information will ensure effective control of feed safety and sustainable veterinary welfare in the fish farming industry.

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A SEARCH FOR GENOMIC VARIANTS ASSOCIATED WITH BODY WEIGHT IN SHEEP BASED ON HIGH DENSITY SNP GENOTYPES ANALYSIS

T.E. DENISKOVA[1] ✉, S.N. PETROV[1], A.A. SERMYAGIN[1], A.V. DOSEV[1], M.S. FORNARA[1], H. REYER[2], K. WIMMERS[2], V.A. BAGIROV[1], G. BREM[1, 3], N.A. ZINOVIEVA[1]

[1]*Ernst Federal Science Center for Animal Husbandry,* 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail horarka@yandex.ru (✉ corresponding author), citelekle@gmail.com, alex_sermyagin85@mail.ru, asnd@mail.ru, margaretfornara@gmail.com, vugarbagirov@mail.ru, n_zinovieva@mail.ru;
[2]*Institute of Genome Biology, Leibniz Institute for Farm Animal Biology (FBN),* Mecklenburg-Vorpommern, 18196 Dummerstorf, Germany, e-mail reyer@fbn-dummerstorf.de, wimmers@fbn-dummerstorf.de;
[3]*Institut für Tierzucht und Genetik, University of Veterinary Medicine (VMU),* Veterinärplatz, A-1210, Vienna, Austria, e-mail gottfried.brem@agrobiogen.de
ORCID:
Deniskova T.E. orcid.org/0000-0002-5809-1262
Petrov S.N. orcid.org/0000-0001-5130-677X
Sermyagin A.A. orcid.org/0000-0002-1799-601
Dotsev A.V. orcid.org/0000-0003-3418-2511
Fornara M.S. orcid.org/0000-0002-8844-177X
Reyer H. orcid.org/0000-0001-6470-0434
Wimmers K. orcid.org/0000-0002-9523-6790
Bagirov V.A. orcid.org/0000-0001-5398-8815
Brem G. orcid.org/0000-0002-7522-0708
Zinovieva N.A. orcid.org/0000-0003-4017-6863
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Abstract

Body weight is one of the most important economically useful traits and is characterized by a complex inheritance pattern. Therefore, a search for the genetic mechanisms that affect body weight is of high scientific interest. This work presents for the first time the results of genome-wide association studies in a population of sheep (*Ovis aries*) from a (Romanov × Katahdin) × Romanov backcross family, whose body weights were recorded at multiple ages and whose SNP profiles were obtained using a high-density DNA chip. We identified 38 SNPs significantly associated with body weight ($p < 0.00001$) and functional candidate genes that affect skeletal muscle growth, bone scaffold formation, and lipid and carbohydrate metabolism. In addition, age-related changes in the set of significantly significant SNPs were observed. Our aim was to search for genomic variants that affect the body weight of (Romanov × Katahdin) × Romanov backcrosses from the resource population at different age periods. The study was performed on 95 sheep from the (Romanov × Katahdin) × Romanov backcross family in 2018-2021 at the Ernst Federal Research Center for Animal Husbandry. Ear notch samples were taken from the backcrosses for genomic DNA extraction using DNA-Extran-2 kits (Syntol LLC, Russia). Animals were genotyped using an Ovine Infinium® HD SNP BeadChip (Illumina, Inc., USA) containing ~ 600 thousand SNP markers. Body weights were recorded at the ages of 6 (BW6), 42 (BW42), 90 (BW90), 180 (BW180) and 270 days (BW270). To study genome-wide associations with body weight, we used regression analysis implemented in PLINK 1.90 (--assoc --adjust --qt-means). To confirm the significance of the identified SNPs and to identify significant regions in the genomes of the studied sheep, the significance threshold was set with Bonferroni correction at a value of $p < 1.09 \times 10^{-7}$, 0.05/459,868. The search for candidate genes located near the identified SNPs was performed using the VEP (Ensembl Variant Effect Predictor) tool of Ensembl genome browser 103 (https://www.ensembl.org/index.html). After quality control, 459,868 SNPs were retained for use in genome-wide association studies (GWAS). The average body weights in the studied sample were 3.28±0.07, 8.03±0.21, 13.74±0.39, 20.19± .51, and 22.51±0.50 kg at the ages of 6, 42, 90, 180, and 270 days, respectively. We found that the set of SNPs associated with the integral indicator of the growth rate, that is, the animal body weight, was different in different aged sheep. Thus, out of 38 identified SNPs, 18 located on OAR2, OAR4, OAR9, and OAR15 were significantly associated with BW6 ($p < 0.00001$); 3 on OAR6 and OAR11 were associated with BW42 ($p < 0.00001$); 2 on OAR10

and OAR19 were associated with BW90 ($p < 0.00001$); 6 on OAR1 and OAR13 were associated with BW180 ($p < 0.00001$), and 6 on OAR1 were associated with BW270 ($p < 0.00001$). Blocks of 3-5 SNPs were found on OAR1, OAR2, OAR4, and OAR5. The significance levels for six SNPs, oar3_OAR4_87887519 ($p < 7.13\times10^{-8}$), oar3_OAR4_87889243 ($p < 1.51\times10^{-7}$), oar3_OAR9_89145258 ($p < 4.95\times10^{-7}$), oar3_OAR1_192662599 ($p < 4.79\times10^{-7}$), OAR1_208070059.1 ($p < 4.79\times10^{-7}$) and oar3_OAR13_31446454 ($p < 6.84\times10^{-7}$), exceeded the threshold for GWAS ($p < 1.09\times10^{-7}$). Along with known candidate genes associated with body weight in sheep, we found new candidate genes whose effects on this trait have not been previously reported. The functional annotation of the identified candidates showed that these genes likely affect skeletal muscle growth, bone frame formation, lipid, and carbohydrate metabolism. The obtained data will be useful for the development of markers and genomic selection programs in sheep breeding.

Keywords: domestic sheep, resource population, SNP markers, DNA chips, GWAS, body weight, candidate genes

The postnatal growth of an animal is the result of complex interactions among genetic factors, nutrient intake, and the endocrine systems [1]. Deepening knowledge of the patterns of growth and development of farm animals is of practical importance for increasing their productivity [2, 3]. Body weight is one of the most important economically useful traits characterized by complex inheritance; therefore, the search for genetic mechanisms that affect body weight formation is of great scientific interest [4, 5].

Based on the results of genome-wide association studies (GWAS) on meat sheep performed using the Illumina OvineSNP50 medium-density DNA chip (Illumina, Inc., USA), Zhang et al. [6] identified the *GRM1*, *MBD5*, *UBR2*, *RPL7*, and *SMC2* genes as potential candidates for involvement in the growth rate of lambs in the postweaning period. The search for genome-wide associations in 1743 sheep revealed OAR6_41936490 on the sixth chromosome (OAR6) as a reliably associated single nucleotide polymorphism (SNP) [7]. Three significant candidate genes (*LAP3*, *NCAPG*, and *LCORL*) associated with growth traits, body frame, body size, and body weight in sheep were located in the region adjacent to this SNP.

Matika et al. [8] performed a genome-wide association study on 600 Scottish black-headed lambs. Phenotypic traits, including bone density, muscle, and adipose tissue content, were evaluated using computed tomography, and genotyping was performed using an Illumina OvineSNP50 medium-density DNA chip. On OAR6, a genomic region was identified that was significantly associated with bone mass ($p < 5.55\times10^{-8}$) and was characterized by an effect on muscle fiber density and fat content.

In addition, QTLs responsible for the development of muscle, fat, and bone components of the lamb body were identified on OAR1, OAR3, and OAR24. Ghasemi et al. [9] searched for genomic associations with body weight at birth in 130 Lori-Bakhtiari sheep using 41323 SNPs and identified *RAB6B*, *TF/serotransferrin*, and *GIGYF2* as potential candidate genes in OAR1. Lu et al. [10] used resequencing of Chinese fine-wool sheep to search for associations with body weight at birth and weaning at the age of 3.5 months and at the age of 12 and 30 months.

In regions adjacent to 113 SNPs that reached the threshold level of significance for genome-wide associations ($p < 0.05$), the *AADACL3*, *VGF*, *NPC1*, and *SERPINA12* genes were identified and annotated; these genes affect skeletal muscle development and lipid metabolism. Four genes, including the MTPN gene, involved in the regulation of skeletal muscle growth, were proposed as potential candidates associated with the body weight of Baluchi sheep at the age of 8 months [11]. Cao et al. [12] recorded the body weight at birth, at weaning, at six months and at one year of age in two generations of two populations of Hu sheep. GWAS based on medium-density SNP profiles and verification of the

identified polymorphisms showed that two SNPs (OARX_76354330.1 and s64890.1) were significantly associated with body weight ($p < 0.05$). Using multivariate analysis, eight new loci, located near the *FAM3C* and *WNT16* genes, were identified as associated with sheep meat productivity [13].

It should be noted that QTL mapping studies can also yield false positive associations. A decrease in the rate of false positive results (false discovery rate, FDR) and an increase in the accuracy of QTL mapping can be achieved using specially created resource populations of farm animals (backcrosses and F_2) [14] obtained from crossing parental lines (or breeds) that have highly divergent phenotypes in terms of growth rate and body weight. In addition, in such a resource population, it is possible to create a reliable base of phenotypes by ensuring that measurements or other phenotypic traits are recorded by the same person, to eliminate the impact of human factors as much as possible.

This work presents for the first time the analysis of genome-wide associations in a resource population of sheep (*Ovis aries*) from (Romanov × Katahdin) × Romanov backcross family whose body weight was recorded at different ages and whose SNP profiles were obtained using a high-density DNA chip. As a result, 38 SNPs were identified that were significantly associated with body weight ($p < 0.00001$) and with functional candidate genes affecting skeletal muscle growth, bone frame, lipid and carbohydrate metabolism. In addition, age-related changes in the profile of significantly significant SNPs were observed.

Our aim was to search for genomic variants that affect the body weight at different ages in sheep from the (Romanov × Katahdin) × Romanov backcross resource population.

Materials and methods. The studies were carried out on 95 sheep of backcrosses from the resource (crossbred) population in 2018-2021. The sheep resource population was a part of the Bank of Genetic Material of Domestic Animals and Poultry biocollection (registered by the Ministry of Education and Science of the Russian Federation No. 498808), created and maintained at the Ernst Federal Research Center for Animal Husbandry.

The creation of a resource sheep population included several stages [15]. In the first stage, the Romanov ewes were crossed with two rams (founders) of the Katahdin US meat breed to obtain F1 crosses. In the second stage, to obtain (Romanov × Katahdin) × Romanov backcrosses, one F_1 ram from each founder was crossed with the Romanov ewes. Mating led to the production of 36 and 32 offspring from each ram, respectively. F_1 lambs from each founder were crossed with two rams of the Romanov breed. As a result, 16 and 10 offspring were obtained. Thus, all backcrosses carry a 25 % share of the genetic material of the Katahdin breed and a 75 % share of the genetic material of the Romanov breed.

Ear notches were collected from the backcrosses for genomic DNA extraction, which was carried out using DNA-Extran-2 kits (Syntol LLC, Russia). The isolated DNA passed quality control in terms of concentration (15 ng/μl and above) according to measurement on a Qubit 4.0 fluorometer (Invitrogen/Life Technologies, USA) and in terms of the ratio of OD_{260}/OD_{280} absorption from 1.8 and above according to analysis on a NanoDrop8000 spectrophotometer (Thermo Fisher Scientific, USA).

The studied animals were genotyped using an Ovine Infinium® HD SNP BeadChip (Illumina, Inc., USA) containing ~ 600 thousand SNP markers. Filtration of SNP markers was performed in PLINK v. 1.90 (16). SNPs with a minor allele frequency (MAF) below 3 % (MAF 0.03), deviating from Hardy-Weinberg equilibrium at $p < 10^{-6}$ (HWE 1e-6), being in linkage disequilibrium (indep-

pairwise 50 5 0.5) or located on the sex chromosomes were removed from the analysis.

Body weight was measured using platform scales MP 300 VEDA F-1 (50/100; 1400×700) Live weight 12 PM (Moscow Weight Plant MIDL, Russia) at the ages of 6 (BW6), 42 (BW42), 90 (BW90), 180 (BW180) and 270 days (BWZhM270). The measurement results were recorded in a database in Microsoft Excel 2017. The mean values (*M*), standard errors (±SEM), standard deviations (±σ) and coefficients of variation (*Cv*, %) were calculated.

To identify genomic associations with body weight, we used regression analysis implemented in PLINK 1.90 (--assoc --adjust --qt-means). To confirm the significance of the SNPs and identify significant regions in the genomes of the studied sheep, a test was performed to test null hypotheses according to Bonferroni at a threshold value of $p < 1.09 \times 10^{-7}$, 0.05/459,868. The data were visualized in the R qqman package [17] in the R software environment [18].

The search for candidate genes located in the region of the identified SNPs was performed using the VEP (Ensembl Variant Effect Predictor) tool [19] in Ensembl genome browser 103 (https://www.ensembl.org/index.html, accessed 11.02.2021) with reference to the sheep (*Ovis aries*) genome assembly Oar_v3.1. Gene Ontology analysis was performed using the DAVID Functional Annotation Bioinformatics microarray analysis tool [20]. The search for probable matches with known QTLs was carried out using the Sheep Quantitative Trait Locus Database (Sheep QTLdb) (https://www.animalgenome.org/cgi-bin/QTLdb/OA/index, date 11.02.2021) [21].

Results. Average indicators of body weight in the studied sample of sheep are presented in Table 1. For this trait, rather high variation was revealed in sheep at all analyzed ages (*Cv* = 21.69-27.86 %).

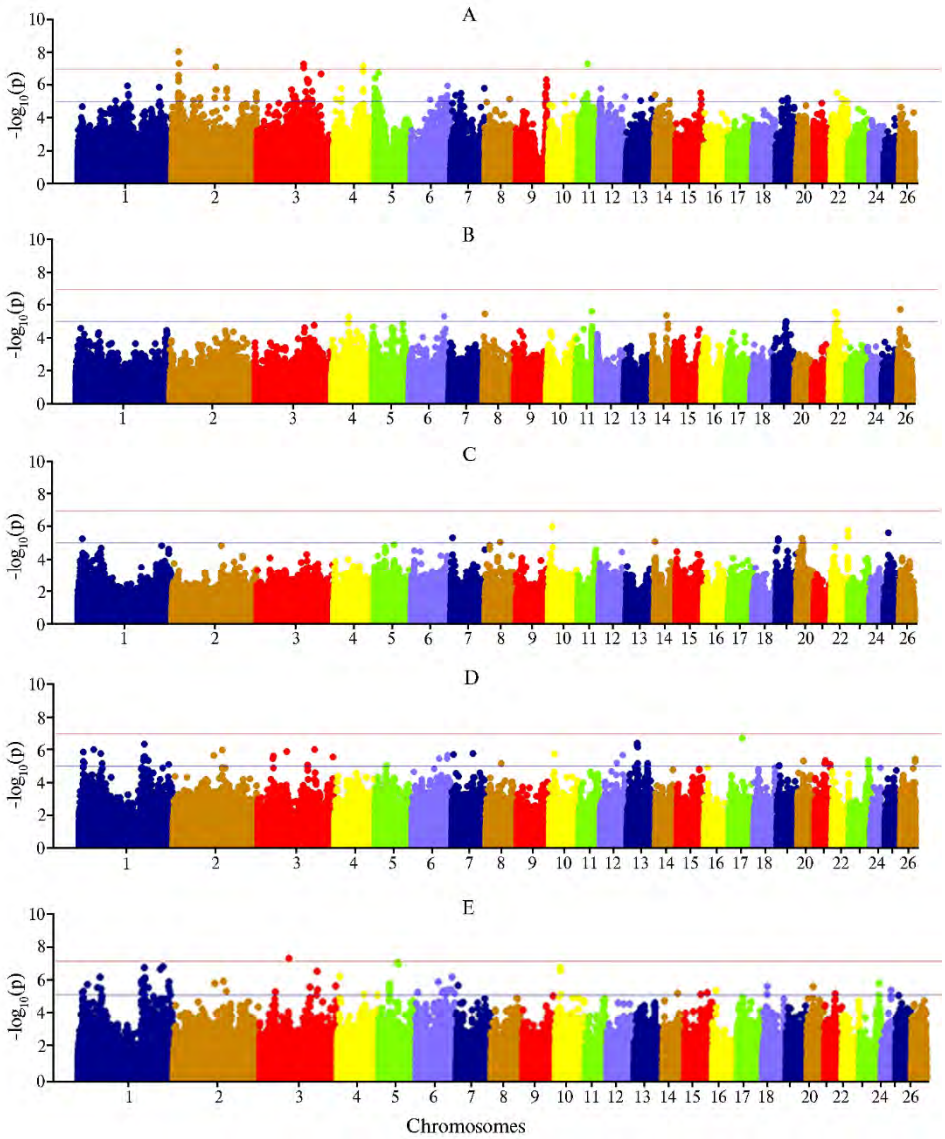
1. Body weight (kg) **and the coefficient of its variability in different age periods in sheep (*Ovis aries*) from a (Romanov × Katahdin) × Romanov family from the resource population** (*n* = 95, Ernst Federal Research Center for Animal Husbandry, Moscow Province, 2019-2021)

Age	*M*±SEM	Min-max	*Cv*, %
6 days	3.28±0.07	1.60-4.83	21.69
42 days	8.03±0.21	3.50-13.00	25.98
90 days	13.74±0.39	3.50-22.10	27.86
180 days	20.19±0.51	9.93-36.80	24.53
270 days	22.51±0.50	12.37-37.80	21.78

Based on this GWAS, it was found that the set of SNPs associated with the integral indicator of the growth rate, that is, the body weight, was not the same in all age periods (Fig., Table 2).

Thus, out of 38 identified SNPs, 18 located on OAR2, OAR4, OAR9, and OAR15 were significantly associated with BW6 ($p < 0.00001$), 3 on OAR6 and OAR11 were associated with BW42 ($p < 0.00001$), 2 on OAR10 and OAR19 were associated with BW90 ($p < 0.00001$), 6 on OAR1 and OAR13 were associated with BW180 ($p < 0.00001$), and 6 on OAR1 were associated with BW270 ($p < 0.00001$).

In addition, significant associations with both BW180 and BW270 were found for 5 SNPs located on OAR1 and OAR3. Blocks of 3-5 relevant SNPs were found on OAR1, OAR2, OAR4 and OAR5. Ultimately, six SNPs were identified as having an association with significance exceeding the threshold value for GWAS ($p < 1.09 \times 10^{-7}$): oar3_OAR4_87887519, oar3_OAR4_87889243, oar3_OAR9_89145258, oar3_OAR1_192662599, oar3_OAR13_31446454, and OAR1_208070059.1.



The results of GWAS for body weight in sheep (*Ovis aries*) from a (Romanov × Katahdin) × Romanov family from the resource population at different ages: A — 6 days, B — 42 days, C — 90 days, D — 180 days, E — 270 days. The upper horizontal line is the significance threshold for genome-wide associations, $-\log_{10}(p) = 1.09\times10^{-7}$; the lower horizontal line is the significance threshold for suggestive associations, $-\log_{10}(p) = 1.02\times10^{-5}$ (n = 95, Ernst Federal Research Center for Animal Husbandry, Moscow Province, 2019-2021).

At the age of 6 days, significant associations of body weight with SNPs located within the *MBD5* gene and in the immediate proximity of the *ORC4* and *ACVR2A* genes on OAR2 were revealed, and these genes were proposed as candidates with functions in postnatal growth in sheep [6]. The *MBD5* gene is involved in the regulation of many endocrine functions, including glucose homeostasis [22]. A study of *MBD5* knockout mice found severe growth retardation, reduced body size, hypoglycemia, and decreased body fat content [22]. Deletions in the human ortholog of *MBD5* cause developmental delay [23] and various malformations of the skeleton [24]. Thus, these studies indicate a significant role of the *MBD5* gene in the regulation of early postnatal growth in mammals. The *ORC4* and *ACVR2A* genes are also involved in growth processes. Mutations in the *ORC4* gene cause

delayed growth and primary osteodysplastic dwarfism [25, 26]. The *ACVR2A* gene regulates bone development [27].

2. Significant ($p < 0.00001$) **SNPs associated with body weight in sheep (*Ovis aries*) from a (Romanov × Katahdin) × Romanov backcross family from the resource population** ($n = 95$, Ernst Federal Research Center for Animal Husbandry, Moscow Province, 2019-2021)

Trait	OAR	Number of SNP	SNP	p	Position	Gene
BW6	2	1	oar3_OAR2_19100665	5.41×10^{-6}	19100665	*SMC2*
BW6	2	1	oar3_OAR2_30932797	5.52×10^{-6}	30932797	*PTCH1*
BW6	2	1	oar3_OAR2_63406327	8.59×10^{-6}	63406327	*ALDH1A1*
BW6	2	1	oar3_OAR2_126584456	6.05×10^{-6}	126584456	*PPP1R1C*, PDE1A*
BW6	2	5	oar3_OAR2_160277567	6.81×10^{-6}	160277567	*MBD5*, ORC4,*
			oar3_OAR2_160295110	1.75×10^{-6}	160295110	*ACVR2A*
			oar3_OAR2_160299881	2.50×10^{-6}	160299881	
			oar3_OAR2_160313744	1.75×10^{-6}	160313744	
			oar3_OAR2_160316296	1.75×10^{-6}	160316296	
BW6	4	1	oar3_OAR4_7542218	7.57×10^{-6}	7542218	*ABCA13*
BW6	4	2	OAR4_24289280.1	1.63×10^{-6}	23180261	*DGKB**
			oar3_OAR4_23286923	7.32×10^{-6}	23286923	
BW6	4	3	oar3_OAR4_87887519	7.13×10^{-8}	87887519	*ASB15**
			oar3_OAR4_87889243	1.51×10^{-7}	87889243	
			oar3_OAR4_87932432	6.71×10^{-5}	87932432	
BW6	9	1	s19680.1	4.46×10^{-6}	86619418	*RIPK2, MMP16*
BW6	9	1	oar3_OAR9_89145258	4.95×10^{-7}	89145258	*ATP6V0D2**
BW6	15	1	oar3_OAR15_74862644	7.80×10^{-6}	74862644	*ARHGAP1, F2*
BW42	6	1	oar3_OAR6_103115026	4.87×10^{-6}	103115026	*EVC*, EVC2*
BW42	11	2	s58053.1	2.49×10^{-6}	47604624	*SCN4A*, ICAM2*
			oar3_OAR11_47604879	2.49×10^{-6}	47604879	
BW90	10	1	oar3_OAR10_12704497	1.08×10^{-6}	12704497	*DGKH*, AKAP11*
BW90	19	1	oar3_OAR19_7100087	5.95×10^{-6}	7100087	*CCR4*
BW180	1	1	oar3_OAR1_43484960	1.03×10^{-6}	43484960	*WLS*
BW180	1	2	oar3_OAR1_192662599	4.79×10^{-7}	192662599	*MB21D2*
			OAR1_208070059.1	4.79×10^{-7}	192689940	
BW180, BW270	1	4	oar3_OAR1_204348376	$4.68\times10^{-6}/3.85\times10^{-6}$	204348376	*FXR1**
			OAR1_220691763.1	$4.68\times10^{-6}/3.85\times10^{-6}$	204351915	
			oar3_OAR1_204368368	$4.68\times10^{-6}/3.85\times10^{-6}$	204368368	
BW180, BW270	1	1	oar3_OAR1_14039930	$6.91\times10^{-6}/5.38\times10^{-6}$	14039930	*HEYL*
BW180, BW270	3	1	oar3_OAR3_220260675	$2.86\times10^{-6}/3.12\times10^{-6}$	220260675	*WNT7B*
BW180	13	1	oar3_OAR13_31446454	6.84×10^{-7}	31446454	*MRC1**
BW180	13	1	oar3_OAR13_61039017	7.75×10^{-6}	61039017	*PLAGL2*
BW180	13	1	oar3_OAR13_61305745	7.17×10^{-6}	61305745	*DNMT3B*
BW270	1	5	oar3_OAR1_185310940	7.76×10^{-6}	185310940	*PARP14**
			oar3_OAR1_185317664	2.52×10^{-6}	185317664	
BW270	1	1	oar3_OAR1_185977490	6.28×10^{-6}	185977490	*ADCY5**
BW270	1	1	oar3_OAR1_186482253	2.52×10^{-6}	186482253	*MYLK**

N o t e. Genes within which the identified SNPs are located are marked with an asterisk; the distance from SNPs located in the intergenic space to the nearest genes was ±400 kb. BW6, BW42, BW90, BW180 and BW270 — body weight at the age of 6, 42, 90, 180 and 270 days, respectively. OAR is a chromosome. The values for 180 days and 270 days are indicated through a slash.

We identified other sheep candidate genes with known functions. Genes *SMC2* on OAR2 and *RIPK2* on OAR9 have been proposed as potential candidates associated with body weight and meat qualities [6[. The PARP14 gene affects the composition of adipose tissue [28]. The *CCR4* gene is part of a complex that regulates energy metabolism and fatty acid metabolism in the skeletal muscles of meat sheep [29].

In addition, we identified candidate genes for growth and development traits whose functions are well described in cattle: *PTCH1* [30] and *DGKH* [31], responsible for body size and growth; *PPP1R1C*, *PDE1A* [32], *PLAGL2* [33], and *DNMT3B* [34], associated with muscle tissue formation; *DGKB* [35], involved in the regulation of insulin secretion; and *ALDH1A1* [36] and *MRC1* [37], involved in lipid metabolism. Mutations in the *ABCA13* gene cause serious disturbances in

osteogenesis in cattle, which supports its importance for bone formation [38]. The *MB21D2* gene affects the mass of internal organs, including the kidneys, in meat-type Simmental cattle [39).

In addition, several genes were found that are responsible for traits directly or indirectly related to body weight in mammals. The *MMP16* [40] and *EVC* [41) genes regulate chondrogenesis, while the *AKAP11*, *ATP6V0D2*, and *WNT7B* genes are associated with osteogenesis in young, actively growing mammals [42-44]. The *HACD2*, *ADCY5*, and *WLS* genes are involved in the regulation of lipid metabolism [45-47]. The *ASB15*, *FXR1*, *HEYL*, and *MYLK* genes regulate skeletal muscle growth [48-51].

Based on the GWAS results, we found that different SNPs were associated with body weight at different ages in the studied sheep population. In their study, Cao et al. [12] also found that the expression of the *CAPN6* gene in the biceps femoris and longus dorsi differs significantly between sheep aged 60 days and sheep aged 6 months. More than half of the associations identified in our study were found in the earliest period of ontogenesis. This is probably due to the biological characteristics of active growth in the last phase of pregnancy and early postnatal life of ruminants, including sheep. For example, the third wave of active myogenesis occurs in the late embryonic or early postnatal period (52), and the number of preadipocytes in brown adipose tissue increases before and after birth in lambs, a phenomenon that is especially important for neonatal survival [53].

Some SNPs identified by the GWAS overlap with QTL regions that were previously discovered by other researchers. Six SNPs (160.2-160.3 and 191.0 Mb) on OAR2 and one SNP (86.6 Mb) on OAR9 were located close to the genomic regions associated with the average daily gain in sheep [6]. The SNP (oar3_OAR15_74862644) on OAR15 was located 400 kb from the QTL, which affects body weight (74.4-74.4 Mb) [7]. In addition, some SNPs overlapped with the QTL regions that were mapped based on microsatellite markers. For example, SNPs OAR4_24289280.1 and oar3_OAR4_23286923 were located within the QTL potentially associated with body weight in sheep on OAR4 [54], and oar3_OAR1_204348376 on OAR1 was associated with BM180 and BM2L70, which is located within the QTL regulating the depth of the muscles above the third lumbar vertebra [55].

Most SNPs on OAR1 (except for oar3_OAR1_14039930) were located within or near QTLs associated with both body weight and muscle mass, lean meat yield, carcass fat content, and carcass bone mass. Interestingly, these QTLs were mapped using 189 microsatellite markers in a population of (Awassi × Merino) × Merino backcrosses descending from a single founder [56]. Because we performed a GWAS using high-density DNA chips in a population of backcrosses originating from two rams (a large variability in body weight is provided), the results of our work are likely to confirm the data of Cavanagh et al. [56], and the corresponding SNPs can potentially be proposed as functional candidates for body weight in sheep.

It should be noted that the identified difference in the SNP sets associated with body weight at different ages is an experimental fact that requires a fundamental study of this pattern. Thus, the question arises whether it is possible to predict the body weight and, therefore, the meat productivity of sheep at an early age by analyzing the genotypes of candidate genes associated with these indicators at later ages. A positive solution to this issue can lead to an increase in the efficiency of the sheep-breeding industry; it will be possible to breed fewer animals with increased meat productivity, and small and slow-growing sheep will be eliminated earlier, thereby reducing the cost of their maintenance and feeding. In

addition, theoretically, it is possible to select sheep embryos with the desired genotypes in the genes responsible for enhanced early postnatal growth (*MBD5*, *ORC4*, *ACVR2A*, *RIPK2* and *SMC2*), followed by cloning and transplantation into recipient ewes. In addition, as the body weight of an animal consists of the sum of the masses of skeletal muscles, bones with cartilage tissue, adipose tissue, and the mass of internal organs, directed introduction or knockout of genes responsible for the formation of one or another component of body weight by genome editing could be used to increase the orientation of sheep production in accordance with consumer demand (for example, leaner meat or with fatty inclusions).

Thus, GWAS for body weight in the (Romanov × Katahdin) × Romanov backcross family allowed the identification of 38 SNPs significantly associated with body weight ($p < .00001$) on OAR1, OAR2, OAR3, OAR4, OAR6, OAR9, OAR10, OAR11, OAR13, OAR15 and OAR19. It was shown that the set of SNPs associated with the integral indicator of the growth rate (body weight) was not the same for all ages. In some ways, this result was expected, since the observed tendency is consistent with the active early postnatal growth in sheep and is probably explained by the incomplete cessation of the action of embryonic growth factors shortly after birth. In our study, we identified SNP markers and genes which can be divided into three groups. The first group included functional candidate genes involved in the regulation of skeletal muscle growth, bone frame formation, and lipid, and carbohydrate metabolism. This group of genes, after validation in a large sample of sheep and identification of the desired genotypes, can be recommended for inclusion in breeding processes soon. The second group consists of SNP markers and genes within the known QTLs associated with body weight in sheep. These genomic regions should be subjected to finer mapping by sequencing to more fully understand the nature of the interactions of the associated genes, the totality of which can be proposed for the development of low-density DNA chips for analyzing the genetic predisposition to more active growth and increased body weight. The third group of identified genes includes those for which an effect on body weight in sheep had not been previously reported. Studying additional SNPs within and around these genes will provide a better understanding of the causal mutations affecting body weight in sheep. Thus, the results of our work create a scientific basis for the selection of candidate genes and SNP markers for use as markers and inclusion in genomic selection programs in sheep breeding.

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SOCIALIZATION AND GENETIC VARIABILITY AS A DRIVER OF DOMESTICATION (BY THE EXAMPLE OF DOG BREEDS)

V.I. GLAZKO[1, 2], G.Yu. KOSOVSKY[2], T.V. BLOKHINA[1], A.A. ZHIRKOVA[1], T.T. GLAZKO[1, 2] ✉

[1]*Timiryazev Russian State Agrarian University—Moscow Agrarian Academy,* 49, ul. Timiryazevskaya, Moscow, 127550 Russia, e-mail vigvalery@gmail.com, aida-cat@yandex.ru, an.zhirkowa@yandex.ru, tglazko@rambler.ru (✉ corresponding author);
[2]*Afanas'ev Research Institute of Fur-Bearing Animal Breeding and Rabbit Breeding*, 6, ul. Trudovaya, pos. Rodniki, Ramenskii Region, Moscow Province, 140143 Russia, e-mail gkosovsky@mail.ru
ORCID:
Glazko V.I. orcid.org/0000-0002-8566-8717
Kosovskii G.Yu. orcid.org/0000-0003-3808-3086
Blokhina T.V. orcid.org/0000-0002-1126-2781
Zhirkova A.A. orcid.org/0000-0001-5985-9395
Glazko T.T. orcid.org/0000-0002-3879-6935
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Abstract

Social activity is the basis of interaction between different species in the process of common niche forming, including animal domestication. The increased social activity is universal characteristic of the "domestication syndrome" for different species (M.A. Zeder, 2017). It is assumed that some elements of this increase are due to a certain neotenization of a number of brain metabolic pathways (M. Somel et al., 2009). This is in good agreement with the data on the association of "domestication syndrome" with the slowing of neural crest cell proliferation (M.A. Zeder, 2015). The syndrome of hypersocialization (Williams-Behren Syndrome — WBS) in humans has been described, associated with hemideletion/hemiduplication of the 7q11.23 region, which includes 25-28 genes whose products are critical for the activity of various aspects of the central nervous system (A. Antonell et al., 2010). It was found that the complex of such genes is located on chromosome 6 in canids, and the domestic dog, considered in recent years as the main model object for studying the genetic mechanisms of domestication (E.A. Ostrander et al., 2019), differs from wolves in the presence of transposon insertions, increased methylation, and reduced gene expression in this region (B.M. von Holdt et al., 2017, 2018; D. Tandon et al., 2019). The aim of this work was to analyze such insertions in the region of the key gene for increased social activity in dogs *WBSCR17* (Cfa6.6 and Cfa6.7) in representatives of different breeds and interspecific hybrids with jackals, as well as finding out the presence of mobile genetic elements in these areas. The detected sequences have high homology to the non-autonomous dispersed nuclear element SINEC2A1_CF (94 % homology) and to two regions of endogenous retrovirus 3 the sequences of which are described in humans and cattle (approximately 80 % homology). Data were obtained on the increased variability of the presence and number of insertions into these areas in dogs of different breeds and hybrids, on the presence of homology sites to endogenous human and bovine retroviruses, as well as a short dispersed nuclear element, species-specific for domestic dogs, SINEC2A1_CF, carrying the hexanucleotide AATAAA which contributes to the completion of transcription. These finding suggest the involvement of retroviruses in the formation of an aggregate niche in the domestication process, which leads to increased variability that contributes to the selection of animals with hypersocialization.

Keywords: domestication, hypersocialization, Williams-Behren syndrome, retrotransposons, dog breeds and hybrids, aggregate niche

Domestication is mainly considered in relation to humans, artificial selection, and as the isolation of an object from the wild with complete control of its life cycle by humans. Also, domestication is an example of mutualism which appears not only between humans and domestic species of plants and animals. This type of relationship is evolutionarily much older and very widespread in nature,

especially among socialized species, for example, among leaf-cutting ants. Another striking example is the mutualism of fungi and plants, algae, bacteria. Mutualistic relationships are developing based on coevolutionary selection for mutant genotypes and lead to behavioral, physiological, morphological changes in both partners during the formation of an aggregate ecological niche [1, 2].

Predisposition to socialization, including reduced aggressiveness, social tolerance, and research tendencies, is a core driver for animal domestication. There is evidence that the socio-cultural factors of human-created niches make a relatively greater contribution to the intraspecific differentiation of cultivated plants and domesticated animals than environmental factors [3]. Interestingly, the supposed ancestral human species bonobos (*Pan paniscus*) significantly differ from chimpanzees in tolerance and altruistic behavior, namely in sharing food with representatives of other species [4].

Since environmental conditions are changing and biodiversity of domesticated animals are narrowing due to various reasons, the elucidation of the mechanisms of domestication becomes important for the management of genetic resources of agricultural species [5].

The oldest domesticated species is the dog (*Canis lupus familiaris*) whose domestication is associated with the hunter-gatherer civilization that preceded the agrarian civilization. It is generally accepted that the longest time of coevolution of humans and dogs in a common niche will allow the identification of key genomic targets of domestication. Therefore, in recent years, the domestic dog has become the leading research model for identifying genes and gene complexes associated with domestication [6, 7].

In humans, Williams-Beuren syndrome (WBS, hypersociability) has been described, which is caused by hemideletion or hemiduplication of 28 genes in the 7q11.23 region [8]. WBS is an autosomal dominant disorder caused by genomic rearrangements due to large region-specific changes and the presence of Alu transposons (short non-autonomous dispersed nuclear elements), which can lead to non-allelic homologous recombination in meiosis [9-11]. The incidence of WBS in the population is approximately $^{1}/_{10000}$ for WBS hemizygosity and $^{1}/20000$ for WBS hemoduplications. Deletion or duplication in WBSCR (Williams-Beuren syndrome critical region) leads to hemizygosity or hemoduplication of 25-28 genes, which explains the seen phenotypic manifestations [12]. Among others, the WBSCR region contains genes encoding transcription regulation factors, for example, GTF2I, GTF2IRD1, BAZ1B, and MLXIPL, as well as signaling molecules FZD9, TBL2, and LIMK1 [13]. It is assumed that the dosage of GTF2I can change the balance of excitation and inhibition [14], which is consistent with numerous evidences indicating an imbalance in the ratio between excitation and inhibition in cortical neurons as the main substrate for the development of the communication network [15, 16].

A block of genes corresponding to the human 7q11.23 region is found on the chromosome 6 of the domestic dog (CFA6). Comparative studies on the mechanisms that determine the increased tendency of the dog to initiate social contacts in comparison with socialized gray wolves have explained this behavior as a type of behavioral neoteny, the preservation of juvenile traits [17], which in itself is potentially the result of transcriptional neoteny in the brain [18]. It was found that structural variants of the WBS genes, particularly for *GTF2I* and its paralogs, underlie stereotyped hypersociability in domestic dogs and foxes [17, 19].

It is known that *WBSCR17* transcripts are predominantly expressed in the cerebellum, hippocampus, thalamus, and cerebral cortex of rats [20], and studies confirm the effect of WBSCR17 on cell morphology and traffic across

cell membranes [21]. WBSCR17 (in humans, GALNT17, N-acetylgalactosaminyltransferase) is highly expressed in the cerebral cortex, participates in the function of lysosomes, cell adhesion, and the formation of the extracellular matrix [22].

Identification of the genetic basis of increased predisposition to social activity and learning ability in dogs is of particular importance in the selection of assistance dogs. For example, when training guide dogs for the blind, more than 60 % of individuals are rejected, mainly due to behavioral problems [23]. It was found that four insertions of retrotransposons (transposable elements, TEs) in genes *WBSCR17* (Cfa6.6 and Cfa6.7), *GTF2I* (Cfa6.66), and *POM121* (Cfa6.83) are associated with variability in the Canine Behavioral Assessment and Research Questionnaire test metrics (C-BARQ, http://www.cbarq.org). One of such insertions in *WBSCR17* turned out to be most closely associated with an increased predisposition to communicate with humans. The identified TEs with localization in introns of the *WBSCR17* and *GTF2I* genes and in the intergenic space are associated with increased methylation of the complex of genes of this chromosomal region, with their decreased expression and variability in the number of TE copies [24, 25].

To date, data have been accumulated indicating significant differences in the distribution of TEs in the domestic dog (*Canis lupus familiaris*), the gray wolf (*Canis lupus*), and the red wolf (*Cuon alpinus*), which are associated with domestication [26]. Thus, TE accounts for 41.75 % of the nucleotide sequences of the dog genome, which is higher than in the gray wolf (39.26 %) and in the red wolf (38.51 %). The most distinct TE components in these genomes are the long interspersed nuclear element LINE1 (L1) and microsatellites both making up 86.1 % of the differentiation between dog and gray wolf and 83.2 % of the differentiation between dog and red wolf. The content of canine-specific L1 Canis1 and L1 Cf in the dog's genome is significantly higher, particularly, almost twice as high as in gray and red wolves. It is assumed that the subfamilies L1 Canis1 and L1 Cf could have been accumulated in the dog's genome during domestication. In the dog genome, the canine-specific short interspersed element nuclear element SINEC Cf occurs in 27.3 million copies which is 1.16 times as much as in the genome of gray wolf and 1.23 times as much as in the genome of red wolf. It can be expected that the copy number of TEs also contributes to the differences in genome methylation profiles in dogs and wolves, as well as to the interbreed characteristics of dogs [27].

In several dog breeds, insertions of the retrotransposon were previously identified, without studies of its origin and homology with other transposons, in the 5 Mb region of chromosome 6 (in humans, deletions in this region are related to WBS syndrome manifestation), generating length variants of genes associated with behavioral responses [17]. However, it should be noted that the genetic basis of behavioral characteristics and molecular mechanisms of structural changes in genes associated with domesticated are poorly studied and require deeper insight.

In this work, as in the studies of von Holdt et al. [17], we used the primers [17] flanking the intron sequences of *WBSCR17*, the key gene determining the increased social activity of dogs and found variability in the presence and number of insertions in the Cfa6.6 and Cfa6.7 regions in representatives of different dog breeds and hybrids with a jackal. The obtained PCR products corresponded to the expected size of fragments in the presence or absence of insertions (555 or 357 bp for Cfa6.6 and 504 or 269 bp for Cfa6.7). Using bioinformatics methods, we isolated the corresponding region in a reference genome of Labrador Retriever and assessed the homology to retrotransposons in this region.

This work aimed to detect insertions in *WBSCR17* locus in dogs of various groups, i.e., the Vietnamese aboriginal breeds and specialized hunting greyhounds and the offspring of an interspecific hybrid of a dog and a jackal (Sulimov dogs —

shalaika breed group), using two pairs of primers to the regions of Cfa6.6 and Cfa6.7, and to analyze nucleotide sequences of the Cfa6.6 and Cfa6.7 regions of the *WBSCR17* locus in the Labrador Retriever reference genome for the presence of retrotransposons of various origins.

Materials and methods. Genetic material from 9 dogs of different breeds of greyhounds, 9 dogs of Vietnamese breeds, and 5 shalaikas (blood and tissue samples) were provided by the Department of Zoology, Timiryazev Russian State Agrarian University—Moscow Agricultural Academy. DNA extraction was performed using the Extran-1 and Extran-2 kits (Syntol LLC, Russia), following the manufacturer's instructions.

The employed primers were forward 5′-CCCCTTCAGCCAGCATATAA-3′, reverse 3′-TTCTCTGGGCTGTCTGGACT-5′ for Cfa6.6 and forward 5′-TGGAGCCATGATTA-GGAAGG-3′, reverse 5′-TAAGGAAGGACCCCATTTCC-3′ for Cfa6.7 [17, 23]. In polymerase chain reaction (PCR), a PCR mixture (Syntol LLC, Russia) was used according to the manufacturer's recommendations. The PCR cycling parameters were i) initial denaturation at 94 °C for 2 min; ii) denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, elongation at 72 °C for 2 min (40 cycles); iii) final elongation at 72 °C for 10 min (a Tertsik amplifier, DNA-technology LLC, Russia). DNA amplification fragments were separated by horizontal electrophoresis in 1.5 % agarose gel stained with ethidium bromide. A DNA marker 100 bp + 1.5 Kb + 3 Kb (12 fragments from 100 to 3000 bp) (NPO SibEnzyme, Russia) was used to determine the size of the amplified fragments.

Using BLASTn software (BLAST: Basic Local Alignment Search Tool, https://blast.ncbi.nlm.nih.gov/Blast.cgi), nucleotide sequences of the *WBSCR17* gene fragments with insertions, a 555 bp fragment for Cfa6.6 and a 504 bp fragment for Cfa6.7, were extracted from a Labrador Retriever reference genome (ROS_Cfam_1.0 Genome Assembly, https://www.ncbi.nlm.nih.gov/assembly/GCF_014441545.1/ and UNSW_CanFamBas_1.0 Genome Assembly, https://www.ncbi.nlm.nih.gov/assembly/GCA_013276365.1) in searching for homology to flanking primers for Cfa6.6 and Cfa6.7 loci on chromosome 6 to determine in these loci the regions homologous to TEs of different origins. The search was performed using the Giri Repbase software (https://www.girinst.org/repbase/) and the CENSOR software (http://www.girinst.org/censor/index.php) [28].

Results. Electrophoretic separation of amplicons derived from the genomic DNA regions flanked by a pair of primers to Cfa6.6 detected fragments of 555-575 bp and 350-375 bp in the Vietnamese dogs and shalaikas (Fig. 1) along with longer fragments up to 850-875 bp in size which were more common in the greyhounds.

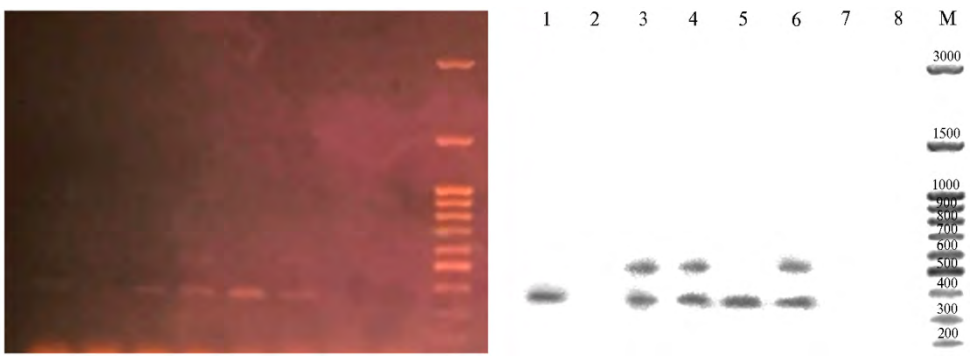

Fig. 1. Electrophoretic spectrum of PCR amplification products of genomic DNA regions flanked by primers to the Cfa6.6 locus in dogs: 1 — Vietnamese Phu Quoc, 2 — Vietnamese wolf-like dog, 3 — Vietnamese Hmong, 4-6 — shalaikas; M — molecular weight marker 100 bp + 1.5 Kb +3 Kb (NPO SibEnzyme, Russia). Fragments of 350-375 and 550-575 bp in length.

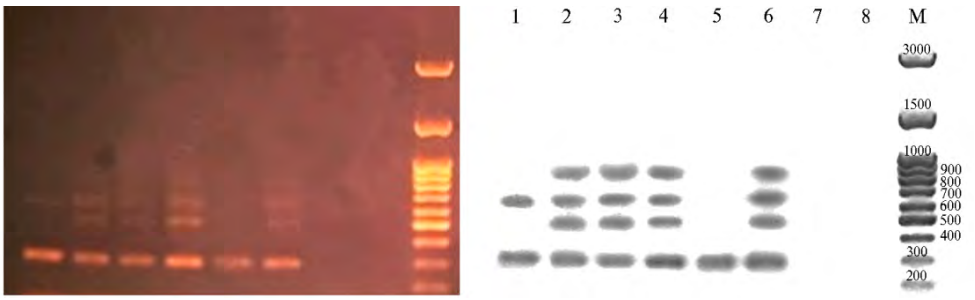

Рис. 2. Electrophoretic spectrum of PCR amplification products of genomic DNA regions flanked by primers to the Cfa6.7 locus in dogs: 1 — Vietnamese Phu Quoc, 2 — Vietnamese wolf-like dog, 3 — Vietnamese Hmong, 4-6 — shalaikas; M — molecular weight marker 100 bp + 1.5 Kb +3 Kb (NPO SibEnzyme, Russia). Fragments of 290-300, 500-510, 650-670, and 850-870 bp in length.

Amplification with a pair of primers to Cfa6.7 resulted in both fragments of the expected values (500-525 bp with insertions and 280-300 bp without insertions) and longer sequences which ranged from 600 to 800 bp in size (Fig. 2).

These data draw us to the conclusion that in the DNA of dogs with the domestication histories which extremely differ in the timing and the degree of influence of artificial selection, there is a pronounced polymorphism of the studied regions in the lengths of genomic fragments flanked by primers to the Cfa6.6 and Cfa6.7 loci. For Cfa6.6 and Cfa6.7, there are predicted regions with the insertions of retrotransposon fragments (555 and 504 bp) and without insertions (357 and 269 bp) and, in some cases, longer regions the presence and the size of which, as we assume, may indicate the appearance of unique insertions and duplications within the genome region under investigation. Our findings are consistent with the results of genotyping reported by other researchers for various dog breeds [23].

As noted above, TE insertions into the *WBSCR17* locus at the Cfa6.6 and Cfa6.7 regions of the domestic dog chromosome 6 of the significantly affect the increase in methylation and a decrease in the expression of a number of genes [23]. To find out which TEs are typical for this region, we compared the sequences of the 555 bp fragment amplified by primers to Cfa6.6, and the 504 bp fragment flanked by primers to Cfa6.7 in sequenced genome of a domestic dog (ROS_Cfam_1.0 Genome Assembly, https://www.ncbi.nlm.nih.gov/assembly/GCF_014441545.1/ and UNSW_CanFamBas_1.0 Genome Assembly, https://www.ncbi.nlm.nih.gov/assembly/GCA_013276365.1). As a result, the nucleotide sequences shown in Figures 3 and 4 were identified.

Then, using the Giri Repbase and the CENSOR software [28], a search was performed for homology to different transposons in Cfa6.6 and Cfa6.7 loci of chromosome 6 (see Fig. 3, 4). It turned out that Cfa6.6 from different sources (ROS_Cfam_1.0 and UNSW_CanFamBas_1.0) contains a sequence (nucleotides 183-370, Table 1) 94 % homologous to a 188 bp fragment of SINEC2A1_CF, a short non-autonomous interspersed nuclear element typically found in dogs.

1. **Analysis of the presence absence of dispersed repeats in the 555 bp fragment of Cfa6.6 region flanked by the primers** (these data were obtained with the use of the Giri Repbase, https://www.girinst.org/repbase/ and the CENSOR software http://www.girinst.org/censor/index.php)

/tmp/censor.57641.tmp/data.ori (SVG Plot; Alignments; Masked)

Name	From	To	Name	From	To	Class	Dir	Sim	Pos/Mm:Ts	Score
/tmp/censor.57641.tmp/data.ori	183	370	SINEC2A1_CF	1	192	NonLTR/SINE/SINE2	d	0.9841	2.0000	1512

N o t e. Figures 3 and 5 show the primer sequences.

5′-(forward)**CCCCTTCAGCCAGCATATAA**ATTAAGGGAACTACACACTTAGTTTTGACCCAGATTTCTTTTTTAGGATGGCAGAAGCACACGCACGCACACACACACACACACACACACACACACAA-
AGTTTCTCTGATGGAAAACACAGAGACCTTCCAGATGAAATGCAGATATAAAAATTCTTTCTGGGATCCCTGGGTGGCGCAGCGGTTTGGCGCCTGCCTTTGGCCCAGGGCGCGATCCTGGAGAC-
CCGGGATCGAGTCCCACGTCGGGCTCCCGGTGCATGGAGCCTGCTTCTCCCTCTGCCTGTGTCTCTGCCTCTCTCTCTCTCTCTCTGTGACTATCATAAATAAATAAAAAATTAAAAAAAAAAAATT-
CTTTCTACTGAAATTTCCAGCCACCCCACCTTCAGACACCAAGACTAAAATGGCTTCTTGGCTAATGTGAGGTACCCACCTTCCTGAGTTATGTAAGCTAGAGTACGGGTAATCGTGAGGTTCTTCC-
TTCTTAGTGTTCTGTGTTGGCATCGGTTGTCTACC**ACTAGTCCAGACAGCCCAGAGAA**(reverse)-3′

Fig. 3. CFA6.6 loci. The sequence identified in the whole genome reference sequence *Canis lupus familiaris* isolate:SID07034| breed: Labrador retriever (chromosome 6; whole genome sequence databases ROS_Cfam_1.0 https://www.ncbi.nlm.nih.gov/assembly/GCF_014441545.1/ and ROS_Cfam_1.0 https://www.ncbi.nlm.nih.gov/assembly/GCF_014441545.1/) **flanked by primers to the CFA6.6 region.** The regions of homology to primers are highlighted in yellow.

5′-(forward)**TGGAGCCATGATTAGGAAGG**AGTCCTTGGAAACTCTGGCTTGTACCCTACATCTGAATTGTGGGGACAGTCTTGTGGGCTAACCCTTAACCTGTGGCATCTGATGCTATCCCTCAGGG-
AGGTAATATCAGGATGGAGTTGAATTACAGGACCTCCAAGCTGGACACACAGAGAATCACTTGGTGTGGGAAACAACATTTGGTGGCCAGGAGCATCAGAAATGGGGTGTTCTCGGGATCCCTGG-
GTGGCGCAGCGGTTTAGCGCCTGCCTTTGGCCCAGGGCGCGATCCTGGAGATCCGGGATCGAATCCCACGTCGGGCTCCCGGTGCATGGAGCCTGCTTCTCCCTCTGCCTGTGTCTCTGCCT-
CTGTCTCTCTCTCTCTGTGACTATCATAAATAAATGAAAAAAAAATTTAAAAAAAAAAAAAAAAAAAAAAGAAATGGGGTGTTCTCTGTGAGTAATGAAGGAGACACATAGGAGAGAAATAGGA**AGGAA-
ATGGGGTCCTTCCTT**(reverse)-3′

Fig. 4. CFA6.7 loci. The sequence identified in the whole genome reference sequence *Canis lupus familiaris* isolate:SID07034| breed: Labrador retriever (chromosome 6; whole genome sequence databases ROS_Cfam_1.0 https://www.ncbi.nlm.nih.gov/assembly/GCF_014441545.1/ and ROS_Cfam_1.0 https://www.ncbi.nlm.nih.gov/assembly/GCF_014441545.1/) **flanked by primers to the CFA6.7 region.** The regions of homology to primers are highlighted in yellow.

A

B

5′-(forward)**CCCCTTCAGCCAGCATATAA**ATTAAGGGAACTACACACTTAGTTTTGACCCAGATTTCTTTTTTAGGATGGCAGAAGCACACGCACGCACACACACACACACACACACACACACAA-
AGTTTCTCTGATGGAAAACACAGAGACCTTCCAGATGAAATGCAGATATAAAAATTCTTTCTGGGATCCCTGGGTGGCGCAGCGGTTTGGCGCCTGCCTTTGGCCCAGGGCGCGATCCTGGAGAC-
CCGGGATCGAGTCCCACGTCGGGCTCCCGGTGCATGGAGCCTGCTTCTCCCTCTGCCTGTGTCTCTGCCTCTCTCTCTCTCTCTCTGTGACTATCATAAATAAATAAAAAATTAAAAAAAAAAAATT-
CTTTCTACTGAAATTTCCAGCCACCCCACCTTCAGACACCAAGACTAAAATGGCTTCTTGGCTAATGTGAGGTACCCACCTTCCTGAGTTATGTAAGCTAGAGTACGGGTAATCGTGAGGTTCTTCC-
TTCTTAGTGTTCTGTGTTGGCATCGGTTGTCTACC**ACTAGTCCAGACAGCCCAGAGAA**(reverse)-3′

Fig. 5. The SINEC2A1_CF insertion position (in red) (A) **and the nucleotide sequence of a 555 bp fragment of the *WBSCR17* locus flanked by the primers to CFA6.6** (highlighted in yellow) **with a region of 94 % homology to the non-autonomous retrotransposon SINEC2A1_CF** (in red) (B). The AATAAA hexanucleotide (transcription termination signal) is marked in blue.

A

B

5′-(forward)**TGGAGCCATGATTAGGAAGG**AGTCCTTGGAAACTCTGGCTTGTACCCTACATCTGAATTGTGGGGACAGTCTTGTGGGCTAACCCTTAACCTGTGGCATCTGATGCTATCCCTCAGGGAG-
GTAATATCAGGATGGAGTTGAATTACAGGACCTCCAAGCTGGACACACAGAGAATCACTTGGTGTGGGAAACAACATTTGGTGGCCAGGAGCATCAGAAATGGGGTGTTCTCGGGATCCCTGGGT-
GGCGCAGCGGTTTAGCGCCTGCCTTTGGCCCAGGGCGCGATCCTGGAGATCCGGGATCGAATCCCACGTCGGGCTCCCGGTGCATGGAGCCTGCTTCTCCCTCTGCCTGTGTCTCTGCCTCTGT-
CTCTCTCTCTCTGTGACTATCATAAATAAATGAAAAAAAAATTTAAAAAAAAAAAAAAAAAAAAAAGAAATGGGGTGTTCTCTGTGAGTAATGAAGGAGACACATAGGAGAGAAATGGA**AGGAAATGGGG-
TCCTTCCTT**(reverse)-3′

Fig. 6. Positions of regions of homology to the region of homology to the endogenous human retrovirus EVR3 (in lilac in the lower part of the figure) **and to the non-autonomous retrotransposon SINEC2A1_CF** (in blue in the lower part of the figure) (A) **and the nucleotide sequence of a 509 bp fragment of the *WBSCR17* locus flanked by the primers** (highlighted in yellow) **to CFA6.7 with regions of homology to the endogenous retrovirus EVR3** (nucleotides 62-231, 435-486, in green) **and to the non-autonomous retrotransposon SINEC2A1_CF** (nucleotides 233-429, in red) (B). The AATAAA hexanucleotide (transcription termination signal) is marked in blue.

Figure 5 shows the positioning of the SINEC2A1_CF transposon (Fig. 5, A) in the revealed Cfa6.6 fragment and its nucleotide sequence (B, in red).

2. Analysis of the presence or absence of dispersed repeats in the 509 bp fragment of Cfa6.7 region flanked by the primers (these data were obtained with the use of the Giri Repbase, https://www.girinst.org/repbase/ and the CENSOR software http://www.girinst.org/censor/index.php)

/tmp/censor.58600.tmp/data.ori (SVG Plot; Alignments; Masked)

Name	From	To	Name	From	To	Class	Dir	Sim	Pos/Mm:Ts	Score
/tmp/censor.58600.tmp/data.ori	62	231	MER21C_BT	741	920	ERV/ERV3	d	0.7771	1.3333	750
/tmp/censor.58600.tmp/data.ori	233	429	SINEC2A1_CF	1	196	NonLTR/SINE/SINE2	d	0.9742	1.5000	1497
/tmp/censor.58600.tmp/data.ori	435	486	MER21C_BT	905	958	ERV/ERV3	d	0.8302	1.1429	312

N o t e. Figures 4 and 6 show the primer sequences.

A 504 bp fragment of the *WBSCR17* locus flanked by primers to CFA6.7 contains sequences homologous to the endogenous retrovirus EVR3 (nucleotides 62-231 and 435-486) and to a 197 bp fragment of SINEC2A1_CF retrotransposon (94 % homology, nucleotides 233-429) (Table 2, Fig. 6).

It should be noted that both fragments homologous to SINEC2A1_CF contain hexanucleotides AATAAA corresponding to the polyadenylation signal which serves as a key regulator of the end of the transcript. This hexamer or a similar sequence is very often found within 30 bp from the 3′UTR-ends [29]. Retrotransposons of the SINE class are found in all genomes in high copy number. Built into genes, they can disrupt expression, alter splicing, or stop transcription. The genome of the domestic dog harbors hundreds of thousands of such insertions, which can significantly affect the transcription of nearby genes or the genes at introns of which such insertions are located [29]. Therefore, a decrease in the expression of genes carrying insertions of these transposons may be due not only to the induced change in the methylation pattern, as suggested by some researchers [23], but also to the presence of the AATAAA hexanucleotide.

The data of Repbase indicate that EVR3 has sequences of homology to the mobile human genetic element MER21C and to a long terminal repeat of one of the endogenous bovine retroviruses [30].

Therefore, the endogenous human retrovirus EVR3 is directly involved in the genomic instability of *WBSCR17*, and its introduction into this locus is associated with a change in the transcription of a group of genes. This may confirm not only the idea of a long-term and directed selection of dogs for interest in contact with humans from the earliest stages of domestication, but also indicate the involvement of retroviruses in their horizontal transfer in and between various species (in particular, among bovine livestock) of a single aggregate ecological niche.

Our findings draw to the following conclusion. A prerequisite for domestication is the formation of a specific niche created by man. The key factor is selection of a plant or an animal to be domesticated. For animals, the key traits are increased social activity and the ability to adapt to conditions modified by humans. To ensure the success of such selection over a relatively short period of domestication, it is necessary to have an increased genetic variability which makes it possible to select the desired phenotypes [31]. Mobile genetic elements may be among tools providing such variability.

The totality of data indicates that the genome of the domestic dog, considered as a model of domestication, differs from the genomes of closely related

wild canines in a certain excess of retrotransposons [26] which make a significant contribution to genetic and genomic variability. The formation of a new niche presupposes a wide exchange between its "inhabitants", including exogenous retroviruses, the direct or altered descendants of which are autonomous retrotransposons which, in turn, involve non-autonomous retrotransposons in this variability. The increased variability of genome regions with a high density of factors which regulate transcription of genes involved in higher nervous activity (in particular, in socialization) with a direct participation of retrotransposons (see Fig. 5, 6) indicates that the generalization of genetic resources through the transfer of genetic material of initial pathogens between species can be the source of interspecific genetic relationships and intraspecific variability during the formation of a common niche. This may also explain the homology to the endogenous human virus ERV3 that we revealed in dogs in the key genome segment the variability of which is associated with hypersociability.

Thus, our findings indicate that polymorphism in the length of the *WBSCR17* gene associated with increased sociability is characteristic of dogs of different origins, as it was also reported by other researchers, and of interspecific hybrids such as shalaikas (Sulimov dogs), a jackal-dog hybrid. The detected polymorphism is due to insertions of transposed sequences. Analysis of such insertions in a reference genome of the domestic dog indicates that they contain nucleotide sequences of a non-autonomous transposon which occurs in the genome of dogs with high frequency, as well as a region of homology to the retrotransposon first described in humans and having homology to the cattle retrotransposon. It can be expected that it was the virome community that aggregated the members of one niche at the genomic level to generate variability enough to select individuals with an increased predisposition to interspecific interactions as a basis for the development of an agrarian civilization.

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IDENTIFICATION OF ANTIBIOTIC RESISTANCE OF THE CATTLE PATHOGEN *Histophilus somni*

S.P. YATSENTYUK[1] ✉, Yu.I. POBOLELOVA[1], D.A. RUDNYAEV[1], A.I. LAISHEVCHEV[2], A.V. KAPUSTIN[2]

[1]*Russian State Center for Animal Feed and Drug Standardization and Quality*, 5, Zvenigorodskoe sh., Moscow, 123022 Russia, e-mail pcr-lab@vgnki.ru (✉ corresponding author), y.pobolelova@vgnki.ru, rudnyaev@vgnki.ru;
[2]*Federal Scientific Centre Skryabin and Kovalenko All-Russian Research Institute of Experimental Veterinary RAS*, 24/1, Ryazanskii Prosp., Moscow, 109428 Russia, e-mail kapustin_andrei@mail.ru, a-laishevtsev@bk.ru
ORCID:
Yatsentyuk S.P. orcid.org/0000-0002-4819-2131
Pobolelova Yu.I. orcid.org/0000-0002-8780-4671
Rudnyuaev D.A. orcid.org/0000-0002-1753-5920
Laishevchev A.I. orcid.org/0000-0002-5050-2274
Kapustin A.V. orcid.org/0000-0003-0136-2487
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Abstract

Histophilus somni is a Gram-negative bacterium of the *Pasteurellaceae* family which is a component of the Bovine Respiratory Disease Complex and the pathogen, causing a multisystem disease — histophilosis. For the treatment of diseases caused by *Pasteurellaceae* bacteria, aminoglycosides, sulfonamides, beta-lactams, tetracyclines and macrolides are most often used, therefore the formation of resistance of *H. somni* to antibiotics of these groups can be expected. This work is the first study of antibiotic resistance of *H. somni* isolated from cattle in the Russian Federation. This work aimed at exploration of the antibiotic resistance of circulating *H. somni* strains by phenotypic and genotypic methods and the evaluation of the PCR method applicability for the prediction *H. somni* resistance to antimicrobial agents. We studied 18 cultures of *H. somni*, the causative agent of histophilosis, isolated in 2018-2019 from biological material (parenchymal organs, washes, sperm) of 145 animals of different breed, age, sex and physiological groups using microbiological method. The cultures were studied using the disk diffusion method for sensitivity to 13 antibiotics of aminoglycosides, beta-lactams, tetracyclines and sulfonamides classes. All obtained isolates were tested by PCR for the presence of genetic determinants of antibiotic resistance, most often found in *H. somni*: *tetH* (resistance to tetracyclines), *bla*$_{OXA-2}$ (resistance to penicillins), *aadA25*, *strA*, *strB*, *aphA1* (resistance to aminoglycosides), *sul2* (sulfonamide resistance). Resistance to aminoglycoside group was most prevalent, i.e., resistance to streptomycin was 50 %, and resistance to neomycin exceeded 40 %. Genes *aadA25*, *strA*, *strB* and *aphA1* were found in the resistant samples. A total of 33 % isolates showed resistance to sulfonamides, all this samples were positive for the *sul2* gene in PCR. The sensitivity to penicillins was quite high (~ 75 %), the sensitivity to beta-lactams approached 100 %. The sensitivity to antimicrobials of the tetracycline group was higher than 80 %. However, neither tetracyclines (*tetH*) nor penicillins (*bla*$_{OXA-2}$) resistance genes were identified during the study. Two isolates were multidrug resistant with resistance to aminoglycosides, beta-lactams and tetracyclines. Also, four samples were resistant to antimicrobial agents of two different groups, i.e., two samples were resistant to aminoglycosides and sulfonamides with *strA*, *strB*, *aadA25*, *aphA1*, and *sul2* genes found, and two samples were resistant to aminoglycosides and beta-lactams with only aminoglycoside resistance genes *aadA25* and *strA* identified. With the exception of samples resistant to tetracyclines and beta-lactams, in which the expected genes were not detected, all observed phenotypes of antimicrobial resistance were consistent with the PCR test results. The combination of genotypic and phenotypic methods for determining antibiotic resistance is necessary for understanding of the resistance mechanisms and increases the efficiency of antibiotic resistance monitoring programs.

Keywords: *Histophilus somni*, PCR, antibiotic resistance, histophilosis, cattle

Histophilus somni (the family *Pasteurellaceae*) is a gram-negative bacterium that is often found in cattle and, as a rule, complicates the severity of respiratory viral diseases [1]. *H. somni* can cause a multisystem disease known as histophilosis. The infection of the upper respiratory tract often precedes damage to other organ

systems. *H. somni* is the most common causative agent of respiratory disease and pneumonia in calves aged 1-2 months and it can also cause lung lesions in feedlot animals. The pathogen can persist for a long time in the host, gradually spreading throughout the herd. In the study of respiratory diseases in cattle in the United States and Canada, it was shown that *H. somni* is the second most common (57 %) bacterial pathogen after *Mannheimia haemolytica* (91 %) [2-5].

Antimicrobials are widely used to treat histophilosis and other bacterial infections of cattle, however, the emergence and spread of antibiotic-resistant strains significantly reduces the effectiveness of antimicrobial therapy. Macrolides, tetracyclines, beta-lactams (penicillins and cephalosporins), aminoglycosides, fenicols and sulfonamides [6, 7] are mostly applied against pathogens of the *Pasteurellaceae* family [6, 7], therefore, the appearance of *H. somni* strains resistant to antibiotics of these groups is expectable. Antimicrobial sensitivity of *H. somni* is currently quite high, but in recent years, pronounced resistance has begun to develop to antibiotics of certain groups [5, 8, 9].

The first investigations of antibiotic resistance of *H. somni* were done by the Upjohn Company (USA) in 1988-1992 [10]. The sensitivity of *H. somni i*solates from cattle lungs in the USA and Canada to beta-lactams, tetracyclines, and macrolides exceeded 90 %, to the aminoglycoside spectinomycin was 87.1 %. Only 35.8 % of the samples were sensitive to the sulfamethazine of sulfonamide group [10]. According Welsh et al. [11], in the USA in 1994-2002, the sensitivity of *H. somni* to ampicillin, cephalothin, and tilmicosin remained high (94-100 %), and to tetracycline and spectinomycin decreased to 88-94 and 65 %, respectively. Portis et al. [12] showed that in vitro sensitivity of *H. somni* from the respiratory complex of cattle to beta-lactams remained close to 90 % during 2000-2009. Also, 90-100 % of *H. somni* isolates were sensitive to florfenicol, while the percentage of *H. somni* isolates sensitive to tetracycline decreased over a 10-year period [12]. Studies of *H. somni* resistance in Australia showed 100 % sensitivity of bacteria to ceftiofur of third-generation cephalosporins, florfenicol, and enrofloxacin, while one sample was resistant to macrolides [13].

From 2012 to 2016, monitoring of *H. somni* in Canada revealed resistance to the aminoglycoside neomycin in 93.6 % of isolates [14]. Lamm et al. [15] reported a wide variability in the sensitivity of the *Pasteurellaceae* family members, the *Pasteurella multocida*, *M. haemolytica*, and *H. somni* to the macrolide tilmicosin (88, 42, and 0 %, respectively). The sensitivity of all three species to antibiotics of the fluoroquinolone group was high (90-98 %) and to tetracycline low (40 %) [15].

Studies of *H. somni* resistance to antibiotics are carried out mainly in the USA and Canada [4, 5, 12]. In Europe, the problem of *Pasteurellaceae* resistance is paid less attention. Despite the fact that the countries of the European Union have implemented two antibiotic resistance monitoring programs — the ARBAO-II organized by the EU in 2003-2005 [16] and VetPath supervised by the European Animal Health Study Center (CEESA, Belgium) in 2002-2006 [17], the resistance of *H. somni* has not been practically studied. Within the framework of these programs, the sensitivity of *P. multocida* and *M. haemolytica* to various antibiotics was investigated. For sensitivity of these bacteria to florfenicol, ceftiofur, and the combination of amoxicillin with clavulanic acid, similar results were obtained in both programs, but the data on the sensitivity to tetracycline are very different [16, 17]. Though *H. somni*, *P. multocida*, and *M. haemolytica* belong to the same family, they demonstrate different sensitivity to antimicrobials [7, 10, 15] which does not allow drawing parallels between them with regard to the development of antibiotic resistance. Russian researchers also evaluated the antibiotic resistance of *P. multocida* and *M. haemolytica* isolated from cattle [18], however, the antibiotic

resistance of *H. somni* has not been studied.

Currently, genetic determinants of antibiotic resistance of bacteria causing cattle respiratory diseases have received much attention [19, 20]. For microorganisms of the *Pasteurellaceae* family that are of veterinary importance (*Pasteurella*, *Mannheimia*, *Actinobacillus*, *Haemophilus*, *Histophilus*), the presence of at least 9 genes associated with resistance to tetracyclines, 5 genes with resistance to beta-lactams, 6 genes for macrolides, and 10 for aminoglycosides are reported [21]. D'Amours et al. [22] who sought for various variants of tetracyclines resistance genes in *H. somni* isolates identified only the *tetH* gene but no other genes. In the papers of Canadian and American researchers, there are data on the integrative conjugative element (ICE) which carries several genes at once encoding the resistance of *H. somni* to antibiotics [14, 19, 25]. *H. somni* has the resistance genes *tetH* for tetracyclines, *bla*$_{OXA-2}$ for penicillins, *strA*, *strB*, *aadA25*, *aphA1*, and *aadB* for aminoglycosides, *sul2* for sulfonamides, *erm(42)*, *mrs(E)-mph(E)* for macrolides, *floR* for florfenicol, and *dfrA14* for trimethoprim [19, 21].

This work is the first to characterize resistance to antimicrobials of *H. somni* strains isolated from cattle in the Russian Federation. Circulation of resistant *H. somni* strains within the Russian livestock farms has been established for the first time.

Our goals were to study the antibiotic resistance of circulating *Histophilus somni* strains using phenotypic and genotypic techniques and to assess if the PCR analysis allows predicting the resistance of *H. somni* to several groups of antimicrobial agents. Assessment of antibiotic resistance was based on molecular identification of genes *blaOXA-2*, *sul2*, *strA*, *strB*, *aadA25*, *aphA1*, and *tetH*.

Materials and methods. *H. somni* cultures were isolated in 2018-2019 from biological material (parenchymal organs, washes, sperm) of cattle of different breeds, age, sex, and physiological groups. The samples were used for culturing on the nutrient medium CM0898B (Oxoid™, Thermo Fisher Scientific, USA) with the supplement FD117 (HiMedia Laboratories Pvt, Ltd., India) and on chocolate agar. Bacteria were grown in 10 % CO_2 at 37 °C for 48 hours. Susceptibility of *H. somni* isolates to streptomycin (10 μg), neomycin 30 (μg), ampicillin (10 μg), amoxicillin (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), tetracycline (30 μg), doxycycline (30 μg) (HiMedia Laboratories Pvt., Ltd., India) were tested by disk diffusion method, susceptibility categories (susceptible, resistant, or intermediate) was determined by growth inhibition zones according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) [23] with modifications.

Bacterial suspensions were prepared in brain-heart broth from 48-hour agar cultures. The turbidity of the suspensions was adjusted to 0.5 McFarland standard (1.5×10^8 CFU/cm^3). For antimicrobial susceptibility testing by disk diffusion, the isolates were incubated for 24 hours at 37 °C in 8-10 % CO_2. The diameter of the inhibition zone was measured with a caliper and expressed in millimeters. All measurements were carried out in 3 replicates.

To identify genetic determinants of antibiotic resistance in *H. somni*, DNA was extracted from a 100 mm^3 suspension using a DNA-sorb-B reagent kit (Central Research Institute of Epidemiology, Russia). *H. somni* isolates were tested for the presence of seven antibiotic resistance genes, *tetH*, *bla*$_{OXA-2}$, *aadA25*, *strA*, *strB*, *aphA1*, and *sul2* by conventional and Real-time PCR [24]. Conventional PCR was carried out according to the following program: 5 min at 95 °C; 10 s at 95 °C, 20 s at 55-64 °C (depending on the primers used), 10 s at 72 °C (40 cycles); 3 min at 72 °C (a Tertsik thermocycler, DNA-Tekhnologiya, Russia). Reaction mixtures for PCR of total 25 mm^3 volume contained 10 mm^3 template DNA, 6 pmol of

each specific primer, dNTPs, and PCR-mixture-2-blue (AmpliSens, Russia). Detection was carried out by electrophoresis in 1.5 % agarose gel. Positive samples were additionally confirmed by Sanger sequencing.

To identify genes *aadA25*, *strA*, *strB*, and *sul2*, TaqMan fluorescent probes were designed and Real-time PCR conditions were optimized. PCR on a Rotor-Gene 6000 (Corbett Research Pty, Ltd., Australia) and a Rotor-Gene Q (Qiagen, Germany) was performed as follows: 15 min at 95 °C; 10 s at 95 °C, 20 s at 63 °C, 10 s at 72 °C (5 cycles without fluorescent signal detection); 10 s at 95 °C, 20 s at 60 °C, 10 s at 72 °C (35 cycles with fluorescent signal detection). Amplification of the *strA* and *strB* genes was carried out simultaneously in a single multiplex real-time PCR assay.

The Real-time PCR was performed in a 25 mm^3 volume reaction mixtures containing 10 mm^3 template DNA, 10 mm^3 of PCR mixture-1 (6 pmol of each specific primer and 3 pmol of each probe, dNTPs, deionized water), 0.5 mm^3 of Taq-F polymerase, and 5 mm^3 of PCR-buffer-Flu (AmpliSens, Russia). The efficiency of DNA extraction was assessed by amplification of internal exogenous controls (IECs). Recombinant plasmids with target inserts (PCR products) into the vector plasmid pAL2-TA (Evrogen, Russia) were used as positive PCR controls.

PCR products were sequenced using Big Dye® Terminator v1.1. Cycle Sequencing Kit (Applied Biosystem, USA), a GeneAmp PCR System 2720 amplifier (Applied Biosystem, USA) and an ABI PRISM 3130 Genetic Analyzer automatic sequencer (Applied Biosystem, USA).

Results. From 145 animals 18 *H. somni* cultures were isolated and identified. Since *H. somni* is a slow-growing organism demanding on the conditions of cultivation, we managed to grow only 12 samples to the counts enough to be evaluated for antibiotic susceptibility.

The investigated isolates were mostly resistant to aminoglycosides. The resistance to streptomycin was 50 %, and resistance to neomycin exceeded 40 % (Table 1). Four samples were additionally tested for susceptibility to kanamycin, gentamicin and spectinomycin. Kanamycin resistance was found in one sample. All 4 samples were susceptible to gentamicin and spectinomycin. In the works of American researchers, only one antibiotic of the aminoglycoside group, the spectinomycin, was included in the panel of antibiotics in a susceptibility testing [10-12, 15]. The susceptibility of *H. somni* isolates to spectinomycin decreased from 86 % in 1988-1992 to 65-86 % in 1994-2002 [10, 11]. Bhatt et al. [14] also showed a high susceptibility of *H. somni* to spectinomycin (87 %), however, Canadian isolates, 22 % of collected during 1980-1990 and 93.6 % collected in 2012-2016, appeared to be resistant to another aminoglycoside antibiotic neomycin, indicating an increase in resistance to this drug.

1. Phenotypic resistance of *Histophilus somni* isolates in antimicrobial susceptibility testing by disk diffusion method

Group of antibiotics	Drug	Number (%)		
		resistant isolates	intermediate isolates	susceptible isolates
Aminoglycosides	Streptomycin	6 (50 %)	–	6 (50 %)
	Neomycin	5 (42 %)	2 (16 %)	5 (42 %)
Beta-lactams	Ampicillin	3 (25 %)	–	9 (75 %)
	Amoxicillin	3 (25 %)	–	9 (75 %)
	Cefotaxime	1 (8 %)	–	11 (92 %)
	Ceftazidime	–	–	12 (100 %)
	Ceftriaxone	–	–	12 (100 %)
Tetracyclines	Tetracycline	–	–	12 (100 %)
	Doxycycline	2 (17 %)	–	10 (83 %)

N o t e. Dashes indicate the absence of isolates resistant to the antibiotic.

In our work, the susceptibility of the isolates to beta-lactam antibiotics, the aminopenicillins and cephalosporins was 75-100 %, being in line with the studies which reported 90-96 % susceptibility of *H. somni* to this group antibiotics in the USA and Canada [10-12]. Note, 25 % of the tested isolates were resistant only to aminopenicillins. One sample was resistant to the 3rd generation cephalosporin cefotaxime, while there was 100 % susceptibility to other 3rd generation drugs, the ceftazidime and ceftriaxone.

According to our data, the susceptibility of the *H. somni* isolates to antibiotics of the tetracyclines group, was also high, reaching 83-100 %. This is consistent with studies conducted in the United States, in which the susceptibility of *H. somni* to tetracycline was 88-100 % [10, 11]. Later, Portis et al. [12] reported that the susceptibility of *H. somni* to tetracycline has been steadily decreasing every year and has almost halved in 9 years, from 83 to 47 %. It should be noted that in another study, the authors reported for 92 % susceptibility of *H. somni* to tetracycline [15] which may be due to both differences in the sample size and the uneven distribution of resistant isolates in North America.

Among six isolates of *H. somni* we additionally tested for susceptibility to the sulfonamide antibiotics group sulphamethoxazole, 33 % were resistant. Approximately the same figures were obtained by Welsh et al. [11] who reported 68-86 % susceptibility to sulfachloropyridizine, also an antibiotic of this group. In an earlier work, more than half of the tested samples (64.2 %) were also shown to be resistant to sulfamethazine [10].

We identified two multi-resistant *H. somni* isolates possessing resistance to three classes of antibiotics, the aminoglycosides, beta-lactams, and tetracyclines. Four isolates showed phenotypic resistance to antibiotics of two classes, namely, two isolates were resistant to aminoglycosides and sulfonamides and two to aminoglycosides and beta-lactams. It should be noted that the multi-resistance of *H. somni* was revealed earlier when studying bacteria isolated from sick animals at feedlots in different states of the United States. In the work of American scientists, 30 % of the studied *H. somni* isolates were resistant to more than seven classes of antibiotics, including aminoglycosides, macrolides, tetracyclines, beta-lactams, fluoroquinolones, lincosamides, and pleuromutilins [25].

To detect genetic determinants of antimicrobial resistance by PCR test, we used the following primers (Table 2).

2. Primers used to detect genetic determinants of antimicrobial resistance in 18 isolates of *Histophilus somni*

Antibiotics	Target gene	Nucleotides 5′-3′	Tm, °C	Reference
Streptomycin/ spectinomycin	*AadA25*	5′-GGCAACGCTATGTTCTCTTGCTTTTG-3′ 5′-TGTACGGCTCCGCAGTGGA-3′	60	This work
Neomycin/ gentamicin	*StrA*	5′-GGCGGCTGATCTGTCTGG-3′ 5′-CAGATAGAAGGCAAGGCGTTC-3′	59	[25]
Neomycin/ gentamicin	*StrB*	5′-CGCGTTGCTCCTCTTCTCCA-3′ 5′-GGCTACATGGCGATCTGCATC-3′	60	[26]
Kanamycin/ neomycin	*aphA1*	5′-TTATGCCTCTTCCGACCATC-3′ 5′-GAGAAAATTCACCGAGGCAG-3′	55	[25]
Sulfonamides	*Sul2*	5′-CCAATACCGCCAGCCCGTCG-3′ 5′-TGCCTTGTCGCGTGGTGTGG-3′	64	[25]
Tetracyclines	*tetH*	5′-CCACCATTATGATCAGTATGTCT-3′ 5′-CATCAGCCATAACAGACCATC-3′	55	This work
Penicillins	*blaOXA-2*	5′-GCAGACGAACGCCAAGCGGA-3′ 5′-CCCGCACGATTGCCTCCCTC-3′	64	[25]

The genes for *H. somni* resistance to tetracyclines *tetH* and penicillins bla_{OXA-2} were not detected in any of the 18 isolates (Table 3). Genes for resistance to aminoglycosides, the *strA* and *strB* (44 % of samples), *aadA25* (39 %), and *aphA1* (11%) were the most common. The sulfonamide resistance gene *sul2* was

detected in 3 of 18 isolates. In contrast to our results, Stanford et al. [20] did not detect the *aadA25* gene in none of the 42 *H. somni* isolates. Neither we nor the authors identified the *bla*$_{OXA2}$ gene in any of the isolates [20]. In another work of Canadian scientists who investigated *H. somni* isolates for the presence of 13 genetic determinants of resistance and 5 genes associated with ICE, 26 % did not contain either ICE genes or resistance genes. The rest 74 % made two groups, one having six resistance genes and the other with nine resistance genes and, moreover, both groups had 4 genes associated with ICE [20].

3. Detection of antibiotic resistance genes in 18 isolates of *Histophilus somni* by PCR test

Group of antibiotics	Resistance gene	Enzyme encoded by resistance gene	Number (%) of positive samples
Aminoglycosides	*aadA25*	Aminoglycoside-3″-adenyltransferase	7 (39 %)
	strA	Aminoglycoside-3″-phosphotransferase	8 (44 %)
	strB	Aminoglycoside -6-phosphotransferase	8 (44 %)
	aphA1	Aminoglycoside -3′-phosphotransferase	2 (11 %)
Tetracyclines	*tetH*	Efflux protein	0
Beta-lactams	*bla*$_{OXA-2}$	Class D beta-lactamase	0
Sulfonamides	*sul2*	Dihydropteroate synthase	3 (17 %)

Whole-genome analysis of seven *H. somni* isolates [20] revealed the *strA*, *strB*, and *sul2* genes to mostly found. These results are consistent with our data on the identification of genes for resistance to aminoglycosides, however, unlike the work of Canadian scientists, we have never found the *tetH* gene. In two isolates with combined resistance to aminoglycosides, penicillins, and cephalosporins revealed in antimicrobial susceptibility testing by disk diffusion, only the genes for resistance to aminoglycosides *aadA25* and *strA* were detected. In two samples phenotyped as resistant to aminoglycosides and sulfonamides, almost all studied resistance genes were detected, except for *tetH* and *bla*$_{OXA-2}$. It is possible that the *strA*, *strB*, *aadA25*, *aphA1*, and *sul2* genes, like the resistance genes described for *H. somni* in other works [20, 25], are located in a mobile gene cassette which can move around within an organism's genome or be transferred to another organism. However, since we did not identify hereby the *tetH* gene, this gene cassette seems to differ from the ICEs previously described [14, 20, 25] and requires further study.

We compared the results that we obtained by microbiological and molecular methods (Table 4).

4. Correspondence of phenotypic and genotypic resistance in 12 *Histophilu somni* isolates

Group of antibiotics	Antibiotics	S phenotype		R phenotype		Correspondence, %
		R genotype	S genotype	R genotype	S genotype	
Beta-lactams	Ampicillin	0	9	0	3	75.0
	Amoxicillin	0	9	0	3	75.0
	Cefotaxime	0	11	0	1	91.7
	Ceftazidime	0	12	0	0	100
	Ceftriaxone	0	12	0	0	100
Tetracyclines	Tetracycline	0	12	0	0	100
	Doxycycline	0	10	0	2	83.3
Aminoglycosides	Streptomycin	0	6	6	0	100
	Neomycin	0	5	7[a]	0	100

N o t e. S — susceptible, R — resistant; [a] — includes isolates of intermediate resistance.

Phenotypic resistance to antibiotics and genetic determinants of resistance have been demonstrated for *H. somni* earlier [5, 20, 25]. By Stanford et al. [20], the correspondence between phenotypic and genotypic resistance for aminoglycosides was extremely high, up to 100 %, which coincides with our findings indicating the occurrence of genetic determinants of aminoglycosides resistance in all strains phenotypically resistant to these antimicrobials. In streptomycin-resistant strains the *aadA25* gene was found. In all five neomycin-resistant strains, the

presence of both *strA* and *strB* genes was detected. Nevertheless, in two strains with intermediate resistance to neomycin, the *strA* and *strB* genes were not detected, but the *aadA25* gene was found. Adenylation of neomycin appears to reduce the effectiveness of this antibiotic, but does not lead to absolute resistance. The *aphA1* gene was found in two samples resistant to both streptomycin and neomycin. Such a high correspondence between the pheno- and genotyping data will make it possible to predict the resistance of isolates to aminoglycosides by molecular methods without time-consuming microbiological procedures.

When comparing genotypic and phenotypic resistance to sulfanilamides, a good agreement was also obtained: in our work it was 100 %, Owen et al. [19] reported 94 %. Both our study and the report of the American authors [19] have shown a significant variability in the phenotype to genotype correspondence for resistance to beta-lactams and tetracyclines. In our study, the *bla*$_{OXA-2}$ gene was not detected in any of the three samples phenotypically resistant to penicillins and cephalosporins. According to the literature data, five different genes encoding beta-lactamases have been found in members of the *Pasteurellaceae* family. However, to date, only *bla*$_{OXA-2}$has been detected in *H. somni* [21], and, therefore, we performed genotyping of resistance to beta-lactam antibiotics only for this gene. It cannot be ruled out that other genes encoding beta-lactamases (*bla*$_{ROB-1}$, *bla*$_{CMY-2}$, *bla*$_{PSE-1}$, or *bla*$_{TEM-1}$) which can be transmitted to *H. somni* from *P. multocida* and *M. haemolytica* by plasmids and mobile genetic elements may occur in the three samples we found to be phenotypically resistant to beta-lactams.

The *tetH* gene which encodes a membrane-associated protein responsible for the active transport of tetracyclines outside the cell (efflux) was also not detected in the *H. somni* isolates we studied, despite the fact that two isolates showed resistance to doxycycline. Apparently, the resistance of these isolates is due to other mechanisms.

Thus, our findings showed *Histophilus somni* to be resistant to antibiotics of the aminoglycoside and sulfonamide groups but highly susceptible (over 90 %) to tetracyclines and 3rd generation cephalosporins, which allows assumption that tetracyclines and 3rd generation cephalosporins will be highly effective in the antibacterial therapy of diseases caused by *H. somni*. A high correspondence occurs between the data of microbiological and molecular detection of resistance to aminoglycosides and sulfonamides, and therefore, the PCR method can be recommended to predict the resistance of *H. somni* to the antimicrobials of these groups. Given published data on the presence of other genes for resistance to macrolides and florfenicol *erm(42)*, *mrs(E)-mph(E)*, and *floR*, and due to the wide use of antibiotics of these groups in the Russian Federation, we believe that the study is antibiotic resistance of *H. somni* must be continued.

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PROPERTIES OF EXPERIMENTAL SAMPLES OF VACCINE AGAINST AVIAN INFECTIOUS CORYZA

M.S. FIRSOVA, A.V. POTEKHIN, VAL.A. EVGRAFOVA, O.V. PRUNTOVA ✉, V.S. RUSALEYEV, R.V. YASHIN

Federal Center for Animal Health Control, FGBU VNIIZZh, mkr. Yurievets, Vladimir, 600901, e-mail firsova@arriah.ru, andrey@biokorm.cat, pruntova@arriah.ru (✉ corresponding author), rusaleev@arriah.ru, evgrafova@arriah.ru, yashin@arriah.ru
ORCID:
Firsova M.S. orcid.org/0000-0002-1531-004X
Potekhin A.V. orcid.org/0000-0002-3529-4809
Evgrafova Val.A. orcid.org/0000-0003-3053-6976
Pruntova O.V. orcid.org/0000-0003-3143-7339
Rusaleyev V.S. orcid.org/0000-0002-4972-6326
Yashin R.V. orcid.org/0000-0002-1385-705X
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Abstract

Avian infectious coryza is caused by the bacteria *Avibacterium paragallinarum* and occurs throughout the world in countries with a well-developed poultry industry, causing significant economic losses to the poultry industry. The specific prevention is the main link in combating infectious rhinitis of chicken. Vaccination of birds provides expressed immunity due to generation of anti-hemagglutinating antibodies. The presented research study is the first to report on immunobiological properties of two formulations of a developed experimental vaccine against avian infectious coryza which contains the formaldehyde-inactivated antigen of a new *A. paragallinarum* strain No. 5111 (serogroup B). The aim of the work was to evaluate the safety, antigenic and protective properties of the absorbed and emulsion-based formulations of a vaccine against chicken infectious rhinitis based on the *A. paragallinarum* strain No. 5111. A whole-cell antigen of the *A. paragalli*narum strain No. 5111 (serotype B-1) inactivated with formaldehyde was used to produce experimental samples of the vaccine for trials. A dose for immunization (0,5 cm^3) contained 10^9 inactivated microbial cells and 3.75 mg of aluminum hydroxide for the absorbed vaccine formulation or oil adjuvant Montanide ISA 70 VG («SEPPIC», France, 70 % wgt) for the emulsion-based formulation. The immunobiological properties of the vaccine were tested on 125 Haysex brown chickens (*Gallus gallus* L.) of 1.5-2.0 months of age which were seronegative to *A. paragallinarum*. The safety of the vaccine samples was tested by injecting chickens in a 2-fold dose (1.0 cm^3). Each sample was injected subcutaneously in the middle third of the neck and intramuscularly in the chest using 5 chickens per each formulation. The clinical status of the birds was observed daily for 42 days. At the end of the experiment, the chickens were slaughtered and the incision of the injection site was visually examined. Three groups of chickens (25 birds each, 75 chickens in total) were assigned to determine protective properties of the vaccine. The birds of group I were immunized with the absorbed formulation of the vaccine, the chickens of group II were injected with the emulsion-based formulation. The birds were injected subcutaneously into the middle third of the neck at a dose of 0.5 cm^3 twice with a 20-day interval. Unvaccinated chickens of group III were used as a control. In 15 days after revaccination, the chickens of groups I, II, and III were infected with a 1-day broth culture of the *A. paragallinarum* strain No. 5111 with a concentration of 5 units according to the optical standard of bacterial suspension turbidity. The clinical status of the chickens was observed during 7 days after infection. The post-mortem examination was performed with a bacteriological analysis of the contents of the nasal sinuses during the experiment and at the end of the experiment. The vaccine antigenicity and the duration of immunity were determined on 30 birds (three groups of 10 birds each). The chickens of group I were immunized with the absorbed vaccine sample, group II — with an emulsion-based sample, and unvaccinated birds of group III served as a control. The vaccine antigenicity was assessed based on humoral antibody level using the hemagglutination inhibition test (HI test). The mild to moderate tissue lesions were observed at the injection site without an obvious inflammatory reaction. For the absorbed formulation, slight subcutaneous swellings and hyperemia were observed in some chickens at the injection site. For the emulsion-based formulation, the formation of connective tissue granules with the vaccine residues without necrotic lesions and an obvious inflammatory reaction of the surrounding tissues occurred at the injection site in all birds. No significant differences in the condition of chicks from vaccinated groups were

observed ($p > 0.05$), but there was a significant difference between the birds of the test and control groups ($p < 0.05$). The level of protection of chickens after double immunization with the adsorbed vaccine and the emulsion-based vaccine was 92 % and 88 %, respectively. Twenty days after the first vaccination with absorbed and emulsion-based formulations, the average antibody titers were below the threshold level ($p > 0.05$). Increased antibody titers in chicken sera were observed only at day 15 post the second immunization. At day 60 post vaccination, the antibody levels in the chicken sera reached their maximum, i.e., 7.5 ± 0.8 $\log_2$ in poultry immunized with the adsorbed vaccine and 8.9 ± 0.7 $\log_2$ in birds immunized with the emulsion-based vaccine ($p > 0.05$). In chickens vaccinated with the vaccine containing aluminum hydroxide gel, a decrease in the antibody titer to 5.5 ± 0.7 $\log_2$ was observed at day 240 while in birds immunized with the emulsion-based vaccine the titer remained at the level of 8.7 ± 0.8 $\log_2$ ($p > 0.05$). No specific antibodies to the causative agent of infectious coryza were detected in chickens of the control group during the entire observation period, including the diseased and convalescence period. Thus, our findings show that the adsorbed and emulsion-based experimental formulations of the developed vaccine against avian infectious coryza are safe and demonstrate high antigenicity and immunogenicity after double administration.

Keywords: avian infectious coryza, *Avibacterium paragallinarum,* antigen, adjuvant, candidate vaccine

Bacterial diseases at industrial poultry farms pose an urgent problem for veterinary medicine. Infectious rhinitis (hemophilia) of chickens caused by *Avibacterium paragallinarum* has recently become a widespread respiratory pathology. The clinical sighs of this acute enzootic infectious disease are catarrhal inflammation of the mucous membranes of the upper respiratory tract and edema in the subcutaneous tissue of the facial part of the head [1-5].

Infectious rhinitis is typical for all countries with developed poultry farming, including the Russian Federation [4-6]. The disease causes serious economic damage to the industry due to growth retardation, loss of egg production (up to 40 %), and costly preventive and recreational measures.

Antigenic diversity of the pathogen is important for the epizootology. The currently recognized nine serovars of *A. paragallinarum* are A-1, A-2, A-3, A-4, B-1, C-1, C-2, C-3, and C-2 which are organized into three serogroups termed A, B, and C. Different serovars and serogroups of the pathogen dominate in different countries [1, 7-9]. In natural illness, birds, as a rule, develop weak and short-term immunity, therefor, recurrences of the disease are possible [6, 10].

Vaccination is the main measures in combating chicken infectious rhinitis as it ensures the development of strong immunity due to generation of antihemagglutinating antibodies. A close relationship was revealed between the protective immunity and the titer of specific antibodies in the hemagglutination inhibition (HI) assay [7, 11, 12]. Vaccination allows significant reduction of antibiotic use which, in turn, helps to prevent the emergence of antibiotic resistance in microorganisms and decreases the residual amounts of antibiotics in poultry products [12-13].

Until the mid-1990s, most commercial vaccines against chicken infectious rhinitis were produced from strains of serogroups A and C, which negatively affected their effectiveness, especially in regions with active circulation of the pathogen of serogroup B. However, over the past 20 years, express methods for the *A. paragallinarum* serotyping have been developed and introduced into laboratory practice, allowing accurate detection of antigenic profiles of the pathogen. Most modern vaccines are positioned as universal, since they contain a set of *A. paragallinarum* strains of serogroups A, B, and C. Individual serotypes within serogroups A and C ensure cross-protection, whereas effectiveness of vaccination against the pathogen of serogroup B directly depends on the antigenic correspondence of the vaccine strain and the epizootic strain circulating in a particular geographic region.

Currently, in Russia, single trivalent vaccine against avian infectious coryza of has been developed and approved the use of which is limited due to its

high reactogenicity. For the prevention of the disease, foreign vaccines are mostly used, which creates the dependence on imported drugs.

Diagnostic studies of pathological material carried out at FGBI ARRIAH identified 12 isolates of *A. paragallinarum* from regions of Russia [2]. Serotyping revealed their belonging to the B-1 serovar. In 2014, two strains were isolated from sick chickens during an outbreak of the disease at large poultry farms which used a commercial trivalent emulsion vaccine against avian infectious coryza. This fact casts doubt on the effectiveness of the vaccine used in the territory of the Russian Federation. In addition, outbreaks of *A. paragallinarum* serotype B-1 have also increased in recent years in Europe and Asia, despite the use of commercial vaccines. The weak cross-protection between strains of serotype B-1 has not yet been explained. Since the studied strains of serotype B-1 provide only partial cross-protection, it is likely that an effective vaccine can be made only from the antigen of a strain isolated in a specific geographic region where this serotype is endemic [7, 10, 14]. The wide distribution of the pathogen of infectious rhinitis of chickens of serotype B-1 in Russia indicates the advisability of using the strain to develop a domestic vaccine [1, 15].

Effective vaccination is largely determined not only by the antigen properties, amount, and the administration routs but also by the appropriate adjuvants [16-18]. A stimulant of nonspecific immunity must primarily be safe [3, 19, 20].

Cultured *A. paragallinarum* No. 5111 of B-1 serovar endemic to the Russian Federation can reach high cell density while maintaining stable hemagglutinating activity and high virulence. Vaccine against avian infectious coryza based this strain in combination with various adjuvants is of great importance for veterinary medicine.

This work it the first to present characterization of the harmlessness, antigenic properties and protective effect of candidate vaccines based on the antigen of a new domestic strain of *A. paragallinarum* No. 5111 of serogroup B.

The work aimed to assess the immunobiological properties of absorbed and emulsion formulations of a candidate vaccine against avian infectious coryza.

Materials and methods. The whole cell antigen of *Avibacterium paragallinarum* No. 5111 (serotype B-1) was inactivated with formaldehyde. The concentration of bacteria in each inoculated dose (0.5 cm^3) was 10^9 microbial cells according to the optical turbidity standard. The absorbed formulation contained 3.75 mg of aluminum hydroxide per dose. The emulsion formulation contained 70 % (w/w) oil adjuvant Montanide ISA 70 VG (SEPPIC, France).

The immunobiological properties of the vaccine were evaluated in 2019 on 125 1.5-2.0-month-old Hisex brown chickens (*Gallus gallus* L.) which were delivered from a poultry farm free from infectious diseases and seronegative to *A. paragallinarum*.

The harmlessness of the formulations was determined by injecting chickens in a 2-fold dose (1.0 cm^3). Each formulation was injected subcutaneously into the middle third of the neck from the dorsal side and intramuscularly into the chest (5 animals per formulation). The examination of clinical state of the birds and the injection site were performed daily for 42 days. At the end of the experiment, the poultry were slaughtered and the tissue condition at the injection site was visually assessed on the incision. The degree of tissue damage after intramuscular injection was assessed according to the Stone's criteria [21]: weak lesions (blanching of the tissues surrounding the injection site with no signs of inflammation and residues of the encapsulated vaccine), moderate lesions (hyperemia and edema

of surrounding tissues ranging in size from 1.0 to 2.0 cm in diameter with the presence of residues of an encapsulated or diffusely distributed vaccine), severe lesions (pronounced tissue inflammation with the formation of granulomas with a diameter of 3.0 to 4.0 cm, the contents of a liquid consistency flow out on a cut or have a cheese-like appearance).

The protective properties of the formulations were assessed on 75 chickens (three groups of 25 animals each). Group I was immunized with the absorbed formulation, group II with the emulsion formulation, group III of unvaccinated chickens was used as a control. The birds were twice injected (20 days apart, subcutaneously into the middle third of the neck from the dorsal side) with a dose of 0.5 m^3. Fifteen days after the revaccination, the chickens were infected with a 1-day broth culture of *A. paragallinarum* No. 5111 (5 U optical turbidity standard). The microbial cell suspension (0.2 cm^3) was administered intranasally. For 7 days since infection, the clinical state of the chickens was monitored daily. The protective properties of the formulations were assessed according to the method proposed by Soriano et al. [22] to determine the *A. paragallinarum* virulence, with the exception that the significant difference between the average score for the experimental and control groups testified to the immunogenicity of the formulation.

The degree of the developed clinical signs in infected birds was scored as follows: 0 points mean no symptoms; 1 point means slight nasal discharge, slight swelling of the nasal sinus region, or both; 2 points mean moderate nasal discharge, moderate swelling of the nasal sinus region, or both; 3 points mean abundant nasal discharge, pronounced swelling of the nasal sinus region, or both; 4 points mean abundant nasal discharge and pronounced swelling of the nasal sinus region, wheezing. When symptoms of disease were detected, the scores for individual animals were summed up and divided by the total number of infected chickens in the group. During the experiment and at its end, a pathoanatomical autopsy was carried out with a bacteriological analysis of the contents of the nasal sinuses from dead and slaughtered chickens. Birds that had no clinical signs, no pathoanatomical changes, and a negative bacteriological test were considered protected from the disease.

The antigenic properties of the vaccine and the duration of immunity were determined using 30 birds (three groups of 10 birds each). Group I was immunized with the adsorbed formulation, group II with the emulsion formulation, group III of unvaccinated birds served as a control. The scheme of immunization and the method of administration of the vaccine were used the same as in studying the protective properties. The antigenic activity of the candidate vaccine formulations was assessed by the titers of humoral antibodies in the hemagglutination inhibition (HI) test [23, 24]. Blood samples were taken from the axillary vein before immunization, 20 days after the first vaccination and 15, 40, 100, 160, and 220 days after revaccination. In HI test, the antibody titers ≥ 4.0 $\log_2$ were considered positives, ≤ 2.0 $\log_2$ as negatives.

The data were processed to determine the arithmetic mean values (M) and standard errors of the mean ($\pm$SEM). The significance of differences was assessed by the Student's t-test. The difference between the values was considered statistically significant at $p < 0.05$.

Results. The first step was to determine the safety of the candidate vaccine formulations. One day after the intramuscular injection of the emulsion formulation, in three chickens, a slight swelling occurred at the injection site which disappeared on its own within 3-5 days.

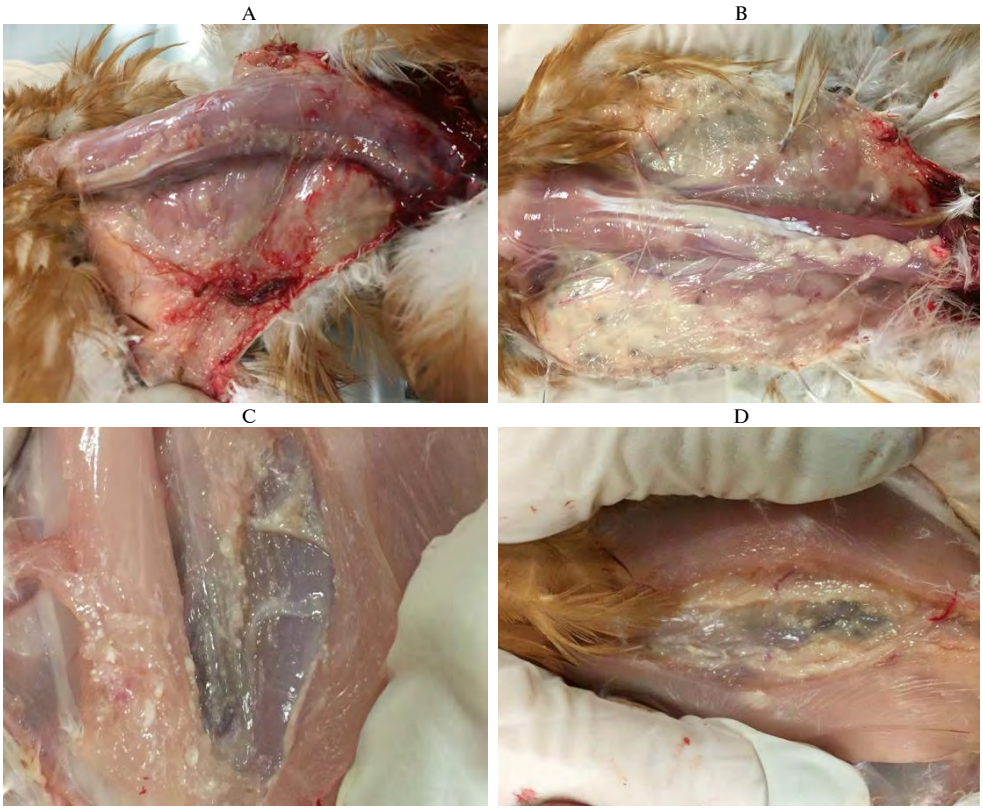

Fig. 1. Hisex brown chickens (*Gallus gallus* L.) injected with an avian infectious coryza candidate vaccine based on *Avibacterium paragallinarum* No. 5111 (serotype B-1) antigen: A — swelling and hyperemia at the site of subcutaneous injection of the absorbed vaccine formulation, B — connective tissue granuloma with vaccine residues at the site of subcutaneous injection of the emulsion vaccine formulation, C — no muscle tissue lesions at the site of intramuscular injection of the he absorbed vaccine formulation, D — encapsulated emulsion residues in the tissues at the site of intramuscular injection of the absorbed vaccine formulation (lab tests).

At the end of the experiment, autopsy of the slaughtered birds revealed tissue lesions form mild to moderate severity without a pronounced inflammation. At the site of subcutaneous injection of the absorbed formulation, slight edema and hyperemia of the subcutaneous tissue was observed in some chickens, whereas for the emulsion formulation, in all birds, there were connective tissue granulomas with vaccine residues though without necrotic lesions and a pronounced inflammation of the surrounding tissues (Fig. 1).

Intramuscular injection of the absorbed vaccine formulation led to a slight tissue damage with no pronounced signs of inflammation and residues of the encapsulated vaccine. With a similar administration of the emulsion vaccine formulation, there were a moderate tissue inflammation, granulomas up to 2.0 cm in diameter, and vaccine residues which looked like small encapsulated droplets located along the muscle fibers. No necrosis and hemorrhages were seen in the surrounding tissues. In totality, these data indicated the safety of both vaccine formulations when administered subcutaneously.

To assess protective properties of the candidate vaccine formulations, the control infection of chickens with 1-day broth culture of *A. paragallinarum* No. 5111 was carried out. One to two days after infection n most birds from the control group and in 3-4 days in some birds of the test groups, the same types of clinical signs (rhinitis, sinusitis, and conjunctivitis) developed. At the initial

stage of the disease, birds showed transparent discharge from the nasal passages and slight one- or two-sided swelling in the nasal sinuses. Subsequently, in sick chickens of the control group, nasal discharge became cloudy and acquired a viscous consistency. The inflammatory exudate often blocked the nasal passages and the birds began to breathe through the mouth. Further development of the disease in chickens from the control group showed pronounced swelling of the nasal sinuses and conjunctival sacs. Most sick birds were depressed with partial or complete refusal of food and water. In some chickens, when the infection developed in the deep parts of the respiratory tract, breathing was accompanied by wheezing. In immunized birds, there were only a slight watery discharge from the nasal passages and subtle one- or two-sided swelling of the nasal sinuses. The average duration of illness in vaccinated chickens was 3-4 days, while in birds from the control group it was 5-7 days (Table).

Protective properties of formulations of a candidate vaccine against avian infectious coryza in Hisex brown chickens (*Gallus gallus* L.) infected with 1-day broth culture of *Avibacterium paragallinarum* No. 5111 (laboratory studies)

Formulation	Birds				Disease severity, the sum of scores per group (M±SEM)	Efficacy, %
	total number	sick	died	in which the pathogen was detected		
Absorbed	25	3	0	3	0.12±0.03	92
Emulsion	25	4	0	4	0.16±0.04	88
Control (no vaccination)	25	23	7	25	3.00±1.00	

When comparing the development of control infection in birds immunized with different vaccine formulations, no significant differences were observed ($p > 0.05$), however, a significant difference occurred between the experimental and control groups ($p < 0.05$). In group I immunized with the absorbed formulation, the protective efficacy was 92 %. The total score characterizing the severity of the disease was 25 times lower compared to the control birds. In group II immunized with the emulsion formulation, the effectiveness was 88 %. In the control birds, clinical signs were observed in 23 chickens, of which 7 birds died.

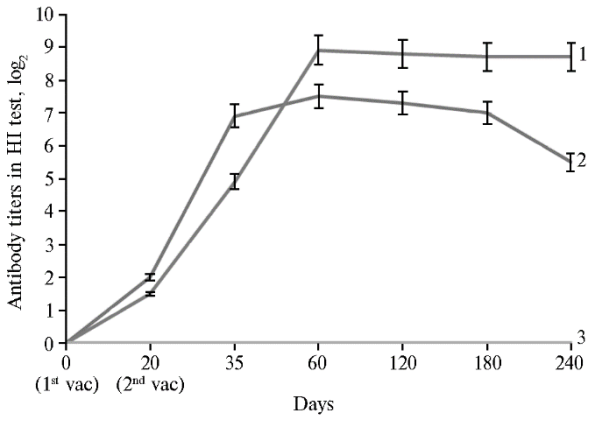

Fig. 2. The titer of humoral antibodies in the hemagglutination inhibition (HI) **test and the duration of immunity in Hisex brown chickens (*Gallus gallus* L.) immunized with two formulations of a candidate vaccine against avian infectious coryza based on the *Avibacterium paragallinarum* № 5111 antigen (B-1 serovar):** 1 — emulsion formulation, 2 — absorbed formulation, 3 — control (no vaccination); 1st and 2nd vaccinations (M±SEM, laboratory tests).

Pathological changes in chickens were observed mainly in the upper parts of the respiratory tract. In the immunized chickens with clinical signs of the disease during life, a small amount of serous exudate was seen in the nasal sinuses. In most of the birds of the control group, the subcutaneous tissue in the facial part of the head had a pronounced edema and a gelatinous consistency. The nasal passages and sinuses in all chickens were filled with fibrinous or fibrinous-purulent exudate. In the conjunctival sacs, serous-purulent exudate with fibrin films was often seen. When distal part of the respiratory tract was affected, fibrinous pneumonia and aerosacculitis usually developed. The

causative agent of the disease was isolated from the inflammatory exudate form the nasal sinuses. The original *A. paragallinarum* No. 5111 strain was isolated from 3 birds of group I, 4 chickens of group II, and from all control birds.

Analysis of the dynamics of the formation of specific antibodies in the blood sera of birds after double immunization testified to the high antigenic activity of both vaccine formulations (Fig. 2).

Twenty days after the first vaccination, in birds vaccinated with both formulations, the average antibody titers were below the threshold value ($p > 0.05$). A significant increase in the blood antibody levels occurred only 15 days after the second immunization. Sixty days after the immunization, the antibody titers in groups I and II were the highest, up to 7.5 ± 0.5 and 8.9 ± 0.2 $\log_2$ ($p < 0.05$), respectively. In 240 days, in chickens immunized with the absorbed formulation, the antibody titers decreased to 5.5 ± 0.6 $\log_2$, while in birds immunized with an emulsion formulation, the titer was 8.7 ± 0.8 log2 ($p < 0.05$). During the entire period, no specific antibodies to *A. paragallinarum* were detected in the control group.

According to the literature, emulsion vaccines are more immunogenic than absorbed vaccines, but when they are used, there is a likelihood of developing local inflammatory reactions and even abscesses. Absorbed vaccines, as a rule, have less reactogenicity for animals; however, when administered subcutaneously, they sometimes cause the formation of connective tissue granulomas [21, 25, 26].

It is known that the effectiveness of inactivated vaccines directly depends on the amount of bacterial antigen per dose. An excessive amount of antigen can suppress the body's immune system up to the development of immunological tolerance and cause undesirable reactions at the injection site. In turn, at an insufficient dose, the antigen does not induce immunological processes in the body [10, 13]. Our experiments showed that the tested vaccine formulations are safe for birds when injected subcutaneously. Evaluating the protective properties confirmed a pronounced immunogenic activity of the formulations. An effective inactivated vaccine must provide protection for at least 80 % of the vaccinated population. In our experiments, the protective activity reached 92 % for the absorbed formulation of the candidate vaccine and 88 % for the emulsion formulation. Similar results were obtained by another scientists. For example, Blackall and colleagues conducted trials to assess the safety and efficacy of inactivated vaccines containing mineral oil and aluminum hydroxide gel as adjuvants. Both types of the drug were injected subcutaneously into the middle third of the neck from the dorsal side. After 3 weeks, the birds were subjected to experimental infection. The emulsion preparation provided 80 % protection, a vaccine based on an aluminum hydroxide gel provided 94 % protection [10, 26]. Our results are also consistent with the data of other authors, who assert that, after 2-fold use, absorbed and emulsion formulations have high antigenic activity against avian infectious coryza [27].

Thus, the tested formulations of a candidate vaccine against avian infectious coryza based on the *Avibacterium paragallinarum* strain No. 5111 (serotype B-1) antigen are harmless when administered subcutaneously to Hisex brown chickens at a 2-fold dose. At the site of injection of the absorbed formulation, some chickens developed slight edema and hyperemia of the subcutaneous tissue. When the emulsion formulation was injected, all birds developed connective tissue granulomas with the presence of vaccine residues

without a pronounced inflammatory reaction of the surrounding tissues. The efficacy of the absorbed formulation was 92 %, of the emulsion formulation 88 %. Both formulations induced production of antihemagglutinating antibodies. The antibody titers were the highest 60 days after vaccination. In birds vaccinated with the absorbed and the emulsion formulations, the antibody titer averaged 7.5±0.8 and 8.9±0.7 $\log_2$, respectively ($p > 0.05$). For early protection of birds from avian infectious coryza, the absorbed vaccine formulation is preferable while the oil adjuvant formulation ensures a more intense and lasting immunity.

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HEMATOLOGICAL PARAMETERS OF EUROPEAN PERCH (*Perca fluviatilis* Linnaeus, 1758) ASSOCIATED WITH PARASITIC INVASIONS

NGUEN THI HONG VAN, S.V. PONOMAREV, Yu.V. FEDOROVYKH ✉, Yu.N. GROZESKU, A.A. BAKHAREVA, I.V. VOLKOVA, V.I. EGOROVA, Yu.V. SERGEEVA

Astrakhan State Technical University, 16, Tatishcheva st., Astrakhan, Russia 414056, e-mail: Hongvannguyen@mail.ru, ya.panama2011@yandex.ru, jaqua@yandex.ru (corresponding author ✉), grozesku@yandex.ru, bahareva.anya@yandex.ru, gridasova@mail.ru, lekaego@mail.ru, js4444@mail.ru
ORCID:
Nguen Thi Hong Van orcid.org/0000-0002-6910-2705
Ponomarev S.V. orcid.org/0000-0002-2899-8672
Fedorovykh Yu.V. orcid.org/0000-0003-0789-1566
Grozesku Yu.N. orcid.org/0000-0002-6691-8696
Bakhareva A.A. orcid.org/0000-0002-7724-2425
Volkova I.V. orcid.org/0000-0001-8945-6669
Egorova V.I. orcid.org/0000-0002-7688-723X
Sergeeva Yu.V. orcid.org/0000-0001-8811-1378
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Abstract

European perch (*Perca fluviatilis* Linnaeus, 1758) is widespread in water basins and has a commercial value. However, in the natural environment, fish are often infected with invasive diseases, which exclude its processing for food purposes. Being in the body of fish, parasites produce toxic substances that affect various organs of fish. Widespread in the Volga Delta pathogenic species, *Eustrongylides excisus*, in large quantities occurs in perch in larval state in the abdominal cavity of the body, in the walls of the intestine, in the liver, in the calf and in the spinal muscle tissue, reaching up to 30 or more parasites per fish. Sexual maturity of the parasite occurs in the stomach of the birds. This disease is peculiar to perch species; larvae of this nematode cause granulomatous inflammatory reaction. In addition, these worms, penetrating deep into the muscles of the perch, spoil the commercial quality of fish, which leads to the culling of fish raw materials and rejection of foodstuff. This work, for the first time, compares the hematological status of infected and healthy perch of natural populations. The work aimed to assess biological and physiological effects of various parasitic invasions on European perch *Perca fluviatilis* (Linnaeus, 1758). Fish of both sexes at the age from 0 to 4 years were caught in natural reservoirs of the Lower Volga (Bolshaya Bolda and Bely Ilmen rivers, Astrakhan region) in September 2016 to June 2019. The total body length, the length to the end of the scale cover, body weight and age were determined, and the Fulton's fatness coefficient was calculated. The skin, fins, mouth, gills, eyes, heart, abdominal cavity, muscles, brain, and spinal cord of fish were examined for parasitological contamination. Blood for analysis was taken from the tail vein in vivo. Nematodes were found in more than 55 % of the caught perch. Nematodes were found in the liver (30 %), abdominal cavity (45 %), intestines (10 %), muscles (15 %), and gills (5 %). Parasites were found free within the body cavity, or encapsulated, with *Eustrongylides excisus* being the most abundant. In the infected fish, the average body weight and body condition coefficient decreased as compared to the healthy fish of the same age. However, the revealed growth retardation in the infected perch was statistically insignificant ($p > 0.05$), while the body condition coefficient was significantly lower than in uninfected fish ($p < 0.01$ for 3-year-old perch). The parasitic infestation of *P. fluviatilis* with *Eustrongylides* nematode caused symptoms of anemia, suppression of erythropoiesis (i.e., a decrease in the proportion of blast forms of the erythrocytic cell series), an increase in the proportion of neutrophils, monocytes, and lymphoblasts in the leucocyte count. In infected perch, the number of red blood cells significantly decreased ($83.01\times10^4\pm4.17\times10^4$ vs. $137.22\times10^4\pm5{,}26\times10^4/mm^3$), as well as the concentration of total protein (40.81 ± 1.19 vs. 48.97 ± 2.07 g/l) and blood cholesterol (5.17 ± 0.28 vs. 6.81 ± 0.30 mmol/l). MCH (mean corpuscular hemoglobin), erythrocyte sedimentation rate, the total number of leukocytes and platelets also increased compared to uninfected fish. The level of pathological red blood cells increased in the infected perch ($9{,}17\pm0{,}23$ % vs. $4{,}87\pm0{,}11$ %). Changes in cell cytoplasm and nucleus, degenerative changes in the cell, changes associated with cell division were the main types of the discovered cell pathology of the infected perch.

Keywords: *Perca fluviatilis*, European perch, parasites, nematodes, hematological parameters, anemia, eustrongylidosis, blood

In terms of cultivation and catch, European perch (*Perca fluviatilis* Linnaeus, 1758) is inferior to salmon (*Salmonidae*) and cyprinids (*Cyprinidae*). The increase in its number is mainly due to the introduction and natural reproduction. For example, in Sweden, European (river) perch is widespread in many water bodies. In Denmark, about 350 thousand two-gram juveniles of this species produced annually by aquaculture methods are exported to Switzerland and Ireland. In Ireland, river perch became a potential aquaculture target as early as in 1995 [1-4]. Importantly, the volume of farmed marketable river perch over the last three years has doubled as compared to previous years. The main producers of European perch are France, Czech Republic, Netherlands, Ukraine, Tunisia, Australia [1, 5].

In Russia, until now, *P. fluviatilis* was considered trash and a low-value commercial fish. This is largely due to the fact that in natural water bodies the perch is largely infested with various parasites, especially the nematodes *Eustongylides* and *Anisakis* [6-10]. Such fish often remains sterile [11], lags behind in growth and development, and its weight is 20-25 % lower than that of uninfected individuals [12].

Changes in culinary habits, the globalization of food supplies, the development of tourism, the spread of alien species are the reasons of a threat the nematodes in fish pose to human health which should not be underestimated [13-15].

Fish farming industry enables rearing healthy river perch free from parasites. However, the farms operating without breeding their own replacement brood stock have to catch producers from natural populations for the subsequent spawning campaign. Hence, there is a need to assess fish-breeding biological parameters and physiological status of female and male perch during their appraisal.

In this study, we have established for the first time a number of hematological and biochemical parameters confirming the physiological status of infected perch from natural populations. The data obtained can be the basis for the development of the physiological and immunological indicators of the European perch both in nature and when rearing in artificial conditions, including within the framework of selection and breeding programs.

The aim of the work was to investigate how parasitic invasions affects biological and physiological characteristics of the European perch *Perca fluviatilis* L. to identify indicators that can be used to assess the health status of fish reared both for market and for the brood stock replacement.

Materials and methods. For the study carried out at the Innovation Center "Bioaquapark — the Scientific and Technical Center of Aquaculture" (Astrakhan State Technical University), the European perch *Perca fluviatilis* (Linnaeus, 1758) individuals of both sexes at the age from 0 (underyearlings) to 4 years (four-year-olds) were caught from natural reservoirs of the Lower Volga (Bolshaya Bolda and Bely Ilmen rivers, Astrakhan Province) from September 2016 to June 2019.

The absolute body length, the length to the end of the scale cover, the body weight and age of the fish were recorded, and the Fulton's condition factor was calculated. The age was determined by the annual scale rings [16]. Parasitological examination of skin, fins, mouth, gills, eyes, heart, abdominal cavity, muscles, brain and spinal cord was performed by a standard technique [17]. Parasites were identified to species using keys and figures [18, 19]. The intensity and extensiveness of the invasion were calculated according to the description [20].

Blood for analysis was taken from the tail vein in vivo [21]. Blood smears fixed with 95 % ethanol were stained with hematoxylin and eosin according to Romanovsky (Guidelines for conducting hematological examination of fish: collection

of instructions for combating fish diseases. Part 2. Moscow, 1999) and examined under a light microscope (BIOMED 6 LED, LLC Biomed, Russia) with immersion (×100). For microphotography, a DMC-510 photo equipment (Panasonic, Japan) was used. For differentiation of the formed elements, the Ivanova's classification (1983) was applied. Morphologically altered erythrocytes were examined [21, 22]. The hemoglobin concentration was measured by the hemoglobin cyanide method using a KFK-3 photoelectric colorimeter (JSC ZOMZ, Russia). Erythrocytes (the number per 1 ml blood) were counted in a Goryaev chamber. The hemoglobin concentration per erythrocyte was calculated. Erythrocyte sedimentation rate (ESR) was assessed using a Panchenkov's apparatus (Methodological instructions for carrying out hematological examination of fish: collection of instructions for combating fish diseases. Part 2. Moscow, 1999).

The indicators of protein and lipid metabolism in healthy and invaded fish were determined by optical densitometry against calibration solutions (a KFK-3 photoelectric colorimeter, Russia). The total protein concentration was measured by the biuret reaction. In biuret test, 0.1 ml of non-hemolyzed serum was poured in tubes with 5 ml of the biuret reagent. The cholesterol concentration was measured colorimetrically as described [23].

The results were processed statistically using Microsoft Excel 2010 and JASP-1 programs (https://jasp-stats.org). The mean values (*M*), standard deviations (±σ), standard errors of the mean (±SEM) were calculated. To confirm the significance of the differences, the Student's t-test was used [24].

Results. Nematodes were found during dissection in more than 55 % of the caught river perch (Table 1). In 5 %, parasites were detected only with a microscope. In infected fish, nematodes colonized the liver (30 %), abdominal cavity (45 %), intestines (10 %), muscles (15 %), and gills (5 %). In the internal organs and in their membranes, parasites were both free and encapsulated, with *Eustrongylides excisus* being the most abundant (see Table 1).

1. Nematode infestation in European perch (*Perca fluviatilis* Linnaeus, 1758) caught from the Bolshaya Bolda and Bely Ilmen rivers (Lower Volga, Astrakhan Province, 2016-2019)

Species	Localization	Extensiveness of invasion, %	Intensity of invasion, the number of nematodes
Anisakis schupakovi	Abdominal cavity, intestines, visceral fat	51	1-19
Eustrongylides excisus	Abdominal cavity, stomach, liver, muscles, visceral fat	60	5-34
Camallanus lacustris	Visceral fat, intestines	30	1-7

Nematodes of *Eustrongylides* spp. inhabit muscles, body cavities, and internal organs of fish [25, 26], including in *P. fluviatilis* [9, 27]. Pathohistological changes in *P. fluviatilis* upon invasion of this parasite are muscle degeneration and necrosis which spread to the sarcoplasm, basal lamina of the sarcolemma, endomysial cells of connective tissue, and capillaries [27]. Immunohistochemical staining revealed numerous proliferating cells in the thickness of the capsule and in the immediate vicinity of the larva of *Eustrongylides* sp., which, as the authors suggest, indicated the initiation of restoration of the muscle damaged by the nematode [27]. Guagliardo et al. [25] described similar histopathological sings, muscle atrophy and chronic inflammatory response in Galaxias maculatus.

Our observations showed that *P. fluviatilis* infestation with *Anisakis schupakovi*, *Eustrongylides excisus*, and *Camallanus lacustris* decreases the average body weight and body condition coefficient compared to healthy fish of the same age. However, the revealed growth retardation in sick individuals was statistically

insignificant ($p > 0.05$), while the body condition coefficient in infected fish was significantly lower than in uninfected ($p < 0.01$) (Table 2).

2. Biological parameters in European perch (*Perca fluviatilis* Linnaeus, 1758) of different ages invaded and uninvaded with parasitic nematodes (*M*±SEM, Bolshaya Bolda and Beliy Ilmen rivers, Lower Volga, Astrakhan Province, 2016-2019)

Indicator	Two-year-olds		Three-year-olds	
	invaded ($n = 17$)	uninvaded ($n = 7$)	invaded ($n = 17$)	uninvaded ($n = 7$)
Body weight, g	47.21±4.19	54.33±5.14	91.77±7.85	106.20±10.06
Absolute body length, cm	15.36±0.32	16.89±0.41	19.68±0.60	20.11±0.72
Length to the end of the scale cover, cm	13.34±0.41	13.92±0.37	16.92±0.47	17.30±0.56
Fulton's condition factor	1.99±0.08	2.01±0.07	1.89±0.09*	2.05±0.10

* The differences between infected and uninfected fish are statistically significant at $p < 0.01$.

In infected fish, a decrease in the hemoglobin concentration (42.35±2.07 g/l) was detected compared to healthy fish (48.40±2.35 g/l), however, these differences were insignificant ($p > 0.05$) (Table 3).

3. Hematological and biochemical parameters in European perch (*Perca fluviatilis* Linnaeus, 1758) invaded and uninvaded with parasitic nematodes (*M*±SEM, Bolshaya Bolda and Bely Ilmen rivers, Lower Volga, Astrakhan Province, 2016-2019)

Indicator	Invaded fish ($n = 17$)	Uninvaded fish ($n = 7$)
Hemoglobin, g/l	42.35±2.07	48.40±2.35
Erythrocyte sedimentation rate (ESR), mm/h	4.50±0.12**	3.34±0.22
Total erythrocyte counts, $\times 10^4$/mm^3	83.01±4.17**	137.22±5.26
Mean corpuscular hemoglobin, MCH, pg	51.03±2.12*	28.34±1.91
Total protein, g/l	40.81±1.19**	48.97±2.07
Cholesterol, mmol/l	5.17±0.28**	6.81±0.30

*, ** The differences between infected and uninfected fish are statistically significant at $p < 0.05$ and $p < 0.01$m respectively.

These data are consistent with available publications. In *Cyprinus carpio* with parasitic invasion, a significant decrease in the hemoglobin concentration was also reported [28]. In hematological studies of *Sander lucioperca* inhabiting the Anzali wetlands (Iran), the hemoglobin concentration increased compared to uninfected fish, but the differences were also insignificant [29]. Many authors reported that the European perch has significant resistance to abiotic and biotic environmental factors, including parasitic invasion [30-34], therefore, the blood hemoglobin concentration changes ambiguously during infection.

The blood erythrocyte counts in infected perch significantly decreased and amounted to $83.01\times10^4\pm4.17\times10^4$ cells/mm3, while in healthy fish this indicator was $137.22\times10^4\pm5.26\times10^4$ cells/mm3 ($p < 0.01$). In the works of other researchers, a decrease in the erythrocyte counts was noted not only in the carp, but also in the African catfish *Clarias gariepinus*, when infected with parasites, which indicated anemia [35]. Due to the decrease in the number of red blood cells, the mean corpuscular hemoglobin (MCH) increased. Differences in this indicator between infected and healthy river perch were significant (51.03±2.12 vs. 28.34±1.91 pg, $p < 0.01$). The ESR also increased (4.50±0.12 vs. 3.34±0.22 mm/h, $p < 0.01$). An increase in ESR, firstly, is a consequence of a decrease in the number of erythrocytes, and secondly, it indicates the development of an inflammatory process in the fish under the influence of parasites [36, 37]. Study of the *Eustrongylides* sp. invasion in the *Channa punctatus* showed that the mean erythrocyte count, hematocrit and hemoglobin concentration were significantly higher ($p < 0.01$) in uninfected fish, while the MCH value significantly increased ($p < 0.01$) in infected fish [26].

In our study, the concentration of total blood protein in healthy perch

P. fluviatilis was 48.97±2.07 g/l while in infected individuals it decreased to 40.81±1.19 g/l ($p < 0.01$). In *C. punctatus*, significant differences in total protein and glucose concentrations were also noted between fish uninfected and infected with *Eustrongylides* sp. [26]. The same authors showed a significant ($p < 0.01$) increase in the activity of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and the cholesterol level in fish infected with *Eustrongylides* sp. compared to uninfected fish [26].

The results of microscopic examination of blood smears showed that the proportion of blast and young cells of the erythrocyte lineage decreased in infected perches (0.47±0.04 %) compared to healthy perches (6.36±0.23 %). This indicates the inhibition of erythropoiesis under the stress effect of the parasite. The proportion of pathologically altered erythrocytes in blood smears increased to 9.17±0.23 % in infected fish vs. 4.87±0.11 % in healthy fish ($p < 0.01$). This increase was mainly due to poikilocytes and schizocytes. This appeared to result from a decrease in the strength of the cell membrane which was a symptom of anemia. Chiocchia et al. [38] showed that under parasitic invasion, swelling of erythrocytes occurred due to a change in the osmotic gradient.

Many works have shown that, upon parasite invasion, the most significant changes occur in the leukocyte formula and blood leukocyte counts [26, 33, 39, 40]. Our study revealed an increase in the number of leukocytes up to 252.12±12.25 cells per 1000 erythrocytes in infected fish vs. 125.4±4.28 cells per 1000 erythrocytes in healthy fish, which is associated with the role of leukocytes in the body, since they are responsible for the protective function of the immune system during parasite invasion [28, 41]. In addition, the number of platelets per 1000 erythrocytes decreased. In the leukocyte formula, shifts towards neutrophils and monocytes occurred (Table 4).

4. Microscopic examination of blood smears of European perch (*Perca fluviatilis* Linnaeus, 1758) invaded and uninvaded with parasitic nematodes (*M*±SEM, Bolshaya Bolda and Bely Ilmen rivers, Lower Volga, Astrakhan Province, 2016-2019)

Trait, indicator	Invaded fish ($n = 17$)	Uninvaded fish ($n = 7$)
Erythropoietic cells, %		
Erythroblasts	0.32±0.27	0.45±0.33
Oxyphilic normoblasts	0.13±1.32*	4.73±1.01
Polychromatophilic normoblasts	0.02±0.71	1.18±0.54
Abnormal erythrocytes, %		
Poikilocytosis	5.9±0.15	3.98±0.14
Pointy end	0.79±0.11	0.59±0.02
Pear-shaped	0.30±0.03	0.28±0.04
Multifaceted	4.11±0.09	3.11±0.78
Nuclear shift to the periphery	1.18±0.02**	0.70±0.13
Schizocytosis	1.82±0.07**	0.19±0.06
Oligochromasia	1.22±0.12	–
Nucleus deformation	0.17±0.05	–
Total proportion of abnormal cells	9.17±0.23**	4.87±0.11
Leukocyte formula, %		
Lymphocytes	75.77±0.98**	90.2±0.54
Neutrophils	3.57±0.32**	0.79±0.11
Monocytes	7.55±0.46**	1.57±0.26
Lymphoblasts	7.27±0.67	5.86±0.37
Myelocytes	3.27±0.74**	0.79±0.12
Promyelocytes	2.57±0.28**	0.79±0.12
Leukocytes per 1000 erythrocytes	252.12±12.25**	125.4±4.28
Thrombocytes per 1000 erythrocytes	2.10±2.01	7. 1±1.89

Note. Dashes indicate that the abnormality was not seen.
*, ** The differences between infected and uninfected fish are statistically significant at $p < 0.05$ and $p < 0.001$, respecetively.

Thus, in more than half of the cases, parasite invasion was found in perches caught from natural reservoirs of the Lower Volga. *Eustrongylides excisus*

was the most frequent species of the detected nematodes. In fish infected with parasitic nematodes, the blood erythrocyte counts, the total protein concentration, and the cholesterol level significant decreased. Infected fish also showed an increase in the mean corpuscular hemoglobin values, ESR, the total number of leukocytes and platelets compared to uninfected fish. Analysis of the blood formed element in infected perch showed suppression of erythropoiesis, which is a sign of anemia. An increase in the proportion of neutrophils, monocytes, and lymphoblasts in the leukocyte formula indicate an increase in leukopoiesis, including neutrophilia in the fish affected by parasite invasion. The resulting informative indicators have the prospect of being used to assess the physiological and immunological state of river perch in aquaculture and when performing selection and breeding work.

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AGE DYNAMICS OF FREE AMINO ACIDS IN THE TISSUES OF THE MEDICINAL LEECH *Hirudo verbana* Carena, 1820 UNDER ARTIFICIAL REPRODUCTION IN AQUACULTURE

L.A. KOVALCHUK[1] ✉, L.V. CHERNAYA[1], N.V. MIKSHEVICH[2]

[1]*Institute of Plant and Animal Ecology, Ural Branch RAS,* 202, ul. 8 Marta, Ekaterinburg, 620144 Russia, e-mail kovalchuk@ipae.uran.ru (✉ corresponding author), Chernaya_LV@mail.ru;
[2]*Ural State Pedagogical University*, 26, pr. Kosmonavtov, Yekaterinburg, 620017 Russia, e-mail mikshevich@gmail.com
ORCID:
Kovalchuk L.A. orcid.org/0000-0003-0467-1461 Mikshevich N.V. orcid.org/0000-0003-2388-4278
Chernaya L.V. orcid.org/0000-0002-3386-9824
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Abstract

Medicinal leeches (*Hirudo verbana* Carena, 1820) used in veterinary medicine, human medicine and pharmacology, are currently bred artificially at bio-factories. The technology of leech culture as a component of aquaculture biotechnics is based on the method of accelerated growth of leeches due to intensive feeding, which allows you to get high-quality products in a fairly short time (8-10 months). Nevertheless, the problem of assessing the physiological status of raised individuals upon artificial reproduction remains not fully addressed. The findings we present herein for the first time allow us to suggest that the age-specific amino acid composition of tissues can be used to assess the physiological state and well-being of medicinal leeches in nature and in aquaculture. The aim of the work is to study the age-dependent content of the free amino acids in the tissues of medicinal leeches artificially bred in aquaculture. Studies were conducted in 2012 on the leech (*Hirudo verbana* Carena, 1820) individuals without signs of diseases which were purchased at the biofactory International Center for Medicinal Leeches (Moscow Province, p. Udelnaya). Leeches were kept in the laboratory in glass vessels with dechlorinated water at 20-22 ºC and fed once a month with fresh bovine blood from healthy animals. The experimental specimens of *H. verbana* was: 5 days (newborn filaments, 0+), 1, 3, 5, 7 and 9 months old, with an average body weight of 0.029, 0.09, 0.25, 0.61, 1.33 and 1.81 g, respectively. Newborn individuals served as a control. The concentration of free amino acids (AA) in the skin and muscle tissue was determined on the analyzer AAA-339M (Mikrotechna, Czech Republic). A total of 105 leech specimens were used, 30 bioassays were prepared, and 660 element determinations were performed. The amino acid composition of the tissues of medicinal leeches *H. verbana* was represented by 23 AA and their derivatives. The dominant AA with an antioxidant orientation, regardless of age, were glutamine and glutamic acid, alanine, valine, leucine, and glycine, the total content of which was 65 % in filaments, and 58 % in 9-month — old individuals of the total fund AA. In the tissues of medicinal leeches, as well as in warm-blooded animals, the full composition of essential amino acids (EAA) was revealed: threonine, valine, lysine, leucine, isoleucine, histidine, arginine, methionine, phenylalanine, tryptophan. With age, the total concentrations of free amino acids decreased ($r = -0.98$ at $p = 0.000$), due to a sharp decrease in the content of arginine (24.0-fold), proline (13.4-fold), isoleucine (12.2- fold), glycine (8.5- fold), lysine (6.8- fold), histidine (6.6-fold), leucine (5.2-fold), ornithine (4.0-fold), glutamic acid and glutamine (3.8-fold), and alanine (2.9-fold). At the age of 5, 7 and 9 months, leeches had a trace content of secondary metabolites: taurine, citrulline and tryptophan ($p < 0.001$). However, the amino acid balance of nitrogen and protein metabolism was not disturbed, since the ratio of essential / non-essential AA did not change significantly and was 0.60 in newborn filaments, 0.73 — in 9-month-old leeches. It was found that the growth and development of *H. verbana* was accompanied by a significant decrease

in the indicator of maturity (glycine/alanine) from 0.75 (newborn filaments) to 0.25 (9 months). The information available in the literature and the data of our research allow us to put forward the concept of using functional amino acids as biomarkers in the development of the scientific bases of technologies for the industrial breeding of these amphibionts.

Keywords: medicinal leeches, leech culture, free amino acids, age, physiological status

In advanced aquaculture, technologies should suit to physiological features and requirements of aquatic organisms during their ontogenesis. The well-being of artificially reproduced species is closely related to their adaptability to environmental conditions. Reliable assessing physiological parameters of animals and their habitat, e.g., using ecological and physiological biomarkers, are necessary to quickly estimate the ecological safety and to create conditions for the sustainable development of aquatic organisms [1, 2]. Currently, work in this area is focused on the search and practical use of biomarkers of immunological, histopathological and physiological parameters of artificially reproduced aquatic organisms.

Available scholar publications and our findings draw us to the concept that functional amino acids (AA) which have a wide spectrum of metabolic activity and play an important role in energy and plastic metabolism in animals can serve as biomarkers [3-5]. Comparing the biochemical composition of 182 freshwater and marine zooplankton species showed the freshwater species to have lower levels of ATP and free amino acids and a higher RNA [6]. In many research papers, the metabolism of copepods, planktonic copepods, and daphnia is investigated [7, 8]. The biological role of free amino acids and their derivatives in marine invertebrates has been shown for muscle proteins of bivalve molluscs [9]. Enhanced amino acid metabolism which provides adaptive resistance to new environmental conditions was revealed in Black Sea mollusk *Anadara kagoshimensis* [10]. Trophic specificity of the amino acid composition of freshwater leech tissues and the key role of essential amino acids in adaptation of blood-sucking hirudinids to extreme habitat conditions are discussed [11, 12].

The age dynamics of free AAs and their derivatives in animal tissues can serve as an informative physiological indicator. This is especially important for mass reproduction when cultivation density is high [13-15]. Changes in the amino acid profile reflecting the role of free AAs occur during larval development of the plaice *Platichthys stellatus* [16]. In early ontogenesis of freshwater neotropical fish *Piaractus mesopotamicus*, high levels of essential amino acids were characteristic. Moreover, these findings indicated the role of taurine in the regulation of fluid osmolality [15]. In mackerel inhabiting the Chinese Sea, metabolically inert taurine in the muscles was seasonally variable [17]. The amino acid composition of commercial fish was influenced by fish nutrition [18]. Modifications of nitrogen metabolism and the role of free amino acids in low-temperature adaptation were studied on the example of an eurythermal pond fish *Perccottus glehni* [19]. These data confirm the importance of free amino acids for regulation of key metabolic processes ensuring the well-being of freshwater organisms in the natural and anthropogenic ecosystems.

Leeches for medical, veterinary and pharmaceutical use are cultivated in biofactories under controlled conditions [20, 21]. The current technology of leech aquaculture (hirudoculture) is based on the methodology developed in Russian back in the middle of the 20th century. Its main stages are the mating of the brood stock leeches, collection of juveniles from mature cocoons and their feeding with bovine blood to the final size [22, 23]. The growth rate of leeches in hirudoculture is exclusively due to growing conditions, e.g., the frequency of water changes, high

cultivation density, spatial limitation, etc., which either positively affect the physiological parameters or, in contrast, cause the high mortality rate of juveniles. However, assessing the well-being of reared leeches remains not fully addressed and the amino acid metabolism have not been given due attention. The ratio of vital free AAs in the tissues as a well-being criterion can contribute to the effective reproduction of medicinal leeches in hirudoculture.

Here, we have presented the first report on the age-specific amino acid profiles as a tool to assess the physiological state and well-being of the medicinal leech both in nature and in aquaculture.

The aim of this work was to determine the free amino acid profile in tissues of cultivated medicinal leeches as dependent on age.

Materials and methods. During cultivation (2012), the pharmacy leech (*Hirudo verbana* Carena, 1820) (purchased at the biofactory "International Center for Medical Leech", Moscow Province, Udelnaya settlement) without signs of diseases were kept in the laboratory in glass vessels with dechlorinated water at 20-22 °C and fed once a month with fresh bovine blood from healthy animals. The free amino acid dynamics was assessed in 5-day-old individuals (newborn filaments, 0+), 1-, 3-, 5-, 7- and 9-month-old leeches with the average body weight 0.029, 0.09, 0.25, 0.61, 1.33, and 1.81 g, respectively. Newborn leeches served as control. The studies were guided by the recommendations of the European convention on protection of the vertebrate animals used for experiment or in other scientific purposes [24, 25].

Free AAs were measured in the musculocutaneous tissue (an AAA-339M analyzer, Mikrotechna, Czech Republic). A 1 g portion of raw biomaterial was homogenized in 3 ml of phosphate buffer (pH 7.4). After two-stage centrifugation (a K-23D refrigerated centrifuge, MLW, Germany; 10,000 rpm for 15 min and 8,000 rpm for 30 min), 0.1 ml of 30 % sulfosalicylic acid was added for deproteinization and neutralized with 0.2 ml of 7 % lithium hydroxide. Norleucine (0.1 ml) was used as an internal standard. The following metabolic groups of AAs were measured: nonessential AAs (NEAs) — alanine, aspartic acid, glycine, glutamic acid + glutamine, proline, serine, tyrosine, cysteine; essential AAs (EAs) — arginine, valine, histidine, isoleucine, leucine, lysine, methionine, threonine, tryptophan, phenylalanine; branched-chain AAs (BCAs) — valine, isoleucine, leucine; aromatic AAs (AAs) — tyrosine, phenylalanine; sulfur-containing AAs (SCAs) — methionine, cysteine, cysteic acid. A total of 105 leeches were tested, 30 biosamples were prepared, 660 biochemical analyses were performed.

Standard software packages, the Microsoft Excel 2007 and STATISTICA 7.0 (StatSoft, Inc., USA) were used for statistical processing. The means (M) and standard errors of the means (±SEM) were calculated. The data were converted into logarithmic form (μmol/100 g) or arcsine form (% of total AAs). To statistically compare multiple values, Fisher's F-test was applied; differences from control were assessed by Dunnett's test (ANOVA). The correlation coefficient r was calculated to evaluate relationships between the free AA levels in leeches and their age. The data were visualized by principal component analysis (PCA) using the statistical environment R (R 3.1.2, packages Vegan and Ade4) [26]. Differences were considered statistically significant at $p < 0.05$.

Results. In *H. verbana*, we detected 23 AAs and their derivatives. The leeches, like warm-blooded animals [1, 2], had a full set of functionally significant essential amino acids (threonine, valine, lysine, leucine, isoleucine, histidine, arginine, methionine, phenylalanine, and tryptophan). The dominant antioxidant AAs, regardless of age, were glutamine, glutamic acid, alanine, valine, leucine, and

glycine which in total constituted 65 % pool of AAs in control (filaments) and 58 % pool in 9-month-old leeches.

The highest rates of amino acid metabolism were characteristic of juvenile leeches at the earliest stages of development (Table 1). Thus, the total concentrations of free AAs in newborns and 1-month-old individuals did not show statistically significant differences ($p > 0.05$), despite the multiple increase in the body weight due to intensive feeding. There was a linear drop in the total concentrations of free AAs ($r = -0.98$ at $p = 0.000$). Due to accelerated growth, in adult leeches aged 9 months, the total AAs decreased 3-fold compared to control ($p < 0.001$).

At early ontogenesis, leeches had a high requirement for amino acids necessary to rapidly increase muscle mass (valine, alanine, ornithine, arginine), to provide reproductive development (glutamine, lysine, histidine, ornithine, lysine), to synthesize elastin and collagen, the main proteins of connective tissues (alanine, glycine, lysine, proline, valine, leucine, threonine), to provide metabolic detoxification (glycine, glutamic acid, cysteine, tryptophan), to normalize carbohydrate and lipid metabolism, and to develop immunity (alanine, taurine, histidine, leucine).

The most significant age-related differences ($F_{5, 24} > 147.74$ at $p < 0.001$) were revealed for amino acids which are key physiological stimulants. With age, amino acid levels declined. There were a 24.0-fold decrease in arginine, a 13.4-fold decrease in proline, a 12.2-fold decrease in isoleucine, an 8.5-fold decrease in glycine, a 6.8-fold decrease in lysine, a 6.6-fold decrease in histidine, a 5.2-fold decrease in leucine, a 4.0-fold decrease in ornithine, a 3.8-fold decrease in glutamic acid and glutamine, and a 2.9-fold decrease in alanine ($p < 0.001$) (see Table 1).

Importantly, in leeches aged 5, 7, and 9 months, the pool of secondary metabolites, namely taurine, citrulline, and tryptophan, was completely exhausted ($p < 0.001$). In hirudoculture, the ontogenesis of leeches was also due to the high supply with specific metabolic groups of AAs, i.e., the branched-chain AAs (valine, leucine, isoleucine) which protect muscle fibers from oxidation and destruction, aromatic AAs (phenylalanine, tyrosine) involved in the synthesis of biogenic amines and neurotransmitters, and sulfur-containing AAs (cysteic acid, cysteine, methionine) possessing immunomodulatory and detoxifying properties (see Table 1).

The balance of nitrogenous and protein metabolism characterized by the ratio of essential to nonessential AAs did not change significantly both in control (EAs/NEAs = 0.60) and in 9-month-old leeches (EAs/NEAs = 0.73) (see Table 1). It is important to note that the pattern we observed for the maturity indicator (MI — the ratio of glycine to alanine) was opposite, that is, the MI values decrease from 0.75 in filamentous leeches to 0.25 in 9-month-old leeches. It is known that in commercial species, an increase in alanine and a decrease in glycine in fish tissues during growth is a stable trait, therefore, the alanine to glycine ratio can be used as an indicator of maturity, namely, MI values of 1.2-1.4 correspond to immature individuals, of 0.3-0.6 to mature ones [27]. According to our data, the MI parameter proposed to determine sexual maturity of fish can also be effective in assessing the age characteristics of leeches in hirudoculture.

We found statistically significant correlations between the age of medicinal leeches and the percentage for both the majority of free AAs and the major metabolic groups (% of the total AAs) in tissues (Fig. 1).

1. Age-dependent profiles of free amino acids (μmol/100 g) **in the tissues of the medical leech *Hirudo verbana* Carena, 1820 grown in hirudoculture** ($n = 30$, $M \pm$SEM)

Amino acid	Age, months						ANOVA	
	0+, control	1	3	5	7	9	$F_{5, 24}$	p
Cysteic acid	49.24±0.49	79.64±0.64	54.48±1.44^{NS}	46.25±1.68^{NS}	41.28±1.24	31.98±1.02	140.67	0.000
Taurine	11.65±0.36	9.39±0.51	6.46±0.50	Trace	Trace	Trace	1201.90	0.000
Aspartic acid	9.95±0.41	126.62±1.92	85.01±4.27	16.07±1.07	4.65±0.44	4.86±0.47	521.48	0.000
Threonine	81.88±1.20	96.20±1.68	58.17±0.85	77.60±3.38^{NS}	98.23±3.66	73.40±2.56^{NS}	42.45	0.000
Serine	91.20±2.86	48.47±1.09	87.78±1.57^{NS}	94.49±3.50^{NS}	104.02±1.22	76.03±2.93	93.99	0.000
Glutamate + Glutamine	608.20±5.79	413.89±2.62	381.01±3.08	285.49±6.89	203.07±1.36	158.33±3.17	1201.5	0.000
Proline	70.74±1.06	28.62±1.00	14.12±0.71	9.48±0.97	7.07±0.43	5.28±0.56	206.85	0.000
Glycine	157.01±2.10	208.32±2.27	134.16±1.60^{NS}	77.47±3.24	27.11±0.58	18.47±1.32	147.75	0.000
Alanine	209.13±1.74	236.69±1.52	205.97±1.54^{NS}	130.04±3.38	97.86±1.61	73.27±2.62	569.51	0.000
Citrulline	9.15±0.42	9.37±0.42^{NS}	4.72±0.35	Trace	Trace	Trace	1189.60	0.000
Valine	93.31±0.85	110.19±1.50	73.09±1.54	96.47±3.30^{NS}	121.77±1.46	91.32±2.12^{NS}	71.53	0.000
Cysteine	6.37±0.42	4.12±0.17	23.55±0.85	15.32±0.71	8.52±0.50	6.35±0.46^{NS}	142.80	0.000
Methionine	23.56±0.84	14.80±0.43	53.29±1.48	36.58±3.40	22.99±0.62^{NS}	18.95±0.56	93.91	0.000
Isoleucine	121.31±1.40	98.36±1.79^{NS}	34.51±0.68	23.90±2.99	13.14±0.72	9.98±0.67	263.72	0.000
Leucine	180.72±2.49	154.04±1.39	130.88±2.61	86.36±3.67	46.1±0.95	34.67±0.62	840.56	0.000
Tyrosine	15.44±0.76	8.80±0.71	27.51±1.12	20.41±1.73^{NS}	16.11±0.60^{NS}	11.77±0.80^{NS}	40.30	0.000
Phenylalanine	30.87±1.40	34.9±0.54^{NS}	56.34±1.11	41.87±2.62	23.76±0.44	17.83±0.81	107.67	0.000

Continued Table 1

Tryptophan	2.82±0.19	22.71±0.77	5.53±0.33	Trace	Trace	Trace	1659.40	0.000
Ornithine	27.43±0.89	27.12±1.01^{NS}	29.20±0.49^{NS}	18.48±1.00	9.15±0.51	6.82±0.49	176.12	0.000
Lysine	70.39±1.49	79.41±1.63^{NS}	79.29±1.01^{NS}	45.46±2.09	13.96±0.54	10.35±0.82	479.28	0.000
Histidine	1.19±0.02	4.68±0.42	11.77±0.43	3.86±0.07	0.89±0.06^{NS}	0.18±0.01	572.90	0.000
Arginine	46.98±1.08	31.17±0.77	22.93±0.66	14.43±1.09	7.72±0.51	1.96±0.07	551.75	0.000
AA pool	1918.51±26.98	1847.58±23.72^{NS}	1579.74±26.41	1140.04±42.57	867.37±13.74	650.80±12.60	418.82	0.000
EAs	653.02±10.66	646.52±10.54^{NS}	525.80±10.71	426.53±19.98	348.56±8.96	258.64±8.24	203.22	0.000
NEAs	1168.02±21.23	1075.54±10.83^{NS}	959.10±14.01	648.79±20.97	468.37±6.50	354.36±7.29	611.56	0.000
BCAs	395.33±4.66	362.59±4.51	238.48±4.83	206.73±9.20	181.02±2.93	135.97±2.09	316.78	0.000
AAs	49.13±2.32	66.49±1.96	89.38±2.52	62.29±3.84	39.83±1.01	29.61±0.48	101.66	0.000
SCAs	90.83±1.45	107.94±1.65	137.78±4.20	98.15±5.58^{NS}	72.79±2.35	56.28±0.31	99.47	0.000
EAs/NEAs	0.60	0.60	0.60	0.66	0.74	0.73		
MI (Glycine/Alanine)	0.75	0.88	0.65	0.60	0.28	0.25		

N o t e. AAs — amino acids, EAs — essential amino acids, NEAs — nonessential amino acids, BCAs — branched-chain amino acids, AAs —aromatic amino acids, SCAs — sulphur-containing amino acids, MI — иmaturity indicator.

^{NS} No statistically significant differences from control (at $p > 0.05$).

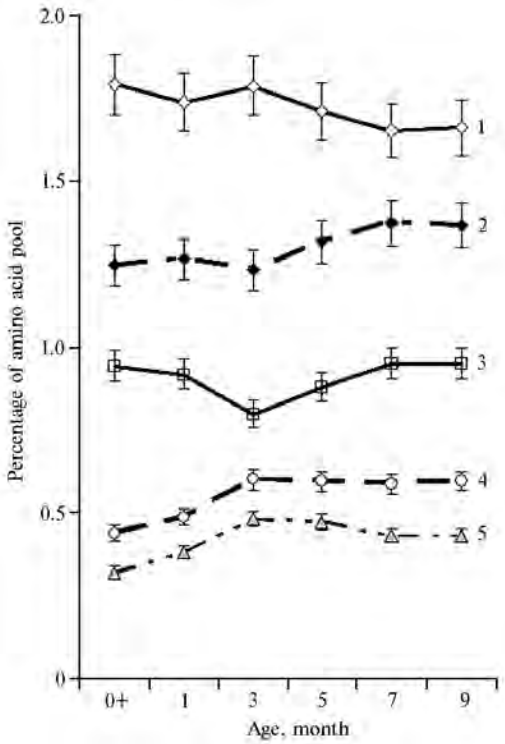

Fig. 1. Age-dependent levels of metabolic groups of amino acids in the tissues of medical leech *Hirudo verbana* Carena, 1820 grown in hirudoculture: 1 — nonessential amino acids, 2 — essential amino acids, 3 — branched-chain amino acids, 4 — aromatic amino acids, 5 — sulfur-containing amino acids ($n = 30$, $M \pm$SEM).

The growth and development of *H. verbana* for 9 months were accompanied by a significant increase in the percentage of tissue valine, methionine, serine, tyrosine, threonine, cysteic acid, cysteine, phenylalanine ($r = 0.55$-0.90), total EAs ($r = 0.86$), BCAs ($r = 0.60$) and SCAs ($r = 0.83$) ($p < 0.05$). As leeches grew, there was a significant decrease in the percentage of tissue arginine, aspartic acid, glycine, glutamate and glutamine, isoleucine, leucine, lysine, ornithine, proline, taurine, tryptophan, and citrulline (r from –0.38 to –0.96) and NEAs pool ($r = -0.85$) ($p < 0.05$). The age specificity of the amino acid content (% of the pool) in the tissues of *H. verbana* was clearly detected using the method of principal component analysis (Fig. 2, Table 2). Figure 2 shows the spatial differentiation of the studied age groups of medicinal leeches according to their physiological needs for individual amino acids. For 7- and 9-month-old leeches, there was an obvious identity of the amino acid composition of tissues.

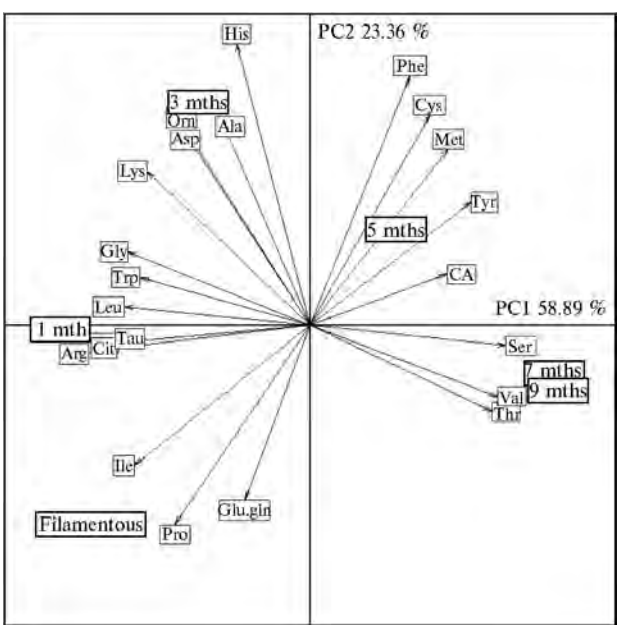

Fig. 2. The PCA analysis of age-dependent amino acid profiles in tissues of medical leech *Hirudo verbana* Carena, 1820 grown in hirudoculture: CA — Cysteic acid, Tau — taurine, Asp — aspartic acid, Thr —

threonine, Ser — serine, Glu.gln — glutamate + glutamine, Pro — proline, Gly — glycine, Ala — alanine, Cit — citrulline, Val — valine, Cys — cysteine, Met — methionine, Ile — isoleucine, Leu — leucine, Tyr — tyrosine, Phe — phenylalanine, Trp — tryptophan, Orn — ornithine, Lys — lysine, His — histidine, Arg — arginine. The arrows indicate the correlations of amino acids with the principal components (n = 30).

The first principal component (PC1, 58.89 % of the total variance) was closely related to amino acids which contribute over 6.02 % of the total variance to age differences, namely, taurine, threonine, serine, glycine, citrulline, valine, leucine, and arginine ($p < 0.001$). The largest contribution to the second principal component (PC2, 23.36 % of the total variance) was made by aspartic acid, proline, alanine, cysteine, phenylalanine, ornithine, and histidine ($p < 0.001$) (see Fig. 2, Table 2), the percentage of which in the leech tissues showed a low age-related variability.

2. Component analysis of the age-dependent concentration of free amino acids (% of the total pool) **in tissues of medical leech *Hirudo verbana* Carena, 1820 grown in hirudoculture** (n = 30)

Amino acid (i = 22)	Loadings, a_{ij}		Contribution = $(a^2_{ij} \times 100)/\lambda_j$, %	
	1	2	1	2
Cysteic acid	0.70***	0.16	3.81	0.52
Taurine	–0.95***	–0.05	6.98	0.04
Aspartic acid	–0.58***	0.63***	2.63	7.74
Threonine	0.91***	–0.29	6.41	1.61
Serine	0.97***	–0.08	7.29	0.12
Glutamate + Glutamine	–0.35	–0.57**	0.93	6.33
Proline	–0.69***	–0.64***	3.67	8.00
Glycine	–0.90***	0.25	6.31	1.18
Alanin	–0.44*	0.70***	1.49	9.65
Citrulline	–0.96***	–0.06	7.09	0.07
Valine	0.94***	–0.24	6.79	1.10
Cysteine	0.59***	0.67***	2.72	8.68
Methionine	0.69***	0.56**	3.64	6.05
Isoleucine	–0.88***	–0.44*	5.98	3.84
Leucine	–0.93***	0.07	6.72	0.09
Tyrosine	0.80***	0.38*	4.90	2.87
Phenylalanine	0.50**	0.80***	1.95	12.37
Tryptophan	–0.83***	0.17	5.35	0.56
Ornithine	–0.64***	0.64***	3.19	7.95
Lysine	–0.81***	0.50**	5.10	4.94
Histidine	–0.36	0.91***	1.02	16.10
Arginine	–0.88***	–0.09	6.03	0.17

N o t e. 1 — PC1, 2 — PC1 (Principal Components; j = 1, 2). Eigenvalues (λ_j) PC1 = 12.95, PC2 = 5.14. PC1 explains 58.89 % of the variance, PC2 explains 23.36 % of the variance.
*, **, *** Statistically significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Our findings disclosed the role of an increased pool of amino acids involved in the regulation of antioxidant metabolic processes due to which the body resistance to hypoxia increases. The antitoxic effect lasts for a 5-month period of growth. High amino acid metabolism during early ontogenesis was also established for other aquatic organisms, e.g., for brackishwater clam *Corbicula japonica*, starry flounder *Platichthys stellatus*, and small-scaled pacu *Piaractus mesopotamicus* [9, 15, 16].

Noteworthy is the high variability in nonessential and essential amino acid levels which is due to the increased requirements for carbohydrate, lipid metabolism and protein synthesis during active growth and development of leeches. This is consistent with the available data both for medicinal leeches and for planktonic species of copepods in which, with age, the tissue amino acid profile changes significantly showing the elevated level of tyrosine involved in the synthesis of hormones [8]. A high requirement for essential amino acids and taurine was found in fry of small-scaled pacu *Piaractus mesopotamicus* [15]. In the larvae of starry

flounder *Platichthys stellatus*, the leucine and isoleucine levels were higher compared to mature fish [16). In mollusks *Corbicula japonica* used for the industrial production of biologically active food additives, the pool of free amino acids depends significantly on the stage of development and, like in medicinal leeches, glutamic acid playing a key role in nitrogen metabolism is the dominant tissue amino acid during the whole life cycle [9].

Our findings revealed a balanced set of essential AAs in *H. verbana*, indicative of the suitability of habitat conditions at different periods of the leech life cycle. A peculiarity of the studied hirudinids is the optimal amino acid balance, as evidenced by the stable EAs/NEAs values during the early ontogenesis (EAs/NEAs = 0.60) and in 9-month-old leeches (EAs/NEAs = 0.73).

In accordance with the available publications, the age-related variability of the amino acid metabolism of aquatic organisms is due to their needs for priority nutrients, and the amino acid composition of tissues and organs objectively reflects the physiological state of individuals at different stages of the life cycle [1, 6, 10], as it is also found out in our research.

Thus, there is a close relationship between the quantitative and qualitative indicators of free amino acids (AAs) in the tissues of the medical leeches *Hirudo verbana* Carena, 1820 and their growth and development, which is due to the multifunctional role and the involvement of AAs in the metabolic processes at different stages of ontogenesis. The informativeness and adequacy of the biochemical parameters in assessing physiological state and adaptive capabilities of medical leeches in aquaculture allows us to suggest these indicators for assessing the physiological status of the leeches during cultivation, in final product of hydroculture, and in the natural environment. This draws us to put forward the concept of using functional AAs as biomarkers in the development of industrial technologies for cultivation of aquaculture objects. Experimental confirmation of the complete balanced set of free AAs characteristic of the tissues of medicinal leeches serves as an additional indication for the use of leech homogenates in medicine, pharmaceuticals and veterinary medicine.

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ON THE MILK EJECTION MECHANISM IN COWS UPON AN INCREASE IN MILK YIELD PER MILKING

V.P. MESHCHERYAKOV ✉

Russian State Agrarian University — Moscow Timiryazev Agricultural Academy, Kaluga Branch, 27, ul. Vishnevskogo, Kaluga, 248007 Russia, e-mail vpmeshcheryakov@mail.ru (✉ corresponding author)
ORCID:
Meshcheryakov V.P. orcid.org/0000-0002-0408-9095
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Abstract

Sympathetic nervous system regulates milk flow through the udder duct system. However, the mechanisms of contraction of the alveoli upon accumulation of different amounts of milk in the udder have not been studied. In the paper, it is found for the first time that the removal of an increased amount of milk is accompanied by a change in the parameters of the udder blood supply caused by an increase in the contractile response of the alveolar complex. Thus, the goal of the paper is to investigate the mechanism of contractile reaction of the alveolar complex and milk removal in cows with an increased milk yield per milking. Nine black-and-white dairy cows of the 2-5th calving were tested, 7 times each, in the first half of lactation (All-Russian Scientific Research Institute of Physiology, Biochemistry and Nutrition of Farm Animals, Kaluga Province). A serial ADU-1 milking machine and a counter sensor (Latvia) were used to record milking parameters. The milk yield per milking ranged from 3.4 to 6.7 kg in the control period and was 24.5 % higher ($p < 0.001$) in the test period. The mammary blood flow (MBF) was assessed by the electromagnetic flowmetry method. A blood flow probe (Nihon Kohden, Japan) was fixed on the external pudendal artery of the udder. The average MBF values were recorded for 3 minutes prior to milking (initial period) and in milking. The points characterizing udder blood supply were marked on the MBF curve, i.e., the beginning of udder irritation, a sharp increase in the blood supply, its maximum and the baseline restoration. The time intervals until a sharp increase in MBF and an increased blood supply period were calculated. The average and maximum MBF values and an increase in mammary blood flow during milking in relation to the initial indicators were determined. The parameters of the udder blood supply were used to assess the contractile response of the alveolar complex. The latent period until milk ejection was determined based on the time from the beginning of udder irritation to a sharp increase in udder blood supply. It was established that the milk yield per milking affects the parameters of milking and the udder blood supply. The increased milk yield per milking led to a shorter period for removal of the first portion of milk ($p < 0.001$), an increase in the average intensity ($p < 0.001$) and maximum intensity ($p < 0.001$) of milk removal as well as an increase in the machine milking period ($p < 0.001$). The increased milk secretion was also accompanied by an increase in the udder blood supply. I.e., the period before the udder blood flow increased sharply shortened ($p < 0.05$), while the average ($p < 0.05$) and maximum ($p < 0.05$) MBF values during milking, as well as period of intensive blood supply to the udder ($p < 0.001$) increased. The increased mammary blood flow during the test period resulted from an increased contractile response of the myoepithelium and alveoli. The intensive contraction of the alveoli, with the increased contraction amplitude and duration, leads to a shorter latent period of milk ejection. The milk ejection and removal are under regulation of the sympathetic nervous system. Probably, the excitation of the udder sympathetic nervous system depends on the amount of accumulated milk. The udder tone during milk ejection determines both the alveoli contraction and the rate of milk movement through the milk ducts and its removal through the nipple. The increased udder tone when low filling with milk resulted in a delayed ejection, delayed of removal first portion of milk, low contractile response of the alveoli, and low milk removal. With an increase in the amount of milk in the udder, its tone decreases, resulting in a shorter latent period of milk ejection and faster removal of the first portion of milk, strengthening the contractile response of the alveoli and increasing the intensity of milk removal. Therefore, the latent period of removal of the first portion of milk can be proposed to characterize the tone of the udder sympathetic nervous system.

Keywords: cows, milk yield per milking, milk removal parameters, udder blood supply, milk ejection, udder sympathetic tone, alveoli, contractile activity

Secretion and motor activity are the udder functions. The sympathetic nervous system directs the functional activity of the udder in cows [1]. The hormones prolactin [2] and serotonin [3] are important to regulate the secretory function. The local mechanism of its regulation becomes apparent during more frequent milking of one of the udder halves during early lactation [4, 5]. Milk production and accumulation in the alveoli occurs continuously. To maintain a high secretory activity of the mammary gland, cows should be milked in a timely manner [6]. In conventional machine milking, milking intervals are relatively constant. The installation of an automatic milking system with more frequent milking leads to changes in the milk composition [7]. There is a positive genetic correlation between the automated milking frequency and milk productivity of dairy cows [8].

The motor function of the udder is realized during milk ejection, providing rapid forcing of alveolar milk into the milk duct system and cisterns under by contracting myoepithelial cells, active milk flow along the duct system and its removal from the udder. To characterize the intensity of milk ejection, intramammary pressure (IMP) [9, 10] and milk removal parameters during conventional milking [11-13] or automated milking [14]. The IMP and milk removal are influenced by the milk yield per milking. The milk yield positively correlates with machine-on time [13] and the milk removal intensity [11, 13]. Milking intensity and duration increase if the milk yield per milking increases [12, 15]. A positive relationship was found between IMP and the milk yield at quarter level [9], and it was also shown that with an increase in the filling of the udder, the maximum IMP value at forcing milk ejection [10].

The mechanisms regulating milk movement along the system of udder ducts are intensively studied. A decrease in the contractile response of the teat sphincter muscles to manual stimulation led the authors to suggested that a change in the tone of the udder sympathetic nervous system plays a role in milk removal [16]. This hypothesis was confirmed experimentally when α- and β-adrenergic receptors were found in the teat [17-20] and in large milk ducts of the cows' udder [19, 20]. The milk removal is regulated by the sympathetic nervous system through the influence on the adrenergic receptors of the smooth muscles of the milk ducts, udder cisterns and teats. Stimulation of α-adrenergic receptors of the udder causes contraction of the teat sphincter [17] and excretory ducts of the udder [21], decreases the milk removal [22] and the peak flow rate during milking [21, 23]. Prazosin blocks the udder α-adrenergic receptors, prevents contraction of the teat sphincter and causes relaxation of the muscles of its sphincter [24]. In response to β-adrenergic agonists, the muscles of the teat sphincter relax [17] and the intensity of milk flow rate during machine milking increases [21, 23]. It is assumed that in the udder, the smooth muscles of the large milk ducts of have a greater effect on the intensity of milk flow rate than the muscles of the teat and its sphincter [21, 23]. The individual ability of cows to milk ejection is determined by the ratio of α- to β-adrenergic receptors in the teat [18]. In the udder parenchyma, the concentrations of adrenoreceptors are low, so the effect of the sympathetic adrenergic system on the alveolar complex may be low [19, 20]. The IMP and milk removal parameters enable early assessment of milk ejection but not the contraction activity of myoepithelium and alveoli.

Earlier, we found a close relationship of the main parameters of milk flow with the udder blood supply before [25] and during machine milking [26]. We assumed [27] that greater blood flow to the udder is due to a higher vascular tone generated by contracting alveoli. The udder blood supply level during milking reflects contractile response of the alveolar epithelial cells.

This paper shows that an enhanced blood supply to the udder due to the contractile activity of alveolar complex provides a higher milk yield.

Our goal was to investigate contractile mechanisms in the alveoli in relation to milk removal when the milk yield per milking increases.

Materials and methods. A crossover trial was performed on nine black-and-white cows (*Bos taurus taurus*) in the first half of the second to fifth lactation (All-Russian Research Institute of Physiology, Biochemistry and Nutrition of Farm Animals, Kaluga Province). Test parameters were measured in each period in a 7-fold repetition. During the control period, the milk yield per milking per cow ranged from 3.4 to 6.7 kg, in the experimental period, the values were 24.5 % ($p < 0.001$) greater due to regulation by between-milking intervals.

Wet hygiene of the teats was carried out for 10 s, then a milking machine ADU-1 (Russia) was attached. The milk removal parameters were calculated based on the records obtained with a bucket meter-sensor (Latvia) [11].

Mammary blood flow (MBF) was estimated by electromagnetic flowmetry. A blood flow probe (Nihon Kohden, Japan) was attached to the external pudendal artery of the udder. The average MBF values were recorded for 3 min before milking (initial period) and during milking. The characteristic points of the MBF curve were i) the beginning of the udder irritation, ii) a sharp increase in the blood flow, iii) its maximum, and iv) the MBF return to the initial level were. The interval until a sharp increase in MBF, the duration of increased blood supply, the average MBF, maximum MBF and its increment during milking compared to the initial level were calculated.

The parameters of the udder blood supply were used to assess the intensity of the contractile response of the alveolar complex. The interval from the beginning of udder irritation to the moment when blood flow sharply increases indicates a latency period of milk ejection [27]. The duration of increased blood supply corresponds to the contractile reaction of the alveoli. The increase in MBF during milking characterizes the amplitude of alveolar compression and expansion.

Statistical processing, correlation and regression analyzes were performed using the Microsoft Excel package. The mean values (M) and standard errors of the means (±SEM) were calculated. Statistical significance of the differences was assessed using the Student's t-test.

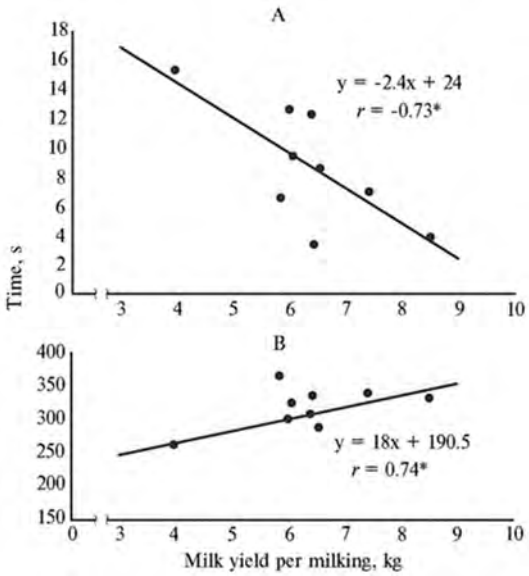
Results. As the milk yield per milking increased, milk removal parameters changed (Table 1), that is, the latency period of milk flow shortened while the average and maximum milk flow rates increased.

1. Milking parameters in black-and-white cows (*Bos taurus taurus*) in the first half of the second to fifth lactation, as influenced by milk yield per milking ($n = 9$, M±SEM, All-Russian Research Institute of Physiology, Biochemistry and Nutrition of Farm Animals, Kaluga Province)

Parameter	Period	
	control	experimental
Milk yield per milking, kg	5.11±0.12	6.36±0.15*
Total milk yield (machine milking), kg	4.51±0.13	5.77±0.16*
Machine stripping, kg	0.61±0.03	0.58±0.03
Milk flow rate, kg/min:		
average	1.19±0.04	1.38±0.04*
maximum	2.23±0.05	2.52±0.05*
Completeness of milking for the first 2 min, %	65.1±2.5	62.6±2.4
Latency period of milk flow, s	12.3±0.8	7.8±0.7*
Milking time, s:		
entire milking period	265.7±6.4	283.0±6.8
machine milking period	186.3±5.0	207.0±5.6*
machine stripping period	79.4±2.8	76.0±2.9

N o t e. Control and experimental periods are described in the *Materials and methods* section.
* Differences from the control are statistically significant at $p < 0.001$.

A higher milk yield per milking drives an increase in the machine milk yield and machine milking time with a clear tendency to lengthen the entire milking period. We did not find the effect of the milk yield on the machine strip yield, machine stripping time and the completeness of milking for first 2 min. In the experiment period, the milk yield per milking and latency period of milk flow correlate ($r = -0.73$; $p < 0.05$). Figure (A) shows calculated parameters of the linear regression for these two variables.



The relationship of the time period to the first portion of milk (A) **and the duration of increased blood flow to the udder** (B) **with milk yield per milking in black-and-white cows (*Bos taurus taurus*) in the first half of the second to fifth lactation** ($n = 9$, All-Russian Research Institute of Physiology, Biochemistry and Nutrition of Farm Animals, Kaluga Province).
* Statistically significant at $p < 0.05$.

The regression equation indicates that as the milk yield per milking increases, the time interval to removal of the cisternal milk fraction shortened. A shorter time to the first milk portion, an increase in the average and maximum milk flow rates with a greater amount of milk being milked indicated a faster milk flow and a higher milk ejection of a cow.

Our findings are consistent with the report of Bruckmaier et al. [10]. In machine milked cows with high udder filling, they revealed greater milk yield and higher milk flow for a longer time. Stimulation of β-adrenergic receptors in the udder of cows causes an increase in milk yield, the maximum milk flow in machine milking [21], and an increase in milk amount and its removal through the catheter [17]. Stimulation of α-adrenergic receptors in the udder resulted in a decrease in milk yield and maximum milk flow [23]. The blockade of α-adrenergic receptors contributed to the enhanced milk removal [24].

With a decrease in the tone of the sympathetic adrenergic nervous system of the mammary gland, teat relaxation accelerates milk removal [17]. The time until the first portion of milk directly depends on the tone of the teat sphincter. In our experiment, milk increased in yield while ejected for a shorter period ($p < 0.001$). A shorter interval to the cisternal milk ejection during the experimental period indicated a lower tone of the teat sphincters and, therefore, of the udder sympathetic innervation.

Obviously, the sympathetic tone of the mammary gland depends on the amount of milk stored in the udder before milking. At low filling, the sympathetic tone before milking is increased. In such cows, milk movement through the ducts and removal through the teat are low-efficient. Milk accumulation in the capacitive system of the gland helps to reduce its tone and thus accelerates milk removal.

The milking period depends on the parameters of milk removal. In case of no changes in the udder tone and milk flow rate in the experiment period, the milking time would increase by 24.5 %, as would the milk yield per milking. However, due to a decreased udder tone and a higher milk flow rate, the milking

time increased only by 6.5 %. Given the functional relationship, milking time should be considered a function of milk flow rate.

Higher milk yield per milking stimulated the udder blood supply (Table 2). In the experimental period, the MBF sharply increased in a shorter time and did not decrease for or a longer time than in the control. When increased milk yield per milking, the average MBF, maximum c and ΔMBF vs. basal MBF exceeded the control.

In the experimental period, we found close positive correlation ($r = 0.74$; $p < 0.05$) between the milk yield per milking and duration of increased blood flow (see Fig., B). The regression equation sows (see Fig., B) that the duration of higher blood flow to the udder increases as the udder filling increases.

2. Blood supply to the udder of in black-and-white cows (*Bos taurus taurus*) in the first half of the second to fifth lactation as influenced by milk yield per milking ($n = 9$, M±SEM, All-Russian Research Institute of Physiology, Biochemistry and Nutrition of Farm Animals, Kaluga Province)

Показатель	Period	
	control	experimental
MBF basal, l/min	2.97±0.10	3.21±0.11
Increase in MBF:		
time to onset, s	89.7±2.0	82.1±2.2*
duration, s	255.2±6.7	305.5±6.3**
MBF during milking, l/min:		
average	3.95±0.11	4.31±0.12*
maximum	4.88±0.18	5.41±0.14*
MBF increment, l/min:		
average	0.98±0.07	1.22±0.07*
maximum	1.99±0.10	2.31±0.11*

N o t e. MBF — mammary blood flow. Control and experimental periods are described in the *Materials and methods* section.
*, ** Differences from the control are statistically significant at $p < 0.05$ and $p < 0.001$, respectively.

The MBF values before milking and during milking in the control (see Table 2) are consistent with our earlier findings [26, 27]. The trend towards an increase in MBF before milking in the experiment was due to an increase in the milk yield per milking. This conclusion is in line with the relationship between MBF in half of the udder and the milk yield per milking [25].

The alveolar contractile response assessed via blood flow parameters showed that the alveolar complex regulates removal of an increased amount of milk. In the experimental period, the latency period of milk flow was 8.5 % shorter ($p < 0.05$) whilst the contractile reaction of the alveoli was 19.7 % longer ($p < 0.001$). In the control, the average and maximum compression amplitudes of the alveolar complex were 0.98±0.07 and 1.99±0.10 conventional units, respectively. The increase in milk yield per milking in the experiment caused a 24.5 % increase in the average compression amplitude ($p < 0.05$) and a 16.1 % increase the maximum compression amplitude ($p < 0.05$). A reduced latency period of milk ejection, the increased amplitudes and longer contraction of the alveoli in the experiment indicate higher contractile activity of the alveolar complex. An increase in the contractile reaction is a response to a decrease in the tone of sympathetic nervous system of the udder under higher degree of the udder filling.

The delay from the start of milking until commencement of milk ejection, a shorter time for reaching maximum intramammary pressure (MIMP), an increase in MIMP during milking with an increase in milking intervals [10] may indicate an increase in milk ejection. According to the authors, the less the filling of the alveoli with milk, the more time it takes for myoepithelial cells to efficiently movement the alveolar milk through the ducts. It is believed that the

sympathetic nervous system influences milk removal not through regulation of milk ejection , but through stimulation and inhibition of adrenergic receptors in large ducts and cisterns of the mammary gland [20].

Due to small number of adrenergic receptors in the udder parenchyma [19, 20], the sympathetic nervous system cannot directly affect the myoepithelial cells. Their contractile activity is regulated by oxytocin the release of which from the neurohypophysis is also controlled by the sympathetic nervous system. In our experiment, the decrease in the latency period and the increase in the contraction amplitude and duration in the alveoli indicate the possible involvement of oxytocin. The release rate and concentration of oxytocin can regulate the contractile activity of the alveolar complex. The release, delivery and blood concentration of the hormone is likely to increase as the amount of milk in the alveoli increases.

Thus, milk yield per milking in dairy cows affects the parameters of milk removal and blood supply to the udder. A 24.5 % increase in milk yield per milking did not affect completeness of milking for the first 2 min, the machine stripping and machine stripping time, but fastened removal of the first cistern milk fraction, increased the milk flow rate and machine milking time. The changes indicate accelerated and more intensive removal of an increased amount of milk. The blood supply to the udder also increased. Namely, period to sharp growth in blood flow to the udder was shorter, the average and maximum mammary blood flow rates increased during milking, and the increased blood supply to the udder lasted longer. The increased blood supply to the udder was due to higher contractile activity of the myoepithelium and alveoli. The amplitude and duration of alveolar contractions increased which shortens the latency period. The sympathetic nervous system influences milk ejection and removal. We assume that the degree of udder filling influences the tone of the udder sympathetic nervous system. Udder tone during milk ejection and removal determines both the alveolar contractile response and the milk movement through the ducts and teats. The increased tone of the udder with low filling leads to the delay from the start of milking until commencement of milk ejection, low contractile response of the alveolar complex and low milk flow rates. As the amount of milk in the udder increase, the udder tone decreases providing a reduction in the latency period and an increase in the contractile reaction and milk flow rate. The latency period necessary for cisternal milk removal can characterize the functional state of the teat sphincter and the tone of the udder sympathetic nervous system.

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PECULIARITIES OF TAXONOMIC AND FUNCTIONAL CHARACTERISTICS OF RUMEN MICROBIOTA OF DAIRY COWS SUFFERED FROM KETOSIS

G.Yu. LAPTEV, E.A. YILDIRIM ✉, T.P. DUNYASHEV, L.A. ILINA, D.G. TYURINA, V.A. FILIPPOVA, E.A. BRAZHNIK, N.V. TARLAVIN, A.V. DUBROVIN, N.I. NOVIKOVA

JSC Biotrof+, 19, korp. 1, Zagrebskii bulv., St. Petersburg, 192284 Russia, e-mail georg-laptev@rambler.ru, deniz@biotrof.ru (✉ corresponding author), timur@biotrof.ru, ilina@biotrof.ru, tiurina@biotrof.ru, filippova@biotrof.ru, bea@biotrof.ru, tarlav1995@biotrof.ru, dubrowin.a.v@yandex.ru, novikova@biotrof.ru

ORCID:

Laptev G.Yu. orcid.org/0000-0002-8795-6659
Yildirim E.A. orcid.org/0000-0002-5846-5105
Dunyashev T.P. orcid.org/0000-0002-3918-0948
Ilina L.A. orcid.org/0000-0003-2789-4844
Tyurina D.G. orcid.org/0000-0001-9001-2432
Filippova V.A. orcid.org/0000-0001-8789-9837
Brazhnik E.A. orcid.org/0000-0003-2178-9330
Tarlavin N.V. orcid.org/0000-0002-6474-9171
Dubrovin A.V. orcid.org/0000-0001-8424-4114
Novikova N.I. orcid.org/0000-0002-9647-4184

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Abstract

In recent years, in the livestock farms of the Russian Federation, a sharp increase in the level of milk productivity in cattle due to the introduction of high amounts of starch into the diet while reducing the proportion of non-starchy polysaccharides negatively affected many functional characteristics of the animal organism, which led, inter alia, to an increase in the prevalence of metabolic diseases. One of these disorders is ketosis which occurs in 23-80 % of highly productive cows in the post-calving period. A negative energy balance in dairy cows during lactation leads to a violation of the coordination of lipid metabolism between adipose tissue, liver, intestines and mammary glands, which leads to ketosis. The microbial community of the rumen plays a significant role in the energy homeostasis of the host, its metabolic and physiological adaptation to periods of lack of energy. In the present research, we describe the pattern of changes of microbial community structure and ongoing microbial metabolic pathways in the rumen of dairy cows with ketosis. The aim of the study was to study the influence of the incidence of ketosis in cows on the composition and metabolic potential of the rumen microbiome using NGS sequencing and quantitative PCR with reverse transcription. The proportion of ketosis in the rumen of the cow increased taxa associated with impaired energy balance, in particular, gluconeogenesis, increased synthesis of lactate, intensification of the pathogenesis process. The abundance of phylum *Bacteroidetes* decreased 1.2 times (at $p \le 0.05$). This phylum includes bacteria related to the formation of propionate and succinate, the main sources of glucose for the gluconeogenesis process and highly valuable for cows succinic and acetic acids. In the rumen of the animal with ketosis, we found an increase ($p \le 0.05$) in the proportion of the genera *Odoribacter* and *Frischella* among which there are pathogens, as well as the presence of the pathogenic species *Bacteroides fragilis.* In the cow with ketosis, non-attributable bacteria from the genus Gp15 completely disappeared from the rumen microbiota. The reconstruction and forecasting of the functional content of the metagenomic community was also carried out using a software package. A significant difference ($p \le 0.001$) was revealed in the expression level of the Ldh-L lactate dehydrogenase gene between animals: the expression level of genes associated with the synthesis of this enzyme in the cow with ketosis was 128±17.9 times higher than in the clinically healthy animal. Using the PICRUSt2 and MetaCyc software package, it was shown that the level of the predicted functional potential with respect to 12 metabolic pathways of the rumen microbiome was increased ($p \le 0.05$) in the cow with ketosis. In particular, there was an increase in the level of predicted metabolic capabilities of the microbiome associated with the implementation of heteroenzymatic lactic acid fermentation, an increase in the possibility of hexitol degradation, the synthesis of O antigens used by pathogenic forms to avoid phagocytosis and to resist the lytic effect of the complement system, etc.

Keywords: ketosis, rumen microbiome, ruminant, NGS sequencing, PICRUSt2, MetaCys, gene expression, metabolic pathways

Bulky feed in the cattle diet stimulate rumen functions, improves cicatricial microflora, digestion and animal health. Nevertheless, such feeds, as compared to concentrated feeds are characterized by a relatively low metabolic energy (ME), which is of fundamental importance in animal feeding. Consumption of voluminous feeds leads to a decrease in milk productivity, early peak lactation and a rapid decline in the lactation curve, which needs compensation for the lack of ME, namely an additional use of dietary concentrates. In recent years, on the livestock farms of the Russian Federation, the milk productivity of dairy cows sharply increased due to high-starch diets with a decrease in non-starchy polysaccharides, which adversely affects physiological functions of animals and, in particular, increases the incidence of metabolic diseases [1, 2]. Several reports [3-5] report ketosis as a metabolic disorder observed in 23-80 % of highly productive cows in the postpartum period. Ketosis is characterized by impaired carbohydrate-fat metabolism and is accompanied by indigestion, hypoglycemia, ketonemia, ketonuria, ketolactia and, as a consequence, damage to the pituitary-adrenal system, thyroid, parathyroid glands, liver, heart, kidneys and other organs [6], which leads to a 10-15 % decrease in milk production on average and reduces productive longevity.

Providing high-yielding dairy cows with nutrients, especially during lactation, requires special attention. During this physiological period, negative energy balance due to excess of energy consumption for milk production over its intake with feed disrupts the coordination of lipid metabolism between adipose tissue, liver, intestines and mammary glands, which leads to ketosis [7, 8]. Therefore, in ruminants, the blood glucose concentration plays a key role in maintaining energy metabolism and preventing metabolic diseases, primarily ketosis.

In ruminants, approximately 90 % of glucose is produced by gluconeogenesis, which occurs mainly in the liver, to a lesser extent in the cortex of the kidneys and in the epithelial cells lining the small intestine. The most important precursors in gluconeogenesis are three-carbon compounds, in particular propionate, pyruvate and lactate which are produced exclusively by cicatricial microflora [9].

Some studies [10, 11] show that the microbial community of the digestive system is crucial for the host energy homeostasis, its metabolic and physiological adaptation to periods of lack of energy. It is important that the energy metabolism of ruminants differs from that of monogastric animals, since energy is supplied mainly due to microbial fermentation in the rumen [12]. In healthy animals, under the action of microbial enzymes, cellulose which is rich in ruminant feed is hydrolyzed in the rumen to cellobiose, then to glucose, and then to lactate [13, 14]. Finally, as a result of a cascade of enzymatic reactions carried out mainly by bacteria of the order *Selenomonadales*, volatile fatty acids (VFA) are synthetized from lactate [15]. The quantitative ratio of lactic, acetic, propionic and butyric acids formed in the rumen depends on the composition of the rumen microbiota [16, 17]. VFAs absorbed in the rumen are transported to the liver and involved in gluconeogenesis which provides the animal with glucose.

software complexes for reconstructing the functional potential of the microbiome profile using 16S metagenomics data are recognized as good bioinformatic tools for phylogenetic analysis of microbial communities [18]. The functions of genes are fairly accurately reproduced on the basis of data from a meta-taxonomic study of DNA sequences [19].

In dairy cows during pregnancy and early lactation, when the risk of ketosis

is the greatest, significant changes in the composition of the rumen microbiota have been revealed [20]. It was also shown that in cows, the number of representatives of the rumen microbial community correlates with the concentration of ketone bodies in the biological fluids [21]. In general, it should be noted that the composition of the rumen microbiota has been studied in sufficient detail. However, there are few studies concerning the structure of the microbial community and microbial metabolic pathways during ketosis in dairy cows [22].

In this work, we have shown that in the rumen of dairy cows with ketosis, shifts in the number of cicatricial microbiota are associated with changes in its functional activity and expression of genes for the synthesis of L-lactate dehydrogenase.

Our goal was to compare the composition and metabolic potential of the rumen microbiome of a clinically healthy cow and a cow with ketosis.

Materials and methods. Two black-and-white Holsteinized dairy cows (No. 1 and No. 2) of the 3rd lactation with milk productivity 11310 and 9800 l/year, respectively, were assigned for the trial (SPK Kobralovsky, Gatchinsky District, Leningrad Province, winter 2020). Based on the clinical signs (lethargy, decreased skin elasticity, ruffled coat, fecal density, rare defecation), animal No. 2 was diagnosed with ketosis.

The cows kept in tie-stalls were fed with the ration which was 26 kg silage, 1.3 kg hay, 2 kg dry beet pulp, 2 kg dry beer grains, 3.5 kg crushed corn, 1.5 kg extruded corn, 3 kg rolled barley, 0.8 kg wheat bran, 0.25 kg feed yeast, 5 kg rapeseed meal, 0.5 kg soybean meal, 110 g chalk, 250 g mineral additives, and 100 g of salt; the digestible energy of the feed was 10.7 mJ per 1 kg dry matter.

The contents of the rumen (10-50 g sample, in 3 replicates from each cow) were aseptically collected manually with a sterile probe.

The content of ketone bodies in milk was determined as described in [23].

Total DNA was extracted from the samples using a Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) according to the attached instructions.

The composition of the rumen bacterial community was analyzed by NGS sequencing on the MiSeq platform (Illiumina, Inc., USA) with primers targeted to the V3-V4 region of the 16S rRNA gene: forward primer 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGG-3′, reverse primer 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′.

Sequencing was performed using Nextera® XT Index Kit (Illiumina, Inc., USA) for generation of libraries, Agencourt AMPure XP (Beckman Coulter, Inc., USA) for the purification of PCR products, and MiSeq® ReagentKit v2 (500 cycle) (Illiumina, Inc., USA) for sequencing. The maximum paired-end read length was 2×250 bp.

Bioinformatic data analysis was performed using the Qiime2 version 2019.10 software (https://docs.qiime2.org/2019.10/). After the initial import of sequences into Qiime2 format, paired read lines were aligned. The sequences were then filtered by quality using the default settings. The noise sequences were filtered using the Deblur method, with the maximum length of trimming sequences of 250 bp (https://msystems.asm.org/content/msys/2/2/e00191-16.full.pdf). For the samples No. 1 and No. 2, 1527 and 5040 sequences were obtained, respectively. To reconstruct phylogeny de novo, the MAFFT software package was utilized (https://mafft.cbrc.jp/alignment/software/), then masked sequence alignment was performed. For taxonomy analysis, reference database GreenGenes 13.5 99 % (https://greengenes.secondgenome.com) was used.

Based on the resultant table of OTUs (operational taxonomic units) using

QIIME 1.9.1 software package plugins (http://qiime.org), the alpha diversity indices were calculated and a graph of the number of OTUs vs. the number of reads was plotted. During the statistical analysis, the diversity indices were not additionally transformed. For rumen microbiota biodiversity, Chao1, Shannon index H, and Simpson index D were calculated [24].

For predictive functional profiling of microbial community metagenome, gene families, and enzymes was performed using the PICRUSt2 software package (v.2.3.0) (https://github.com/picrust/picrust2) [18] according to the recommended scenario (default settings). The nearest-sequenced taxon index (NSTI) of the closest taxon for both samples was 0.3, which allows for prediction of metabolic pathways. For the analysis of metabolic pathways and enzymes, the MetaCyc database (https://metacyc.org/) was used. MetaCyc metabolic pathway profiles were assessed after normalization of ASV (Amplicon Sequence Variants) abundance with base 2 logarithm [18].

Total RNA was extracted from cicatricial samples using Aurum Total RNA kit (Bio-Rad, USA) according to the manufacturer's instructions.

Using iScript RT Supermix kit (Bio-Rad, USA), a reverse transcription reaction was performed to obtain cDNA [25]. The relative expression of genes *ldb0101* and *ldh-L* in bacteria capable of lactic acid fermentation was assessed by qPCR. Amplification reaction with primers targeted to the *ldb0101* gene associated with the synthesis of D-lactate dehydrogenase (F 5′-GCGGGATCCGATGACTAAAATTTTTGCT-3′ and R 5′-GCGTGTCGACTTAGCCAACCTTAACTGG-3′) and the *ldh-L* gene for L-lactate dehydrogenase (F 5′-CATCAAAAAGTTGTGTTAGTCGGCG-3′ and R 5′-TCAGCTAAACCGTCGTTAAGCACTT-3′) were performed using SsoAdvanced™ Universal SYBR® Green Supermix kit (Bio-Rad, USA) according to the manufacturer's protocol. Amplification programm: 1 min at 95 °C (1 cycle); 15 s at 95 °C, 1 min at 50 °C (45 cycles).

Relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method [26]. Primers to the universal gene encoding the ribosomal 16S subunit of prokaryotes (F 5'-AGGCCTTCGGGTTGTAAAGT-3′, R 5'-CGGGGATTTCACATCTCACT-3') were used as a reference control.

Mathematical and statistical processing was performed using the Microsoft Office Excel 2003 and R-Studio software packages (Version 1.1.453) (https://rstudio.com). Differences were considered statistically significant at $p < 0.05$ in the Student's *t*-test.

Results. Analysis of the of ketone bodies in milk revealed a significant excess over the reference values (0.9±0.04 g/l for No. 2 vs. reference values of 0.06-0.08 g/l) and confirmed ketosis in one of the dairy cows diagnosed by the clinical signs. We did not find ketone bodies in the milk of a clinically healthy cow. Bovine ketosis is characterized by the accumulation of ketone bodies, mainly beta-hydroxybutyrate, acetoacetate and acetone, which can be measured in body fluids including blood, urine, and milk [27].

Analysis of taxonomic diversity based on the data of 16S metagenomic NGS sequencing of DNA from the rumen contents of both cows revealed 21 phyla of microorganisms of which *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* dominated (Fig. 1). The abundance of *Bacteroidetes* and *Firmicutes* members in the rumen of dairy cows is in line with earlier data of other authors [28-30]. However, the proportion of representatives of *Bacteroidetes* detected in our study was significantly higher compared to the data of these researchers. Such differences can be associated with the composition of the diets, the milk yield, and other factors [31]. Interestingly, in a cow with a diagnosis of ketosis, compared to clinically healthy cows, the representation of bacteria of the phylum *Bacteroidetes* decreased 1.2

times ($p \leq 0.05$) while the phyla *Firmicutes* and *Proteobacteria* increased 2.1 times ($p \leq 0.001$) and 3.8 times ($p \leq 0.01$). Earlier Grum et al. [21] also demonstrated a decrease in the counts of the phylum *Bacteroidetes*, the order *Bacteroidales* and the family *Prevotellaceae* whilst the concentration of ketone bodies in biological fluids of cows increased. Similar results have also been reported for the *Prevotellaceae* family [32, 33].

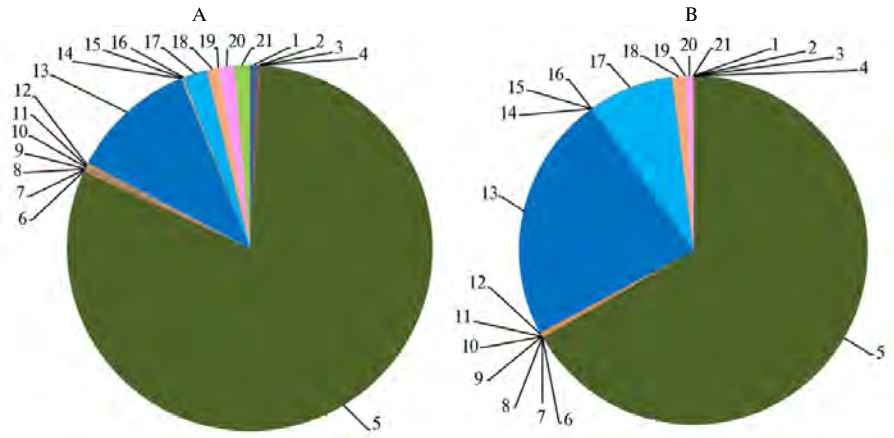

Fig. 1. Abundance of bacterial phyla in rumen of the 3rd lactation black-and-white Holsteinized cows: A — the clinically healthy cow, B — the cow diagnosed with ketosis; 1 — *Acidobacteria*, 2 — *Actinobacteria*, 3 — *Aquificae*, 4 — *Armatimonadetes*, 5 — *Bacteroidetes*, 6 — *Candidatus_Saccharibacteria*, 7 — *Chlamydiae*, 8 — *Chloroflexi*, 9 — *Cyanobacteria*, 10 — *Elusimicrobia*, 11 — *Euryarchaeota*, 12 — *Fibrobacteres*, 13 — *Firmicutes*, 14 — *Latescibacteria*, 15 — *Lentisphaerae*, 16 — *Planctomycetes*, 17 — *Proteobacteria*, 18 — *Spirochaetes*, 19 — *Synergistetes*, 20 — *Tenericutes*, 21 — *Verrucomicrobia* (SPK Kobralovsky, Gatchinsky District, Leningrad Province, 2020).

A detailed analysis of the cows' microbiomes at the genus level revealed significant differences in 13 bacterial families (*Acidaminococcaceae*, *Anaeroplasmataceae*, *Bacteroidaceae*, *Bifidobacteriaceae*, *Enterococcaceae*, *Eubacteriaceae*, *Lachnospiraceae*, *Lactobacillaceae*, *Orbaceae*, *Porphyromonadaceae*, *Rikenellaceae*, *Ruminococcaceae*, *Succinivibrionaceae*) and 26 genera, including *Bacteroides*, *Bifidobacterium*, *Butyrivibrio*, *Coprococcus*, *Dysgonomonas*, *Enterococcus*, *Eubacterium*, *Frischella*, *Lachnospira*.

In the rumen of the cow diagnosed with ketosis, as compared to the clinically healthy cow, we detected a lower proportion of representatives of the phylum *Bacteroidetes*, i.e., bacteria of the families *Bacteroidaceae* ($p \leq 0.05$) and *Porphyromonad*aceae ($p \leq 0.05$) (Fig. 2, A). In particular, the number of bacteria of the genus *Bacteroides* spp. belonging to the *Bacteroidaceae* family decreased 2.1-fold times ($p \leq 0.05$) and of the genus *Dysgonomonas* spp. from the family *Porphyromonadaceae* decreased 3.6-fold ($p \leq 0.01$). For example, *Dysgonomonas* species, in particular *D. gadei* and *D. hofstadii* completely disappeared from the rumen of the cow diagnosed with ketosis. Among bacteria of the genus *Bacteroides*, the species *B. caecigallinarum* and *B. eggerthii* were not detected in the rumen of the cow with metabolic disorders. It is known that many members of the genera *Bacteroides* and *Dysgonomonas* produce fatty acids in the rumen, including significant amounts of propionate [34]. As noted above, VFAs resulted from cicatricial fermentation are utilized as energy resources for animals; moreover, propionate is the main source of glucose for gluconeogenesis [8]. Therefore, propionate is of great physiological importance for ruminants, and a decrease in the number of these microorganisms in one of the cows in our study could become a provoking factor for ketosis.

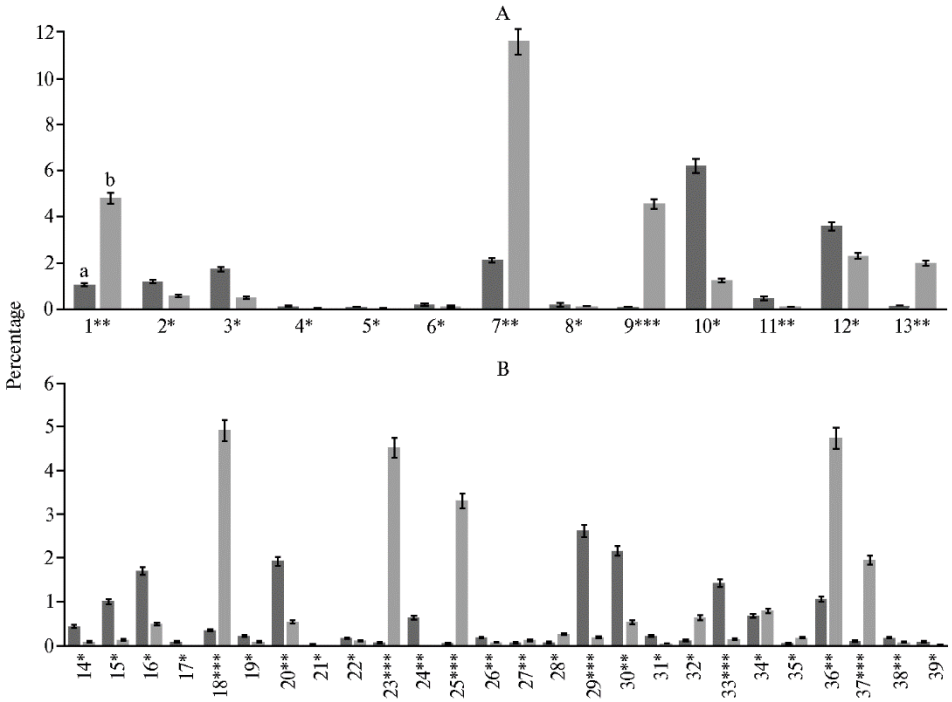

Fig. 2. Bacterial taxa which significantly differ in abundance in rumen of the 3rd lactation black-and-white Holsteinized cows: A — families, B — genera; a — the clinically healthy cow, b — the cow diagnosed with ketosis; 1 — *Acidaminococcaceae*, 2 — *Anaeroplasmataceae*, 3 — *Bacteroidaceae*, 4 — *Bifidobacteriaceae*, 5 — *Enterococcaceae*, 6 — *Eubacteriaceae*, 7 — *Lachnospiraceae*, 8 — *Lactobacillaceae*, 9 — *Orbaceae*, 10 — *Porphyromonadaceae*, 11 — *Rikenellaceae*, 12 — *Ruminococcaceae*, 13 — *Succinivibrionaceae*; 14 — *Alistipes*, 15 — *Anaeroplasma*, 16 — *Bacteroides*, 17 — *Bifidobacterium*, 18 — *Butyrivibrio*, 19 — *Coprococcus,* 20 — *Dysgonomonas*, 21 — *Enterococcus*, 22 — *Eubacterium*, 23 — *Frischella*, 24 — Gp15, 25 — *Lachnospira*, 26 — *Lactobacillus*, 27 — *Odoribacter*, 28 — *Oribacterium*, 29 — *Parabacteroides*, 30 — *Paraprevotella*, 31 — *Porphyromonas*, 32 — *Pseudobutyrivibrio*, 33 — *Saccharofermentans*, 34 — *Selenomonas*, 35 — *Shuttleworthia*, 36 — *Succiniclasticum*, 37 — *Suc-cinivibrio*, 38 — *Lactobacillus*, 39 — *Bifidobacterium* (SPK Kobralovsky, Gatchinsky District, Leningrad Province, 2020).

*, **, *** Differences between the sick and health cow statistically significant at $p \leq 0.05$, $p \leq 0.0$, and $p \leq 0.001$, respectuvely.

In rumen, propionate is synthetized via the acrylate pathway or the succinate pathway [35]. Bacteria of the genus *Megasphaera* are the main producers of propionate via the acrylate pathway [36, 37]. In our study, the proportion of *Megasphaera* bacteria was trace (from 0.006±0.0005 to 0.01±0.0007 %) (see Fig. 2, B) while two genera, the *Bacteroides* spp. and *Dysgonomonas* spp. capable of propionate production via the succinate pathway rather than the acrylate pathway [38-40] were much more abundant, from 0.47±0.03 to 1.7±0.2 % and from 0.500±0.034 to 1.9±0.3 %. Macy et al. [41] described similar route for the propionate synthesis.

In the cow diagnosed with ketosis, the number of the *Porphyromonadaceae* family of the phylum *Bacteroidetes* was 5.1 times lower ($p \leq 0.05$) than in the clinically healthy cow. In particular, in this family, bacteria of the genus *Parabacteroides* decreased 15.9 times ($p \leq 0.001$). The dominant representative of this genus, *P. chinchillae* fell from 2.2±0.13 to 0.01±0.0004%. Earlier, Wang et al. [42] showed that the synthesis of succinate which is the main metabolite of *Parabacteroides* bacteria was associated with a decrease in hyperglycemia in mice through activation of intestinal gluconeogenesis. Thus, members of the *Porphyromonadaceae* family may be associated with ketosis resistance in animals.

A decrease in the ruminal abundance of the genus *Paraprevotella* (the family *Prevotellaceae*) represented mainly by *P. clara* ($p \leq 0.01$), in the sick cow could also cause metabolic disorders. It was shown that *P. clara* and *P. xylaniphila* from the human intestine produce succinic and acetic acids. In cows, these compounds are highly valuable end products of glucose metabolism [43].

In the rumen of the sick cow, we found an increase ($p \leq 0.05$) in two genera among which there are pathogens, namely *Odoribacter* of *Porphyromonoadaceae* family and *Frischella* of *Orbaceae* family. The pathogenic species *Bacteroides fragilis* was also more abundant. In particular, the *Odoribacter denticanis* that we found in the sick cow is the causative agent of human abdominal abscess [44]. Interestingly, bacteria of the genus *Frischella* have not been previously detected in the rumen. The genus *Frischella* that we discovered were represented in the rumen of cows by the only species, the *F. perrara*. The *F. perrara* are present in the intestines of the honey bee where it induces scab, a disease that manifests itself as a dark streak on the epithelial surface of the pylorus [45]. *Bacteroides fragilis* was isolated from tissues of 105 aborted cow fetuses. The histopathological lesions included placentitis and bronchopneumonia [46].

An increase in the abundance of pathogenic forms could also provoke ketosis, since, as noted, the so-called secondary ketosis accompanies infectious and gastrointestinal diseases.

Perhaps the lower number of pathogenic forms in the rumen of a clinically healthy cow was associated with the displacement of these microorganisms by the indigenous normal flora (genera *Bifidobacterium*, *Alistipes*, etc.) with antimicrobial activity. For example, in the clinically healthy cow, we identified bacteria of *Bifidobacteriaceae* family (0.07±0.004 %) known for their pronounced antimicrobial properties [47] while these microorganisms were absent in the sick cow. The increased abundance ($p \leq 0.01$) of *Rikenellaceae* family from the phylum *Bacteroidetes* in the rumen of a cow without metabolic disturbances could also have a positive effect on the health of the animal. Recently, a study was conducted that showed a direct relationship between a representative of this family *Alistipes finegoldii* and a decrease in the manifestations of colitis [48]. Other researchers [49] have identified in the *Alistipes* spp. the ability to produce anti-inflammatory sphingolipids. Bacteria of the genus *Anaeroplasma* (phylum *Tenericutes*) the number of which also increased in the rumen of a clinically healthy cow ($p \leq 0.05$) are known to induce the anti-inflammatory cytokine TGF-β which enhances the intestinal barrier by increasing the synthesis of mucosal IgA [50]. The genus *Coprococcus* (phylum *Firmicutes*) which was also more abundant ($p \leq 0.05$) in the rumen of the healthy cow were associated with a decrease in the incidence of rectal cancer [51].

The complete absence of non-attributable bacteria from the genus Gp15 in the sick cow indicates a positive role of these bacteria in regulation of microbiological and metabolic processes of the digestive system. This conclusion is of fundamental interest, since at present, the functional features of the genus Gp15 are practically uncharacterized not studied as these microorganisms are unculturable.

1. Alpha diversity of ruminal microbial community in the 3rd lactation black-and-white Holsteinized cows based on NGS sequencing (*M*±SEM, SPK Kobralovsky, Gatchinsky District, Leningrad Province, 2020)

Cows	OTU	Chao1	Shannon H	Simpson D
Clinically health cow	96.0±5.15	97.1±4.92	6.1±0.54	0.98±0.06
Cow diagnosed with ketosis	125.0±6.40*	126.0±6.16*	5.8±0.41	0.97±0.05

N o t e. OTU — operational taxonomic unit.
* Differences between the cows are statistically significant at $p \leq 0.05$.

Table 1 shows the alpha biodiversity indices of the rumen microbial community in two cows, clinically healthy and sick. When comparing the Shannon's and Simpson's diversity indices, we did not establish statistically significant differences between the animals. The Chao1 index which estimates the absolute number of OTUs in the community was 1.3 times higher ($p \leq 0.05$) for the rumen microbiota of the cow diagnosed with ketosis.

Similar data were obtained when calculating the OTUs. As can be seen from Figure 3, a flatter curve of OTU accumulation was characteristic for the rumen microbiome of the healthy cow as compared to the sick cow. Therefore, it can be assumed that in ketosis, distribution of species as per abundance in the ruminal community is less uniform while the abundance of the represented species is greater compared to clinically healthy animals.

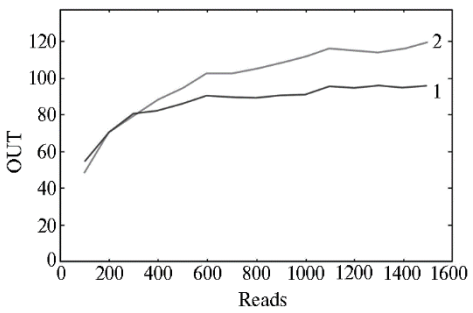

Fig. 3. Operational taxonomic units (OUTs) vs. reads in NGS sequencing analysis of ruminal microbiota of the 3rd lactation black-and-white Holsteinized cows: 1 — the clinically healthy cow, 2 — the cow diagnosed with ketosis (SPK Kobralovsky, Gatchinsky District, Leningrad Province, 2020).

Production of lactate in the rumen is a key stage in the initiation of metabolic disorders in ruminants [52]. Using quantitative reverse transcription PCR, we studied the expression of bacterial genes associated with the production of lactate dehydrogenases. In the rumen, lactate can be synthetized from pyruvate by two different NAD-linked lactate dehydrogenases, the key bacterial enzymes of lactic acid fermentation. The lactate dehydrogenase is involved in production of L(+) lactate (EC 1.1.1.27) and is encoded mainly by the *ldh-L* gene [53, 54], another lactate dehydrogenase (EC 1.1.1.28) is involved in production of D(–) lactate [55]. The following genes known to date are involved in the synthesis of this enzyme: *ldb0101*, *ldb0813*, *ldb1010*, *ldb1147*, and *ldb2021*. Evaluation of the expression of genes *ldh-L*, *ldb0101*, *ldb0813*, *ldb1010*, *ldb1147* and *ldb2021* should give an adequate understanding of the lactate synthesis in the rumen of cows. We chose qPCR with reverse transcription to study gene expression due to the generally recognized reproducibility, high sensitivity and specificity of this method for assessing the expression of bacterial genes [56-58].

No expression of genes *ldb0813*, *ldb1010*, *ldb1147*, and *ldb2021* occurred in the rumen of both cows. We did not find any significant differences between animals in expression level of the *ldb0101* gene of lactate dehydrogenase EC 1.1.1.28 (Fig. 4, A). Nevertheless, a significant difference ($p \leq 0.001$) was found between animals in the expression of the *ldh-L* lactate dehydrogenase EC 1.1.1.27 gene. In the cow diagnosed with ketosis, the expression of genes associated with the synthesis of this enzyme was 128 ± 17.9 times higher than in the clinically healthy animal (see Fig. 4, B). The obtained results are logical, since in case of unbalanced feeding cows can indeed experience dysbiotic disorders of the cicatricial microflora [59], and the equilibrium can shift towards the lactate, an intermediate fermentation product in the rumen. Excess lactate correlates with decreased rumen pH and lactic acidosis [59]. Under acidosis conditions, as a result of suppression of VFA producers sensitive to a decrease in pH, e.g., *Selenomonas ruminantium* and *Megasphaera elsdenii* [8], the rate of propionate production in the rumen decreases, therefore, the gluconeogenesis in animals can slow down, increasing the risk of ketosis.

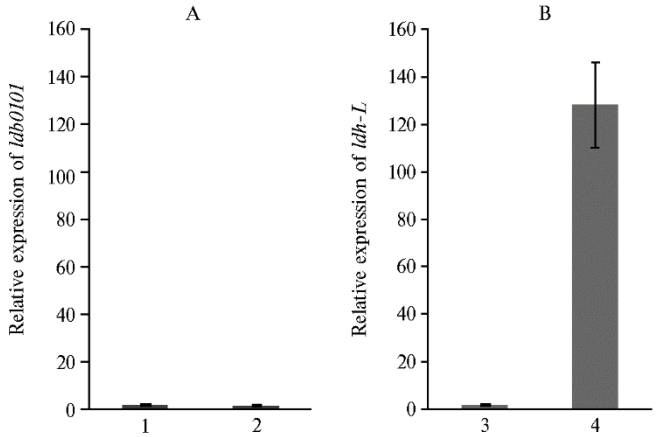

Fig. 4. Relative expression of genes involved in synthesis of lactate dehydrogenases in the rumen of the 3rd lactation black-and-white Holsteinized cows (vs. the reference prokaryotic gene of 16S rRNA): A — a gene involved in D(–) lactate metabolic pathway, B — the gene for L-lactate dehydrogenase; 1 — the clinically healthy cow, 2 — the cow diagnosed with ketosis (SPK Kobralovsky, Gatchinsky District, Leningrad Province, 2020).

Reconstruction and prediction of the functional activity of the rumen community based on metagenomic analysis of nucleotide sequences of 16S rRNA genes was carried out using the PICRUSt2 software package and MetaCyc. We annotated various metabolic pathways (232 in total) of which in 12 pathways a predicted functional potential was significantly ($p \leq 0.05$) enhanced in the cow diagnosed with ketosis as compared to the clinically healthy animal (Fig. 5).

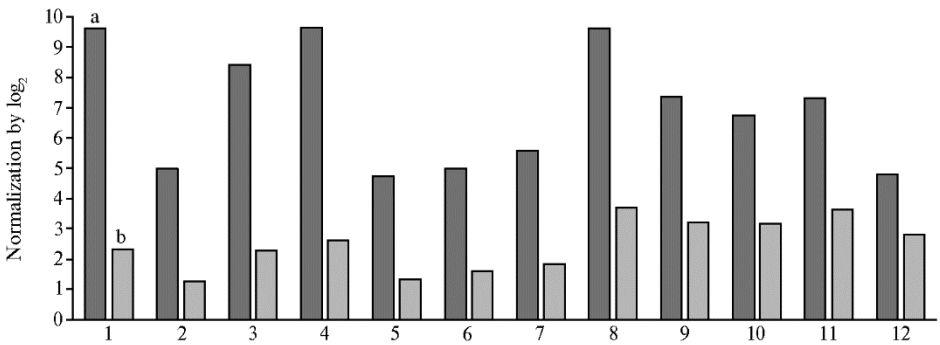

Fig. 5. Functional annotation ($p \leq 0.05$) **of metabolic pathways in ruminal metagenomic community of the 3rd lactation black-and-white Holsteinized cows:** a — the cow diagnosed with ketosis, b — the clinically healthy cow; 1 — utilization of sucrose, 2 — biosynthesis of methaquinones, 3 — utilization of fructose, 4 — biosynthesis of structural blocks for the synthesis of O-antigen, 5 — degradation of myo-, chiro- and scylloinositol, 6 — biosynthesis of (R, R)-butanediol, 7 — degradation of 4-aminobutanoate, 8 — utilization of glucose and glucose-1-phosphate, 9 — degradation of hexitols, 10 — fermentation of hexitols to lactate, 11 — synthesis of vitamin B_{12}, 12 — heteroenzymatic lactic acid fermentation (SPK Kobralovsky, Gatchinsky District, Leningrad Province, 2020).

In particular, as compared to the clinically healthy cow, the predicted metabolic potential of the microbiota associated with heteroenzymatic lactic acid fermentation (60) increased in the sick cow which is consistent with the data on the expression of the L-lactate dehydrogenase EC 1.1.1.27 gene (see Fig. 4, B).

Moreover, in the rumen microbiota of both cows, the analysis identified genes encoding most of the critical enzymes of the lactic acid fermentation pathway, i.e., fructokinase (EC 2.7.1.4), glucose-6-phosphate isomerase (EC 5.3.1.9), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), phosphoketolase (EC 4.1.2.9), phosphate acetyltransferase (EC 2.3.1.8), D-lactate dehydrogenase (EC 1.1.1.28),

and L-lactate dehydrogenase (EC 1.1.1.27) (see Fig. 5). The abundance of genes associated with the synthesis of D-lactate dehydrogenase and L-lactate dehydrogenase in the cow diagnosed with ketosis was 2.0 times and 1.5 times higher ($p \leq 0.05$) than in the clinically healthy cow (Fig. 6).

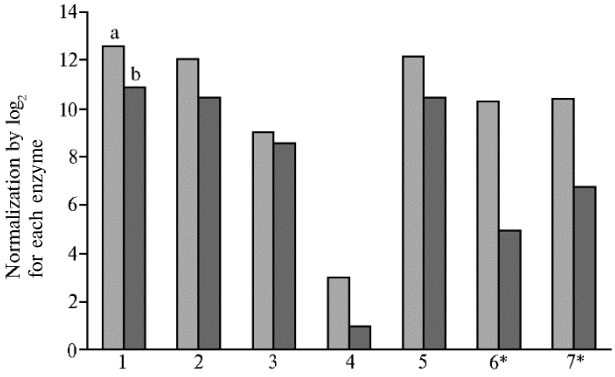

Fig. 6. Functional annotation ($p \leq 0,05$) **of enzymes predicted in the ruminal metagenomic community of the 3rd lactation black-and-white Holsteinized cows:** a — the cow diagnosed with ketosis, b — the clinically healthy cow; 1 — fructokinase, 2 — glucose-6-phosphate isomerase, 3 — glucose-6-phosphate dehydrogenase, 4 — phosphoketolase, 5 — phosphate-acetyltransferase, 6 — D-lactate dehydrogenase, 7 — L-lactate dehydrogenase (SPK Kobralovsky, Gatchinsky District, Leningrad Province, 2020).
* Differences between sick and clinically healthy animals are statistically significant at $p \leq 0.05$.

Interestingly, despite the revealed increase in metabolic potential associated with the production of lactate in the rumen of the animal suffering from ketosis, the abundance of lactate-producing lactic bacteria from the *Lactobacillaceae* family was higher ($p \leq 0.05$) in the rumen of the clinically healthy cow (see Fig. 2, A). This may be due either to the fact that different types of lactobacilli exhibit unequal functional activity of genomes and the ability to produce lactate, or microorganisms of other taxa possess this ability. For example, as can be seen from Figure 2, A, in the rumen of the cow suffering from ketosis, as compared to the healthy animal, the proportion of bacteria of the *Lachnospiraceae* family increased 5.6-fold ($p \leq 0.01$). The abundance of bacteria of this family, namely of the genera *Oribacterium*, *Pseudobutyrivibrio* and *Shuttleworthia* increased 4.5-fold, 6.3-fold and 5.6-fold ($p \leq 0.05$) in the rumen of the cow diagnosed with ketosis. We revealed that, due to the disease of the animal, a new species of the genus *Pseudobutyrivibrio*, the *P. xylanivorans* appears in the rumen. It is known that the main end products of metabolic fermentation in these bacteria are organic acids, including lactate [61-63]. Similarly, the number of microorganisms of the genus *Succinivibrio* from the family Succinivibrionaceae, capable of producing lactate in addition to acetate, succinate, and formiate also increased ($p \leq 0.001$) in the rumen of the cow with health problems [64].

Returning to Figure 5, it should be emphasized that, in the cow with impaired health as compared to the clinically healthy animal, the metabolic capabilities of the microbiota associated with the utilization of glucose and glucose-1-phosphate increased 2.6 times ($p \leq 0.05$). The VFAs produced by the microflora and absorbed in the rumen then are transported to the liver and involved in gluconeogenesis to provide the host animal with glucose. The concentration of blood glucose plays a key role in maintaining energy metabolism and preventing metabolic diseases, especially ketosis. It is well known that, in addition to VFA-synthesizing bacteria, many other microorganisms, for example, members of the *Enterobacteriaceae* family, actively utilize glucose and glucose-1-phosphate as sources of nutrients, forming alcohols as the main product. Thus, *Escherichia coli* is capable of culture on exogenous glucose-1-phosphate as the only carbon source [65]. This can lead to a decrease in the amount of nutrient substrate for bacteria of the order *Selenomonadales* and impairment of VFA synthesis.

In addition, in the cow suffering from ketosis, the functional potential of the cicatricial microbiota associated with the biosynthesis of butanediol increased 3.0 times ($p \leq 0.05$) (see Fig. 5). In the rumen, alpha-acetolactate decarboxylase synthesizes butanediol from pyruvate which is necessary for glucose synthesis during gluconeogenesis. As a result, in the cow diagnosed with ketosis, the concentration of pyruvate which is highly valuable for the production of glucose could be reduced. This can also create prerequisites for metabolic disorders. The metabolic function of butanediol itself is not well understood although there are indications that it may play a role in preventing intracellular acidification [66].

Interestingly, in the cow diagnosed with ketosis, the potential degradation of hexitols in the rumen content also increased 2.3 times ($p \leq 0.05$). The diets enriched with hexitols, e.g., D-mannitol, D-sorbitol, and galactitol was associated with the accumulation of glycogen in the mammalian liver [67]. Therefore, the degradation of hexitols could provoke ketosis which occurs under carbohydrate deficiency and is associated with mobilization of glycogen stores from the liver.

Importantly, in cicatricial microbiota of the sick cow, there was a 3.6-fold increase ($p \leq 0.05$) in metabolic capabilities of myo-, chiro- and scyllo-inositol degradation, a 3.0-fold increase in 4-aminobutanoate degradation ($p \leq 0.05$), and a 3.7-fold increase in biosynthesis of O-antigen structural blocks ($p \leq 0.05$).

Metabolic degradation of myoinosite was originally described for *Klebsiella aerogenes* [68]. Myoinositol and other stereoisomers of the hexahedral alcohol inositol in the form of various inositol phosphates and phosphatidylinositol lipids act as important signal transmitters in intracellular signaling cascades, participating in the regulation of intracellular calcium content, signal transmission from the insulin receptor, lipid cleavage and reduction of neurotransmitter cholesterol concentration. A decrease in the synthesis of myoinositol in the rumen is associated with a decrease in the formation of hepatic lipoproteins. During the lactation period of dairy cows, lipid mobilization is extremely intense, and the need for myoinositol may exceed its availability. Thus, an increase in the degradation of this compound by the cicatricial microflora could participate in the initiation of ketosis as a metabolic disorder.

The structural blocks of O-antigen polymers are oligosaccharide subunits that serve as a component of the outer membrane of some gram-negative bacteria [69]. A number of obligate and opportunistic microorganisms, e.g., *Salmonella enterica*, *Francisella tularensis*, and *Burkholderia cepacia* use O-antigens to avoid phagocytosis and resist the lytic action of the complement system [70-72]. The ability to synthesize O-antigens revealed in the cicatricial microbiota during ketosis is important, since there are not only primary (metabolic) ketosis arising from disturbances in feeding and maintenance regimens but also secondary ketosis arising as a concomitant disease in animals suffering from gastrointestinal diseases and some infectious diseases [73]. This is consistent with the increased ($p \leq 0.05$) abundance of *Odoribacter* genera of the *Porphyromonoadaceae* family, *Frischella* genera of the *Orbaceae* family comprising pathogens and the pathogenic species *Bacteroides fragilisthat* that we reveled in the rumen of the sick cow.

We discovered the potential increase in the degradation of 4-aminobutanoate in the cow diagnosed with ketosis, which is another negative factor, since 4-aminobutanoate serves as the main inhibitory neurotransmitter in the mammalian brain [74]. It is known that many bacteria can synthesize 4-aminobutanoate as an intermediate in the degradation of putrescine. The first evidence of bacterial degradation of 4-aminobutanoate was found when studying the metabolism of the bacterium *Pseudomonas fluorescens* [75] which can grow on 4-

aminobutanoate as the only source of carbon and nitrogen [76].

So, combination of molecular and bioinformatics methods allowed us to study in detail the rumen microbiome in cows and to predict its functional potential depending on the state of health. In the rumen of a cow with ketosis, the proportion of taxa associated with impaired energy balance, in particular gluconeogenesis, increased L-lactate synthesis, and increased pathogenesis, is higher. Therefore, an increase in the abondance of these microorganisms could cause or exacerbate ketosis, or both. In the cow suffered from ketosis, the expression of genes associated with the synthesis of lactate dehydrogenase (EC 1.1.1.27) increased 128±17.9 times compared to the clinically healthy animal. The obtained estimate of gene expression is consistent with the data on the metabolic potential of the cicatricial microbiota predicted from metagenomic analysis of nucleotide sequences of 16S rRNA genes. Using the PICRUSt2 and MetaCyc software package, 232 metabolic pathways have been annotated. In 12 metabolic pathways, the predicted functional potential is enhanced in the cow diagnosed with ketosis as compared to the clinically healthy cow. In particular, the number of genes associated with synthesis of D-lactate dehydrogenase and L-lactate dehydrogenase, with utilization of glucose and glucose-1-phosphate, with biosynthesis of butanediol and degradation of hexitols was increased. These findings can be used to predict metabolic disorders in cows, in particular ketosis. However, further research is needed to confirm whether there really is a causal relationship between the occurrence of ketosis, changes in the abundance of specific microorganisms in the rumen, their metabolic potential, and gene expression in the microbial community of the digestive system. Perhaps this will allow for an approach to optimization of the microbial community composition to correct ketosis. In particular, probiotic preparations for direct regulation of the abundance and composition of the rumen microbiota may be helpful.

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INFLUENCE OF PHYTOLYTIC AND PROTEOLYTIC ENZYMES ON CONVERSION OF WHEAT AND CORN GRAIN POLYMERS

L.V. RIMAREVA, M.B. OVERCHENKO, E.M. SERBA ✉, N.I. IGNATOVA, N.V. SHELEKHOVA

All-Russian Research Institute of Food Biotechnology — a Branch of Federal Research Center for Nutrition, Biotechnology and Food Safety, 4b, ul. Samokatnaya, Moscow, 111033 Russia, e-mail serbae@mail.ru (✉ corresponding author), lrimareva@mail.ru, mb_over@mail.ru, ignatova59@list.ru, satella@mail.ru
ORCID:
Rimareva L.V. orcid.org/0000-0003-3097-0836
Overchenko M.B. orcid.org/0000-0003-0191-5897
Serba E.M. orcid.org/0000-0002-1660-2634
Ignatova N.I. orcid.org/0000-0002-8416-7478
Shelekhova N.V. orcid.org/0000-0001-7735-2942
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Abstract

Grain, in addition to starch, hemicelluloses and protein, is known to be phytic acid and its salts. It has been shown that the use of phytolytic enzymes promotes the release of phosphorus, improves the digestibility of feed nutrients and increases the meat productivity of livestock and poultry. However, the catalytic effect of phytases and their combinations with proteases on degradation of polysaccharides and protein polymers in grain raw materials, on the release of cations and anions, especially in grain wort for the production of alcohol, has been little studied. This work shows for the first time that phytolytic enzymes improve qualitative estimates, rheological properties and change the ionic composition of grain wort by not only releasing phosphates and metal cations, but also by increasing the concentration of organic salt anions. The combined action of phytolytic and proteolytic enzymes on polymers of grain raw materials provides their catalytic degradation to soluble carbohydrates, nitrogenous substances, cations and anions to produce the enriched grain wort. This work aimed to assess the degree of phytolytic and proteolytic conversion of high-molecular-weight polymers in grain wort. For grain wort, 50 g portions of wheat (*Triticum* sp.) or corn (*Zea mays*) grain flour put into Erlenmeyer flasks were added with 150 cm^3 of water and incubated in a water bath. At grain batching for starch dextrinization, a thermo-stable α-amylase was added (0.6 units/g starch). For starch conversion to sugars and hydrolysis of non-starch polysaccharides in the control, we used glucoamylase (9.0 units/g starch) and xylanase (0.15 units/g raw material). Phytase (from 1.0 to 2.5 units/g raw material) and a proteolytic enzyme preparation (0.1 units/g raw material) were also added. The control was added neither with phytase, nor the proteolytic enzyme. The profiles of the main polymers of grain raw materials, the grain wort concentration, the content of reducing carbohydrates (RC) were determined according to the techno-chemical instructions for the control of alcohol production, the amine nitrogen (NH_2^+) concentration was measured by a method based on the ability of amino acids to form soluble copper compounds with a suspension of copper phosphate. The dynamic viscosity of the grain wort was evaluated by vibrational viscometry. The study of the ionic composition of the grain mix and wort was carried out using a PrinCE-560 series capillary electrophoresis system (PrinCE Technologies B.V., Netherlands) equipped with a conductometric detector. The optimal dosage of phytase was 1.5 units/g raw material which ensures the maximum release of ions identified by capillary electrophoresis and the effective conversion of polysaccharides, protein and phytin substances of the grain. It was found that the phytolytic enzymes contributed to a decrease in the viscosity of wheat and corn wort of more than 20 %, a 9.5-11.3 % increase in the concentration of reducing carbohydrates, and a 2.1-2.4-fold increase in the concentrations of released ions. The concentration of amino nitrogen in the wort did not change significantly. It was shown that as a result of the phytolytic action, the concentration of not only metal phosphates and cations, but also anions of organic salts, such as oxalates, malates, citrates, and succinates (in wheat wort) and oxalates, malates, citrates, and lactates (in corn wort) increased. A more significant effect of phytase for corn wort was revealed: the concentration of phosphates in the nutrient medium increased 3.9 times vs. 1.6 times for wheat wort, the levels of

potassium and magnesium ions were 12 % and 22 % higher, respectively, as compared to the control in which the phytase was not used. The optimal composition of the enzyme complex is proposed which ensures the effective hydrolysis of polysaccharides, phytin and protein substances of processed raw materials. The synergistic effect of phytolytic and proteolytic enzymes enhances the catalytic hydrolysis of high molecular weight polymers of plant origin and enriches the grain wort with carbohydrates, nitrogenous substances, phosphates, and minerals in bioavailable form. Biocatalytic treatment of grain with the developed enzyme complex which, along with amylases and xylanase, contains phytase and proteases, provides a 16.8 % and 18.8 % increase in the concentration of reducing carbohydrates in wheat and corn wort, a 1.7-fold and 1.9-fold increase in amine nitrogen and a decrease in wort viscosity by 41.7 % and 44.7 %, respectively.

Keywords: phytase, protease, wheat raw material, corn raw material, biocatalysis, ions, cations, phosphorus, grain wort

The agro-industrial complex of the Russian Federation annually processes more than 100 million tons of agricultural raw materials. Alcohol, starch, brewing and feed manufacturers are large consumers of grain. Innovative technologies assist in increasing commercial profitability of grain processing and are also targeted to solve environmental problems and to produce competitive food and feed products [1-3]. These technologies are effective due to broad substrate specificity of biocatalysts, providing deep hydrolysis of high molecular weight polymers of grain raw materials [4-6]. It was previously shown that the synergism of amylolytic, proteolytic and hemicellulolitic enzymes improves grain quality, enriches the wort with carbohydrates, nitrogenous substances, and increases the yield of the target product [4].

Grain contains, in addition to starch, hemicelluloses, cellulose, and proteins, phytic acid and its salts — phytates which are the principal storage form of mineral phosphorus in plants [7-9]. The amount of phytic compounds in grain differs between crops and also depends on seed quality and growing conditions. For example, the amount varies from 0.4 to 3.9 % for wheat and from 0.7 to 2.8 % for corn [10, 11]. Phosphorus in plants is approximately 80 % phytic acid, or *myo*-inositol-1,2,3,4,5,6-hexakisphosphate ($InsP_6$) in which inositol-bound phosphorus is not bioavailable [12, 13]. In addition, phytates, exhibiting a high negative charge, bind metal cations [9, 14, 15] and also form rather strong complexes with proteins and carbohydrates [16, 17]. Phytases allows the release of valuable components of raw materials and increases its bioavailability [13].

Phytase which is present in plants during their growth promotes the catalytic degradation of phytic acid [18], but its amount is insufficient for the complete release of phosphorus. Phytases synthesized by microorganisms are attracting more and more attention. The most promising producers are fungi of the genus *Aspergillus* (6) and recombinant strains of yeast and bacteria [19-21].

In recent investigations which focus on raw grain processing for various purpose, the catalytic efficiency of phytases is attracting increasing interest. Considerable attention is paid to phytases as tools to improve the digestibility of feed nutrients. It is shown that phytases promote the breakdown of phytic compounds, the release of phosphorus and other trace elements, which leads to an increase in the growth and meat productivity of animals and poultry [22-24]. The interaction of phytic acid and its salts with substances that make up food raw materials and food products has been under consideration [16, 17, 24-26].

Investigations of sorghum and maize lager beer brewing using phytases have shown the potential for improving the nutritional value of yeast during grain wort fermentation [8, 10, 27, 28]. It has been confirmed that, under conditions of anaerobic fermentation, phosphorus is assimilated by yeast, mainly in the initial phase. Young actively multiplying yeast cells contained 2 times more phosphorus compared to the end of fermentation. However, the catalytic efficiency of phytases in raw grain processing for alcohol, especially the composition of a complex of

hydrolytic enzymes with different substrate specificity have practically not been studied [4]. There are only preliminary data on the positive effect of the *Aspergillus awamori* phytase on yeast growth and alcoholic fermentation of grain wort [28].

In the presented work, we showed for the first time that in grain wort, due to phytase activity, the concentration of anions of organic salts increases in addition to a release of phosphates and metal cations. The combined action of phytases and proteolytic enzymes increases the degree of catalytic destruction of grain biopolymers resulting in soluble forms of carbohydrates, nitrogenous substances, cations and anions. This contributes to the production of enriched grain wort with good rheological properties. Hereof, the optimal enzymatic complex is proposed to ensure effective hydrolysis of polysaccharides, phytic and protein substances in grain and to maximum accumulate reducing carbohydrates, amine nitrogen, phosphates and minerals in a bioavailable form.

This work aimed to assess effects of phytase and proteolytic enzyme preparations on the conversion of wheat and corn high molecular weight polymers in preparing grain wort.

Material and methods. Alcoholic fermentation was performed in 2019-2020 using traditional enzyme preparations (EP) of different substrate specificity. These were Amylex® 5T (Genencor, USA; heat stable α-amylase, 2000 AAU/cm^3), Diazyme® X5 (Genencor, USA; glucoamylase, 8000 GlAU/cm^3, 140 AAU/cm^3), Tegazyme RT 75L (Lyven SA, France; xylanase, 3600 XAU/cm^3), Protoorizin (VNIIPBT, Russia; proteases, 620 PAU/cm^3), Phytaflow (Novozymes, Denmark; phytase, 30,000 PhAU/cm^3).

For wheat (*Triticum* sp.) or corn (*Zea mays* L.) grain wort, 750 cm^3 Erlenmeyer flasks, each with 50 g of grain flour and 150 cm^3 of water (1:3) were mixed and incubated in a PE-4300 water bath (Ekros, Russia) according to the "soft" enzymatic-hydrolytic processing, i.e., the batches were allowed for 30 min at 40-50 °C, then kept for 120 min at 85 °C, stirring occasionally. After cooling to 60 °C, the batches were treated with EP for 60 min. At mixing flour and water, the thermostable α-amylase was used (0.6 AAU/g starch) for starch dextrinization. In the control batches, glucoamylase (9.0 GlAU/g starch) and xylanase (0.15 XAU/g raw material) were added for saccharification of starch and hydrolysis of non-starch polysaccharides. In the experimental batches, along with amylase, glucoamylase and xylanase, phytase (1.0, 1.5, and 2.5 PhAU/g raw material) and proteases (0.1 PAU/g raw material) were added. The concentration of soluble solids in wheat wort was 21.1 %, in corn 21.8 %.

The major grain polymers, wort gravity, and reducing carbohydrates (RC) were measured as described [29], the amine nitrogen (NH_2^+) quantifying was based on the ability of amino acids to form soluble copper complexes with copper phosphate suspension [30]. Dynamic viscosity of wort was assessed by vibrational viscometry (an SV-10 sinusoidal vibration viscometer, A&D Co., Ltd., Japan) with Win-CT Viscosity software. The ionic composition of batches mix and wort was studied using a PrinCE-560 series capillary electrophoresis system (PrinCE Technologies B.V., Netherlands) equipped with a conductometric detector [31].

Statistical processing of the data obtained in at least three replicates was carried out using the Student's *t*-test at $p < 0.05$ (Statistica 6.0, StatSoft, Inc., USA). The mean values (*M*) and standard errors of the means (±SEM) were calculated.

Results. To study the effect of phytase on wheat and corn wort quality (Fig. 1-3), the phytase was used at a dosage of 1.0 PhAU/g raw material, previously established for rye wort [28]. The phytase contributed to a significant ($p < 0.05$)

increase in the concentration of reducing carbohydrates in grain wort, by 9.5-11.3 % compared to the control, but practically did not affect the protein hydrolysis (see Fig. 1, A, B). The concentration of amine nitrogen in the wort significantly increased ($p < 0.05$) only when proteases were part of the enzymatic complex, i.e., 1.7-fold for wheat wort and 1.9-fold for corn wort (see Fig. 1, B).

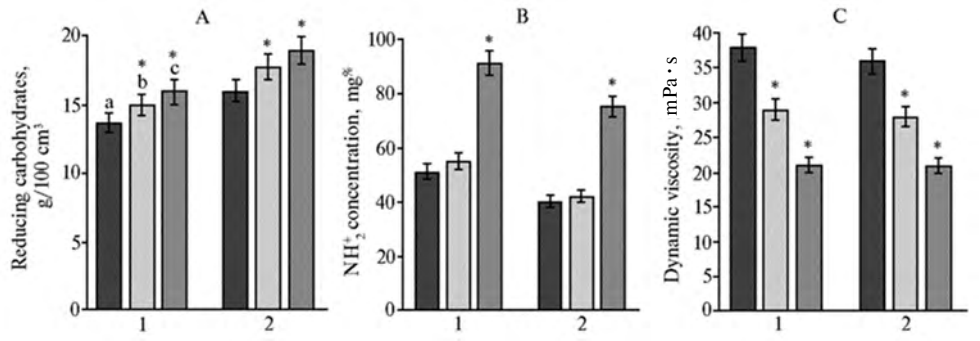

Fig. 1. Reducing carbohydrates (A), NH_2^+ **concertation** (B) **and dynamic viscosity** (C) **of wheat** (1) **and corn** (2) **wort as influenced by different enzyme preparations:** a — control, b — control + phytase (1.0 PhAU/g raw material), c — control + phytase (1.0 PhAU/g raw material) + protease (0.1 PAU/g raw material). See *Materials and methods* section for details.
* Differences form the control (a) are statistically significant at $p < 0.05$.

The rheological properties of the grain wort revealed in this study confirmed our earlier data for rye wort [28]. Phytase caused a 23.7 % decrease in viscosity for wheat wort and a 22.2 % decrease for corn wort ($p < 0.05$) (see Fig. 1, B). A complex of enzymes, including amylase, glucoamylase, xylanase, phytase, and protease, provided a deeper hydrolysis of polysaccharides. The RC level was 16.8 % higher in wheat wort and 18.8 % higher in corn wort as compared to the control ($p < 0.05$) (see Fig. 1, A). The wort viscosity decreased more significantly, by 41.7-44.7 % ($p < 0.05$) (see Fig. 1, C).

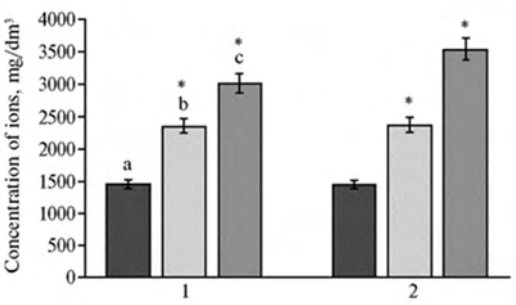

Fig. 2. Total ions in wheat (1) **and corn** (2) **wort as influenced by different enzyme preparations:** a — grain batch, b — control, c — control + phytase (1.0 PhAU/g raw material). See *Materials and methods* section for details.
* Differences b—a, c—a, and c—b are statistically significant at $p < 0.05$.

It is known that salts of phytic acid, being strong chelating agents, form stable protein-phytate complexes with proteins and also bind metal cations, which can negatively affect the functions of hydrolytic metal-dependent enzymes [32]. In our experiment, phytase had a significant effect on the increase in the total concentration of ions in the wheat and corn wort (Fig. 2). Ion concentrations increased 2.1-2.4-fold ($p < 0.05$) as compared to flour-water mix and 1.3-1.5-fold as compared to the control ($p < 0.05$).

Enzyme complexes containing phytase significantly ($p < 0.05$) increased the proportion of released phosphates in the pool of identified ions as compared to the control, 40.8 % vs. 31.2% for wheat and 30.8 % vs. 11.3 % for corn (Fig. 3). The proportion of potassium and magnesium ions remained practically unchanged.

We also investigated the effect of the dosage of phytase in the enzyme complex (1.0; 1.5; 2.5 PhAU/g raw material) on the quality parameters of the wheat and corn wort (Fig. 4).

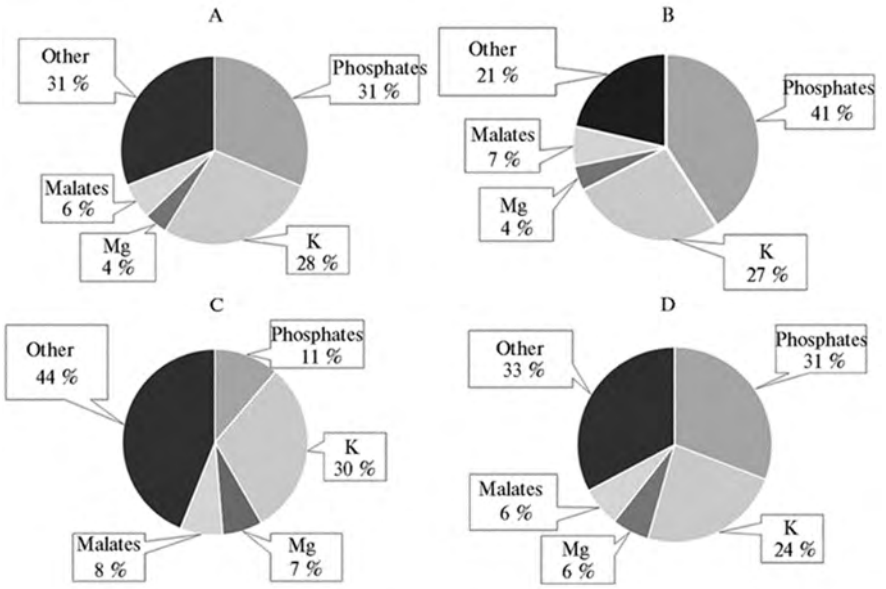

Fig. 3. Content of principal identified ions (% of total pool) **in wheat** (A, B) **and corn** (C, D) **wort as influenced by different enzyme preparations:** A, C — enzyme complex without phytase (control), B, D — enzyme complex with phytase (control + phytase 1.0 PhAU/g raw material). See *Materials and methods* section for details.

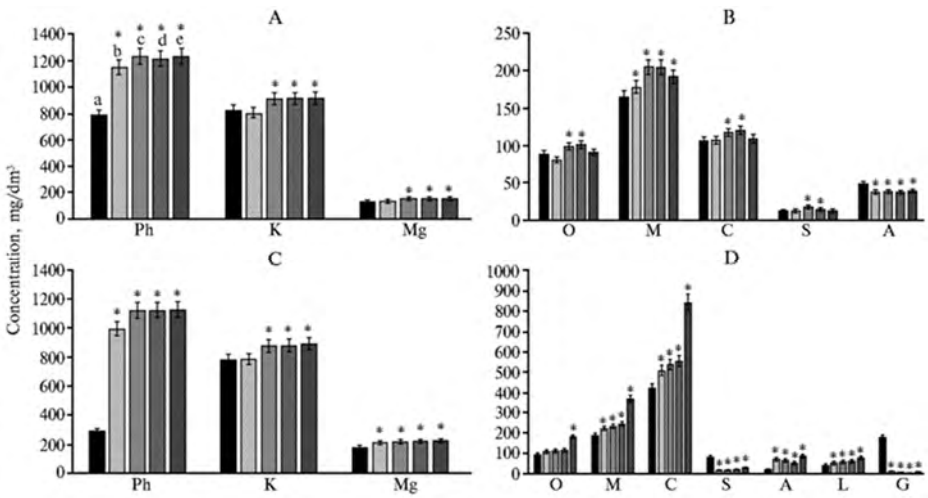

Fig. 4. Profiles of inorganic and organic ions in wheat (A, B) **and corn** (C, D) **wort as influenced by different enzyme preparations:** a — control, b — control + phytase (1.0 PhAU/g raw material), c — control + phytase (1.5 PhAU/g raw material), d — control + phytase (2.5 U PhA/g raw material), e — control + phytase (1.0 PhAU/g raw material) + protease (1.0 PAU/g raw material); Ph — phosphates, K — potassium, Mg — magnesium, O — oxalates, M — malates, C — citrates, S — succinates, A — acetates, L — lactates, G — glycolates. See *Materials and methods* section for details.
* Differences form the control (a) are statistically significant at $p < 0.05$.

Phytase promoted the release of phosphorus (see Fig. 4). Thus, in wheat wort upon treatment with phytase-containing enzyme complex, the phosphate concentration varied from 1145 to 1232 mg/dm^3 depending on the phytase dosage, which exceeded 1.5-1.6-fold ($p < 0.05$) the control (see Fig. 4, A). In addition, there was a slight increase in the concentration of potassium and magnesium ions, by 11 % and 17 % ($p < 0.05$) for a dosage of 1.5 1.5 PhAU/g raw material. Phytase had a more pronounced effect on the corn wort: the concentration of phosphates

in the nutrient medium increased ($p < 0.05$) 3.4-3.9-fold (991-1124 mg/dm^3 vs. 290 mg/dm^3) (see Fig. 4, C). The concentration of potassium and magnesium ions increased by 12 and 22 %, respectively ($p < 0.05$). The data obtained showed that the optimal dosage of phytase was 1.5 PhAU/g raw material. Further increase in its concentration to 2.5 PhAU/g practically had no effect on the grain wort quality parameters.

Phytase and an increase in its dosage influenced not only the concentration of phosphorus, potassium and magnesium ions in the wort, but also other identified ions. As compared to the control, the content of anions of organic salts (see Fig. 4, B, D) increased. Particularly, malates (succinic acid salts) amounted to 178.2-205.1 vs. 165.4 mg/dm^3 for wheat and 224.1-367.8 vs. 189.66 mg/dm^3 for maize. Citrates amounted to 510.1-844.4 vs. 422.8 mg/dm^3 in the corn wort whilst practically did not change in the wheat wort. The concentration of lactic acid salts (lactates) in the corn wort increased 1.4-2.0 times ($p < 0.05$) depending on the dosage of phytase. However, phytase led to a decrease ($p < 0.05$) in the glycolic acid salts in corn wort (6.3-10.9 mg/dm^3 vs. 178.4 mg/dm^3 in the control). When processing wheat, lactates and glycolate in the wort were practically absent.

With addition of proteases, we observed a definite tendency to an increase in the concentration of phosphorus, potassium and magnesium ions in the grain wort (see Fig. 4, A, C).

The potential of phytases to increase the level of bioavailable phosphorus and feed and food digestibility has been actively investigated [13, 16, 22, 23]. Nevertheless, the catalytic action of phytases has not been sufficiently studied, as well as their synergism in terms of conversion of polysaccharides and protein polymers of grain and the release of cations and anions, especially in fermented grain wort.

Here we have shown the positive effect of phytase and proteolytic enzymes on wheat and corn wort composition and ion profiles. Apparently, not only phosphorus but also carbohydrate and protein polymers were released due to biocatalytic hydrolysis of phytates which are strong chelating agents capable of forming stable protein-phytate complexes with proteins [33]. This contributes to a better access of hydrolytic enzymes to substrates. Also, the release of metal cations from the active centers of these enzymes may increase their catalytic activity and, therefore, destruction of grain wort polymers.

Our findings indirectly confirm reports which proved the role of phytic acid in the inhibition and regulation of the catalytic ability of metalloprotein enzymes. Of these, xanthine oxidase has been more studied [33, 34]. We have shown that the complex of enzymes which, along with carbohydrases, contains phytase and proteases promotes a deeper hydrolysis of polysaccharides and protein substances. In wheat and corn wort, there was a significant ($p < 0.05$) increase in RC concentration (by 16.8 %and 18.8 %), amine nitrogen (1.7-fold and 1.9-fold) and a decrease in viscosity (by 41.7 % and 44.7 %).

Thus, a positive effect of phytases on rheological properties, ion profiles and bioavailable phosphorus in wheat and corn wort has been established. The use of phytase contributes to a significant release of ions, up to 2 times as much as in grain mix. Due to phytase, there was an increase in organic salts, the oxalates, malates, citrates, succinates for wheat wort and oxalates, malates, citrates, lactates for corn wort. In wheat and corn wort, phosphates increased 1.6 times and 3.9 times, potassium by 11 % and 12 %, magnesium by 17 % and 22 % as compared to the control. Phytase decreased the viscosity of grain wort by more than 20 %. For catalytic conversion of wheat and corn biopolymers, the optimal dosage of phytase, ensuring maximum accumulation of ions, is 1.5 PhAU/g raw material.

The synergistic action of phytases and proteases enhances catalytic hydrolysis of high-molecular-weight polymers and enriches grain wort with carbohydrates, nitrogenous substances, bioavailable phosphates and minerals. Our findings indicate that further experiments are necessary to assess effects of phytase complex on amylolytic and proteolytic processing of grain raw materials, on yeast metabolism, growth and reproduction during ethanol fermentation, and on digestibility of grain feeds fed to farm animals.

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THE EFFECTS OF ENZYMATIC HYDROLYSATES OF FEATHERS AND COLLAGEN IN DIETS FOR BROILER CHICKS (*Gallus gallus* L.) ON MEAT QUALITY

V.I. FISININ[1], V.S. LUKASHENKO[1] ✉, I.P. SALEEVA[1], V.G. VOLIK[2], D.Yu. ISMAILOVA[2], E.A. OVSEYCHIK[1], E.V. ZHURAVCHUK[1]

[1]*Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS,* 10, ul. Ptitsegradskaya, Sergiev Posad, Moscow Province, 141311 Russia, e-mail fisinin@land.ru, lukashenko@vnitip.ru (✉ corresponding author), saleeva@vnitip.ru, ovseychik@vnitip.ru, evgeniy_20.02@mail.ru;
[2]*All-Russian Research Institute of Poultry Processing Industry — Branch of Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS,* 1, Rzhavki, Solnechnogorsk Region, Moscow Province, 141552, e-mail volik@dinfo.ru, dilaramis08@mail.ru
ORCID:
Fisinin V.I. orcid.org/0000-0003-0081-6336
Lukashenko V.S. orcid.org/0000-0002-0107-8235
Saleeva I.P. orcid.org/0000-0002-7446-1593
Volik V.G. orcid.org/0000-0002-1798-2093
Ismailova D.Yu. orcid.org/0000-0003-3918-8752
Ovseychik E.A. orcid.org/0000-0002-2312-1388
Zhuravchuk E.V. orcid.org/0000-0002-2951-0659

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Abstract

The deficit of feed-grade protein for the productive animals and poultry is presently propelling the interest toward the use of the hydrolysates of slaughter wastes as the potential protein sources. The slaughter wastes of poultry (feathers, intestines, blood, heads, shanks, meat-bone residues of the deboning) can mount up to one third of the initial bodyweight. Earlier we have developed the two-stage technology of easily digestible protein additives involving the hydrolysis and fermentation of protein from poultry slaughter wastes (primarily keratin from feathers and collagen from meat-bone residue). The study presented is a pioneer evidence that the substitution of these additives for fishmeal in diets for broilers provides more rapid growth and does not deteriorate the protein, fatty and amino acid profiles, concentrations of non-peptide, peptide and residual nitrogen, technological properties of meat. The study was performed in 2019 and aimed at the comparative evaluation of meat quality at 38 and 49 days of age in floor-housed broilers fed standard diet and three experimental diets with these hydrolysates. The broilers (cross Ross-308, 35 birds per treatment) kept in conditions of the Center for Genetics & Selection "EPH Zagorskoye" (Moscow Province) were allotted to four treatments partially slaughtered at 38 days of age and partially at 49 days. Control treatment 1 was fed a standard broiler diet with fishmeal; in experimental treatments fishmeal was substituted by enzymatic hydrolysate of feathers (treatment 2), enzymatic hydrolysate of collagen (treatment 3), mixture of these two hydrolysates with addition of probiotic Bacell M (0.2 %), and bran (0.5 %) as a source of vegetable fiber (treatment 4). It was found that supplementation of diets with the hydrolysates of keratin and collagen significantly improved live bodyweight in broilers by 8.78-10.89 % in compare to control ($p \le 0.001$). The content of peptide nitrogen in breast muscles in treatments 1 and 4 tended to grow with the increase in slaughter age (in treatment 1 from 0.07 at 38 days to 0.27 % at 49 days; in treatment 4 from 0.07 to 0.35 %). The number of protein fractions (with molecular weights from 100 to < 20 KDa) in meat increased with slaughter age with all studied diets. Protein of breast muscles contained more isoleucine, leucine, valine, and phenylalanine at 38 and 49 days of age in compare to thigh meat with all studied diets. The digestibility of essential amino acids in breast muscles in treatment 4 grew from 81.78 % with slaughter at 38 days to 90.29 % at 49 days. Evaluation of biological value of meat revealed better balanced ratios of the essential amino acids in breast muscles in control treatment at 38 days of age (the difference in the amino acid score 70.53 %) and in treatment 4 at 49 days of age (the difference in the amino acid score 66.48 %). In thigh meat this difference was higher in treatment 3 at 38 days of age (59.69 %) and in treatment 4 at 49 days of age (61.43 %). There were no significant differences between the treatments in concentrations of fatty acids in meat. The conclusion was made that the use of new protein additives based on the enzymatic hydrolysates of feathers and collagen in diets for broilers does not deteriorate the parameters of meat quality.

Keywords: broiler chicks, feed additives, feathers, collagen, enzymatic hydrolysates, live bodyweight, meat quality, amino acid score, fatty acids, essential nutrients, technological traits

In broiler chickens, productivity and meat quality depend largely on the nutritional value of their rations [1-5]. Poultry diets can significantly affect digestion. The quality of broiler meat [6-10] is largely due to dietary animal proteins, along with grain ingredients common in most poultry feeds [5, 11-14].

Modern poultry crosses with high meat production are very demanding for balanced rations. Therefore, broiler chickens can completely realize their genetic potential only if whole range of essential amino acids are provided, that is, feeds contain ingredients of animal origin [15, 16]. Probiotics [17-19] and prebiotic compounds support digestive health, increase the digestibility of feeds and assimilation of nutrients [20, 21]. In Russia, daily maintenance requirements of protein and amino acids in poultry were determined for well-digestible corn-soybean feed with fish meal as a source of animal protein. Currently, feed mixtures for poultry includes ingredients with a relatively low availability of nutrients, the wheat, barley, sunflower meal, meat and bone meal. Fish meal [14, 22, 23] remains an expensive and scarce component. This limitation may decrease the growth rate, meat productivity and meat quality in broilers.

In the poultry industry, slaughter and processing waste (feathers, intestines, blood, heads, legs, meat and bone residue) make up 25-30 % of the body weight. These by-products are the sources of animal protein [24, 25]. Mechanical deboning chicken carcasses or their parts results in 27-40 % bone residue which is 15-20 % bones and 25-30 % whole proteins, the ash-to-protein ratio is 0.7. Most of the protein and minerals are in the bone tissue while moisture and fat are in the pulp [26, 27].

Replacing traditional sources of protein in the poultry diet with hydrolysates of by-products of animal husbandry [28-30], including poultry farming [31-33], are generating considerable interest. Another reason is that low molecular weight peptides derived from enzymatic hydrolysis of proteins from poultry by-products exhibit regulatory functions and antimicrobial, antioxidant, antihypertensive, and immunomodulatory activities [34, 35].

In our previous works, we have developed a two-stage technology based on enzymatic hydrolysis of feathers or bone collagen to produce feed additives, being up to 89 and 84 % digestible protein [28, 32], and proposed broiler feed formulations containing these additives. The present study proves for the first time that the developed dietary additives fasten live weight gain in broilers. Moreover, the produced meat meets quality requirements in protein, amino acid and fatty acid profiles, non-protein, peptide and residual nitrogen concentrations, and in technological parameters.

The work aimed to evaluate meat quality parameters in broilers fed the basal ration and three experimental diets based on enzymatic hydrolysates of feathers and collagen.

Materials and methods. Feathers from gutted broilers as a keratin-containing material and bone residue after mechanical deboning as a collagen-containing material were processed in two stages, the hydrothermal hydrolysis in a thin layer and enzymatic hydrolysis.

For feeding trials, Ross 308 cross broiler chickens (*Gallus gallus* L.) were assigned to four dietary treatments (35 broilers per group): group I (control) fed basal diet (BD) with fishmeal as the protein source, group II fed BD with replacement of fishmeal by the feather hydrolysate equal in protein content, group III fed BD with replacement of fishmeal by the collagen hydrolysate equal in protein content, and group IV fed a mixture of both enzymatic hydrolysates added with 0.2

% probiotic preparation Bacell-M (Biotehagro LLC, Russia) and 0.5 % vegetable dietary fiber (wheat bran). The litter floor rearing was used according to the welfare recommendations for the cross (vivarium of the Breeding and genetic center Zagorskoe EPH FSC VNITIP RAS, Moscow Province, 2019).

Breast and thigh meat of 38- and 49-day-old broilers were collected for analytical studies.

To measure water- and salt-soluble proteins, a 10 g crushed meat sample (breast, thigh) was poured with 60 ml of distilled water (or 3.5 % NaOH solution), allowed for 10 min, and then centrifuged at 2500 rpm for 10 min. The supernatant was poured into a 250 ml volumetric flask. The procedure was repeated thrice, pouring the supernatant into the same flask. The volume was adjusted to 250 ml with water (or 3.5 % NaOH), 50 ml of the resulting centrifugate were mixed with 50 ml 20 % trichloroacetic acid (TCA), allowed for 40 min and filtered through a paper filter. The precipitate on the filter was washed with 10 % TCA and water. The filter with the precipitate was burned in a combustion tube. The amount of nitrogen was determined by the Kjeldahl method.

To determine alkali-soluble proteins, a 5 g crushed s meat ample (breast and thigh) was poured with 50 ml 0.1 N. NaOH, mixed, allowed for 16-18 h, and centrifuged for 10 min at 2500 rpm. The supernatant fluid was poured into a 250 ml volumetric flask. The procedure was repeated thrice in total. The centrifugate volume was adjusted to 250 ml with 0.1 N. NaOH. A 25 ml aliquot was burned in a combustion tube. The amount of nitrogen was determined by the Kjeldahl method.

To quantify non-protein and residual nitrogen, 2 g crushed meat sample (breast and thigh) was poured with 20 ml of distilled water, mixed, allowed for 10-15 min, and filtered through a paper filter into a 100 ml volumetric flask. The procedure was repeated fourfold in total. The filtrate volume was adjusted to 100 ml with water, 30 ml of 20 % TCA was added to 30 ml of the filtrate, mixed and filtered through a paper filter. A 25 ml aliquot was burned in a combustion tube and the residual nitrogen was determined by the Kjeldahl method. The filter cake was also burned and non-protein nitrogen was determined according to the Kjeldahl method.

Crude protein content was determined according to ISO 5983-2: 2009, hydrogen ion concentration (pH) according to ISO 2917-749.

Protein fraction profiling was performed using one-dimensional SDS-PAGE. In microcentrifuge tubes, A 50 µl aliquot of the extract in a microcentrifuge tube was added with 5 0 µl of a solubilizing solution (10 %glycerol, β-mercaptoethanol, 0.02 % bromophenol blue, 0.5 M Tris-HCl, 2 % SDS) and allowed for 5 min in a thermostat at 95 °C. The supernatant after centrifugation (Eppendorf 5402R, Eppendorf, Germany; 10000 rpm, 7 min) was separated for 2.5 hours by denaturing electrophoresis in 12.5 % polyacrylamide gel with 0.1 % SDS under a constant current and 60 V for concentrating gel and 130 V for separating gel (an electrophoretic chamber VE-10, LLC Helikon Company, Russia). Thermo Scientific™ PageRuler™ Unstained Broad Range Protein Ladder (a mix of 11 proteins of 250, 150, 100, 70, 50, 40, 30, 20, 15, 10, and 5 kDa, Thermo Scientific, USA) was used to estimate protein sizes.

To compare amino acid profiles, 50 g meat samples were homogenized (a BUCHI Mixer B-400, BÜCHI Labortechnik AG, Germany), dried in a drying oven, and placed in a Soxhlet extractor to remove fats. Sample preparation and controlled oxidation of cystine to cysteic acid and methionine to methionine sulfone were carried out according to GOST 32195 (ISO13903). A 10.0±0.1 mg portion of a dried and defatted sample was hydrolyzed with concentrated

hydrochloric and propionic acids (50:50) for 18 h at 110 °C. The resulting solution was evaporated to dryness on a rotary evaporator. Then 1 cm^3 of pH 2.2 buffer was added to the flask and the sample was transferred quantitatively into a vial. Precolumn derivatization was performed in an HPLC autosampler using ortho-Phthalaldehyde (OPA) for primary amino acids and 9-Fluorenylmethyl chloroformate for secondary amino acids. The ratio of derivatives to the sampled volume was 1:10.

Total amino acids were separated formed by Reversed-phase high-performance liquid chromatography (RP-HPLC) (Agilent 1260 Infinity LC, ZORBAX C18 PA column, 3.5 μm, 4.6×150 mm, Agilent Technologies, USA). Eluent A was acetonitrile:methanol:water (45:45:10), eluent B was 10 mM Na_2HPO_4 and 10 mM $Na_2B_4O_7$ (pH 8.2), a standard elution mode for 25 min, λ = 338 nm for primary amino acids, λ = 262 nm for secondary amino acids.

Free amino acids were extracted with diluted hydrochloric acid (GOST 32195-2013 — ISO 13903:2005). Nitrogen-containing macromolecules extracted together with amino acids were precipitated with sulfosalicylic acid and filtered off. The filtrate was acidified to pH 2.2. Amino acids were separated by ion exchange chromatography, reacted with ninhydrin, and their concentrations were measured photometrically at λ = 570 nm.

Fatty acid profiling was performed by gas chromatographic analysis in a VNIIMP modification [36]. Crude fat was extracted for 4 h with ether in a Soxhlet extractor from subcutaneous adipose tissue and abdominal fat. A 1-10 g samples were treated for 3-24 h with a mixture of 10 ml chloroform and 10 ml methanol according to the modified Folch method with 1 % KCl to dissolve lipid components. The samples were clarified using paper filters and evaporated to dryness. Then 0.01 g of the residue was mixed with 3 ml of 15 % acetyl chloride in methanol, incubated for 2 h at 100 °C, and KOH in methanol was added to pH 5.0-6.0. A 3 ml portion of saturated NaCl solution and 3 ml of hexane were added to the mixture, allowed for 3-5 min, and 0.2 ml was taken for analysis from a transparent hexane fraction containing methyl esters of fatty acids.

The methyl esters were analyzed chromatographically in a nitrogen flow at sample volume 1 μl, temperature gradient from 100 to 260 °C (10°C/min), gas flow mixing 1:100, and detector temperature 250... 300 °C (an Agilent 7890 instrument, Agilent Technologies, USA, with a flame ionization detector and an HP-Innowax 60 m×0.32 mm×0.5 μm capillary column). The fatty acid concentrations were calculated by internal normalization method using a standard mixture of fatty acid methyl esters Supelco® 37 Component (Sigma-Aldrich, USA).

The moisture binding capacity was determined by the Grau-Hamm method modified by Zhuravskaya [37].

To assess the biological value of proteins, their amino acid score difference coefficients (AASDC, %) were calculated:

$$\text{AASDC} = \frac{\sum_{j=1}^{N} \Delta\text{AASD}}{n} \times 100\ \%,$$

where ΔAASD is the difference in an amino acid score which is calculated as $\Delta\text{AASD} = C_i - C_{min}$ (C_i stands for score excess of the *i*-th essential amino acid, %; C_{min} is minimum score of the essential amino acid for test protein as compared to reference protein, %); *n* — number of essential amino acids. Biological value (BV) was calculated as BV, % = 100 % – AASDC.

Statistical processing was performed using the Statistica 10.0 software package (StatSoft, Inc., USA). The results are presented as a weighted mean *M*

with standard deviation (±SD). The significance of differences in mean values satisfying normal distribution and equality of variances was assessed by one-way ANOVA analysis of variance using Duncan's test. The critical level of significance of the null statistical hypothesis (p) was 0.05.

Results. Group II fed on a diet with feather hydrolysate gained the maximum weight. On average, in 38-day old cocks and hens, it was 2391 and 2183 g, being 8.78 % ($p \leq 0.01$) and 9.70 % ($p \leq 0.001$) compared to the peers from the control group, and by day 49, the weight was also 9.35 % ($p \leq 0.001$) and 10.89 % ($p \leq 0.001$) higher than in the control. In group III and group IV during, the birds exceeded the control one by 1.81 % and 2.35 % and by 3.87 % and 4.96 %, respectively. Probably, the accelerated weight gain is due to an increase in free peptides and amino acids in the hydrolysates. Available hydrolyzed proteins are known to improve feed conversion efficiency.

Table 1 shows changes in meat protein fractions during growing period.

1. Meat protein fraction profiling and nitrogen content in Ross 308 cross broiler chickens (*Gallus gallus* L.) fed diets based on feather and collagen hydrolysates ($n = 35$, M±SD, vivarium of the Breeding and genetic center Zagorskoe EPH FSC VNITIP RAS, Moscow Province, 2019)

Group	Protein, %				Nitrogen, %		
	WS	SS	AS	total	peptide	residual	non-protein
38-day old broilers							
Breast							
I (control)	3.06±0.05	2.63±0.05	17.0±0.1	22.9±1.8	0.07±0.01	0.14±0.03	0.21±0.03
II	2.75±0.05	2.31±0.05	16.3±0.2	22.4±1.8	0.25±0.03	0.02±0.01	0.27±0.03
III	2.75±0.05	2.81±0.05	17.8±0.1	24.3±1.9*	0.20±0.02	0.02±0.01	0.22±0.02
IV	1.55±0.10	2.19±0.05	15.8±0.1	20.2±1.6	0.07±0.01	0.14±0.03	0.21±0.03
Thigh							
I (control)	2.31±0.05	1.94±0.05	14.9±0.1	19.7±3.0	0.06±0.01	0.10±0.03	0.16±0.02
II	1.94±0.05	1.69±0.05	15.7±0.2	19.9±3.0	0.19±0.02	0.02±0.01	0.21±0.01
III	2.44±0.05	1.75±0.10	15.9±0.1	20.9±1.7	0.14±0.01	0.02±0.01	0.16±0.03
IV	2.56±0.05	2.00±0.10	14.3±0.1	19.3±1.5	0.23±0.03	0.02±0.01	0.25±0.02
49-day old broilers							
Breast							
I (control)	3.13±0.05	0.69±0.05	17.9±0.2	21.9±1.8	0.27±0.02	0.03±0.01	0.30±0.03
II	2.00±0.10	0.63±0.05	19.0±0.2	23.0±1.8	0.35±0.01	0.03±0.01	0.38±0.03
III	2.44±0.05	0.44±0.05	20.8±0.1	24.0±1.9	0.24±0.02	0.03±0.01	0.27±0.02
IV	3.44±0.05	0.63±0.05	18.0±0.2	23.1±1.8	0.35±0.03*	0.04±0.01	0.39±0.03
Thigh							
I (control)	2.13±0.05	0.50±0.05	15.7±0.1	19.0±2.8	0.31±0.01	0.03±0.01	0.34±0.03
II	2.25±0.05	0.69±0.05	14.8±0.1	18.6±2.8	0.29±0.01	0.03±0.01	0.32±0.02
III	2.56±0.05	0.69±0.05	15.8±0.2	20.2±1.6	0.37±0.02	0.03±0.01	0.40±0.03
IV	2.88±0.05	0.69±0.05	14.0±0.2	17.8±2.7	0.38±0.02	0.02±0.01	0.40±0.03

Note. For a description of the groups, see the *Materials and methods* section. WS — water-soluble, SS — salt-soluble, AS — alkali-soluble.
* Differences from the corresponding control are statistically significant at $p \leq 0.05$.

Protein level indicates a nutritional value of meat, the daily protein requirement for an adult is 110-160 g, approximately 60 % being animal proteins. Proteins differ in amino acid composition, structure, solubility and biological functions. Water-soluble proteins are mainly sarcoplasmic proteins, e.g., myogen, globulin, myoglobin, and also nucleoproteins while salt-soluble proteins are mainly myofibrillar proteins, e.g., myosin, actin, and actomyosin. Eady et al. [38] extracted salt-soluble and water-soluble proteins at pH 5.4, 6.4, 6.9, 7.2, 7.5, 8.0, and 9.0. The protein concentration and SDS-PAGE analysis showed that post-mortem aging and the pH of the extraction buffer affect both the total amount and profiles myofibrillar and sarcoplasmic proteins recovered from deboned broiler breast fillets. Tropomyosin and troponin, the regulatory proteins, and alkali-soluble proteins, mainly stromal proteins, including collagen, elastin, as well as glycoproteins mucin and mucoid, have also been extracted [39].

In our experiment (see Table 1), from day 38 to day 49, the proportion of salt-soluble proteins in both the pectoral and femoral muscles decreased, while the ratio of water-soluble to alkali-soluble protein fractions did not change. It should be noted that myogen and myoglobulin, which are classified as water-soluble proteins, are partially saline-extractable and a significant part of water-soluble proteins and salt-soluble proteins are extracted with alkali. Perhaps the proportion of water-, salt- and alkali-soluble proteins is not correct to characterize age-related changes in poultry meet quality.

The level of peptide nitrogen changed with the age of birds (see Table 1). For breast meat, in groups I (control) and IV, the peptide nitrogen accounted for 0.07 % on day 38, reaching 0.27 % in group I ($p \leq 0.05$) and 0.35 % in group IV ($p \leq 0.05$) on day 49. A similar but statistically insignificant trend was seen in the femoral muscles. For residual and non-protein nitrogen, the changes were statistically significant (see Table 1).

Evaluation of the technological properties of meat (pH and moisture-binding capacity, MBC) in broilers fed basal and experimental diets did not reveal meanable differences in pH between the pectoral and femoral muscles in both age periods. All studied samples corresponded to pH 6.0-6.8 adopted for poultry meat (Table 2). Raising broilers up to 49 days of age increased MBC values regardless of the diet.

2. pH and moisture-binging capacity (MBC) **of meat of Ross 308 cross broiler chickens (*Gallus gallus* L.) fed diets based on feather and collagen hydrolysates** (n = 35, M±SD, vivarium of the Breeding and genetic center Zagorskoe EPH FSC VNITIP RAS, Moscow Province, 2019)

Group	pH		MBC, %	
	at day 38	at day 49	at day 38	at day 49
Breast				
I (control)	6.65±0.01	6.69±0.01	53.75±0.22	54.53±0.20
II	6.38±0.01	6.18±0.01	52.41±0.24	60.86±0.27
III	6.61±0.01	6.33±0.01	60.23±0.27*	63.83±0.26*
IV	6.52±0.01	6.87±0.01	62.18±0.29*	64.66±0.26*
Thigh				
I (control)	6.71±0.01	6.52±0.01	53.83±0.29	54.21±0.18
II	6.80±0.01	6.51±0.01	53.51±0.27	60.15±0.20
III	7.01±0.01	6.79±0.01	60.90±0.26*	62.23±0.20*
IV	6.71±0.01	6.52±0.01	53.83±0.29	54.21±0.18

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

A similar effect has been described in 60-, 90-, 120-, 150- and 180-day-old Da Heng broilers when, with age, the loss of breast meat drainage decreased [40]. The authors associate the influence of the slaughter time with the age-related morphological traits of the of muscle fibers, e.g., diameter, cross-sectional area of myofibrils, packing density in the muscle fiber. The larger body weight and breast mass of chickens is due to the larger diameter and area of the myofiber and the lower myofiber density in old birds than in young birds. The authors noted the effect of age on all meat quality characteristics of chicken breast muscles ($p < 0.05$). They often observed an increase in pH, a decrease in MBC, a greater shear force, and darker and redder meat color. Biochemical and molecular mechanisms underlying the dependence of meat quality on muscle characteristics still need to be studied [40].

In our trial, the highest MBC values were characteristic of white and red meat from group III fed collagen hydrolysate ($p \leq 0.05$) and from group IV fed a mixture of feather and collagen enzymatic hydrolysates added with 0.2 % probiotic preparation Bacell-M ($p \leq 0.05$). Note that the ability to retain moisture during

storage and processing determines the suitability of raw meat for a wide range of manufactured products, except for raw smoked and dry-cured. A decrease in this ability is associated with protein denaturation [41]. Bowker et al. [41] noted that differences in moisture retention in broiler breast fillets were not due to differences in denaturation of myofibrillar proteins. They suggested that denaturation of sarcoplasmic proteins to myofibrils may affect moisture retention in breast meat. The increase in MBC noted in our trial indicates an improvement in the technological properties of broiler meat due to experimental diets.

One- and two-dimensional denaturing PAGE of meat proteins is widely used to compare proteomic profiles under the influence of various factors. Figure 1 and Table 3 show protein fraction profiling in our trial

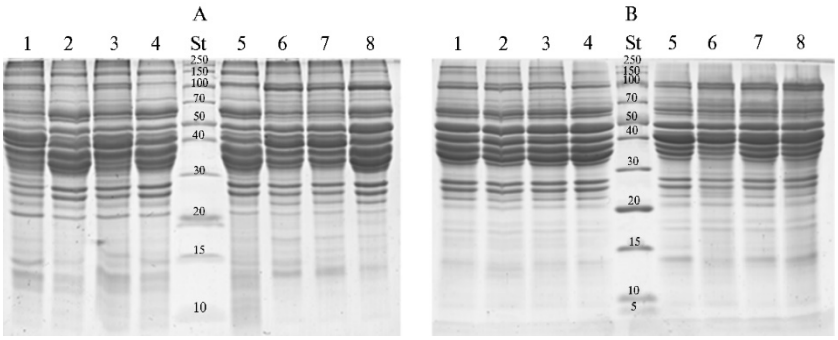

Fig. 1. 1D SDS-PAGE of meat samples of 38-day-old (A) **and 49-day-old** (B) **Ross 308 cross broiler chickens (*Gallus gallus* L.) fed diets based on feather and collagen hydrolysates:** 1 — thigh (group I, control); 2 — thigh (group II); 3 — thigh (group III); 4 — thigh (group IV); 5 — breast (group I, control); 6 — breast (group II); 7 — breast (group III); 8 — breast (group IV). St — Thermo Scientific™ PageRuler™ Unstained Broad Range Protein Ladder, 250, 150, 100, 70, 50, 40, 30, 20, 15, 10 and 5 kDa, Thermo Scientific, USA) (n = 35, M±SD, vivarium of the Breeding and genetic center Zagorskoe EPH FSC VNITIP RAS, Moscow Province, 2019).

3. The number of protein fractions in meat of 38-day-old and 49-day-old Ross 308 cross broiler chickens (*Gallus gallus* L.) fed diets based on feather and collagen hydrolysates (1D SDS-PAGE; vivarium of the Breeding and genetic center Zagorskoe EPH FSC VNITIP RAS, Moscow Province, 2019)

Molecular weight, kDa	Group I (control)		Group II		Group III		Group IV	
	breast	thigh	breast	thigh	breast	thigh	breast	thigh
38-day old broilers								
> 100	6	7	7	5	6	7	7	5
100-40	11	12	12	10	11	12	12	10
39-20	10	10	9	9	9	10	10	9
< 20	17	14	11	12	11	14	11	12
In total	44	43	39	36	37	43	40	36
49-day old broilers								
> 100	6	5	6	7	6	7	6	7
100-40	12	11	12	11	12	11	14	14
39-20	10	10	9	11	9	11	14	9
< 20	14	10	11	12	11	12	14	12
In total	42	36	38	41	38	41	48	43

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

On day 38, in group I (control) and III group fed the collagen hydrolysate diet, the protein fraction profiles were the most abundant and represented mainly by low molecular weight peptides (see Fig. 1, Table 3). On day 49, the number of fractions was maximum in group IV (pectoral muscles), On day 49, the number of fractions was maximum in group IV (pectoral muscles), with a 33 % increase in peptide molecules of < 40 kDa as compared to the age of 38 days. This attracts special attention as many low molecular weight peptides are bioactive. Thus, Hou et al. [35] note that maize and soybean meal diets added with 2-8 % of animal

protein hydrolysates (e.g., pig intestines, salmon entrails, or poultry tissue) or soy protein hydrolysates can provide desirable growth rates and feeding efficiency in weaning pigs, calves, and poultry in the first day of life. Protein hydrolysates appear to be promising in optimizing the nutrition of productive animals.

The main tendency we observed was that with age, the total number of protein fractions decreased in the control group while increased the experimental groups, to the greatest extent in group IV fed a mix of feather and collagen hydrolysates with 0.2 % probiotic preparation Bacell-M (see Fig. 1, Table 3).

On day 38, amino acid profiles were the most balanced in the pectoral muscles in group I and in the femoral muscles in group III (Fig. 2). On day 49, both pectoral and femoral muscles in group IV exhibited the maximum. The diagram reflects specific effects of the feather hydrolysate, the collagen hydrolysate, and a combination of both on the balance of amino acids in breast and thigh, and moreover, the effects are also age-dependent (see Fig. 2). Summarizing, we can conclude that in raising to 38 days of age, replacement of fishmeal with collagen hydrolysate (group III) provides a better balance of femoral muscle proteins. In raising to 49 days of age, the amino acid profiles are more balanced when fishmeal is replaced with a mixture of collagen and feather hydrolysates in combination with the probiotic preparation Bacell-M (group IV).

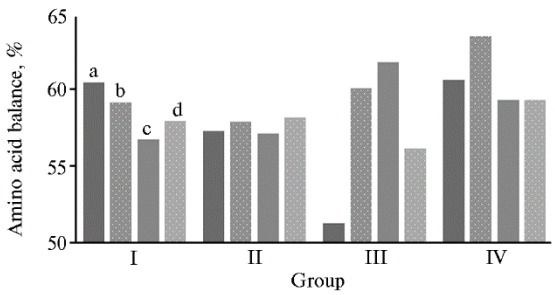

Fig. 2. Amino acid balance in meat of 38-day-old and 49-day-old Ross 308 cross broiler chickens (*Gallus gallus* L.) fed diets based on feather and collagen hydrolysates: a — 38 days, breast, b — 49 days, breast, c — 38 days, thigh, d — 49 days, thigh. Deviations from mean values ±2 %, for a description of the groups, see the section *Materials and methods* (vivarium of the Breeding and genetic center Zagorskoe EPH FSC VNITIP RAS, Moscow Province, 2019).

Protein assessment for amino acid balance is mandatory when determining the nutritional value and digestibility of food products and their ingredients [42]. The study of meat physicochemical properties and nutritional value in Chinese 817 Crossbred chicken compared to commercial import broilers (AAB) and laying hens revealed the advantage of this new cross in terms of the nutritional value of meat (breast and thigh). In this cross, lysine and leucine predominate among the essential amino acids, while glutamic and aspartic acids were the major nonessential amino acids.

Animal protein is a concentrated sources of essential amino acids in the human diet. A wide range of non-traditional feed ingredients is being studied for the ability to improve essential amino acid profiles in poultry meat. Haščík et al. [43] found a higher tyrosine concentration ($p \leq 0.05$) in breast muscles in the poultry fed with feed supplemented with probiotics and propolis extract. Considering the composition of amino acids and a relatively high score of essential amino acids, the breast meat of chickens treated with a probiotic and propolis extract seems to be a promising source of proteins with an increased ($p \leq 0.05$) content of phenylalanine and tyrosine (76.27 %) compared to the untreated chickens (73.49 %).

Optimizing the pattern of essential limiting amino acids in poultry diets affects directly not only growth performance and meat quality, but also immune status of the birds [11]. The titer of antibodies against the Newcastle disease virus

gradually increased as the dietary levels of energy and protein increased. Better weight gain and antibody titer are believed to confirm health. Moreover, the best immune response may be due to better use of nutrients, including for an immune response [11].

In broilers, depending on the diet composition and slaughter age (Table 4), various essential amino acids were limiting. The lowest score showed valine (group I regardless of age), leucine (group II, 38 days of age), tyrosine (group III regardless of age and group IV, 38 days of age) in the pectoral muscles and cystine (group I regardless of age), lysine (group II, 38 days of age), valine (group III regardless of age and group IV, 38 days of age), cystine (group IV, 49 days of age) in the femoral muscles.

4. Score (%) of essential amino acids in meat of 38-day-old and 49-day-old Ross 308 cross broiler chickens (*Gallus gallus* L.) fed diets based on feather and collagen hydrolysates: (n = 35, vivarium of the Breeding and genetic center Zagorskoe EPH FSC VNITIP RAS, Moscow Province, 2019)

Amino acid	38 days of age				49 days of age			
	I (control)	II	III	IV	I (control)	II	III	IV
				Breast				
Threonine	99.35	157.38	63.78	118.80	127.85	127.18	128.13	99.58
Tyrosine	140.48	145.57	53.70	81.78	85.35	85.09	86.96	105.39
Cystine	100.77	89.31	76.00	137.08	77.31	73.62	80.15	143.15
Valine	81.22	91.96	74.08	84.16	86.76	86.08	86.66	90.90
Methionine	133.00	115.68	99.14	126.00	112.09	110.68	111.73	131.82
Phenylalanine	101.49	95.32	100.11	104.35	119.70	115.16	116.00	106.46
Isoleucine	108.08	120.53	149.18	118.80	160.95	160.88	161.45	114.73
Leucine	109.80	72.07	121.10	113.16	127.86	129.81	130.36	90.29
Lysine	133.38	90.09	172.09	131.42	145.29	143.87	144.69	139.31
Tryptophan	99.35	157.38	177.00	277.20	127.85	127.18	166.70	216.50
				Thigh				
Threonine	110.13	121.85	143.55	121.75	75.00	88.70	122.53	89.90
Tyrosine	136.48	131.09	174.74	137.43	105.26	123.87	163.57	124.57
Cystine	70.08	127.54	92.00	115.62	145.77	74.46	159.92	77.77
Valine	75.94	90.46	85.16	77.72	73.68	77.42	68.32	78.66
Methionine	112.77	118.77	93.50	124.82	110.05	117.32	139.50	120.00
Phenylalanine	98.54	77.41	104.76	106.43	106.68	95.89	89.65	97.19
Isoleucine	97.48	89.20	92.10	117.88	128.95	112.90	100.25	113.78
Leucine	101.99	94.04	103.90	111.77	121.06	92.17	102.54	92.30
Lysine	119.67	71.27	111.35	130.95	156.95	106.55	125.11	105.22
Tryptophan	298.70	211.10	253.60	279.80	236.80	258.10	203.00	264.00

Note. For a description of the groups, see the *Materials and methods* section.

In 38-day old birds of group IV fed a mix of keratin hydrolysate, collagen hydrolysate and a probiotic preparation, the cystine level in meat was 37-43 % higher compared to the control. The level of tryptophan in pectoral muscles in all experimental birds was 1.9-2.9 times higher compared to the control birds fed fishmeal-based diet. At the same age (38 days), cystine in the femoral muscles increased in group II and groups IV, being 65-82 % higher than in the control, but with fattening up to 49 days, its amount decreased.

In general, replacement of fishmeal increased scores for cystine and tryptophan and improved the balance of breast and thigh meat for essential amino acids (see Table 4). Both at 38 and 49 days of age, isoleucine, leucine, valine, and phenylalanine responsible for the growth and development of muscle tissue were higher in the breast muscles than in the thigh muscles. Other works also indicate that femoral and pectoral muscles differ in amino acid profiles [44]. Our findings are consistent with the report on an increase in growth rates and the efficiency of poultry feeding due to alternative feed additives derived from protein-containing poultry by-products [43].

Protein digestibility is a key characteristic of biological value. With age of birds, digestibility of thigh meat increased by approximately 4 % but was lower

than for breast meat (Table 5), though the thigh meat accumulated 8 % more essential amino acids.

5. Meat digestibility (%) in Ross 308 cross broiler chickens (*Gallus gallus* L.) fed diets based on feather and collagen hydrolysates (n = 35, vivarium of the Breeding and genetic center Zagorskoe EPH FSC VNITIP RAS, Moscow Province, 2019)

Group	Breast		Thigh	
	38 days of age	49 days of age	38 days of age	49 days of age
I (control)	81.22	77.31	70.08	73.68
II	72.07	73.62	71.27	74.46
III	53.70	80.15	75.16	68.32
IV	81.78	90.29	77.72	77.77

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

A food is considered a complete protein when score of all essential amino acids is 100 %. For calculation, score of each essential amino acid in the "ideal protein" is taken as 100 % to determine the percentage of compliance for individual protein of the product or the total protein of the diet. The rate of at least one amino acid less than 100 % delays growth and development. The limiting amino acid becomes the major determinant. The breast protein in the first three groups cannot be considered complete. In group IV, the digestibility of essential amino acids of breast protein from day 38 to day 49 increased from 81.78 % to 90.29 %, being higher than in other groups. Given the 5 % measurement accuracy, it can be concluded that on day 49 in group IV, breast meat protein was complete. However, in the same group IV, the thigh meat digestibility did not change with age (77.72-77.77 %). The only change was that cystine became the limiting amino acid. In general, the digestibility of essential amino acids in group IV remained higher than in other groups, regardless of the meat type and the slaughter age.

We calculated indicators of meat protein quality [44], i.e., the amino acid score (AAS, %), amino acid score difference coefficient (AASDC, %) (see Table 4), and biological value (BV, %). The lower the AASDC value, the higher the protein quality. In group I and group II, on day 38, 29.47 % and 41.46 % of amino acids of breast meat were not utilizable, on day 49, 39.79 % and 42.34 % (Table 6). In group III and group IV, on the contrary, the excess of essential amino acids decreased. So, at day 38 of age, the AASDC value in breast meat was 54.92 % and 47.50 %, decreasing to 41.13 % and 33.52 % at day 49. Thence, in breast meat, the profile of essential amino acids is more balanced in group I on day 38 (BV = 70.53 %) and in group IV on day 49 (BV = 66.48 %).

6. Meat quality parameters (%) in Ross 308 cross broiler chickens (*Gallus gallus* L.) fed diets based on feather and collagen hydrolysates (n = 35, vivarium of the Breeding and genetic center Zagorskoe EPH FSC VNITIP RAS, Moscow Province, 2019)

Group	Breast				Thigh			
	38 days of age		49 days of age		38 days of age		49 days of age	
	AASDC	BV	AASDC	BV	AASDC	BV	AASDC	BV
I (control)	29.47	70.53	39.79	60.21	52.10	47.90	52.34	47.66
II	41.46	58.54	42.34	57.67	42.00	58.00	40.28	59.72
III	54.92	45.08	41.13	58.87	40.31	59.69	59.12	40.88
IV	47.50	52.51	33.52	66.48	54.70	45.30	38.57	61.43

N o t e. AASDC — amino acid score difference coefficient, %; BV — biological value, %. For a description of the groups, see the *Materials and methods* section.

A different trend was seen in the femoral muscles. In group I, we did not reveal any changes from day 38 to day 49 of feeding. However, the AASDC values indicate a 1.5-fold level of excess essential amino acids in the femoral muscles. In group II, there was a decrease in AASDC, and the AASDC value for the femoral muscles differed, albeit insignificantly, from that for the pectoral muscles. In group

III, there was a tendency to an increase in AASDC from 40.31 % to 59.12 % in thigh meat vs. almost equal decrease in AASDC in breast. In group IV, the AASDC clearly decreased between day 38 and day 49, from 47.50 % to 33.52 % for breast meat and from 54.70 % to 38.57 % for thigh meat. Therefore, the breast meat in group III (BV = 59.69 % on day 38) and in group IV (BV = 61.43 % on day 49) is the most balanced in essential amino acids.

Fatty acid content is also an indicator of poultry meat quality, which can vary across breeds, depending on diets and supplements [45, 46]. Our results showed that the largest proportion of fatty acids was palmitic fatty acid (saturated), oleic fatty acid (unsaturated ω-9), and linoleic fatty acid (unsaturated ω-6), for day 38 and day 49, 16.49 % and 21.50 %, 25.63 % and 33.57 %, and 32.39 % and 45.68 %, respectively. Unsaturated fatty acids, especially oleic and linoleic acids, are essential nutrients for humans. Here, the differences in oleic acid content between groups were 7.94 % for four groups of 38-day-old broilers and 2.49 % for 49-day-old broilers. Such differences can be explained by diets based on different sources of animal protein.

To summarize, a keratin hydrolysate (experiment) instead of fishmeal (control) in diets of broiler chickens increases body weight by 9-10 %, a collagen hydrolysate by 3.80 %, a combination of both added with the probiotic preparation Bacell-M by 4.96 %. Regardless of the diet, meat water-binding capacity (WBC) increases in the broilers of 49 days of age vs. 38 days of age. The birds fed the collagen hydrolysate or a combination of both hydrolysates with the probiotic Bacell-M have maximum meat WBCs, i.e., +4.3 % and +12.4 % compared to the control. The essential amino acid profiles were more balanced in breast meat of the control broilers on day 38 (biological value BV = 70.53 %) and of broilers fed a mix of both hydrolysates with Bacell-M on day 49 (BV = 66.48 %). In the same feeding group, on day 38, the digestibility of the breast meat essential amino acids in 38-day old birds was as that in control birds whilst in 49-day old broilers it was 16.8 % higher compared to the control. The digestibility of essential amino acids in the thigh meat in almost all experimental groups exceeded that in the control, particularly, by 10.9 % in group fed both enzymatic hydrolysates in combination with Bacell-M. Therefore, the diets based on keratin and collagen hydrolysates provide growth performance, as well as required biological quality and technological characteristics of broiler meat.

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FRACTAL ANALYSIS OF FREQUENCY-TAXONOMIC PROFILE OF BROILER'S GUT MICROBIOTA FOR STUDYING THE INFLUENCE OF PROBIOTICS ON BIRD DEVELOPMENT

N.I. VOROBYOV[1] ✉, I.A. EGOROV[2], I.I. KOCHISH[3], I.N. NIKONOV[3], T.N. LENKOVA[2]

[1]*All-Russian Research Institute for Agricultural Microbiology*, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail Nik.IvanVorobyov@yandex.ru (✉ corresponding author);
[2]*Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS,* 10, ul. Ptitsegradskaya, Sergiev Posad, Moscow Province, 141311 Russia, e-mail Olga@vnitip.ru, dissovet@vnitip.ru;
[3]*Skryabin Moscow State Academy of Veterinary Medicine and Biotechnology*, 23, ul. Akademika K.I. Skryabina, Moscow, 109472 Russia, e-mail zoo-kafedra@yandex.ru, ilnikonov@yandex.ru

ORCID:
Vorobyov N.I. orcid.org/0000-0001-8300-2287
Egorov I.A. orcid.org/0000-0001-9122-9553
Kochish I.I. orcid.org/0000-0001-8892-9858
Nikonov I.N. orcid.org/0000-0001-9495-0178
Lenkova T.N. orcid.org/0000-0001-8026-3983

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Abstract

The article provides theoretical and empirical data about the probiotic effect on the microbiota bioconsolidation in the broiler intestines of the Smena cross. The results of such studies can be used to improve the quality and volume of meat products in large-scale production. The probiotics have been used to improve feed digestibility and accelerate bird development. To stimulate the transformation of plant substrates in the bird intestines, probiotics were used instead of antibiotics. The probiotics contained the bacteria *Lactobacillus plantarum* and *Lactobacillus fermentum*. The study goal is to develop a methodology for fractal analysis of the frequency-taxonomic profiles of operational taxonomic units (OTUs) of the microbiota into the bird intestines. Using the fractal methodology, the index of microbiom bioconsolidation of the bird intestines was calculated, which characterizes the biosystem self-organization of microflora and the efficiency of biochemical transformations of plant substrates in the bird intestines. In the experiment, the microflora was studied in one control and two experimental groups of birds. The OTUs profiles were obtained by the molecular genetic NGS method (Next Generation Sequencing). The key concept of fractal analysis of OTUs profiles was the concept of the elementary OTU fractal. The elementary OTU fractal is three OTUs, the frequencies of which form a geometric numerical sequence (for example, {0.5; 0.25; 0.125}). The OTU profiles may contain several elementary OTU fractals combined into one larger OTU megafractal. We assume that if the number of OTUs combined into the OTU megafractal increases, then biochemical transformations of plant substrate are carried out more efficiently and on a large scale, and the bird macroorganism receives more nutrients. Therefore, we define the bioconsolidation index of the broiler microbiome as the ratio of the number of OTUs in the OTU megafractal to the total number of OTUs in the OTU profiles. The fractal portraits of OTU profiles were used to identify elementary OTU fractals. The elementary OTU fractals were identified by the linear arrangement of three OTU images on fractal portrait. The fractal analysis confirmed that the bacterial probiotics increase the microbiom bioconsolidation in the bird intestines. The microbiome bioconsolidation index in the experimental groups of birds (0.82...0.86) was higher than this index in the control group of birds (0.55). According to the results of fractal analysis, probiotic No. 1 (with *Lactobacillus plantarum*) is not recommended for use, and probiotic No. 2 (with *Lactobacillus fermentum*) is recommended for use.

Keywords: frequency-taxonomic profile, fractal portrait, intestinal microbiota, biosystem consolidation index, broilers, dietary probiotics, *Lactobacillus*.

Annual volumes of pedigree and hybrid young poultry import to the Russian Federation poses a risk of the uncontrolled spread of infectious diseases of various etiologies. Currently, active studies are being conducted to replace imported chickens with domestic highly productive genetic crosses marked with slow-feathering (*K*) or rapid-feathering (*k*) gene alleles.

Feeding is equally important and should regard the response of the bird microbiome to individual dietary components as a factor to significantly increase the meat quality and broiler performance in commercial poultry.

The feed digestibility for poultry depends entirely on the enzymatic activity of the intestinal microbiota [1-4]. Change in keeping conditions or diets, dietary antibiotics, pro- or prebiotics [5-8] and feed contamination with mycotoxins [9-11] force gut microbiota to rearrange its destructive biosystems in the abundance and profiles of microbial genotypes. As a result, the bird intestine microbiota can maintain the biotransformation of different plant substrates at the most efficient level [12]. This allows most of the nutrient resources to be redirected to the development of the poultry.

Biosystemic self-organization of gut microbial communities is necessary for transformation of substrates into nutrient and their timely delivery to the macroorganism. Due to intestinal microbial biosystem, birds can gain live weight faster and reach maximum egg production. In addition, microbial biosystems can protect against pathogenic microflora [13]. Therefore, the biosystem self-organization of gut microbiota is a factor, ensuring better performance and high egg production in poultry.

The macroorganism and microbiota of the bird's intestines form an integral biosystem in which they interact to develop and survive together. As the feed composition changes, the configuration and genotype composition of gut microbial biosystem of a bird undergo changes to the most effectively transform a variety of plant substrates into nutrients [14-16]. Therefore, partial or complete participation of genotypes in the functioning of intestinal microbial biosystem can serve as a quantitative indicator of biochemical transformation of plant substrates and protection of the host organism from pathogenic microflora [17-19].

Only a part of the gut microbiota is incorporated into the biosystem. In the self-organization of biosystems, only those genotypes are selected that are capable of performing the required biochemical transformations of forage substrates most efficiently and with the lowest energy and resource consumption [4, 18, 19]. Therefore, the self-organization and bioconsolidation of microorganisms ensure the implementation of required biochemical transformations of organic substrates with maximum intensity and in a certain order, that is, in an organized manner, in stages and with the lowest energy and resource costs on the part of the macroorganism. In such conditions, the bird receives all the necessary nutrients and develops rapidly.

In response to feed composition, the gut microbial biosystem rearranges its enzymatic profiles, metabolic pathways and connections between components [3, 13]. To stimulate the activity of microbial biosystems and increase the efficiency of transformation of plant substrates in the bird's intestines, various feed additives are used, e.g., antibiotics, exogenous enzymes, prebiotics, probiotics, synbiotics, and phytobiotics [4]. However, the ability of pathogenic microorganisms to acquire antibiotic resistance [2] led to the ban on their use in poultry feed in the EU countries since 2006. In this regard, the use of other types of feed stimulating and protective additives replacing antibiotics becomes relevant [6].

Molecular methods provide more information about gut microbiota of birds. To date, detailed frequency-taxonomic profiles of operational taxonomic units (OTU profiles) comprising thousands of OTUs are available [21]. For the 16S rRNA gene, the OTU profiles are only 10 % identified to genus and species according to international taxonomic information databases, and the rest OTUs add to the list of unidentified genotypes, possibly representing new species and genera [21-23]. OTU profiles provide taxonomic information on cultured and uncultured microorganisms and quantitative estimates for each microbial genotype, from minor to major member of the intestinal microbiota. For multivariate statistical analysis of quantitative and taxonomic information of the OTE profile, fractal analysis is best suited.

Fractal analysis of biological data is a class of multivariate statistical analyzes. Therefore, with its help, from the entire set of actual molecular genetic data, it is possible to extract information about the features of the biosystem organization of microbiomes in birds and to study the effect of probiotics on the self-organization of microbial biosystems. As applied to OTU profiles, the key concept of fractal analysis is an elementary fractal OTU, taking into account the special power-law ratio of the frequencies of three OTUs [24, 25]. This key concept stems from fundamental power-law quantitative relationships that are reflected in the relative sizes of elements in nature, including in plants (for example, tree fractals) and in microbiological biosystems.

Obviously, in birds, any changes in feeding and keeping are reflected in changes in the OTU profiles and their fractal characteristics. We believe that a decrease in the number of elementary OTU fractals and in the number of microbial genotypes in OTU fractals is a sign of weaker biosystem interactions of microorganisms and less effective biochemical transformations they carried out. Therefore, using the fractal analysis of the OTU frequency-taxonomic profiles and determining the bioconsolidation index of the gut microbiota in birds, it will be possible to assess the influence of probiotics on the biosystem self-organization of microbial communities and on the poultry grow and development.

The aim of the study was to develop a methodology for fractal analysis of frequency-taxonomic profiles of operational taxonomic units (OTUs) of the gut microbiota in birds. The fractal technique will allow calculation of the bioconsolidation index for the microbiota, and this index, in turn, is supposed to be used to study the effect of probiotics on the biosystem self-organization of microflora and the efficiency of biochemical transformations of plant substrates in the intestines of birds.

Our goal was to develop methods for fractal analysis of the frequency-taxonomic profiles of operational taxonomic units (OTUs) to characterize gut microbiota of birds. The fractal technique will allow calculation of the gut microbiota bioconsolidation index to assess it as a tool in investigation of how probiotics affect biosystem self-organization of microflora and biochemical transformations of plant substrates in the intestines of birds.

Description of the technique. Materials and methods of the verification test. To verify the method of fractal analysis of OTE profiles, a feeding trial was carried out (the Zagorskoe EPH, Moscow Province, 2018). Broiler chickens of the Smena cross were assigned for three feeding groups, 25 birds each. Control group 1c fed a basal diet (BD, Table 1), experimental group 2e fed the BD supplemented with *Lactobacillus plantarum*-based probiotic No. 1 (10^7 CFU/g, 1 kg/t feed), and experimental group (3e) fed the BD supplemented with *Lactobacillus fermentum*-based probiotic No. 2 (10^7 CFU/g, 1 kg/t). The chickens were kept in

AviMax cage batteries (Big Dutchman, Germany). The poultry was raised up to 36 days of age in accordance with the recommendations (FSC VNITIP RAS).

1. Composition (%) of basal diet (BD) supplemented with potential probiotic preparations for Smena cross broiler chickens in feeding groups (n = 25, Zagorskoe EPH, Moscow Province, 2018)

Ingredient	Days of age					
	1-21			22-36		
	1c	2e	3e	1c	2e	3e
Corn	60.00	–	–	60.00	–	–
Wheat	–	63.58	65.59	–	60.00	61.92
Soybean meal	26.21	16.63	16.52	17.12	17.52	17.43
Corn gluten	3.50	7.17	6.87	7.52	5.44	5.18
Fish flour	4.59	4.00	4.00	–	–	–
Sunflower meal	–	–	–	6.98	4.92	4.86
Sunflower oil	2.00	4.46	2.76	3.83	7.53	5.92
Limestone	1.46	1.58	1.58	1.49	1.53	1.53
Monocalcium Phosphate	0.91	0.82	0.82	1.35	1.23	1.23
Lysine monochlorohydrate	0.23	0.50	0.50	0.50	0.50	0.50
DL-methionine	0.29	0.31	0.31	0.25	0.30	0.30
Threonine	0.09	0.17	0.17	0.12	0.15	0.15
Salt	0.22	0.28	0.28	0.34	0.38	0.38
Cellobacterin-T	–	–	0.10	–	–	0.10
Premix	0.50	0.50	0.50	0.50	0.50	0.50

N o t e. 1c — control group, 2e, 3e — experimental groups. For 2e, BD was supplemented with probiotic No. 1 (10^7 CFU *Lactobacillus plantarum* per g), for 3e, BD was supplemented with probiotic No. 2 (0^7 CFU *Lactobacillus fermentum* per g), both at a dosage of 1 kg/t feed. Dashes indicate that the ingredient was removed from the diet.

On day 36, the broilers were weighed. Weighing results were processed by standard methods of analysis of variance using Microsoft Excel 2010 software. Parametric (Student's t-test) and nonparametric (Wilcoxon-Mann-Whitney method) statistical methods were used. The mean values (M) and standard errors of the means (±SEM) were calculated.

The caecum contents were aseptically collected after slaughter of chickens aged 36 days (in three replicates for each group) with strict adherence to sampling techniques and immediately frozen. The cecal microbiota was studied using next generation sequencing (NGS) technique [26] (the International Laboratory of Molecular Genetics and Poultry Genomics, Moscow). Total DNA was extracted using Genomic DNA Purification Kit (Fermentas, Inc., Lithuania). In PCR reaction, eubacterial primers 343F 5'-CTCCTACGGRRSGCAGCAG-3' and 806R 5'-GGACTACNVGGGTWTCTAAT-3' were used (Verity DNA amplifier, Life Technologies, Inc., USA). Metagenomic sequencing of amplified 16S rRNA gene fragments was performed (MiSeq Reagent Kit v3, MiSeq device, Illumina, Inc., USA). The resulting reads were subjected to bioinformation processing (CLC Bio GW 7.0 platform, Qiagen N.V., the Netherlands). Taxonomic analysis was performed using the RDP Classifier program (https://rdp.cme.msu.edu/classifier/classifier.jsp) using information databases.

Table 2 shows the taxonomic groups of microorganisms in the obtained OTU profiles of the intestinal microbiota in broiler chickens.

2. OTU frequency-taxonomic profiles and taxonomic groups of gut microbiota in 36-day-old Smena cross broiler chickens under probiotic feeding trials (n = 25, Zagorskoe EPH, Moscow Province, 2018)

No. OTU	Microorganisms
1	Bacillus
2	Lactobacillus*
3	Bifidobacteria
4	Cellulolyticus. Lachnospira
5	Cellulolyticus. Ruminococcus
6	Cellulolyticus. Clostridium
7	Cellulolyticus. Bacteroides

Continued Table 2

8	Cellulolyticus. Eubacterium
9	Conditionally pathogenic. Enterobacteriaceae*
10	Conditionally pathogenic. Actinomycetes*
11	Staphylococcus
12	Campylobacter
13	Pseudomonas
14	Proteobacteria
15	Tenericutes
16	Eripsipelotrichs
17	Uncultivated

N o t e. OTU — operational taxonomic unit. Lactobacilli (OTU No. 2), opportunistic Enterobacteriaceae (OTU No. 9), and Actinomycetes (OTU No. 10) are indicator groups of microorganisms to assess feed digestibility and the protection of poultry from pathogens.

Fractal analysis of OTU profiles. To minimizing energy and resource consumption, it is more profitable to decompose plant substrates by several microbial genotypes combined into a biosystem. This ensures the timely and sufficient enzymatic flows generated by microorganisms for destruction of plant substrates. In this case, both the number of enzymes and the number of microbial genotypes that generate enzymes will presumably be in one-to-one correspondence with the number of target restriction sites in decomposed organic molecules. Therefore, in an optimized destructing biosystem, there should be the same ratios between the numbers (frequencies) of microbial genotypes and the numbers (frequencies) of target restriction sites in organic molecules. This means that, in optimal microbial biosystems, the frequencies of genotypes and the frequencies of OTUs in OTU profiles, as well as restriction sites in decomposed organic molecules should be described by fundamental fractal power relations. On the basis of this statement, the definition of an elementary fractal OTU was formulated and a fractal analysis of the OTU profiles was carried out.

Determination of an elementary OTU fractal. If three OTE frequencies are ordered in a geometric numerical sequence, for example {0.5; 0.25; 0.125}, or in the logarithmic form the arithmetic numerical sequence{$log_2(0.5) = -1$; $log_2(0.25) = -2$; $log_2(0.125) = -3$}, then these OTUs represent an elementary OTU fractal, and the corresponding microbial genotypes belong to the biosystem part of the microbiota. In this case, it is allowed to combine several elementary OTU fractals into a larger OTU mega-fractal.

OTU mega-fractals give an idea of the genotypic composition of biosystems in the gut microbiota of birds and of the number of microbial genotypes realizing individual development strategies outside the biosystem. The ratio of fractal to out-of-fractal OTUs characterizes the microbial resource of the bird microflora involved in the biochemical transformation activity. We assume that a decrease in the number of OTUs in OTU mega-fractals, and hence, of genotypes in microbial biosystems, signals a decrease in the productivity of gut biochemical transformations in birds and, as a consequence, a delay in their development.

Fractal portrait of the OTE profile. For visual detection of OTE mega-fractals and their mathematical accounting, you can use the fractal portrait of the OTU profile. The construction of OTU fractal portraits greatly facilitates the detection of elementary OTU fractals.

Before constructing a fractal portrait, it is necessary to arrange the OTU profile in descending order of frequency values (Table 3). This is necessary to determine the OTU with the maximum frequency (p_{max}), that is, the OTU ranked first in the ordered OTU profile. After that, the procedure for constructing a fractal portrait of an OTU profile will be reduced to placing points (or any geometric figures) on the coordinate plane, which represent each OTU by their position on the portrait.

3. OTUs frequencies ranked in descending order in feeding groups of 36-day-old Smena cross broiler chickens under probiotic feeding trials (n = 25, Zagorskoe EPH, Moscow Province, 2018)

Group					
1кс		2e		3e	
No. OTU	frequency, %	No. OTU	frequency, %	No. OTU	frequency, %
6	26.7	5	39.4	5	31.7
7	25.3	6	20.8	6	22.3
5	24.5	7	19.3	7	19.3
17	14.8	17	13.8	17	13.6
2	3.2	2	2.3	2	4.1
14	2.0	4	1.2	14	3.2
4	1.8	14	1.2	9	1.3
10	0.6	10	0.6	4	1.0
16	0.5	3	0.4	1	0.9
9	0.4	16	0.3	10	0.8
8	0.2	8	0.2	13	0.4
		9	0.2	16	0.4
		15	0.2	11	0.3
		1	0.1	15	0.3
				3	0.2
				8	0.1
				12	0.1

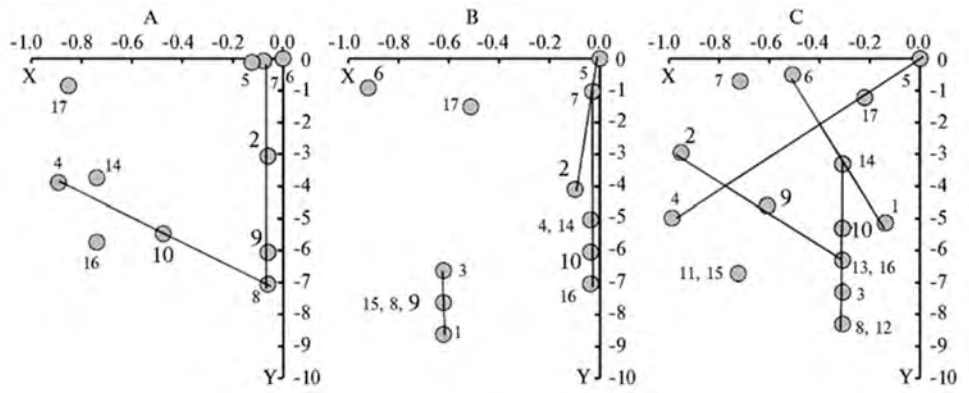
N o t e. OTU — operational taxonomic unit. OUT numbering corresponds to Table 2. 1c — control group, 2e, 3e — experimental groups. For 2e, BD was supplemented with probiotic No. 1 (10^7 CFU *Lactobacillus plantarum* per g), for 3e, BD was supplemented with probiotic No. 2 (0^7 CFU *Lactobacillus fermentum* per g), both at a dosage of 1 kg/t feed.

To determine each OTU position on the portrait (each OTU point), it is necessary to calculate their Y- and X-coordinates using the following formulas:

$$Y_i = log_2(p_i/p_{max}),\ X_i = \text{fractional part } log_2(p_i/p_{max}), \quad (1)$$

where p_i is the frequency OUT with serial number i in the OTU profile (see Table 3).

In accordance with the formulas (1) and the Table 3, we calculated the coordinates of the OTU points and constructed fractal portraits of the OTU profiles for the intestinal microbiota of the three groups of broilers under study (Fig.).



Fractal portraits of OTU (operational taxonomic unit) **profiles of gut microbiota in three feeding groups of 36-day-old Smena cross broiler chickens under probiotic feeding trials:** A — control group 1c (basal diet BD, see Table 1), B — experimental group 2e (BD + probiotic No. 1, *Lactobacillus plantarum*, 10^7 CFU/g), C — experimental group 3e (BD + probiotic No. 2, *Lactobacillus fermentum*, 10^7 CFU/g) (n = 25, Zagorskoe EPH, Moscow Province, 2018).

Y and X coordinates of OTU were calculated (1). The numbers near the circles correspond to the OTU numbering from Table 2. The numbers of lactobacilli (OTU No. 2), opportunistic Enterobacteriaceae (OTU No. 9) and Actinomycetes (OTU No. 10) are enlarged, since these are indicator groups of microorganisms to assess feed digestibility and poultry protection from pathogens. Segments of straight lines connecting the points indicate elementary OTU fractals.

In fractal portraits (see Fig.), some points are connected by segments of

straight lines. In this way, elementary OTU fractals are distinguished in the portraits, which does not contradict the definition of the elementary OTU fractal of the. For example, the logarithms of the frequencies for OTU No. 3, 9, 1 (see Fig., B) make an arithmetic series {–6.62; –7.62; –8.62}. Therefore, they represent an elementary OTU fractal and in the portrait should be connected by a straight-line segment. For similar reasons, the elementary OTU fractals are highlighted by straight-line segments, namely, the OTU Nos. 4, 10, 8 (see Fig., A) with frequencies logarithm series {–3.89; –5.48; –7.06}, OTU Nos. 10, 13, 3, 8 (see Fig., C) with {–5.31; –6.31; –7.31; –8.31}, etc. In the portraits, the elementary OTU fractals are combined into one OTU mega-fractal (see Fig. A, C) and into two OTU mega-fractals (see Fig., B).

Microbiota bioconsolidation index. Fractal portraits of OTU profiles (see Fig.) represent OTU mega-fractals of different genotype and quantitative composition. We believe that the number of OTUs in the of OTU mega-fractals (and hence the number of genotypes in the microbial biosystem) reflects the intensity of biochemical transformations of organic substrates in the bird's intestines performed by the part of microbiota arranged in a biosystem. Consequently, the more genotypes of the microbiota form the biosystem for biochemical transformation of plant substrates, the more efficient and large-scaled these transformations are, and the more nutrients the macroorganism receives. Hereof, we define the bioconsolidation index of the intestinal microbiota *Ind* in broilers as the ratio of the OUT number in the mega-fractal to total OUT in the OUT profiles:

$$Ind = N_F/N_0, \quad (2)$$

where N_F, N_0 are the number of OTUs in OUT mega-fractals and total OUT in the OUT profiles. Table 4 shows the results of bioconsolidation index calculations.

4. The mean live mass and bioconsolidation index of microbiota in 36-day-old Smena cross broiler chickens under probiotic feeding trials (n = 25, Zagorskoe EPH, Moscow Province, 2018)

Parameter	Group		
	1c	2e	3e
Average body weight, kg	2.15±0.02	2,05±0,02	2,14±0,02
Bioconsolidation index *Ind*	0.55±0.02	0,86±0,02	0,82±0,02

N o t e. 1c — control (basal diet BD, see Ttable 1), 2e, 3e — experimental groups. For 2e, BD was supplemented with probiotic No. 1 (10^7 CFU *Lactobacillus plantarum* per g), for 3e, BD was supplemented with probiotic No. 2 (0^7 CFU *Lactobacillus fermentum* per g), both at a dosage of 1 kg/t feed.

Discussion. Fractal analysis of the OTU frequency-taxonomic profiles of the broiler intestinal microbiota provides information on the size of OTU mega-fractals and on the number of microbial genotypes in the intestines that are arranged into a biosystem. The microbiota bioconsolidation index (*Ind*) is calculated as the ratio of the number of OTUs in OTU mega-fractals to the total number of OTUs in the frequency-taxonomic profiles of the intestinal microbiota. Equality to 1 of the microbiota bioconsolidation index (*Ind* = 1) means that all microbiota genotypes form biosystem to provide biotransformation, and equality to 0 (*Ind* = 0) means the absence of the biosystem organization of the microbiota in broilers. Based on the fractal analysis of the OTU frequency-taxonomic profiles, it was found that in broilers from the experimental groups the value of the microbiota bioconsolidation index (*Ind* = 0.86 in group 2e and *Ind* = 0.82 in group 3e) exceeds the microbiota bioconsolidation index in the control (*Ind* = 0.55). This means that the use of *L. plantarum* and *L. fermentum* bacteria as probiotics promotes better self-organization of the poultry intestinal microbiota.

However, the average body weight of broilers in the control and experimental groups (see Table 4) do not correlate with microbiota bioconsolidation

indices. In addition, the average body weight of broilers in the experimental group 2e (2.05±0.02 kg) was even less than in the control group 1c (2.15±0.02 kg). Perhaps this is a consequence of the unsuccessful self-organization of microbial biosystems in the intestines of birds from group 2e, which was provoked by the probiotic preparation No. 1. In contrast to the fractal portrait B (see Fig.), portraits A and C (see Fig.) contain one OTU mega-fractal each. In addition, attention should be drawn to the fact that the indicative OTUs No. 2, 9, 10 (lactobacilli, enterobacteria and actinomycetes) are separated in two unrelated OTU mega-fractals (see Fig., B) while in fractal portraits A and B (see Fig.) these OTUs are grouped in one OTU mega-fractal. Probably, the location of indicative OTUs in different OTU mega-fractals, and hence the location of indicative genotypes in different biosystems, affects the decrease in the efficiency of conversion of organic substrates and delayed development of broilers in group 2e.

Thus, here we propose methods for fractal analysis of the frequency-taxonomic profiles of operational taxonomic units (OTUs) of the intestinal microbiota in birds to assess the influence of food factors on the biosystemic self-organization of microflora and the efficiency of biochemical transformations of plant substrates. Based on the calculated bioconsolidation index of the intestinal microbiota for broilers fed diets supplemented with various experimental probiotics, *Lactobacillus fermentum* is recommended for use, since these bacteria contribute to the better self-organization of microorganisms into functional biosystems, promote the development of birds and may reduce incidence of diseases.

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