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EXPRESSION OF GENES ASSOCIATED WITH ECONOMIC TRAITS OF BROILER CHICKENS (*Gallus gallus domesticus*), AS INFLUENCED BY VARIOUS PARATYPICAL FACTORS

(review)

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Abstract

Commercial production of broiler chicken meat is based on the use of early maturing high-yielding crosses created by geneticists and breeders. The original lines of modern broiler chickens were obtained through artificial selection, primarily in terms of feed efficiency, conversion and growth rate (W. Fu et al., 2016). Progressive genetic research, breeding and feeding techniques combined with effective veterinary control ensure production of high quality poultry meat (A.A. Grozina, 2014). From 1957 to 2001, the time for broiler chickens to reach market weight decreased 3-fold, while feed intake decreased too (M. Georges et al., 2019). Expression study of genes involved in broiler growth and development, nutrient assimilation, and resistance to pathogens is necessary for successful selection of birds with desirable qualities (K. Lassiter et al., 2019). The aim of the review is to analyze the diversity of genes and their activity in the formation of economically useful traits of broiler chickens and factors influencing their expression. The article presents an overview of the genes involved in growth and development (*GH*, *IGF-1*, *GHR*, *MYOD1*, *MYOG*, *MSTN*), nutrient assimilation (*SLC2A1*, *SLC2A2*, *SLC2A3*, *SLC2A8*, *SLC2A9*, *SLC2A12*, *SLC6A19*, *SLC7A1*, *SLC7A2*, *SLC7A5-7*, *SLC15A1*, *SLC38A2*), immune response (*IL1B*, *IL6*, *IL8L2*, *IL16*, *IL17A*, *IL18*, *TNF-α*, *AvBD1-AvBD14*). A somatotropic growth hormone (GH)—insulin-like growth factor 1 (IGF-1)—growth hormone receptor (GHR) axis is a pathway to regulate skeletal growth rate and body size (L.E. Ellestad et al., 2019). Analysis of the gene *GH, GHR,* and *IGF-1* expression and selection for high growth rate in broiler chickens can increase growth hormone binding activity, IGF-1 synthesis in the liver, and therefore body weight (S. Pech-Pool et al., 2020). Myogenesis is mediated by a number of factors and genes, including myogenic regulatory factors (*MRF*), myogenic differentiation factor 1 (*MYOD1*), myogenin (*MYOG*) the expression of which may vary depending on the feed ingredient and specific additives. Dietary proteases significantly increase the expression of *MYOD1* and *MYOG* genes in pectoral muscle, *GH* and *IGF-1* in liver and improve growth performance (Y. Xiao et al., 2020). Genes associated with nutrient absorption and their expression affect transport proteins, leading to accelerated nutrient delivery to the intestinal epithelium, circulatory system, and then to all organs and tissues. In turn, their expression can depend on various feed additives. Solute carrier family (SLC) proteins involved in amino acid transport comprises SLC6A19 (B0AT1) and SLC38A2 (SNAT2) sodium-dependent carriers of neutral amino acids; SLC7A1 and SLC7A2 carriers of cationic amino acids (cationic amino acid transporter — CAT: CAT1, CAT2); SLC7A5-7 L-type amino acid transporter (LAT: LAT1, gLAT2) (J.A. Payne et al, 2019; C.N. Khwatenge et al., 2020; N.S. Fagundes et al., 2020). Immunity gene expression (*IL1B*, *IL6*, *IL8L2*, *IL16*, *IL17A*, *IL18*, *TNF-α*, *AvBD1-AvBD14*) initiating the synthesis of immune response factors is affected by *Escherichia coli*, *Salmonella* spp., *Pseudomonas aeruginosa*, *Clostridium perfringens*, *Listeria monocytogenes*, *Eimeria* spp. infections (G.Y. Laptev et al., 2019; T. Nii et al., 2019). The modulating effect of temperature on gene expression was also revealed. Increased rearing temperature (39 °C) leads to a significant increase in expression of *IL6*, *IL1b*, *TNF-α*, *TLR2*, *TLR4*, *NFkB50*, *NFkB65*, *Hsp70* and *HSF3* genes in spleen and liver tissues (M.B. Al-Zghoul et al., 2019). Various feed additives (prebiotics, probiotics, synbiotics, phytobiotics and amino acids) are being

sought that act via modulation of gene expression and may maintain the physiological condition of birds, prevent the development of diseases, promote faster growth without compromising health and thus improve poultry productivity.

Keywords: broiler chickens, productivity, gene expression, growth, immunity, feed additives

Global demand for animal products is expected to increase by 70% by 2050. Meeting this demand will require the use of scientific advances, the use of modern technologies with minimal impact on the environment and improving the quality of raw materials of animal origin (mainly by genetic methods). Global livestock productivity, measured by carcass weight and egg production since the 1960s, has increased by 20-30% as a result of developments in nutrition, genetics, and disease control [1].

Farm animals are excellent model organisms for genetic studies of phenotypic evolution. Domestic animals have developed adaptations at the gene level to new environmental conditions and have been subjected to strict human selection, which has determined amazing phenotypic transformations in their behavior, morphology and physiology. The search for genetic changes that underlie phenotypic changes provides an opportunity to take a different look at the general mechanisms through which genetic variation determines phenotypic diversity.

Historically, domestic chickens were bred for two purposes — meat and eggs. Throughout the 20th century, specialized crosses of broiler chickens and laying hens were created and improved in order to improve productivity both in terms of growth and reproductive properties. This approach, coupled with the introduction of new genomic selection methods, has become most effective in achieving maximum productivity [2].

The original lines of modern broiler chickens are obtained through careful artificial selection, primarily for feed efficiency and growth rate, the traits that are of primary importance for the economic efficiency of the industry [3]. Progressive genetic research, breeding technologies and nutrition, combined with effective veterinary control, make it possible to produce high-quality poultry meat [4]. From 1957 to 2001, the time to reach market weight of broiler chickens decreased 3 times while feed consumption also decreased [1].

Feed conversion is an important genetic trait that determines economic efficiency, since 70% of the cost of raising animals is on feed. Determining the mRNA expression of genes involved in broiler growth and development, nutrient absorption, and pathogen resistance is essential for the successful selection of birds with desirable traits [5].

The purpose of this review was to analyze the diversity of genes and their activity in the formation of economically useful traits in broiler chickens and the factors influencing the expression of these genes.

Genes associated with growth of broiler chickens. Growth rate, weight and body parameters are determined by genotype and environmental factors, including nutrition. Along with genes (Table 1), the nervous and endocrine systems play a significant role in regulating broiler chicken growth [6].

Among the components of the neuroendocrine system, the somatotropic axis receives the most attention [7]. The main regulator of skeletal growth rate and body size is the growth hormone (GH)—insulin like growth factor 1 (IGF-1) pathway. These hormones stimulate tissue growth, regulate the metabolism of proteins, lipids and carbohydrates, and maintain homeostasis [6]. The effect of growth hormone on the body can be carried out directly through activation of the growth hormone receptor (GHR) or indirectly through its messenger IGF-1, produced in the liver and promoting the growth of muscle tissue [8].

1. Genes associated with growth of broiler chickens

Gene	Protein	Function	Biomaterial	Reference
GH	Growth hormone	Postnatal tissue growth, including skeletal muscle	Pituitary gland, liver, skeletal muscles	[6]
IGF-1	Insulin-like growth factor 1	Muscle and bone growth	Liver, skeletal muscles	[9]
GHR	Growth hormone receptor	Binding of growth hormone and activation of signal transduction leading to growth	Liver, skeletal muscles	[10, 11]
MYOD1 (MYOD)	Myogenic differentiation factor 1	Growth and development of skeletal muscles	Skeletal muscles	[12, 13]
MYOG	Myogenin	Growth and development of skeletal muscles	Skeletal muscles	
MSTN	Myostatin	Suppression of skeletal muscle growth and differentiation	Skeletal muscles	[14]

Analysis of the expression of *GH*, *GHR* and *IGF-1* genes in fast-growing broilers and slow-growing laying hens showed a significant difference. Thus, in slow-growing chickens, high expression of GH mRNA in the pituitary gland and low expression of *GHR* and *IGF*-1 mRNA in the liver and muscles were noted, while in broilers the values were opposite. Slow-growing chickens presumably had reduced GH binding activity in the liver, and this could be caused by downregulation of growth hormone receptors in the liver by increasing the amount of growth hormone in the plasma. Selection for high growth rate in broiler chickens could increase growth hormone binding activity, hepatic IGF-1 synthesis, and hence body weight [6].

Muscle growth, or myogenesis, is a complex, precisely regulated process [12]. Myoblasts participate in the formation of skeletal muscles of broiler chickens. Their differentiation is controlled by myogenic regulatory factors (MRFs) [11]. These factors are involved in the proliferation and differentiation of myoblasts (13), as well as in the regulation of skeletal muscle development and promote their growth. The MRF family includes myogenic differentiation factor 1 (MYOD1) and myogenin (MYOG) [12].

Myostatin (MSTN) is a protein of the transforming growth factor beta (TGF-β) superfamily, which is secreted by skeletal muscles and acts as a potent inhibitor of muscle tissue growth and differentiation. Mutations in the *MSTN* gene cause myofiber hypertrophy which leads to increased muscle mass [15]. A striking phenotypic example of the manifestation of such a mutation are Belgian Blue cows, in which a natural mutation in the *MSTN* gene was identified [16].

Some ingredients in the diet can affect the expression of genes associated with body weight growth in broiler chickens. Thus, when protease was added to the diet, the expression of the *MYOD1* and *MYOG* genes in the pectoral muscles, as well as the *GH* and *IGF-1* genes in the liver significantly increased, along with improved growth performance [11]. Feeding chickens creatine in combination with pyruvate has been shown to reduce myostatin expression in breast muscles [14].

Genes associated with nutrient utilization. Broiler chickens exhibit rapid growth and development while meeting energy and nutrient requirements [17]. Poultry growth and productivity depend to some extent on the ability of the intestines to digest and absorb nutrients [18]. The main site of their absorption is the small intestine [19]. Transport of essential nutrients, the proteins, carbohydrates and fatty acids in the small intestine is performed by carrier proteins that are expressed in enterocytes. Improving the transport of nutrients due to the activation of genes encoding transport proteins can lead to an accelerated entry of these substances into the intestinal epithelium, the circulatory system, and then to all organs and tissues.

Once broken down into peptides and amino acids, proteins are transported to the small intestine [20]. Amino acid transport is carried out by carriers of the solute carrier family (SLC): SLC6A19 (B0AT1) and SLC38A2 (SNAT2), the sodium-dependent carriers of neutral amino acids; SLC7A1 and SLC7A2, the

cationic amino acid transporter (CAT); SLC7A5-7, the L-type amino acid transporter (LAT) [21].

Cationic amino acid transporters perform bidirectional transport to exchange cationic amino acids such as lysine, arginine and histidine between organs. CAT1 (cationic amino acid transporter 1), CAT2 (cationic amino acid transporter 2) and LAT2 (Y+L amino acid transporter 2) are involved in the transport of arginine and lysine. SNAT2 (sodium-coupled neutral amino acid transporter 2) transports L-glutamine to maintain homeostasis. LAT1 (L-type amino acid transporter 1) carries out the outflow of neutral amino acids (leucine, isoleucine, methionine) and the influx of aromatic ones (phenylalanine, tyrosine, tryptophan) [17, 22]. In turn, the peptides are transported to the small intestine via peptide transporter 1 (PEPT1), located within the membrane of epithelial cells [20].

Correction of the diet with various additives may affect the expression of genes associated with nutrient transport (Table 2). Thus, 0.5 g/kg cricket chitin added to the basal diet of Cobb 500 broiler chickens increased the relative expression of the *SLC15A1* gene mRNA on day 42 of growing, while 0.5 g/kg dietary cricket chitosan, on the contrary, decreased this parameter [18]. The addition of 0.03-0.09% protease to the basal diet of Ross 308 cross broilers increased the expression of the genes of the amino acid transporters *SCL6A19*, *SLC7A1*, *SLC7A2*, *SLC7A6*, *SLC7A7* and the peptide transporter *SLC15A1* [11].

Polysaccharides, broken down into glucose, fructose and galactose, are absorbed by enterocytes lining the microvilli of the small intestine [23]. Thus, in the form of monosaccharides, they enter the bloodstream and from there are transported into cells using membrane glucose transporter proteins (GLUT) [24]. In mammals, GLUT4 is a well-studied protein that serves as a major insulin-dependent transporter in skeletal muscle and adipose tissue [25] and is responsible for rapid glucose transport following insulin production by the pancreas [26]. However, the absence of GLUT4 has been revealed in chickens and broiler chickens, and only GLUT1, GLUT2, GLUT3, GLUT8, GLUT9 and GLUT12, the genes of which are expressed in skeletal muscles [27, 28], hypothalamus, liver, heart, adipose tissue, kidneys [23] and small intestine [20] have been partially described and characterized.

However, it has been established that in birds the insulin-induced glucose transporter protein GLUT12 may be an analogue of the GLUT4 transporter in mammals [27]. Expression of the transporter genes GLUT1, GLUT8 and GLUT12 depends on the stage of ontogenesis. Thus, during embryogenesis and within 5 days after hatching, the *SLC2A1* gene was expressed in the pectoralis major muscle, while the *SLC2A8* gene was expressed after hatching. The expression of *SLC2A12* gradually increased from day 12 of embryonic development to day 5 after hatching. In the sartorius muscle, the expression of *SLC2A1* and *SLC2A8* remained unchanged, while the *SLC2A12* expression also gradually increased during early muscle development after chick hatching [28].

The addition of dried beer grains fermented by *Bacillus subtilis*, *Lactobacillus rhamnosus* and *Saccharomyces cerevisae* to the basal diet increased the expression of vector genes (*SLC2A1*, *SLC2A2*, *SLC7A1*, *SLC7A2*, *SLC7A5*, *SLC15A1*) in broiler chickens of the Ross 308 cross [20].

Different tissue specificity of glucose transporters was found in chickens, with mRNA expression of the *SLC2A1* gene being high in the hypothalamus, *SLC2A2* and *SLC2A9* in the liver, *SLC2A3* in skeletal muscle, and the *SLC2A8* gene was equally expressed in all tissues studied, including abdominal fat. Moreover, in chickens with high body weight, the expression of these genes was higher than in chickens with low body weight [25].

2. Genes involved in nutrient transport in broiler chickens

Gene	Protein	Function	Biomaterial	Reference
SLC2A1	Hexose transporter (GLUT1)	Transport of glucose, fructose, galactose	Skeletal muscle, small intestine, liver, hypothalamus, abdominal fat	[20, 25, 28]
SLC2A2	Hexose transporter (GLUT2)		Small intestine, liver, hypothalamus, abdominal fat	[20, 25]
SLC2A3	Hexose transporter (GLUT3)		Liver, hypothalamus, abdominal fat	[25]
SLC2A9	Hexose transporter (GLUT9)			
SLC2A8	Hexose transporter (GLUT8)		Skeletal muscles	[28]
SLC2A12	Hexose transporter (GLUT12)			
SLC15A1	Peptide transporter (PEPT1)	Peptide transport	Small intestine and pectoral muscles	[11]
SLC38A2	Amino acid transporter (SNAT2)	Transport of neutral amino acids		[22]
SLC6A19	Amino acid transporter (B0AT1)			
SLC7A1	Amino acid transporter (CAT1)	Transport of cationic amino acids		
SLC7A2	Amino acid transporter (CAT2)			[11, 17]
SLC7A5	Amino acid transporter (LAT1)	Transport of L-amino acids		[22]
SLC7A6	Amino acid transporter (γLAT2)	Transport of γ-L-amino acids		[11, 17]
SLC7A7	Amino acid transporter (LAT3, γLAT1)			[11]

Addition of 2% sugarcane bagasse to the basal diet of Ross 308 chickens upregulated the expression of *SLC7A1* (*CAT1*) in the duodenum, jejunum, and ileum, and *SLC6A19* (*B0AT1*) in the ileum only. Downregulation of the *SLC2A2* (*GLUT2*) gene in the small intestine was observed. Birds fed a coarse corn diet had increased expression of *SLC7A6* (*γLAT2*) in the jejunum and *SLC7A7* (*γLAT1*) in the ileum. However, feed additives did not affect the expression of the *SLC7A2* (*CAT2*), *SLC2A1* (*GLUT1*) and *SLC15A1* (*PEPT1*) genes [19].

Genes associated with immunity. The expression of immunity genes in broiler chickens (Table 3) is influenced by infection with microorganisms *Escherichia coli*, *Salmonella* spp., *Pseudomonas aeruginosa*, *Clostridium perfringens*, *Listeria monocytogenes*, *Eimeria* spp. and others, initiating the synthesis of immune response factors [29, 30]. The poultry gastrointestinal tract is a major entry point for pathogens that can cause enteric infections, which result in significant economic losses due to treatment costs, reduced growth, and premature mortality [31]. The protective barrier functions in the intestine are provided by the mucous layer covering the epithelium, tight junctions between epithelial cells, and factors of innate (macrophages, cytokines and antimicrobial peptides) and acquired (T- and B-lymphocytes and secreted IgA) immunity [30].

3. Genes involved in the immune response in broiler chickens

Gene	Protein	Function	Biomaterial	Reference
IL1B, *IL6*, *IL8L2*, *IL16*, *IL17A*, *IL18*	Proinflammatory cytokines (interleukins: IL1β, IL6, IL8, IL16, IL17 и IL18)	Attraction of immune cells to the site of infection, development of inflammation	Spleen, small intestine, macrophages, cloacal bursa	[29, 33, 36, 39]
TNF-α	Proinflammatory cytokine — tumor necrosis factor	Development of an inflammatory response	Spleen	[29]
AvBD1-14	Gallinatsin (Gal-1-14)	Antimicrobial action	Bone marrow, respiratory tract, skin, small intestine, liver, genitourinary organs, spleen, thymus, cloacal bursa, red blood cells	[38, 41, 42]

Cytokines are small extracellular signaling proteins that play a significant role in the development of the immune system, as well as in the formation of the immune response to pathogens or environmental stressors, such as changes in the temperature of poultry rearing. It is known that in vertebrates, cytokines are secreted by all types of cells, the immune cells, blood cells, connective tissue, spleen, thymus, etc. In birds, tumor necrosis factor α (tumor necrosis factor-alpha, TNF-α) and interleukins (IL) 1β, 6, 8, 16, 17, and 18 act as proinflammatory cytokines, that is, they contribute to the development of the inflammatory response during bacterial, viral, and protozoal infections [32]. Moreover, IL8 is a chemokine that induces chemotaxis in immunocompetent cells such as macrophages and monocytes [30].

It has been proven that pathogenic microorganisms stimulate the expression of pro-inflammatory cytokines in broiler chickens. Thus, an increase in the expression of IL6 was found in the ileum and cecum of Ross 308 cross chickens infected with *Campylobacter jejuni* [33], and when chicken embryo fibroblasts were infected with the reticuloendotheliosis virus (REV) [34]. In broiler chickens, the *Eimeria tenella* indection increases in the expression of IL6 and IL8 in the spleen and cecum [35], *Salmonella enteritidis* increases IL1B and IL8 levels in enterocytes and macrophages, while exposure to sodium butyrate at a subinhibitory concentration reduces bacterial colonization due to gene suppression proinflammatory cytokines [36]. Addition of deoxynivalenol at a concentration of 5 mg/kg to the diet of Ross 308 chickens increased the expression of *IL6* and the tight junction protein *claudin 1* (*CLDN1*) in the duodenum [37].

A modulating effect of temperature on gene expression was revealed.

Increased poultry rearing temperature (39 °C) leads to a significant increase in the mRNA expression of *IL6, IL1β, TNF-α, TLR2, TLR4, NFκB50, NFκB65, Hsp70* and *HSF3* genes in spleen and liver tissues [38]. Reduced rearing temperature (gradual decrease to 20 °C) can lead to a slight increase in *IL2*, *IL6* gene mRNA expression in the spleen, indicating the ability to adapt to cold [39]. Antimicrobial peptides defensins divided into three classes, the α-, β- and θ-defensins, play a significant role in immunity; α- and θ-defensins are found in mammals, while β-defensins, also known as gallinacins (Gal), are found only in birds.

Defensins kill a wide range of bacteria, fungi and viruses and can stimulate the acquired immune response against pathogens. Currently, in chickens 14 β-defensins (from AvBD1 to AvBD14) have been identified the genes of which are expressed in the bone marrow, respiratory tract, skin, digestive tract, liver, genitourinary and immune organs (spleen, thymus, cloacal bursa), and also in erythrocytes. In addition, another group of defensins, the ovodefensins (gallins), was described in birds, which have antimicrobial activity against *E. coli*, are expressed in the oviduct and its membrane, and are present in egg albumen [40-42].

When chicken cells were infected in vitro with the intestinal commensal *Lactobacillus johnsonii*, *Bacteriodes doreii*, the expression of the *IL1B* and *IL6* genes increased, while the expression of *AvBD8-AvBD10* remained almost unchanged. These results suggest that commensal gut bacteria do not induce the *AvBD8-AvBD10* gene expression. However, when *E. coli* and *Enterococcus faecalis* were cultured with artificially synthesized defensins (AvBD6, AvBD9, and AvBD10), opportunistic bacteria were suppressed [40].

It was found that 14 *AvBD* genes are expressed depending on the bird breed and tissue type. The degree of acquired and innate immunity varies among breeds [39].

The influence of the feed factor on the expression of genes potentially significant when raising broiler chickens. Additional sources of nutrients (prebiotics, probiotics, synbiotics, phytobiotics, vitamins, enzymes, amino acids, minerals, fatty and organic acids) play an important role in maintaining the health of broiler chickens. They promote the growth and development of poultry, support the functioning of normal intestinal microflora, have an antimicrobial effect against pathogenic microflora, and strengthen the immune system [43]. Table 4 provides a summary of the effects of various feed additives on the expression of genes of potential importance in broiler chicken production.

4. Expression of genes potentially significant in raising broiler chickens as influenced by various substances

Feed additive	Gene	Function	Reference
Oligosaccharides of the raffinose family	*CD3*, *chB6*	Increased expression	[44]
Lactobacillus plantarum and oligosaccharides of the raffinose family	*IL1β*, *IL6*, *IL8*, *IL18*	Increased expression	[45]
Mannanoligosaccharides	*PEPT1*	Increased expression	[46]
Thyme extract	*GH*, *IGF-1*	Increased expression	[47]
β-Glucan	*IL1*, *IL18*, *TNF-α*	Increased expression	[48-50]
	AvBD1, *AvBD2*, *AvBD4*, *AvBD6*, *AvBD9*, *AvBD4*, *AvBD9*	Decrease in expression	
Galactooligosaccharides	*IL17A*, *IL1β*, *AvBD1*, *GLUT1*	Increased expression	[51]
	IL10, *GLUT2*	Decrease in expression	[52, 53]
Inulin	*GHR*, *IGF-1*	Increased expression	[54, 55]
	IL6, *IL8*, *IL18*	Decrease in expression	
Lactobacillus spp.	*IL1β*, *IL6*	Decrease in expression	[56]
ДНК *Lactobacillus acidophilus*	*IL18*	Increased expression	[57]
Bacillus amyloliquefaciens	*IL1β*	Increased expression	[58]
Lactobacillus salivarius and galactooligosaccharides	*IL1β*, *IL6*, *IL18*	Increased expression in the spleen	[45]
	IL1β, *IL8*	Decreased expression in the cecum	

Continued Table 4

Lactococcus lactis subsp. *lactis* 2955 and inulin	*IL6*, *IL8*, *IL18*	Decrease in expression	[55]
Lactobacillus reuteri, *Enterococcus faecium*, *Bifidobacterium animalis*, *Pediococcus acidilactici* and fructooligosaccharide	*IL1β*	Decrease in expression	[59, 60]
	IL10	Increased expression upon infection *Clostridium perfringens*	
	IL1β, *IL10*	Reduced expression during infection *Salmonella enterica* ser. *Enteritidis*	
Essential oils of garlic, lemon, thyme, eucalyptus (Intebio, BIOTROF, Russia) (infection of *S. enterica* ser. *enteritidis*)	*AvBD10*, *IL6*, *IL8L2*	Increased expression followed by decrease	[29]
	AvBD9	No influence	
Thymol (infection of *S. enterica* ser. *Typhimurium*)	*IL10*	Increased expression	[61]
	IL6	Decrease in expression	
Chestnut tannins	*IL6, IL10*	Increased expression	[62]
	IL1β, IL8	No influence	
Licorice extract (infection of *Campylobacter jejuni*)	*IL1β*	Decrease in expression	[63]
Essential oils of mint, star anise, cloves	*IL18*	Decrease in expression	[64]
Basil	*GH*	Increased expression	[65]
	GHR	No influence	
Sage, chamomile, wall germander, marjoram	*IGF-1*	Increased expression	[66]
Dogwood cherry extract	*GLUT-1*, *GLUT-2*	Increased expression	[67]
Methionine	*LAT1*	Reduced expression in methionine deficiency	[22]
	SNAT2, *CAT1*	Increased expression in methionine deficiency	
	B0AT1	Increased expression under methionine deficiency and normal levels	
	MYOD, *MYOG*	No influence	[68]
Methionine and cysteine	*IGF-1*	Increased expression	[69]
L-arginine	*MYOD*, *MYOG*	Increased expression	[70, 71]
	IL8, *TNF-α*	No influence	

Prebiotics. Prebiotics improve and support the functioning of the intestine by stimulating the growth of the number and biodiversity of beneficial microorganisms and reducing the spread of pathogenic microorganisms, and also have a positive effect on lymphoid tissue and innate immunity of the intestine. Prebiotics are fructooligosaccharides (FOS), galactooligosaccharides (GOS) and raffinose (RFO) family of oligosaccharides, extracted from various plants; mannanoligosaccharides (MOS) from the cell walls of the yeast *Saccharomyces cerevisiae*; β-glucan from the cell walls of yeast or fungi [45, 49, 72].

Injection of RFO into Cobb 500 broiler embryos at increasing concentrations (1.5, 3.0, and 4.5 mg) directly proportionally increased the expression of *CD3* and *chB6* which serve as markers of T- and B-cells [44].

Some polysaccharides, such as β-glucans, affect genes associated with immunity. Thus, the introduction of 0.1% β-glucan into feed induces the expression of the *IL1*, *IL18* and *TNF-α* genes. In turn, the increased content of TNF-α in poultry stimulates the appearance of CD8+ lymphocytes (T-killer cells) [48, 49], and such a response may depend on infection. In Cobb cross broiler chickens infected with *Salmonella enteritidis* and consuming β-glucan, the expression of *AvBD1*, *AvBD2*, *AvBD4*, *AvBD6*, *AvBD9* genes increases while in birds free from infection, on the contrary, the *AvBD4* and *AvBD9*expression in the spleen decreased. Consequently, β-glucan exhibits immunostimulant properties, providing protection against pathogen infection [50].

Dietary galactooligosaccharide (GOS) modulated the immune response by increasing the expression of the cytokine IL17A, improving growth performance [51], and decreasing *IL10* expression in the ileum and cecum of Ross 308 chickens [52]. Administration of GOS activated the *IL1β*, *IL10*, *AvBD1*, *GLUT1* and *GLUT2* genes in the jejunum and cecum of Ross 308 broilers while suppressing the glucose transporter gene *GLUT2* expression in the duodenum [53].

In broiler chickens fed postbiotics (metabolic products of *Lactobacillus plantarum* RG14 and *L. plantarum* RI11) and inulin (fructooligosaccharide), hepatic *GHR* and *IGF-1* mRNA expression and final body weight increased [54].

Probiotics. Probiotics are live strains of microorganisms that, when administered in adequate quantities, have a positive effect on health, intestinal functioning, prevent the proliferation of pathogenic microflora, and generally promote the growth of the macroorganism. Probiotics are widely used in feeds, especially those intended for animals with a simple monogastric stomach. Probiotic strains include microorganisms of the genera *Bacillus*, *Enterococcus*, *Lactobacillus*, *Bifidobacterium*, *Pediococcus*, *Streptococcus*, *Saccharomyces* and *Kluyveromyces* [73].

Probiotic based on *Lactobacillus* spp. is able to reduce the expression of *IL1β* and *IL6* in the cecum of Arbor Acres cross broiler chickens infected with *Salmonella typhimurium*. Suppression of expression by probiotics is most likely due to reduced intestinal colonization by pathogens. Treatment of chicken cecal cells with *Lactobacillus acidophilus* DNA increased *IL18* expression [56, 57, 74].

A probiotic consisting of three strains of *Bacillus amyloliquefaciens* increased *IL1β* expression in the ileum on day 21 in Cobb 500 broilers infected with *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella* oocysts [58].

Synbiotics. Synbiotics are a combination of pre- and probiotics that have beneficial effects on the health, create a protective barrier in the digestive tract and promote the growth of beneficial intestinal microorganisms [73].

Administration of *Lactobacillus salivarius* in combination with GOS to Cobb broiler chicken embryos on day 12 of incubation significantly increased the expression of *IL1β* in the spleen on day 7 and *IL6* and *IL18* on day 21. In the cecum, this combination led to a decrease in the expression of *IL1β* and *IL8* on day 42 [45]. Inulin enriched with 1000 CFU of *Lactococcus lactis* subsp. *lactis* 2955, as well as the prebiotic alone, can reduce the expression of genes *IL6*, *IL8*, *IL18* associated with immunity. Moreover, the suppression was more pronounced in the cecum than in the spleen, and increased with the age of broilers [55].

A dietary synbiotic containing four live strains *Lactobacillus reuteri*, *Enterococcus faecium*, *Bifidobacterium animalis* and *Pediococcus acidilactici* and the prebiotic fructooligosaccharide reduced the content of *IL1β* mRNA, while increasing the *IL10* mRNA in Cobb 500 broiler chickens infected with *Clostridium perfringens* [59]. This supplement reduced the *IL1β* and *IL10* mRNAs when chickens were infected with *Salmonella enterica* ser. *enteritidis* [60].

Phytobiotics. Phytobiotics are bioactive substances of plant origin, including phenolic, nitrogen-containing and organosulfur compounds, alkaloids, phytosterols and carotenoids. They are found in fruits, vegetables, grains and legumes, nuts, herbs and essential oils. Phytobiotics can be used as antimicrobials for protection against pathogenic bacteria, viruses and fungi. They are added to feed to improve health, productivity, the quality of poultry meat and eggs and as growth stimulants. Phytobiotics can also act as prebiotics and provide nutrients for beneficial gut bacteria [74, 75].

A phytobiotic containing a mixture of garlic, lemon, thyme and eucalyptus essential oils increased the *AvBD10*, *IL6* and *IL8L2* expression in Ross 308 chickens on day 1 after infection with *S. enterica* ser. *enteritidis* followed by a decrease in expression. At the early stages of infection, the phytobiotic stimulated the immune response to the pathogen and then suppressed the inflammatory response [29]. Feeding broiler chickens of the Ross 308 cross with 1% thymol nanoemulsion (a phenolic compound from thyme essential oil) increased the expression of *IL10*, decreased the expression of *IL6* and improved growth during infection with *S. enterica* ser. *typhimurium* [61]. Thyme extract had no effect on the expression of *GH* and *IGF1* genes in Cobb 500FF broilers [47].

Adding dietary chestnut tannins significantly increased the expression of the cytokines *IL6* and *IL10* in Ross 308 chickens on days 2 and 6 of feeding, while no significant increase was observed for the proinflammatory cytokines *IL1β* and *IL8*. This phytobiotic product has the potential to support growth and feed conversion efficiency [76]. The *Glycyrrhiza glabra* (licorice) extract added to the diet of Ross 308 broilers increased bodyweight gain and improved feed conversion. Moreover, infection of chickens with *Campylobacter jejuni* led to a decrease in the *IL1β* expression [63]. A supplement containing the essential oils of *Mentha arvensis* (mint), *Illicium verum* (star anise), and *Syzygium aromaticum* (clove) also increased bodyweight gain and improved feed conversion but decreased the *IL18* mRNA content in Ross 308 broilers [64].

In Rose cross broiler chickens fed basil, the expression of the *GH* gene in the liver increased significantly with an increased weight and improved feed conversion. Broiler productivity was higher due to stimulating synthesis and release of growth hormone. However, basil had no effect on the growth hormone receptor GHR [65]. The use of a powder preparation from medicinal plants *Salvia officinalis* (sage), *Matricaria chamomilla* (chamomile), *Teucrium polium* (felty germander) and *Origanum majorana* (marjoram) led to an increase in the expression of the *IGF1* gene in Ross 308 broiler chickens, which may favor the development of immunity [66]. Ross 308 broiler chickens fed 200 mg/kg cornelian cherry extract showed increased expression of the glucose transporter genes *GLUT1* and *GLUT2* and the highest weight gain [67].

Amino acids. Amino acids perform the main physiological function in the body, they participate in protein synthesis necessary to construct tissues and organs. The use of amino acids as a feed additive has a positive effect on poultry productivity [43].

The essential amino acids methionine and arginine must be present in the chicken diet. Methionine is involved in the DNA methylation, the elimination of reactive oxygen species, and affects growth performance and breast yield in broilers [68, 69]. Methionine deficiency (0.28% methionine) slowed down the growth of broiler chickens of the Arbor Acres and Cobb 500 crosses, decreased feeding efficiency and *LAT1* expression in the kidneys, and activated the expression of the *SNAT2* and *CAT1* transporter genes [22, 68]. Co-injection of methionine and cysteine in ovo increased *IGF1* expression in newly hatched Ross 308 chicks [69].

Arginine is involved in maintaining the immune system, improves growth performance and reduces abdominal fat percentage in chickens [43]. Injection in ovo of L-arginine (100 $\mu g \cdot ml^{-1} \cdot egg^{-1}$, 1000 $\mu g \cdot ml^{-1} \cdot egg^{-1}$ and 2500 $\mu g \cdot ml^{-1} \cdot egg^{-1}$) increased the *MYOD* and *MYOG* expression in Ross 1040 broiler chickens, but has no significant effects on the *IL8* and *TNFα* expression [70]. A mixture of palm and sunflower oils together with L-arginine and vitamin E fed at a dosage of 50 mg/kg, increased productivity and changed the expression of cytokines in Cobb 500 broiler chickens which may have a positive effect on immune function [71].

Thus, the commercial production of broiler chicken meat is based on the use of early maturing, highly productive crosses. However, such a bird has weak resistance and is exposed to various environmental factors that can affect growth rate, weight, appetite, feed digestion and may cause various diseases. Currently, there is a search for feed additives for poultry to maintain its physiological state, prevent diseases, help accelerate growth without compromising health, and improve productivity performance by changing the transcriptional activity of various genes. Many works have shown that the expression of genes involved in the poultry growth and development (*GH*, *IGF1*, *GHR*, *MYOD1*, *MYOG*, *MST*), nutrient utilization (*SLC2A1*, *SLC2A2*, *SLC2A3*, *SLC2A8*, *SLC2A9*, *SLC2A12*, *SLC6A19*, *SLC7A1*, *SLC7A2*, *SLC7A5*-7, *SLC15A1*, *SLC38A2*), immune response (*IL1β*,

IL6, *IL8L2*, *IL16*, *IL17A*, *IL18*, *TNF-α*, *AvBD1-AvBD14*) are influenced by various factors, including prebiotics, probiotics, synbiotics, phytobiotics and amino acids as feed supplements.

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Bos taurus β-CASEIN: PROTEIN STRUCTURE, GENE POLYMORPHISM, EFFECT ON THE HUMAN GASTROINTESTINAL TRACT

(review)

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Abstract

High-quality food products play an important role in a healthy lifestyle in the modern world. Cow's milk and milk products contain all the essential nutrients. The main components of milk are water, fat, protein, lactose, minerals, vitamins, etc. (P.C. Wynn et al., 2013). With the advent of A2 milk on the market, which reduces the symptoms of lactose intolerance and has good digestibility, the population's demand for this product began to grow. A2 milk is obtained from cows of the A2 genotype for β-casein. Cow's milk is 3.5 % protein (P. Feng et al., 2020). Casein is the most important protein component of milk and makes up about 80 % of the total protein composition of cow's milk. Casein consists of four fractions, the αs1, αs2, β and κ. β-Casein is a protein that is one of the main ones in cow's milk and makes up most of all casein. β-Casein consists of 209 amino acid residues, of which 16.7 % is proline, evenly distributed over the polypeptide, which limits the formation of the α-helix (S. Pattanayak, 2013). More than 95 % of casein in milk is in micellar form. The β-casein gene has 13 allelic variants, among which types *A1* and *A2* are the most studied, differing in the sequence of amino acids in the primary structure. Since the primary structure of the protein is different, A1 and A2 β-caseins broken down in the human gastrointestinal tract form different bioactive peptides. For *A1* allele, β-casomorphin-7, a peptide consisting of seven amino acid residues is formed. The level of this peptide is 4 times higher in A1 milk than in A2 milk. When using milk containing β-casein type A1, 12 h after consumption, people may experience bloating, abdominal pain, flatulence, heaviness in the stomach, changes in the frequency and consistency of stools, in some cases, symptoms of celiac disease. Consumption of milk containing type A1 β-casein leads to a significantly longer transit time through the gastrointestinal tract (6.3 hours longer), inflammation of the small intestine and inflammation of the gastric mucosa compared to drinking milk containing A1 type of β-casein. People with lactose intolerance have adverse gastrointestinal symptoms after drinking milk, which may be associated with the presence of β-casein A1 in milk, and not with lactose itself (H. Brüssow, 2013; D. Hu et al., 2014). Unlike variant A1, β-casein A2 increases 2-gold the natural production of glutathione, one of the most important antioxidants of the human body.

Keywords: casein, β-casein, A1 milk, A2 milk, cow's milk, milk proteins

Cow's milk and dairy products are an important part of the traditional diet of people in many countries around the world. The variety of dairy products is due to the chemical composition of milk, the main components of which are water, fat, protein, lactose, minerals, and vitamins [1].

In the modern world, disease prevention and a healthy lifestyle play an important role, so agricultural and food companies are interested in supplying high-quality products to the market [2-4]. With the advent of A2 milk on the US

market in 2015, which reduces the symptoms of lactose intolerance and has good digestibility, public demand for this product began to grow. A2 milk is produced by cows of the A2 genotype for β-casein [5, 6].

Currently, molecular genetics methods identify genes that control certain useful traits of farm animals [7-9]. The study of polymorphism of milk protein genes, in particular β-casein is of significant interest for population genetic research.

The purpose of the presented review is to summarize new data on β-casein of cow's milk which have been obtained over the past few years due to a more in-depth study of both the biochemical properties of milk and dairy products, and the genetic characteristics of animals.

The digestibility of milk depends on its protein composition. Cow's milk is 3.5% protein on average [10, 11]. The protein composition significantly influences the nutritional value of milk and, in combination with all its constituent components, determines its technological properties [10]. The milk protein composition is complex and varies in structure, physicochemical properties and biological functions. There are three groups of milk proteins, the caseins, whey proteins and fat globule membrane proteins [11].

Casein is a group of phosphoproteins and makes up approximately 80% of the protein in cow's milk. The molecular weight of the casein fraction is 19000-25000 Da [12-14]. The bovine casein genes are located on chromosome 6q31-33 [15-17]. There are four main casein fractions, the αs1 (18% of total caseins), αs2 (8-11%), β (25-35%) and κ (18-15%) [18-20]. That is, the most common is β-casein [21].

β-Casein and its structure. Beta casein is a protein that accounts for 30 to 35% of all milk proteins [15]. Its molecules are the most hydrophobic and contain a large amount of proline. The β-casein molecule has a negatively charged hydrophilic region from 1 to 43 a.a. and a hydrophobic segment with a high proline content in the region from 44 to 209 a.a. This determines the similarity of β-casein with the structure of surfactants. The solubility of β-casein in water is better than that of other caseins, especially at low temperatures [22-24].

β-Casein is an amphiphilic phosphoprotein with an isoelectric point pH 4.8-5.1. It has a highly hydrophilic negatively charged N-terminal region and a hydrophobic C-terminal region [25-27]. It differs from α-casein in its pronounced dependence on temperature and the temperature dependence of its solubility in the presence of calcium ions. β-Casein is more hydrophobic than α- or κ -casein. It contains five phosphoserine residues in the hydrophilic region which give it a net negative charge under the neutral pH of milk [26, 28].

β-Casein is a chain of 209 amino acid (a.a.) residues of which 16.7% is proline evenly distributed throughout the polypeptide, which limits the formation of an α-helix.

More than 95% of the casein in milk is in micellar form. Casein micelles are nearly spherical particles ranging in size from 30 to 300 nm. Each micelle contains from several thousand to hundreds of thousands of molecules of all types of caseins. The properties and chemical composition of micelles ensure their high stability and stability in the absence of tertiary protein structure [29, 30].

The structure of the casein micelle has not been fully established. Two models based on submicelles and internal structure have been proposed. The internal structure model corresponds to a continuous three-dimensional network of folded αs1-casein polypeptide chains, to which æ-casein binds via micellar calcium phosphate; β-casein is held on the carcass by weak hydrophobic bonds and can easily leave the micelle and re-enter it. According to the submicelle model, micelles are composed of hundreds of discrete particles, the submicelles with a diameter of 10-20 nm in which casein molecules are hydrophobically bonded to

each other. There are two types of submicelles, the F2 and F3. F2 submicelles consist of αs1- and æ-caseins, F3 of α- and β-caseins. Submicelles are retained within micelles mainly with the participation of colloidal calcium phosphate (Fig. 1).

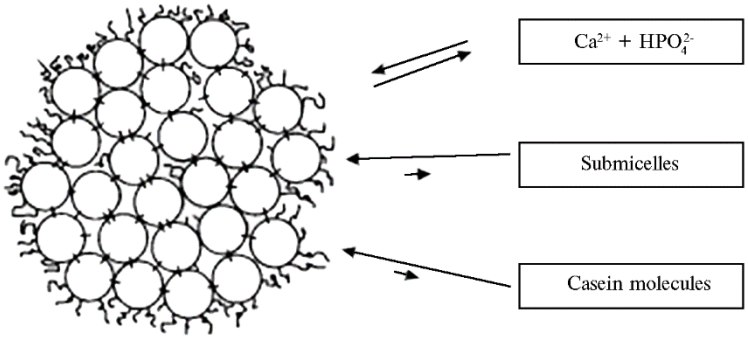

Fig. 1. Model of β-casein micelle from cow's milk. Consists of spherical submicelles bound to each other by calcium phosphate (29, 30).

The of β-casein submicelle formation is reversible. Their stability is affected by temperature, salt concentration and pH. β-Casein submicelles are more stable than low molecular weight surfactant micelles, similar to diblock copolymer micelles [29-31].

β-Casein molecules tend to self-associate in solution due to the large hydrophobic part in their structure. They remain as monomers at low temperatures (up to 5 °C) at concentrations above the critical micelle concentration, and with increasing temperature they self-associate due to hydrophobic interactions in the core of the spherical aggregate, leaving a hydrophilic outer coating [32]. The degree of polymerization of the β-casein molecule directly depends on temperature [28, 33]. Regarding the hydrophilic region, which gives a high negative charge density, β-caseins are able to bind strongly by ionic interactions the divalent ions, especially Ca^{2+}, through phosphoserine, aspartic acid, glutamic acid and free carboxyl groups. The addition of $CaCl_2$ and an increase in temperature lead to the formation of large β-casein aggregates linked by divalent bridges established with residual phosphoserine groups, which is a reversible reaction when the temperature is decreased [31, 34].

Studies carried out using various methods (circular dichroism spectroscopy and Fourier-transform infrared spectroscopy) have shown a conformational change in the structure of β-casein which creates a more hydrophobic environment. C-terminal amino acids have been shown to provide the hydrophobic environment necessary for protein self-association and the formation of micellar aggregates [30-32].

The self-association of β-casein is micelle-like. Both ionic strength and temperature increase the amount of polymer present, that is, they increase the association constant and the degree of association [31]. The number of monomeric protein constituents in these micelle-like structures — almost spherical polymers ranges from 15 at an ionic strength of 0.1 mol/l (pH 7 and 20 °C) to 52 at 0.110 mol/l (pH 6.7 and 37 °C). In this case, structures containing β-casein directly are in a molten globule-like state [29, 32]. Submicelles with these properties have been used to prepare encapsulates of therapeutic hydrophobic molecules.

β-Casein is the most active of all milk proteins, it is the first of the caseins to position itself at the oil-water interface, the presence of phosphoserine in the structure gives thickness and steric stability to the adsorbed layer surrounding the milk protein. β-Casein serves as a precursor to peptides with various biological activities, small peptides with antihypertensive properties [31, 35].

Polymorphism of β-casein. In cow's milk, 13 different forms of β-

casein have been identified, and genetic variants for this protein are A1, A2, A3, B, C, D, E, F, H1, H2, I, and G [36, 37]. Genetic variants of β-casein can be detected by electrophoretic separation under acidic or alkaline conditions [38-40]. Variants A can be distinguished from B, C and D under alkaline conditions and from each other only under acidic conditions [34, 41, 42]. In breeding, it is important to identify connections between genotypes of milk protein genes and traits of cow productivity. Thus, genetic variant B of β-casein is associated with increased fat and casein content in milk, variant A is associated with increased milk yield [43-45].

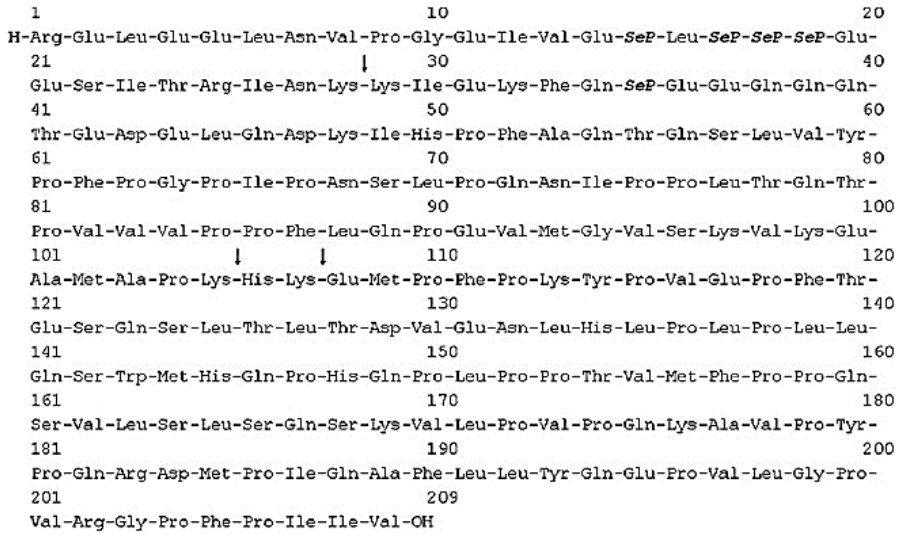

Fig. 2. The primary sequence for the most common genetic variant of β-casein A2, the β-CN A2-5P (35, 61, 62). SeP residues identified as phosphorylated are in italics and bold.

Among the genetic variants of β-casein, A1 and A2 are the most studied [46-48]. A1 differs from A2 in only one amino acid, at position 67, the replacement of cytosine with adenine led to the replacement of proline with histidine [49-51]. Detection and quantitation of A2 β-casein is mandatory for A2 dairy products [14, 25, 52]. β-Casein A2 is believed to be the original β-casein protein because a point mutation caused the emergence of β-casein A1 in European cattle several thousand years ago [53-55]. β-Casein A1 is the most common type found in cow's milk produced in Europe (except France), USA, Australia and New Zealand [56-58]. The primary sequence for the most common A2 variant, the β-CN A2-5P [34, 59, 60], is shown in Figure 2.

Caseins and whey proteins (β-lactoglobulin and β-lactalbumin) are the two main groups of milk proteins [61-63]. Casein genes are candidate genes for milk protein biosynthesis [41, 64, 65]. A common source of bioactive peptides are proteins found in cow's milk. Bioactive peptides are released through the enzymatic hydrolysis of caseins and whey proteins by gastrointestinal enzymes. In vitro, the bioactive peptide β-casomorphin-7 is produced by digestion of β-casein A1, but not β-casein A2 [66-68].

Effect of β-casomorphin-7 on the function of the gastrointestinal tract. Since β-casomorphine-7 has morphine-like activity and is believed to cause diseases such as type 2 diabetes mellitus [69], sudden neonatal death syndrome [70], coronary heart disease and cardiovascular diseases [71, 72], increases the risk of cancer diseases [73], may contribute to the development of autism in children [74], studying the frequency of genetic variants A1 and A2 is of interest.

In 12 h after consuming milk containing type A1 β-casein, people experience bloating, abdominal pain, flatulence, heaviness in the stomach, changes in stool frequency and consistency, and in some cases symptoms of celiac disease [75-78]. Consumption of such milk is associated with a significant increase in colon transit time (6.6 h longer) and gastrointestinal transit time (6.3 h longer) compared to milk of A1 type β-casein and causes inflammation in the small intestine and gastric mucosa [79-81].

Milk containing A1 β-casein causes greater worsening of gastrointestinal symptoms and increased gastrointestinal transit time in lactose-intolerant individuals than in lactose-tolerant individuals, whereas milk containing A2 β-casein does not worsen symptoms of lactose intolerance [82-84]. This suggests that in some people with lactose intolerance, adverse gastrointestinal symptoms after consuming milk may be related to the presence of β-casein A1 in milk rather than lactose itself [76, 85, 86].

Milk containing A2 β-casein reduces acute gastrointestinal symptoms compared to milk containing A1 β-casein [76, 87]. In the human body, β-casein A2 approximately doubles the natural production of glutathione, an antioxidant that is involved in detoxification processes [88-90].

In β-casein A2, the presence of proline instead of histidine prevents hydrolysis of the peptide bond between amino acid residues 66 and 67 and inhibits the production of β-casomorphin-7 [91, 92]. During sequential gastrointestinal digestion of milk containing β-casein A1, the amount of β-casomorphin-7 formed is 4-fold higher than during the digestion of A2 milk [93-95]. Unlike the A1 allele, which can be a risk factor for human health, the A2 allele has breeding value [96-98].

Thus, cow's milk contains all the nutrients necessary for humans, including an average of 3.5% protein most of which is casein. The most common is β-casein. More than 95% of the casein in milk is in micellar form. At least 13 genetic variants of β-casein are described. Types A1 and A2 are the most studied and differ from each other by one amino acid at position 67 of the amino acid chain, histidine in A1 vs. proline in A2. The presence of histidine causes the release of the bioactive peptide β-casomorphin-7 during proteolysis of β-casein A1 in the intestine, while proline in protein A2 prevents rupture of the polypeptide sequence at this critical site. The bioactive peptide β-casomorphine-7 has morphine-like activity and affects the gastrointestinal tract and central nervous system of humans. It is believed that there is a correlation between the consumption of milk containing A1 β-casein and the incidence of human diseases such as type 1 diabetes, coronary heart disease and gastrointestinal digestive discomfort. Further study of the β-casein gene and the proteins it encodes will provide a more complete knowledge on metabolism of proteins of cow's milk and dairy products containing β-casein A1 and A2 and their impact on human health.

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THE USE OF PREBIOTICS BASED ON OLIGO- AND DISACCHARIDES IN POULTRY FARMING — a mini review

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Abstract

In meat poultry farming, technologies of chick feeding and growing allow getting a carcass ready for sale for a short period (35-42 days). Such a high growth rate is due not only to proper feeds, but also to various feed additives (E.V. Yaskova et al., 2015). The ban of antibiotics-based growth stimulants in the European Union determines the search for alternative natural substances that provide similar effects. A promising group of such substances is prebiotics (D.S. Uchasov et al., 2014) which provide an increase in the efficiency of nutrient utilization, have a positive effect on the blood morphobiochemistry, poultry natural resistance, productivity, meat quality and economic efficiency (I.V. Chervonova, 2016). This mini review systematizes data on disaccharides as potential modulators of the intestinal microbiome profile and growth stimulants of broiler chickens when antibiotics are rejected. Several groups of substances with a prebiotic effect are widely used as ingredients of premixes and compound feeds. Currently, mono-, oligo-, di- and polysaccharides are being studied as promising prebiotics. The search for new biologically active substances with a multifactorial effect on broiler chickens is relevant. Feed additives used in poultry farming contain components with prebiotic properties. These components are oligo- and disaccharides (maltose, lactose, sucrose, lactulose, fructooligosaccharides, galactooligosaccharides, soy oligosaccharide), polysaccharides (cellulose, pectins, inulin, dextrin, etc.), monosaccharides (xylitol, raffinose), amino acids (arginine, valine, glutamic acid), antioxidants (vitamins A, E, C, carotenoids, selenium salts), organic acids (citric, acetic, propionic), plant and microbial extracts (carrot, corn, rice, garlic, potato, yeast), algae extracts. The prebiotic preparations based on organic acids (lactic, lemon, fumaric, formic) and lactulose are mostly used (E.V. Shatskikh et al., 2008). Natural prebiotics include fructans (fructooligosaccharides, short-chain fructooligosaccharides, oligofructose, inulin), mannooligosaccharides from *Saccharomyces cerevisae*, soy oligosaccharides and galacto- or transgalactooligosaccharides (D. Charalampopoulos et al., 2009). Lactulose, a synthetic structural isomer of lactose (4-O-β-D-galactopyranosyl-D-fructofuranose) consists of fructose and galactose linked by a β-1,4-glycoside bond. It is an odorless white crystalline substance highly soluble in water. Synthetic disaccharides are 1.5 times sweeter than lactose and can crystallize from an alcoholic solution. β-Glycoside bonds in disaccharides are not hydrolyzed by digestive enzymes (H. Rutloff et al., 1967). Therefore, disaccharides pass through the stomach and small intestine without degradation and, being unchanged, reach the large intestine (L.N. Skvortsova, 2010). In addition, lactulose has the highest index of prebiotic activity. It stimulates lacto- and bifidobacteria in the large intestine, promotes the restoration of normal microbial profile, declines pH in the colon, inhibits conditionally pathogenic microbes, improves the absorption of nutrients, and increases immunity (V.S. Buyarov et al., 2012; V.S. Buyarov et al., 2015). Commercial feed additives based on oligo- and disaccharides as a prebiotic component may contain various substances, including trehalose, lactulose, and inulin. All of them have restorative, immunostimulating, therapeutic and prophylactic properties, contribute to the restoration of intestinal microbial community, change the final microbial products, and prevent the occurrence of inflammation and infectious diseases (C. Schumann, 2002; K.M. Tuohy et al., 2002; J.H. Cho et al., 2014).

Keywords: broiler chickens, disaccharides, prebiotics, lactulose

According to some estimates, by 2050 the world's population will reach 9 billion people [1]. As a result, the demand for food, especially for livestock products,

is growing and will continue to grow. Therefore, innovative approaches and methods are needed to intensify food production of animal products while simultaneously reducing costs, but maintaining quality and safety, that is, the development of optimization strategies [2].

In poultry farming, birds reach maturity in a short time, which corresponds to the growing needs for animal protein. Global poultry meat production doubled from 2009 to 2021, especially in developing countries [3]. Due to modern feeding and housing technologies in poultry farming, it takes 35-42 days to produce carcasses ready for sale. Such a high growth rate is ensured both by complete feeds and feed additives of various types [4]. The ban on antibiotic-based growth promoters in the European Union since January 1, 2006 has spurred the search for alternative natural substances that provide similar effects. In addition, the intensification and optimization of poultry farming technologies should not adversely affect the quality and safety of poultry products.

Prebiotics are substances that have a positive effect on the host by selectively stimulating metabolic activity and the growth of beneficial intestinal microbiota [5]. It is believed that prebiotic drugs will be in demand due to the lack of negative impact on product quality and human and animal health unlike antibiotics [6].

There is a massive data on prebiotics in the scientific literature. Published results indicate that such drugs in the poultry diets provide an increase in the feed nutrient utilization and improve blood morpho-biochemical parameters, natural resistance, productivity, quality of products and economic indicators [7]. However, in most cases, information on different groups of substances is not systematized and is fragmented.

Several groups of substances have a prebiotic effect [8]. These are oligosaccharides (soy oligosaccharides, fructooligosaccharides, galactooligosaccharides), monosaccharides (xylitol, raffinose, sorbitol, xylobiose), disaccharides (lactulose), polysaccharides (cellulose, hemicellulose, pectins, gums, mucilage, inulin), peptides (soybean, milk), enzymes (saccharomyces proteases, β-galactosidases of microbial origin), amino acids (valine, arginine, glutamic acid); antioxidants (vitamins A, C, E, carotenoids, glutathione, Q10, selenium), fatty acids (eicosapentaenoic acid), organic acids (acetic, citric), plant and microbial extracts (carrot, potato, corn, rice, pumpkin, garlic, yeast) and other products (lecithin, para-aminobenzoic acid, lysozyme, lactoferrin, lectins, extracts of various algae).

Some substances with prebiotic properties (enzymes, amino acids, vitamins) are already widely used in premixes and mixed feed formulations. Mono-, oligo-, di- and polysaccharides are considered as potential prebiotics. These compounds constitute an indigestible component of the diet that can be utilized by the intestinal microflora followed by a beneficial effect on the host [9]. The search for new bioactive substances that can have multiple effects remains relevant for modern broiler poultry farming. The use of compounds that have prebiotic effects is a way to improve gut health and animal performance without antibiotic growth promoters. This group of substances includes oligosaccharides, in particular lactulose. Lactulose is one of the promising disaccharides for prebiotic use. It is a synthetic structural isomer of the milk sugar lactose. Lactulose has the highest index of prebiotic activity, it stimulates the growth of lacto- and bifidobacteria in the large intestine, inhibits the opportunistic microbiota, helps restore normal microbiota, reduce the pH of the colon contents, improve utilization of nutrients, and increase immunity [10].

The purpose of this review was to systematize data on the effectiveness of prebiotics by the example of disaccharides (lactulose) as potential regulators of the gut microbiome composition and growth stimulants for broiler chickens when avoiding the use of antibiotics.

Structure, properties, and classification of disaccharides.

According to the chemical structure, most prebiotics are carbohydrates, non-fermentable poly- and disaccharides. In the large intestine, due to activity of the microorganisms that utilize prebiotics, organic acids (acetic, propionic, butyric, lactic) and hydrogen are produced. These acids are important for the macroorganism, they ensure the constancy of positive microbiota and pH in the intestinal lumen, the absorption of water and calcium, sodium, chlorine, magnesium ions, have a bactericidal and fungicidal effect, serve as natural metabolites and are completely assimilated in the animal's body, supplying it with additional energy [11, 12].

A disaccharide is two monosaccharide units joined by an acetal or ketal bond [13]. The glycosidic bond connects two monosaccharides and can be either α-glycosidic in case of α configuration of the anomeric hydroxyl group in the sugar, or β-glycosidic for the β configuration [14]. The three most common disaccharides are maltose, lactose, and sucrose [15] (Fig. 1). Maltose is a reducing sugar derived from starch hydrolysis by α-amylase [16]. Lactose is also a reducing sugar that consists of a D-glucosyl unit and an α-D-galactopyranosyl unit linked by a β-(1,4)-glycosidic bond. Lactose is a constituent of milk and dairy products, such as skim milk and whey [17]. Sucrose consists of glucose and fructose linked by an α-(1,2)-glycosidic bond (see Fig. 1). Maltose, lactose and sucrose are hydrolyzed by maltase, lactase and sucrase into their constituent monosaccharide units. The α-glucosidase complexes maltase-glucoamylase and sucrase-isomaltase, present in the brush border of the small intestine, cleave glycosidic bonds in maltose and sucrose, respectively, with most of the maltase activity occurring in the sucrase-isomaltase complex [18].

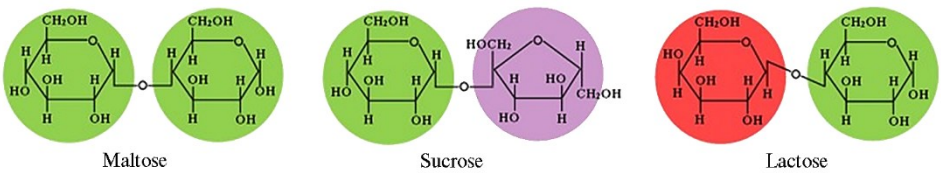

Fig. 1. Chemical structure of disaccharides (18).

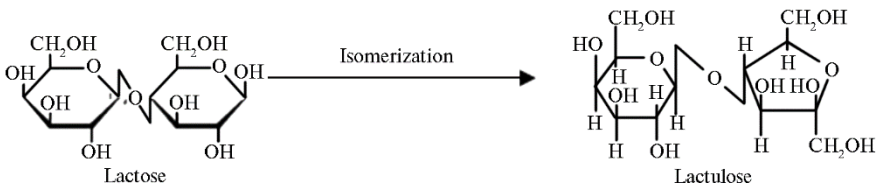

Fig. 2. Scheme of the isomerization for production of lactulose (20).

The synthetic disaccharide lactulose (4-O-β-D-galactopyranosyl-D-fructofuranose), consisting of two sugar molecules fructose and galactose linked together by a β-1,4-glycosidic bond, also belongs to the class of oligosaccharides, a subclass of disaccharides (Fig. 2). It is a white crystalline substance, odorless, highly soluble in water. Synthetic disaccharides are 1.5 times sweeter than lactose and can crystallize from an alcohol solution. The β-glycosidic bond of the disaccharide is not hydrolyzed by digestive enzymes [19]. Once in the body, this disaccharide passes through the stomach and small intestine without degradation, that is, the peculiarity of lactulose is that it can reach the large intestine unchanged [20].

The production of lactulose by chemical and enzymatic methods has been reported. The disadvantage of the chemical method is the need to use high temperatures and strong acids to purify the product, which can lead to environmental pollution [21]). On an industrial scale, lactulose is produced by chemical isomerization of lactose in an alkaline environment [22]. Since the 1950s, lactulose has been recognized as a bifidogenic factor when added to the diet [23].

1. Chemical and physiological characteristics of sugars and other glycemic carbohydrates (24)

Carbohydrate	Type	Digestive enzyme	In the intestinal lumen	In blood	Glycemic index	Possible metabolization options
Sucrose	Disaccharide: glucose-fructose, α-1,2 bond	Sucrase	Glucose, fructose	Glucose, lactate, fructose	65	Used as a source of energy, stored as glycogen and/or converted to other metabolites. Partially converted to lactic acid and glucose, which are used as energy sources or stored as glycogen, and fatty acids are used as energy sources or triacylglycerol, stored as lipids
Isomaltulose	Disaccharide: glucose-fructose, α-1.6 bond	Isomaltulase	Glucose, fructose	Absent	32	
Lactose	Disaccharide: glucose-galactose, α-1-4 bond	Lactase	Glucose and galactose	Glucose and galactose	45	Used as energy sources, stored as glycogen and/or converted to other metabolites
Maltose	Disaccharide: glucose-glucose, α-1,4-glycosidic linkage	Maltase	Glucose	Glucose	105	
Trehalose	Disaccharide: glucose-glucose, α-1,1-glycosidic linkage	Trehalase	Glucose	Glucose	70	

2. Feed additives based on oligo- and disaccharides

Feed addiive	Manufacture	Dosage	Reference
Trehalose	100ING.RU — online distributor of ingredients and raw materials for food and other industries	2 g/kg feed	https://100ing.ru/category/tregaloza/
Trehalose (Tre)	Hayashibara Co., Ltd, Japan	0.25, 0.50 and 0.75 % of diet	[35]
Inulin (chicory extract dry)	SENSUS BV, the Netherlands	1 g/kg feed	[36]
Inulin (powder)	Beneo, China	1 g/200 ml water	https://100ing.ru/product/inulin-poroshok-500-gr/10878/
	Jarrow Formulas, Inc., USA	1 g/200 ml water	https://100ing.ru/product/inulin-beneo-gr-orafti-1-kg/7421/
Lactulose	VTF, Russia	1 g/kg feed	https://vtf.ru/goods/stm/
Lactovit	North Caucasus Research Institute of Animal Husbandry, Russia	1 ml/100 g bodyweight	[37]
Lactoflex	Povolzhsky Research Institute of Production and Processing Meat and Dairy Products RAAS, Russia	0,1-0,3 g/kg bodyweight	http://volniti.ucoz.ru/
Ecofiltrum	OAO ABBA RUS, Russia	0,4-1,6 kg/g feed	[10, 38, 39]
Lactumin	Lactoprot Deutschland GmbH, Германия	200 mg/kg bodyweight	[40]
Todikam-Lact	Povolzhsky Research Institute of Production and Processing Meat and Dairy Products RAAS, Russia	200 mg/kg bodyweight	http://volniti.ucoz.ru/

In contrast to chemical methods, the production of lactulose using enzymes such as α-galactosidase or cellobiose-2-epimerase has several advantages. This technology is specific, safe and environmentally friendly. However, the production of lactulose using α-galactosidase is not cost-effective because it requires fructose and lactose and the reaction only occurs at high substrate concentrations. The enzymatic production of lactulose using cellobiose-2-epimerase provides high yield of lactulose from a single lactose substrate [23].

The dietary fiber present in disaccharides may vary (α- or β-glycosidic linkage) that affects the rate of digestion and absorption. Table 1 shows the chemical and molecular parameters, the digestion, absorption, distribution, and metabolization of some carbohydrates [24].

Mechanism of action (pharmacokinetics) of lactulose. The metabolism of indigestible sugars with the participation of intestinal microbiota, provides the macroorganism with several advantages [25]. Intestinal condition improves, which is associated with an increase in the abundance of bifidobacteria (bifidogenicity) and suppression of putrefactive processes. Absorption of minerals increases, in particular calcium, magnesium and iron, which affects the state of the skeletal system and hematopoiesis (reduction of anemia) [26] and immunomodulation occurs.

Let us consider the mechanism of action of disaccharides using lactulose as an example. Compared to lactose, it has superior sweetness and high solubility, meaning it is a sugar that can be functionally useful and used in various food industries.

The therapeutic and prophylactic properties of lactulose include stimulating the growth of beneficial microflora, inhibiting the development of pathogenic bacteria, and protecting against intestinal infections. It also promotes the synthesis of vitamins and the absorption of minerals, reduces cholesterol in the blood, prevents the formation of liver stones, and is effective in the treatment of liver and kidney diseases [27, 28].

Lactulose is metabolized by colon bacteria to monosaccharides and then to volatile fatty acids, hydrogen and methane. It reduces the production and absorption of ammonia in the intestines in three ways. First, the metabolism of sugars causes a laxative effect by increasing gas production and osmolality, which leads to a decrease in the transit time of the contents through the intestines and a decrease in pH in the intestinal lumen. Second, lactulose promotes a higher ammonia uptake by colon bacteria, which use ammonia as a source of nitrogen for protein synthesis. Third, lowering intestinal pH facilitates the conversion of ammonia (NH_3) produced by intestinal bacteria to ammonium (NH_4^+) [28], an ionized form that cannot cross biological membranes. Finally, lactulose causes a decrease in ammonia production in the intestine. An acidic environment destroys bacteria that produce urease and participate in the formation of ammonia. The unabsorbed disaccharide also inhibits glutaminase activity, which blocks the intestinal absorption of glutamine and its metabolism to ammonia. There is also an improvement in lipid metabolism, a decrease in renal nitrogen excretion (similar to the effect of dietary fiber), activation of hormone production, an effect on the central nervous system and gut-associated lymphoid tissue [29].

Thus, resistance to the effects of gastric juice and digestive tract enzymes is the main feature of disaccharides, which determines their physiological function.

Feed additives based on prebiotic oligo- and disaccharides. In poultry farming, feed additives for various purposes are used. Their components can be prebiotics, e.g., oligo- and disaccharides (lactulose, fructooligosaccharides, galactooligosaccharides, soy oligosaccharide), polysaccharides (cellulose, pectins,

inulin, dextrin), monosaccharides (xylitol, raffinose), amino acids (arginine , valine, glutamic acid), antioxidants (vitamins A, E, C, carotenoids, selenium salts), organic acids (citric, acetic, propionic), plant and microbial extracts (carrot, corn, rice, garlic, potato, yeast), extracts of various algae. The most popular prebiotics are based on organic acids (lactic, citric, fumaric, formic) and lactulose [30]. Natural prebiotics include fructans (e.g., fructooligosaccharides, short-chain fructooligosaccharides, oligofructose, inulin), followed by mannooligosaccharides (derived from *Saccharomyces cerevisae*), soy oligosaccharides, and galacto- or transgalactooligosaccharides [31].

Commercially available feed additives based on oligo- and disaccharides may contain various substances as a prebiotic component, including trehalose, lactulose, and inulin (Table 2). All of them have general strengthening, immunostimulating, therapeutic and prophylactic properties, help restore intestinal microflora, and prevent the occurrence of inflammations and infectious diseases [32-34].

Lactulose. Interest in this type of oligosaccharide may be due to the numerous beneficial properties that lactulose exhibits [41]. Acting as a prebiotic, lactulose promotes growth, improves digestion and strengthens the bird's immunity [42].

Lactulose was approved by the Food and Drug Administration (FDA, USFDA) in the USA in 1977 [32] and is currently used in both medicine and the food industry [43], including including as a functional food. Based on oligosugars, food products and their components have been created that have a pronounced positive functional effect on the human and animal body in general and on the intestinal microbiome in particular [41].

The main function of lactulose as a prebiotic is to improve intestinal microflora. Under the influence of lactulose, the number of bifidobacteria and lactobacilli in the gastrointestinal tract increases, and clostridia, salmonella and Escherichia coli decrease [44].

An increase in the number of goblet cells when taking lactulose may be associated with the growth of bacteria that determine the dynamics of mucin. The results of histomorphological studies provide new insight into the potential prebiotic effects of lactulose in broilers [45]. It was shown that in birds fed 0.2% dietary lactulose, the number of lactobacilli increased on day 28 and *E. coli* decreased compared to the control birds [34].

Prebiotics remove potentially pathogenic bacteria from the intestines or reduce their number by enriching the microbial population with beneficial strains. This improves the state of the intestinal tract and can have a positive effect both on metabolism in general and on organ-specific biochemical processes [46]. If necessary, lactose can be replaced with lactulose (4-O-β-D-galactopyranosyl-D-fructose) [47, 48]. It has been shown that lactulose in combination with tetraammonium bromide salt improves the main indicators of livestock products while simultaneously increasing resistance to internal and external infectious factors [49].

Trehalose. Trehalose (Tre) feed additives have a wide range of properties, including increasing growth rate (possibly by improving innate immune responses, such as suppressing Toll-like receptors and inflammatory cytokines in the chick duodenum [50]. Tre is a glucose-glucose disaccharide with an α,α-1,1-glycosidic bond and is found in a variety of organisms, including bacteria, yeast, fungi, and invertebrates [35].

Interestingly, despite numerous reports on the ability of trehalose to stabilize proteins upon cooling and heating, the mechanism of interaction of trehalose with proteins has not yet been studied [51]. To explain the nature of the interaction of the disaccharide with protein molecules, various hypotheses have been put forward. However, none of them has been confirmed so far. It has been

established that in the presence of water, trehalose forms pastes and exhibits tropism towards protein molecules, but does not form hydrogen bonds with them [52].

Inulin. Inulin is a prebiotic found in many plants. It reaches the large intestine unchanged, where it is fermented by beneficial bacteria. Inulin also inhibits the growth of pathogenic bacteria. Inulin consumption by chickens increases yield at slaughter, but little is known about the effects of inulin on poultry meat [53].

If its dietary intake is insufficient, inulin-containing supplements can be recommended. Regular use of the functional additive inulin modifies the composition of microbial associations in the intestinal tract, improves the functioning of the digestive system and immune system, inulin reduces the severity of inflammatory and infectious processes, and is effective in metabolic syndrome [54].

A study of the effects of inulin (alone and in combination with isomaltooligosaccharide and fructooligosaccharides) showed that the abundance of lactobacilli in the intestines of birds fed supplemented diets was higher than those fed the same diets without prebiotics [55, 56].

Imbalance in the intestinal microbial assiiations can affect the functions of the liver, adipose tissue, kidneys and pancreas. In chickens fed at an early age a combination of *Lactococcus lactis* with inulin and the prebiotic galactooligosaccharide, the overall activity of pancreatic enzymes significantly increased followed by an increase in bodyweight. Moreover, both synbiotics have been shown to have a positive effect on the activity of two liver enzyme markers (alanine aminotransferase and aspartate aminotransferase) [57]. Increased activity of pancreatic amylase, lipase, and trypsin may be due to the production of additional enzymes by gut bacteria, which contributes to improved nutrient absorption and weight gain [58].

So, early maturity and high productivity of poultry are ensured both by complete feeds and feed additives. Prebiotics can serve as an alternative to antibiotic-based growth promoters banned in the European Union. Several groups of substances have a prebiotic effect, and many probiotics have already found wide practical use. Oligo- and disaccharides, polysaccharides, monosaccharides, amino acids, antioxidants, organic acids, plant and microbial extracts, algae extracts can be prebiotic components of feed additives for poultry farming. Prebiotics based on organic acids and lactulose are often used. The most common natural prebiotics are fructans (fructooligosaccharides, short-chain fructooligosaccharides, oligofructose, inulin), mannooligosaccharides (derived from *Saccharomyces cerevisae*), soy oligosaccharides, and galacto- or transgalactooligosaccharides. Here, we consisder the benefits of lactulose, a synthetic structural isomer consisting of two sugar molecules (fructose and galactose) linked by a β-1,4-glycosidic bond. Synthetic disaccharides are 1.5 times sweeter than lactose. The β-glycosidic bond of the disaccharide is not hydrolyzed by digestive enzymes, so lactulose enters the large intestine unchanged. Lactulose has the highest index of prebiotic activity. It stimulates the growth of lacto- and bifidobacteria in the large intestine, helps restore normal microflora, reduces pH, inhibits opportunistic microflora, improves the absorption of nutrients, and increases immunity. Commercially available feed additives based on oligo- and disaccharides may contain trehalose, lactulose, and inulin as a prebiotic component. All such drugs have restorative, immunostimulating and therapeutic and prophylactic properties.

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DNA ANALYSIS OF MYOSTATIN, LEPTIN AND CALPAIN 1 GENE POLYMORPHISM IN RUSSIAN CATTLE POPULATION OF ABERDEEN ANGUS BREED

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Abstract

Beef meat is characterized by high nutritional value and unique amino acid composition (V.S. Kolodyaznaya et al., 2011; D. Pighin et al., 2016). Specialized beef cattle breeds in particular Aberdeen Angus ensures good acclimatization ability and high productivity both in Russia and broad (R. Toušová et al., 2015; V.M. Gabidulin et al., 2018; A.I. Otarov et al., 2021). Modern strategies of increasing efficiency of meat cattle farming include animal genotyping for genetic determinants of high productivity and targeted selection (V.F. Fedorenko et al., 2018; S.A. Terry et al., 2020). This paper is the first to report the development of a PCR-RFLP-based test for detection of Arg4Cys *LEP* and *CAPN1*_316 allele polymorphisms. This test was used for genotyping of the Aberdeen-Angus cattle population. The frequencies of the different polymorphism genotypes are evaluated and the influence of the *LEP* and *CAPN1* polymorphisms on animal fattening qualities. The purpose of the study was to develop the test systems for revealing the leptin *LEP* gene c.466 C→T polymorphism and calpain 1 *CAPN1* gene polymorphism rs17872000, to genotype Aberdeen Angus cattle population for these genes and the myostatin *MSTN* gene, and to investigate the links between different allele variants and fattening qualities of animals. Bulls (n = 64) and heifers (n = 81) of the population of Aberdeen-Angus young cattle (*Bos taurus*) (OOO Happy Farm, Medynsky District, Kaluga Province) born from March 2020 until May 2021 were selected for the study. Weaning of calves from mothers was carried out at the age of 6-9 months, depending on the development of a particular calf. The live weight was determined at the age of 6, 8, 12 and 15 months. Blood samples were collected for genotyping. DNA was isolated using DNA-Extran 1 kit (ZAO Syntol, Russia). The test for genotyping was developed based on the polymerase chain reaction—restriction fragment length polymorphism (PCR-PDRF) method. PCR amplification was carried out on a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories Singapore) followed by RFLP analysis of amplification products to differentiate the SNP alleles. Specific endonucleases with restriction sites in mutant alleles were found using the NEBcutter v2.0 program (https://nc2.neb.com/NEBcutter2/). PCR-RFLP products were analyzed using 2 % agarose gel electrophoresis. The test system for the F94L *MSTN* polymorphism has been developed earlier (E.N. Konovalova et al., 2021). To verify the *CAPN1*_316 amplification, Sanger sequencing was performed for three genotypes. In developing the diagnostic test for of Arg4Cys *LEP* и *CAPN1*_316 polymorphisms, we used the sequences of NM_174259 and AJ512638.1 (NCBI, https://www.ncbi.nlm.nih.gov/). The polymorphism F94L *MSTN CC* genotype occurred in 98.77 % of investigated animals. For Arg4Cys *LEP* polymorphism, *CT* genotype prevailed with a frequency of 42.19 % for bulls and 45.68 % for heifers. For *CAPN1*_316 polymorphism, the *GG* genotype predominated and accounted for 51.56 % of bulls and 69.14 % of heifers. The frequencies of the desirable productivity alleles among bulls and heifers were 0.00 and 0.01, respectively, for *A* F94L *MSTN*, 0.49 and 0.51 for *C* Arg4Cys *LEP*, and 0.20 and

0.28 for *C CAPN1*_316. The bulls with Arg4Cys *LEP TT* genotype demonstrated the highest efficiency of live weight gain from birth to 8 months, the 778 g per day which is significantly higher ($t = 2.18$) compared to *CC* (748 g). For *CAPN1*_316 polymorphism, in heifers from birth to 8 months, there was a trend to higher daily weight gain in animals of *CC* genotype, 770 g vs. 720 g for *GC* genotype and 730 g for *GG* genotypes. However, in the post-weaning period, the observed trends changed. The 12-month-old bulls with the Arg4Cys *LEP CC* genotype had a significantly higher live weight compared to *CT* bulls, The *CAPN1*_316 *GG* heifers of 8 to 15 months of aged showed a significantly higher live weight gain compared to *CAPN1*_316 *CC*, 790 g vs. 740 g. The developed test systems ensures detection of the Arg4Cys *LEP* and *CAPN1*_316 polymorphisms and can be used for genotyping and selecting beef cattle of desirable genotypes.

Keywords: cattle, Aberdeen Angus breed, productivity markers, SNP, myostatin, leptin, calpain 1

Beef is characterized by a high protein content, balanced in six essential amino acids, and the presence of two limiting amino acids, the leucine and threonine, which makes it most suitable for dietary nutrition [1]. Beef is an important source of linoleic acid, which has anticarcinogenic activity, and contains transisomers of fatty acids that help prevent coronary heart disease [2].

Currently, there are approx. 1 billion cattle in the world for meat production. Compared to poultry and swine, beef cattle have a significantly lower efficiency in converting feed into muscle tissue. However, it should be taken into account that beef cattle produce high-quality protein mainly from those feeds that are either not used or make up a small proportion in the diet of pigs and poultry [3].

In addition to feeding, housing and breeding, modern strategies for improving the efficiency of beef production focus on the genetic traits of animals that have a significant impact on their health and productivity [3]. Nowadays. genotyping techniques developed due to modern advances in molecular biology are used to establish the genetic determinants of high animal productivity for targeted selection of carriers of economically valuable alleles to involve them in breeding [4].

Specialized beef cattle breeding in Russia has undergone dynamic development in the last decade due to special program and significant government support [5]. Because of high nutritional value, increasing the proportion of beef from specialized beef cattle breeds is important for improving the quality of food supply for the population [3].

Aberdeen Angus are a highly productive beef cattle breed that have become widespread throughout the world [5]. Animals of this breed are distinguished by high growth energy. The average daily increase in live weight is more than 1000 g, and in the best individuals, it can reach 1300-1500 g [6]. In addition, characteristic features of the Aberdeen Angus breed, which are transmitted through crossing with other breeds, include polling, tender, fine-fiber meat with well-defined marbling [7, 8].

In the Kaluga Province, as in many regions of the Russian Federation, beef cattle is one of the priority for the development of livestock breeding. Necessary infrastructure, favorable natural and climatic conditions, a stable sales market, and grant supports promote a significant increase in the number of specialized beef cattle, from 24,411 animals in 2016 to 104,459 animals by 2020, or by 427.9% [9]. The dynamic development of the industry in this region, in particular at Peasant Happy Farm LLC (Kaluga Province, Medynsky District) which currently specializes in breeding Aberdeen Angus cattle, requires the modern genetic methods for breeding, including genotyping for genes associated with productive traits. These genes in beef cattle include myostatin (*MSTN*), leptin (*LEP*) and calpain 1 (*CAPN1*).

Myostatin plays a key role in skeletal muscle development. After animal birth, the myostatin gene negatively regulates skeletal muscle growth and development by limiting the number and size of muscle fibers [10]. Myostatin and its

effect on meat yield have been intensively studied, primarily with regard to loss-of-function mutations leading to increased skeletal muscle mass and appearance of the double-muscle phenotype which has been described in many species, including cattle, sheep, pigs, rabbits, and humans [11].

In cattle, the *MSTN* gene, also known as differentiation factor 8 (*GDF8*), is located on chromosome 2 (BTA2), and of the nine *MSTN* mutations currently known in *Bos taurus* [12], two have been found in the Aberdeen Angus breed. These are deletion 11 bp (nt821del11) which causes the genetic defect of double muscularity (M1) and single nucleotide polymorphism F94L (c.282C>A), causing the amino acid replacement of phenylalanine with leucine. Both mutations have a similar effect on intensive muscle growth, however, F94L, unlike nt821del11, is not associated with increased bodyweight of the calf at birth, which allowed foreign experts to recommend to include it in breeding programs to improve the accuracy of genomic prediction [13-15]. Previously, a fairly high frequency of occurrence of the desired allele *A* (0.97-1.00) in Russian populations of Limousin cattle was shown [16]. This polymorphism can be considered a promising genetic marker for selecting animals with increased muscle mass.

Quite interesting is the leptin gene (*LEP*) which is also called the obesity gene. The *LEP* gene produces the hormone of the same name which is secreted by adipocytes. Its connection with the feed intake and energy balance in mice and humans has been established [17]. In cattle, this gene is located on chromosome 4 (BTA4). The Arg4Cys *LEP* c.466 C→T polymorphism, also referred to as LEP73, R4C and R25C, is of immediate interest. It is located at a 73 bp distance from the beginning of exon 2 and causes the amino acid substitution of arginine for cysteine at position 4 of mature leptin [18-20]. The *T* allele of this SNP is associated with increased leptin mRNA expression which directly affects feeding behavior, determines the greatest efficiency of feed consumption and, as a consequence, formation of carcasses with a high fat content [21]. The *C* allele is associated with less fat deposition in the carcass, resulting in leaner carcasses produced from animals carrying this genotype [22].

Protein hydrolysis is known to be closely related to muscle growth during fattening and meat tenderness after slaughter. Calpain 1 (CAPN1) is an important protease that hydrolyzes proteins in myofibers [23]. In this regard, one of the polymorphisms of the calpain 1 gene, a single nucleotide substitution c.947G>C *CAPN1*_316 located on BTA29 is being considered. Due to this SNP, a variant of the calpain 1 protein is encoded that causes the weakening of connections between muscle fibers. This provides the uniform distribution of intramuscular fat and, thereof, greater marbling, tenderness and juiciness of meat [24-27].

The study of genes associated with meat productivity and the development of test systems for identifying preferential allelic variants remain relevant. It is necessary to take into account that the availability and ease of genotyping method is important for mass use in breeding beef cattle.

In this work, test systems were created based on the PCR-RFLP method (polymerase chain reaction—restriction fragment length polymorphism) to identify allelic variants of the Arg4Cys *LEP* and *CAPN1*_316 polymorphisms. The developed tests were used for genotyping of the Aberdeen Angus cattle population. The frequencies of occurrence of various genotypes on the studied polymorphisms were calculated, and the influence of polymorphisms in the leptin and calpain 1 genes on the fattening qualities of animals was assessed.

Our goal was i) to develop test systems for detecting polymorphisms of the leptin (*LEP*) gene c.466 C→T and calpain 1 (*CAPN1*) gene rs17872000, ii) to investigate the Aberdeen Angus cattle population for these genes and the myostatin gene (*MSTN*), and iii) to reveal the relationship of allelic variants with

the fattening qualities of animals.

Materials and methods. The work was carried within the framework of scientific cooperation of Ernst Federal Research Center for Animal Husbandry — VIZh with Timiryazev Moscow Agricultural Academy. In 2021-2022, a purebred population of young Aberdeen-Angus cattle (*Bos taurus*) of the Happy Farm LLC (Kaluga Province, Medynsky District) (n = 145) was investigated. The representative groups were bulls (n = 64) and heifers (n = 81) born between March 2020 and May 2021.

Animals were kept loose in groups, in open walking areas with light windproof canopies and feeding tables. A feed mixer was used to distribute a complete feed twice a day, providing animals with the necessary nutrients in accordance with the standards for each full-age group. Automatic heated drinking bowls were installed at the sites. Calves were weaned from their mothers at 6-9 months of age depending on theier growthe rate.

The young animals were weighed on electronic scales with fixation REUS-A-U (Tenzosila LLC, Russia) at the age of 6, 8, 12 and 15 months.

DNA for genotyping was extracted from blood sampled on September 12, 2021 and November 21, 2021 using a Vacuette blood collection system (K2E Sep tubes with EDTA and separating gel) (Greiner Bio-One, Austria). DNA was isolated using the DNA-Extran 1 kit (ZAO Synthol, Russia) in accordance with the manufacturer's protocol. Since the polymorphism of the genes under study is caused by simple single nucleotide polymorphisms (SNPs), the polymerase chain reaction method followed by restriction fragment length polymorphism analysis (PCR-RFLP) was used to develop test systems.

To amplify the mutation regions, oligonucleotide primers were selected using the Primer3Plus software (https://www.primer3plus.com/):

Polymorphism	Primer sequence (5′→3′)	Restriction endonuclease (site)
Arg4Cys *LEP*	LPN_F: TGATAGCCATGGCAGACAGC LPN_R: CCTCCCTACCGTGTGTGAGA	HpyCH4V (TG↓CA)
*CAPN1*_316	CAPN316_F: TGAACTACCAGGGCCAGATG CAPN316_R: ACAGGGTGGTGTTCCAGTTG	BstDSI (C↓CRYGG)

PCR was carried out in the following mode: 3 min at 95 °C (initial denaturation); 30 s at 95 °C (denaturation), 40 s at 61 °C (annealing) and 30 s at 72 °C (elongation) (35 cycles); 4 min at 72 °C (final elongation) (a Bio-Rad T100 thermal cycler, Bio-Rad Laboratories, Singapore). If amplification was successful, the alleles of the studied SNPs were detected by RFLP analysis using restriction endonucleases that recohnize specific sites in the DNA sequences of mutant alleles. Restriction endonucleases were chosen in the NEBcutter v2.0 program (https://nc2.neb.com/NEBcutter2/). An enzyme (1 u.a.) was added to the sample and incubated for 8 h. The enzymes HpyCH4V and BstDSI (SibEnzyme LLC, Russia) were used in accordance with the manufacturer's recommendations.

PCR-RFLP products were analyzed by electrophoresis in a 2% agarose gel for 30 min at 125 V using a molecular weight marker with 100 bp increments (ZAO Syntol, Russia).

A test system for analyzing the F94L *MSTN* polymorphism was developed previously [16].

To verify correctness of PCR for the polymorphism of the calpain 1 gene *CAPN1*_316, the amplification products of three putative genotypes were sequenced by Sanger method with the equipment and protocol of JSC Evrogen, Russia. The resulting sequences were processed using UGENE Pro V.38.1 9 software (http://ugene.net/ru/).

Genotype frequencies and average daily bodyweight gains were calculated by common methods [28, 29].

Statistical analysis was carried out according to standard methods using

the Microsoft Excel 2013 software package [30, 31]. The statistical significance of differences was assessed by Student's *t*-test. The difference was considered statistically significant at $t \geq 1.96$ and $p < 0.05$.

Results. As a result of primer design, optimized temperature-time regime of PCR and choice of restriction enzymes, test systems for the analysis of polymorphisms of the leptin (Arg4Cys *LEP*) and calpain 1 (*CAPN1*_316) genes were developed (Fig. 1, 2).

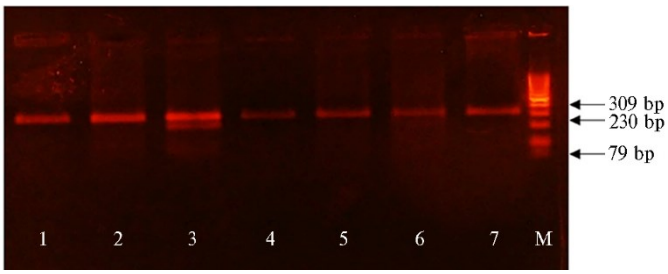

Fig. 1. Genotyping of young cattle (*Bos taurus*) of the Aberdeen Angus breed based on the polymorphism of the leptin gene Arg4Cys *LEP*. The top fragment is 309 bp long and corresponds to the wild type allele *C*, lower fragment 230 and 79 bp correspond to mutant allele *T*; 1, 2, 4-7 — animals with the *CC* genotype, 3 — with the *CT* genotype. M — molecular mass marker in 100-bp increments (ZAO Syntol, Russia) (Peasant Happy Farm, Kaluga Province, 2020-2021).

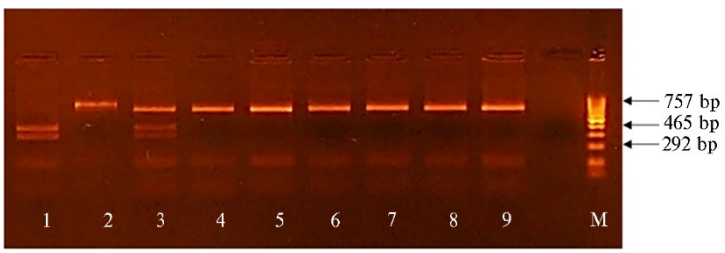

Fig. 2. Genotyping of young cattle (*Bos taurus*) of the Aberdeen Angus breed based on the polymorphism of the calpain 1 gene *CAPN1*_316. The top fragment is 757 bp long and corresponds to the wild type allele *G*, lower fragments 465 and 292 bp correspond to mutant allele *C*; 1 — animal with the *CC* genotype, 3 — with the GC genotype, 2, 4-9 — with the *GG* genotype. M — molecular mass marker in 100-bp increments. (ZAO Syntol, Russia) (Peasant Happy Farm, Kaluga Province, 2020-2021**).**

When developing a test system to detect the *CAPN1*_316 polymorphism, we used the sequence NM_174259.2 (National Center for Biotechnology Information, NCBI, https://www.ncbi.nlm.nih.gov/gene/). It was expected that for the mutant allele a DNA fragment of 267 bp would be amplified, which upon restriction would produce fragments of 164 and 103 bp. However, the resulting DNA fragments turned out to be much larger, which prompted us to conduct a comparative BLAST analysis of the NM_174259.2 sequence with the whole genome assembly (NCBI >281661-UMD3.1.1) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The analysis showed 100% identity of the compared sequences, and the sizes of the obtained amplicons corresponded to the sequence >281661-UMD3.1.1. That is, when analyzing *CAPN1*_316, a DNA fragment of 757 bp in size was amplified, which, in the presence of a mutant allele, was cut into two fragments of 465 and 292 bp in size (see Fig. 2).

Sanger sequencing of the amplified fragment of the *CAPN1*_316 mutation region revealed the desired DNA sequence and three possible genotypes for this polymorphism (Fig. 3), finally eliminating false results.

When developing a test system for diagnosing Arg4Cys *LEP* polymorphism, the NCBI AJ512638.1 sequence was used. As expected, PCR amplified a DNA fragment of 309 bp in size, which, in the presence of a mutation, was cut by the corresponding restriction endonuclease into two fragments of 230 and

79 bp in length. Thus, the developed test system ensures can clearly identify the *TT*, *CT* and *CC* genotypes of Arg4Cys *LEP*.

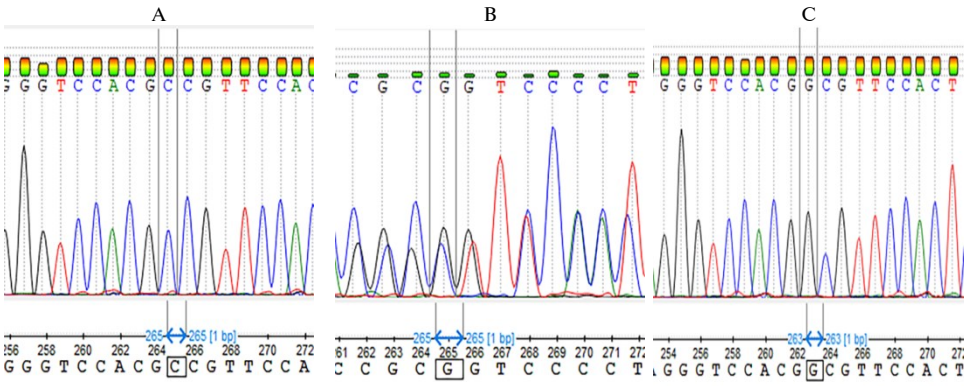

Fig. 3. Results of Sanger sequencing of the PCR fragment of the calpain 1 gene *CAPN1*_316 mutation region in young cattle (*Bos taurus*) of the Aberdeen Angus breed: A — *CC* genotype, B — *GC* genotype, C — *GG* genotype (Peasant Happy Farm, Kaluga Province, 2020-2021).

When genotyping young Aberdeen Angus breeds, we found that all the studied genes were polymorphic, but the frequency of homozygous and heterozygous genotypes among bulls and heifers varied widely (Table 1).

1. Results of Aberdeen Angus young cattle (*Bos taurus*) genotyping for polymorphisms F94L *MSTN*, Arg4Cys *LEP* and *CAPN1*_316 (Peasant Happy Farm, Kaluga Province, 2021-2022)

Polymorphism	Genotype/allele	Frequency of occurrence	
		bulls (*n*/%)	heifers (*n*/%)
F94L *MSTN*	*CC*	64/100	80/98.77
	CA	0/0.0	1/1.23
	*AA**	0/0.0	0/0.0
	C	1.00	0.99
	*A**	0.00	0.01
Arg4Cys *LEP*	*TT*	23/35.93	23/28.39
	CT	27/42.19	37/45.68
	CC	14/21.88	21/25.93
	T	0.51	0.49
	C	0.49	0.51
*CAPN1*_316	*GG*	33/51.56	56/69.14
	GC	26/40.63	18/22.22
	*CC**	5/7.81	7/8.64
	G	0.80	0.72
	*C**	0.20	0.28

N o t e. The allele and genotype desired for selection are marked with an asterisk.

Thus, based on the F94L MSTN polymorphism, two of three putative genotypes were identified, i.e., the homozygous for the wild-type allele (*CC*) and heterozygous (*CA*) which was found in only one heifer (1.23%). The *AA* genotype was not found among the studied animals.

For Arg4Cys *LEP*, all three expected genotypes were identified, while *CC* and *CT* in the groups of bulls and heifers were detected with a similar frequency (21.88 and 25.93%, 42.19 and 45.68%, respectively; 35.93% of bulls and 28.39% of heifers had the *TT* genotype.

For *CAPN1*_316, all three expected genotypes were also found, but the frequency of occurrence of the *CC* genotype, desirable for breeding, was relatively low for bulls and heifers, 7.81 and 8.64%, respectively. Most of the animals were homozygous for the *G* allele. In total, 40.63% of bulls and only 22.22% of heifers (that is, 2 times less) were heterozygous.

The frequency of the desired allele *A* for F94L *MSTN* among bulls and heifers was 0.00 and 0.01, respectively, of *C* for Arg4Cys *LEP* 0.49 and 0.51, of *C*

for *CAPN1*_316 0.20 and 0.28 (see Table. 1).

Analysis of the bodyweight dynamics allowed us to conclude that the animals under study were highly productive, while the bodyweight of bull calves at 8 months met the minimum breed requirements for elite class animals, and heifers exceeded the requirements of class I by 8.7% [32]. Thus, the average bodyweight at birth was 23.73±0.51 kg for bulls and 22.18±0.38 kg for heifers. At the age of 6 months it reached 181.18±1.98 kg in bulls and 168.38±1.47 kg in heifers, at 8 months 220.69±2.85 and 201.14±1.89 kg, at 12 months 334.11±4.25 and 317.00±13.4 kg, and at 15 months 409.38±4.81 and 368.89±4.48 kg. The average daily weight gain was over the period from birth to 8 months 803 g for bulls and 733 g for heifers, from 8 to 15 months 886 and 784 g. The analysis of the productivity of young animals indicated that favorable conditions were created on the farm to realize the productivity potential and the possibility of obtaining representative data when comparing indicators in animals with different genotypes for the *MSTN*, *LEP* and *CAPN1* genes.

2. Dynamics of bodyweight (kg) **of the Aberdeen-Angus young cattle (*Bos taurus*) depending on genotypes for the Arg4Cys *LEP* and *CAPN1*_316 polymorphisms** (*M*±SEM, Peasant Happy Farm, Kaluga Province, 2021-2022)

SNP	Genotype	Age, months				
		0	6	8	12	15
		Bulls				
Arg4Cys *LEP*	*TT* (*n* = 23)	22.74±0.70	185.36±3.26	225.79±4.77	337.52±3.70	414.83±4.88
	CT (*n* = 27)	24.59±0.86	179.13±2.85	214.45±3.80	327.33±2.36	403.37±3.72
	CC (*n* = 14)	23.71±1.31	179.95±4.86	219.79±7.20	341.57±6.19	417.57±7.55
t	*CC* и *CT*	0.62	0.15	0.66	2.13*	1.69
	CT и *TT*	1.67	1.21	1.86	2.27*	1.87
	CC и *TT*	0.73	0.75	0.69	0.56	0.31
*CAPN1*_316	*GG* (*n* = 33)	23.79±0.71	179.66±2.64	218.06±3.98	334.79±2.63	412.18±3.58
	GC (*n* = 26)	23.73±0.79	184.75±3.27	224.04±4.57	337.19±3.61	413.35±4.56
	CC (*n* = 5)	23.40±2.72	172.73±5.69	207.86±7.63	313.60±11.29	358.80±14.46
t	*CC* и *CG*	0.14	1.83	1.82	1.99*	1.82
	CG и *GG*	0.05	1.21	0.99	0.54	0.20
	CC и *GG*	0.16	1.10	1.18	1.83	1.77
		Heifers				
Arg4Cys *LEP*	*TT* (*n* = 23)	21.16±0.51	171,18±2,62	205,42±3,26	313,90±3,44	368,84±5,27
	CT (*n* = 37)	22.13±0.53	166,56±2,21	199,01±2,73	316,28±2,53	370,28±2,53
	CC (*n* = 21)	23.48±0.90	167,00±3,62	199,08±4,86	320,57±2,83	367,81±4,51
t	*CC* и *CT*	1.29	0,10	0,01	1,13	0,48
	CT и *TT*	1.31	1,35	1,51	0,56	0,25
	CC и *TT*	2.23	0,94	1,08	1,50	0,15
*CAPN1*_316	*GG* (*n* = 56)	22.39±0.48	168,03±1,94	200,92±2,49	318,37±1,87	369,80±2,94
	GC (*n* = 18)	22.88±0.80	164,64±3,54	197,85±4,58	312,65±4,43	368,53±5,18
	CC (*n* = 7)	19.50±0.50	176,14±2,07	207,24±5,55	317,00±2,77	366,00±6,42
t	*CC* и *CG*	3.60*	2,80*	1,30	0,83	0,31
	CG и *GG*	0.53	0,84	0,59	1,19	0,21
	CC и *GG*	4.18*	2,86*	1,04	0,41	0,54

* The differences are statistically significant at $t \geq 1.96$ and $p < 0.05$.

The study of the dynamics of live weight should be divided into two periods, before weaning from birth to 8 months and after weaning dueing 12-15 months, associated with the technology of raising calves (Table 2).

According to the Arg4Cys *LEP* polymorphism in the group of bulls up to 8 months, the greatest tendency to increase bodyweight was in animals with the *TT* genotype, which at the same time had the lowest birth weight. Thus, bulls with the *TT* genotype were heavier than their counterparts with the *CC* genotype by 5.41 kg at 6 months of age, and by 6.00 kg at 8 months of age. A similar trend was revealed in heifers with the *TT* genotype, at the age of 6 months they weighed 4.18 kg more than their *CC* counterparts, and at 8 months 6.34 kg more. At birth, heifers with the *TT* genotype weighed 2.32 kg less compared to the *CC* genotype ($t = 2.23$).

In the post-weaning period (see Table 2), the observed trend changed: at

the age of 12 months, bulls with the *TT* genotype were inferior in bodyweight to those homozygous for the *C* allele by 4.05 kg, but were heavier than their analogues with the *CT* genotype by 10.19 kg ($t = 2.27$). At 15 months, the difference between the *TT* and *CC* genotypes was 2.74 kg in favor of homozygotes for the *C* allele. The minimum bodyweight was in heterozygous animals. In the heifers at 12 months, there was also an advantage of animals with the *CC* genotype over *TT* genotype by 6.67 kg. At 15 months, heifers with the *TT* genotype again weighed 1.03 kg more than their *CC* counterparts.

It is noteworthy that animals heterozygous for the *LEP* gene polymorphism had the lowest bodyweight values from birth to 15 months in both sex and age groups.

For the *CAPN1*_316 polymorphism in the bulls, the highest live weight was in heterozygous animals, at the age of 6 and 15 months it was 172.73±5.69 and 358.80±14.46 kg, respectively (see Table 2). However, this superiority was not statistically significant and only indicated a trend. The animals of the *CC* genotype showed the lowest rates, their bodyweight at 12 months was 21.18 kg lower compared to the *GG* genotype *(t* = 1.99). In the heifers, a statistically significant advantage was observed for animals with the *C* allele of *CAPN1*_316 in terms of bodyweight at birth and at the age of 6 months. Thus, in animals with the ***CC*** genotype, despite a significantly less weight at birth (by 2.89 kg vs. *GC*, $t = 3.60$ and by 3.38 kg vs. *GG*, $t = 4.18$), at 6 months of age haf more weight (by 8.11 kg vs. *GG*, $t = 2.86$ and by 11.5 kg vs. *GC*, $t = 2.80$).

In the Arg4Cys *LEP* locus, the *T* allele in the homozygous state had the most pronounced effect on the average daily weight gain from birth to 8 months of age. Among bulls, individuals with the *TT* genotype gained 778 g daily, which was 50 g more compared to *ST* bulls ($t = 2.18$) and 30 g more compared to *CC* bulls. In heifers under 8 months of age, a similar trend occurred, the animals with the *TT* genotype gained 755 g vs. 725 g for the *ST* genotype and 720 for the *CC* genotype.

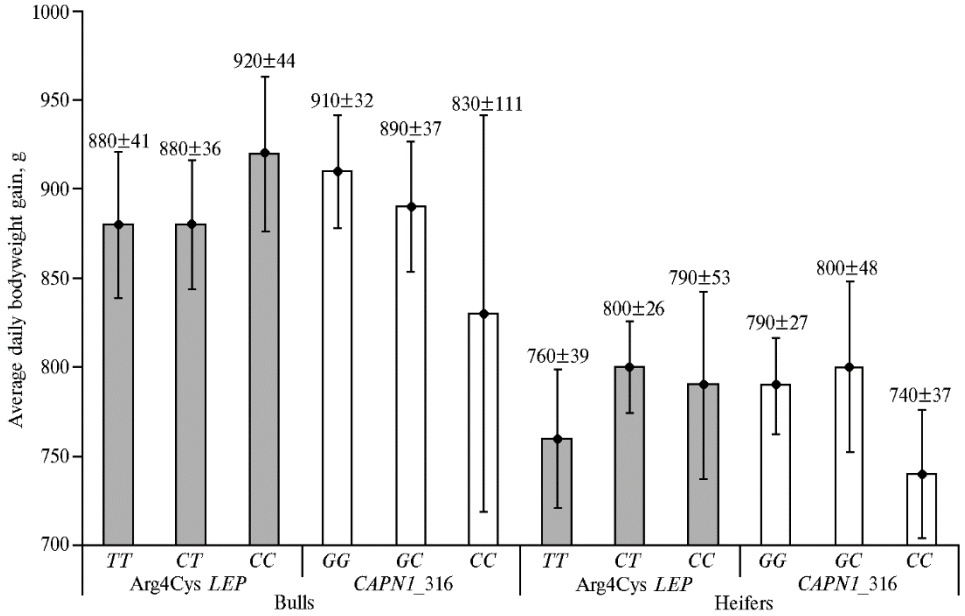

Fig. 4. Average daily bodyweight gain in the Aberdeen-Angus young cattle (*Bos taurus*) depending on genotypes for polymorphisms of the leptin Arg4Cys *LEP* and calpain 1 *CAPN1*_316 genes (*M*±SEM, Peasant Happy Farm, 2021-2022). See Table 2 for sample sizes.

At the *CAPN1*_316 locus, a positive effect of the *C* allele in the homozygous

state was found among heifers. In average daily weight gain, the *CC* cariers under the age of 8 months were superior to *GG* and *GC* by 40 and 50 g, respectively.

However, in the post-weaning period (8-15 months), the observed trends changed. For Arg4Cys *LEP*, there was an advantage in average daily weight gain in the *CC* bulls. They gained 40 g/day more compared to the *CT* and *TT* genotypes. Among heifers, the largest average daily weight gain occurred in heterozygous animals. For *CAPN1*_316, the maximum values among heifers were recorded for the *GC* genotype, and among bulls for the *GG* genotype (Fig. 4).

The developed test systems, based on a technically relatively simple and least expensive method of PCR-RFLP analysis, is the most accessible means of animal genetic identification for use in any Russian or foreign molecular genetic laboratory without any restrictions. It should be noted that the number of genotype identification systems based on fluorescent detection is growing every year. PCR-RFLP method was used to detect the Arg4Cys *LEP* polymorphism [23]. To ,detect the *CAPN1*_316 polymorphism, along with PCR-RFLP [27], real-time PCR or matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) methods were used [24-26]. The main advantages of fluorescent methods are the avoiding of agarose gel electrophoresis step and automation, which reduces analysis time for high-throughput genotyping. The risk of cross-contamination in the PCR laboratory is also reduced [33]. However, it should be noted that when conducting quantitative PCR analysis, differences in amplification efficiency can lead to a significant error, therefore, with the spread of quantitative analysis, the requirements for the purity of the DNA preparations are increasing. Thence, a complete abandonment of simpler methods based on traditional PCR-RFLP seems impractical [33].

Im this works, we genotyped the Russian population of Aberdeen Angus cattle for the myostatin, leptin and calpain 1 genes using DNA tests developed by us. It was shown that the animals contain both wild-type and mutant alleles associated with the productivity performance of beef cattle. For the F94L *MSTN* polymorphism, almost all animals were homozygous for the wild-type allele. For the Arg4Cys *LEP* and CAPN1_316 polymorphisms, wild-type alleles occurred with a high frequency.

The literature data analysis allows us to conclude that the effect of F94L *MSTN* polymorphism is similar to the mutation that causes the genetic defect of double muscularity (M1), but without affecting bodyweight at birth [13-15]. Allele *A* is associated with 5.5% larger silverside area (a valuable part of the carcass) and 2.3% larger areas of the *longissimus dorsi* muscle cross sections at 10-11 ribs, last rib, or between the first and second lumbar vertebrae [13]. In turn, the latterare indirectly associated with increased meat content in the carcass [13].

The phenotypic effects of the F94L *MSTN A* allele were partially recessive. Therefore, the *AA* F94L *MSTN* genotype may produce an intermediate (non-double) muscle phenotype, which is valuble for beef cattle breeding [13]. Therefore, this SNP is considered a very promising genetic marker of meat productivity performance [13-15].

For the Arg4Cys *LEP* polymorphism, it is difficult to definitively indicate which allele is more preferable for selection. On the one hand, the mutant *T* allele associated with higher leptin expression determines better weight gain and fattening qualities which probably explains the high bodyweight of young Aberdeen Angus cattle in our study. On the other hand, the *C* allele is associated with leaner meats which may be beneficial in dietary nutrition [22].

Our data on the frequency of the *T* allele are in lines with other reports. The *T* allele in the population we studied turned out to be relatively frequent and amounted to 0.49-0.51, depending on the animal sex. In the study of Aberdeen

Angus population by F.C. Buchanan et al. [23], this figure was 0.58, being higher compared to the Charolais (0.34), Hereford (0.55) and Simmental (0.32) breeds. The research team of N.P. Gerasimov [19], like us, revealed a significant proportion of heterozygous animals. The *CT* genotype frequency was 50.0% in young animals and 53.3% in adults of the breeding stock. *CT* Arg4Cys *LEP* heifers stood out in terms of meat productivity and showed a tendency to increase pre-slaughter weight by 1.67-2.63% and carcass weight by 0.76-0.85%)compared to the *CC* and *TT* genotypes [19]. In contrast to our findings for the perios from birth to 8-month age, the *TT* Arg4Cys *LEP* cows had minimal productivity parameters [19], however, in the post-weaning period, a similar trend was observed in animals from the Peasant Happy Farm.

For the *CAPN1*_316 polymorphism, a clear idea has already been formed that the *C* allele, associated with increased tenderness of meat, is more preferable for breeding [23, 25, 26]. In our work, when studying the *CAPN1*_316 polymorphism, a significantly larger DNA fragment was amplified. Sanger sequencing of the resulting PCR product revealed the nucleotide sequence NM_174259.2. In this case, there is a possibility of alternative splicing which does not result in excision of non-coding intronic sequences [34, 35]), apparently determining the larger size of the amplified region. This fact requires further study to assess the productive qualities of animals. Our results are of interest, showing the positive effect of the *TT* Arg4Cys *LEP* genotype on the cattle weight gain from birth to 8 months.

It is known that the bodyweight of young animals at the age of 205 days is a characteristic of the milk production of mother cows [36]. However, we cannot exclude the influence of the calves' own genotypes on their bodyweight. Thus, it was found that in carriers of the *CC* genotype for the leptin gene, the feeling of satiety occurs later and the digestibility of feed is higher, which ultimately determines greater bodyweight [17, 21, 22]. Apparently, our data also indicate the influence of the Arg4Cys *LEP* genotype on the average daily weigh gain in calves from birth to weaning.

The study of the *CAPN1*_316 polymorphism led to contradictory results. The *CC CAPN1*_316 heifers aged 6 months had a statistically significantly greater weight compared to heifers with the *GG* genotype, and at the age of 12 months, this trend persisted. Howeverm the *CC CAPN1*_316 bulls at the age of 12 months were inferior in weight to their *GG* peers. E. Casas et al. [25] and S.N. White et al. [26] also did not find a dependence of fattening traits on the genotype for *CAPN1*_316. Probably, this polymorphism has a greater effect on the meat quality parameters, and in order to identify the desired genotype of the Aberdeen Angus breed for *CAPN1*_316, further study of slaughter parameters, in particular the histological and physical-technological characteristics of muscle tissue, is necessary.

The change in the influence of the Arg4Cys *LEP T* allele on the average daily weight gain from 8 to 15 months remains controversial. To explain such differences in fattening performance, in our opinion, the role of leptin as a hormone with multiple effects should be considered. Leptin is involved in the regulation of eating behavior, affects feed digestibility, reproductive and immune functions [37]. It is believed that leptin expression and blood levels are subject to circadian rhythms and vary depending on feeding conditions [37]. Leptin transmits signals through specific leptin receptors (LepR) of two types, the expressed and secreted, which are located on the membranes of target cells. The secreted receptors competitively bind, reducing its interaction with expressed isoforms and, thereof, the effects leptin mediates [38]. In humans, the importance of this mechanism has been established [39]. It ensures changes in the bioavailability of leptin,

especially in the first years of life. Before the onset of puberty, the concentration of soluble LepR continuously decreases [39]. In our opinion, in cattle, as they reach sexual maturity, the main function of leptin becomes the regulation of reproductive qualities and the immune system. Further research is required to verify this assumption. In this regard, the report by M. Zhang et al. [40] is of interest. In their work, a correlation analysis of complex genotypes and the manifestation of economically valuable traits in foxes revealed the interaction between the genes of leptin and its receptor with a synergistic effect [40].

Continuing research will provide tools for genotyping cattle populations for productivity polymorphisms and identifying genetic markers that have the strongest associations with desirable traits. This will help improve the predictive accuracy of genomic selection and accelerate selective improvement in herds [41, 42]. To increase the profitability of beef cattle breeding, the selection of young animals with better fattening potential is also promising.

Thus, on an Aberdeen Angus cattle population as an example, we developed test systems for DNA diagnostics of the Arg4Cys *LEP* and *CAPN1*_316 polymorphisms and performed beef cattle genotyping to select carriers of desired genotypes. Among the animals tested, *CC* F94L *MSTN*, *TC* Arg4Cys *LEP* and *GG* *CAPN1*_316 genotypes were the most common. For rapid fattening of animals under the age of one year, the *TT* Arg4Cys *LEP* genotype is desireable due to a significant superiority to peers in terms of average daily weight gain. The DNA tests we have developed require inexpensive and readily available reagents. This simplifies testing in molecular genetic laboratories to identify individuals with the desired genotypes for productivity.

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BIOCHEMICAL STATUS AND PRODUCTIVE PARAMETERS OF PIGS (*Sus scrofa domesticus*) IN MODELING STRESS AND ITS CORRECTION

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Abstract

A peculiarity of living organisms is the internal constancy maintained by self-regulation mechanisms. In higher animals, the functions of control and regulation of biochemical reactions are performed by the neuro-endocrine system. With its help the organism perceives various influences of external and internal environment and reacts to them by means of hormones. In this regard, biomarkers of stress level are primarily the content of hormones, as well as blood concentrations of metabolites and their correlation. The use of dihydroquercetin, vitamins C and E in nutrition can help to reduce the negative effects of stress. In the present work we have established for the first time the positive influence of additional feeding of antioxidant complex on the adaptation of pigs under stress by hormonal regulation and strengthening of antioxidant status of the organism. The aim of the work was to evaluate the effect of feeding a complex of adaptogens DHQEC (dihydroquercetin and vitamins E, C) on the biochemical status and productive qualities of pigs under stress modelling. Fattening experiments were performed on 34 pigs (*Sus scrofa domesticus*) F_2 [(Large White × Landrace) × Duroc] (Ernst Federal Research Center for Animal Husbandry, 2022-2023). Body weight (BW) of piglets aged 99 days at the beginning of the experiment was 40.7-41.0 kg. The duration of the fattening period was 90 days. During the preliminary period, piglets were distributed into four groups by the paired-analogues method: I control (C–, without dietary DHQEC) (9 animals), II control (C+, with dietary DHQEC) (9 animals), III experimental (E–, without dietary DHQEC) (8 animals), IV experimental (E+, with dietary DHQEC) (8 animals). Each stall sized 2.4×2.25 m (5.4 m^2) with 1.05 m feeding front. That is, with four pigs per stall (groups E– and E+) instead of 3 pigs per stall (groups C– and C+), there was a decrease in the stall area per pig from 1.8 to 1.35 m^2, and the feeding front from 0.35 to 0.26 m (according to GOST 28839-2017, the norm is at least 0.3 m per pig). DHQEC contained DHQ (Ecostimul-2, AO Ametis, Russia; DHQ 72-73 %, 32 mg/kg of feed), vitamin E (INNOVIT E60, MEGAMIX, Russia, 10 mg/kg of feed), and vitamin C (Tiger C 35, Anhui Tiger Biotech Co., Ltd., China, 35 mg/kg of feed). Animals from groups C+ and E+ received dietary DHQEC (0.025 % by weight of mixed fodder) throughout the whole period of the tests. Young animals were weighed individually every decade. To assess the clinical, physiological and metabolic status of the organism at the end of the growing period, as well as during transfer to final fattening and before slaughter, blood samples were taken from the jugular vein in the morning before feeding. Calcium, phosphorus, magnesium, aspartate aminotransferase, alanine aminotransferase activity, alkaline phosphatase, total bilirubin, creatinine, cholesterol, glucose, total protein, albumin, chloride and urea were determined in blood serum. To assess antioxidant status, the total amount of free water-soluble antioxidants was determined amperometrically in serum samples. The serum concentrations of total and free thyroxine

(T_{4t} and T_{4f}) and triiodothyronine (T_{3t} and T_{3f}), as well as thyroid hormone, cortisol, adrenaline, insulin-like growth factor-1, and melatonin were also determined by solid-phase enzyme-linked immunosorbent assay. BW of pigs after starvation was evaluated immediately before slaughter. After slaughter, the carcass was weighed, the slaughter yield, thickness of the skin, muscle eye area, and pH were determined 45 min after slaughter and after 24 h of storage. It was found that with increased competition for feed table, DHQEC provides a significant decrease in cortisol ($p = 0.014$) and adrenaline ($p = 0.09$) in piglets during the final fattening. Due to competition for feed, the melatonin concentration decreased ($p = 0.01$), while DHQEC in E+ group normalized the melatonin level to the values for the 1st and 2nd blood draws. Stress had a negative effect on some metabolic processes indicators of which are biochemical blood parameters (blood concentration of triglycerides, cholesterol, bilirubin, AsAT). At final fattening, there were significant shifts in the animal hormonal status. In piglets in groups C compared to E, the concentration of T_{4t} ($p = 0.02$), T_{3t} ($p = 0.05$), T_{3f} ($p = 0.004$) decreased together with an increase in the thyroid hormone (TTG) production ($p = 0.05$). Dietary DHQEC somewhat smoothed the negative influence of the modelled factor. The lowest values of T_{4t}, T_{4f}, T_{3t}, T_{3f} were rebealed in the E+ group. It should be noted that the TTG content and integral thyroid index (ITI) in the E+ group decreased to 0.46 mME/l and 69.7 units vs. 0.51 mME/l and 263.8 units in the E– group, while the conversion of T_{4f} to T_{3f} decreased 1.73 times. With increasing BW of animals (70 kg and more), the effect of limitation of machine area and feeding front was stronger, which was manifested in the decrease of ADG in the E– group in the last fattening period ($p < 0.05$). Under the influence of DHQEC during the final fattening, there was a tendency to increase the amount of insulin-like growth factor-1 (IGF-1) in groups C+ and E+ compared to groups C– and E– (163.7 and 162.8 vs. 141.0 and 142.1 ng/ml, respectively, $p = 0.14$), which correlates with higher ADG. The obtained results indicate that DHQEC supplementation, having antioxidant activity, can improve growth parameters and, apparently, exhibit tissue-specific regulation of IGF-IR mRNA transcription. At the end of the experiment there was an increase in the blood melatonin (MT) concentration in animals from groups C– and C+ ($p < 0.05$), and dietary DHQEC did not affect the change of this index. A statistically significant decrease in MT content in E– and E+ groups compared to the control groups (292.2 and 179.8 pg/ml vs. 457.6 and 458.7 pg/ml, $p = 0.01$) should be noted. Therefore, competition for feed led to a decrease in this index, and feeding of DHQEC in E+ group normalised its values to those at the 1st and 2nd blood draws. Stress had a significant effect on the quality of production. The adaptogen-antioxidant complex DHQEC significantly improves the adaptive abilities of pigs and reduces the influence of stress factors on production performance.

Keywords: adaptogen, dihydroquercetin, vitamin, stress, young pigs, hormones, blood biochemistry, slaughter performance

A characteristic feature of a living organism is its ability to maintain a constant internal environment through self-regulation mechanisms. In higher animals, the functions of controlling and regulating biochemical reactions are performed by a complexly organized neuroendocrine system. Due to it, the body perceives the influences of the external and internal environment and reacts to them through biochemical signals, the hormones [1].

Stress is one of the key factors affecting the body of pigs under modern intensive fattening technologies. Physiological stress increases an individual's vigilance and provides the effort necessary to exhibit behavioral responses. They are associated with increased heart rate and the secretion of stress hormones such as cortisol and catecholamines (adrenaline and norepinephrine). An animal's premortem physiological and behavioral responses can significantly alter meat quality by influencing muscle energy metabolism, including metabolite and glycogen content [2]. In pigs, biomarkers of chronic and acute stress are numerous and include content of hormones [3], antioxidants [4], some metabolites [5] and their ratio [6, 7].

Under stress, the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis are mainly activated due to the production of catecholamines and glucocorticoids [8]. E. Petrosus et al. [9] identified a pattern of changes in the intestinal microbiome and susceptibility to disease due to increased levels of cortisol and catecholamines in piglets during weaning. C.-H. Yu et al. [10] found that before weaning stress in hybrid piglets was modulated, there were no differences in plasma cortisol levels between groups. After weaning, piglets increased the plasma cortisol concentration ($p < 0.05$) compared to animals not exposed to

stress (230±50 vs. 120±50 nmol/l).

Thyroid hormones affect the functions of almost all organs and systems (including the heart, central nervous system, autonomic nervous system, gastrointestinal tract), bone health, metabolism, and activate genes that accelerate metabolism and thermogenesis. Increased metabolic rate is associated with increased oxygen and energy consumption. Thyroid hormones also play a key role in fertility, development, tissue differentiation, and fetal growth, and they regulate cellular metabolism and calcium levels [11-13].

At large pig farms, even relatively comfortable conditions very often adversely affect the physiological and biochemical processes in animals. The thyroid gland, as the most important regulatory element of homeostasis, quickly responds to the influence of endogenous and exogenous factors by changing secretory activity [14].

The stress response is closely related to meat quality. The intravital mechanisms of its deterioration under stress and the long-term effects of stress causing spoilage of meat obtained from animals are still not well understood, although the problem itself is obvious [15]. Before slaughter, animals are subjected to potentially stressful procedures that may include food deprivation, collection and mixing of animals, transport to the slaughterhouse, and waiting before slaughter. Some of these factors are physical or physiological (food deprivation, fatigue or pain), others are psychological (presence of strangers, separation from members of the rearing group, encounter with individuals with whom the animal has not previously been exposed) [2].

Meat characteristics such as PSE (pale, soft, exudative) and DFD (dark, firm, dry) are closely related to glycolysis and oxidative stability in stress-induced tissues. Oxidative stress, which is accompanied by a decrease in intracellular antioxidant capacity and an increase in the production of reactive oxygen species (including free radicals), leads to changes in the metabolism of glycogen, glucose, structural modifications of cell membranes and a decrease in meat quality. Acute stress can cause PSE; with chronic stress, meat acquires signs of DFD which are formed before slaughter, which ultimately causes huge economic losses in pig production [1].

Oxidative stress is a consequence of an imbalance of pro-oxidants and antioxidants, leading to cell and tissue damage. Depletion of antioxidant systems becomes one of the causes of oxidative stress, which causes an avalanche of production of reactive oxygen species (ROS) or free radicals [16]. Recently, there has been increased interest in studying the role of free radicals, the reactive oxygen and nitrogen species (ROS and RNS). On the one hand, ROS and RNS are formed as a result of natural physiological processes and are necessary to maintain the functions of the immune system, cell signal transmission and hormone synthesis, on the other hand, oxidative stress caused by high concentrations of free radicals may lead to damage to DNA, proteins, and membrane lipids. The required amount of ROS in the body is maintained by the antioxidant system [17]. The production of ROS in mammals is caused by the activity of endogenous prooxidant enzymes NADPH oxidase, xanthine oxidase, peroxisomes and cytochrome P450. Their production is balanced by endogenous antioxidant enzymes, including superoxide dismutase, catalase, glutathione peroxidase, reduced glutathione, and heme oxygenase 1 (HO-1) [18]. These antioxidant defense systems are directly regulated by nuclear factor erythroid 2-related factor 2 (Nrf2). Excessive oxidative damage can be controlled with exogenous antioxidants such as vitamins C and E, polyphenols, carotenes, flavonoids, omega-3 fatty acids, and N-acetylcysteine (NAC) [19, 20].

Supplementation with dihydroquercetin and vitamins C and E may reduce the negative effects of stress [21, 22]. However, their effect on the synthesis of hormones and the oxidative stability of meat in fattened young pigs exposed to stress has been practically not studied. It is also of interest to study the relationships between biochemical blood markers (including hormones), growth performance of animals during the fattening period, slaughter performance and meat quality when modeling unfavorable environmental conditions.

This work was the first to establish the positive effect of feeding a complex of antioxidants (dihydroquercetin and vitamins E, C) on pig adaptation to stress conditions due to the optimization of metabolic processes and hormonal status via enhancing antioxidant protection which contributed to the production of higher quality meat.

The purpose of the work is to assess the effect of feeding the adaptogen complex DHQEC (dihydroquercetin and vitamins E, C) on the biochemical status and productive qualities of pigs when modeling stress.

Materials and methods. The experiments were carried out in 2022-2023 (the Ernst Federal Research Center for Animal Husbandry − VIZh). Thirty-four piglets (*Sus scrofa domesticus*) F_2 [(Large White × Landrace) × Duroc] of 40.7-41.0 kg weight and 99-day age, were subjected to 90-day fattening, 40 days for the 1st fattening period and 50 days for the 2nd fattening period.

Experiments were performed in accordance with the fundamentals and principles of proper care and maintenance of laboratory animals [23-26]. All piglets were kept in the same conditions that met zoohygienic requirements (with the exception of simulated factors during the test periods). Feeding was carried out according to modern standards [27] using group self-feeders.

During the preliminary growing period, piglets were assigned to four groups according to age, bodyweight and average daily gain, group I (control, $n = 9$, C–, DHQEC–), II (control, $n = 9$, C+, DHQEC+), III experimental ($n = 8$, E–, DHQEC–), IV ($n = 8$, E+, feeding with DHQEC+).

Each pen was 2.4×2.25 m (5.4 m^2) with 1.05 m feeding front. That is, when placing 4 pigs per pen (groups E– and E+) instead of 3 pigs per pen (groups C– and C+) the pen area per pig decreased from 1.80 to 1.35 m^2, the feeding front from 0.35 m to 0.26 m (with a norm of at least 0.3 m/animal according to GOST 28839-2017). This causes additional competition for feed during fattening and was a technological and feed stress factor.

The DHQEC composition corresponds to new information on action and feeding standards of dihydroquercetin [22, 28], the standards for the use of vitamins in pig feeding [27], the synergistic effect of antioxidants, activation of antioxidant defense, immunostimulation, our previous data on DHQEC (2019 -2022) [21, 29] and other reports [30, 31].

Dietary DHQEC was DHQ (Ekostimul-2, JSC Ametis, Russia, No. PVR-2-9.9/02502; DHQ 72-73%), vitamin E (INNOVIT E60, MEGAMIX, Russia), and vitamin C (Tiger C 35, PVI-2-2.15/04504, Anhui Tiger Biotech Co., Ltd., China) added to feed at 32 mg/kg, 10 mg/kg, and 35 mg/kg, respectively [32]. In lab conditions, the ingredients were mixed with crushed wheat grain and added to the feed (paddle mixer type SV-2.2; AgroPostavka, Russia).

Animals from groups C+ and E+, in addition to the basal diet, were fed DHQEC (0.025% of the feed weight) hroughout the test. The DHQEC added to feed did not change the amount of consumed energy and essential nutrients in animals [27].

The young animals were weighed individually every ten days (a REUS-A-U scales, Tenzosila LLC, Russia). The absolute and average daily weight gain was

calculated for each group during the fattening periods and for the entire experiment in general.

To assess the clinical, physiological and metabolic status, blood was taken from the jugular vein in the morning before feeding three times. The first blood sampling was before the beginning of the experiment (n = 15, animals were randomly selected from a set further used to form groups), the second sampling was when transferred to final fattening (n = 20, 5 animals from each group), and the third was before slaughter (n = 34, all animals). Each blood sample was divided into four vacuum tubes (10 ml EDTA and 5 ml serum) for biochemical and hematological analysis, antioxidant status (AOS) assessment and measurement of hormone levels.

The number of erythrocytes, leukocytes, hemoglobin content, hematocrit were determined (a hematological analyzer ABC VET, Horiba ABZ, France) with Unigem reagents (Reamed, Russia). Blood serum and plasma were separated by centrifugation (3000 rpm for 15 min, lab centrifuge UC-1412A, ULAB, China) and stored at –20 °C. Serum aspartate aminotransferase (AsAT), alanine aminotransferase (AlAT), alkaline phosphatase (ALP), total protein (TP), albumin (ALB), creatinine (CREA), urea (URO), total bilirubin (TOBIL), calcium (Ca), phosphorus (P), magnesium (Mg), cholesterol (CHOL), glucose (GLU), chlorides (CL) were measured (an automatic biochemical analyzer Erba Mannheim automatic XL-640, Erba systemic reagents; Erba Lachema s.r.o., Czech Republic). To assess the antioxidant status (AOS), the total amount of free water-soluble antioxidants (FWSA) was measured in blood serum amperometrically (a chromatograph Tsvet-Yauza 01-AA, NPO Khimavtomatika, Russia).

To measure hormone concentrations, blood samples were centrifuged (a Hettich ROTOFIX 32 device, ANDREAS HETTICH GmbH, Germany) for 10 min at 4500 rpm. Blood serum was collected with an automatic pipette Techno F1 (Lenpipet, Russia) and transferred into 1.5 ml Eppendorf tubes. Tubes with serum were placed on a Multi Bio RS-24 multirotator (BioSan, Latvia) for 10-15 min. The concentration of total and free thyroxine (T_{4t} and T_{4f}) and triiodothyronine (T_{3t} and T_{3f}), thyroid-stimulating hormone (TSH), cortisol (CORT), adrenaline (ADR), insulin-like growth factor 1 (IGF-1), melatonin (MT) was determined by enzyme-linked immunosorbent assay in accordance with the manufacturer's recommendations (an automatic microplate photometer Immunochem-2100, High Technology, Inc., USA).

The integral thyroid index (ITI, the ratio of the amount of thyroid hormones to their pituitary regulator) and the peripheral conversion index (PCI, characterizes the tissue conversion of thyroxine into its biologically more active metabolite triiodothyronine) were calculated: $ITI = (T_{3f} + T_{4f})/TSH$; $PCI = T_{4f}/T_{3f}$.

The control slaughter was performed in accordance with the Rules for veterinary inspection of slaughter animals and veterinary and sanitary examination of meat and meat products (approved by the Main Directorate of Veterinary Medicine of the USSR Ministry of Agriculture on December 27, 1983). Immediately before slaughter, the live weight (LW) of pigs after fasting was determined. After slaughter, the fresh carcass weight (without head, legs, tail, internal organs and internal fat according to GOST 31476-2012) and the slaughter yield (the ratio of the fresh carcass weight to the live weight before slaughter) were determined. The thickness of the backfat was measured with a metal ruler above the spinous processes between the 6th and 7th thoracic vertebrae, not counting the skin thickness. The area of the transverse section of the *longissimus dorsi* muscle at the

10-11th ribs was determined by making an imprint on paper, followed by measurement with a planimeter as per methodological recommendations [33]. pH was measured (Testo 205 device, Testo SE & Co. KGaA, Germany) 45 min after slaughter and after 24 h of storage.

The experimental data were processed biometrically by one- and two-way analysis of variance (ANOVA) using Student's, Dunnett's and Tukey's tests in the STATISTICA 13RU program (StatSoft, Inc., USA). Arithmetic mean values (*M*), standard errors of the means (±SEM) and significance level (p) were calculated. The differences were considered statistically significant and the existence of a relationship between the parameters was accepted at a significance level not exceeding 0.05.

Results. Based on the weighing and feed consumption, we determined the absolute and average daily weight gain in piglets (ADG) and feed costs per unit of gain (Table 1, Fig. 1).

1. Bodyweight of crossbred piglets (*Sus scrofa domesticus*) F_2 [(large white × Landrace) × Duroc] and feed costs when fed dietary complex of adaptogens DHQEC (dihydroquercetin and vitamins E, C) with modeling stress (*M*±SEM, physiological yard of the Ernst Federal Research Center for Animal Husbandry — VIZh, 2022-2023)

Parameter	Group			
	C– (*n* = 9)	C+ (*n* = 9)	E– (*n* = 8)	E+ (*n* = 8)
Growing period (preliminary period)				
Length of period, days	30	30	30	30
Average daily gain, g	735.56±20.67	735.93±11.42	730.0±20.03	737.92±22.36
1st fattening period				
Length of period, days	40	40	40	40
Live weight at the beginning of fattening, kg	40.97±0.77	40.74±0.39	40.80±0.64	40.91±0.72
Live weight at the end of the 1st fattening period, kg	78.99±1.64	78.83±0.45	78.16±1.06	79.03±1.78
Gross gain, kg	38.02±1.16	38.09±0.26	37.36±0.72	38.11±1.40
Average daily gain:				
total, g	950.56±29.05	952.22±6.49	934.06±18.06	952.81±34.93
to control (C–), %	100.0	100.2	98.3	100.2
to control (C+), %	99.8	100.0	98.1	100.0
2nd fattening period				
Length of period, days	50	50	50	50
Live weight at the end of fattening, kg	126.83±1.98	128.13±1.14	125.99±1.29	128.41±2.83
Gross gain, kg	47.84±1.15	49.30±1.19	47.83±1.15	49.39±1.58
Average daily increase:				
total, g	984.38±20.33	1014.54±22.30	958.19±17.41*	991.71±38.97
to control (C–), %	100.0	103.1	97.3	100.7
to control (C+), %	97.0	100.0	94.4	97.7
For the entire period				
Length of period, days	90	90	90	90
Gross gain, kg	85.87±1.72	87.39±1.26	85.19±1.26	87.50±2.59
Average daily increase:				
total, g	968.84±18.58	986.08±13.40	946.99±10.10	973.36±30.95
to control (C–), %	100.0	101.8	97.7	100.5
to control (C+), %				
Feed costs for the entire period				
Total, kg	283.0	283.0	283.0	283.0
Compound feed:				
total, kg/kg gain	3.31±0.07	3.24±0.05	3.33±0.05	3.26±0.10
to control (C–), %	100.0	97.9	100.6	98.5
to control (C+), %	102.2	100.0	102.8	100.6

Note. For a description of the groups, see the Materials and methods section.
* Differences from the control (group C+) are statistically significant by Student's *t*-test at $p < 0.05$.

During the growing period, ADG was 730.0-737.9 g. For the 1st fattening period, animals from the test group E– under simulated external conditions and with a decrease in the feeding front showed a tendency to decrease the ADG value, 934.1 g vs. 950.6-952.8 g in the control and test group E+, or by 1.7-1.9%. It should be noted that the effect of DHQEC was not noticeable under standard

housing in group C+, but in group E+, ADG was comparable to the control, which indicates a positive effect of DHQEC when the feeding front was limited (see Table 1).

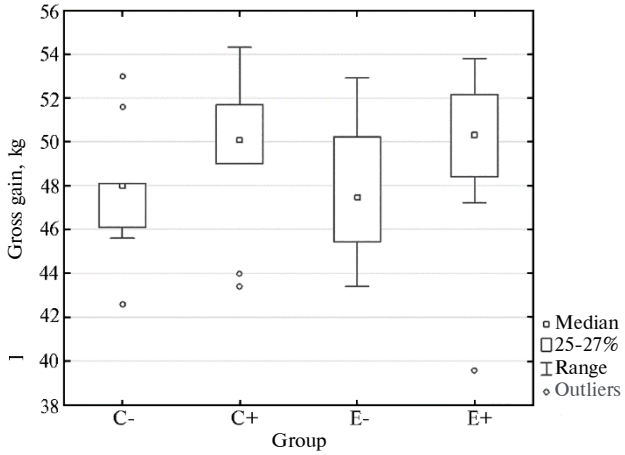

Fig. 1. Absolute bodyweight gain in crossbred piglets (*Sus scrofa domesticus*) F_2 [(Large White × Landrace) × Duroc] during the 2nd fattening period when fed dietary complex of adaptogens DHQEC (dihydroquercetin and vitamins E, C) with modeling stress (physiological yard of the Ernst Federal Research Center for Animal Husbandry — VIZh, 2022-2023). For a description of the groups, see the Materials and methods section.

During the 2nd period of fattening, ADG differed slightly from that during the 1st period and amounted to 958.2-1014.5 g, which may indicate both a greater influence of environmental factors and increased competition. The standard keeping of animals from group C– provided ADG = 984.4 g ($p = 0.37$ vs. the 1st period), and dietary DHQEC (group C+) provided better adaptation ($p = 0.02$ vs. the 1st period) and a 3.1% increase in ADG vs. group C–.

With an increase in LW (70 kg or more), the effect of limiting the area of the pen and the feeding front became more pronounced ($p = 0.87$ vs. the 1st period), causing competition for food in groups E– and E+ (see Fig. 1). ADG in group E– was 958.2 g, or 2.7% less compared to group C– and 5.6% ($p < 0.05$) less than in group C+. Dietary DHQEC under a limited feeding front (group E+) contributed to the ADG preservation ($p = 0.82$ vs. the 1st period).

Thus, the main effect of DHQEC as part of compound feeds was a stable ADG at 3.31-3.33 kg feed per 1 kg ADG in amimals not fed DHQEC vs. 3.24-3.26 kg feed per 1 kg ADG in thosed fed with DHQEC.

Biochemical analysis showed (Table 2) that during the 1st period of fattening, the blood TP content in E– pigs compared to other groups was higher (72.1 vs. 68.1-68.5 mmol/l, $p > 0.05$), the urea content was lower (4.5 vs. 4.6-5.3 mmol/l, $p > 0.05$), and the A/G ratio was the lowest (1.13 vs. 1.16-1.25, $p < 0.05$). This may indirectly indicate worse nitrogen utilization under stress. Feeding DHQEC contributed to improved parameters in the E+ pigs. A similar pattern occurred during the final fattening.

The TOBIL in pigs under feeding stress (E–, E+) was 1.15 and 1.20 μmol/l, respectively, or 33.7 and 39.5% higher ($p < 0.05$) than in group C– which could be due to increased oxidative stress.

The blood concentrations of chlorides in groups E– and E+ compared to C– and C+ was 108.8 and 109.6 mmol/l vs. 107.2 and 108.3 mmol/l, or 1. 5 and 1.2% more ($p > 0.05$). In group E–, there was a tendency to increase the content of cholesterol and triglycerides compared to the control ($p < 0.10$). In this group, animals had the highest AlAT concentration (69.4 IU/l) compared to the rest (58.80-59.5 IU/l, $p > 0.05$).

2. Blood morphological and biochemical parameters of crossbred piglets (*Sus scrofa domesticus*) F_2 [(Large White × Landrace) × Duroc] when fed dietary complex of adaptogens DHQEC (dihydroquercetin and vitamins E, C) with modeling stress (M±SEM, physiological yard of the Ernst Federal Research Center for Animal Husbandry — VIZh, 2022-2023)

Parameter	Beginning of the test (n = 15)	1st fattening period				2nd fattening period			
		group							
		C– (n = 5)	C+ (n = 5)	E– (n = 5)	E+ (n = 5)	C– (n = 9)	C+ (n = 9)	E- (n = 8)	E+ (n = 8)
Total protein, g/l	65.20±0.77	68.14±2.63	68.52±1.17	71.24±2.20	68.40±2.00	79.60±1.87	78.57±1.20	81.99±1.57	79.56±1.47
Albumin (A), g/l	35.10±0.50	37.78±1.44	37.28±0.71	37.64±0.95	36.74±2.05	45.02±0.92	45.47±0.52	45.50±0.83	44.28±1.04
Globulin (G), g/l	30.20±0.51	30.36±1.25	31.24±0.91	33.60±1.49	31.66±0.75	34.58±1.36	33.10±1.02	36.49±1.31	35.29±0.92
Creatinine, μmol/l	114.80±1.74	134.38±1.82	134.18±3.94	139.16±12.94	133.14±5.88	168.08±5.46	168.20±5.83	169.98±7.80	164.73±4.25
Urea, mmol/l	5.08±0.29	5.27±0.64	4.64±0.31	4.54±0.29	4.83±0.12	7.26±0.38	6.95±0.25	6.71±0.58	6.41±0.53
AsAT, IU/l	37.10±1.98	40.24±7.09	36.18±3.12	37.74±4.08	33.84±3.71	75.76±9.03	60.71±5.26	78.30±16.69	76.51±11.77
AlAT, IU/l	57.10±2.33	58.80±4.95	58.92±2.56	69.36±7.01	59.54±5.80	82.30±4.76	80.34±4.48	75.23±5.14	85.64±5.24
Alkaline phosphatase, IU/l	259.50±18.71	249.80±29.06	221.40±11.17	248.60±18.95	250.60±22.11	241.44±10.94	233.11±11.59	220.50±8.68	272.25±22.56
Total bilirubin, μmol/l	1.05±0.04	0.86±0.07	0.90±0.06	1.15±0.07**	1.20±0.10	3.79±0.40	4.15±1.00	3.43±0.90	4.35±1.00
Total cholesterol, mmol/l	2.50±0.11	2.67±0.14	2.52±0.10	2.97±0.13†	2.67±0.12	2.97±0.11	2.80±0.11	3.06±0.12	3.11±0.12
Triglycerides, mmol/l	0.37±0.02	0.39±0.04	0.39±0.06	0.44±0.03†	0.34±0.05	0.40±0.03	0.41±0.03	0.57±0.09	0.47±0.04
Glucose, mmol/l	5.80±0.13	6.18±0.30	6.53±0.49	6.38±0.66	6.06±0.56	5.59±0.39	5.56±0.28	5.57±0.18	5.72±0.25
Calcium, mmol/l	3.04±0.02	3.05±0.03	3.15±0.04†	3.05±0.06	3.05±0.09	3.10±0.04	3.10±0.05	3.14±0.03	3.13±0.08
Phosphorus, mmol/l	3.41±0.07	3.09±0.03	3.03±0.11	3.15±0.08	3.30±0.15	3.09±0.07	2.97±0.13	3.12±0.06	2.99±0.09
Magnesium, mmol/l	0.96±0.07	1.27±0.08	1.17±0.06	1.20±0.07	1.29±0.06	1.05±0.03	1.00±0.06	1.02±0.05	1.04±0.02
Iron, rmol/l	35.80±1.75	29.11±3.22	24.34±2.59	29.13±1.67	26.69±0.98	21.70±1.90	23.50±2.21	21.87±1.78	24.15±3.30
Chlorides, mmol/l	108.30±0.40	107.16±0.91	108.34±0.47	108.84±0.58	109.58±1.19	109.74±0.48	109.31±1.35	112.21±0.76**	111.86±1.11†
Leukocytes, $\times 10^9$/l	24.20±0.89	32.21±1.73	29.17±1.99	31.54±1.71	29.71±2.59	23.67±1.71	23.45±1.07	23.61±1.46	23.63±2.04
Red blood cells, $\times 10^{12}$/l	11.00±0.27	5.62±0.26	5.55±0.11	5.60±0.20	5.36±0.15	12.03±0.12	11.40±0.36	12.16±0.38	11.59±0.32
Hemoglobin, g/l	106.20±2.48	124.42±2.22	122.92±2.15	129.20±2.35	120.74±6.23	151.53±0.89	141.34±3.64**	145.54±4.76	139.76±2.84***
Hematocrit, %	59.4±1.48	29.91±0.71	29.60±0.44	31.28±0.81	29.79±1.47	74.34±1.01	68.93±1.60**	72.49±1.78	69.00±1.35**
A/G	1.17±0.02	1.25±0.02	1.20±0.04	1.13±0.04*	1.16±0.08	1.31±0.05	1.38±0.04	1.26±0.05	1.26±0.04
Ca/P	1.16±0.03	1.27±0.01	1.35±0.06	1.25±0.02	1.20±0.07	1.30±0.03	1.37±0.07	1.30±0.03	1.36±0.03
TWSA, mg/l	10.76±0.26	13.79±1.34	17.0±1.63	12.04±0.80*	9.60±0.54**	10.93±1.21	9.54±1.61	11.23±1.30	10.98±1.17

N o t e. AsAT — aspartate aminotransferase, AlAT — alanine aminotransferase, TWSA — total amount of water-soluble antioxidants. For a description of the groups, see the Materials and methods section.

*, **, *** Differences to control (C–) are statistically significant by Student's t-test, respectively, at $p < 0.05$, $p < 0.01$, $p < 0.001$; † — tendency towards reliability ($p < 0.1$).

3. The content of various hormones in the blood serum of crossbred piglets (*Sus scrofa domesticus*) F_2 [(Large White × Landrace) × Duroc] when fed dietary complex of adaptogens DHQEC (dihydroquercetin and vitamins E, C) with modeling stress (*M*±SEM, physiological yard of the Ernst Federal Research Center for Animal Husbandry — VIZh, 2022-2023)

Group	Parameter			
	CORT, nmol/l	ADR, ng/ml	IGF-1, ng/ml	MT, pg/ml
Beginning of the test				
General sample (n = 8)	66.62±16.24	2.59±0.56	178.20±3.74	201.42±34.46
1st fattening period				
C– (n = 5)	69.02±24.72	2.98±0.39	161.84±14.41	209.88±83.26
C+ (n = 5)	66.33±12.86	1.84±0.50	156.53±5.99	221.18±69.98
E– (n = 5)	47.73±5.11	3.05±0.80	174.52±4.96	326.73±75.46
E+ (n = 5)	44.14±11.40	3.48±0.75	165.56±11.52	246.88±60.82
Average over groups (n = 20)	56.80±8.92	2.85±0.30	164.61±4.83†	251.17±35.02
p-value, group factor 1 (C, E)	0.085	0.197	0.294	0.342
p-value, group factor 2 («–»,«+»)	0.794	0.580	0.486	0.644
p-value, group factor 1 × фактор группы 2	0.970	0.236	0.858	0.540
2nd fattening period				
C– (n = 5)	98.49±23.70	34.21±27.36	140.99±12.54	457.62±102.96
C+ (n = 5)	75.23±22.44	13.80±9.97	163.68±7.46	458.67±100.00
E– (n = 5)	149.81±20.79	66.44±0.59	142.05±11.48	292.15±72.75
E+ (n = 5)	68.88±29.16	2.10±0.68	162.81±20.77	179.78±17.21
Average over groups (n = 20)	98.10±16.83*	25.29±10.23†	152.38±6.86	347.06±45.95†
p-value, group factor 1 (C, E)	0.248	0.655	0.995	0.014
p-value, group factor 2 («–»,«+»)	0.014	0.085	0.138	0.501
p-value, group factor 1 × фактор группы 2	0.144	0.375	0.946	0.493

Note. For a description of the groups, see the Materials and methods section. CORT — cortisol, ADR — adrenaline, IGF-1 — insulin-like growth factor 1, MT — melatonin.
* Differences vs. the previous period are statistically significant by Studen's t-test at $p < 0.01$; †— tendency towards reliability ($p < 0.1$).

4. Content of thyroid hormones and thyroid-stimulating hormone (TSH) **in blood serum of crossbred piglets (*Sus scrofa domesticus*) F_2 [(Large White × Landrace) × Duroc] when fed dietary complex of adaptogens DHQEC (dihydroquercetin and vitamins E, C) with modeling stress** (*M*±SEM, physiological yard of the Ernst Federal Research Center for Animal Husbandry — VIZh, 2022-2023)

Group	Parameter					Index	
	T_{4t}, nmol/l	T_{4f}, pmol/l	T_{3t}, nmol/l	T_{3f}, pmol/l	TSH, mIU/l	ITI	PCI
			В начале опыта				
General sample (n = 8)	51.44±4.19	19.86±1.64	2.30±0.10	5.25±0.49	0.32±0.12	127.70±48.15	3.92±0.55
			1-й период откорма				
C– (n = 5)	55.06±4.48	20.89±1.13	2.38±0.13	5.48±0.48	0.23±0.06	139.76±36.24	3.86±0.29
C+ (n = 5)	52.45±5.58	21.79±1.51	2.36±0.19	5.26±0.72	0.23±0.11	296.23±197.73	4.32±0.60
E– (n = 5)	59.10±4.32	20.91±1.32	2.11±0.18	5.49±0.79	0.25±0.10	168.62±69.17	3.93±0.38
E+ (n = 5)	51.16±2.02	20.03±1.17	2.36±0.16	5.49±0.65	0.21±0.08	199.05±87.53	3.74±0.38
Average over groups (n = 20)	54.44±1.72	20.91±0.49	2.30±0.07	5.43±0.24	0.23±0.03	200.91±44.15	3.96±0.16
p-value, group factor 1 (C, E)	0.69	0.38	0.32	0.80	0.94	0.70	0.44
p-value, group factor 2 («–»,«+»)	0.11	0.99	0.40	0.82	0.77	0.29	0.67
p-value, group factor 1 × фактор группы 2	0.38	0.68	0.44	0.99	0.98	0.63	0.64
			2-й период откорма				
C– (n = 5)	41.90±3.00**	19.07±1.52	2.23±0.10	4.91±0.09	0.21±0.12	206.61±80.00	3.88±0.27
C+ (n = 5)	38.64±4.02**	17.64±1.89†	2.13±0.24	3.66±0.93	0.22±0.07	144.69±70.27	6.09±2.13
E– (n = 5)	36.84±4.57***	18.34±1.57	2.08±0.26	3.10±0.99**	0.51±0.26	263.78±257.37	7.42±2.10††
E+ (n = 5)	27.97±3.37***	16.15±2.05†	1.43±0.28**	1.38±0.32***	0.46±0.19*	69.68±39.09†	12.85±2.30***
Average over groups (n = 20)	36.34±1.84	17.80±0.69	1.97±0.11	3.26±0.39	0.35±0.07	171.19±54.02	7.56±1.04
p-value, group factor 1 (C, E)	0.02	0.42	0.05	0.004	0.05	0.93	0.007
p-value, group factor 2 («–»,«+»)	0.09	0.19	0.09	0.050	0.90	0.23	0.060
p-value, group factor 1 × фактор группы 2	0.02	0.50	0.03	0.003	0.30	0.63	0.004

N o t e. For a description of the groups, see the Materials and methods section. T_{4t} — total thyroxine, T_{4f} — free thyroxine, T_{3total} — total triiodothyronine, T_{4cfree} — free triiodothyronine, ITI — integral thyroid index, PCI — peripheral conversion index.

*, **, *** Differences vs. the previous period are statistically significant by Studen's t-test at $p < 0.05$, $p < 0.01$, $p < 0{,}001$, respetively; †— tendency towards reliability ($p < 0.1$).

5. Pre-slaughter, slaughter and meat quality parameters in crossbred piglets (*Sus scrofa domesticus*) F_2 [(Large White × Landrace) × Duroc] when fed dietary complex of adaptogens DHQEC (dihydroquercetin and vitamins E, C) with modeling stress (*M*±SEM, physiological yard of the Ernst Federal Research Center for Animal Husbandry — VIZh, 2022-2023)

Parameter	Group				p-value
	C–	C+	E–	E+	
	n = 9	*n* = 9	*n* = 8	*n* = 8	
Live weight after fasting, kg	124.06±1.95	125.13±1.14	124.99±1.48	126.16±2.31	0.87
Carcass length, cm	117.22±1.59	114.78±1.95	115.00±1.65	114.63±2.18	0.72
Weight of steamed carcass, kg	83.01±0.44	82.45±0.26	82.93±0.42	83.22±0.28	0.80
Slaughter yield, %	74.97±0.46	73.95±0.29†	75.01±0.36	75.01±0.32	0.12
Head weight (with tongue), kg	8.06±0.21	8.77±0.38	8.03±0.20	8.40±0.30	0.22
Leg weight, kg	1.92±0.06	1.86±0.04	1.88±0.03	1.96±0.08	0.56
Liver weight, kg	1.59±0.03	1.66±0.03	1.70±0.03*	1.70±0.05†	0.15
Kidney weight, kg	0.40±0.01	0.41±0.02	0.41±0.02	0.41±0.02	0.97
Heart weight, kg	0.46±0.02	0.50±0.02	0.49±0.02	0.49±0.01	0.25
Spleen weight, kg	0.22±0.01	0.24±0.02	0.21±0.01	0.19±0.01*	0.06
Lung weight, kg	0.81±0.04	0.81±0.08	0.88±0.08	0.77±0.04	0.65
Total mass of offal, kg	13.46±0.23	14.24±0.34	13.60±0.17	13.91±0.33	0.21
Yield of by-products, % by weight of the steamed carcass	14.48±0.22	15.41±0.44†	14.51±0.17	14.71±0.26	0.11
Thickness of the backfat between the 6th and 7th thoracic vertebrae (without skin thickness), mm	28.67±1.67	27.56±1.91	28.38±1.99	24.50±1.46†	0.36
Thickness of backfat on the loin (without skin thickness), mm	16.56±0.80	16.89±2.31	19.50±1.10*	15.50±2.00	0.42
pH of the longissimus dorsi muscle 45 min after slaughter, units	5.88±0.11	6.11±0.10	5.92±0.12	6.05±0.11	0.37
pH of the longissimus dorsi muscle after 24 h storage, units	5.67±0.02	5.72±0.02*	5.73±0.03†	5.70±0.03	0.21
Carcass category according to GOST R 31476-2012	2nd	2nd	2nd	2nd	
The loin eye size, cm^2	58.75±3.02	64.70±2.69	58.68±2.62	64.80±4.23	0.32
WHC, %	70.96±1.81	67.07±0.89†	68.48±1.20	67.42±1.32	0.18
TWSA, мг/г	0.11±0.01	0.12±0.01	0.11±0.01	0.10±0.00	0.45

N o t e. For a description of the groups, see the Materials and methods section. WHC — water holding capacity. TWSA — total amount of water-soluble antioxidants.

* Differences from control (C–) are statistically significant by Studen's *t*-test at $p < 0.05$, † — tendency towards reliability ($p < 0.1$).

In pigs fed DHQEC, the blood concentration was 24.3 and 26.7 μmol/l vs. 29.1 μmol/l in the control, or 16.5 and 8.2% less ($p > 0.05$) and the hemoglobin concentration was 122.9 and 120.7 g/l vs. 124.4 and 129.2 g/l, or 1.2 and 6.6% less ($p > 0.05$). This is obviously due to more intense oxidation and reduction processes in the body. Exposure to a stress factor in the 1st period of fattening led to greater consumption of water-soluble antioxidants (TWSA), and feeding DHQEC (C+ group) probably ensured their accumulation.

In the 2nd period of fattening (before slaughter), animals from groups E– and E+ had a lower blood content of urea (by 7.6 and 7.8%), a higher blood content of TP (by 3.0 and 1.3%) and lower A/G values (by 3.8 and 8.7%). In group E–, ALP and AlAT were 220.5 U/l vs. 233.1-272.3 U/l and 75.2 IU/l vs. 80.3-85.6 IU/l ($p > 0.05$). Despite the fact that in groups C– and E– the iron concentration was lower, in groups C+ and E+, the hemoglobin decreased statistically significantly (141.3 and 139.8 g/l vs. 151.5 and 145.5 g/l, respectively, or by 6.7 and 3.9%, $p < 0.01$). Hematocrit also fecreased (68.9 and 69.0% vs. 74.3 and 72.5%, or by 7.2 and 4.8%, $p < 0.01$). The concentration of chlorides was 2.3% higher ($p < 0.01$), and there was a tendency to increase (by 42.5%) in blood triglycerides ($p = 0.08$) in group E–, which indicated that E– pigs were under stronger stress compared to other groups. In the E+ group fed DHQEC, these indicators improved and were 0.3 and 17.5% lower than the values in the E– group ($p > 0.05$).

Calculation of the total values by growth periods indicated a trend towards an increase in cortisol content in group C– compared to previous periods, 98.1

nmol/l vs. 66.6 and 56.8 nmol/l, or by 47.3 and 72.7 % ($p < 0.01$). At the end of fattening, the amount of cortisol increased in group E– compared to the C– control, 149.81 vs. 98.49 nmol/l, or by 52.1% ($p < 0.05$) (Table 3, data are also presented in graphical form in Fig. 2, see http://www .agrobiology.ru).

To assess the functional state of the thyroid gland, the integral thyroid index and the peripheral conversion index are of interest, characterizing the quantitative ratio of thyroid hormones and their pituitary regulator and the conversion of thyroxine to triiodothyronine. In the 1st period of fattening, no significant changes were found in the blood concentration of thyroid hormones of animals from groups C+, E–, E+ compared to group C– (Table 4, see Fig. 2, http://www.agrobiology.ru). The T_{4t} indicator showed a slight downward trend ($p = 0.11$) in pigs fed DHQEC (groups C+ and E+), which may indicate that the body retained the energy necessary for intensive growth. Maintaining lower thyroid hormone levels may be a mechanism for reducing metabolic demands. Under the influence of increasing stress in groups E– and E+ at the end of fattening, compared to groups C– and C+, the blood concentration of T_{4t} ($p = 0.02$), T_{3t} ($p = 0.05$), T_{3f} ($p = 0.004$) decreased while TSH production ($p = 0.05$) and PCI ($p = 0.007$) were higher.

Weighing the animals before slaughter (after fasting) showed that in pigs in groups C+ and E+ LW was 125.1 and 126.1 kg vs. 124.1 and 125.0 kg in groups C– and E– (a decrease by 0.8 and 0.9%, respectively, $p > 0.05$) (Table 5). The weight of the fresh carcass had no intergroup differences ($p = 0.80$). The slaughter yield was higher in groups C–, E–, E+ compared to C+by 1.0; 1.1 and 1.1%, respectively ($p = 0.12$). In the C+ group, the specific yield of offal was 14.2% vs. 13.5-13.9% ($p = 0.21$).

The influence of stress factors in groups E was expressed in an increase in LW compared to C– and C+ (1.70 kg vs. 1.59 and 1.66 kg, or 6.9 and 2.4% more, $p < 0 .05$) and a tendency towards a decrease in spleen weight (0.21 and 0.19 kg in groups E– and E+ vs. 0.22 and 0.24 kg in groups C– and C+, or by 4.5 and 20.8% less, $p = 0.06$). In group E–, the stress led to an increase in fat depot: the thickness of the back fat on the lower back significantly increased compared to C– (by 17.8%, $p < 0.05$). DHQEC provided a tendency to a decrease in the thickness of the backfat both between the 6th and 7th thoracic vertebrae, and on the lower back. It should be noted that feeding DHQEC did not significantly affect the increase in pH_{45} (6.11 and 6.05 in groups C+ and E+ vs. 5.88 and 5.92 in groups C– and E–, or 3.9 and 2. 2% more, $p = 0.37$). A significant increase in the pH of the *longissimus dorsi* muscle occurred after 24 h in C+ pigds compared to C– pigs, by 0.9% ($p < 0.05$). An increase in pH occurred with a greater water-holding capacity of meat from animals of C– and E– groups compared to C+ and E+ ($p = 0.18$), while the pH value was the highest in group C–. The quality of carcasses is characterized by the area of the muscle eye, which was 67.07 and 64.80 cm^2 in groups C+ and E+, being larger than in C– and E– by 10.1 and 10.4%, respectively.

In modern intensive livestock farming, the use of bioactive substances of both natural and synthetic origin to reduce the impact of stress factors is becoming increasingly important [31, 34-36]. A study performed on newborn buffaloes treated and not treated with vitamin E (DL-α-tocopherol acetate) confirmed that vitamin E supplementation resulted in improved growth, metabolic and endocrine profiles [37].

Cortisol is a steroid hormone, the main representative of glucocorticoids, which is produced in the zona fasciculata of the adrenal cortex under the control of adrenocorticotropic hormone (ACTH) of the pituitary gland. Its production depends on the combination of incoming neuronal and humoral stimuli, as well

as the blood cortisol content (given the negative feedback) [9]. Cortisol regulates the metabolism of proteins, fats, carbohydrates, water and electrolytes, is involved in the regulation of inflammatory reactions and the response to stress factors, and is involved in the development of adaptation syndrome [38, 39]. By affecting protein metabolism, cortisol increases protein synthesis in the liver and inhibits it in muscle, bone and lymphoid tissues. The catabolic and antianabolic effects release amino acids, which are taken up by the liver, deaminated, and converted into carbohydrates [40, 41].

Under stress, glucocorticoids help increase the amount of glucose in the blood. In our studies, the glucose content in animals of the experimental groups remained within the normative values and had no intergroup differences (see Table 2). The creatinine content that characterizes the activity of the creatine phosphokinase and the rate of muscle mass gain was also normal in all animals. This corresponded to high increases in the livestock's fat mass.

In E– animals, there was a tendency to increase the amount of triglicerides during the 1st fattening period (0.44 mmol/l vs. 0.34-0.39 mmol/l, $p < 0.10$), which indicates their stress. AsAT activity (a marker of damage to the liver and cardiovascular system) was normal in all pigs throughout the experiment, but the highest values were characteristic of group E– (78.3 IU/l vs. 60.7-76.5 IU/l at the end of fattening, $p > 0.05$). Dietary DHQEC had a clear effect on the AsAT (E+ vs. E– and C+ vs. C– by 10.0 and 10.3%, respectively) (see Table 2).

It is important that at the 1st stage of fattening, feed stress led to a slight decrease in the blood cortisol content (47.7 and 44.1 nmol/l in E– and E+ vs. 69.0 and 66.33 nmol/l in C– and C+, or by 30.9 and 33.5%) (see Table 3, Fig. 2). We suggest that the simulated stress caused by competition for food could provoke additional physical activity, which provided some exercise for the animals. However, with increased food competition, feeding DHQEC led to a significant decrease in the blood cortisol level during the final fattening ($p = 0.014$).

Adrenaline is synthesized in the adrenal medulla, is a derivative of the amino acid tyrosine and belongs to the group of catecholamines. Adrenaline enhances the response of the nervous system and hormones. In addition, ADR reduces digestive-related responses that are not directly associated with stress [42]. At the beginning of the experiment and in the 1st period of fattening the blood adrenaline concentration was low (2.59 and 2.85 ng/ml, respectively), and at slaughter it tended to change ($p = 0.07$). Importantly, we did not identify a relationship between the blood adrenaline concentration between for a certain group, but there was a clear tendency towards a decrease in the parameter in animals fed DHQEC in groups C+ and E+ compared to groups C– and E– (8.60±5.14 vs. 50.32±20.91, or 5.9-fold, $p = 0.09$). Feeding DHQEC significantly contributed to the stress resistance, as it is evidenced by the levels of adrenaline and cortisol before slaughter (see Table 3, Fig. 2).

Supplementation of diets with bioactive ingredients of various origins can promote the secretion of insulin-like growth factor 1 (IGF-1) which affects animal and poultry the productivity performance [43]. Feeding plant extracts can improve growth performance and nutrient utilizaion in growing pigs, reduce fecal gas emissions, and increase blood components associated with the immune response such as leukocytes and lymphocytes, as well as serum IGF-1 concentrations [44].

G. Liu et al. [45] reported that supplementation of plant extracts resulted in an increase in average daily weight gain, serum IGF-1 content and IGF-1 receptor mRNA content in tissues (stomach, duodenum, muscle) in pigs. In our study, the amount of IGF-1 in pig blood serum at the beginning of the experiment was 178.2 ng/ml (see Table 3, Fig. 2). At the age of 140 days, we did not note

intergroup differences, but there was a general trend towards a decrease in IGF-1 content ($p = 0.10$). On day 180 it remained ($p = 0.03$). However, under the influence of DHQEC, the IGF-1 content had a slight tendency to increase during the final fattening period, in the C+ and E+ groups compared to the C– and E– groups (163.7 and 162.8 vs. 141.0 and 142.1 ng/ml, or by 16.1 and 14.6%, $p = 0.14$), which correlated with more intense increases in body weight. The results we obtained indicated that DHQEC, having antioxidant properties, improved growth performance and, apparently, tissue-specifically regulated the amount of IGF-IR mRNA. In addition, these data may be important for determining the physiological role of the IGF-1 system in marker control of pig growth.

Melatonin is the most effective antioxidant in the body. It has both direct antioxidant activity and indirect activity, stimulating other antioxidant systems. MT directly binds hydroxyl, superoxide anion, hydrogen peroxide, singlet oxygen, perioxynitrite and nitric oxide. MT also stimulates the activity of a cascade of enzymes, most notably intracellular superoxide dismutase, glutathione peroxidase, and catalase [46]. Endogenous MT is capable of modulating the amount of NO in mitochondria and, as a consequence, the circadian rhythm of oxidative phosphorylation and glycolysis in vivo. We did not find statistically significant differences in the content of melatonin in the blood serum between the first and second sampling (see Table 3), that is, stress and handling of the animals during blood sampling did not affect the change in MT concentration. However, in group E–, the MT value was the highest in the 1st fattening period (326.7 pg/ml) compared to the control groups (209.9 and 221.2 pg/ml, $p > 0.05$). Feeding DHQEC in the E+ group led to a decrease in MT to 246.9 pg/ml, or by 24.4% compared to E–. It is interesting that at the end of the experiment, the blood MT concentration in groups C– and C+ increased compared to the 1st fattening period by 218.2 and 207.4% ($p < 0.05$), but feeding DHQEC did not affect this parameter. It should be noted that there was a statistically significant decrease in MT in the E– and E+ groups compared to the controls by 36.2 and 60.8%, respectively ($p = 0.01$), that is, increased feed competition led to a decrease but not to an increase in the MT content. Moreover, feeding DHQEC in the E+ group normalized the indicator to the values of the 1st and 2nd blood samplings.

Consumer preferences for meat are generally determined by its physical characteristics, while nutritional value is related to its chemical composition. Exogenous melatonin negatively affects muscle pH and rate of water loss in finishing pigs, but this may be somewhat dependent on melatonin dose [47]. Melatonin injections also affect pork quality [48].

In the work of Y. Zhou et al. [30], the use of DHQ (15 mg/kg LW) increased the blood concentrations of testosterone derivatives, antioxidants (melatonin and betaine), unsaturated fatty acids (docosahexaenoic acid, DHA) and beneficial amino acids (proline). Thus, the increase in melatonin levels in piglets in our experiment was obviously a necessary response to stress and pre-slaughter holding, while in pigs from the E+ group the melatonin concentration did not change which indicates a significant role of the DHQEC complex in the pre-slaughter period.

Thyroid function and thyroid hormone activity are considered critical for maintaining performance in companion animals [49]. Increased secretion of thyroid hormone activates metabolism and therefore heat production. S. Zhang et al. [50] showed that the blood concentrations of cortisol, T_3, T_4 in growing pigs with 25 kg LW decreased as the ambient temperature increased from 18 to 32 °C, and the amount of T_4 at 18 °C exceeded the values at 27, 29 and 32 °C ($p < 0.05$).

Knowledge of the mechanisms underlying fluctuations in TSH and thyroid

hormone levels is important for understanding animal adaptation processes [51]. Genetic factors account for up to 65% of interindividual variation in TSH and thyroid hormone levels [52, 53], but other factors may also influence thyroid function. These are age and sex [54, 55], internal factors (microbiota) [56], stress [57], drug use [20] and environment [58, 59].

In our experiment, during final fattening, significant changes occurred in the hormonal status of the animals (Table 4). Under the influence of stress (groups C vs. groups E), T_{4t} ($p = 0.02$), T_{3t} ($p = 0.05$), T_{3f} ($p = 0.004$) concentrations decreased, which was accompanied by increased TSH production ($p = 0.05$). The functions of the thyroid gland, pituitary gland and hypothalamus are coordinated, and this ensures control of the content of thyroid hormones. If there is not enough thyroid hormone in the blood, the pituitary gland increases the production of thyroid-stimulating hormone (TSH), which stimulates the production of hormones by the gland. As soon as the level of thyroid hormones is restored, TSH synthesis slows down and its amount approaches normal. At that, PCI (an indicator of tissue conversion of thyroxine) increased several times ($p = 0.007$), which additionally indicates a strong influence of stress on the animal's body. Feeding DHQEC during nutritional stress somewhat smoothed out the negative impact of the modeled factor, leading to additional regulation and reduction of the body's metabolic losses. The lowest T_{4t}, T_{4f}, T_{3t}, T_{3f} values were identified in the E+ group. It should be noted that in the E+ group, TSH and ITI indicators decreased to 0.46 mIU/l and 69.7 units vs. 0.51 mIU/l and 263.8 units, or by 9.8 and 73.6%, in group E– with a 1.73-fold decrease in the T_{4f} to T_{3f} conversion (see Table 4, Fig. 2).

Under simulated stress, the DHQEC supplement provided the best physiological response during the test period (see Table 2). The combination of DHQ with vitamins C and E enhanced mechanisms of antioxidant protection. During the 1st period of fattening, under the influence of stress, water-soluble antioxidants (WSA) were consumed. According to a two-factor analysis, $p < 0.001$ for group E vs. group C, $p = 0.74$ for groups "–" vs. groups "+", and $p = 0.03$ for two factors (see Table 2). The effect of stress in groups E was clear (a decrease in the amount of water-soluble antioxidants compared to group C+). During final fattening, both factors (stress and feeding DHQEC) did not affect the blood WSA level ($p > 0.05$).

As already noted, the quality of meat depends on the physiological and behavioral reactions of the animal before slaughter [39, 60], including changes in the content of metabolites and glycogen [61, 62]. We assessed the quality of pork according to GOST 7269-2015 for most important prameters, the meat water holding capacity (WHC), color, pH. In samples of muscle tissue from groups C+, E–, E+, there was a slight decrease in WHC ($p = 0.18$) compared to group C– (see Table 5). The pH value of muscle tissue samples from group C+ (5.72 ± 0.02) was better ($p < 0.05$) than that of group C– (5.67 ± 0.02). This may resulted from the use of DHQEC which prevented the decrease in pH after slaughter due to improving animal stress resistance and antioxidant effects ex vivo. Under simulated stress, the pH value of muscle tissue samples from group E– also tended to improve compared to the control group C– ($p < 0.10$). Previously, we showed [29, 32] that modulation of social and technological stresses is accompanied by animal body training which apparently we also noted in this experiiment.

In 24 h after slaughter, the pH values of the muscle tissue samples in E+ group were comparable to that for C+ group, 5.70 ± 0.03 and 5.72 ± 0.02, respectively ($p > 0.05$) which indicates action of DHQEC. In terms of pH, meat samples in group E+ were not inferior to those of group C+.

Nowadays, the use of bioactive anti-stress drugs of natural origin is more

preferable [63]. Studies on several biochemical parameters in pigs of different genotypes indicate that local animals are more adapted to changing environmental conditions [64]. The use of feed products, in particular antioxidant additives, can significantly increase the body's resistance and reduce stress load [65] which is achieved through hormonal regulation of functions and an improvement in the body's antioxidant status.

Thus, simulated stress (additional feed competition during fattening) led to production of stress hormones. The antioxidant defense was significantly reduced, as evidenced by a decrease in the blood content of melatonin and free antioxidants of F_2 piglets [(Large White × Landrace) × Duroc]. Stress had a negative impact on metabolism, in particular, on blood biochemical parameters, e.g., the concentrations of total protein, triglycerides, cholesterol, chlorides, total bilirubin, and aspartate aminotransferase. Under stress, the concentration of thyroid hormones also decreased while the production of thyroid-stimulating hormone increased and the peripheral conversion index (PCI, characterizes the tissue conversion of thyroxine to triiodothyronine) increased. Stress significantly affected meat quality. A complex of antioxidants (dihydroquercetin and vitamins E, C), used as a feed additive, improved metabolic processes, including protein and mineral metabolism, increased the body's protective functions, nutrient utilization, and animal adaptability. The effect of the antioxidant feed additive was evident throughout the entire fattening period. As a result, the characteristics of the resulting products (slaughter performance, meat quality) were improved.

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THE LEVEL OF SPERM PLASMA PROTEIN OXIDATIVE MODIFICATION ASSESSED IN STALLIONS (*Equus ferus caballus* L.) OF DIFFERENT AGES

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Abstract

Cryopreservation of stallion semen is a modern widespread method in horse breeding for preserving the genetic material of animals. Freezing and thawing reduces the reproductive characteristics of spermatozoa. Oxidative stress that causes damage to macromolecules is a factor contributing to damage to germ cells. With age, the oxidative stress and the amount of damaged proteins increase. In this work, for the first time, we quantified products of oxidative modification of proteins (OMP) in semen plasma in stallions of different ages. There is a significant increase in the content of protein carbonylation products in older animals compared to younger ones, mainly due to neutral aldehyde derivatives. This study is the first to assess the reserve-adaptive potential (RAP) of the seminal plasma of stallions. It was found that the ability to withstand oxidative stress in young stallions is significantly higher than in older stallions. The aim of this study was to assess the level of spontaneous OMP, induced OMP and the RAP values for stallion spermatozoa as influenced by the animal age. The study was carried out in 2020 on 40 purebred Arabian and Soviet draft stallions (*Equus ferus caballus* L.) (AO Tersk breeding stud No. 169, Stavropol Territory; Perevozsky and Pochinkovsky stud farms, Nizhny Novgorod Province). Three ejaculates of each stallion were collected with a 48-hour interval. The stallions of group I (n = 20) were from 14 to 21 years of age (mean age 15.8±1.9 years), of group II (n = 20) from 3 to 5 years of age (4.3±0.6 years). In each ejaculate, the volume and concentration of spermatozoa in 1 ml of semen was determined. Then the ejaculate was divided into two parts, one was diluted with lactose-chelate-citrate-yolk (LCCY) medium in a ratio of 1:3 and the progressive motility (PM) and survival of spermatozoa were determined at 4 °C. To assess the survival of spermatozoa during hypothermic storage of sperm, its PM was determined with a 24-hour interval up to a decrease in PM to 5 %. Sperm was frozen in liquid nitrogen vapor in 18 ml aluminum tubes according to the standard of the All-Russian Research Institute for Horse Breeding and stored in liquid nitrogen at –196 °C. The cryopreserved sperm was thawed in a water bath at 40 °C for 90 s, followed by the determination of the spermatozoa PM and survival at 4 °C. Another part of the ejaculate was centrifuged at 3500 rpm for 20 min. After microscopy of the supernatant, aliquots of seminal plasma free of spermatozoa were frozen in 2.0 ml Eppendorf tubes at –18 °C. To quantify the OMP, we used the spectrophotometric analysis of 2,4-dinitrophenylhydrazones formed by the interaction of protein carbonyl derivatives (aldehydes and ketones) with 2,4-dinitrophenylhydrazine. The total amount of carbonyl derivatives was recorded in a native sample of biological material (spontaneous OMP) and after in vitro induction of protein oxidation of biological material with a reaction mixture containing solutions of iron(II) sulfate and hydrogen peroxide (metal-catalyzed induced OMP). From metal-catalyzed and spontaneous OMP, RAP was evaluated to characterize the OS resistance. Spectrophotometric measurements were carried out at 14 wavelengths, at 260-280 nm for neutral aldehyde-dinitrophenylhydrazones, at 258-264 and 428-520 nm for basic aldehyde-dinitrophenylhydrazones, at 363-370 nm for neutral ketone-dinitrophenylhydrazones, and at 430-434 and 524-535 nm for basic ketone-dinitrophenylhydrazones. Statistically significant differences in sperm quality between animals of two age groups were found only in the survival rate of spermatozoa during hypothermic storage of diluted ($p < 0.05$) and

cryopreserved ($p < 0.01$) sperm. The total amount of spontaneous OMP products in the seminal plasma of older stallions was statistically significantly higher than in young stallions (531.7 and 384.3 ODU/g protein, respectively, $p < 0.05$). In addition, in group I, there was a shift in the absorption spectrum towards neutral aldehyde derivatives the content of which in animals of group II was significantly lower (367.6 and 255.8 ODU/g protein, $p < 0.05$). The evaluation of metal-catalyzed (induced) OMP also revealed a higher total amount of carbonyl derivatives in older stallions, but its increase under the influence of an oxidizing mixture was much higher vs. the initial spontaneous OMP in young stallions. The RAP value for the seminal plasma of young stallions significantly exceeds that of mature stallions ($p < 0.05$), which can positively affect the reproductive characteristics of native and cryopreserved sperm.

Keywords: *Equus ferus caballus*, stallions, sperm, seminal plasma, cryopreservation, oxidative stress, protein oxidative modification

Oxidative stress (OS) leads to sperm damage [1, 2] and a decrease in the quality of native and cryopreserved sperm [3-5]. The cause of OS development is excessive production of reactive oxygen species (ROS), depletion of the body's antioxidant capacity, or a combination of these factors. As a result of the predominance of pro-oxidant processes over the antioxidant capabilities of cells, ROS interact with basic macromolecules, causing irreversible damage to nucleic acids, lipid peroxidation (LPO), and oxidation of proteins, including the most important enzymes and structural proteins [6].

Quantification of oxidative modification of proteins (OMP) is one of the markers of the OS severity, since this indicator reflects the degree of damage to amino acid residues of proteins by free radicals of oxygen and nitrogen, as well as lipid peroxidation products [7]. OMP products appear in cells earlier than other derivatives of oxidative damage to macromolecules, they are stable and accessible for laboratory detection [8].

Sperm and sperm plasma proteins, like other proteins in the body of stallions, are prime targets for ROS and RNS due to their high sensitivity to free radicals [9, 10]. Numerous and structurally diverse free radicals are produced from enzymatic and non-enzymatic redox reactions, photochemical and ionizing effects. Oxidative modification of amino acid residues of proteins yields carbonyl derivatives, the aldehydes and ketones. Aldehydes are early markers of the oxidative destruction of proteins, and ketone derivatives are later ones [6, 8, 9]. It is important to remember that OMP occurs not only because of an increase in the concentration of free radicals, but also as a result of a shift in the balance between antioxidant and prooxidant systems in favor of the latter.

With age, the production of ROS increases in the body and a significant amount of OS products accumulates. This is associated with mitochondrial dysfunction, high production of ROS under depletion of the body's antioxidant capacity, disruption of the proteasome system, and degradation of damaged proteins [11].

Here, for the first time, we measured the content of products of oxidative modification of proteins in the spermoplasm of stallions of different ages and revealed a statistically significant increase in the content of protein carbonylation products in aged animals compared to younger ones, mainly due to neutral aldehyde derivatives. The reserve-adaptation potential (RAP) of stallion spermoplasm has been studied for the first time. Our investigation revealed that the ability to withstand oxidative stress in young stallions is significantly higher than in older ones.

The purpose of this study was to evaluate spontaneous and induced oxidative modification of proteins, and to assess the reserve-adaptation potential of spermoplasm in stallions of different ages.

Materials and methods. In 2020, sperm of 40 stallions (*Equus ferus caballus* L.) of purebred Arabian and Soviet draft breeds (JSC Tersky Pedigree Stud

No. 169, Stavropol Territory; Perevozsky and Pochinkovsky Stud Farms, Nizhny Novgorod Province) was obtained during the breeding season (March-April) for a mare in heat using an artificial vagina. Three ejaculates were obtained from each stallion with an interval of 48 h. During the experiment, the feeding and housing conditions complied with the established standards for the stallions.

To assess the age dependence of OMP and reserve-adaptation potential (RAP) of spermoplasm, stallions were assigned to two groups, $n = 20$ each, in group I, animals aged from 14 to 21 years (15.8±1.9 years), in group II, from 3 to 5 years (4.3±0.6 years).

After sperm collection, each ejaculate was filtered through a sterile gauze pad to remove the secretion of the vesicular glands. The volume of ejaculate (in ml) after filtration was assessed using a graduated cylinder. Sperm concentration was measured (an SDM1 photometer, Minitube GmbH, Germany). Then the ejaculate was divided into two parts, one was diluted at 1:3 with lactose-chelate-citrate-yolk (LCY) medium, and the progressive motility (PM) and survival of sperm at 4 °C were assessed.

Progressive motility (PP) was assessed using the Argus CASA system (ArgusSoft Ltd., Russia) and a Motic BA 410 microscope (Motic, China) in a Makler chamber at 37 °C.

To assess the survival of sperm during hypothermic storage of sperm, their progressive motility was determined at 24-hour intervals until the PP decreased to 5%. Sperm was frozen in liquid nitrogen vapor in 18 ml aluminum tubes using standard technology of the All-Russian Research Institute of Horse Breeding and stored in liquid nitrogen at –196 °C [12]. After thawing in a water bath at 40 °C for 90 s, the progressive motility and survival of cryopreserved spermatozoa at 4 °C were determined.

The other part of the ejaculate, immediately after sperm collection, was centrifuged at 3500 rpm for 20 min (ELMI CM-6M, ELMI, Latvia). After microscopy of the supernatant, aliquots of sperm-free seminal plasma were frozen in 2.0 ml Eppendorf tubes at –18 °C until testing.

To quantify OMP, spectrophotometric analysis of 2,4-dinitrophenylhydrazones resulting from the reaction of protein carbonyl derivatives (aldehydes and ketones) with 2,4-dinitrophenylhydrazine was performed as per a patented procedure [13]. The total amount of carbonyl derivatives was assessed in a native biomaterial to measure the actual content of carbonyl derivatives formed in vivo due to spontaneous OMP and after in vitro induction of protein oxidation with a reaction mixture containing iron(II) sulfate and hydrogen peroxide (metal-catalyzed induced OMP). The oxidizing mixture promoted additional formation of carbonyl derivatives. If in the native sample there was a reserve of antioxidant systems and there were few amino acid residues that could quickly oxidize, then the induced OMP had minimal difference from the spontaneous one. Comparison of metal-catalyzed and spontaneous OMP levels gives estimates of the reserve-adaptive potential (RAP), that is, the ability to withstand OS.

Neutral aldehyde-dinitrophenylhydrazones (ADNPHn) was assessed at 260-280 nm, basic aldehyde-dinitrophenylhydrazones character (ADNPHb) at 258-264 and 428-520 nm, neutral ketone-dinitrophenylhydrazones (KDNPHn) at 363-370 nm, basic ketone-dinitrophenylhydrazones (KDNFGb) at 430-434 and 524-535 nm (SF-2000 spectrophotometer, OKB Spectr, Russia). The area under the curve of the absorption spectrum of dinitrophenylhydrazine (DNPH) derivatives of protein carbonyl derivatives (S) was measures. The obtained extinction values were expressed as optical density units E_{OD} per 1 g of spermoplasm protein content determined by the Lowry method.

Statistical analysis was performed using Statistica 13.3 (StatSoft, Inc.,

USA) and Microsoft Office Excel 2016. Normality of distribution was determined by the Shapiro-Wilk test. The statistical significance of differences between independent samples was assessed by the Mann-Whitney U test. The result was considered statistically significant at $p < 0.05$. Data are presented as median (*Me*) and quartiles (Q1; Q3).

Results. A comparative analysis of spermograms (Table 1) revealed that the rate of sperm survival during hypothermic storage was significantly lower in older stallions than in young animals. Both diluted and cooled sperm (66.0 h for adult stallions, 107.0 h for young stallions, $p < 0.05$) and sperm thawed after cryopreservation (17.3 h and 49.0 h, respectively, $p < 0.01$) showed similar patterns. A similar dependence of sperm survival on age was found not only in stallions [14, 15], but also in men [16, 17] and in bulls [18]. The likely reason for this is the accumulation of irreversible damage to nucleic acids with age, which negatively affects reproductive properties [14, 19].

1. Comparative characterization of sperm quality in purebred Arabian and Soviet draft stallions (*Equus ferus caballus* L.) of different ages (Me [Q1; Q3]; AO Tersky Pedigree Stud No. 169, Stavropol Territory; Perevozsky and Pochinkovsky Stud Farms, Nizhny Novgorod Province, 2020)

Parameter	Group I	Group II
Number of stallions, n	20	20
Age, years	15.8 [14.0; 21.0]	4.3 [3.0; 5.0]
Native and diluted sperm ($n = 60$)		
Ejaculate volume, ml	47.4 [15.0; 80.0]	61.9 [39.0; 105.0]
Sperm concentration, million/ml	153.9 [83.0; 245.0]	159.1 [105.0; 214.0]
Progressive mobility, %	48.0 [35.0; 60.0]	50.3 [40.0; 65.0]
Sperm survival rate, h	66.0 [36.0; 120.0]	107.0* [84.0; 120.0]
Frozen-thawed sperm ($n = 60$)		
Progressive motility, %	17.1 [10.0; 30.0]	21.8 [13.0; 33.0]
Sperm survival rate, h	17.3 [6.0; 72.0]	49.0** [12.0; 84.0]

* and ** Differences between sperm survival during hypothermic storage of diluted semen in stallions of groups I and II are statistically significantat $p < 0.05$ and $p < 0.01$, respectively.

The sperm survival during hypothermic storage (4 °C) is one of the main quality indicators. Spermatozoa survival is their ability to maintain progressive motility after hypothermic storage of diluted chilled or cryopreserved sperm. The survival of sperm is statistically significantly correlated with their progressive motility and the frequency of mares' pregnancies [20].

The total spontaneous OMP of the spermoplasm of stallions of group I was statistically significantly higher than in group II (531.7 and 384.3 E_{OD}/g protein, respectively, $p < 0.05$) (see Table 1). This indicates more pronounced oxidative stress in group I and more significant damage to amino acid residues of spermoplasmic proteins in older stallions compared to young animals. Besides, in group I, a shift in the absorption spectrum towards neutral aldehyde derivatives occurred, and their content in group II was lower (367.6 vs. 255.8 E_{OD}/g protein, $p < 0.05$).

A small amount of ROS in sperm is necessary for the transmission of intracellular signals, hyperactivation and acrosomal reaction, which provides fertilizing ability. However, excess production of ROS inevitably leads to the development of OS and damage to macromolecules [21].

OMB of spermoplasm causes changes in their secondary and tertiary structure which negatively affects the functional state of proteins which means the morphological and reproductive characteristics of sperm deteriorate (22). Damaged proteins can undergo processes of aggregation and fragmentation due to protein-protein interactions.

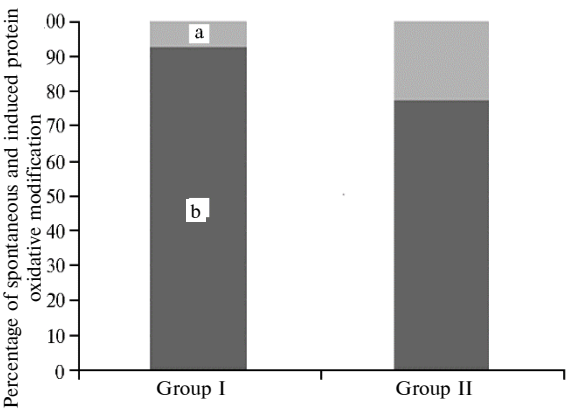
Accumulation of OMP products, protein aggregates and derivatives resistant to proteolysis disrupts cell metabolism, leading to apoptosis or necrosis [22,

23]. It should be noted that antioxidant and proteolytic systems prevent the development of oxidative stress and OMP, therefore, some damage to protein molecules is reversible. Enzymatic antioxidant systems include superoxide dismutase, glutathione reductase, catalase, and peroxidase. Non-enzymatic antioxidants are vitamins E and C, glutathione, carotenoids, ubiquinone and other metabolites [21, 22]. However, with age, pro-oxidant systems increasingly predominate over protective ones, and the degree of OMP is growing. Besides, antioxidant enzymes themselves, like other proteins, are subject to oxidation which disrupts their functioning [24].

2. Absorption spectrum (EOD/g protein) **of products derived from spontaneous and metal-catalyzed protein oxidative modification** (OMP) **in spermoplasm of purebred Arabian and Soviet draft stallions (*Equus ferus caballus* L.) of different ages** (Me [Q1; Q3]; AO Tersky Pedigree Stud No. 169, Stavropol Territory; Perevozsky and Pochinkovsky Stud Farms, Nizhny Novgorod Province, 2020)

Carbonyl derivatives	Group I (aged from 14 to 21 years; n = 20)		Group II (aged from 3 to 5 years; n = 20)	
	spontaneous OMP	induced OMP	spontaneous OMP	induced OMP
SADNFGn	367.6 [162.8; 500.5]	374.7 [236.5; 785.9]	255.8 [122.8; 328.1]	315.3 [193.8; 349.5]
SKDNFGn	83.9 [47.4; 131.5]	105.3 [74.9; 386.4]	69.9 [30.4; 84.3]	76.4 [66.0; 122.5]
SADNFGb	64.6 [40.7; 141.1]	84.0 [66.1; 323.4]	49.6 [29.5; 71.1]	71.4* [62.4; 98.6]
SKDNFGb	11.3 [6.9; 25.2]	13.8 [10.8; 43.0]	9.6 [5.1; 12.3]	11.8 [11.4; 19.4]
Stotal	531.7 [264.6; 787.6]	562.8 [364.2; 1519.7]	384.3 [172.6; 490.4]	484.7* [334.9; 598.4]

N o t e. SADNFGn, SKDNFGn, SADNFGb, SKDNFGb are the area under the curves of the absorption graphs of neutral aldehyde dinitrophenylhydrazones, neutral ketone dinitrophenylhydrazones, basic aldehyde dinitrophenylhydrazones, basic ketone dinitrophenylhydrazones, respectively. Stotal — the total area of the figure formed when plotting the absorption curve of DNPH derivatives of proteins carbonyl derivatives at different wavelengths.
* Differences between metal-catalyzed and spontaneous protein oxidative modification in stallion spermoplasm are statistically significant at $p < 0.05$.



Reserve adaptive potential of spermoplasm in purebred Arabian and Soviet draft stallions (*Equus ferus caballus* L.) of different ages: a — induced oxidative modification of proteins, b — spontaneous protein oxidative modification; Group I — adult stallions (from 14 to 21 years of age), Group II — young stallions (from 3 to 5 years of age) (*Me* [Q1; Q3]; AO Tersky Pedigree Stud No. 169, Stavropol Territory; Perevozsky and Pochinkovsky Stud Farms, Nizhny Novgorod Province, 2020).

The predominance of neutral aldehyde derivatives in older stallions (Table 2) indicated oxidative damage to the amino acid residues of cysteine, glutamine, asparagine, tryptophan, tyrosine, methionine, leucine, and proline. Due to oxidation of the thiol group of cysteine, disulfide cross-links are formed [21, 25]. In this case, the structural and functional state of proteins containing cysteine residues changes. A number of CRISPS (cysteine-rich secretory proteins) are present in the sperm of stallions [26]. CRISPs, found in the testes and epididymis of mammals, are involved in fertilization. During spermatogenesis, the CRISP2 protein is incorporated into the acrosome, where it is believed to participate in the adhesion of germ cells to Sertoli cells. CRISP2 also serves as part of the sperm tail and is involved in the regulation of flagellar beating. The CRISP3 is found in seminal fluid isolated from the prostate, and its function is unknown [26-28]. Oxidative modifications of cysteine residues can negatively affect the functioning of these proteins.

An assessment of induced OMP also revealed a higher total amount of

carbonyl derivatives in group I, but an increase in the amount of carbonyl derivatives when exposed to an oxidizing mixture was much higher compared to the initial level of spontaneous OMP in young stallions (see Table 2, Fig.). Our findings indicate that in the spermoplasm of young stallions there are amino acid groups that could potentially be subject to oxidative stress. However, this did not happen in vivo, probably due to the active antioxidant systems that prevent damage to protein molecules [29]. This is also confirmed by the fact that during induced oxidation, the proportion of neutral and basic aldehyde derivatives (the early markers of oxidative destruction) increased significantly. The results obtained indicate high RAP in young stallions.

In group I, the added oxidative mixture increased the content of carbonyl derivatives (see Fig.), but not so significantly when compared to the initial values. This indicates a significant accumulation in vivo of damaged amino acid radicals of sperm plasma proteins and depletion of RAP in adult stallions.

Thus, the total oxidative modification of spermoplasm proteins in older purebred Arabian and Soviet draft stallions is higher than in younger stallions ($p < 0.05$). The reserve-adaptive potential of the spermoplasm of young stallions significantly exceeds that of older animals ($p < 0.05$), which can positively influence the reproductive quality parameters of native and cryopreserved sperm. A higher content of sperm plasma protein carbonylation products in older stallions semen indicates oxidative stress which may cause a decreased sperm viability.

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BIOCHEMICAL AND MOLECULAR GENETIC INDICATORS OF ANTIOXIDANT PROTECTION AND IMMUNITY IN MALE CHICKS (*Gallus gallus domesticus*) OF DIFFERENT GENOTYPES

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Abstract

A comparative study of the relationship between the antioxidant protection (AOP) and immunity in poultry of various genotypes is relevant for clinical and physiological assessment of health status and the search for combinations of genotypes to obtain new crosses. In this work, for the first time, differences in biochemical and molecular genetic indicators of antioxidant protection and immunity were established for the Russian White, Ross 308 cross, and Russian White × Cornish cockerels. Correlations were revealed between the expression of some genes for AOP and immunity enzymes in caecum and liver tissues, and the average daily weight gain. The aim of the work was to assess the factors of immunity and antioxidant status, nonspecific immunity indicators, and gene expression levels for enzymes involved in antioxidant protection and immune response in male chickens (*Gallus gallus domesticus*) of different genotypes. The studies were carried out in 2022 at physiological yard of the Ernst Federal Research Center for Animal Husbandry. Blood samples were taken from Russian White cockerels (RW, $n = 28$), Ross 308 cross broilers ($n = 9$) and Russian White × Cornish cockerels (CORN × RW, $n = 128$) at slaughter at the age of 9 weeks. The TBA test with thiobarbituric acid to measure the TBA-active products (TBA-AP) was performed with Agat-Med kits (Russia). The activity of ceruloplasmin (CP) was determined by the Revin method, the amount of total water-soluble antioxidants (TWSA) amperometrially (a TsvetYauza-01-AA with an amperometric detector, Khimavtomatika, Russia), the ratio of TBA-AP to the CP was calculated. TAWSA was evaluated as equivalents to gallic acid using calibration solutions with a mass concentration of 0.2, 0.5, 1.0 and 4.0 mg/dm^3 prepared from 100 mg/dm^3 gallic acid. A solution of orthophosphoric acid (0.0022 mol/dm^3) was used as an eluent. Other indicators of antioxidant status were determined with commercial kits (Elabscience Elabscience Biotechnology, Inc., China). Reduced glutathione (E-BC-K096-M), superoxide dismutase (SOD) (E-BC-K020-M), catalase (E-BC-K031-M) and total antioxidant status (TAS) (E-BC-K219-M) were measured by ELISA test (an Immunochem-2100 microplate photometer, High Technology Inc., USA). Nonspecific immunity (i.e., bactericidal activity BA and lysozyme activity LA) of RW ($n = 12$), CORN × RW ($n = 68$) and Ross 308 ($n = 9$) male chicks were determined (a microbiological analyzer Multiskan FC, ThermoFisher Scientific Inc., Finland). Analysis of relative gene expression was performed using real-time PCR. Tissue samples of the caecum and liver were taken from RW ($n = 10$), Ross 308 ($n = 9$), and CORN × RW ($n = 11$) cockerels, 30 samples of each tissue. The relative expression of the genes responsible for antioxidant protection (catalase *CAT*, glutathione peroxidase *GSH-Gpx*, heme oxygenase 1 *HO-1*, superoxide dismutase *SOD*, related transcription factor 2, NF-E2 *Nrf2*) and involved in the immune response (avian beta defensin 9 *AvBD9*, interleukin 6 *IL6*, interleukin 8 *IL8*) was assessed. Total antioxidant status (TAS) of broilers was lower

than that of analogues, which was confirmed by the maximum content of TBA-AP, 4.27 vs. 3.04 μmol/l for RW ($p < 0.05$) and 2.79 μmol/l ($p < 0.01$) for CORN × RW, with a minimum content of ceruloplasmin (37.78 mg/l), and, accordingly, a higher TBA-AP/CP ratio. In the blood of Ross 308 cross males, the maximum TWSA was detected (49.78 mg/l at $p < 0.001$ compared to RW), which was due to the maximum amount of reduced glutathione among analogues (38.26 μmol/l at $p < 0.001$ compared to RW and $p < 0.001$ compared to CORN × RW). The blood activity of catalase in broilers was also high (100.50 U/l at $p < 0.05$ compared to RW and $p < 0.01$ compared to CORN × RW). However, their antioxidant system must work at the maximum to neutralization of reactive oxygen species (ROS). Our data on the expression of AOP and immunity genes confirmed these conclusions. In the caeca of broilers, the genes *CAT* and *GSH-Gpx* expression was 5 times higher compared to egg breed cockerels ($p = 0.0007$ and $p = 0.0008$, respectively), *HO-1* 2 times higher ($p = 0.01$), *SOD* higher by 40 %. In the liver of broilers, there was a decrease in the genes *SOD* and *GSH-Gpx* expression by 5-6 times compared to RW ($p = 0.005$ for both genes), *CAT* expression increased by 27 %, and *HO-1* by 42 times ($p = 0.001$). In broilers, the blood lysozyme concertation and activity were the highest (0.47 μg/ml and 3.14 AU/TP, $p < 0.001$) with a decrease in the percentage of lysis (36.1 vs. 45.6-48.7% in other cockerels, $p < 0.05$) with the minimum BA among analogues. This is confirmed by the fact that the expression of pro-inflammatory cytokines (primarily IL-8) which inhibit humoral immunity was generally lower in the studied broiler tissues while it increased in males of other genotypes. This could lead to a decrease in the humoral response. The average daily weigh gain of poultry highly correlated with the *CAT* ($r = 0.998$ at $p = 0.03$) and *AvBD-9* ($r = 0.999$ at $p = 0.016$) expression in the caecum. In the caecum, high correlations were found between the expression of *CAT* and *AvBD-9* ($r = 0.999$ at $p = 0.014$), *IL6* and *HO-1* ($r = 0.999$ at $p = 0.1$), which confirms the relationship between AOP and bird health. Ross 308 cross broilers showed a higher accumulation of lipid peroxidation products. This highlights the feasibility of using nutritional factors to reduce oxidative stress and increase the antioxidant potential of the body to improve the quality of poultry products.

Keywords: antioxidant status, immunity, chickens, broilers, genotypes, gene expression, *CAT*, *GSH-Gpx*, *HO-1*, *SOD*, *Nrf2*, *AvBD9*, *IL6*, *IL8*

Poultry of modern genotypes have a high productivity potential, but it is often not fully realized because of stresses of various etiologies. Oxidative stress, which results from an imbalance in the formation and detoxification of free radicals as a result of disturbances in feed, climatic, technological and biological growing conditions, negatively affects health, growth performance and product quality. The effects of various stresses on the bird's body were described in our previous work [1]. It has been established that climatic and other maintenance factors determine behavioral, physiological and immune reactions in the body, affect antioxidant and biochemical status, and productivity. The works of various authors are devoted to studying the influence of stress of various etiologies, including oxidative stress, on the poultry body and product quality [2-5]. It has been shown that oxidative stress can impair health status, growth performance, and meat quality [6-9].

Reactive oxygen species (ROS) are produced in mitochondria [10]. During normal physiological processes in the body, the production and clearance of ROS are in dynamic equilibrium [11]. However, when this equilibrium is shifted, ROS can lead to oxidative damage, pathological processes, and even cell death [12]. Oxidative stress induces sensitivity to ROS, signaling through certain pathways, and activation of target genes, such as those encoding antioxidant defenses and certain inflammatory mediators.

There is a relationship between antioxidant defense systems and the body's natural resistance. Thus, an increase in free radical reactions of lipid peroxidation (LPO) leads to disruption of the function of processing antigenic information and the synthesis of antibodies. By damaging cellular structures, oxidative stress can trigger or enhance the inflammatory response caused by immune cells and mediators [13]. A number of immunomodulators block the lipid peroxidation of plasma and subcellular membranes, protecting them from the action of peroxides and free radicals, which are mostly formed in metabolically active cells (macrophages, neutrophils), and thereby maintaining the normal structure and function of membranes [14].

The body contains a variety of antioxidant molecules that counteract and neutralize ROS and other oxidants. Antioxidant defense systems can be divided into two categories, the enzymatic and non-enzymatic. They can also be classified as endogenously produced or exogenously produced dietary antioxidants [15].

It is known that the antioxidant protection and immunity status are influenced by exo- and endogenous factors. The features of physiological and biochemical processes in the body, which determine the intensity of metabolism and growth rate of a bird, largely depend on its origin and direction of productivity [16]. Many researchers associate the antioxidant properties of poultry products with the poultry breed [17, 18]. However, little is known about the effect of genotype on antioxidant defense in poultry. Of interest is a comparative study of the relationship between the processes of antioxidant protection and immunity in poultry of different genotypes, which is important in the clinical and physiological assessment of health status and the search for combinations of genotypes to obtain new crosses. An integrated approach, including biochemical, microbiological and molecular genetic methods, provides a deeper understanding of the mechanisms of immunity formation and antioxidant protection, which is necessary to obtain high-quality poultry products.

In this work, for the first time, differences in biochemical and molecular genetic indicators of antioxidant protection and immunity were established in males of the White Russian breeds, the Ross 308 cross and crosses of the Russian White and Cornish breeds. Correlations were revealed between the expression of some genes of AOD enzymes and immunity in the cecum of the intestine, liver tissue and the average daily increase in live weight, as well as between the relative expression of genes.

The purpose of the work was to reveal factors invlolved in the formation of immunity and antioxidant status, indicators of nonspecific immunity, gene expression for enzymes involved in antioxidant protection and the development of the immune response in cockerels (*Gallus gallus domesticus*) of different genotypes.

Materials and methods. The study was carried out on cockerels (*Gallus gallus domesticus*) of the Russian White breed (RW, $n = 28$), broilers of the Ross 308 cross ($n = 9$) and Russian White × Cornish crossbreds (CORN × RW, $n = 128$). The birds were kept in the same conditions (the physiological yard of the Ernst Federal Research Center for Animal Husbandry — VIZh, 2022). For the biochemical analysis of pro- and antioxidant status, blood samples were collected at slaughter at the age of 9 weeks.

In blood serum, the concentration of products that react with thiobarbituric acid (TBA-AP) was determined using Agat-Med kits (Russia), ceruloplasmin activity (CP) according to the Revin method [19], the total content of water-soluble antioxidants (TWSA) amperometrically (a TsvetYauza-01-AA device with an amperometric detector, Khimavtomatika, Russia), the ratio of TBK-AP to CP by calculation.

To determine TWSA, the strength of the electric current that occurs during the oxidation of molecules on the surface of the electrode at a certain potential was measured. TWSA was estimated in equivalents of gallic acid. For this purpose, working solutions for calibration with a mass concentration of 0.2, 0.5, 1.0 and 4.0 mg/dm^3 were prepared from 100 mg/dm^3 solution of gallic acid. Orthophosphoric acid solution (0.0022 mol/dm^3) was an eluent. Other indicators of antioxidant status, namely the content of reduced glutathione (E-BC-K096-M), the activity of superoxide dismutase (SOD) (E-BC-K020-M), catalase (E-BC-K031-M) and total antioxidant status (TAS) (E-BC-K219-M) was determined by ELISA test using an Immunochem-2100 microplate photometer (High Technology, Inc., USA) and commercial kits (Elabscience Biotechnology, Inc., China) according to

the protocols suggested by the manufacturers.

Indicators of nonspecific immunity of males RW (n = 12), CORN × RW (n = 68) and Ross 308 (n = 9), the blood serum bactericidal (BSBA) and lysozyme activity were determined using a Multiskan FC microbiological analyzer (ThermoFisher Scientific, Inc., Finland) [20]. Serum lysozyme activity (LA) was characterized by the percentage of lysis, the amount of lysozyme in 1 ml of blood serum (lysozyme, μg/ml) and specific activity units (AU) per 1 mg of protein (AU/TP) [21].

Relative gene expression analysis was performed by real-time PCR. Tissue samples of the caeca and liver were collected from males RW (n = 10), Ross 308 (n = 9) and CORN × RW (n = 11) (a total of 30 samples of each tissue). The relative expression was determined for genes responsible for antioxidant protection (catalase — *CAT*, glutathione peroxidase — *GSH-Gpx*, heme oxygenase 1 — *HO-1*, superoxide dismutase — *SOD*, related transcription factor 2 NF-E2 — *Nrf2*) and involved in the immune response (avian beta-defensin 9 — *AvBD9*, interleukin 6 — *IL6*, interleukin 8 — *IL8*). Samples were prepared as per the Instructions for sanitary and microbiological control of carcasses, poultry meat, poultry products, eggs and egg products at poultry farming and processing enterprises (https://meganorm.ru/Data2/1/4293751/4293751517.pdf). Samples were placed in IntactRNA solution (Evrogen, Russia) and stored at –20 °C. The studies were carried out in 3 repetitions.

Total RNA from the samples was isolated using the Aurum Total RNA kit (Bio-Rad, USA) according to the manufacturer's instructions. Homogenization of samples were homogenized (a Precellys Evolution homogenizer, Bertin Technologies SAS, France). Using the iScript™ RT Supermix kit (Bio-Rad, USA), a reverse transcription reaction was performed to obtain cDNA on an RNA template.

Amplification was carried out using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, USA) in accordance with the manufacturer's protocol [22] (a DTlight detecting amplifier, NPO DNA-Technology, Russia). Amplification mode and conditions are 5 min at 95 °C (pre-denaturation); 30 s at 95 °C, 30 s at 60 °C, 30 s at 70 °C (40 cycles) [23]. Relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method [24]. The bird beta-actin protein gene *ACTB* was a reference gene. The primer sequences (5′ → 3′) were for *SOD* F: CGGGCCAGTAAAGGTTACTGGAA, R: TGTTGTCTCCAAATTCATGCACATG; for *GSH-Px* F: GCATCCGCTTCCACGACTTCCT, R: CCGCTCATCCGGGTCCAACAT; for *HO-1* F: GGTCCCGAATGAATGCCCTTG, R: ACCGTTCTCCTGGCTCTTGG, for *CAT* F: ACCAAGTACTGCAAGGCGAA, R: TGAGGGTTCCTCTTCTGGCT; for *Nrf2* F: AAAACGCTGAACCACCAATC, R: GCTGGAGAAGCCTCATTGTC; for *AvBD-9* F: AACACCGTCAGGCATCTTCACA, R: CGTCTTCTTGGCTGTAAGCTGGA; for *IL6* F: AGGACGAGATGTGCAAGAAGTTC, R: TTGGGCAGGTTGAGGTTGTT; for IL8 F: GGAAGAGAGGTGTGCTTGGA, R: TAACATGAGGCACCGATGTG.

The average daily weight gain was determined by weighing performed strictly before feeding. We used analytical scales PR224 (Ohaus Corp., USA) for 1-day-old chickens, electronic kitchen scales VT-8008 (Vitek, Russia) for chickens aged from 1 day to 3-4 weeks, and an electronic steelyard MT-1645 (MARTA, China) for adults.

Mathematical processing of the results was carried out with the software packages Microsoft Office Excel 2003, STATISTICA 10 (Statistica 13RU, StatSoft, Inc., USA) using descriptive statistics and correlation analysis methods. Mean values (M), standard errors of means (±SEM), standard deviations (±SD), coefficients of variation (Cv), and Pearson correlation coefficients (r) were calculated. Correlations at r up to 0.2 were considered very weak, 0.2-0.5 weak, 0.5-

0.7 medium, 0.7-0.9 high, more than 0.9 very high. The scatter of data values was considered insignificant at $Cv < 10\%$, medium at $Cv = 10\text{-}20\%$, and significant at $20\% < Cv \leq 33\%$. Differences were statistically significant at $p < 0.05$, highly significant at $p < 0.01$, $p < 0.001$.

Results. The intensity of lipid peroxidation is determined both by radical and peroxide formation and by the state of endogenous antioxidant defense, therefore, determining the antioxidant activity of these systems is of practical importance [25]. Often, to assess the state of lipid peroxidation, the reaction with thiobarbituric acid (TBA) is widely used. The TBA test is based on the ability of TBA to react with malondialdehyde (MDA), a low molecular weight compound that serves as an intermediate in the enzymatic oxidation of arachidonic acid and as an end product in the oxidative degradation of lipids [26, 27].

LPO products, in particular MDA, exhibit cytotoxic, mutagenic and carcinogenic properties. The consequences of their exposure include, for example, loss of cell proliferation potential, changes in gene expression, mutations, molecular heterogeneity, disruption of intercellular communication, and organ dysfunction [28]. In addition, MDA is one of the end products of the peroxidation of polyunsaturated fatty acids in the human body and a marker of oxidative stress.

1. Parameters of antioxidant protection and lipid peroxidation in cockerels (G*allus gallus domesticus*) of different genotypes (physiological yard of the Ernst Federal Research Center for Animal Husbandry — VIZh, 2022)

Parameter	*M*	min	max	±SEM	±SD	*Cv*, %
Russian white breed ($n = 28$)						
TBA-AP, μmol/l	3,04*	2,26	4,10	0,08	0,43	14,22
CP, mg/l	62,53	38,00	117,00	3,43	18,14	29,00
TWSA, mg/l	45,20	29,43	75,54	2,02	10,67	12,61
Reduced glutathione, μmol/l	23,84***	11,47	37,74	2,04	7,62	31,96
SOD, U/ml	19,45***	17,58	20,19	0,22	0,83	4,25
Catalase, U/l	52,25*	6,67	196,06	14,16	52,97	101,37
TAS, mmol/l	0,69	0,28	0,95	0,04	0,15	22,09
TBA-AP/CP	0,05					
Cross Ross 308 broilers ($n = 9$)						
TBA-AP, μmol/l	4,27	1,95	6,87	0,57	1,70	39,78
CP, mg/l	37,78†††	25,00	66,00	3,95	11,84	31,34
TWSA, mg/l	49,78	42,28	59,46	1,61	4,82	9,69
Reduced glutathione, μmol/l	38,26	29,83	48,84	2,23	6,69	17,48
SOD, U/ml	15,22	10,89	18,03	0,76	2,27	14,90
Catalase, U/l	100,50	46,06	217,27	17,69	53,07	52,80
TAS, mmol/l	0,64	0,36	0,99	0,07	0,21	33,15
TBA-AP/CP	0,11					
Crosses of Russian White and Cornish breeds						
TBA-AP, μmol/l	2,79**	1,33	5,23	0,06	0,72	25,72
CP, mg/l	41,94††	23,00	78,00	0,99	11,16	26,62
TWSA, mg/l	41,03***	22,80	73,55	0,97	11,00	26,81
Reduced glutathione, μmol/l	22,02***	7,86	44,91	2,01	10,26	46,59
SOD, U/ml	19,30***	16,57	21,21	0,20	1,04	5,41
Catalase, U/l	32,94**	11,81	61,81	6,68	17,69	53,72
TAS, mmol/l	0,78†	0,59	1,37	0,03	0,16	20,27
TBA-AP/CP	0,07					

N o t e. TBA-AP — thiobarbituric acid reacting products, TWSA — total amount of water-soluble antioxidants, CP — ceruloplasmin, SOD — superoxide dismutase, TAS — total antioxidant status

*, **, *** Differences from broilers are statistically significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; †, ††, ††† — compared to the Russian White breed at $p < 0.05$, $p < 0.01$, and $p < 0{,}001$, respectively.

In our study, the maximum blood content of TBA-AP was in broilers, 4.27 μmol/l vs. 3.04 μmol/l in RW males ($p < 0.05$) and 2.79 μmol/l in CORN × RW ($p < 0.01$) (Table 1).

Attention to ceruloplasmin is due to the fact that it is one of the main scavengers of extracellular free radicals. Ceruloplasmin specifically inhibits damage to various biomolecules [29]. The blood content of ceruloplasmin in RW birds was 62.53 mg/l, in CORN × RW crossbreds 41.94 mg/l, and in broilers 37.78 mg/l. The difference was statistically significant between RW and broiler

groups ($p < 0.001$), as well as between RW and CORN × RW ($p < 0.01$).

TWSA in birds ranged within 45.20-49.78 mg/l with the maximum value in broilers (a significant difference with CORN × RW, $p < 0.001$). In these birds, the parameter showed low variability. In RW males, TWSA was 45.20 mg/l with moderate variability. In CORN × RW, TWSA was the minimum and highly variable (41.03 mg/l). The maximum TWSA in broilers vs. other genotypes was most likely associated with an increase in the amount of reduced glutathione and other water-soluble antioxidants the content of which we neglected.

The amount of reduced glutathione and catalase activity in broilers were the highest among the analyzed genotypes, 38.26 μmol/l (at $p < 0.001$ compared to RW and $p < 0.001$ compared to crossbreeds) and 100.50 U/l (at $p < 0.05$ and $p < 0.01$). SOD activity, on the contrary, turned out to be minimum, 15.22 U/ml (at $p < 0.001$ compared to RW and $p < 0.001$ compared to crossbreeds). TAS in broilers was lower than in analogues, which can be explained by the high consumption of various antioxidants for interaction with LPO products TBA-AP. When assessing the relationship between lipid peroxidation and antioxidant defense (AOD) based on the ratio of a number of components of these systems, we noted that the TBA-AP/CP ratio in broilers, due to low CP activity, was higher than in birds of other genotypes. That is, the antioxidant system in broilers of the Ross 308 cross is the most vulnerable, despite the high TWSA level and catalase activity. This fact is confirmed by the minimum TAS value which characterizes the state of all antioxidants in the body (30).

Thus, the high growth rate of broilers makes them more susceptible to oxidative stress, which can negatively affect meat quality. According to I.F. Gorlov et al. [31], the oxidative changes in chilled meat depends on the reactivity of the poultry antioxidant system and the formation of lipid peroxidation products. The weakening of antioxidant activity and activation of free radical oxidation of lipids in the blood of broiler chickens enhance the processes of meat oxidation [31]. In addition, stress increases the production of free radicals which can inactivate essential antioxidant enzymes, causing autocatalytic irreversible oxidation [32]. In this regard, the synthesis of new antioxidant enzymes is the most important response to stress conditions, which can explain the higher activity of catalase, the content of reduced glutathione and TWSA in broilers.

M. Madkour et al. [33] noted a decrease in the activity of catalase and superoxide dismutase in the liver tissues of broilers under heat stress at an early age compared to poultry raised under normal conditions.

K.S. Ostrenko et al. [34] propose to replenish the need for antioxidants with a decrease in the ability of laying hens to adapt to changing environment due to long-term selection for maximum egg productivity. In the work of these authors, when using the dietary water-soluble antioxidant dihydroethoxyquin, the content of lipid peroxidation products and holesterol in lipoprotein fractions of various densities decreased [34].

Nonspecific resistance is the ability to maintain optimal functioning of organs, systems, or the entire body, both under stereotypical conditions and all kinds of influences [35]. The decisive link in the chain of environment—body response—productivity should be considered the genotype [36]. When studying the indicators of nonspecific immunity in cockerels, we identified differences in indicators depending on the genotype of the bird (Table 2).

The resistance of a bird is largely determined by the humoral immunity level. Lysozyme, performing important biological functions in the body (bactericidal properties, stimulating effects on phagocytosis, neutralization of some microbial toxins, anti-inflammatory effect) serves as one of the key humoral factors

of innate immunity. The lysozyme gene is gradually activated in mature macrophages [37].

In broilers, we found the highest blood content and highest activity of lysozyme (0.47 μg/ml and 3.14 AU/TP, $p < 0.001$) and a decrease in the percentage of lysis (36.1% vs. 45.6-48.7 % in other cockerels, $p < 0.05$). That is, the mechanisms of humoral immunity activation in birds of different genotypes differ. V.G. Ovsyannikova et al. [38] reported that humoral factors of innate immunity are involved in the acute algogenic process, providing preventive protection against pervasion of microbial agents. This response does not last long, but it should be noted that in broilers it was stronger.

2. Parameters of nonspecific immunity in cockerels (G*allus gallus domesticus*) of different genotypes (physiological yard of the Ernst Federal Research Center for Animal Husbandry — VIZh, 2022)

Parameter	*M*	min	max	±SEM	±SD	*Cv*, %
Russian white breed ($n = 12$)						
Lysis, %	48.70*	32.87	64.59	2.56	8.85	18.17
Lysozyme, μg/ml serum	0.36*	0.22	0.45	0.02	0.06	16.53
Lysozyme activity, AU/TP	2.42†	1.27	3.32	0.16	0.56	23.09
BSBA, %	55.32*†	37.10	66.00	2.44	8.47	15.31
Cross Ross 308 broilers ($n = 9$)						
Lysis, %	36.10	16.07	56.92	4.94	14.82	41.05
Lysozyme, μg/ml serum	0.47	0.34	0.80	0.05	0.15	31.71
Lysozyme activity, AU/TP	3.14	2.02	6.17	0.43	1.28	40.92
BSBA, %	35.14	5.20	54.00	7.09	21.27	60.51
Crosses of Russian White and Cornish breeds ($n = 68$)						
Lysis, %	45.60*	9.38	91.50	2.29	18.87	41.38
Lysozyme, μg/ml serum	0.25***	0.05	0.50	0.01	0.10	41.94
Lysozyme activity, AU/TP	1.34***	0.07	3.16	0.11	0.89	66.14
BSBA, %	37.99	16.40	69.90	1.44	11.90	31.33

Note. BSBA is blood serum bactericidal activity.
*, ** Differences from broilers are statistically significant at $p < 0.05$ and $p < 0{,}001$; † — compared to crossbred poultry at $p < 0{,}001$.

Cytokines are polypeptides or glycoproteins that are synthesized and secreted primarily by immune cells. Cytokines are involved in nonspecific resistance reactions, cellular and humoral immunity [39]. Interleukins, cytokines, and tumor necrosis factor are the main inflammatory mediators secreted by immune cells to control the inflammatory response. Proinflammatory cytokines (including IL-6 and IL-8) enhance cellular immunity and inhibit humoral immunity [40], while playing a major role in the formation of antiviral defense.

As will be shown below, in broilers the expression of genes for pro-inflammatory cytokines (primarily IL-8) was generally lower, and in Russian White males and crossbreds it increased in the tissues of the caecum and liver; this could reduce the humoral response, expressed as a decrease in BSBA (see Table 2). IL-6, by suppressing the secretion of pro-inflammatory cytokines, acts as an anti-inflammatory factor.

V.I. Fisinin et al. [41] showed that lysozyme titer, which correlates with the amount of cytokines and glucocorticoid hormones, could serve as a marker of stress. Cytokines mediate interactions between cells and perform various biological functions, in particular they regulate cell growth, differentiation and maturation, the immune response, and are involved in inflammation and wound healing. Decreased cytokine levels may slow down the differentiation of stem cells into mature immune cells and lead to decreased disease resistance [42]. Sanitary and bacteriological indicators of the microclimate at a poultry farm affect the general clinical condition and safety of the livestock. Resistance parameters should be considered depending on whether the poultry is used for meat or eggs To ensure optimal performance, it is important to monitor and, if necessary, modulate the immune response to maintain homeostasis.

3. Relative expression of genes in the cecum of the intestine and in the liver of cockerels (G*allus gallus domesticus*) of different genotypes (physiological yard of the Ernst Federal Research Center for Animal Husbandry — VIZh, 2022)

Genotype	Gene							
	CAT	*GSH-Gpx*	*HO-1*	*SOD*	*AvBD9*	*IL6*	*IL8*	*NrF2*
Cecum								
RW (C) (n = 10)	1	1	1	1	1	1	1	1
CORN × RW (n = 11)	2.42±0.169	2.84±0.257	2.18±0.163	2.04±0.219	1.82±0.095	1.87±0.093	5.18±0.692	1.16±0.157
Ross 308 (n = 9)	5.06±0.274	4.73±0.270	2.05±0.216	1.41±0.155	3.22±0.189	1.76±0.215	0.67±0.052	1.58±0.210
Liver								
RW (C) (n = 10)	1	1	1	1	1	1	1	1
CORN × RW (n = 11)	1.14±0.110	1.57±0.155	119.63±9.005	1.33±0.132	25.52±4.294	3.43±0.371	1.58±0.178	0.52±0.100
Ross 308 (n = 9)	1.27±0.099	0.15±0.066	42.19±3.486	0.18±0.012	19.15±1.391	0.98±0.100	0.46±0.090	0.96±0.129

N o t e. RW is Russian White breed (control — C), Ross 308 are broilers of cross Ross 308, CORN × RW is cross between Russian White and Cornish breeds; *CAT* — catalase, *GSH-Gpx* — glutathione peroxidase, *HO-1* — heme oxygenase 1, *SOD* — superoxide dismutase, *NrF2* — related transcription factor 2, *AvBD9* — avian beta defensin 9, *IL6* — interleukin 6, *IL8* — interleukin 8. The results in graphical form are presented at http://www.agrobiology.ru.

Adaptation to stress occurs at the level of genes, which are called vitagens. These include genes responsible for the synthesis of protective molecules, including heat shock proteins, immunoglobulins, and antioxidant enzymes [43]. Studying the expression of these genes is a new approach to the diagnosis and prevention of stress at the molecular level [44]. Our studies have shown that the transcriptional activity of the analyzed genes depends not only on the genotype of the bird, but also on the organ and tissue in which these genes are expressed (Table 3).

The biological role of superoxide dismutase (SOD) is to catalyze the dismutation of superoxide radical into hydrogen peroxide. Gluthione peroxidase catalyzes the reaction and reduces hydrogen peroxide to water using reduced glutathione as a co-substrate. Tissue-specific expression and activity of *SOD* and *GSH-Gpx* are influenced by various factors, including genetic, nutritional, and stress-related [45]. Heme oxygenase 1, known as heat shock protein-32 (HSP32), is responsible for the degradation of heme to produce carbon monoxide, biliverdin and free iron. Like HSP70, HO-1 is a stress-inducible, one of the three HO isoforms described to date, providing a critical protective mechanism in systems that are responsible for adaptation to oxidative, inflammatory, and cytotoxic stress [46].

In most tissues, HO-1 is expressed at relatively low levels and can be induced by various insults associated with oxidative stress (heme, ultraviolet radiation, heavy metals, cytokines, hydrogen peroxide, nitric oxide NO, glutathione depletion) [47].

In the intestinal cecum of birds of three genotypes we studied, the expression of the *CAT*, *GSH-Gpx*, *HO-1*, and *SOD* genes differed. In CORN × RW compared to RW, the gene relative expression was higher, e.g., for *GSH-Gpx* 2.84-fold ($p = 0.006$), for *CAT* 2.42-fold ($p = 0.004$), for *HO-1* 2.18-fold ($p = 0.01$), and for *SOD* 2.0-fold ($p = 0.02$). In broilers, the relative expression of *CAT* and *GSH-Gpx* increased 5-fold vs. egg poultry ($p = 0.0007$ and $p = 0.0008$, respectively), for *HO-1* the relative expression was 2 times higher ($p = 0.01$), for *SOD* 40% higher. The data obtained are consistent with the blood catalase activity and reduced glutathione in the broilers.

Differences in the expression of genes associated with antioxidant defense were also found in the liver of coccerels. In CORN × RW, the expression was higher than in RW, for *HO-1* by 119 times ($p = 0.001$), for *SOD* by 33%, for *GSH-Gpx* by 57%, and for *CAT* by 14%. In the broilers, a 5-6-fold decrease in the relative expression of the antioxidant defense genes *SOD* and *GSH-Gpx* occurredv compared to the RW group ($p = 0.005$). The relative expression of *CAT* in the broilers turned out to be 27% higher, *HO-1* 42 times higher ($p = 0.001$).

The Nrf2 transcription factor is best known as one of the main participants in the development of cellular responses to xenobiotics and oxidative stress. Recent studies have identified new Nrf2 target genes and identified several additional functions of Nrf2 that extend beyond its redox properties, including regulation of inflammation, autophagy, metabolism, proteostasis, and protein denaturation responses. Nrf2 has become a major target of research related to inflammation, metabolism, cancer prevention, and treatment because its functions are more extensive than originally thought ([48].

In the cecum, the expression of the transcription factor Nrf2 in the broilers was 1.5 times higher than in the control. In the liver tissues of CORN × RW birds, *Nrf2* expression was 2 times lower than the control ($p = 0.02$), and in the broilers it was comparable to the control.

The nonspecific immune system includes β-defensins and interleukins. IL-6 is a multifunctional cytokine that is involved in inflammatory responses. IL-8 (a member of the CXC sub-family of chemokines) serves as a chemoattractant for leukocytes, the activation of which leads to proinflammatory responses such as

oxidative burst and increased cell death. IL-8 was first isolated from fibroblasts in chickens. It is known that under stress there is an increase in the amount of interleukins, which is caused by the development of inflammation. Stress can alter regulation of the immune system by increasing the activity of interleukins, namely IL-6, a major mediator of inflammatory and immune responses [49, 50].

In the cecum of CORN × RW cockerels compared to egg-bred birds, the expression of *AvBD9* and *IL6* genes was 1.8 times higher (p = 0.005 and p = 0.004, respectively), of *IL8* 5 times higher (p = 0.009). In the liver of CORN × RW, a similar pattern occurred, the relative expression of *IL6* was greater than that in RW by 3.43 times (p = 0.006), *AvBD9* by 25.50 times (p = 0.009), *IL8* by 1.56 times (p = 0.05).

In the ceca, the relative expression of *IL6* in broilers was higher than in RW by 1.76 times (p = 0.03), *AvBD9* by 3.22 times (p = 0.002). Moreover, the expression of *IL8* which is activated during infections, was approximately 30% lower in broilers compared to egg-laying birds. In the liver of broilers, the expression of *AvBD9* was 19.15 times higher than that in RW (p = 0.001), but the expression levels of the *IL6* and *IL8* genes were loer than in Russian White poultry.

4. Correlations (*r*) between the relative expression of antioxidant defense and immunity genes depending on their location and in connection with growth intensity of cockerels (G*allus gallus domesticus*) of different genotypes (*n* = 30, physiological yard of the Ernst Federal Research Center for Animal Husbandry — VIZh, 2022)

Parameter	Gene							
	CAT	*GSH-Px*	*HO-1*	*SOD*	*AvBD-9*	*IL6*	*IL8*	*Nrf2*
				Cecum				
ADWG	0.998 (p = 0.03)	0.993	0.733	0.275	0.999 (p = 0.016)	0.722	–0.189	0.992
CAT		0.987	0.700	0.228	0.999 (p = 0.014)	0.688	–0.235	0.997
GSH-Px	0.987		0.807	0.384	0.990	0.798	–0.073	0.970
HO-1	0.700	0.807		0.855	0.715	0.999 (p = 0.10)	0.530	0.639
SOD	0.226	0.384	0.855		0.250	0.863	0.893	0.148
AvBD-9	0.999 (p = 0.014)	0.990	0.715	0.250		0.704	–0.214	0.995
IL6	0.688	0.798	0.999 (p = 0.1)	0.863	0.704		0.543	0.927
IL8	–0.235	–0.073	0.530	0.893	–0.214	0.543		–0.314
Nrf2	0.997	0.970	0.639	0.148	0.995	0.630	–0.314	
				Liver				
ADWG	0.989	–0.690	0.223	–0.776	0.622	–0.131	–0.587	0.049
CAT		–0.577	0.362	–0.677	0.728	0.014	–0.463	–0.096
GSH-Px	–0.577		0.552	0.992	0.139	0.808	0.991	–0.757
HO-1	0.362	0.552		0.441	0.903	0.937	0.658	–0.963
SOD	–0.677	0.991	0.441		0.012	0.726	0.966	–0.667
AvBD-9	0.728	0.139	0.903	0.012		0.696	0.270	–0.752
IL6	0.014	0.808	0.937	0.726	0.696		0.880	–0.997
IL8	–0.463	0.991	0.658	0.966	0.270	0.880		–0.837
Nrf2	–0.096	–0.757	–0.963	–0.667	–0.752	–0.997	–0.837	

Note. ADWG — average daily live weight gain, *CAT* — catalase, *GSH-Gpx* — glutathione peroxidase, *HO-1* — heme oxygenase 1, *SOD* — superoxide dismutase, *Nrf2* — related transcription factor 2, *AvBD9* — avian β-defensin 9, *IL6* — interleukin 6, *IL8* — interleukin 8.

We also calculated Pearson correlations between the relative expression of immunity genes and AOD in different tissues (in the cecum and in the liver) and in connection to the growth rate of birds of all three genotypes (Table 4).

Average daily live weight gain (ADWG) in poultry showed tight correlation with the expression of the *CAT* (*r* = 0.998 at p = 0.03) and *AvBD9* (*r* = 0.999 at p = 0.016) genes in the cecum. In the cecum, high correlations were also noted between the expression of the *CAT* and *AvBD9* (*r* = 0.999 at p = 0.014), *IL6* and *HO-1* (*r* = 0.999 at p = 0.1) genes. Thus, the genes for antioxidant protection and

immunity are closely related to each other, which confirms the connection between the body's AOD and poultry health. V.G. Narushin et al. [51] determined correlations between the expression of certain immune genes and biochemical and immunological blood parameters in laying hens. The most informative biochemical and immunological blood parameters were the content of urea, urea nitrogen, glucose and the activity of IgG2 immunoglobulin.

Our work did not establish significant correlations between ADWG and the expression of genes associated with AOD and immunity in the liver of birds (see Table 4). Here, we also did not find statistically significant correlations between the expression of these genes. This requires research, which we propose to conduct using additional dietary components to increase the antioxidant and immune status of poultry and improve product quality.

So, on cockerels of cross Ross 308, the Russian White breed and crosses of the Russian White and Cornish breeds, it is shown that the bird's genotype determines the state of the body's antioxidant system, which influences the accumulation of lipid peroxidation (LPO) products, the function of the enzymatic component of antioxidant defense (AOD), general antioxidant status and immune response. In broilers of the Ross 308 cross, a higher accumulation of lipid peroxidation products occurs which imposes special tension in the antioxidant system and can be compensated by a high total content of water-soluble antioxidants and catalase activity. Data on the expression of genes for antioxidant enzymes and immunity in the cecum and in the liver of birds confirm the results of biochemical blood tests. We are the first to establish correlations of the expression of some genes for AOD enzymes and immunity in the cecum and liver tissue with the average daily weight gain of cockerels, as well as between the relative expression of genes, which confirms the connection between AOD and poultry health. Our findings indicate that studying and applying ways to reduce oxidative stress and additionally protect the antioxidant system of intensively growing poultry are relevant. Next, we plan to explore ways to reduce the impact of stress (including oxidative stress) on poultry health and meat quality through nutritional factors.

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MILK TRYPSIN CLEAR INCREASES UNDER BOVINE MASTITIS SIMULTANEOUSLY WITH INFLAMMATION GENE EXPRESSION

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Abstract

Mastitis is one of the most serious problems in dairy farming. Mastitis often affects high-yielding cows, with a 10-15 % reduction in productivity and irreversible mammary gland dysfunction. In clinical course pathology has clear diagnostic signs. The main known methods of diagnostics of subclinical forms of mastitis (mastitis tests) are based on the determination of somatic cells in milk, the number of which correlates with inflammation, but the development of methods for early diagnosis of mastitis and pre-mastitis state of cows remains relevant. Biochemical parameters and morphological profiles of animal blood, expression of genes associated with inflammation are also examined in mastitis. However, the presence of enzymes in animal milk has not been fully studied. Trypsin is considered as a hormone-like substance capable of influencing metabolism and being a marker of inflammatory processes in animals and humans. Previously, we have shown the role of trypsin in experimental toxicosis of chickens and dietary changes. In the presented study we have for the first time revealed trypsin in the milk of cows, the increase in its activity in mastitis was established and compared with changes in other indicators used to assess the state of animals in pathology. The aim of the present work is to detect trypsin activity in milk of healthy and mastitis-affected cows and to determine the number of somatic cells in milk, relative expression of genes associated with inflammation, as well as morpho-biochemical blood parameters. The results obtained on Ayrshire cows (*Bos taurus*), 10 lactating cows without clinical signs of mastitis and 15 cows with clinical signs of mastitis (SGC Smena — a branch of the FSC VNITIP RAS, Moscow Province, 2022), showed that in the milk, the activity of genes associated with inflammation and the trypsin activity varied depending on the mammary gland health. In mastitis this index increased compared to the norm by 106.6 % ($p \le 0.05$), whereas trypsin activity in blood serum of healthy and mastitis cows had no significant differences. Of the biochemical parameters of cow blood, the most informative were the concentration of glucose, calcium and phosphorus. We found that in blood serum of mastitic cows the amount of glucose increases by 67.4% ($p < 0.05$), calcium by 38.8% ($p < 0.05$), the concentration of phosphorus, on the contrary, decreases by 23.8 % ($p < 0.05$) compared to healthy animals. In the blood morphological profile at mastitis leukocytosis is observed, there is a decrease in immunoreactivity by 42,5 % ($p < 0.05$), the ratio of lymphocytes and neutrophils by 20.4 % ($p < 0.05$), the number of eosinophils by 57.4 % ($p < 0.05$) and basophils by 33.3 % ($p < 0.05$), while the number of monocytes increases by 46.5 % compared to the control ($p < 0.05$). The expression of genes of monocyte chemotactic protein 1 and monocyte chemotactic protein 2 increased 5.5-fold, tumor necrosis factor alpha 3.9-fold, interleukin 4 and interleukin 8 2.9-fold and 14-fold, respectively, in cows with mastitis compared to healthy cows. Thus, we found that cow's milk contains trypsin, which is not inferior to the enzyme in the blood serum of animals in terms of activity (48.2±3.8 units/l). In inflammation of the mammary gland confirmed by instrumental and molecular genetic methods, trypsin activity in milk increases, which can be used in the development of diagnostic methods for pre-mastitis and early stages of mastitis.

Keywords: cows, mastitis, milk trypsin, mastitis diagnostic methods

Mastitis (inflammation of the mammary gland) is one of the most common diseases of dairy cattle. The pathology causes economic losses due to decreased milk production and poor milk quality [1-3]. To combat bovine mastitis and reduce the damage caused by this disease, a search is underway for more advanced and highly sensitive methods for diagnosing and treating the disease [4].

In ongoing studies, much attention is naturally paid to morphological and biochemical blood tests [5, 6], but the reliability of the parameters as prognostic indicators has not been proven [6]. Another modern approach is the assessment of the expression of genes associated with inflammation [7-9]. But even in this case, the results, regardless of connection with other indicators, are not yet considered as unambiguous [8].

It is known that active digestive enzymes are present in the blood of animals and humans [10-12]. The ability of enzymes to penetrate into the blood due to the structural features of pancreatic cells was described by Soviet scientists back in 1973 [13]. Blood enzymes are considered as possible markers in the diagnosis of mastitis in cows [14]. Such indicators include N-acetyl-beta-D-glucosaminidase, lactose, haptoglobin and serum amyloid A in cow milk [15]. It has previously been shown that only the alkaline phosphatase activity test is reliable in the early diagnosis of subclinical bovine mastitis, but not the lactate dehydrogenase and aspartate aminotransferase tests [15].

Some enzymes enter milk since they are synthesized in the cells of the mammary gland. Other enzymes are produced by various microorganisms found in milk, which, during vital activity, release substances that alter milk composition and properties. Lipase, lactase, phosphatase, reductase, peroxidase, catalase, trypsin, trypsinogen, and lysozyme were detected in human milk [14]. The scientific literature contains information on comparative analysis of enzyme activity in breast and cow's milk, as well as in human colostrum and regular milk [15]. There are evidences to support the view that lipase and ribonuclease probably enter milk from the blood; lysozyme is released from secretory epithelial cells; lactate and malate dehydrogenases, glucose-6-phosphate dehydrogenase and lactose synthetase are synthesized in the mammary gland; lipase, diastase, protease and lysozyme, stimulated by bile salts, are present in quantities sufficient to break down milk substrates [15]. Lipase activity in human milk is being studied [16].

Among the enzymes, in our opinion, trypsin, which is considered as a hormone-like substance, deserves detailed attention [17]. Trypsin affects metabolism and, we believe, can be a marker in the diagnosis of inflammatory processes in animals and humans [18]. Previously, we identified changes in trypsin activity during experimental chicken toxicosis [19] and depending on diets [20]. Although the transcriptomic responses of bovine mammary gland cells to mastitis-causing pathogens have been studied [8], the relationship between such signs of mastitis as the number of somatic cells in milk and the level of expression of immune genes associated with mammary gland inflammation is still unknown.

In this work, trypsin activity was discovered for the first time in cow's milk, and its changes during mastitis were established. New data have been obtained on the expression of inflammatory genes during cow mastitis. Thus, the transcriptional activity of the genes for monocyte chemotactic protein 1 increased by 5.5 times, monocyte chemotactic protein 2 by 9 times, tumor necrosis factor by 3.9 times, genes of interleukin 4 and interleukin 8 by 2.9 times and 14-fold.. Trypsin activity as a putative indicator of the mammary gland state is being discussed.

The goal of the work was to identify trypsin activity in the milk of healthy and mastitic cows and to investigate its probable association with the number of

somatic cells, the relative expression of inflammatory genes, and morpho-biochemical blood parameters.

Materials and methods. Physiological experiments were carried out on 25 Ayrshire cows (*Bos taurus*) at the farm of the Smena State Center, a branch of the Federal Scientific Center All-Russian Research and Technological Institute of Poultry — FSC VNITIP RAS, Moscow Province) in 2022. Group I (control) was healthy lactating cows without clinical signs of mastitis (n = 10), and group II with clinical signs of mastitis (n = 15). The mastitis was confirmed by kenotest, viscometric study (milk analyzer Somatos-Mini, LLC VPK Sibagropribor, Russia) and flow cytometry (automatic analyzer CombiFoss 7 DC, FOSS, Denmark). Flow cytometry determines the total somatic cell counts (SCC) and differential somatic cell counts (DSCC, the proportion of lymphocytes and polymorphonuclear neutrophils in the total number of cells) [21]. All tests were performed as recommended by the manufacturing companies. Milk was collected in the morning into sterile 15 ml tubes and no later than in 3 h, the enzyme activity was measured. Milk samples were prepared by centrifugation in microtubes for 5 min at 14,000 rpm (an Eppendorf 5430R centrifuge, Eppendorf, Germany). After centrifugation, the top layer containing fat was removed. For assay, the second layer after the fat was collected. Trypsin activity was measured (a biochemical analyzer SINNOWA 3000M (SINNOWA Medical Science & Technology Co., Ltd, China) by a kinetic method using Na-benzoyl-DL-arginine-p-nitroanilide (BAPNA, ACROS ORGANICS, Switzerland) as a substrate in accordance with description [22].

Blood was taken from the tail vein into vacuum tubes for collecting venous blood with a coagulation activator (filler silicon oxide SiO_2). To obtain comparable results, trypsin activity in blood serum was determined in the same way as in milk (a biochemical analyzer SINNOWA 3000M, SINNOWA Medical Science & Technology Co., Ltd, China) [22]. Blood biochemical parameters were examined (an automatic biochemical analyzer BioChem FC-120, High Technology, Inc., USA) with reagent kits for total protein, glucose, cholesterol, calcium, phosphorus, alkaline phosphatase assay (High Technology, Inc., USA).

Blood morphology was examined using an automatic hematology analyzer MicroCC-20Plus (High Technology, Inc., USA). Blood tests were performed on healthy cows and cows with mastitis (at least 2 times in each cow).

Gene expression analysis was performed using quantitative reverse transcription polymerase chain reaction (RT-PCR). Milk was sampled from each lobe of the udder of 6 cows (No. 1-3, clinically healthy animals, No. 4-6, animals with signs of mastitis), 24 samples in total. The samples were stabilized in IntactRNA solution (JSC Evrogen, Russia) according to the manufacturer's recommendations and stored at –20 °C.

The samples were homogenized (Precellys Evolution homogenizer, Bertin Technologies, France). Total RNA was isolated using the Aurum Total RNA kit (Bio-Rad, USA) according to the manufacturer's instructions. To obtain cDNA on an RNA template, a reverse transcription reaction was carried out with the iScript™ Reverse Transcription Supermix kit (Bio-Rad, USA). The gene amplification reaction (a DTlight detecting amplifier, NPO DNA-Technology, Russia) was carried out with primers described [23] using the SsoAdvanced™ Universal SYBR® Green Supermix kit (BioRad, USA) in accordance with the manufacturer's protocol [24]. The amplification mode and conditions for the analysis were as follows: 5 min at 95 °C (preliminary denaturation); 30 s at 95 °C, 30 s at 60 °C, 30 s at 70 °C (40 cycles) [25]. Relative expression was determined by the $2^{-\Delta\Delta CT}$ method [26]. The housekeeping gene *RPL19*, encoding ribosomal protein L19

(RPL19), was the reference gene.

Statistical processing included calculation of the mean value (*M*) and standard deviation (±SD) using Microsoft Excel. The significance of differences was assessed by Student's *t*-test. Differences were considered statistically significant at $p < 0.05$. Correlation analysis was performed according to Pearson using the Microsoft Excel computer program.

Results. Cows with clinical signs of mastitis, additionally confirmed by kenotest and viscometry, were selected for the study. Healthy animals without signs of mastitis served as controls.

SSC and DSSC values are used as biomarkers in breast health monitoring. These indicators were assessed in the milk of healthy and mastitic cows by flow cytometry.

1. The total number of somatic cells and the proportion of lymphocytes and polymorphonuclear neutrophils in the milk of healthy (group I) **and mastitic** (group II) **Ayrshire cows (*Bos taurus*)** (farm of the SGC Smena — a branch of the Federal Scientific Center VNITIP RAS, Moscow Province, 2022)

Parameter	Group I ($n = 10$)		Group II ($n = 15$)		t_d	p
	M±SD	*Cv*, %	*M*±SD	*Cv*, %		
Number of somatic cells, $\times 10^3$/ml	176.6±53.4	82.4	584.9±122.7	286.9	3.05	0.0089
Proportion of lymphocytes and polymorphonuclear neutrophils in the total number of cells, %	49.8±2.8	11.3	76.8±6.4	14.8	3.87	0.0046

N o t e. The total number of samples accounted for at least 20 in group I and at least 30.in group II.

In our experiment, in the milk of healthy cows, the number of somatic cells (SCC) was 176.6×10^3/ml, which is 3.31 times less ($p < 0.01$) than in the milk of animals with mastitis. The range of phenotypic variability (*Cv*, %) indicates significantly less variability of this indicator in the milk of healthy animals. The DSCC rate in healthy animals was 49.8%, while in the milk of sick animals it was 76.8%, or 1.54 times more. The difference between the compared groups was highly significant ($p < 0.01$). Noteworthy is the fact that in the compared groups the range of phenotypic variability for DSCC was close. Therefore, this indicator is more stable than the total number of somatic cells in milk.

2. Blood and milk trypsin activity in healthy (group I) **and mastitic** (group II) **Ayrshire cows (*Bos taurus*)** (*M*±SD; farm of the SGC Smena — a branch of the Federal Scientific Center VNITIP RAS, Moscow Province, 2022)

Parameter	Group I ($n = 10$)				Group II ($n = 15$)			
	Milk (by udder lobes)							
Activity, U/l	LA	LP	RP	RA	LA	LP	RP	RA
	51.0±10.3	51.0±7.7	47,0±6,2	44,0±6,5	111,0±18,3	76,0±11,5	82,0±12,1	130,0±14,5
On average	48.2±3.8				99.6±7.3*			
UH	1.1	1.1	1,2	1,3	0,5	0,7	0,6	0,4
On average	1.20				0.55			
	Blood serum							
Activity, U/l	57.9±2.5				52.4±3.1			

N o t e. UH — udder health coefficient, LA — left anterior lobe of the udder, LP — left posterior lobe of the udder, RP — right posterior lobe of the udder, RA — right anterior lobe of the udder. Samples from each animal were
* Differences from group I are statistically significant at $p < 0.05$.

We detected trypsin activity both in blood serum and in cow's milk (Table 1). Within the sample, the activity of this enzyme turned out to be a stable indicator. To assess the health of the mammary gland, we propose the following formula: ZMZH = TK/TM, where ZMZH is the coefficient of breast health; TK — trypsin activity in the blood, units/l; TM — trypsin activity in milk, units/l. The breast index of 1.1 and above indicates normal state of the mammary gland; when the breast index is < 1.1, a deviation from the norm occurs, caused by inflammation in the mammary gland.

The results showed that there were no significant differences in trypsin activity in the blood serum of healthy and mastitic cows. However, trypsin activity in milk clearly corresponded to the health status of the mammary gland, fluctuations from the average sample value were due to milk fat. Thus, in cows with mastitis, trypsin activity in milk increased by 106.6% ($p < 0.05$) compared to values in healthy animals.

Analysis of the correlation between trypsin activity in milk and in blood serum in cows showed that in healthy animals this relationship is stable, moderate and positive ($r = 0.43$; $p < 0.05$), and in case of mammary gland pathology it became moderate negative ($r = -0.45$; $p < 0.05$). Therefore, in animal husbandry practice, to diagnose the early stage of mastitis, an udder health assessment coefficient (UH) can be proposed, consisting of two interrelated indicators. The coefficient is calculated as the ratio of trypsin activity in blood to trypsin activity in fresh animal milk according to the formula given above.

To assess the general health of the animals, we performed biochemical blood tests (Table 3).

3. Blood biochemical parameters in healthy (group I) **and mastitic** (group II) **Ayrshire cows (*Bos taurus*)** (M±SD; farm of the SGC Smena — a branch of the Federal Scientific Center VNITIP RAS, Moscow Province, 2022)

Parameter	Group I (n = 10)	Group II (n = 15)
Total protein, g/l	102±5.2	92±1.9
Albumin, g/l	52±4.2	62±3.2
Glucose, mmol/l	2.2±0.14	3.7±0.12*
Cholesterol, mmol/l	3.6±0.65	4.0±0.28
Calcium, mmol/l	2.2±0.06	2.5±0.07*
Phosphorus, mmol/l	2.1±0.18	1.6±0.13*
Alkaline phosphatase, U/l	249±12.7	180±30.9

N o t e. The total number of samples accounted for at least 20 in group I and at least 30.in group II.
* Differences from group I are statistically significant at $p < 0.05$.

In this case, the most informative indicators were the blood levels of glucose, calcium and phosphorus. The results showed that in the blood of cows with mastitis, the amount of glucose increases by 67.4% ($p < 0.05$), calcium by 38.8% ($p < 0.05$), while the phosphorus content, on the contrary, decreases by 23.8% ($p < 0.05$) compared to healthy animals.

To assess the immune status of animals, we examined blood morphological blood parameters (Table 4).

4. Hematological parameters in healthy (group I) **and mastitic** (group II) **Ayrshire cows (*Bos taurus*)** (M±SD; farm of the SGC Smena — a branch of the Federal Scientific Center VNITIP RAS, Moscow Province, 2022)

Parameter	Group I (n = 10)	Group II (n = 15)
White blood cells (WBC), $\times 10^9$/l	5.0±0.42	9.6±0.93*
Neutrophils (Neu), %	43.4±3.51	49.5±6.78
Lymphocytes (Lym), %	44.9±1.30	40.7±4.60
Monocytes (Mon), %	4.3±0.40	6.3±0.83*
Eosinophils (Eos), %	6.8±0.53	2.9±0.18*
Basophils (Bas), %	0.6±0.06	0.4±0.04*
Red blood cells (RBC), $\times 10^{12}$/l	5.5±0.09	5.4±0.12
Hemoglobin concentration (HGB), g/l	91.0±0.51	85.0±1.22*
Hematocrit (HCT), %	26.6±0.25	25.0±0.38*

N o t e. The total number of samples accounted for at least 20 in group I and at least 30.in group II.
* Differences from group I are statistically significant at $p < 0.05$.

The number of leukocytes in the blood of cows with mastitis increases by 92.0% ($p < 0.05$). To determine the level of stress, we used as a basis the Harkavi index [27], calculated as the ratio of the relative content of lymphocytes to the relative content of neutrophils. Despite the fact that the percentage of neutrophils and lymphocytes are within physiological norms, the calculated index indicates

stress in animals. In healthy cows, the indicator is 1.03, in cows with signs of mastitis 0.82. The decrease in the coefficient is associated with a decrease in the number of lymphocytes by 9.4%. Calculation of the immunoreactivity index (IIR) according to D.O. Ivanov (2014) by the formula IIR = (L + E)/M, where L are lymphocytes, E are eosinophils, M are monocytes [28] showed a 42.5% decrease in this indicator under mastitis. With inflammation of the mammary gland, we noted a decrease in the number of eosinophils (by 57.4%; $p < 0.05$), monocytes (by 31.5%; $p < 0.05$) and basophils (by 33.3%; $p < 0.05$).

Oxidation processes in cows with mastitis were less intense due to a 6.6% ($p < 0.05$) decrease in the level of hemoglobin that carries oxygen to cells. In animals with mastitis, the hematocrit decreased by 6.0% ($p < 0.05$) compared to healthy cows, which also negatively affects metabolism.

5. Relative gene expression in milk of healthy (group I) **and mastitic** (group II) **Ayrshire cows (*Bos taurus*)** (*M*±SD; farm of the SGC Smena — a branch of the Federal Scientific Center VNITIP RAS, Moscow Province, 2022)

Group	*MCP-1*	*MCP-2*	*TNF-α*	*INF-γ*	*IL2*	*IL4*	*IL8*	*Casp6*
I ($n = 3$)	1	1	1	1	–	1	1	–
II ($n = 3$)	5.48±0.68	9.17±0.67	3.85±0.51	0.72±0.33	–	2.85±0.26	14.17±1.60	–

N o t e. A milk sample was taken from each lobe of the udder, a total of 24 samples in the experiment. Dashes mean that gene expression was not detected.

When comparing the expression of the genes *MCP-1*, *MCP-2*, *TNF-α*, *INF-γ*, *IL2*, *IL4*, *IL8*, *Casp6* in healthy and mastitic cows, we found an increase in the transcriptional activity of almost all genes associated with the development of inflammation. It is known that inflammation can result from the interaction of multiple regulatory pathways or biological processes [29]. However, at present, knowledge about the expression of inflammatory and regulatory cytokines in cells present in cow's milk is insufficient. Previous studies have shown that proinflammatory cytokines are often considered as promising biomarkers of mastitis, including specific ones for determining the status and etiology of the disease [8]. In particular, it has been reported that during the early stages of mastitis, the level of inflammatory cytokines increases faster than the total number of somatic cells in milk [9]. However, the use of the number of somatic cells in milk as a selection trait to increase resistance to mastitis in cattle has given limited results [30], therefore, information on molecular markers of susceptibility/resistance to mastitis is considered promising for identifying cattle genetically resistant to mastitis [31-33].

The etiology of mastitis, in addition to mechanical damage during milking, is often associated with various infections that affect the host body in the early stages of the disease, causing, in particular, the secretion of cytokines. Moreover, the production of different cytokines in response to different infections is not the same, which can serve as a differentiating factor in the etiology of mastitis [34-36]. Thus, the development of mastitis is most often associated with bacterial infections, but viruses, microscopic algae and fungi can also act as pathogens [37-40]. In particular, algae of the genus *Prototheca* have been reported as the third most common causative agent of mastitis after members of the genera *Streptococcus* and *Staphylococcus* [41].

Internal mammary ductal epithelial cells play a key role in recognizing mastitis-causing pathogens through toll-like receptors (TLR2 and TLR4) [42, 43]. TLRs influence the transcription factor NF-κB which controls the expression of immune response, apoptosis, and cell cycle genes, particularly tumor necrosis factor-α (TNF-α) genes and the interleukins IL1β, IL6, and IL8 [44-46]. It is well known that serum cytokines such as interferon, tumor necrosis factor α, IL17, IL6, and IL4 play a key role in inflammatory processes, suggesting their possible

involvement in the pathological process of mastitis in cattle [47, 48].

Cytokines are important for intercellular communication. Known processes that are stimulated or inhibited by cytokines include cell differentiation, proliferation, remodeling, degeneration, regeneration, and even cell death. It has been reported that in mastitis, along with an increase in the number of somatic cells, the level of secreted cytokines (interleukins IL1, IL2, IL4, IL5, IL6, IL8, IL10 and IL12) in milk increases [49].

In our study (see Table 5), in cows with mastitis, there was an increase in the expression of monocyte chemotactic protein 1 and monocyte chemotactic protein 2. These are cytokines belonging to the group of CC-chemokines. In sick cows, the expression of *MCP-1* increased 5.5-fold, *MCP-2* 9-fold. Monocytes play a leading role in inflammation. The accumulation of monocytes is due to their adhesion and migration under the influence of chemoattractants, in particular the recently described chemotactic cytokines (chemokines) [50, 51]. The monocyte-specific chemokine MCP-1 (monocyte chemoattractant protein 1) is synthesized by activated monocytes/macrophages and vascular wall cells and, by binding to its receptor CCR2, regulates the adhesion and migration of monocytes on matrix proteins and endothelium. MCP-2 has unique functional properties compared to other chemokines, including MCP-1. Other research teams have shown in in vitro models of mastitis that bovine mammary epithelial cells can express the chemokines CXCL6 (also called GCP2) and CCL8 (also called MCP2) in response to certain bacterial cell components [52, 53]. In general, the expression of monocyte chemotactic protein 1 and monocyte chemotactic protein 2 in mastitis has been little studied, so our data are of significant interest in connection with the issues under discussion.

The expression of tumor necrosis factor α in cows with mastitis increased by 3.9 times compared to healthy ones (see Table 5). TNF-α is a proinflammatory cytokine produced primarily by macrophages. Depending on the location of its release and the receptor with which it binds, TNF-α can perform various functions, e.g., to stimulate synthesis of other cytokines and cause inflammatory reactions, to control vital processes in the cell and maintain tissue homeostasis [54, 55]. In this regard, in combination with other cytokines, TNF-α plays an important clinical role in cattle, mediating immune inflammatory reactions (mastitis, endotoxic shock, endometritis). Cytokines, particularly TNF-α, participate in the development of metabolic diseases, e.g., acidosis [56]. TNF-α is involved in inflammation [57] and regulates a number of physiological functions, including appetite, fever, energy metabolism, and endocrine activity [58]. Various agents such as viruses, parasites, bacteria, and endotoxins induce TNF-α production [59].

We also found that the expression level of interleukins 4 and 8 in cows with mastitis increased by 2.9 times and 14 times, respectively (see Table 5). Interleukins are polypeptides produced by cells involved in immune and inflammatory responses [60].

The main producers of IL4 in the mammary glands of cattle are T- and B-lymphocytes, eosinophils and basophils, mast cells, plasma cells [60, 61], as well as epithelial cells, which together form the basis of the type II immune response [62]. IL4 has been reported to regulate innate immunity and have an inhibitory effect on IFN-β in dairy cows [60]. In our study, along with increased IL4 expression, we noted a trend towards increased expression of IFN-β, a cytokine secreted by various cells involved in innate and adaptive immune responses [63], as well as by antigen-presenting cells involved in the elimination of pathogens [64, 65]. Interestingly, in other studies, the IL4 content in milk during mastitis, on the contrary, decreased (66), therefore, this issue requires further study.

Interleukin 8 (IL8) [67] is an inflammatory cytokine produced by a variety of cell types, e.g., lymphocytes, neutrophils, monocytes, macrophages, and epithelial cells [68], including bovine mammary epithelial cells [69]. At the site of inflammation, IL8 is involved in the recruitment and activation of neutrophils [70]. During the acute phase of coliform mastitis, the concentration of IL8 in cow's milk increases significantly [71]. The chemotactic activity of IL8 was detected in the mammary gland secretions of cows with mastitis during intramammary infection with *Staphylococcus aureus*, but not in healthy cows [72]. Thereof, interleukin 8 is believed to provide the infiltration of neutrophils into mammary gland secretions during mastitis. Interleukin 8 also alters milk protein composition by inhibiting milk-specific protein secretion and the influx of whey proteins [72]. Overall, IL8 is considered a potent mediator of inflammation and is also involved in the recruitment of leukocytes to sites of infection [73]. Polymorphisms of the *IL8* gene under mastitis are being studied [74, 75]. It was reported that one of the polymorphisms, the +472 A>G in *IL8* is associated with a high SSC in the milk of cows infected with *S. aureus* [76].

Since blood trypsin activity is related to the content of nitric oxide metabolites [77], it is possible that trypsin is involved in the inflammatory response in breast tissue. Moreover, there is a method for diagnosing mastitis by the content of nitrite (NO^{2-}) and nitrosothiols (RSNO) in milk [4]. The method for detecting these compounds in biological objects is widely described in special literature [78]. Evidences suggest that trypsin is not only a pancreatic enzyme, but also a signaling molecule involved in metabolism and maintaining the health of organs and tissues.

For a long time it was believed that trypsin is synthesized only in the pancreas. Results from a study of human and mouse nonpancreatic tissue samples showed [79] that the trypsin gene is expressed at high levels in the pancreas, spleen, and significantly in the small intestine. In situ hybridization and immunohistochemical analysis revealed trypsin expression in epithelial cells of skin, esophagus, stomach, small intestine, lung, kidney, liver, extrahepatic bile ducts, in spleen cells and neurons. In the spleen, trypsin is found in macrophages, monocytes and lymphocytes in the white pulp, in the brain — in nerve cells of the hippocampus and cortex [79]. Such a wide distribution in the body suggests a general role of trypsin in maintaining normal functions of epithelial cells, the immune defense system, and the central nervous system [79].

Receptors (PAR2) activated by trypsin have also been discovered [80]. Trypsin is the main PAR2 activating protease that initiates inflammatory signaling [81]. The PAR2 receptor is located on apical and basolateral membranes of intestinal epithelial cells [82, 83] and can be stimulated by trypsin, tryptase, and bacterial proteases [84]. PAR2 is also present in the membrane of immune cells, stromal and endothelial cells. Systemically, PAR2 stimulation promotes blood coagulation, adhesion, and leukocyte extravasation [85]. Thus, it can be assumed that the increase in trypsin activity in the milk and blood of cows with mastitis is associated with activation of PAR2 receptors in mammary epithelial cells and blood leukocytes.

In this work, we for the first time identified trypsin activity in the milk of healthy cows and noted its significant increase in mastitis, which indicates a connection between trypsin and the inflammation in the mammary gland. Previously, we studied the role of trypsin in experimental toxicosis of chickens [19] and under changes in diets [20]. Scholar publications note the multiplicity of trypsin localization [80] and functions [84, 85], including signaling [17]. Therefore, trypsin activity may serve as a marker of homeostasis disorders. The connection between trypsin activity in milk, on the one hand, and mastitis, including its physiological and

biochemical signs, on the other hand, has not been previously studied.

We additionally performed biochemical and morphological blood analyzes and measured SCC, DSCC and the expression level of the main immune genes in milk. It turned out that in cows with mastitis, along with a 2-fold increase in trypsin activity, SCC, DSCC and the expression of the *MCP-1*, *MCP-2*, *TNF-α*, *IL4* and *IL8* genes increased manifold. The revealed multiple increases in trypsin activity and gene expression give reason to believe that by combining these indicators, it is possible to develop a test with sensitivity sufficient for early diagnosis of subclinical forms of mastitis and pre-mastitis state. Comparison of these indicators over time, starting with pre-mastitis period, and with mastitis of different etiologies will provide new knowledge about the mechanisms of development and course of this pathology. At the next stage of research, we also plan to determine the expression of the trypsin gene in healthy and mastitic cows.

Thus, the results of our research draw us the following conclusions. In healthy cows, trypsin is discovered in milk for the first time. In the milk, its activity amounted to 48.2±3.8 units/l, being comparable to that in blood serum. During inflammation of the mammary gland, somatic cell count (SCC) in milk increases 3.3-fold ($p < 0.01$) vs. healthy animals, and trypsin activity increases 2.0-fold. In the blood of mastitic cows, the glucose content is 67.4% higher ($p < 0.05$), total calcium 38.8% higher ($p < 0.05$), while the phosphorus, on the contrary, 23.8 % lower ($p < 0.05$) than in healthy animals. Therefore, for these blood biochemical parameters, the body's response to the pathology is significantly lower than for SCC and trypsin in milk. In mastitic cows, the ratio of lymphocytes and neutrophils decreases by 20.4%, immunoreactivity by 42.5%, the number of eosinophils by 57.4%, basophils by 33.3%; the number of monocytes increases by 46.5%. This is also much lower than changes in SCC and trypsin activity in milk under mastitis. Compared to healthy animals, in cows with mastitis, the expression of genes associated with inflammation was much higher, in particular 5.5-fold for monocyte chemotactic protein 1, 9-fold for monocyte chemotactic protein 2, 3.9-fold for tumor necrosis factor, 2.9-fold for interleukin 4 and 14-fold for interleukin 8. The large differences identified in trypsin activity in milk and the expression of genes associated with inflammation in norm and in mastitis create prospects for the development of a diagnostic test with sensitivity sufficient for the early detection of subclinical forms of mastitis and pre-mastitis state.

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EFFECTS OF DIETARY FIBER ON MINERAL METABOLISM AND CAECAL MICROBIAL DIVERSITY IN BROILER CHICKENS (*Gallus gallus* L.) FED A SEMI-SYNTHETIC DIET

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Abstract

Various additives used in poultry diets can change the mineral status of the body. Dietary fiber has long been considered an anti-nutritional factor due to adverse effects on feed intake and nutrient absorption. However, with increasing evidence, it has been found that dietary fiber has a positive effect on nutrient digestion, fermentation, and absorption processes in poultry. In this work, for the first time, data were obtained on the influence of dietary fibers, the microcrystalline cellulose, lactulose and chitosan on mineral metabolism and caecal microbiocenosis of broiler chickens fed a semi-synthetic diet. A decrease in the accuulation of toxic microelements in the body of a bird was demonstrated, as well as a change in the microbial community of the caecum. Experiments on the Arbor Acres cross broiler chickens (*Gallus gallus* L.) were carried out in the vivarium (the FSC BSA RAS). A total of 150 of week-old broiler chickens were divided into 5 groups of analogues ($n = 30$ each). The duration of the experiment was 35 days. The first control group C_1 was fed with a semi-synthetic diet (SS). The second control group C_2 received a semi-synthetic diet deficient in trace elements (DSS). For dietary fibers, test group I was fed with dietary microcrystalline cellulose (E460, 0.25 g/kg feed), test group II with dietary lactulose (1 g/kg feed), and test group III with dietary chitosan (0.5 g/kg feed). In feed and biomaterial of broilers, 25 chemical elements were assayed: Ca, Cu, Fe, Li, Mg, Mn, Ni, As, Cr, K, Na, P, Zn, I, V, Co, Se, Ti, Al, Be, Cd, Pb, Hg, Sn, Sr by atomic emission spectrometry and mass spectrometry techniques. Microbial biodiversity of the caecum was assessed on day 42. NGS sequencing was performed using a MiSeq platform (Illumina, Inc., USA). In test group I, the dietary fiber led to a statistically significant increase in the calcium (by 23,4 %. $p \le 0.05$) vs. C_2. In test group III, there was a 1.5-forl decrease in the indicator ($p \le 0.05$) vs. C_1 and a 26.3 % decrease ($p \le 0.05$) vs. C_2. The lithium content increased 1.7 times ($p \le 0.05$) vs. C_1 when chitosan was added to a semi-synthetic diet deficient in trace elements. The concentration of manganese and cobalt significantly ($p \le 0.05$) decreased in all test groups vs. C_1. In group I, the amount of selenium increased 2.35 times ($p \le 0.05$) vs. C_1 it decreased 1.74 times ($p \le 0.05$) vs, C_2. In the same group, the iodine level increased 1.74 times and 1.5 times ($p \le 0.05$) vs. control groups. In test groups II and III, selenium decrease 4.64 times and 4.55 times ($p \le 0.05$) vs. C_2. The concentration of arsenic in group II exceeded C_1 1.63 times ($p \le 0.05$), and in group III, its concentration, on the contrary, decreased 1.58 times and 2.0 times ($p \le 0, 05$) vs. C_1 and C_2. The dietary fiber scontributed to the removal of toxic elements. In test group I and group III, the concetration of strontium decreased ($p \le 0.05$) by 25.7 and 45.9 %, respectively, vs. C_1. For C_2, a decrease in the amount of strontium by 22.2 and 43.4 % was similarly revealed ($p \le 0.05$). In group I, the counts of *Rikenellaceae* increased 6.3 and 6.8 times, *Lachnospiraceae* 12 and 4.9 times, *Ruminococcaceae* 2.1 times and 3. 9 times compared to C_1 and C_2, respectively. In group II, the abundance of *Lactobicallaceae* decreased 6 times, the number of *Rikenellaceae* increased 6.2 times, *Lachnospiraceae* 9.57 times, *Ruminococcaceae* 3.1 times compared to C_1. In group III, there was a decrease in the content of *Lactobicallaceae* by 13.3 and 1.55 times compared to C_1 and C_2. The number of *Rikenellaceae* increased 5.5 times, *Lachnospiraceae* 11.8 times, *Ruminococcaceae* 3.5 times compared to C^1. Thus, dietary fibers added to a semi-synthetic diet led to a decrease in the content of macroelements in the body of Arbor Aikres cross

broiler chickens, the elimination of toxic elements, and increased the counts of *Rikenellaceae* and *Lachnospiraceae* taxa with a simultaneous decrease in the number of *Lactobacillaceae* in the intestine.

Keywords: semi-synthetic diet, dietary fiber, metabolism, mineral metabolism, microbiome, caecum

Over the past few decades, poultry feeding concepts have undergone significant changes, driven by the transition from domestic to industrial feed production [1]. This became possible due to assessment of nutritional needs and the metabolic role of nutrients in birds.

Since the 1950s, numerous experiments have been conducted to determine the protein and essential amino acid requirements of poultry [2, 3] and the ideal protein ratio in diets [4]. With the five synthesized essential amino acids now available, it is possible to formulate a balanced semi-synthetic diet in which the crude protein content is provided by the most limiting amino acid sourced from feed proteins [5]. In addition, a semi-synthetic diet may ensure balanced feeding by compensating for deficiencies in certain nutrients [6]. The use of a semi-synthetic diet will allow a more complete assessment of the effects of feed additives or other dietary components on the mineral metabolism and microbial diversity of the bird's gut, and will facilitate the study of genetic and environmental variations in the population.

Compiling semi-synthetic diets must consider the microelement status of the body that depends on the exogenous intake of microelements from feed during normalization of the intestinal chyme composition [7]. As a result, and due to the body's desire for a constant internal environment, absorption processes alter which leads either to normalization of the content of certain elements, or to microelement deficiency [8]. In turn, the intensity of absorption depends on many factors, particularly on normal functioning of the intestinal microbiota which can modify the bioavailability of microelements through their accumulation in microbial cells and changes in intestinal pH [9].

Various dietary additives used in poultry can alter the mineral status of the body. For example, dietary fiber has long been considered antinutritional due to its adverse effects on feed intake and nutrient digestibility. However, scresearchers later discovered that dietary fiber has a positive effect on the digestion, fermentation, and absorption [10]. Moderate amounts of fiber in diets also modify growth performance and improve gut health by modulating beneficial microbiota in the colon and enhancing immune function [11].

This paper is the first to reveal a decrease in the content of toxic microelements and a change in the microbial community of the cecum of broiler chickens fed a semi-synthetic diet supplemented with microcrystalline cellulose, lactulose and chitosan.

Our goal was to study the effect of dietary fiber on mineral metabolism and microbiocenosis of the cecum in broiler chickens fed a semi-synthetic diet.

Materials and methods. For experiment, 150 Arbor Acres cross broiler chickens (*Gallus gallus* L.) of 1-week age were divided into 5 groups, $n = 30$ each (the vivarium of the Federal Scientific Center BST RAS, https://цкп-бст.рф/).

During the experiment, all birds were kept under the same conditions and all manupulations were in accordance with the instructions and recommendations of Russian Regulations, 1987 (Order No. 755 on 08/12/1977 the USSR Ministry of Health) and The Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, D.C., 1996). All efforts were made to minimize bird suffering and reduce the number of samples used (Protocol No. 1 of 05/21/2021).

Of 35-day experiment, the preparatory and test periods were 7 and 28 days, respectively. During the test period, the first control group (C_1) was fed a semi-synthetic diet (SS), the second control group (C_2) was fed a micronutrient-

deficient diet (SSD), in treatments, the birds were fed SDD added with 0.25 g microcrystalline cellulose (E460) per 1 kg feed (group I), 1 g/kg lactulose (group II), and 0.5 g/kg food-grade chitosan (group III). The chickens can drink distilled water without restriction. A semi-synthetic diet (C1) was as recommended by M.L. Scott et al. [12] and a semi-synthetic diet deficient in microelements (C2) was modified by us. Feed samples were prepared by stepwise mixing.

The bird was decapitated under nembutal ether on day 42. Carcasses were ground whole, and bulk samples were subjected to analysis for 25 chemical elements: Ca, Cu, Fe, Li, Mg, Mn, Ni, As, Cr, K, Na, P, Zn, I, V, Co, Se, Ti, Al, Be, Cd, Pb, Hg, Sn, Sr using atomic emission and mass spectral methods. The biomaterial was ashed (a microwave decomposition system MD-2000, PerkinElmer, Inc., USA) and the content of elements in the ash was measured (an Elan 9000 mass spectrometer and an Optima 2000 V atomic emission spectrometer, PerkinElmer, Inc., USA).

The microbial biodiversity of the bird's cecum was assessed on day 42 at the Institute of Cellular and Intracellular Symbiosis, Ural Branch of the Russian Academy of Sciences, Orenburg (https://ikvs.info/tskp/). For DNA extraction, samples were incubated at 37 °C for 30 min in 300 μl of sterile lysis buffer (20 mM EDTA, 1400 mM NaCl, 100 mM Tris-HCl, pH 7.5; lysozyme solution of 100 mg/ml concentration, 50 μl). The purity of the DNA preparations was assessed by electrophoresis in a 1.5% agarose gel with photometry (NanoDrop 8000, Thermo Fisher Scientific, Inc., USA). DNA concentration was measured fluorometrically (a Qubit 2.0 device with high sensitivity for dsDNA determination, Life Technologies, USA).

DNA libraries for sequencing were created using the Illumina protocol (Illumina, Inc., USA) with primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21 to the variable region V3-V4 of the 16S rRNA gene [24]. NGS sequencing was performed using a MiSeq platform (Illumina, Inc., USA) with the MiSeq Reagent Kit V3 PE600 (Illumina, Inc., USA) at the Center for Shared Use of Scientific Equipment "Persistence of Microorganisms" (Institute of Cellular and Intracellular symbiosis Ural Branch RAS, Orenburg). The resulting operational taxonomic units (OTUs) were classified with a VAMPS online tool and the RDP database (http://rdp.cme.msu.edu). Some OTUs were aligned using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the databases for nucleotide sequence nr/nt (NCBI, https://www.ncbi.nlm.nih.gov/) and aligned ribosomal RNA gene sequences SILVA (https://www.arb-silva.de).

Statistical processing was carried out using the Statistica 10.0 program (StatSoft, Inc., USA). Results are submitted as arithmetic means (M) and standard errors of the mean (±SEM). Differences were considered statistically significant at $p \leq 0.05$ (Student's t-test). The USEARCH v8.0.1623_win32 software package (https://www.drive5.com/usearch/download.html) was used for bioinformatic processing of sequencing data. Processing included merging of paired reads in operational taxonomic units, filtering of reads by quality and length (minimum size of 300 bp), removal of chimeras, doubletons and singletons, clustering of reads in OTUs at a similarity level 97% [26].

Results. Table 1 shows the composition of the diets for the broilers.

1. Composition (g/100 g of feed) **of a semi-synthetic diet** (SS) **and a semi-synthetic microelement deficient diet** (SSD) **of Arbor Acres cross broiler chickens (*Gallus gallus* L.)** (vivarium of the Federal Scientific Center for Biological Systems and Agrotechnologies RAS)

Ingredient	SS	SSD
Casein	20	20
Gelatin	5	5
Cellulose	3	3

Continued Table 1

Vegetable oil	3	3
Choline chloride	0.2	0,2
Glucose	1.25	1,25
Rice	61.38	61,38
Methionine	0.1	0,1
Cystine	0.2	0,2
$CaHPO_4 \cdot H_2O$	1.8	1,8
$CaCO_3$	1.45	1,45
KH_2PO_4	1.013	1,013
KCl	0.21	0,21
Na_2CO_3	0.555	0,555
$MnCl \cdot 4H_2O$	0.04	–
$FeSO_4 \cdot 7H_2O$	0.05	–
$MgSO_4 \cdot 7H_2O$	0.615	0,615
KJ	0.001	0,001
$CuSO_4 \cdot 5H_2O$	0.001	–
$ZnCl_2$	0.016	–
$CoCl_2$	0.0002	–
$NaMoO_4 \cdot 2H_2O$	0.0008	–
Na_2SeO_3	0.000015	–
Vitamin mixture	0.052	0,052

N o t e. Composition of the vitamin mixture (mg/100 g of feed): B_1 — 2.5, B_2 — 1.5, B_6 — 0.6, B_{12} — 0.002, Ca-pantothenate — 2.0, biotin — 0.06, folic acid — 0.4, K_3 — 0.5, C — 25.0, PP — 15.0, A — 1000 IU, D_3 - 360 IU, E — 0.5 IU. Dashes mean that the ingredient was not added.

When identifying chemicals in biological substrates, it was first necessary to study their accumulation in the body of chickens in order to draw conclusions about the accumulation of macro- and microelements.

The dietary fiber in the broilers' diet led to a statistically significant increase in calcium content in experimental group I by 23.4% ($p \le 0.05$) vs. C_2. In group III, on the contrary, we noted its decrease by 1.5 times ($p \le 0.05$) compared to C_1 and by 26.3% ($p \le 0.05$) vs. C_2. In general, there was a tendency towards a decrease in the content of all macroelements in the group that additionally received chitosan, including a statistically significant decrease in the amount of phosphorus by 17.5% ($p \le 0.05$) vs. C_1 (Table 2).

2. Content (g/bird) **of macroelements, essential, conditionally essential microelements and toxic elements in the body of Arbor Acres cross broiler chickens *(Gallus gallus* L.) fed a semi-synthetic diet added with various dietary fibers** (n = 30, M±SEM; vivarium of the Federal Scientific Center for Biological Systems and Agricultural Technologies RAS)

Element	Group				
	C_1	C_2	I test	II test	III test
			Macronutrients		
Na	12.9±1.03	12.7±1.01	13.4±1.21	13.3±1.23	11.7±1.07
P	63.3±3.12	60.6±2.86	69.6±3.21	68.5±3.68	52.2±2.69[a]
K	35.2±1.89	35.8±2.11	35.3±1.58	35.3±2.11	32.8±3.11
Ca	91.9±5.42	80.7±2.31	99.6±3.14[b]	89.3±2.89	59.5±3.11[ab]
Mg	4.1±0.65	3.9±0.72	4.1±0.81	4.3±0.78	3.6±0.98
			Microelements		
Li	0.2±0.02	0.2±0.01	0.2±0.03	0.1±0.02	0.3±0.01[a]
B	0.9±0.03	1.3±0.04	1.1±0.02	0.9±0.01	0.4±0.03[ab]
Si	488.3±23.12	458.9±31.83	454.1±34.17	545.8±24.61	398.6±31.64
V	0.7±0.03	0.7±0.04	0.7±0.02	0.9±0.02	0.5±0.03
Cr	4.4±1.11	4.4±1.09	4.6±1.23	6.0±2.11	5.3±3.12
Mn	16.9±1.32	8.8±2.11	9.1±2.36[a]	11.2±3.12[a]	9.5±4.17[a]
Fe	916.6±30.10	883.4±29.86	960.5±35.44	956.2±41.2	852.6±34.97
Co	4.1±1.45	0.4±0.02	0.5±0.03[a]	0.3±0.03[a]	0.2±0.04[a]
Ni	6.1±1.21	5.6±1.45	5.2±1.24	4.5±2.11	5.1±2.45
Cu	24.6±2.36	21.5±2.45	20.5±3.11	29.2±2.58	17.8±3.12
Zn	383.4±25.64	339.9±18.95	376.7±21.37	377.5±31.20	283.6±29.34
As	0.19±0.00	0.24±0.001	0.20±0.001	0.31±0.002[a]	0.12±0.001[ab]
Se	4.7±1.24	19.5±2.36	11.2±3.12[ab]	4.2±2.86[b]	4.3±3.14[b]
I	3.3±1.32	3.9±1.45	5.8±2.11[ab]	4.7±3.11	3.7±2.87
			Toxic elements		
Sr	29.2±1.31	27.9±1.87	21.7±2.23[ab]	23.9±3.11	15.8±2.89[ab]
Cd	0.12±0.001	0.14±0.001	0.13±0.001	0.13±0.001	0.12±0.001

Continued Table 2

Sn	0.03±0.001	0.12±0.001	0.03±0.001[b]	0.03±0.001[b]	0.03±0.001[b]
Hg	0.03±0.001	0.03±0.001	0.03±0.001	0.03±0.001	0.03±0.001
Pb	1.2±0.63	0.6±0.03	0.6±0.02[a]	0.6±0.03[a]	0.6±0.04[a]
Al	1.6±0.74	1.1±0.68	0.5±0.02[ab]	0.7±0.01[ab]	0.7±0.03[ab]

N o t e. For a description of the groups, see the Materials and methods section.
[a, b] Differences from C1 and V2 are statistically significant at $p \leq 0.05$.

Adding chitosan into the SSD contributed to an increase in the amount of lithium by 1.7 times ($p \leq 0.05$) vs. C1. The boron content in test group III decreased by 2.19 times ($p \leq 0.05$) and 3.15 times ($p \leq 0.05$), respectively, compared to the two control groups (see Table 2). The manganese content decreased in all experimental groups ($p \leq 0.05$) vs. C1, in group I 1.86-fold, in roup II 1.50-fold, and in group III by 1.77-fold. A similar trend was for cobalt in the test groups, its amount statistically significantly decreased 7.98 times, 12.70 times and 16.90 times, respectively, compared to C1.

In group I, we recorded an increase in the amount of selenium ($p \leq 0.05$) by 2.35 times compared to C1 and a decrease ($p \leq 0.05$) by 1.74 times compared to C2. In the same group, a significant ($p \leq 0.05$) increase in iodine content was revealed (1.74 times and 1.50 times vs. both control groups). In groups II and III, the amount of selenium decreased by 4.64 and 4.55 times ($p \leq 0.05$) compared to C2. In the As accumulation group II exceeded C1 by 1.63 times ($p \leq 0.05$), and in group III, on the contrary, it is 1.58 times and 2.00 times ($p \leq 0.05$) less vs. C1 and C2.

Absorption, distribution and toxicity of heavy metal compounds depend both on the biological features of the digestive organs and the physicochemical properties of the absorbed substances, their interaction with feed components and on the presence of various additives in feed. As is known, the protein content in the diet affects the absorption of toxic elements in the body. In our case, the addition of dietary fiber contributed to the active elimination of toxic elements (see Table 2). The strontium content in groups I and III decreased by 25.7 and 45.9%, respectively ($p \leq 0.05$) vs. C1. Compared to C2, the parameter decreased by 22.2 and 43.4% ($p \leq 0.05$). The content of tin in birds from the test groups was 4.0 times less ($p \leq 0.05$) vs. C2, of lead 2.0 times less ($p \leq 0.05$) compared to C1. Data for aluminum were similar. A statistically significant ($p \leq 0.05$) decrease in its content was noted in all experimental groups, e.g., in group I by 3.19 and 2.22 times ($p \leq 0.05$) compared to C1 and C2, respectively, in group II by 2.51 and 1.74 times ($p \leq 0.05$), in III by 2.23 and 1.55 times ($p \leq 0.05$).

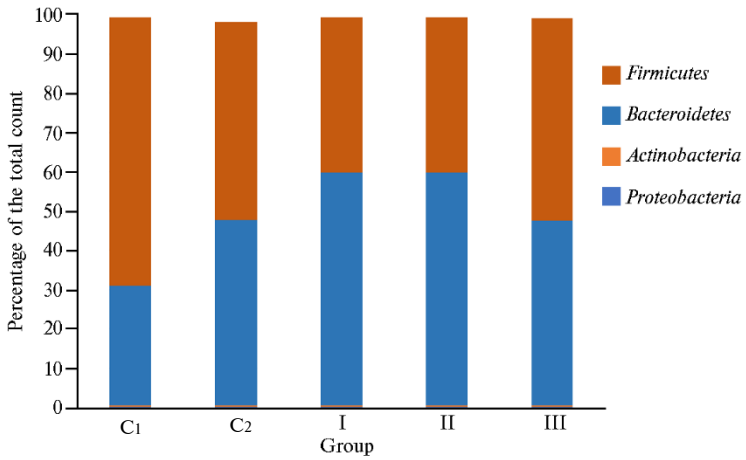

Fig. 1. Microbial profile of the cecum in the Arbor Acres cross broiler chickens (*Gallus gallus* L.) of fed a semi-synthetic diet added with various dietary fibers (n = 30; vivarium of the Federal Scientific Center for Biological Systems and Agricultural Technologies RAS). For a description of the groups, see the Materials and methods section.

In the microbial profile of the cecum contents in groups C_1 and III, we revealed the dominance of the phylum *Firmicutes*, while in groups I and II, the *Firmicutes* abundance was 39.7 and 39.8%, respectively. The number of *Bacteroidetes* in C_1 was 30.5%, or 16.8, 28.9, 28.9 and 16.6% less than in C_2, I, II and III groups, respectively. The abundance of other taxa did not exceed 3% (Fig. 1).

At a lower taxonomic level, C_1 group was dominated by the family *Lactobicallaceae* (61.6%); *Bacterodaceae* (26.2%), *Ruminococcaceae* (4.5%) and *Rikenellaceae* (4.2%) were also represented. In C_2 group, the bacteria of the families *Rikenellaceae* (26.5%), *Lactobicallaceae* (22.7%), *Bacterodaceae* (20.6%) and *Lachnospiraceae* (16.8%) had the greatest abundance. In group C_2 compared to group C_1, the number of *Lactobicallaceae* decreased 2.71 times, while the *Rikenellaceae* increased 6.30 times, *Lachnospiraceae* 12 times, *Ruminococcaceae* 2.10 times, and *Enterobacteriaceae* 5.60 times.

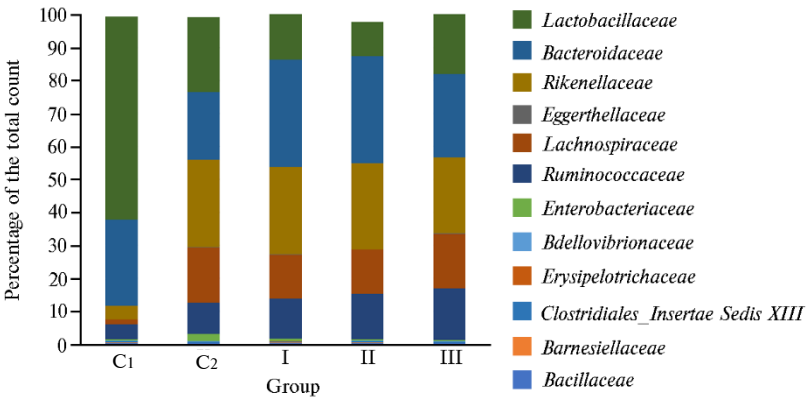

Fig. 2. Bacterial families found in the cecum of Arbor Acres cross broiler chickens (*Gallus gallus* L.) fed a semi-synthetic diet added with various dietary fibers (n = 30, vivarium of the Federal Scientific Center for Biological Systems and Agricultural Technologies RAS). For a description of the groups, see the Materials and methods section.

In group I, the number of *Lactobicallaceae* decreased 4.5 times, *Bacterodaceae* increased by 3.8 and 9.4%, *Rikenellaceae* 6.3 and 6.8 times, *Lachnospiraceae* 12 and 4.9 times, *Ruminococcaceae* 2.1 and 3.9 times compared to C_1 and C_2. In group II, when lactulose was added to the diet, the abundance of *Lactobicallaceae* decreased 6 times, and the number of representatives of *Bacterodaceae* increased by 6.5 and 12.1% compared to C_1 and C_2. The number of *Rikenellaceae* increased 6.20 times, *Lachnospiraceae* 9.57 times, and *Ruminococcaceae* 3.10 times vs. C_1. In group III, when chitosan was added, a decrease in the abundance of *Lactobicallaceae* was 13.30-fold and 1.55-fold, respectively, compared to C_1 and C_2. The abundance of *Rikenellaceae* increased 5.5-fold, *Lachnospiraceae* 11.8-fold, and *Ruminococcaceae* 3.5-fold vs. C_1 (Fig. 2).

The NGS sequencing showed that at genera level, in the cecum contents of the broilers from the group receiving SS diet, on day 42 the majority were represented by *Lactobacillus* (59.8%), *Bacteroidetes* (25.9%), *Alistipes* (4.2%) (Fig. 3, A). In group C_2, fed SSD deficient in minerals, representatives of the genus *Alistipes* dominated (26.5%, or 22.3% higher than for C_1). The number of *Lactobacillus* was lower by 39.5%, *Bacteroidetes* by 8.5% vs. C_1. Unclassified representatives of the microbial community accounted for 11.2%. Also genera *Mediterraneibacter* (7%), *Merdimonas* (5.6%), *Limasilactobacillus* (2.1%), and *Intestinomonas* (1.9%) were present in group C_2 (see Fig. 3, B).

In group I, according to the metagenomic sequencing data, representatives of the genus *Alistipes* dominated in the cecum contents (28.9%, or 24.7 and 2.4% highercompared to C_1 and C_2, respectively). Bacteria of the genus *Bacteroidetes*

accounted for 27.5%, which is 10.1% higher than in C_2, unclassified microorganisms accounted for 9.4%. The proportion of *Lactobacillus* was 47.6 and 8.1% lower than in groups C_1 and C_2, respectively. The number of bacteria of the genus Intestinomonas (10%) turned out to be 9.8 and 8.1% higher than in C_1 and C_2 (see Fig. 3, C).

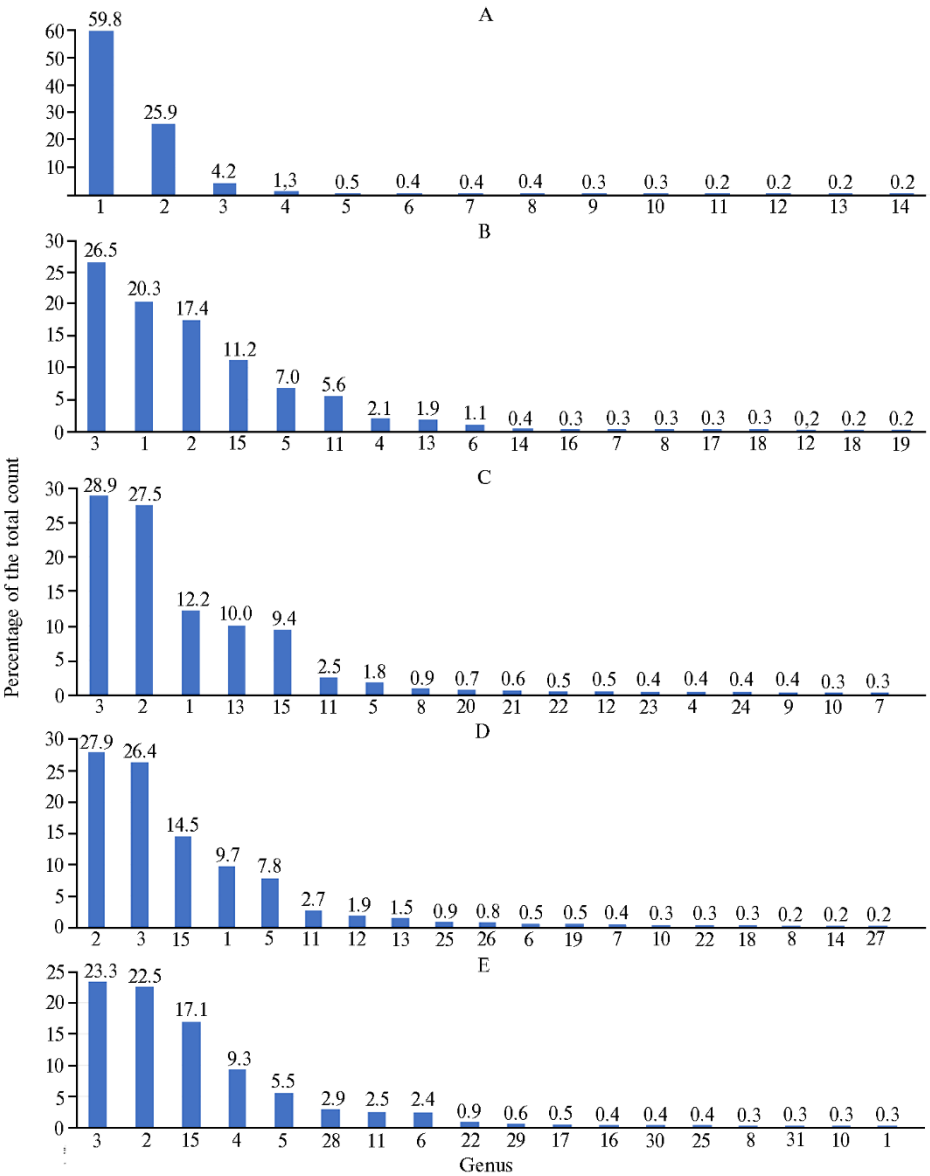

Fig. 3. Cecum microbiome genera composition in Arbor Acres cross broiler chickens (*Gallus gallus* L.) fed a semi-synthetic diet added with the of various dietary fibers: 1 — *Lactobacillus*, 2 — *Bacteroides*, 3 — *Alistipes*, 4 — *Limosilactobacillus*, 5 — *Mediterraneibacter*, 6 — *Faecalibacterium*, 7 — *Pseudoflavonifractor*, 8 — *Ligilactobacillus*, 9 — *Enterobacter*, 10 — *Rubneribacter*, 11 — *Merdimonas*, 12 — *Subdoligranulum*, 13 — *Intestinimonas*, 14 — *Neglecta*, 15 — unclassified, 16 — *Frisingicoccus*, 17 — *Eisenbergiella*, 18 — *Lachnospiraceae incertae sedis*, 19 — *Monoglobus*, 20 — *Fournierella*, 21 — *Ruthenibacterium*, 22 — *Coprobacter*, 23 — *Dysosmobacter*, 24 — *Catabacter*, 25 — *Anaerotignum*, 26 — *Clostridium XVIII*, 27 — *Anaeromasillibacillus*, 28 — *Weisella*, 29 — *Blautia*, 30 — *Bacillus*, 31 — *Ihubacter*; A — first control group (K_1), B — second control group (K_2), C — I test group, D — II test group, E — III test group ($n = 30$, vivarium of the Federal Scientific Center for Biological Systems and Agricultural Technologies RAS). For a description of the groups, see the Materials and methods section.

In group II, the most numerous taxa were *Bacteroidetes* and *Alistipes*. The number of *Bacteroidetes* was 10.5% higher than in C_2. The proportion of *Alistipes* bacteria was 22.2% higher compared to C_1. Unclassified microorganisms accounted for 14.5% of the total number. The abundance of *Lactobacillus* was 50.1 and 10.6% lower than in groups C_1 and C_2, respectively, of *Mediterraneibacter* was 7.3% higher than in C_1. Bacteria of the genus *Merdimonas* accounted for 2.7%, *Subdoligranulum* for 1.9%, *Intestinmonas* for 1.5%. Bacteria of other genera accounted for no more than 1% of the total number (see Fig. 3, D).

In group III, the most numerous bacteria were the genus *Alistipes* (23.3%, or 19.1% higher than in group C_1) and *Bacteroidetes* (22.5%, or 5.1% higher than in C_2). In this group, we revealed 17.1% of unidentified representatives of the bacterial community. The proportion of *Limosilactobacillus* bacteria was 9.3%, which is 8.0 and 7.2% higher compared to groups C_1 and C_2. In this group additionally fed chitosan, representatives of the genera *Weisella* (2.9%), *Merdimonas* (2.5%), *Faecalibacterium* (2.4%) were identified. Bacteria of other genera accounted for less than 1% (see Fig. 3, E).

Overall, we showed that the addition of dietary fiber led to a decrease in the accumulation of heavy metals in broiler chicken tissues, which may also be due to the excretion from the body with dietary fiber and a decrease in absorption in the intestine. This observation makes it promising to develop dietary fiber-based supplements for improving the health of farm animals under constantly increasing anthropogenic load on the environment.

Fiber is known to reduce mineral absorption. Metals bound by indigestible substances, mainly fiber, remain unavailable for absorption. Fiber can be hydrolyzed by colonic bacteria to release metals, but absorption will not occur and the metals will be excreted in the feces. Therefore, it is the fiber content of feed that can largely ensures the availability of minerals [13]. Due to the rise in cost of traditional feed components, new ingredients are needed that will reduce the cost of poultry feed. The use of dietary fiber is being considered as a solution [14].

The effectiveness of new feed substrates must not only be assessed according to generally accepted parameters. Special attention should be paied to the metabolism of chemical elements. Dietary fiber has a significant impact on mineral metabolism, impare of which can lead to various disorders [1], and, conversely, balance in chemical elements ensures increased productivity of animals and poultry [15]. Fermented dietary fiber helps reduce intoxication because it strengthens the intestinal barrier wall, normalizes its motor activity, and restores microbiota [6]. When analyzing the microbial profile of the cecum contents, it is noteworthy that the number of *Lactobacillaceae* decreased significantly in group C2 and in the test groups, which may be due to a lack of minerals necessary for growth. Importantly, no significant increase in the abundance of opportunistic microflora was recorded. Note, the abundance of cellulolytic bacteria from the taxa *Rikenellaceae* and *Lachnospiraceae* increased. This is due to an increase in the content of difficult to decompose components in the diet of broilers.

Note that dietary fibers, as anti-nutrients, have not been considered for a long time as an additive to the diet of animals and poultry. There are works [16, 17] that show a strong negative correlation between the fiber content in the diet and the digestibility of proteins and fats. Dietary fiber is not hydrolyzed by digestive enzymes of the small intestine, but can be partially fermented by the microflora of the gastrointestinal tract [16, 17]. The end products of microbial fermentation are various gases (H_2, CO_2, CH_4), lactic acid and short-chain fatty acids. Dietary fiber remains almost completely undigested, but when it is fermented into short-chain fatty acids, the energy produced can be used by host animals [18]. A

number of studies have shown that adding moderate amounts of various sources of fiber to the diet is beneficial. Diets high in fiber, especially insoluble fiber, have been shown to reduce disease incidence in poultry [19]. Dietary fiber improved the functions of the digestive organs, especially the stomach [20], increased the secretion of bile acids and enzymes [21], and changed the intestinal microflora [22]. This led to more efficient use of nutrients and an increase in animal growth rates [23]. Additionally, fiber in poultry diets may have a positive effect on gut health by preventing the adhesion of pathogenic bacteria to the epithelial mucosa [24], which is consistent with our findings.

Our data are consistent with reports supporting the trend toward the use of fiber as an alternative to antibiotics as growth promoters. Previously, antibiotics were widely used in poultry feed for the prevention and treatment of diseases. However, the indiscriminate use of antibiotics can lead to their residual content in meat and the selection of antibiotic-resistant forms of microorganisms. With the ban or strict regulation of the use of antibiotics in feed as growth promoters in the global poultry industry, an increased incidence of intestinal disorders in poultry has been documented [25]. Therefore, alternatives to antibiotics are being sought, feed formulations with easily digestible ingredients and enzyme additives are being developed, and the use of various feed processing methods is being considered in order to ultimately improve the growth performance of poultry.

Moderate amounts of fiber in the diet have been considered as one alternative to improve nutrient absorption and growth performance. Y.P. Li et al. [26] found that low-fiber diets do not provide full utilization of feed proteins and birds receive less energy than from high-fiber diets. In addition to feed additives, e.g., probiotics, prebiotics, and plant extracts, feed ingredients or feed components, e.g., fiber, hold promise for developing nutritional strategies to reduce gastrointestinal morbidity and improve poultry productivity [23]. However, we note that the available data on improved nutrient absorption when feeding dietary fiber are contradictory. For example, M. Houshmand et al. [27] examined the ability of fiber to compensate for calcium deficiency in poultry diets. There was no deficiency in the second group when using the low-calcium diet and the fiber-supplemented diet. That is, fiber is beneficial as a nutritional supplement to improve poultry growth performance and nutrient utilization. However, adding fiber does not always improve growth performance and nutrient absorption. In experiments of A. Sadeghi et al. [28], the authors found that intestinal villi length decreased in birds fed dietary fiber. This caused a decrease in the absorption of nutrients in the jejunum and an increase in their excretion, which is consistent with our findings.

Based on our data, methods can be developed to modulate the microbial profile of poultry intestines in order to use inexpensive feeds containing difficult-to-degrade fiber. It is important to note that when introducing dietary fiber into a diet deficient in minerals, we did not notice any severe dysbiotic processes in poultry.

Thus, dietary fibers (microcrystalline cellulose, lactulose, edible chitosan) added to the semi-synthetic diet of Arbor Acres cross broiler chickens leads to a decrease in the accumulation of macroelements in the bird's body, promotes the elimination of toxic chemical elements and an increase in the number of taxa *Rikenellaceae* and *Lachnospiraceae* with a simultaneous decrease of *Lactobacillaceae* in the intestines. The strontium content in poultry consuming cellulose and edible chitosan decreased by 25.7 and 45.9%, respectively ($p \leq 0.05$) vs. C_1 control (a semi-synthetic diet). A decrease by 22.2 and 43.4% ($p \leq 0.05$) was detected compared to C_2 control (a semi-synthetic diet deficient in microelements). In test groups, the comtent of tin reduced by 4.0 times ($p \leq 0.05$) vs. C_2, of lead by 2.0

times ($p \leq 0.05$) vs. C_1. In all test groups, the aluminum content decreased statistically significantly ($p \leq 0.05$). In group I fed cellulose, the number of *Lactobicallaceae* decreased by 4.5%, while the number of *Bacterodaceae* increased by 3.8 and 9.4%, *Rikenellaceae* by 6.3 and 6.8 times, *Lachnospiraceae* by 12.0 and 4.9 times, *Ruminococcaceae* by 2.1 and 3.9 times compared to C_1 and C_2. In group II, when lactulose was added to the feed, the number of *Lactobicallaceae* decreased by 6 times, and the number of *Bacterodaceae* increased by 6.5 and 12.1% compared to C_1 and C_2. The number of *Rikenellaceae* increased by 6.2 times, *Lachnospiraceae* by 9.57 times, and *Ruminococcaceae* by 3.1 times vs. C_1. In group III, when broiler chickens were fed chitosan, the number of *Rikenellaceae* increased by 5.5 times, *Lachnospiraceae* by 11.8 times, and *Ruminococcaceae* by 3.5 times vs. C_1. In general, the ability of dietary fiber to influence the cecum microbiome composition in broilers has been revealed. We believe that based on the biotic relationships between bacteria, targeted improvements in poultry productivity will be possible.

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METABOLIC PARAMETERS AND METHANOGENESIS IN THE RUMEN LIQUID IN in vitro TESTING EXPERIMENTAL DIETS SUPPLEMENTED WITH PHYTOBIOTICS AND $CoCl_2$

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Abstract

Dietary bioactives that increase the efficiency of feed nutrient use can provide sustainable and safe livestock products. Some bioactives are modifiers of rumen function in ruminants. These compounds are mostly administered separately. This paper is the first to describe the metabolic changes during in vitro incubation of the Kazakh white-headed bull ruminal liquid (RL) with feed compositions (biosubstrates) containing phytopreparations and cobalt chloride. The most effective combinations and dosages of these additives are evaluated. It was found out that *Artemisiae absinthil herba* (2.0 g/kg DM) + $CoCl_2$ (1.5 mg/kg DM) increases digestibility of feed dry matter (by 2.1 %), the activity of digestive enzymes and the concentration of metabolites in the RL while decreases methane production by 33.9 %. *Salviae folia* (1.6 g/kg DM) + $CoCl_2$ (1.5 mg/kg DM) provide the maximum reduction in methane emissions (by 46.3 %). Plant preparations increase the activity of RL amylase 2.6-4.0-fold and RL proteases 3.6-fold compared to control. Our goal was to reveal the effect of herbal preparation and cobalt chloride combination on the metabolic changes assessed in RL by in vitro technique. The experiments were carried out in 2021 at the BST RAS (Orenburg). Rumen liquid (RL) was sampled from four Kazakh white-headed bulls (*Bos taurus taurus*) weighing 250-265 kg at the age of 9-10 months. The samples were collected 12 h after feeding, through a chronic rumen fistula. The control ration (variant I) was 70 % coarse feed (mixed meadow hay) and 30 % concentrated feed (crushed barley). Test rations II was added with $CoCl_2$ (1.5 mg/kg DM; OOO NPK Ascont+, Russia), III with *Salviae folia* (1.6 g/kg DM), IV with *Artemisiae absinthil herba* (2.0 g/kg DM), V with *Salviae folia* (1.6 g/kg DM) + $CoCl_2$ (1.5 mg/kg DM), and VI with *Artemisiae absinthil herba* (2.0 g/kg DM) + $CoCl_2$ (1.5 mg/kg DM). Each RL sample was tested 4-fold (n = 16). Feed samples weighing 500 mg in polyamide bags were incubated for 48 h at 39.5 °C in a mixture of buffer solution with RL. At the end of incubation, the samples were rinsed and dried at 60 °C to a constant mass. The coefficient of digestibility in vitro of dry matter was calculated. Air samples were taken separately from each container to determine the methane content by gas chromatography (a Crystallux-2000M device, OOO NPF Meta-chrome, Russia). The amount of volatile fatty acids (VFA) in the RL was determined by gas chromatography with flame ionization detection (a gas Crystallux-4000M chromatograph). The concentration of various forms of nitrogen was determined by the Kjeldahl method (the Millab company equipment, Italy). Amylase activity was measured by Smith-Roy method modified by Anosone for high activity enzymes in the pancreatic juice. Proteolytic activity was assessed colorimetrically (λ = 450 nm) by destruction of Hammarsten Grade casein. The dry matter of biosubstrates was determined by drying to a constant mass at 60 °C. It was found that *Salviae folia* and *Artemisiae absinthil herba* shifted the fermentation during incubation towards propionate and butyrate. *A. absinthil herba* increased the intensity of nitrogen metabolism in RL during incubation, while total nitrogen content increased by 11.6 %, non-protein nitrogen by 144.3 %, ammonia by 71.4 %, and urea by 31,7 % ($p < 0.05$). Phytomaterials significantly increased the activity of amylase, proteases, and the concentration of VFA, but also increased the methane emission. Combinations of phytomaterials and cobalt chloride had a positive effect on the fermentation processes in the "artificial rumen". The maximum effect was revealed when using *A. absinthil herba* and cobalt chloride. There was an increase in the digestibility of dry matter with a decrease in methane formation by 2.1 %, and an increase in the

activity of digestive enzymes and the volatile fatty acid concentration.

Keywords: *Artemisiae absinthil herba*, *Salviae folia*, phytobiotics, cobalt chloride, nitrogen, volatile fatty acids, methane, digestive enzymes, "artificial rumen", beef cattle

The emergence of bacterial resistance and the abandonment of the use of feed antibiotics as growth promoters [1] have led to the need for searching natural and safe alternatives, such as probiotics, prebiotics, mineral components or phytobiotics [2, 3].

Through secondary metabolism, plants produce a variety of organic compounds that can be beneficial to animals. Phytobiotics have been shown to exhibit high biological activity. They have been investigated as modifiers of rumen function in ruminants [4-6]. Thus, the addition of a mixture of plant extracts had a positive effect on ruminal fermentation and growth performance in bulls consuming large amounts of feed concentrates [7]. Another study [8] found a positive effect of neomycin and oregano leaves on the severity of gastrointestinal diseases, as well as the mortality of newborn calves.

Plants and their bioactive compounds with antimicrobial properties have been found to improve feed utilization and animal productivity by altering microbial fermentation in the rumen [9]. However, currently plant products are used in the feed industry mainly as additives, flavorings and appetite stimulants [10]. Despite many studies, mostly in vitro, on the potential use of phytobiotics [6, 7], there is little information on their use in combination with other substances to improve metabolic processes and stimulate growth.

Previously, in in vitro experiments, we tested herbal remedies *Salviae folia*, *Inulae rhizomata et radices*, *Artemisiae absinthil herba*, *Scutellaria baicalensis*, *Origanum vulgare* and selected samples, the sage leaves and wormwood grass that showed the greatest functional activity [11]. Bioactive substances of herbal preparations, such as alkaloids, flavonoids, saponins, tannins, phenolic compounds, terpenoids and essential oils, optimize protein metabolism, reduce methane production and acidosis, which ultimately improves fermentation in the rumen [12]. To correct the effect of herbal preparations on methane production and fermentation in the rumen, it is necessary to investigate the effectiveness of using their compositions with chemical elements.

Cobalt is a promising chemical element because it is important for the microbial population in the rumen of ruminants, in particular for cellulolytic microorganisms. In addition, the production of vitamin B_{12}, vital for the host and protozoa, increases with the amount of cobalt available through bacterial synthesis [13-15]. A mixture of essential plant ingredients and organic cobalt in small ruminants]16] helped reduce the formation of methane and ammonia in the rumen and improve fermentation, and the form and amount of cobalt had a toxic effect on the number of methanogenic bacteria [17].

This paper describes for the first time changes in metabolic parameters in rumen fluid in vitro when herbal remedies and cobalt chloride were added to diet samples (biosubstrates), and the most effective combinations and dosages of these additives were determined. When using the *Artemisiae absinthil* herba complex (2.0 g/kg DM) and $CoCl_2$ (1.5 mg/kg DM), an increase in the digestibility of dry matter, the activity of digestive enzymes and the content of metabolites in the rumen fluid, as well as a decrease in methane formation were revealed. The use of the *Salviae folia* complex (1.6 g/kg DM) and $CoCl_2$ (1.5 mg/kg DM) led to the maximum reduction in in vitro methane formation.

The purpose of the work is to study in vitro the effect of herbal preparations and cobalt chloride (separately and in combination) on changes in metabolic parameters in the rumen fluid of bulls ("artigicial rumen method").

Materials and methods. The experiments were carried out in 2021 at the Shared Use Center of the BAT RAS (Orenburg). The material for the study was obtained from bulls (*Bos taurus taurus*) of the Kazakh white-headed breed ($N = 4$),

with an average weight of 250-265 kg at the age of 9-10 months. The animals were kept individually on a leash in standard cages, fed twice a day, access to water was unlimited. Feeding was carried out with regards to the recommendations of A.P. Kalashnikov et al. [18].

Rumen fluid (RF) was collected 12 h after feeding, through a chronic rumen fistula (d = 80 mm; Ankom Technology Corp., USA) with a rubber hose (outer diameter 40 mm) into a 3-liter thermos. Transportation was carried out at 4-8 °C for 20-30 min. RF, preheated to 39 °C, was used immediately upon arrival at the laboratory.

The studies were carried out in vitro using an Ankom DaisyII incubator (Ankom Technology Corp., USA) according to a special technique [19, 20]. Before use, RF samples were filtered through 4 layers of gauze and mixed with a buffer solution (1:4). The chemical composition of the buffer solution corresponded to saliva and maintained the pH of the "artificial rumen" close to physiological range (pH 6.0-6.5). Before mixing, the buffer solution was heated to 39 °C and saturated with CO_2.

Control diet (sample I) was 70% roughage (mixed-grass meadow hay) and 30% concentrated feed (crushed barley). Five test samples were added with phytosubstances and chemical elements, sample II with $CoCl_2$ (1.5 mg/kg DM; NPK Askont+ LLC, Russia), sample III with *Salviae folia* (1.6 g/kg DM), sample IV with *Artemisiae absinthil herba* (2.0 g/kg DM), sample V with *Salviae folia* (1.6 g/kg DM) + $CoCl_2$ (1.5 mg/kg DM), sample VI with *Artemisiae absinthil herba* (2.0 g/kg DM) + $CoCl_2$ (1.5 mg/kg DM). The choice of dosages of herbal preparations and chromium chloride was based on previous studies and recommendations [21, 22]. Each RF sample from 4 animals was tested 4 times (n = 16). *Salviae folia* (LSR-005376/07, Krasnogorskleksredstva JSC, Russia) contained 1.3-2.5% essential oil, consisting of D-α-pinene, cineole (~ 15%), α- and β-thujone, D-borneol and D-camphor. Alkaloids, flavonoids, tannins, oleanolic and ursolic acids were also found in the leaves of this plant [21]. *Artemisiae absinthil herba* (LSR-000171/08, LLC PKF FITOFARM, Russia) contained sesquiterpene lactones, bitter glycosides (absinthine, anabsinthine, artabsin, etc.), which give the plant a peculiar bitter taste, saponins, flavonoids, phytoncides, ascorbic acid, resinous and tannin substances, potassium salts, artemisetin, essential oil (0.2-0.5%), carotene, organic acids (malic, succinic) [21].

Feed samples (500 mg) were placed in polyamide bags and incubated in a mixture of a buffer solution with rumen fluid for 48 h at 39.5 °C. At the end of incubation, the samples were washed and dried at 60 °C to constant weight.

The in vitro dry matter digestibility coefficient was calculated as the difference in the feed sample mass with the bag before and after incubation:

$$K = (A - B)/C \times 100\%,$$

where K is the digestibility coefficient of the feed dry matter, %, A is the weight of the feed sample with bag before incubation, mg, B is the weight of the food sample with the bag after incubation, mg, C is initial weight of the food sample without the weight of the bag before incubation, mg.

After incubation, air samples were collected separately from each incubator container into special glass 200 ml^3syringes with rubber stoppers to determine the methane content by gas chromatography (a Kristallyuks-2000M device, OOO NPF Meta-Chrome, Russia). The amount of volatile fatty acids (VFA) in the RF was determined by gas chromatography with flame ionization detection (a Kristallyuks-4000M gas chromatograph). The content of various forms of nitrogen was assessed (a Millab equipment, Italy) according to Kjeldahl.

Amylase activity was measured by the Smith-Roy method modified by Anoson to determine high enzyme activity in pancreatic juice [23] and was expressed in mg of digested starch · ml^{-1} · min^{-1}. Amylase activity estimates were based on the hydrolysis of starch paste. By measuring the color intensity of a starch solution with

iodine reagent on KFK-3-01 (JSC ZOMZ, Russia), the rate of hydrolysis of the paste (amylase substrate) was determined. The activity of proteolytic enzymes was assessed by the amount of digested purified Hammersten casein with colorimetric control (λ = 450 nm) [24]. The technique was based on the colorimetric determination of casein concentration on KFK-3-01. The dry matter (DM) of biosubstrates was determined by drying to constant weight at 60 °C.

Animal care and experimental studies were carried out in accordance with the instructions and recommendations of regulations, the Order of the USSR Ministry of Health No. 755 of August 12, 1977 "On measures to further improve organizational forms of work using experimental animals" and Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, D.C., 1996). During the experiments, measures were taken to minimize animal suffering and reduce the number of prototypes.

The data were processed using the SPSS Statistics 20 program (IBM, USA). Mean values (*M*) and standard errors of means (±SEM) were calculated. The statistical significance of differences between the experimental and control groups was determined by Student's *t*-test; differences were considered significant at $p \leq 0.05$ and $p \leq 0.01$.

Results. The composition and nutritional value of the diet of the animals from which ruminal fluid were sampled are shown in Table 1.

1. Composition and nutritional value of the daily diet (per 1 animal) **of the Kazakh white-headed bulls (*Bos taurus taurus*) producing rumen liquid for in vitro tests** (Center for shared equipment use BAT RAS, 2021)

Ingredient	Amount
Mixed grass hay, kg	4.5
Legume hay, kg	3.2
Concentrates, kg	1.8
Table salt, kg	0.035
Vitamin A, thousand IU	30.0
Vitamin D, thousand IU	2.7
The diet contained:	
dry matter, kg	8.78
exchange energy, MJ	70.0
crude protein, kg	0.52
crude fiber, kg	2.46
neutral detergent fiber, kg	0.55
acid detergent fiber, kg	0.41
hemicellulose, kg	0.15
crude fat, kg	0.24
organic matter, kg	8.20
calcium, g	45.0
phosphorus, g	33.0

The digestibility of the dietary DM with the inclusion of phytochemicals increased in sample III by 1.3% ($p \leq 0.05$) and decreased in sample IV by 2.9% vs. control (Fig. 1). Cobalt chloride increased the digestibility of dry matter by 1.5%. The best digestibility was haracteristic of composition *Artemisiae absinthil herba* + $CoCl_2$, being 2.1% higher vs. control ($p \leq 0.05$). For *Salviae folia* + $CoCl_2$, the value was 1.3% higher than the control. These findings are indirectly supported by previous studies that found that essential oils from certain *Artemisiae* species improved in vitro rumen fermentation and dry matter digestibility [25]. This fact is explained by the likely increase in the content of terpenes, which are present in significant quantities in the wormwood extract. Feed intake is inversely related to dietary terpene concentrations, and ruminants cannot consume terpenes above a threshold [26]. Terpenoid extracts of wormwood suppress rumen microbiota and reduce the rate of cellulose digestion [27]. Additionally, J.P. Wu et al. [28] noted a change in rumen fermentation and better absorption of substances when adding a mixture of oregano (*Origanum vulgare* L., *Lamiaceae*) essential oils and cobalt lactate. In our experiment, the similar

effects occurred for *S. folia* (*Lamiaceae*) and *A. absinthil herba*.

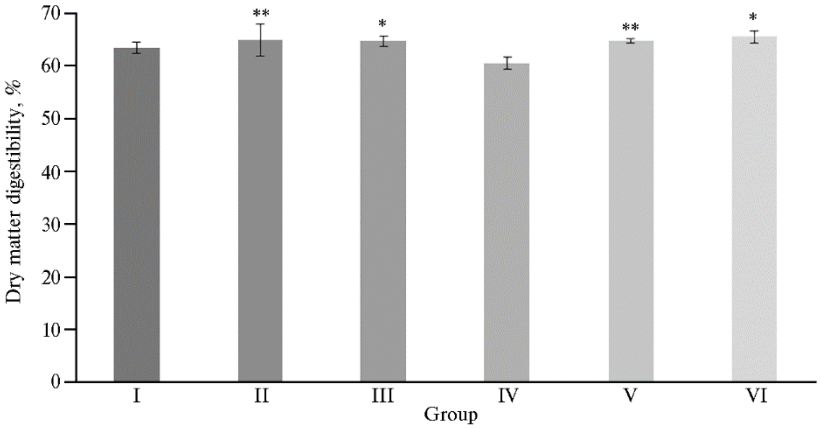

Fig. 1. Dry matter in vitro digestibility of diet composition added with herbal remedies and $CoCl_2$ after 48-hour incunation in the rumen fluid collected from Kazakh white-headed bulls (*Bos taurus taurus*): I — control, II — $CoCl_2$ (1.5 mg/kg DM), III — *Salviae folia* (1.6 g/kg DM), IV — *Artemisiae absinthil herba* (2.0 g/kg DM), V — *Salviae folia* (1.6 g/kg DM) + $CoCl_2$ (1.5 mg/kg DM), VI — *Artemisiae absinthil herba* (2.0 g/kg DM) + $CoCl_2$ (1.5 mg/kg DM) (n = 16, M±SEM, Center for shared equipment use BAT RAS, 2021).
* and ** Differences from control are statistically significant at $p \le 0.05$ and $p \le 0.01$, respectively.

Artemisiae absinthil herba increased the content of total nitrogen by 11.6% ($p \le 0.05$) vs. control. In sample II, the total nitrogen decreased by 10.6%, in III by 22%, in V by 19.7%, in VI by 14.4% ($p \le 0.05$). A direct relationship was found between protein nitrogen and the metabolism of total nitrogen (Table 2). The content of non-protein nitrogen in the test samples, on the contrary, was higher than in the control, by 76.7% in sample III ($p \le 0.05$), by 144.3% in samples IV ($p \le 0.05$) by 28.3% in samples V, by 16.7% in samples VI.

2. Nitrogen content after in vitro incubation of diet samples added with herbal remedies and $CoCl_2$ in the rumen liquid of the Kazakh white-headed bulls (*Bos taurus taurus*) (n = 16, M±SEM, Center for shared equipment use BAT RAS, 2021)

Treatment	N, mg%				
	total	non-protein	ammonia	urea	protein
I (control)	92.4±1.71	21.0±1.13	4.2±0.90	4.1±1.51	71.4±2.02
II ($CoCl_2$, 1,5 мг/кг СВ)	82.6±1.12	19.6±1.42	3.5±0.91	6.4±1.20*	63.0±1.33
III (*Salviae folia*, 1,6 г/кг СВ)	72.1±1.83*	37.1±1.31*	5.9±0.51*	6.0±0.81	35.0±1.21*
IV (*Artemisiae absinthil herba* 2.0 g/lg DM)	103.1±1.61*	51.3±1.71*	7.2±1.81	5.4±1.92	51.8±1.43
V (*Salviae folia* 1.6 g/kg DM + $CoCl_2$ 1.5 mg/kg DM)	74.2±1.41*	26.9±1.32	3.9±0.73	4.5±1.13	47.3±1.71*
VI (*Artemisiae absinthil herba* 2.0 g/kg DM + $CoCl_2$ 1.5 mg/kg DM)	79.1±1.22*	24.5±1.53	6.7±0.82*	4.5±1.31	54.6±1.51*

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

The content of various bioactive substances in plants [29, 30] which are eaten by animals, contributes to changes in enzymatic processes in the rumen. There may also be a dependence on the dosage of the administered components. Thus, a high content of wormwood in the diet of sheep led to an increase in the amount of ammonia and nitrogen in the rumen [31]. Cobalt is necessary for enzymes of rumen microorganisms involved in nutrient metabolism [32, 33].

The likely mechanism of action of herbal substances is their ability to inhibit the activity of ammonia-producing bacteria in the rumen, with a corresponding change in the nitrogen content of the rumen fluid [34]. In addition, a decrease in the concentration of nitrogenous substances may be due to an increase in the proteolytic activity of microorganisms, which we will discuss hereinbelow.

The content of ammonia nitrogen turned out to be maximum with *Artemisiae absinthil herba* used both separately and with $CoCl_2$, 7.2±1.81 and 6.7±0.82 mg%, respectively (see Table 2).

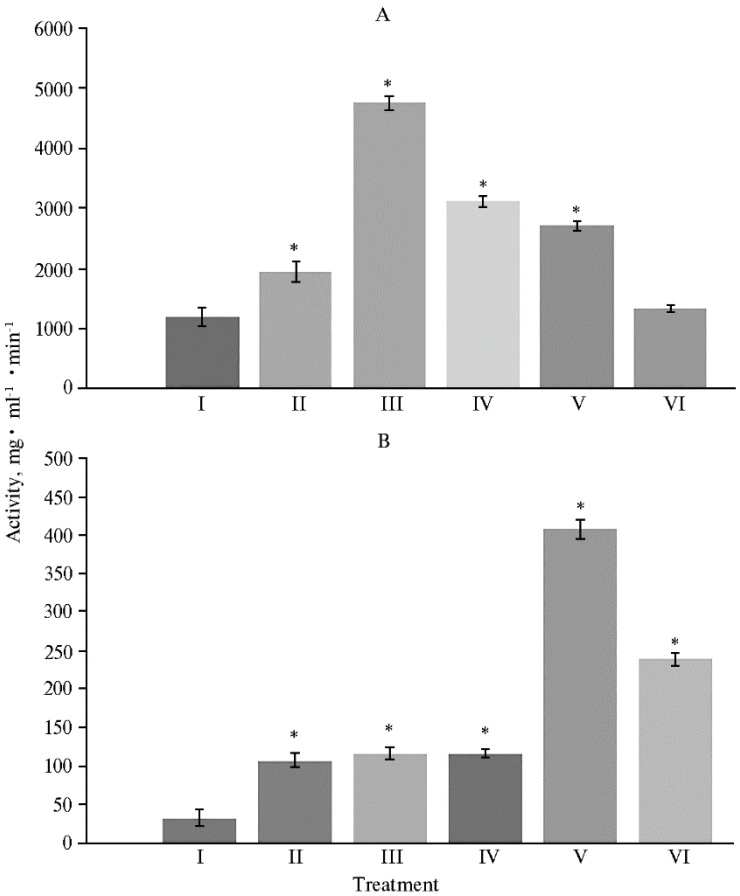

Fig. 2. Activity of amylase (A) **and proteolytic enzymes** (B) **after in vitro incubation of diet samples added with herbal remedies and $CoCl_2$ in the rumen liquid of the Kazakh white-headed bulls (*Bos taurus taurus*):** I — control, II — $CoCl_2$ (1.5 mg/kg DM), III — *Salviae folia* (1.6 g/kg DM), IV — *Artemisiae absinthil herba* (2.0 g/kg DM), V — *Salviae folia* (1.6 g/kg DM) + $CoCl_2$ (1.5 mg/kg DM), VI — *Artemisiae absinthil herba* (2.0 g/kg DM) + $CoCl_2$ (1.5 mg/kg DM) (n = 16, M±SEM, Center for shared equipment use BAT RAS, 2021).
* Differences from control are statistically significant at $p \leq 0.05$.

The introduction of phytonutrients into the micro-diet led to an increase in the activity of digestive enzymes, amylase and proteases in RF in vitro (Fig. 2, 3). Thus, amylase activity was higher vs. control in sample II by 64.5% ($p \leq 0.05$), in sample III by 303.7% ($p \leq 0.05$), in sample IV by 164.6% ($p \leq 0.05$), in sample V by 130.7% ($p \leq 0.05$), in sample VI by 11.9%. The maximum amylolytic activity was recorded for *S. folia*. Our results confirm previous data, according to which decoctions of the aerial parts of *Salvia aegyptiaca* and *Salvia verbenaca* showed lower activity towards α-amylase [35]. There is also an opposite opinion [36] that an aqueous solution of *Salvia eriophora* inhibited the enzyme α-amylase due to the presence of phenolic substances (fumaric and caffeic acid, epicatechin) in the extract.

In the control, protease activity was 32.7±0.12 mg · ml^{-1} · min^{-1}. When *S. folia* and $CoCl_2$ were added together, the protease activity showed maximum (12.4 times higher than the control, $p \leq 0.05$). In sample VI, proteolytic activity was 7.3 times higher than in the control ($p \leq 0.05$). Cobalt chloride increased the protease activity

by 3.2 times ($p \leq 0.05$), and the phytosubstances *S. folia* and *A. absinthil herba* by 3.4 times ($p \leq 0.05$).

In the available literature, data on this issue are contradictory. Thus, salvianolic acid, the most common bioactive component of *Salvia miltiorrhiza*, can inhibit metalloproteinase [37]. High proteolytic activity could be associated with the dominant effect of cobalt. It is known that aqueous metal complexes with cobalt enhance proteolytic activity [38]. As for Artemisiae, these grass species contain nitrogenous metabolites [39] which probably contribute to the activation of proteases of rumen microorganisms.

Our studies have shown that the herbal substances of *S. folia* and *A. absinthil herba* can shift rumen fermentation towards propionate and butyrate prodution. When using *A. absinthil herba*, we noted an increase in nitrogen metabolism in the RF with an increase in total nitrogen by 11.6%, non-protein nitrogen by 144.3%, ammonia nitrogen by 71.4%, and urea nitrogen by 31.7% ($p \leq 0.05$). It has previously been shown that herbal substances shift rumen fermentation towards propionate and reduces ammonia concentrations and methane production due to effects of bioactive substances such as terpenoids [40, 41], essential oils [42, 43] and tannins [44] on rumen microorganisms. The increase in the amount of nitrogenous substances may also be associated with the presence of similar metabolites in Artemisiae [39].

3. Volatile fatty acid concentration after in vitro incubation of diet samples added with herbal remedies and $CoCl_2$ in the rumen liquid of the Kazakh white-headed bulls (*Bos taurus taurus*) (n = 16, M±SEM, Center for shared equipment use BAT RAS, 2021)

Treatment	Volatile fatty acids, mmol/l				
	acetic	propionic	butyric	valerian	nylon
I (control)	0.070±0.0002	0.010±0.0001	0.020±0.0001	0.030±0.0004	0.008±0.0006
II ($CoCl_2$, 1.5 mg/kg DM)	0.250±0.0050**	0.014±0.0003	0.008±0.0020	0.020±0.0011	0.006±0.0010
III (*Salviae folia*, 1.6 g/kg DM)	0.070±0.0020	0.050±0.0040*	0.060±0.0030*	0.041±0.0050	0.014±0.0003
IV (*Artemisiae absinthil herba* 2.0 g/lg DM)	0.080±0.0010	0.020±0.0010	0.170±0.0020*	0.063±0.0040	0.030±0.0010
V (*Salviae folia* 1.6 g/kg DM + $CoCl_2$ 1.5 mg/kg DM)	0.250±0.0030	0.260±0.0020**	0.160±0.0030*	0.190±0.0020**	0.140±0.0020**
VI (*Artemisiae absinthil herba* 2.0 g/kg DM + $CoCl_2$ 1.5 mg/kg DM)	0.460±0.0060**	0.36±0.00400*	0.180±0.0050*	0.030±0.0001	0.050±0.0002**

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

The VFA content in the control and test treatmrnts with the addition of phytochemicals turned out to be quite low (Table 3). Cobalt chloride increased the concentration of acetic acid when testing samples II and V by 3.6 times ($p \leq 0.01$), sample VI by 6.6 times ($p \leq 0.01$) vs. control. The concentration of propionic acid also increased in all test variants with $CoCl_2$, in the presence of *S. folia* by 26 times ($p \leq 0.01$), *A. absinthil herba* by 36 times ($p \leq 0.05$). Results suggest that cobalt chloride may increase microbial methylmalonyl-CoA mutase activity, thereby increasing the amount of propionic acid in rumen contents [45].

The amount of butyric acid was higher copared to control in all variants with phytosubstances, in sample III by 3.0 times, in sample IV by 8.5 times, in sample V by 8.0 times, in sample VI by 9.0 times ($p \leq 0.05$). Propionic acid is the only gluconeogenic VFA produced in the rumen, which is absorbed and metabolized to succinate through a series of reactions [46, 47]. However, cobalt chloride at a dosage of 1.5 mg/kg DM had no effect on the production of propionate and contributed to a decrease in the amount of butyrate and an increase in methane formation by 51.2% ($p \leq 0.05$). Vitamin B_{12} serves as a growth factor for ruminal bacteria such as *Prevotella ruminicola* and *Methanomicrobium mobile*. *Prevotella ruminicola* increases the content of propionic acid in the rumen, which contributes to an increase in the methane concentration.

Combinations of phytosubstances and cobalt chloride had a positive effect on in vitro fermentation processes in RF. The best effect ocurred when using *A. absinthil herba* and cobalt chloride, that is, the digestibility of DM increased, while methane production decreased by 33.9%, the activity of digestive enzymes increased, as well as the concentration of VFAs in the rumen fluid (see Tables 3, 4).

Controlling greenhouse gas emissions is critical to industrial beef production. Thus, polyphenols of phytobiotics can significantly influence the microbiome of the digestive system of ruminants, including reduced abundance of methanogenic archaea [48]. Due to their natural origin and safety, essential oils are increasingly used to modify the microbiome, especially to reduce methane production in ruminants [34].

The methane concentration in our tests increased vs. control by 51.2% with cobalt chloride, and by 16.5% ($p \leq 0.05$) with *Salviae folia*. Phytosubstances + cobalt chloride contributed to a reduction in methane synthesis in samples V and VI by 46.3 and 33.9%, respectively ($p \leq 0.05$) (see Table 4).

4. Methane emission after in vitro incubation of diet samples added with herbal remedies and $CoCl_2$ in the rumen liquid of the Kazakh white-headed bulls (*Bos taurus taurus*) (n = 16, M±SEM, Center for shared equipment use SAT RAS, 2021)

Treatment	Methane concentration	
	CH_4, g/m^3	CO_2 equivalent/g
I (control)	12.1±2.2	302.5±1.1
II ($CoCl_2$, 1.5 mg/kg DM)	18.3±2.3*	457.5±3.1*
III (*Salviae folia*, 1.6 g/kg DM)	14.1±1.9*	352.5±2.4
IV (*Artemisiae absinthil herba* 2.0 g/kg DM)	9.4±2.4	235.0±1.9*
V (*Salviae folia* 1.6 g/kg DM + $CoCl_2$ 1.5 mg/kg DM)	6.5±1.2*	162.5±2.3
VI (*Artemisiae absinthil herba* 2.0 g/kg DM + $CoCl_2$ 1.5 mg/kg DM)	8.0±1.4*	200.0±2.5

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

Some papers have reported that phytochemicals are effective in reducing ruminal acetate and ammonia concentrations and methane production in small ruminants and beef cattle [49-51]. In our experiments, the amount of methane decreased only with *A. absinthil herba* by 22.3%, and with *S. folia*, on the contrary, it increased by 16.5% ($p \leq 0.05$).

A likely mechanism for reducing methanogenesis may be inhibition of the enzymes responsible for this process. Methyl-CoM reductase is known to play a significant role in methanogenesis [51]. Finding target molecules to inhibit this enzyme in ruminants is an important task. There is evidence that phytochemicals have better affinity for hydrogen bonds and can be used to reduce methanogenesis [52].

It is possible that a decrease in methanogenesis also results from changes in the populations of methanogenic bacteria in the rumen fluid. It was previously found that *A. capillaris* extract reduced methane emissions by 14% ($p < 0.05$) after 48 h of incubation with a reduce in the abundance of methanogen communities (ciliates and methanobacteria populations) [53]. A similar effect was observed under the influence of a mixture of essential oils and cobalt carbonate [16]. In addition, some sources and dosages of cobalt had toxic effects on the abundance of methanogenic bacteria [17].

The amount of CH_4 and CO_2 equivalent were maximally reduced when using the *Salviae folia* and $CoCl_2$ composition ($p \leq 0.05$). The molar fractions of acetate, propionate and butyrate in this variant increased significantly compared to the control, while the intensity of nitrogen metabolism in RF was high. It was found that the introduction of cobalt into a micro-diet without herbal substances did not reduce methane formation and worsened the DM digestibility of the diet. It has previously been noted that the total content and individual quantity of some short-chain fatty acids, the acetate:propionate ratio, pH and overall gas production in the rumen of ruminants are significantly dependent on certain plant substrates (wormwood, chamomile, fumitory and mallow) [54], which was also observed in our experiments.

Thus, when using herbal preparations and cobalt chloride (individually and in

combination) as part of biosubstrates, changes in metabolic parameters in the rumen fluid of bulls in vitro ("artificial rumen") were not uniform. Phytosubstances significantly increased the activity of amylase, proteases, the concentration of volatile fatty acids in the rumen fluid and activated nitrogen metabolism, but also increased the methane emission. The combination of *Artemisiae absinthil herba* and *Salviae folia* with $CoCl_2$ reduced methane production and enhanced metabolization. For the complex of *Artemisiae absinthil herba* (2.0 g/kg DM) and $CoCl_2$ (1.5 mg/kg DM), the activity of digestive enzymes and the content of metabolites in the rumen fluid in vitro increased and methane emission decreased by 33.9%, while DM digesability was 2,1% higher compared to control. The complex of *Salviae folia* (1.6 g/kg DM) and $CoCl_2$ (1.5 mg/kg DM) maximally reduced methane production (by 46.3%). Herbal preparations contributed to an increase in amylase activity in the rumen fluid by 2.6-4.0 times and protease activity by 3.6 times when compared to the control. However, we did not identify patterns of dose-dependent changes in metabolic parameters in the rumen fluid when combining herbal preparations and cobalt chloride, so we plan to continue studying the effect of herbal substances (individually or in combination with other substances) on the ruminant rumen microbiome.

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ON THE MATING OF DOMESTICATED AND WILD SABLES (*Martes zibellina* Linnaeus, 1758) TO GENERATE GENOTYPES WITH VARIOUS FUR COLORING

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Abstract

Sable (*Martes zibellina* L.) skins have always been in high demand on the fur market. At present, there is a trend towards an increase in interest in the skins of wild sables vs. cage-bred sables. According to experts from fur farms and auction houses, this id due to the inferior fur quality the cage-bred sables have during domestication, e.g., the hairline has become thicker and coarser, the fur is less silky, with a low variability in color and tone. According to the preferences of buyers at international fur auctions and the recommendations of auction house experts, sable skins with a fur color points 5 (brown with a golden tin), 6 (the color is somewhat lighter than for 5 points), and 7 (beige, sandy-golden with a dark brown ridge), of medium tone and chestnut shade are currently the most in demand. The assortment of sable breeding products can be expanded by matting with wild animals. This report presents the first results of obtaining hybrid animals with a fur color of 5-7 points from crossing purebred sables with individuals from the wild (females and males of the Yakut, Irkutsk and Yenisei ridges). The work was carried out at OOO Savvatyevo Animal Breeding Plant. The sables caught in Siberia was brought to the farm for mating with cage-breeding sable in 2021. According to the results of whelping in May 2022, two groups of sables were formed, the control (purebred puppies from mating caged-bred male and female sables, 35 males, 30 females) and experimental (crossbred puppies from mating purebred and wild sables, 39 males, 30 females). The resultant reproduction showed that when wild males mate with purebred females, the reproductive performance of females is not inferior to those in crossing with cage-bred males. In crossbred and purebred offspring, the average yield of puppies per successful female was 3.9 sables. Purebred male progeny is inferior to crossbreds in live weight (1279.6±17.8 g, 1560.0±68.5 g, $p \leq 0.001$) and body length (45.6±0.5 cm, 47.8±0.6 cm, $p \leq 0.01$). Purebred female progeny is somewhat superior to hybrid females in terms of live weight (1138.7±25.4 g, 1111.3±18.7 g, $p \geq 0.01$), but body length and chest girth behind the shoulder blades did not reveal differences. Purebred males and females complete their growth earlier than hybrids. Purebred young animals have two variations in the coloring covering hair, almost black and dark brown. In crossbred males and females, the covering hairs are dark brown, brown, light brown, almost black. Purebred and crossbred sables show no significant differences in the color of the base of downy hairs. Crossbred males and females have a greater variability in the color of the tops of downy hair compared to the purebred control. The quality of the hairline of crossbred males is 0.08 points higher than that of crossbred females. In color, crossbred males are lighter than females by 0.34 points, and the males are closer to the desired color variation. By tone, the resulting young sables approach the desired one, and by shade, they correspond (2.03 points for females and 2.07 points for males). Among the resulting males, there are fewer individuals with gray hair (10.5 %) vs. resulting females (13 %), a gray spot is present (average score for females 4.67, for males 4.52). Evaluation of parental pairs with regard to the main economically important traits showes that the best desired type of coloration of crossbred young animals results from mating light males with dark females of heterogeneous pairs. The

mother sable coloration does not influence the coloration of daughters and sons while males better transmit their traits by color to offspring, for sons, $r = 0.61$ ($p \leq 0.001$), for daughters, $r = 0.72$ ($p \leq 0.001$).

Keywords: sable, skins, coloring, tone, shade, furs, sable farming, breeding, selection

Sable (*Martes zibellina* Linnaeus, 1758) is a valuable fur-bearing animal [1, 2] of the order *Carnivora*, the family *Mustelidae*, the genus *Martes*, the subgenus *Martes* [1, 3-5]. The subgenus *Martes* on the territory of Eurasia also includes forest martens (*M. martes* L., 1754), stone martens (*M. foina foina* Erxleben, 1777), and yellow-throated marten (*M. flavigula* Bjddaert, 1785) [1, 5, 6]. Outside Eurasia, there are two more members of the subgenus *Martes* [7]. When crossing the area of sable and marten, their hybrids, kidus, are encountered [1, 8, 9]. Sable appearance (an elongated body, a small head, and short legs) is typical of the *Mustelidae* family. The body length of males varies within 40-55 cm, the tail length is 20 cm, and the weight of an individual is on average up to 1.5 kg. Females are slightly smaller than males. Ears up to 5.5 cm long, blunt. The limbs are relatively short and wide. Winter hair is quite thick, lush, silky, and shiny. The color in different parts of the area varies from yellowish-brown to dark brown, almost black. The head is usually lighter than the back. In the neck there may be a light, rounded spot, sharply limited or with vague edges [1]. In natural populations, the color of the sable's hair is extremely variable, from very light to pitch-black with many transitional variations [10]. Black sable (with color ranging from dark brown to resinous black) is rare in nature. Much more often, you come across light-colored individuals with fur coloring from sandy yellow to dark brown tones. The proportion of individuals with light coloration in some natural populations reaches almost 100% [11].

Significant variability in the color of the sable is associated with the individual, age, and sex characteristics of the animals, as well as the geographic areas of their habitat. Even the color of the same animal does not remain constant throughout the year. In summer, it is darker than in winter, mainly due to a decrease in the proportion of downy hair in the hairline [10]. With great individual variability in fur color within the same population, in the same areas, sometimes even in the same broods, there are very dark, very light (close to straw color) and medium-colored individuals [1].

Based on the color and shades of the lower part of the downy hair on sable skins, 29 variants were identified, which can be assigned to 6 groups based on the main color. In the color and shades of the upper part of the downy hair, 38 variants were identified, which were combined into 11 groups. In addition, the lighter the upper part of the downy hair, the larger part of the hair length it occupies [10]. Based on the nature of intraspecific variability in color, silkiness and degree of softness of the hair in the Russian sable, geographic races are distinguished (historically they are called ridges). In 1938, 8 ridges were described: Tobolsk, Altai, Yenisei, Minusinsk, Barguzin, Amur, Yakut, and Kamchatka. This geographic variability is characterized by a gradual darkening of the fur color of sables from west to east. In the north of the range, as well as in the highlands with extreme winter weather conditions, populations with lighter fur have formed [1]. According to N.N. Bakeeva et al. [1], of the 18 previously designated subspecies, four currently exist. The Altai subspecies (large in size, relatively dark fur color, 3.08 point color index; the population is 26% dark, 66% medium and 8% light) is distributed throughout Altai and in the northeastern part of Tuva. Sakhalin subspecies is very small and light-colored form, color index of 2.60 points; the population is 16% dark, 78% medium, 6% light and separated from mainland individuals by the Strait of Tartary. Kamchatka subspecies (individuals of particularly large sizes, fur color index is 3.2 points, belongs to the dark subspecies); the area of the subspecies is

not limited to the Kamchatka Peninsula, it lives in the adjacent continental territories (the basins of the Apuki and Penzhina rivers). The Tobolsk subspecies is represented by large individuals, the lightest in color (color index 1.78 points; the population is 26% dark, 66% medium, and 8% light); the area covers the entire Cis-Ural region, namely, the right bank of the Pechora River basin, the upper reaches of the Kolva and Vishera rivers, as well as the Urals and Trans-Urals. In other populations occupying the interior regions of the sable's range, it is very difficult to find boundaries, although some morphological differences between them are often expressed quite reliably. Consequently, it is difficult to confirm the reality of separating other groups of sables (Yenisei, Tunguska, Angara, Kuznetsk, Sayan, Chikoy, Barguzin, Shantar and Kuril) into subspecies; they can be considered as local morphs [1].

In the past, sable was widespread from the forest zone of Eastern Europe to northern Asia. This species was found in Belarusian, Polish, and Lithuanian forests. However, as a result of the influence of anthropogenic factors (taiga logging, deforestation, fires, plowing of land, grazing, hunting), the sable disappeared from the territory of Europe, and behind the Ural ridge its once continuous area fell into a number of areas isolated from each other [1, 12-14]. By the beginning of the 20th century, sable became an endangered species. It was necessary to take emergency government measures to protect the sable, control and prohibit hunting, create nature reserves and sanctuaries, artificially resettle the animals into empty lands, and develop cage-based sable farming [14-17]. The measures taken have yielded positive results. The able population was 701 thousand individuals ш 1960, 723 thousand individuals in 1978, and 1180 thousand individuals by 1988. In the 1980s, active sable hunting resumed. Over the past years, the number of sable in the Russian Federation has been stable and accounted to 1400-1500 thousand individuals. According to the Ministry of Natural Resources and Ecology of the Russian Federation, in 2020 there were 1546.0 thousand individuals. The main commercial resources of the species are concentrated in the Far Eastern (796.0 thousand individuals) and Siberian (675.7 thousand individuals) federal districts, Krasnoyarsk Territory (332.6 thousand individuals), the Republic of Sakha (Yakutia) (256.2 thousand individuals), Khabarovsk Territory (200.0 thousand individuals), Irkutsk Region (200.0 thousand individuals) [18]. The habitat area of the sable in Russia is about 7 million km^2 [19]. The current range of the sable, in addition to the Russian Federation, includes China [20-23], North Korea [24], Japan [25], Mongolia [26, 27], and Kazakhstan [28, 29].

For a long time, attempts to breed sable in cages remained unsuccessful due to the peculiarities of its reproductive strategy, which differs significantly from that of most predators [30-32]. The rut usually occurs from mid-June to early August. During estrus, which occurs in the summer, there are several periods of sexual heat with an interval of 8-10 days, of which only the last ends with provoked ovulation. Pregnancy lasts 7.5-8 months, and after the latent period (late February-early March), the blastocyst is implanted into the uterine wall. The total duration of the period of development and formation of the embryo after implantation (true pregnancy) is only 30-35 days [1, 30, 31].

Russia became the first and only country where industrial technology for the production of sable skins was developed, and to this day Russia remains the leader in this production. Domestic sable breeding has a long history. Commercial sables were used to form cage populations and for breeding [11, 32].

Regular sable breeding in Russia began in 1931 [11, 30-32]. The ancestor of cellular sable breeding was the Pushkin State Animal Farm (currently the Federal State Unitary Enterprise Russian Sable) which later bred black sable (the

animals differed from wild animals in having a more saturated black hair color) [31-33].

For the first time, P.A. Manteuffel managed to obtain offspring from a sable under cage conditions in 1929 at the Moscow Zoo [31]. In the same year, K.G. Tuomainen obtained sable puppies in Solovetsky Pushkhoz [31, 32]. To create a cage population of sable, the gene pool of nine natural populations of animals different in size and fur color from different regions of Siberia, the Urals (including those belonging to the so-called low-value ridges), and from Tuva was used [34-36]. A total offspring accounted for apprx. 100 individuals in 1929 and 129 individuals in 1930. In 1931, the creation of a unique herd began, from which the entire population of cage-bred sables in Russia originates [32, 34].

In the USSR, at the first stage, individuals from different ridges were crossed, and the Barguzin dark females initially turned out to be the worst in terms of reproduction rates. In 1935, to improve the herd, animals were brought to the Pushkin State Animal Farm from the Barguzinsky Reserve (13 females, 2 males) and the Povenets Farm (Karelian Autonomous Soviet Socialist Republic) (25 females and 20 males). In 1936, 280 sables from the reorganized Aleksandrovsky State Animal Farm were additionally brought together with 25 females and 37 males of the Barguzin Ridge caught in the wild nature [32]. In the Pushkinsky fur farm in 1936, dark males accounted for less than 30% of the population, in 1940-1941 there were 48% dark males [32]. Breeding the "ideal sable" with a large body size, dark coloring and silky fur was complicated by the fact that the initial population consisted mainly of light-colored animals (brown and sandy-yellow with a large throat patch). In addition, the behavior of the animals turned out to be wild; the females generally did not reproduce at all. Animals of the Amur ridge predominated. There were fewer Yenisei, Ural and Altai animals. To consolidate the dark color, a decrease in fertility was allowed [33]. To speed up selection for darkening fur, dark Barguzin males were crossed with females from the Yenisei Ridge, and the darkest females were selected from the offspring to be again crossed with Barguzin males. Crossing Barguzin males with Amur Ridge females gave good results. In addition to targeted crossings of sables from different ridges, lines and families with dark colors, thick fur and the largest fertility were created [32]. In 1940, 70 females and 70 males of raised young animals were transferred from the Pushkinsky fur farm to the Krasnoyarsky fur farm (Krasnoyarsk Territory). In the first post-war years, young animals from the Pushkinsky animal farm supplied the sable breeding farms of the Saltykovsky (Moscow Province), Biryulinsky (Tatar Autonomous Soviet Socialist Republic) and Beloyarsky (Krasnoyarsk Territory) state farms [32]. In the early 1970s, the Saltykovsky state farm approved and further constantly used the influx of blood from Barguzin sables caught in the wild. For 30 years, the farm selected sables with the coloration and fur structure characteristic of the Barguzin Ridge wild sables with simultaneous selection for larger size, better fur quality, and higher reproductive potential [31].

Sable color is determined by the zonal coloring of the underfur, the color of the guard hairs, the size and color of the throat patch, and the appearance of gray hair. Many of these traits are inherited independently of each other. Until 2015, no genetically confirmed mutations in hair color were found in sables. It was believed that the general diversity of color in nature and during cage breeding of sables is due to modifier genes that suppress pigment formation in the hair to varying degrees [36].

Sables are characterized by a lighter coloration of the head compared to the body due to natural suppressor genes that inhibit pigment production in the hair. Black-headed individuals with the same color of the head and body resulted from long-term selection for darkening the color of the fur at the Pushkinsky fur

farm [36, 37]. Black-headedness arises when modifier genes (black-headedness polygenes) remove the suppression of pigment formation encoded by natural suppressor genes. Black-headedness is inherited as a quantitative trait and positively correlates with the overall color of the hair coat the darkening of which accelerates when common sables are crossed with black-headed sables [37, 38]. According to the requirements of OST 1010-86, the 5-point scor of fur color corresponded to the darkest individuals of the black or dark brown type. Animals that did not meet these requirements were culled during selection. Since in almost all breeds and types of fur-bearing animals, heterozygous individuals, as a rule, have a worse color rating than homozygous ones, possible carriers of mutant color genes were removed from the herd. In 1991, when wild caught sables of standard color were added to the fur farm population (six males in total, five of them from Kamchatka), animals began to appear with some lightening of color (with beige, pastel and gray shades) [39-41].

Over 25 generations of commercial domestication of sables on specialized fur farms, the de novo appearance of animals with extensive white spotting or piebalds on the paws, tip of the tail, muzzle and body occurred. The size and color of the spots vary greatly. The color of the spots can be not only white, but also yellow of varying intensity. Based on the inheritance of this phenotype, it was suggested that sables have two mutations that determine white spotting [30]. One mutation occurs in a dominant gene and is expressed both in hetero- and homozygous states. Another mutation appears to affect a semidominant gene with a recessive lethal effect. In a heterozygous state, it causes white spotting, and in a homozygous state, it causes white hair coloration. Spotted sables often have blue eyes, and the nasal planum is partially or completely depigmented. Homozygous individuals die at the prenatal or postnatal stage. There was only a single case of welping when a young sable lived to 45 days of age [30].

The first pastel-colored sable was born from a pair of black sables in 2005 (Pushkinskoye animal farm). These parents previously had nine black puppies from three crosses. A genealogical analysis of these parents showed that all their ancestors for three generations had black fur. A.D. Manakhov et al. [41] did not identify either homozygous or heterozygous *TYRP1b* sables in natural populations and suggest that the pastel variant of the sable arose de novo in the Pushkinskoye animal farm. Parents of pastel coloring, both in homogeneous selection of pairs and when mating with black sables, in most cases produce offspring separated by hair color. According to G.A. Kuznetsova [39, 40], pastel color is due to a dominant mutation. *PP* is homozygous genotype for pastel color, *Pp* is heterozygous. Thus, the pastel coloration of the hair in caged sables is the first proven mutation in this species. In the *Pp* genotype, the dominant mutant gene can alter the color tone, two mutant *PP* genes increase the effect. In addition, the effect of associated genes, in particular modifier genes, is possible (41).

The modern Russian caged sables can be divided into phenotypic groups depending on the degree of color lightening due to an increase in the expression of modifier genes. These are standard purebred, characteristic of the black sable breed, with the absence of activity of modifier genes; standard crossbreds of the first generation from crossing purebred black and pastel animals (including those with lightened ears, muzzle or head); standard lightened ones (like lavender, smoky and other shades that lighten the color); dark pastels; mid-tone pastels; light pastels; ferret type; palominoids (with high expression of modifier genes, the birth of white sables is possible) [40, 41].

Currently, by catching, breeding and selection of sable in Russia, the breeds Black Sable (1969, patent holder is the Federal State Unitary Enterprise Russian Sable), Saltykovskaya 1 (2007, patent holder is the Saltykovsky Breeding

State Farm JSC), Saltykovskaya silver (2020, patent holder is the Saltykovsky Breeding State Farm JSC, Vavilov Institute of General Genetics) and the breed type Pushkin Amber (2018, patent holder is the Federal State Unitary Enterprise Russian Sable) [31, 42, 43]. However, international auctions in recent years show that interest in commercial sable skins is currently growing; their share of those offered for sale averages 86%, while caged sable skins are sold on average at 27% [43, 44, 45]. According to experts from fur farms and auction houses, the reason is a decrease in the fur quality during sable domestication, the hair has become thicker and coarser, less silky, with low variability in color and tone, therefore, an expansion of the product range is required. The most in demand at present are sable skins with 5-7 point colors, medium tone, chestnut shade, with silky fur [43, 44]. Expanding the range of sable breeding products is possible by mating cage-bred sables with individuals from wild populations.

In this work, for the first time in the history of modern Russia, we analyzed the results of mating cage-bred and commercial sable. The indicators of the reproductive capacity of commercial males (Yakut, Irkutsk and Yenisei ridges) and females of the Saltykovskaya 1 breed, bred at the Savvatyevo Animal Farm LLC (Tver Province), are assessed. A comparative analysis of the growth dynamics of purebred and crossbred sable puppies is carried out. New requirements for grading (individual assessment) of crossbred young animals have been developed. A comprehensive assessment of the body size, fur quality and hair color of crossbred young animals was carried out. The selection of parental pairs to produce crossbred sables with competitive skin products that meet modern market requirements are analyzed.

The purpose of the study was 1) to compare rutting and whelping peculiarities of the cage-bred and wild sables, 2) to assess the growth rate of purebred and crossbred young sables during the growing period and 3) to evaluate the effectiveness of parent selection when mating a wild sable with a cage-bred sable to create the technology of sable selection based on color and hair quality.

Materials and methods. The work was carried out at LLC Zveroplemzavod Savvatyevo (Tver Province, 2021-2022). In 2020-2021, wild sables *Martes zibellina* L. (2 females and 10 males), caught in Siberia (Yakut, Irkutsk and Yenisei ridges) was brought to the farm for mating with cage-bred sable, which was carried out in July 2021. The most highly productive domesticated females aged from 2 to 9 years with an average progeny of 3.5-5.5 welps were selected for mating with wild males. Females Nos. 5190002 and 5200004 were wild (brought from the Kemerovo region and the Republic of Sakha-Yakutia, respectively). Male No. 5180001 from the natural population (did not cover any females), wild females No. 5200004, 5190002, and purebred females No. 3150516, 2180360, 2150620, 3150026, 3150078, 1180150, 1190132 which did not produce offspring were excluded. As a result, the sample included 9 wild males and 18 purebred females.

In April 2022, the females gave birth. Based on the results of whelping in May 2022, two groups of sables were formed, group I was purebred puppies (35 males, 30 females) from mating purebred males and females (control), group II was crossbred puppies (39 males, 30 females) from mating purebred and wild sables. The groups were formed according to the date of the welp birth, the mother's age and her average fertility in previous years.

From May to October 2022, the growth rates of purebred and crossbred young animals were compared. Control and test yong sables after weaning f (at 45 days of age) was measured and weighed every 15 days until 60 days of age and then every 30 days until 150 days of age. Body length, chest circumference behind the shoulder blades were measured with tape, an accuracy of 0.5 cm. Young

animals were weighed on a Vibra AJ-620 lE electronic scale (SHINKO DENSHI Co., Ltd., Japan), with an accuracy of 10 g. The absolute and relative increase in live weight and body length were calculated monthly.

Individual characterization of young sables aged 6 months and their parents was carried out in October 2022. The young sables were assessed for body size, build, fur quality, coloring and additional traits (throat patch, gray hair), and the parents was assessed for coloring. We used OST 1010-86 "Industry standard. Farm animals. Fur-bearing animals of cage breeding. Zootechnical requirements for grading (assessment)" (Moscow, 1986) with additions developed by us. For purebred and crossbred young animals (taking into account the requirements for grading crossbred young animals of the desired type), size, quality of pubescence, and quality of hair color were assessed. When grading young animals, special attention was paid to the desired type of color characteristic of wild sable (5-7 point color, medium tone, chestnut shade).

In November 2022, the results of mating and whelping were assessed according to the number of males who covered and did not cover females, the number of covered females, the number of empty females, the number of successfully whelped females, fertility, the number of animals for removing, the yield of young animals.

In December 2022, based on zootechnical documentation, we analyzed the results of the selection of parents for matings wild sable with cage-bred sable and assessed the correlation between the coloration of parents and young animals (Pearson's correlation coefficient).

Variation statistics was used for data processing (the Microsoft Excel computer program and statistical analysis packages Statistica 6.0, StatSoft, Inc., USA). Means (M) and standard errors of the mean (±SEM) were calculated. The significance of differences was assessed using Student's t-test. Differences were considered statistically significant at $p \leq 0.05$.

Results. The frequency of mutations is known to increase under conditions beyond the optimal habitat, under stress and physiological discomfort [46]. Populations living in the peripheral pessimum of the area are at the limit of the adaptive capabilities which should lead to a more rapid accumulation of mutations. Yakutia has the most severe conditions of the sable's range where the likelihood of encountering individuals with non-standard hair color may be the highest. Currently, among sable skins from different regions of Yakutia, proportion of light-colored skins is high [46]. There are especially many individuals with light fur in the northwestern and western populations [46]. In almost all ecological and geographical zones, there was a shift in the color ratio towards lightening. According to N.N. Osipova et al. [46], the selection involving sables from the northern taiga regions of Yakutia, especially from areas with a high frequency of non-standard coloring, could have great prospects for Russian fur farms [46]. We used sables from the Yakut, Irkutsk and Yenisei ridges in crossbreeding (schemes are submitted in Table 1).

Table 2 presents the results of whelping in the experimental batch of sables in 2022. Of the 9 males in the rut in 2021, only 8 covered females (see Table 2). Male No. 5190011 had the best rutting results, covering 8 females, 6 of which whelped. Males Nos. 5190003, 5200013 and 5200009 each mated with 2 females, but only every second of them whelped. Male No. 5180001 did not cover a single female. In total, of the 25 covered females, 72% gave birth, the rest (28%) were empty. As a result, 77 puppies were obtained from 18 females, 6 of which died before registration.

1. Scheme of mating wild male sable *Martes zibellina* L. with females of different origins (OOO Zveroplemzavod Savvatyevo, Tver Province, 2021)

Male No.	Female No.	Female age in 2021	Average female fertility for previous years	Origin of females
5190011	3130566	8 years	4,0	OOO Vostok, Krasnodar Province
	3120714	9 years	4,0	OOO Vostok, Krasnodar Province
	2180488	3 years	4,0	FSUE Russian Sable, Moscow Province
	2180360	3 years	4,5	FSUE Russian Sable, Moscow Province
	3150516	6 years	4,0	OOO Znamenskoye Animal Farm, Tver Province
	2150820	6 years	4,4	FSUE Russian Sable, Moscow Province
	1150338	6 years	4,6	OOO Plemzverocomplex Magistralny, Altai Territory
	1150054	6 years	4,6	OOO Plemzverocomplex Magistralny, Altai Territory
5190001	3170006	4 years	2,0	OOO Znamenskoye Animal Farm, Tver Province
	3160206	5 years	4,5	OOO Znamenskoye Animal Farm, Tver Province
	1150142	6 years	4,2	OOO Plemzverocomplex Magistralny, Altai Territory
5200011	4180174	3 years	4,0	OOO Zveroplemzavod Savvatyevo
	3150034	6 years	5,2	OOO Znamenskoye Animal Farm, Tver Province
	2150620	6 years	3,8	FSUE Russian Sable, Moscow Province
	1150214	6 years	4,0	OOO Plemzverocomplex Magistralny, Altai Territory
5190007	3170380	4 years	3,3	OOO Znamenskoye Animal Farm, Tver Province
	2150608	6 years	2,6	FSUE Russian Sable, Moscow Province
	1190098	2 years	2,0	OOO Plemzverocomplex Magistralny, Altai Territory
	5190002	2 years	4,0	Captured in Nyurengri, Republic of Sakha-Yakutia
5190003	3160032	5 years	3,5	OOO Znamenskoye Animal Farm, Tver Province
	3150026	6 years	2,2	OOO Znamenskoye Animal Farm, Tver Province
5200013	3170454	4 years	4,0	OOO Znamenskoye Animal Farm, Tver Province
	3150078	6 years	4,4	OOO Znamenskoye Animal Farm, Tver Province
5200009	1180150	3 years	5,5	OOO Plemzverocomplex Magistralny, Altai Territory
	3150562	6 years	4,0	OOO Znamenskoye Animal Farm, Tver Province
5190005	1190132	2 years	0,0	OOO Plemzverocomplex Magistralny, Altai Territory
5190009	5200004	Age unknown	No pregnancy	Caught in Mariinsk, Kemerovo Province
5180001	Did not mate with females			

N o t e. Females of the Saltykovskaya 1 breed were used.

2. Whelping during matings of wild male sables *Martes zibellina* L. with females of different origins (OOO Zveroplemzavod Savvatyevo, Tver Province, 2022)

Male No.	Mated/gave birth	Female No.	Date of birth	Fertility	Died before registration	Offspring
5190011	8/6	2180488	14 April	5	0	5
		3120714	18 April	3	0	3
		3130566	05 April	4	1	3
		1150054	12 April	4	0	4
		1150338	02 April	6	0	6
		2150820	28 March	7	4	3
		3150516	No pregnancy			
		2180360	No pregnancy			
			M	4.8		3.0
5190001	3/3	1150142	09 April	6	0	6
		3160206	10 April	4	0	4
		3170006	14 April	4	0	4
			M	4.7		4.7
5200011	4/3	1150214	01 April	5	1	4
		2150620	No pregnancy			
		3150034	15 April	4	0	4
		4180174	19 April	4	0	4
			M	4.3		3.0

Continued Table 2

5190007	4/4	2150608	11 April	4	0	4
		3170380	17 April	4	0	4
		1190098	14 April	2	0	2
		5190002	14 April	4	2	0
			M	3.3		3.3
5190003	2/1	3150026	No pregnancy			
		3160032	19 April	4	0	4
			M	4.0		2.0
5200013	2/1	3150078	No pregnancy			
		3170454	11 April	4	0	4
			M	4.0		2.0
5200009	2/1	3150562	20 April	3	0	3
		1180150	No pregnancy			
			M	3.0		1.5
5190005	1/0	1190132	No pregnancy			
5180001	Did not mate with females					

N o t e. For animal numbers, see Table 1. Females of the Saltykovskaya 1 breed were used (female No. 5190002 was caught in the wild). M is the average offspring of a male from all covered females and the average yield of offspring. Fertility is the number of puppies (living and dead) of a successfully whelped female. Decline before registration is the number of puppies that did not survive to be weaned from their mother (45 days of age). Output is the number of puppies that survived until November 1 of the current year (counting date for registration).

3. Reproductiveness of purebred mothers (*Martes zibellina* L.) of the control and test progeny from mating with males of different origins (OOO Zveroplemzavod Savvatyevo, Tver Province, 2022)

Number	Group I (control)	Group II (test)
Successfully whelped females	18	18
Puppies	73	77
Average fertility	4.1	4.3
Puppies died before registration	2	6
Live puppies	71	71
Live puppies per successfully whelped females	3.9	3.9

N o t e. Females of the Saltykovskaya 1 breed were used. In the control group, 35 males and 30 females (purebred animals) were born, in the experimental group 39 males and 30 females (crossbred animals).

We noted the best average fertility indicators in females covered by male No. 5190011 (4.8 puppies) and male No. 519001 (4.7 puppies), good results were in females covered by male No. 5200011 (4.3 puppies). In terms of progrny per the main female (4.7 puppies), male No. 519001 was the leader. In male No. 5190011, due to two empty females and a 17.2% loss of young animals, the observed output decreased to 3 puppies, which may be due to the heavy load (the male covered 8 females with polygamy of the species 1:4).

Our results of mating of wild males with purebred females correspond to the reproduction rates of cage-bred sables [47, 48].

An assessment of the reproductive ability of purebred mothers from whom sables of the control and test groups were born in 2022 (Table 3) showed that with minor differences in average fertility and the number of puppies in favor of mothers of crossbred puppies from wild fathers, the number of dead crossbred puppies was higher. As a result, the yield of puppies by November and the average yield of puppies per successful female in both groups were the same (see Table 3).

4. Dynamics of live weight and measurements of sables *Martes zibellina* L. in the offspring from mating wild sables with sables of the Saltykovskaya 1 breed (*M*±SEM, OOO Zveroplemzavod Savvatyevo, Tver Province, 2022)

Group	Parameter	June	July	August	September	October	November
Group I (control):							
females	weight, g	637.5±25.5	855.9±21.4	994.2±15.6	1145.8±100.9	1120.7±16.1*	1138.7±25.4
(*n* = 30)	length, cm	30.5±0.5	37.3±0.7	40.7±0.3	41.9±0.3	42.6±0.2	42.4±0.3
	girth, cm	18.4±0.4	20.2±0.2	20.4±0.2	20.7±0.2	20.8±0.2	–
males	weight, g	679.8±34.8	967.6±34.5	1158.5±17.7	1221.4±14.0	1267.7±12.9	1279.6±17.8
(*n* = 35)	length, cm	30.9±0.6	37.7±0.8	43.2±0.37	44.7±0.3	45.7±0.3	45.6±0.5
	girth, cm	19.1±0.4	22.18±0.16	22.3±0.1	22.7±0.2	22.8±0.1	–

Continued Table 4

Group II (test):							
females,	weight, g	614.4±29.3	820.1±28.1	956.7±18.5	1022.8±14.8	1065.3±15.8	1111.3±18.7
(n = 30)	length, cm	30.3±0.4	36.4±0.8	40.2±0.4	41.7±0.3	42.7±0.2	42.9±0.2
	girth, cm	18.6±0.4	19.8±0.3	20.1±0.2	20.5±0.2	21.0±0.2	–
males,	weight, g	746.3±26.8	1048.0±33.5	1246.0±17.2**	1309.5±12.8***	1480.5±28.0***	1560.0±68.5***
(n = 39)	length, cm	32.1±0.2	38.9±1.0	44.0±0.4	45.4±0.3	46.5±0.3	47.8±0.6**
	girth, cm	20.3±0.4*	21.9±0.2	22.2±0.1	22.1±5.4	22.8±0.2	–

Примечание. Dashes in the table mean that no measurements were taken.
*, **, *** Differences between groups are statistically significant at $p \le 0.05$, $p \le 0.01$, and $p \le 0.001$, respectively.

5. Absolute and relative increase in body weight and size in sables *Martes zibellina* L. offspring from mating wild sables with sables of the Saltykovskaya 1 breed (M±SEM, OOO Zveroplemzavod Savvatyevo, Tver Province, 2022)

Group	Body weight gain		Body length increase	
	absolute, g	relative, %	absolute, cm	relative, %
		Aged 60 days		
I (control):				
females (n = 30)	4.6±0.1	16.2	0.11±0.03	9.12
males (n = 35)	6.4±0.2	19.7	0.18±0.03	14.59
II (test):				
females (n = 30)	4.6±0.1	16.7	0.13±0.05	10.44
males (n = 39)	6.6±0.2	18.9	0.17±0.04	13.11
		Aged 90 days		
I (control):				
females (n = 30)	5.1±0.2	15.2	0.04±0.02	2.95
males (n = 35)	2.1±0.1	5.4	0.05±0.01	3.47
II (test):				
females (n = 30)	2.2±0.1	6.9	0.05±0.02	3.73
males (n = 39)	2.1±0.1	5.1	0.05±0.02	3.18
		Aged 120 days		
I (control):				
females (n = 30)	–0.8±0.1	–2.2	0.02±0.01	1.67
males (n = 35)	1.5±0.1	3.8	0.03±0.01	2.24
II (test):				
females (n = 30)	1.4±0.1	4.2	0.03±0.01	2.40
males (n = 39)	5.7±0.2	13.1	0.04±0.01	2.42
		Aged 150 days		
I (control):				
females (n = 30)	0.6±0.1	1.6	–0.01±0.01	–0.47
males (n = 35)	0.4±0.1	0.9	0.00±0.00	–0.22
II (test):				
females (n = 30)	1.5±0.1	4.3	0.01±0.01	0.47
males (n = 39)	2.7±0.1	5.4	0.04±0.01	2.80

Crossbred males had the maximum average bodyweight at the beginning of the experiment (June) and until its end (November). In purebred males in June, the average bodyweight was 66.5 g less (the differences are not significant). In November, this difference in males of the test and control groups was 280.4 g ($p \le 0.001$). This is confirmed by the absolute and relative bodyweight gain (Table 5).

Crossbred females from group II at the beginning of the experiment were inferior in bodyweight to the control ones by 23.1 g (the differences are not significant). In purebred females, a sharp increase in bodyweight occurred from August to September, but in October this figure decreased and in November stabilized. The absolute and relative increase in bodyweight in females of the control group in September were –0.8 g and –2.2%, respectively (see Table 5). In crossbred females, the bodyweight changed more smoothly, without sudden jumps. In November, the control females exceeded the test ones in bodyweight by 27.4 g (the differences are not significant).

At the beginning of the experiment, males from group II exceeded males from the control group by 1.2 cm in body length (see Table 4, the differences are not significant). There were no significant fluctuations in body length in males of both groups from June to October. However, in October the crossbred males continued to grow, while in the control males the body length stabilized and by

November practically did not change. This is evidenced by the absolute and relative increase in body length (see Table 5). Initially, for males of the control group there were 0.18 g and 14.59%, respectively, for males of the test group 0.17 g and 13.11%. At the end of the experiment, the figures were 0 g and –0.22% for control males, and 0.04 g and 2.8% for test males. At the end of the experiment, the difference in body length between caged and crossbred males was 2.2 cm in favor of the latter ($p \leq 0.01$).

There was no significant difference in body length between control and test females throughout the study.

The data presented in Table 4 show that at the beginning of the experiment, crossbred males exceeded males in the control group in chest girth behind the shoulder blades by 1.2 cm ($p \leq 0.05$). However, from June to July, purebred males had a more accelerated increase in this trait value. Since July, in males of the control group, the chest girth behind the shoulder blades has stabilized; in crossbred males, it has decreased in September, which is apparently due to a decrease in bodyweight during this period. In control and crossbred females, the chest girth behind the shoulder blades increased until July, and then it stabilized.

It is known that the color of the awn and underfur is controlled by multiple genes. Very complex, polygenic inheritance of color in sable has not yet been sufficiently studied [1, 37]. In nature, the color of the fur varies from straw-yellow, sand, orange to pitch-black with many transitional options [10]. Currently, in accordance with OST 1010-86, the desired color type for dark brown sables is dark brown to almost black, uniform throughout the body. The down should be dark gray with a blue tint, evenly colored along the entire length of the hair. The mark for coloring is reduced if the tops of the down hair are dark brown, chestnut, the coverts are brown or light brown, the color of the sides and body is lighter, the color of the down is gray of varying intensity with light chestnut tops. When sorting sable skins at the Soyuzpushnina auction, the skin color is assessed by colorftion from 1 (darkest) to 10 (lightest), by tone (1 meams dark, 2 meams medium, 3 meams light) and shade (1 meams blue, 2 meams chestnut, 3 meams red). Gray hair is also assessed, dividing the skins into six categories, dull (without gray hair), 1 meams light gray, 2 meams gray, 3, 4, 5 meams bright gray [45].

We have changed the color requirements for crossbred sables, regarding the desired type. The color of the awn, tone (color of the down), shade (color of the tops of the down hair), gray hair, and throat patch were assessed separately. In grading young animals of groups I (control) and II (test) we studied the color of the hairline elements (Table 6).

The young animals of the control group had two variations in the color of the covering hairs, the almost black (47% in females, 81% in males) and dark brown (53% in females, 19% in males). In group II, in males and females, this trait varied within four color categories. In crossbred test females and males, dark brown color predominated (38 and 60%, respectively). The lightened color of the awns was more pronounced in crossbred females (31% brown, 23% light brown) than in males (28% brown, 8% light brown). The greatest diversity in the downy hair color occurred in males of the control and test groups (5 color categories identified). In females of the control group, 4 variations in the color based of the down were observed. The least rang of variation occurs in female of test group. The most common color variations were gray (40-73%) and dark gray (13-31%). Gray-brown coloration of the down base was observed in 16%, 8% and 7% of cases, respectively, in control males, crossbred males and control females. Gray with a blue tint down base was rare (4% of control and crossbred males). Thus, we did not find any significant differences in the color of the downy hair base in

control and crossbred sables, but males turned out to be the most variable in the coloration of the downy hair base compared to females.

6. Distribution (%) of hair color variants in the offspring from mating wild sables (*Martes zibellina* L.) with sables of the Saltykovskaya 1 breed (OOO Zveroplemzavod Savvatyevo, Tver Province, 2022)

Hair element	Color	Group I (control)		Group II (test)	
		females (n = 30)	males (n = 35)	females (n = 30)	males (n = 39)
Covering hair	Almost black	47	81	8	4
	Dark brown	53	19	38	60
	Brown	0	0	31	28
	Light brown	0	0	23	8
Down bases	Gray	73	45	69	40
	Dark gray	13	27	31	28
	Light gray	7	8	0	20
	Taupe	7	16	0	8
	Gray with a blue tint	0	4	0	4
Down tops	Plain	40	42	8	8
	Chestnut	53	31	53	32
	Brown	7	27	16	28
	Beige	0	0	15	4
	Orange tint	0	0	8	0
	whitish	0	0	0	12
	Light gray	0	0	0	16

Crossbred males expressed the greatest diversity in the color of the tops of downy hair (see Table 6). In animals of group II, six variations of this trait were found. In crossbred females from group II, five color variants were identified. In control males and females, the trait varied within three color categories. Brown coloration of the tops of downy hair was the most common in control and crossbred females (53% in both groups). Plain tops were most common in control males and females (42% and 40%, respectively). These data indicate that crossbred young sables (both males and females) are more variable in the color of the downy hair tops. The uniformity of the downy hair top color in control males and females indicates that the uniformity of down color is one of the main target trait when breeding dark sables. At all sable breeding farms the darkest individuals with underfur without zonal coloring were selected for many decades [31, 33, 38, 44].

In assessing the fur quality, hair color and additional traits (gray hair, throat patch) of crossbred young animals (Table 7), special attention was paid to the color parameters characteristic of wild sable (5-7 point color, tone 2 medium, down 2 with chestnut tops) which are now desired traits for breeding.

7. Results of assessment of crossbred sables *Martes zibellina* L. in the offspring from mating wild sables with cage-bred sables (OOO Zveroplemzavod Savvatyevo, Tver Province, 2022)

Quality		Color		Tone		Shade		Gray		Throat spot	
points	%	designation	%	points	%	points	%	points	%	points	%
Crossbred females (group II, n = 30)											
5	30.0	1	23.4	1	43.3	1	6.7	0	86.7	5	80.0
4	66.7	2	20.0	2	46.7	2	83.3	1	6.7	4	13.3
3	3.3	3	30.0	3	10.0	2/3	6.7	2	3.3	2	6.7
		4	23.3			3	3.3	3	3.3		
		6	3.3								
M = 4.26	100	M = 2.66	100	M = 1.67	100	M = 2.03	100	M = 0.23	100	M = 4.67	100
Crossbred males (group II, n = 38)											
5	42.1	1	7.9	1	26.3	2	94.7	0	89.5	5	81.6
4	50.0	2	21.0	2	50.0	2/3	5.3	1	10.5	4	2.6
3	7.9	3	44.7	3	23.7					3	2.6
		4	18.4							2	13.2
		5	5.4								
		6	2.6								
M = 4.34	100	M = 3.00	100	M = 1.97	100	M = 2.05	100	M = 0.10	100	M = 4.52	100

Grading of the crossbred young animals (see Table 7) showed that the quality of hair in males is 0.08 points higher than in females. In color, crossbred males are 0.34 points lighter than females and closer to the desired color. The resulting young animals were close to the desired sables in tone and were also consistent in shade (2.03 points for females and 2.07 points for males). Among the resulting crossbred males, there are fewer individuals with gray hair than among females (10.5% vs. 13.0%). In caged sables, for which the throat spot is undesirable, it is practically absent, but was passed on to the resulting youngs (average score for females 4.67, for males 4.52) (see Table 7).

It was previously shown that in pastel individuals the intensity and shades of pigmentation vary significantly. The color of the guard hairs is from very light brown to dark brown, sometimes similar to the usual dark sable, the underfur is from dark gray to light gray, almost blue, with the tops of the hairs from light brown to brown. Moreover, the color tone of the awn almost always correlates with the color of the underfur [39, 40]. Our experiment established a similar correlation.

8. Distribution (%) of color among young crossbred sables *Martes zibellina* L. at different types of mating of commercial sables with cage-bred sables (OOO Zveroplemzavod Savvatyevo, Tver Province, 2022)

Mating depending on the color of the awns of the parents (mother × father)	*n*	Awn color in young animals					
		1	2	3	4	5	6
1 × 1	6	83.3	0	0	16.7	0	0
1 × 2	13	23.1	30.7	23.1	23.1	0	0
1 × 6	26	0	11.5	65.4	19.3	3.8	0
1 × 7	4	0	0	0	25.0	25.0	50.0
2 × 2	10	20.0	60.0	20.0	0	0	0
2 × 6	5	0	20.0	40.0	40.0	0	0
3 × 6	4	0	0	50.0	50.0	0	0

In variants with heterogeneous selection of parents according to hair color (mother × father: 1 × 6, 1 × 7, 2 × 6, 3 × 6; Table 8), that is, when light males are mated with dark females, individuals predominate in the offspring have intermediate or lightened coloring of the guard hairs, characteristic of males. With homogeneous selection (1 × 1, 2 × 2, see Table 8) when dark females are mated with dark males, the resulting young sables have a darker hair color. However, with this type of selection, in addition to dark animals, individuals with the color 4 are selected, which corresponds to a light brown. It is obvious that the intensity of the color of the covering hair is inherited as a quantitative polygenic trait. The same was noted by G.A. Kuznetsov, K.V. Kharlamov [36] and G.A. Kuznetsov [37]. Sables are characterized by a lighter coloration of the head compared to the body, which is due to the action of natural suppressor genes that inhibit the development of pigmentation in the hair. Black-headed individuals, in which the color of the head and body do not differ, resulted from many years of selection for darkening the color of the fur at the Pushkinsky fur farm [36, 37].

The results of studies on producing lightened sable progeny (2004-2010) indicate that palomin and pastel colors are not caused by oligogenic mutations, but by the action of modifier genes that alter the degrees of lightening of the standard color. It is possible that modifiers are recessive polygenes with incomplete expression. Homozygosity for these genes ensures the birth of white puppies who die in the first days of life, and survivors with a smaller set (or reduced expression) of genes have a light pastel color [39, 40]. According to A.P. Nyukhalova et al. [38], the degree of darkening of the hairline is also controlled polygenically and is inherited as a recessive epistasis [38]. Since sables homozygous for black color genes obviously do not exist, it is natural that even the blackest individuals are carriers of part of the genes in a heterozygous state and, when mating with each

other, produce both their own types and various deviations. Light shades are, as a rule, found in animals heterozygous for color genes [38]. This means that previously, in order to preserve the existing color, animals heterozygous for recessive color genes, and sometimes for dominant ones, were constantly culled from the herd. This selection technology excludes the preservation of individuals with a different hair color. Therefore, it is natural that in fur farms until recently there were no sables with officially registered mutant color types [39]. The purpose of our research is to preserve and improve existing sable breeds and expand the range of competitive skin products that meet modern market requirements.

9. Correlation between the coloration of parents and crossbred young animals during mating of females of the Saltykovskaya 1 breed and wild male sables (*Martes zibellina* L.) (OOO Zveroplemzavod Savvatyevo, Tver Province, 2022)

Parent—offspring	*n*		Correlation coefficient
	parents	offspring	
Mothers—daughters	18	30	$r = 0.051$ ($p \geq 0.05$)
Mothers—sons	18	35	$r = 0.098$ ($p \geq 0.05$)
Fathers—daughters	8	30	$r = 0.72$ ($p \leq 0.001$)
Fathers—sons	8	35	$r = 0.61$ ($p \leq 0.001$)

The Table 9 shows that there is practically no correlation in color between mothers and daughters, and the same for sons (the color of the mother does not affect the color of the sons). Vice versa, the males influence the coloration of both sons ($r = 0.61$ at $p \leq 0.001$) and daughters ($r = 0.72$ at $p \leq 0.001$), and the correlation is even stronger for daughters. That is, males are better able to pass on hair color to their offspring.

In conclusion, the inheritance of lightened coloration in matings of wild and purebred sable must be further studied to find out what wild sable genotypes can ensure the desired hair color in the population of Savvatyevo Zveroplemzavod LLC. It is equally important to study in detail how the softness and silkiness of the hair is inherited.

Thus, in mating with wild sable males, the reproduction rates of purebred females are not lower than for cage-bred sables. On average, the yield per successfully whelped female for both groups was 3.9 puppies. In the resulting offspring, crossbred males were superior to purebred males in terms of growth (by 280.4 g at $p \leq 0.001$ and 2.2 cm at $p \leq 0.01$ for bodyweight and body length, respectively). Cage females slightly exceeded crossbreds in bodyweight (by 27.4 g, $p \leq 0.01$), but did not differ in body length and chest circumference behind the shoulder blades. Purebred offspring (males and females) of sables finished growing 2 months earlier than crossbred animals. Purebred young animals have two variations in the color of the covering hairs, almost black and dark brown. In crossbred males and females, the trait varies within four color categories (dark brown, brown, light brown, almost black). We did not find any significant differences in the color of the downy hair base between purebred and crossbred sables, but all males turned out to be more variable than females in the downy hair base coloration. Crossbred males and females are more variable in the color of the downy hair top. In control (purebred) males and females the observed uniformity of coloring downy hair tops indicates that uniformity of downy hair coloring is one of the main selection trait when breeding dark sables. Crossbred males have a higher quality of hair than females, the fur is lighter and closer to the desired color. A similar trend can be seen in tone. In terms of shade, crossbred females are more consistent with the target indicator than males. Among crossbred males there are fewer individuals with gray hair than among females. The throat spot has been transmitted to both females and males. The probability of obtaining the expected color in crossbred young animals is higher when light males are mated with dark females. The mother

color does not affect the color of daughters and sons, while fathers are better at transmitting their color to the offspring (for sons $r = 0.61$, for daughters $r = 0.72$; $p \leq 0.001$).

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THE ROLE OF FUNGI IN THE ETIOLOGY OF MASS SKIN LESIONS IN SABLES *Martes zibellina* L. 1758 IN TOMSK REGION

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Abstract

Mass skin lesions in sables *Martes zibellina* in Siberia have been known since the 18th century, but their etiology is still not well understood. The disease affects up to 62 % of hunted sables, causing damage to the skin and causing serious economic loss. One of the versions suggests the participation of microscopic fungi in the occurrence of this dermatosis. In the present work, it was established for the first time that keratinophilic dermatophyte fungi of various species are involved in the etiology of skin disease in sables, some of which were discovered in the territory of the Russian Federation for the first time. The aim of the work was to reveal and identify clinically significant fungi in sables with clinical manifestations of skin diseases. Pathological material (hair, crusts) was taken from the affected areas of the skins of wild sables (*Martes zibellina* L. 1758), hunted during the 2018-2019 hunting season in various areas of the Tomsk region. A total of 28 samples of pathological material were studied. Mycological examination included a Wood's lamp test, direct microscopy of the pathological material, inoculation on mycological media, followed by identification of isolated fungal cultures. Inoculation was performed on DTM-Expert, a differential diagnostic medium for dermatophytes (FNTs VIEV RAS, Russia) and on Sabouraud medium with chloramphenicol (HiMedia Laboratories Pvt. Ltd., India). The incubation was carried out under aerobic conditions at 26-28 °C, the incubation period was up to 21 days. To study the cultural and morphological features, the cultures were re-inoculated on Sabouraud agar in Petri dishes, incubated for 10-14 days. For molecular genetic identification, isolated colonies of fungi grown on Sabouraud's medium were selected for 10 days at 26-28 °C. DNA was isolated using the GeneJET Plant Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The resulting DNA was used to carry out the polymerase chain reaction (PCR). Regions of the internal transcribed spacer (ITS) of the ribosomal RNA gene were sequenced. Phylogenetic analysis of the obtained nucleotide sequences was performed using the SeqMan application (DNASTAR Lasergene v.7.1.0, https://www.dnastar.com/software/lasergene/). Sequence alignment with those available in the GenBank database was performed using the Standard Nucleotide BLAST software package (http://www.ncbi.nlm.nih.gov/BLAST/). The resulting nucleotide sequences of particular interest were deposited to the GenBank NCBI database. When examining the affected sables, skin lesions were found, which were localized mainly in the back, waist,

and sides. They were characterized by loss of guard hairs, alopecia, formation of crusts and scabs. Dark spots were often observed in the area of lesions from the side of the skin. Lesions were observed both in males and females, mainly in young animals. During visual examination, samples of pathological material were sticky bundles of hair (downy, less often guard hair) with dried crusts and scales at the base. The result of the fluorescent test with a Wood's lamp in all samples was negative. Microscopy revealed bundles of downy hairs stuck together and a large amount of purulent debris, which made it difficult to detect fungal elements. As a result of cultural mycological analysis, 51 cultures were isolated, 18 taxa (species and genera) of fungi were identified. At the same time, keratinophilic dermatophyte fungi (*Arthroderma cuniculi*, *Chrysosporium carmichaelii*, *Chrysosporium* spp.) were isolated from 12 % of the samples, probably acting as etiological agents of dermatosis. Growth of dermatophytes was observed only on the DTM-Expert selective differential diagnostic medium; fast-growing non-dermatophyte fungi grew on ordinary media. Non-dermatophyte fungi with keratinolytic properties were also isolated — *Scopulariopsis brevicaulis* (16 %), *Acremonium* spp. (14 %), *Aspergillus* spp. (36 %), which can act as secondary opportunistic pathogens.

Keywords: pathogenic fungi, animal mycoses, dermatomycoses, dermatophytes, *Arthroderma*, *Chrysosporium*, *Martes zibellina*, sable

In recent decades, a sharp increase in the incidence of opportunistic mycoses has been noted among domestic and wild animals. Some mycoses are highly contagious, capable of causing death, and affecting large populations in nature [1]. Typical examples are amphibian chytridiomycosis, white nose syndrome (WNS) in bats, and snake fungal disease (SFD) [2-4]. Mycogenic infections cause significant damage to the biodiversity of natural ecosystems. When commercial animals are affected, mycoses also lead to significant economic losses.

Therefor, a detailed study of the mycobiota of wild animals which can be both contaminants and saprobionts, and potentially pathogenic species of fungi (pathobionts), becomes important. Research on the human and animal microbiome has focused on prokaryotes, while fungi have been studied to a much lesser extent [5]. The species composition of the mycobiota of wild animals in the Russian Federation and abroad has not been sufficiently studied [6].

Some representatives of animal mycobiota can be dangerous to humans, that is, they have epidemiological significance. Thus, pathogens of adiaspiromycosis are capable of causing respiratory mycoses [7]. In 2015, a new species of *Emmonsia* spp. was discovered, causing not only respiratory but also disseminated mycoses, and a sharp increase in incidence was noted (8). In 2018, one of the first ecological-epidemiological studies to detect fungal pathogens in wild animals was undertaken in Brazil. Fungi were found in 102 of 1063 samples, including pathogenic species in 89 samples [9].

In Russia, the *Mustelidae* species with valuable fur, including sable (*Martes zibellina* L. 1758), are of great commercial importance.

The first mention of a skin disease in free-ranging sables in Siberia was found back in the 18th century, but its etiology remained unclear for many years. The disease is recorded throughout the species' range. The only Russian work by N.D. Stepanenko written in the late 1960s, and devoted to mycogenic infections of wild sables was closed to readers for a long time and republished only in 2007 [10]. It describes a massive fungal infection of sables, which occurs in the form of chronic skin lesions and causes enormous economic damage to the fur trade. N.D. Stepanenko and his colleagues were the first to conduct a mycological study of affected skins. In most samples, fungi of the genus *Cephalosporium* (syn. *Acremonium*) were isolated which were indicated as the causative agents of the disease.

In the 1960s, skin defects known as "pockmarks" affected up to 70% of wild Siberian sables [10]. Currently, skin lesions are still widespread. O.Yu. Tyutenkov et al. [11] inspected more than 2 thousand sable skins in procurement organizations and coududcted a questionnaire survey of hunters. In the bulk sample, a significant proportion of affected skins was identified, 53.5±1.1% [11]. According to the authors' report, massive skin lesions of sables are caused by fungi, but

proper mycological diagnostic studies have not yet been carried out.

This work for the first time finds out that the etiology of skin disease in sables involves keratinophilic dermatophyte fungi of various species some of which were discovered for the first time in the Russian Federation.

The goal of the work was detection and identification of clinically significant microscopic fungi in sables with signs of skin diseases.

Materials and methods. Pathological material (hair, crusts) was taken from the affected areas of the skins of wild sables (*Martes zibellina*) during hunting season of 2018-2019 in various areas of the Tomsk region, 3 samples from Kolpashevo District, 19 samples from Bakcharsky District, 6 samples from southern regions (Tomsk, Kozhevnikovsky and Shegarsky). A total of 28 samples of pathological material were studied.

Mycological examination included a Wood's lamp test, direct microscopy of pathological material, inoculation on mycological media, followed by identification of isolated fungal cultures. Inoculation was carried out on the differential diagnostic medium for dermatophytes DTM-Expert (FSC VIEV RAS, Russia) and on Sabouraud's medium with chloramphenicol (HiMedia Laboratories Pvt. Ltd., India).

The cultures were incubated under aerobic conditions at 26-28 °C up to 21 days. To study cultural and morphological traits, cultures were reseeded by stab-inoculation into Sabourau's agar in Petri dishes and incubated for 10-14 days. Crushed drop preparations of fungal cultures were microscoped in a dry Micro-optix MX 100 microscope system (West Medica, Austria) at a magnification of ×100 and ×400.

Species phenotypic identification of fungi was carried out with the key [12].

For direct microscopy, pathological material were prepared in a 15% solution of potassium hydroxide (KOH) and microscoped at ×100 and ×400.

For molecular genetic identification, individual colonies of fungi grown on Sabouraud medium for 10 days at 26-28 °C were selected. DNA was isolated using the GeneJET Plant Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The resulting DNA was used for polymerase chain reaction (PCR).

The internal transcribed spacer of ribosomal RNA (ITS) were amplified with primers (5′→3′) ITS1Fwfun TTGGTCATTTAGAGGAAGTAAAAGTC, ITS1Rvfun CTGCGTTCTTCATCGATGC. Amplification (an amplifier DTprime 5, DNA-Technologies LLC, Russia) was run as follows: 5 min at 94 °C; 15 min at 94 °C, 20 s at 50 °C, 20 s at 72 °C (35 cycles); 5 min at 72 °C.

Regions of the internal transcribed spacer of the ribosomal RNA gene (ITS) were sequenced with the same primers using the Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, USA).

For double-strand sequencing, the same primers were used as for PCR. The nucleotide sequence was determined (an ABI Prism 3100 automatic sequencer, Applied Biosystems, USA) according to the manufacturer's instructions.

Phylogenetic analysis of the obtained nucleotide sequences was performed with the SeqMan application (DNASTAR Lasergene v.7.1.0, https://www.dnastar.com/software/lasergene/). The Standard Nucleotide BLAST software package (http://www.ncbi.nlm.nih.gov/BLAST/) was used to align sequences with those available in the GenBank database. Nucleotide sequences of particular interest have been deposited in the NCBI GenBank database https://www.ncbi.nlm.nih.gov/genbank/).

Results. When examining the sable skins, lesions were discovered, which were localized mainly in the back, lower back, and sides. They were characterized by loss of guard hairs, alopecia, crusts and scabs formation. Hairline was of uneven length, hair pulled out in clumps. Dark spots were often observed in the area of

lesions on the mesternal side. Some of the lesions were hidden by the undercoat and were revealed by palpation (tubercles, crusts). Lesions were observed in both males and females, mainly in young animals.

Upon visual examination, samples of pathological material were sticky tufts of hair (downy, less often guard hair) with dried crusts and scales at the base. The result of the Wood's lamp fluorescent test was negative in all samples.

During microscopy, tufts of sticky downy hair and a large amount of purulent debris were observed, making it difficult to detect fungal elements, which is why spores and fungal mycelium could not be detected in the samples.

By cultural mycological analysis of 28 samples of pathological material, fungi were isolated from 25 samples (89.2%). In total, 51 cultures were isolated, 18 taxa (species and genera) of fungi were identified (Fig. 1).

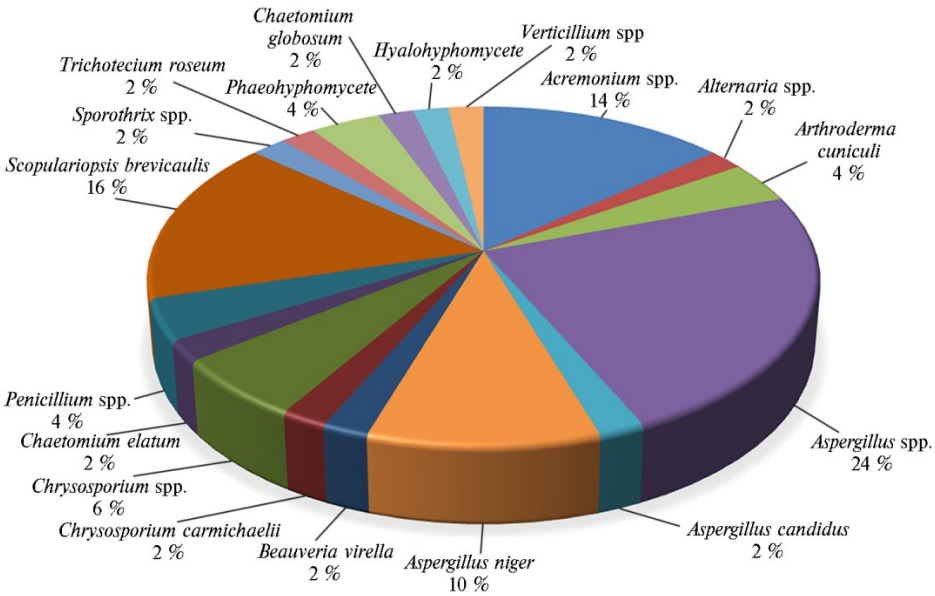

Fig. 1. Taxonomic composition and percentage of fungi isolated from samples of pathological material from affected areas of wild sable skins (*Martes zibellina* L. 1758) (Tomsk Province, 2018-2019)**.**

The mycobiota of skin lesions was dominated by fungi of the genus *Aspergillus* (36%, *Aspergillus* spp. 24%, *A. niger* 10%, *A. candidus* 2%). The most common species also were *Scopulariopsis brevicaulis* (16%) and *Acremonium* spp. (14%). In addition, several species of keratinophilic dermatophyte fungi have been isolated, e.g., *Arthroderma cuniculi* (4%), *Chrysosporium carmichaelii* (2%), and *Chrysosporium* spp. (6%). In total, the proportion of keratinophilic fungi was 12%. The share of other fungal taxa is 2-4% (*Alternaria* spp., *Beauveria virella*, *Chaetomium elatum*, *Chaetomium globosum*, *Penicillum* spp., *Sporothrix* spp., *Trichotecium roseum*, *Verticillium* spp., *Phaehyphomycete*, *Hyalohyphomycete*).

The occurrence of fungi ranged from 0 to 4 species per sample. From most samples, two species were identified. Of particular interest are two isolates of dermatophyte fungi (isolates No. 1.1-19 and No. 3.11-19), initially identified by morphological characteristics as *Trichophyton* spp. Both cultures were isolated from sables of the Bakchar region. On days 7-9 of growth, both isolates caused reddening of the DTM-Expert medium and formed white velvety-powdery colonies characteristic of dermatophyte fungi (Fig. 2).

When subcultured by stab-inoculation into Sabouraud's agar, white velvety-woolly colonies, slightly convex, with ciliated, hyaline edges, were observed. The reverse side of the colonies was light brown. The diameter of the colonies was

30-40 mm on day 14 of growth. Microscopy revealed hyaline branching mycelium and numerous oval and drop-shaped unicellular microconidia. Multicellular spores (macroconidia) were not observed. During long-term incubation of cultures, the formation of spirally convoluted hyphae was observed.

Fig. 2. Growth of the dermatophyte *Trichophyton* spp. No. 3.11-19, isolated from samples of pathological material from the affected areas of the skins of wild sables (*Martes zibellina* L. 1758), on the selective medium DTM-Expert.

To clarify the species identity, molecular genetic identification of *Trichophyton* spp. isolates No. 1.1-19 and No. 3.11-19 was carried out by sequencing the ITS region (Table).

According to ITS sequencing, both isolates of *Trichophyton* spp. (No. 1.1-19 and No. 3.11-19) showed the greatest homology (100 and 97.7%, respectively) with the strain *Trichophyton* spp. IFM 41172, isolated from a badger. The isolates showed significant similarity with the strain *Trichophyton* spp. NWHC 44736-43-02- 01B, isolated from a gopher snake. The third closest homologue was *Arthroderma cuniculi* CBS 492.71, isolated from a human (see Table). Thus, the closest homolog identified to species for both isolates is *Arthroderma cuniculi.*

As can be seen from the dendrogram based on sequenced ITS regions of *A. cuniculi* isolates No. 1.1-19 and *A. cuniculi* No. 3.11-19 (Fig. 3), they form a separate cluster together with the strain *Trichophyton* spp. IFM 41172, adjacent to clusters of three *A. cuniculi* and two *A. tuberculatum* strains.

Another isolate (No. 3.5-19), presumably a dermatophyte, was phenotypically identified as *Chrysosporium* spp. When growing on Sabouraud's agar, white, uniformly colored colonies, velvety fluffy, convex, with radial folding, were observed. The edges were smooth. The reverse side of the colonies was yellow to light brown, with radial furrows. The diameter of the colonies on day 14 of growth was 30-40 mm. The culture produced numerous oval and round microconidia characteristic of the genus *Chrysosporium*. When sequencing the ITS region, an isolate of *Chrysosporium* spp. No. 3.5-19 showed 100% homology with the strain *Chrysosporium carmichaelii* E00083342, isolated from a human nail (GenBank: KC923439.1, https://www.ncbi.nlm.nih.gov/nucleotide/KC923439.1).

In addition to the three mentioned cultures, three more were identified by morphological features as *Chrysosporium* spp., however, their species identity requires further molecular clarification.

The ITS sequences of three dermatophyte isolates identified by sequencing were deposited in the GenBank NCBI under numbers MN534766.1 (*A. cuniculi* 1.1-19), MN653980.1 (*A. cuniculi* 3.11-19), MT556012.1 (*C. carmichaelii* 3.5-19).

The clinical signs of skin lesions in sables in the present study is in many ways similar to the description previously made by other authors. The lesions were characterized as "pockmarks," "bald patches", "scabs", "cut and matted hair", and hair loss in tufts [10]. Such descriptions are characteristic of a chronic inflammatory process. Direct microscopy of the pathological material was uninformative due to the large amount of purulent debris. The cultural method of mycological analysis showed a significant species diversity of fungi that make up the mycobiota of skin lesions of wild sables. The vast majority of the studied samples (89.2%) contained from 1 to 4 species of fungi. Moreover, 88% of the isolated fungi turned out to be non-dermatophytic molds. Obviously, they play the role of contaminants for the skin of sables. Scopulariopsis brevicaulis was a common species (16%). This species, although not a dermatophyte, has keratinolytic activity [13] and is capable of causing superficial mycoses in humans [14] and animals 15]. Isolation may indicate its certain etiological significance as an opportunistic pathogen.

Results of BLAST analysis of ITS sequences of *Trichophyton* spp. isolates No. 1.1-19 and No. 3.11-19 from affected areas of wild sable skins (*Martes zibellina* L. 1758) (Tomsk Province, 2018-2019)

Closest strain	Homology, %		Accession number in GenBank	Isolated from
	isolate No. 1.1-19	isolate No.3.11-19		
Trichophyton sp. IFM 41172.	100.00	97.73	AB458161.1 https://www.ncbi.nlm.nih.gov/nuccore/AB458161.1	Badger (*Meles meles*)
Trichophyton sp. NWHC 44736-43-02-01B	93.24	92.56	KX148667.1 https://www.ncbi.nlm.nih.gov/nuccore/KX148667.1	Bullsnake (*Pituophis catenifer*)
Arthroderma cuniculi CBS 492.71	90.59	90.94	NR_077138.1 https://www.ncbi.nlm.nih.gov/nuccore/NR_077138.1	Human, skin lesions

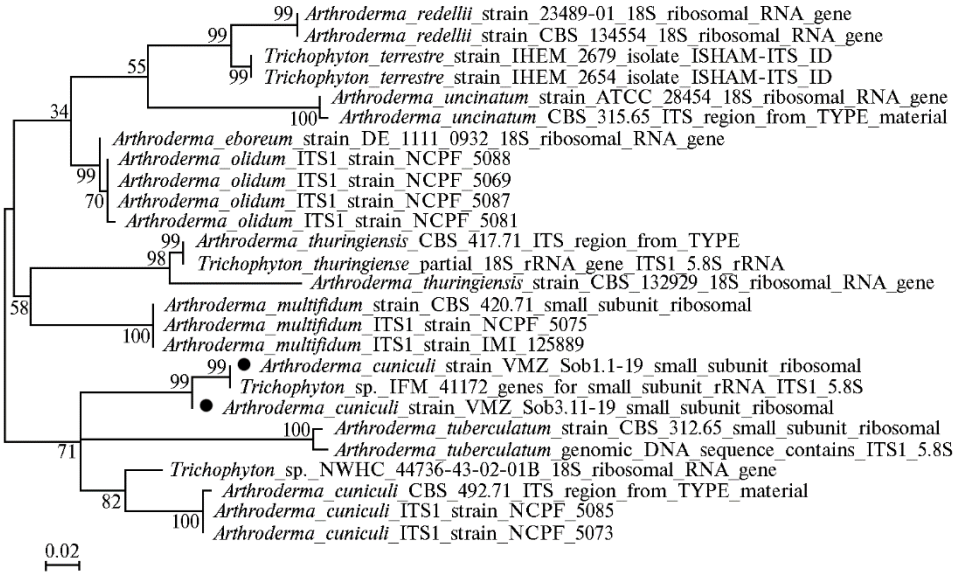

Fig. 3. Phylogenetic dendrogram based on ITS region sequencing of isolates *Arthroderma cuniculi* No. 1.1-19 and *A. cuniculi* No. 3.11-19 (marked with circles) **from affected areas of wild sable skins (*Martes zibellina* L. 1758)** (Tomsk Province, 2018-2019)

Representatives of the genus *Acremonium* (14% of pool) deserve special attention. Samples from some animals contained two morphotypes of *Acremonium* spp. (white and yellow morphotypes). Their species identity requires clarification. Fungi of the genus *Acremonium* also have keratinolytic activity [16] and cause diseases in humans [17] and animals [15].

In the study by N.D. Stepanenko [10], it was the fungi of the genus *Acremonium* (according to the old nomenclature *Verticillium*) that were isolated from most samples and recognized as the main etiological factor of the disease. In our opinion, this taxon may play a certain role in the pathogenesis of the disease, but does not serve as the main (primary) causative agent.

The most likely pathogens leading to skin lesions are keratinophilic dermatophyte fungi (genus *Onygenales*) with 12% share in the mycobiota. Two detected isolates of *Arthroderma cuniculi* form a separate cluster along with the strain *Trichophyton* sp. IFM 41172 from a badger. The molecular genetic characteristics of the *A. cuniculi* strains we isolated and their taxonomic position require further study.

The genus *Arthroderma* (family *Arthrodermataceae*) is the largest genus of dermatophyte fungi, which includes 27 species [18]. The species *A. cuniculi* is poorly studied, and its ecological niche and clinical significance are not yet entirely clear. It was isolated and first described in 1963 [19]. It was isolated in several cases both from wool and from soil in the habitats of animals, in particular hares (https://www.ncbi.nlm.nih.gov/nuccore/KT155576.1). Phylogenetical relatives have been isolated from snake skin lesions (https://www.ncbi.nlm.nih.gov/nuccore/KX148667.1) and also from human in case of dermatophytosis lesions (https://www.ncbi.nlm.nih.gov/nucleotide/NR_077138.1), indicating the pathogenic potential of *A. cuniculi*. In our opinion, this species may be the main etiological factor in skin infections in sables. We discovered *A. cuniculi* for the first time in Russia, and for the first time it was isolated from sables. It is possible that *A. cuniculi* is zooanthropophilic and poses a threat to humans, like many other dermatophyte species that infect animals [20].

In addition, keratinophilic fungi of the genus *Chrysosporium* were isolated from sables. One isolate, the *Chrysosporium carmichaelii* was identified to species

by sequencing. The species identity of three other isolates of *Chrysosporium* spp. requires clarification. The ecology of the species *C. carmichaelii* has not been sufficiently studied. It has been isolated from soil and dust [21], from bats, and from a human fingernail (https://www.ncbi.nlm.nih.gov/nucleotide/KC923439.1), indicating its pathogenic potential. Fungi of the genus *Chrysosporium* are known as causative agents of superficial and deep mycoses in humans and animals [22] and can be considered as possible causative agents of skin infections in sables. According to available publications, here we submit the first report of the detection of *C. carmichaelii* isolation in the Russian Federation.

Importantly, keratinophilic dermatophyte fungi, such as *A. cuniculi* and *Chrysosporium* spp., are fastidious and slow growing in culture; their growth is easily suppressed by fast-growing molds. Cultures of *A. cuniculi* were obtained only on the selective medium for dermatophytes DTM-Expert, recently developed at the Federal Scientific Center All-Russian Institute of Experimental Veterinaty RAS (Moscow) [23]. Most cultures of *Chrysosporium* spp. were also isolated on the DTM-Expert medium, while on the standard Sabouraud's medium their growth was apparently inhibited by fast-growing molds.

We hypothesize that due to the lack of selective media for dermatophytes N.D. Stepanenko [10] failed to isolate dermatophyte fungi from sables, but found only non-dermatophyte fungi with a predominance of the genus *Acremonium* which were taken as infectious agents. Although in our work the proportion of dermatophyte fungi was 12%, in reality their prevalence in sables may be higher, but not in all cases such fungi can be isolated from clinical material. The true distribution of pathogenic fungi can be further studied using modern highly sensitive diagnostic techniques, in particular metagenomic sequencing [24].

There are only a few publications devoted to the study of the mycobiota of mustelids in terms of various diseases. In Czechoslovakia, a high prevalence (from 30 to 73%) of adiaspiromycosis, a respiratory disease caused by species of the genus *Emmonsia* (formerly classified as genus *Chrysosporium*), was diagnosed among mustelids. Main pathogen *Emmonsia parva* is a typical saprotroph that lives in soil and on plant debris [25]. In the UK, adiaspiromycosis in the wild was diagnosed in almost a third (28%) of animals of different species examined; the main pathogen detected was *Emmonsia crescens*, which can also infect humans [26]. It cannot be excluded that species of the genus Emmonsia, morphologically close to the genus *Chrysosporium*, also circulate in the population of Siberian sables, which may be revealed in further studies.

It should be noted that in world literature, infectious diseases of sables are covered very poorly, since in nature this species lives only in Russia, Kazakhstan, Mongolia, China, Korea and Japan.

Thus, from sables with skin lesions caught in the Tomsk region, representatives of 18 different taxa of fungi were isolated, including keratinophilic dermatophyte fungi (*Arthroderma cuniculi*, *Chrysosporium carmichaelii*, *Chrysosporium* spp.), probably acting as etiological agents of mass dermatosis. The species *A. cuniculi* and *C. carmichaelii* have not been previously diagnosed in the Russian Federation, and this is the first report of their occurrence in sables. Non-dermatophytic fungi with keratinolytic properties (*Scopulariopsis brevicaulis*, *Acremonium* spp., *Aspergillus* spp.) may play the role of secondary opportunistic pathogens in this disease. Studies have demonstrated the presence of dermatophyte fungi in the wild, indicating the need for further investigation of the prevalence of clinically significant fungi in wild animals. It is advisable to develop a set of measures to combat fungal infections of wild animal that cause significant economic damage.

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BLOOD BIOCHEMICAL PARAMETERS IN RESERVOIR HOSTS UNDER IXODID TICK-BORNE BORRELIOSIS

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Abstract

Currently, there is a wide distribution of natural foci of ixodid tick-borne borreliosis (ITB, Lyme disease) in Europe, Asia, Australia and America, as well as high infection rate of people and animals. Studies have shown that this new natural focal infection occupies a leading position in terms of morbidity and socio-economic damage. Many pathogenic microorganisms, including *Borrelia burgdorferi*, which cause disease in humans and animals, persist in certain natural foci. Ticks feed on different animals at different stages of development. The size of the tick population mainly depends on the number of adult reservoir hosts. The research, for the first time, revealed in ITB infection reservoir hosts with diagnostically significant titers of antibodies to *B. burgdorferi* the blood serum biochemical parameters which indicate the pathological effect of the pathogen on the body of wild animals, the development of multiple organ failure and harm to their health. The aim was to study the natural foci of ixodid tick-borne borreliosis and to obtain new data on the epizootology of the disease, including an assessment of the effect of parasitism of ixodid ticks infected with *B. burgdorferi* on the blood chemical composition of reservoir hosts, the mountain hare and moose. Blood serum from adult males of mountain hare (n = 11) was used, including 5 samples that had diagnostically significant titers of antibodies to *B. burgdorferi* in the indirect immunofluorescence assay (IDIF) (1:40 and 1:80), and 6 samples that did not have diagnostically significant titers of antibodies to *B. burgdorferi* (control). We also studied blood serum from moose (n = 114) of different sex and age groups (animals aged 6-7 months and adults), including 24 samples with diagnostically significant titers of antibodies to *B. burgdorferi* and 90 samples from clinically healthy animals (Kirov region), including individuals whose sera in the IDIF did not have diagnostically significant titers of antibodies to *B. burgdorferi*. Animals were hunted during scientific shooting during the autumn hunting seasons of 2005-2020. Blood samples for laboratory studies were taken from the jugular vein immediately after the animal was shot. Anti-borreliosis antibodies in blood serum was detected in IDIF test using *B. afzelii* corpuscular antigen (strain Ip-21) and fluorescein isothiocyanate (FITC) labeled luminescent immune serum against globulins of various animal species (rabbit, dog, bull, pig, chicken). Biochemical studies of blood serum were performed using a semi-automatic analyzer Biochem SA (High Technology Inc., USA) with a set of reagents (Eco-Service, Russia) to measure the concentration of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, lactate dehydrogenase (LDH), alpha-amylase, total protein, albumin, total bilirubin, direct bilirubin, creatinine and cholesterol. It was found that the animals with diagnostically significant titers of antibodies to *B. burgdorferi* had statistically significant ($p < 0.05$) differences in the activity of AST, ALT, alkaline phosphatase, the content of total protein, total and direct bilirubin, creatinine. Thus, in a hare with diagnostically significant titers of antibodies to *B. burgdorferi*, AST activity was 342.2 % higher compared to animals without titers. An increase in AST activity in moose was noted, by 35.0 % in young females, by 35.3 % in young males, by 31.2 % in adult females, and by 24.0 % in adult males. ALT activity in hare with diagnostic titers to *B. burgdorferi* was 32.8 % higher compared to the control. An increase in ALT activity was also found in moose,

by 53.8% in young females, by 90.4 % in young males, by 188.6 % in adult females, and by 173.9 % in adult males. In hare, the value of the de Ritis coefficient testified to the predominance of the heart pathology, and in moose, on the contrary, to liver damage. An increase ($p < 0.05$) in the activity of alkaline phosphatase during borreliosis in adult moose was noted, by 132.5 % in females and by 206.3 % in males, and a decrease in the enzyme activity in young females. In hare, an increase in the content of total bilirubin by 42.4 % was revealed, in young female moose by 86.1 %, in young males by 121.9 %, in adult females by 118.8 %, in adult males by 70.4 %. In addition, the content of direct bilirubin increased in male moose, by 59.1 % in young and by 102.8 % in adults. The amount of total protein in all groups of animals with diagnostically significant antibody titers to *B. burgdorferi* increased: in hare by 123.6 %, in young female moose by 24.3 %, in young males by 53.5 %, in adult females by 76.7 %, in adult males by 19.9%. In hares with diagnostic titers of antibodies to *B. burgdorferi*, the renal failure led to a 40.7 % increase for creatinine, the end product of metabolism. Univariate analysis (ANOVA) allowed us to establish a significant effect of *B. burgdorferi* on the increase in serum concentrations of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total protein, total and direct bilirubin, and creatinine. The data obtained indicate multiple organ failure in reservoir hosts under borreliosis and pathological effects of *B. burgdorferi* on animals.

Keywords: *Borrelia burgdorferi,* ixodid ticks, reservoir hosts, *Alces alces*, *Lepus timidus*, blood biochemistry, blood serum

Ixodid tick-borne borreliosis (ITB, Lyme disease) is a relatively new group of naturally focal vector-borne infectious diseases. The pathogen was discovered in 1982 [1]. ITB is a typical spirochetosis with the clinical and pathogenetic features of this group of infectious diseases. The ability of spirochetes to persist for a long time in the body of humans and animals leads to the formation of a chronic process that occurs with systemic damage to organs [2, 3].

The pathogen enters the body with the saliva of the tick. Its primary accumulation occurs in the basal and papillary layers of the epidermis, which is accompanied by vascular changes and manifests itself as erythema migrans, which sometimes serves as the only marker of the acute period of the disease [3]. Skin lesions (pathognomonic signs of the disease in humans) in sick animals are observed only in isolated cases due to the presence of fur and skin pigmentation.

As Borrelia accumulate, they spread hematogenously and lymphogenously from the primary focus throughout the body, entering internal organs and other areas of the skin (secondary erythema). Generalization of the infection is clinically accompanied by symptoms of general intoxication and damage to various organs (brain and spinal cord with involvement of the meninges, liver, kidneys, heart, spleen, muscles, joints, lymph nodes in the inflammatory process) [3].

Currently, for the countries of Europe, Asia, Australia and America, the relevance of ITB is determined by the wide distribution of natural foci, as well as the high infection rate of people and animals. Studies have shown that this new natural focal infection occupies a leading place in terms of morbidity and socio-economic damage [2-4].

To date, no specific prevention measures have been developed for the disease, and in nature, ixodid ticks have practically no natural enemies. As a result, only the human factor remains the main regulator of the numbers of these arthropods [5)]. On the territory of the Russian Federation, a decrease in the area of acaricidal treatments is the main reason for the epidemiological problems with tick-borne infections [6]. In addition, the expansion of borreliosis foci is caused by the reduction of arable land, the abandonment of intensive agriculture, and the increase in suburban construction and landscaping of urban areas. There is a mosaic growth of forests and the formation of a favorable environment for ixodid ticks and their hosts [7-10].

Many pathogenic microorganisms, including *Borrelia burgdorferi*, which cause diseases in humans and animals, persist in the body of reservoir hosts. This reservoir function is closely related to the association between the animal species and the pathogen, which must remain viable without interfering with the survival

of the host. The specificity of arthropod vectors for different animal species is also important in the transmission of the pathogen and is of particular importance for the development of a predictive model of disease risk.

At different stages of development, ticks feed on different types of animals. Tick-borne pathogens are transmitted to susceptible organisms from small to medium-sized mammals and birds (mainly by nymphs and larvae), while tick population size is primarily dependent on the number of adult reservoir hosts. Medium-sized mammals such as the mountain hare (*Lepus timidus*, Linnaeus 1758), and large ungulates such as moose (*Alces alces*, Linnaeus 1758), white-tailed deer (*Docoileus virginianus* Zimmermann, 1780), cattle (*Bos taurus taurus* Linnaeus, 1758) and horses (*Equus caballus* Linnaeus, 1758), serve as feeders for all stages of tick development. Large ungulates are the primary food source for adults and are not capable of pathogen accumulation but are nonetheless important for pathogen transmission because they provide food for large numbers of adult females, contributing to increased tick numbers [11]. Large wild and domestic animals are considered incompetent reservoirs, meaning ticks that feed on them can infect each other when feeding together [12]. In addition, incompetent reservoirs serve as a supporting reservoir for all stages of mite development. Reducing the population of incompetent reservoirs can reduce potential transmission, the prevalence of Borrelia, and the risk of disease in humans.

Incompetent reservoirs determine the increase in tick numbers in the area where they live, and if competent hosts also have high numbers, the risk of disease in humans increases significantly. Studies have shown that in areas inhabited by European roe deer (*Capreolus capreolus* Linnaeus, 1758) and cattle, Ixodes ticks are found in greater numbers [11, 13-15] and the number of reported cases of borreliosis is higher [16]. In general, for a correct and complete interpretation of the epizootology of any anthropozoonotic disease, the causative agent, vector and reservoir hosts should be considered as environmental system. Humans are always incidental hosts, and their risk of infection is based on the presence of competent and incompetent reservoir hosts [17, 18].

Biochemical blood parameters are widely used to assess the condition of the body of mammals, determine the presence of parasites [19], and can also indicate the state of the feed supply [20]. L.B. Keith et al. [21] and I.M. Keith et al. [22] studied parasites of the American hare (*Lepus americanus* Erxleben, 1777) in North America. V. Haukisalmi et al. [23, 24] studied parasites of voles in northern Finland at different stages of the population cycle. Their results suggest that parasites do not have any obvious effect on population cycles.

Borrelia persistence has been confirmed in some vertebrate species. Bacteremia is subject to changes that depend on the health of the host and the viability of the pathogen [18]. According to D.C. Duffy et al. [25] and T. Boulinier et al. [26], if a large number of infected ticks parasitize reservoir hosts, the reproductive dynamics of host populations may be disrupted.

At present, the pathological effect of Borrelia on the human body and some species of domestic animals has been well studied, but it has not been established whether *B. burgdorferi* has any effect on the body and on the population of hosts and reservoir hosts of ixodid ticks as a whole. In the literature available to us, we have not found data regarding the effect of *B. burgdorferi* on competent and incompetent reservoir hosts, including on biochemical blood parameters.

In this work, for the first time, biochemical parameters of blood serum were established in reservoir hosts with ITB, which have diagnostically significant titers of antibodies to *B. burgdorferi* that indicate the pathological influence of the pathogen on the body of wild animals, the development of multiple organ failure

and harm to health.

Our goal was to study the natural foci of ixodid tick-borne borreliosis and obtain new data on the epizootology of the disease, including assessing the effect of parasitism of ixodid ticks infected with *Borrelia burgdorferi* on the blood chemical composition of the reservoir hosts, the mountain hare and moose.

Materials and methods. Blood serum from adult male white hare (n = 11) was used, including 5 samples that in the indirect immunofluorescence assay (IDIF) had diagnostically significant titers of antibodies to *B. burgdorferi* (1:40 and 1:80), and 6 samples that did not have diagnostically significant antibody titers to *B. burgdorferi* (control). We also examined blood serum from moose (n = 114) of various sex and age groups (young animals aged 6-7 months and adults), including 24 samples with diagnostically significant antibody titers to *B. burgdorferi*, and 90 samples from clinically healthy animals in the Kirov Province, including individuals whose blood serum did not have diagnostically significant antibody titers to *B. burgdorferi* in the IDIF test.

The animals were caught by shooting during the autumn hunting seasons of 2005-2020, the white hare in the Kotelnichsky District of the Kirov Province with the center in the village of Sloboda (58°39′58″N, 50°43′42″E), the moose on the territory of the scientific and experimental hunting farm (Zhitkov All-Russian Institute of Hunting and Fur Farming), located in the Slobodsky District of the Kirov Province with its center in the village Rogovoe (58°33′04″N, 50°43′42″E). All animals were wild and moved freely, feeding on local vegetation.

Immediately after shooting the animal, blood for laboratory studies was sampled from the jugular vein (*venae jugularis*) into UNIVAC vacuum tubes (Eiliton LLC, Russia) with a coagulation activator (4 ml each) and centrifuged (a Liston C 2204 centrifuge, Liston LLC, Russia) for 20 min at 1500 rpm.

Anti-borreliosis antibodies in blood serum were detected in the indirect immunofluorescence assay (IDIF) using the corpuscular antigen of *B. afzelii* (strain Ip-21) and luminescent immune serum labeled with fluorescein isothiocyanate (FITC) against globulins of various animal species (rabbit, dog, bovine, pig, chicken). IDIF test was performed according to the recommendations of E.I. Korenberg et al. [27]. The results were recorded using a luminescent microscope LUMAM (JSC LOMO, Russia; an immersion lens). Titers of specific antibodies at a dilution of 1:40 or higher were considered diagnostically significant [28, 29].

Biochemical studies of blood serum (a semi-automatic Biochem SA analyzer, High Technology, Inc., USA) were carried out with a set of reagents (Eco-Service, Russia) for quatification of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, lactate dehydrogenase (LDH), alpha-amylase, total protein, albumin, total bilirubin, direct bilirubin, creatinine and cholesterol.

Statistical analysis was performed using Microsoft Excel Office 2019 and Statgraphics 19-X64 software by generally accepted methods [30]. Mean values (M), standard deviations (±SD), medians (Me), percentiles (25% and 75%) were calculated. To compare parameters between groups, the nonparametric Wilcoxon-Mann-Whitney test (U) was used. Relationships between traits were assessed using Spearman rank correlation. To assess the effect of *B. burgdorferi* on blood biochemical parameters, one-way analysis of variance (ANOVA) was used. The influence of the factor was considered statistically significant at $p < 0.05$.

Results. Biochemical parameters of blood serum of clinically healthy wild animals and individuals with diagnostically significant titers of antibodies to *B. burgdorferi* (1:40 and 1:80) are given in Tables 1-3.

1. Blood biochemical parameters of adult male mountain hare (*Lepus timidus* Linnaeus, 1758) with diagnostic values of antibodies to *Borrelia burgdorferi* and animals with the antibody titers below the cutoff titer values (Kirov Province, Kotelnichsky District, 2005-2020)

Parameter	Animals with diagnostic antibody titers (n = 5)	Animals without diagnostic antibody titers (n = 6)
Aspartate aminotransferase, U/l:		
min-max	44.2-115.9	15.6-25.8
M±SD	85.5±31.09	21.8±4.20
Me	99.3*	22.4
25 %-75 %	61.2-106.8	19.3-25.2
Alanine aminotransferase, U/l:		
min-max	61.2-91.2	39.4-69.8
M±SD	77.5±12.96	56.9±11.02
Me	78.1*	58.7
25 %-75 %	68.0-88.9	51.5-63.7
Lactate dehydrogenase, U/l:		
min-max	697.0-1242.5	620.1-902.1
M±SD	889.9±210.40	753.5±112.1
Me	821.6	740.5
25 %-75 %	786.3-902.1	670.3-838.2
α-Amylase, U/l:		
min-max	138.5-294.1	163.9-261.5
M±SD	212.7±68.21	228.4±35.69
Me	237.5	236.6
25 %-75 %	145.0-248.5	219.6-252.4
Total protein, g/l:		
min-max	96.1-162.7	58.3-76.7
M±SD	131.5±28.66	64.1±7.17
Me	136.9*	61.2
25 %-75 %	108.3-153.8	59.3-66.7
Albumen, g/l:		
min-max	63.2-88.5	56.2-86.9
M±SD	77.6±9.70	70.7±11.27
Me	79.3	69.7
25 %-75 %	73.6-83.4	63.6-77.8
Total bilirubin, mmol/l:		
min-max	3.3-7.8	1.6-3.6
M±SD	5.0±1.85	2.8±0.66
Me	4.2*	3.0
25 %-75 %	3.7-6.0	2.7-3.2
Direct bilirubin, mmol/l:		
min-max	0.1-2.5	0.2-1.0
M±SD	1.1±0.87	0.7±0.34
Me	1.0	0.8
25 %-75 %	0.8-1.1	0.5-1.0
Creatinine, mmol/l:		
min-max	100.2-144.6	72.2-124.3
M±SD	124.8±16.5	96.0±18.70
Me	129.1*	91.8
25 %-75 %	119.2-131.1	86.3-106.7
Cholesterol, mmol/l:		
min-max	0.1-0.3	0.1-0.4
M±SD	0.3±0.09	0.2±0.09
Me	0.3	0.2
25 %-75 %	0.2-0.3	0.2-0.3

* Differences between the groups are statistically significant at $p < 0.05$.

We identified a strong correlation between some biochemical parameters in animals with diagnostically significant titers of antibodies to *B. burgdorferi*. In mountain hare, there were correlations between AST activity and the total bilirubin content ($r = -1$, $p = 0.00$), total protein and direct bilirubin ($r = -1$, $p = 0.00$). In young female moose correlations were between AST and ALT ($r = -1$, $p = 0.00$), AST and total protein ($r = 1$, $p = 0.00$), total and direct bilirubin ($r = 1$, $p = 0.00$); in young male moose between total protein and creatinine ($r = 0.89$; $p = 0.02$). Unlike control animals, in young female moose with diagnostically significant titers of antibodies to *B. burgdorferi*, a strong positive correlation ($r = 1$; $p = 0.00$) ocurred between the activity of alkaline phosphatase and the total protein content,

while a strong negative correlation ($r = -1$; $p = 0.00$) occurred between AST activity and total bilirubin, AST and direct bilirubin, AST and creatinine.

2. Blood biochemical parameters of young moose (*Alces alces* Linnaeus, 1758) with diagnostic values of antibodies to *Borrelia burgdorferi* and animals with the antibody titers below the cutoff titer values (Kirov Province, Kotelnichsky District, 2005-2020)

Parameter	Femails		Mails	
	with diagnostic antibody titers ($n = 3$)	clinically healthy ($n = 20$)	with diagnostic antibody titers ($n = 7$)	clinically healthy ($n = 20$)
Aspartate aminotransferase, U/l:				
min-max	197.5-240.4	117.5-219.1	166.7-349.4	120.2-207.5
M±SD	216.0±22.07	161.2±28.30	238.7±76.33	160.0±30.92
Me	210.1*	155.6	209.3*	154.7
25 %-75 %	203.8-225.2	146.9-178.9	189.5-283.1	131.6-193.5
Alanine aminotransferase, U/l:				
min-max	78.0-112.4	47.2-72.0	70.8-173.1	48.0-71.2
M±SD	95.2±17.17	60.8±6.42	116.7±37.08	58.7±6.74
Me	95.1*	61.9	108.8*	57.2
25 %-75 %	86.6-103.8	56.8-63.9	90.3-141.7	54.8-64.7
Alkaline phosphatase, U/l:				
min-max	176.2-221.6	165.2-296.0	153.8-470.8	169.2-270.8
M±SD	199.1±22.70	230.4±40.8	289.2±111.48	222.2±31.14
Me	199.4	232.0	261.8	219.1
25 %-75 %	187.8-210.5	197.7-269.2	210.0-359.1	200.9-253.8
Total protein, g/l:				
min-max	68.1-84.3	46.5-83.0	72.0-178.6	45.5-86.1
M±SD	76.5±8.13	61.6±9.91	121.4±41.03	66.4±13.53
Me	77.1*	62.0	108.3*	70.5
25 %-75 %	72.6-80.7	53.7-67.0	90.6-154.7	53.2-77.8
Albumen, g/l:				
min-max	34.6-53.1	33.0-49.1	34.6-54.6	32.2-53.3
M±SD	44.7±9.37	40.9±4.48	46.5±8.22	42.9±6.94
Me	46.5	41.8	49.2	42.9
25 %-75 %	40.5-49.8	38.2-43.7	40.8-52.7	36.0-50.0
Total bilirubin, mmol/l:				
min-max	13.1-25.6	5.6-10.9	9.9-25.5	5,6-10,1
M±SD	17.9±6.77	7.9±1.54	18.1±5.29	8,4±1,11
Me	14.9*	8.0	18.7*	8,4
25 %-75 %	14.0-20.3	6.3-9.1	15.1-21.2	7,6-9,3
Direct bilirubin, mmol/l:				
min-max	2.1-3.5	1.4-3.7	2.1-5.7	0.9-3.8
M±SD	2.9±0.70	2.6±0.60	3.7±1.33	2.4±0.9
Me	3.2	2.6	3.8*	2.4
25 %-75 %	2.6-3.3	2.3-3.1	2.5-4.5	1.8-2.8
Creatinine, mmol/l:				
min-max	149.7-209.6	117.7-180.7	110.5-215.6	109.3-180.4
M±SD	185.2±31.47	152.8±20.3	156.0±43.51	149.1±23.78
Me	196.4	155.1	132.4	142.9
25 %-75 %	173.0-203.0	139.8-169.5	124.8-192.0	133.3-173.0

* Differences between the groups are statistically significant at $p < 0.05$.

In moose with diagnostically significant antibody titers, a strong correlation ($r = 1$; $p = 0.00$) was idetnitied between adult and young females, young females and adult males, adult females and males for alkaline phosphatase; between young and adult females, young females and adult males for total protein; and between young and adult females, young females and adult males for total bilirubin.

The one-way analysis (ANOVA) estimates the effect of *B. burgdorferi* on blood biochemical parameters of reservoir hosts. In the mountain hare, there was a statistically significant effect of *B. burgdorferi* on increasing the activity of AST ($p = 0.00$; 73.67% influence rate), ALT ($p = 0.01$; 47.44% influence rate), total protein content ($p = 0.00$; 77.77% influence rate), total bilirubin ($p = 0.02$; 44.1% influence rate), and creatinine ($p = 0.02$; 44.44% influence rate). In young female moose, *B. burgdorferi* had a significant effect on increasing the activity of AST ($p = 0.00$; 32.59% influence rate), ALT ($p = 0.00$; 69.17% influence rate), on total protein ($p = 0.02$; 22.48% influence rate), total bilirubin ($p = 0.00$; 65.57%

influence rate), creatinine (p = 0.02; 21.85% influence rate); in young male moose on an increase in the activity of AST (p = 0.00; 37.65% influence rate), ALT (p = 0.00, 65.64% influence rate), on total protein (p = 0.00; 53.57% influence rate), total bilirubin (p = 0.00; 71.7% influence rate), direct bilirubin (p = 0.00; 24.88% influence rate). In adult female moose, *B. burgdorferi* infection caused an increase in activity of AST (p = 0.02, 20.34% influence rate), ALT (p = 0.00, 62.49% influence rate), alkaline phosphatase (p = 0.00, 77.89% influence rate), in total protein (p = 0.00, 64.26% influence rate), total bilirubin (p = 0.00, 67.61% influence rate); in adult male moose an increase in activity of AST (p = 0.01, 14.19% influence rate), ALT (p = 0.00, influence rate 52.72%), alkaline phosphatase (p = 0.00, 67.83% influence rate), in total protein content (p = 0.00, 30.77% influence rate), total bilirubin (p = 0.00, 55.65% influence rate), direct bilirubin (p = 0.00, 46.12% influence rate).

3. Blood biochemical parameters of adult moose (*Alces alces* Linnaeus, 1758) with diagnostic values of antibodies to *Borrelia burgdorferi* and animals with the antibody titers below the cutoff titer values (Kirov Province, Kotelnichsky District, 2005-2020)

Parameter	Femails		Mails	
	with diagnostic antibody titers (n = 4)	clinically healthy (n = 20)	with diagnostic antibody titers (n = 10)	clinically healthy (n = 30)
Aspartate aminotransferase, U/l:				
min-max	301.6-326.9	157.1-341.6	198.6-452.3	162.3-342.5
M±SD	317.3±11.43	253.8±52.38	301.2±74.05	250.9±47.52
Me	320.2*	244.1	301.6*	243.3
25 %-75 %	312.3-325.2	219.6-296.0	247.4-338.2	208.4-299.4
Alanine aminotransferase, U/l:				
min-max	56.9-223.4	29.4-55.4	58.8-299.8	30.4-53.2
M±SD	133.9±71.36	43.6±7.35	141.5±80.23	41.9±6.33
Me	127.5*	44.2	111.2*	40.6
25 %-75 %	91.0-170.4	39.1-49.7	89.2-153.5	38.3-46.9
Alkaline phosphatase, U/l:				
min-max	109.1-195.2	49.0-89.1	124.5-438.1	46.6-88.3
M±SD	156.3±37.1	69.3±12.62	226.2±96.12	69.9±11.31
Me	160.5*	69.0	215.6*	70.4
25 %-75 %	137.7-179.2	57.5-79.3	152.7-251.3	61.4-79.2
Total protein, g/l:				
min-max	62.2-104.5	45.4-70.1	63.2-94.1	59.4-79.4
M±SD	92.7±20.36	57.4±7.48	79.8±13.34	68.1±4.93
Me	102.0*	57.8	82.4*	68.7
25 %-75 %	91.5-103.2	52.4-62.0	66.7-92.1	63.4-71.4
Albumen, g/l:				
min-max	39.8-69.9	30.9-53.8	34.9-59.6	33.4-53.2
M±SD	49.8±13.61	43.7±5.62	44.5±7.58	42.3±5.65
Me	44.8	44.9	44.3	41.5
25 %-75 %	42.8-51.8	40.2-46.5	39.9-46.8	38.8-46.6
Total bilirubin, mmol/l:				
min-max	9.8-24.7	6.7-10.7	10.1-26.5	6.5-11.5
M±SD	17.9±6.30	8.6±1.02	16.3±5.2	9.1±1.43
Me	18.6*	8.5	15.6*	9.2
25 %-75 %	15.1-21.5	8.1-9.3	12.3-18.8	8.3-10.1
Direct bilirubin, mmol/l:				
min-max	2.1-3.9	1.6-3.7	2.3-10.7	1.3-4.0
M±SD	3.1±0.77	2.6±0.55	6.0±2.90	2.7±0.79
Me	3.2	2.7	5.7*	2.8
25 %-75 %	2.7-3.6	2.2-2.9	3.5-8.2	2.1-3.3
Creatinine, mmol/l:				
min-max	144.12-202.3	156.4-225.7	135.4-229.6	126.3-223.2
M±SD	174.96±25.94	183.93±18.59	183.98±33.98	182.1±23.66
Me	176.71	185.7	169.5	181.2
25 %-75 %	159.16-192.51	169.6-195.35	162.75-219.27	167.5-200.1

* Differences between the groups are statistically significant at $p < 0.05$.

It should be noted that the entire territory of the Kirov Province in landscape and climate terms is favorable for the reproduction and maintenance of the population of ixodid ticks and their reservoir hosts [31].

Currently, the epidemiological situation regarding ITB in the Kirov Province remains unfavorable and the incidence exceeds the average for the Russian Federation. Thus, in 2020, this figure was 2.2 times higher than the Russian average (2.85 cases of the disease per 100 thousand population) and amounted to 6.29 cases per 100 thousand population. The highest incidence was registered in the Uninsky District (53.79 cases per 100 thousand population). The childhood morbidity rate was 2.7 times higher than the average for Russia (1.75 cases per 100 thousand population) and amounted to 4.71 cases per 100 thousand child population. Borreliosis has been registered in Kirov and 19 districts. In 2020, the virology laboratory of the Center for Hygiene and Epidemiology in the Kirov Region examined 1,815 ticks, 1,673 from people and 142 from environmental objects. Among ticks from people, 42.4% were positive for borreliosis (46.8% in 2018). Of ticks from environmental objects, 43.7% were positive for borreliosis (71.8%i in 2018, 49.2% in 2019) [32].

Our earlier serological and bacteriological studies [33-36] found out that the borrelia IDIF positive animals (mountain hare, fox *Vulpes vulpes* Linnaeus, 1758, elk, raccoon dog *Nyctereutes procyonoides* Gray, 1834, black grouse *Lyrurus tetrix* Linnaeus, 1758, capercaillie (*Tetrao urogallus* Linnaeus, 1758) and animals from internal organs of which borrelia were isolated on the BSK-H medium (badger *Meles meles* Linnaeus, 1758, wild boar *Sus scrofa* Linnaeus, 1758) serve as feeders for ixodid ticks and the reservoir hosts, distributors and accumulators of borreliosis pathogens (Fig. 1).

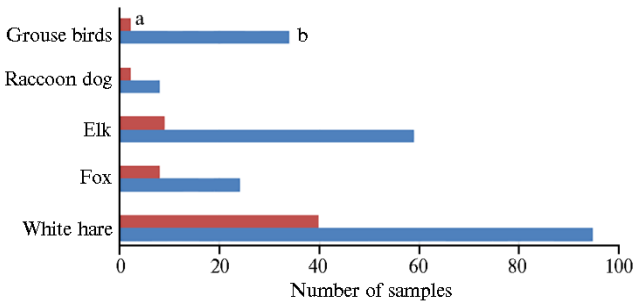

Fig. 1. Results of a study of blood serum of some animals for borreliosis in the reaction of indirect immunofluorescence: a — positive samples, b — studied samples [34, 36].

The high infestation of the mountain hare (Fig. 2) is explained by the greater likelihood of its contact with ticks due to high activity during the spring rutting period, abundant multi-layered fur, and sizes corresponding to the layer of high concentration of ticks on vegetation.

We believe that the number of white hare will make it possible to predict the epizootic situation regarding ITB, since even its slight fluctuations can affect the number and infection of tick vectors by increasing the likelihood of *Borrelia* horizontal transmission. A decrease in the number of reservoir hosts, on the contrary, will lead to a reduction in the population of the vector and, as a consequence, the pathogen. Similar data on the dynamics of parasitic systems in natural foci of ITB are given by Yu.V. Kovalevsky et al. [37].

H.E. Lyubeznova et al. [31] analyzed the dependence of tick infection with Borrelia on the number of rodents, white hare and fox. When comparing the number of mouse-like rodents and the infection of ticks with Borrelia, an average correlation was revealed ($r = 0.4$, $p < 0.05$) with a shift after 1 year. With an increase in the population of the white hare ($r = 0.8$, $p < 0.01$) and fox ($r = 0.72$, $p < 0.01$) in the second and third years, an increase in tick infection occurred.

Fig. 2. Hungry (1) **and feeding** (2) **ixodid ticks on a mountain hare (*Lepus timidus*, Linnaeus 1758).** Photo by I.A. Domsky.

Human borreliosis is characterized by polymorphism of clinical signs and the predominance of chronic forms [38-40]; for animals, it is a latent form. This makes it difficult to make a diagnosis.

Currently, borreliosis in dogs is the most studied. Clinical signs are reported in 5-20% of cases [41]. An acute disease begins after several months, and sometimes years, of a prosperous period. Borreliosis sugns are fever, lameness, sore muscles and joints, migratory arthritis, enlargement and swelling of the lymph nodes. Most often, one joint is affected, usually from the side of the tick bite [42]. Neurological disorders, diseases of the heart, liver, kidneys, bladder and eyes are recorded somewhat less frequently in the acute period [43]. However, nephritis is a lethal form of borreliosis in dogs and develops in them at the age of 3 years and older [44]. According to N.S. Pustovit [45], symptoms of renal failure and pathology of the urinary tract were detected in 21% of seropositive dogs.

In cattle, borreliosis is also asymptomatic. The dominant clinical signs are lameness and joint swelling, and less commonly, erythematous skin rash, fever, laminitis, abortion, weight loss, and decreased productivity [46-48]. The birth of calves with severe internal organ pathologies in infected cows has been reported [48]. In cows, the presence of *B. burgdorferi* has been proven in joint fluid, blood, urine, feces and milk [47, 49].

Biochemical research methods provide significant assistance in assessing the effect of a pathogen on the body [50]. Thus, in everyday practice, when diagnosing liver diseases, generally accepted biochemical tests for bilirubin content, aminotransferase activity, and alkaline phosphatase are used. Traditionally, these tests are combined to diagnose clinical and biochemical syndromes (cytolysis, cholestasis, etc.) [50].

We found that animals in whose blood sera diagnostically significant titers of antibodies to *B. burgdorferi* (1:40 and 1:80) were detected by IDIF had statistically significant differences ($p < 0.05$) in the activity of AST, ALT, alkaline phosphatase, the content of total protein, total bilirubin, direct bilirubin and creatinine from the control group.

Cytolysis syndrome (violation of the integrity of hepatocytes) is caused by impaired permeability of cell membranes, disintegration of membrane structures, necrosis of hepatocytes with the release of enzymes into the plasma, which entails an increase in the activity of AST and ALT. In this case, increased amounts of both fractions of bilirubin are determined in the blood serum [50]. In the mountain hare with diagnostically significant antibody titers to *B. burgdorferi*, AST activity was 342.2% higher compared to animals without antibody titers. An increase in enzyme concentration was also noted in moose, by 35.0% in young females, by 35.3% in young males, by 31.2% in adult females, and by 24.0% in adult males. ALT activity in the mountain hare with diagnostic titers to B. burgdorferi was higher by 32.8% compared to animals without titers. An increase in ALT activity was also found in moose, by 53.8% in young females, by 90.4% in young males, by 188.6% in adult females, and by 173.9% in adult males.

ALT is a predominant marker of liver disease. Since ALT is localized in the cytoplasm, and AST is localized in the mitochondria, the AST index increases to a lesser extent in liver diseases. In addition, a significant increase in AST activity indicates severe damage to hepatocytes and serves as one of the early markers of disorders in the heart muscle [50].

In clinical practice, the de Ritis coefficient (ratio of AST to ALT) is used for the differential diagnosis of liver and myocardial diseases. Since ALT activity predominantly increases in liver diseases, this coefficient decreases. In cardiac pathology, on the contrary, an increase in AST activity predominates and the de Ritis coefficient increases [50]. In a mountain hare with diagnostically significant titers of antibodies to *B. burgdorferi*, the de Ritis coefficient was 1.25, in animals without them it was 0.38, which indicated heart pathology. In moose, the opposite results were obtained. In young females, the de Ritis coefficient was 2.20, in young males 1.92, in adult females 2.50, in adult males 2.71. In animals from the control group, these values were respectively 2.51, 2.70, 5.52, and 5.99. Thereof, our data indicated liver damage in this reservoir host species.

Cholestasis syndrome is caused by both a violation of the biliary function of hepatocytes and damage to the bile canaliculi (intrahepatic cholestasis), and a disorder of the outflow of bile through the hepatic and common bile ducts due to their obstruction (extrahepatic cholestasis). Both forms are characterized by increased activity of alkaline phosphatase and some other excretory enzymes, hypercholesterolemia, and hyperbilirubinemia [50]. We revealed a statistically significant ($p < 0.05$) increase in alkaline phosphatase activity in adult moose, by 132.51% in females and by 206.32% in males. The increase may be due to cholestasis of any etiology and localization (hepatitis, cirrhosis when intrahepatic, obstructive jaundice when extrahepatic). Cholestasis is doubtful under normal alkaline phosphatase level [50]. A decrease in the activity of this enzyme was also revealed in young females that might be associated with some slowdown in growth rate and a decrease in osteoblastic activity under infection and possible lack of energy. T. Soveri et al. [51] note that parasites influence blood parameters of the mountain hare. Thus, *Trichostrongylus retortaeformis* causes a decrease in alkaline phosphatase activity.

The nature of pigment metabolism disorders is assessed based on the results of a study of serum bilirubin. With parenchymal (hepatic) jaundice in patients with hepatitis, cirrhosis and other liver diseases, hepatocytes are damaged and bilirubin conversion is impaired. We detected an increase in the content of total bilirubin in all groups of animals with diagnostically significant titers of antibodies to *B. burgdorferi*. In the white hare, the indicator increased by 42.42%, in young female moose by 86.12%, in young males by 121.91%, in adult females by 118.77%, in adult males by 70, 44%. In addition, in young male moose the amount of direct bilirubin was increased by 59.09%, in adults by 102.85%.

Note that disruption of the uptake of free bilirubin by the liver cell and its binding to glucuronic acid causes an increase in the amount of free (indirect) bilirubin in the blood. The release of bilirubin glucuronide (direct bilirubin) from the liver cell into the bile capillaries, caused by inflammation, destruction, necrosis and a decrease in the permeability of hepatocyte membranes, leads to regurgitation of bile back into the sinusoids and into the general bloodstream and, accordingly, to an increase in the content of bound (direct) bilirubin in the blood. Disorder of hepatocyte function is accompanied by a loss of the ability of the liver cell to capture and metabolize urobilinogen absorbed in the intestine, which enters the general bloodstream in large quantities and is excreted in the urine. As a consequence, with parenchymal jaundice, the blood content of both free (indirect) and bound (direct) bilirubin increase [50].

Determination of total protein in blood serum is used to diagnose liver, kidney, and cancer diseases. Increased protein content is recorded during the development of acute and chronic infectious diseases and autoimmune pathologies.

In our study, an increase in the total protein content was recorded in all groups of studied animals that had diagnostically significant titers of antibodies to *B. burgdorferi*, in the mountain hare by 123.6%, in young female moose by 24.3%, in young males by 53.5%, in adult females by 76.7%, in adult males by 19.9%. Literature data on the content of total protein in the blood serum of animals infected with *B. burgdorferi* are contradictory. N.S. Pustovit [45] notes that total blood protein increases in infected animals. O.A. Laktyushina [52] found its decrease in dogs with borreliosis.

The renal failure leads to an increase in the content of the final product of metabolism — creatinine. We observed an increase in this indicator by 40.7% in hares with diagnostically significant antibody titers to *B. burgdorferi*. According to T. Soveri et al. [51] who studied the blood biochemical parameters of the mountain hare, in clinically healthy animals the total protein was 54.0±5.40 mmol g/l, creatinine 92.0±12.90 mmol/l. These figures are consistent with our data. T. Soveri et al. [51] also found that high host animal densities facilitate parasite transmission, and poor feed supply may reduce host resistance to various diseases.

According to V.I. Starostina et al. [53], in people with borreliosis, the content of total protein and AST activity in the blood increase. N.N. Vorobyova et al. [54, 55] found that 31.1% of people infected with Borrelia have a moderate enlargement of the liver, which is accompanied by an increase in the activity of ALT, AST, alkaline phosphatase, and hyperbilirubinemia in the blood serum. M.V. Savelyeva et al. (56) also report an increase in the activity of ALT and AST in 52.9% of people with ITB. According to D.V. Dmitrenko (57), liver pathology in borreliosis manifests itself as anicteric hepatitis which is clinically inconspicuous (minor dyspeptic disorders, moderate increase in liver size). It is detected in patients most often during a biochemical blood test as a moderate increase in the number of transaminases (usually ALT) and/or hyperbilirubinemia which indicates cytolysis syndrome. The results of biochemical studies showed in 50% of sick dogs an increased blood AST content which ranged from 44 up to 87.0 IU/l. ALT was elevated in 30% of cases and ranged from 75.0 to 112.0 IU/l; 30% of dogs showed an increase in creatinine levels from 153.2 to 212.0 mmol/l [49].

P.V. Aksenova [58], studying the blood of free-living bison, found antibodies to *B. burgdorferi* in 16.4% of samples. In Belovezhskaya Pushcha, from 20.0 to 30.0% of bison were seropositive for *B. burgdorferi*. However, the clinical signs of the disease have not been established. The results of our research are also confirmed by the data of N.S. Pustovit [45] who notes that in infected animals the levels of AST, ALT, alkaline phosphatase, creatinine, total protein, and bilirubin increase.

Thus, indirect immunofluorescence assay identified diagnostically significant titers of antibodies to *Borrelia burgdorferi* (1:40 and 1:80) in blood serum of white hare and moose, considered as reservoir hosts of *B. burgdorferi*. Infected amimals statistically significantly differ ($p < 0.05$) from control animals in blood aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase activity and in total protein, total and direct bilirubin, and creatinine levels. In animals with diagnostically significant titers of antibodies to *B. burgdorferi*, some blood parameters correlate, that is, in mountain hare, AST activity with total bilirubin, total protein with direct bilirubin; in young female moose, AST activity with ALT activity, AST activity with total protein, total bilirubin with direct bilirubin; in young male moose, total protein with creatinine. Unlike control animals, in young female moose with diagnostically significant antibody titers to *B. burgdorferi*,

blood alkaline phosphatase activity and total protein correlate positively while correlations between AST activity and total bilirubin, direct bilirubin, and creatinine are negative. In moose with diagnostically significant antibody titers, there are strong correlations for alkaline phosphatase activity between adult and young female, young females and adult males, adult females and males, for total protein level — between young and adult females, young females and adult males, for total bilirubin — between young and adult females, young females and adult males. One-way ANOVA analysis revealed a significant effect of *B. burgdorferi* on increasing biochemical blood parameters, including the AST, ALT, alkaline phosphatase, total protein, total and direct bilirubin, and creatinine concentrations Our data indicate pathology of the cardiovascular system, kidneys and liver in the mountain hare and, mainly, liver pathology in moose under borreliosis. An increase in the concentration of total protein with normal albumin levels in reservoir hosts occurs due to an increase in globulin fractions which indicates acute and/or chronic infection. A significant change in the blood biochemical parameters in reservoir hosts with diagnostically significant titers of antibodies to *B. burgdorferi* indicates its pathological effect on animals, multiple organ failure and irreparable harm to health.

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BIOLOGICAL PROPERTIES OF AFRICAN SWINE FEVER VIRUS ASFV/Kaliningrad 17/WB-13869

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Abstract

African swine fever (ASF) is a contagious viral disease of domestic pigs and wild boars of all ages and breeds. To date, the infection is endemic in many European and Asian countries including the Russian Federation. Previously ASF virus isolated and studied by Russian scientists were characterized as highly virulent, with 100 % mortality and assigned to genotype II. However, data on the detection of ASFV with reduced virulence and mortality were later reported, thus further analysis of modern isolates is of high importance. In this work, for the first time, we report the biological properties of the ASF virus (named ASFV/Kaliningrad 17/WB-13869) isolated on the territory of the Kaliningrad region of the Russian Federation. The bioassay was carried out on six large white breed pigs (*Sus scrofa domesticus* L.) weighing 15-20 kg, the experiment was performed in a BSL-3 animal facility at the Federal Center for Animal Health (FGBU ARRIAH). Pigs Nos. 3-6 were infected intramuscularly with ASFV/Kaliningrad 17/WB-13869 (genotype II serotype 8) that was isolated from the tubular bone of a wild boar carcass (Bagrationovskiy District, Kaliningrad Province) at a dose of 10 HAD/head. Two uninfected pigs (Nos. 1, 2) were kept in-contact with the infected ones. Clinical signs and body temperature of experimental animals were registered daily. The presence and severity of clinical signs and pathological changes were expressed quantitatively (the sum of scores for a number of indicators). Clinical score was based on assessment of body temperature, weight, behavior, appetite and water consumption, the state of the digestive and respiratory systems, skin and mucous membranes, the presence of nasal discharge and vomiting. Pathological anatomical autopsy assessed changes in the organs of the spleen, kidneys, liver, lung, submandibular and mesenteric lymph nodes. Points were assigned on a scale of severity of recorded signs from 1 to 3 (the most severe). Blood sampling (5.0 cm^3 from each animal) was carried out until the death of pigs on the 0th, 3rd, 6th, 10th, 13th and 19th days after the start of the experiment. Samples taken from dead animals included; spleen, kidneys, liver, lung, submandibular and mesenteric lymph nodes. Samples and blood were used to prepare 10 % suspensions in sterile saline using an automatic homogenizer, then centrifuged at 400 g (Sigma Laborzentrifugen GmbH, Germany) for 2 min. The resulting supernatant was used for DNA extraction. Blood serum samples were tested for the presence of Anti-ASFV antibodies using ELISA test systems INgezim PPA Compac (Ingenasa, Spain) and ID Screen (IDvet, France), and immunoperoxidase method (IPM). Real-time PCR detected ASFV genome starting from the 3rd day after infection, while IPM detected anti-ASFV antibodies 1-2 days before the death of infected animals, no anti-ASFV antibodies were detected in the serum of animals by ELISA test systems throughout the experiment. Maximum clinical score was registered in animals with a sub-acute form of the disease (21 and 35 points, respectively), while minimum with hyper-acute disease form (6 and 8 points). As a result, ASFV/Kaliningrad 17/WB-13869 virus isolate was characterized as highly virulent, capable of causing ASF in pigs in forms from hyperacute to subacute with mortality up to 100 % of infected and contact animals, which is similar to the clinical picture caused by ASF virus isolates from the Russian Federation in 2007-2018.

Keywords: African swine fever, wild boars, bioassay, laboratory diagnostic methods, clinical signs, pathological changes

African swine fever (ASF) is a contagious viral disease of wild boars and domestic pigs of all ages and breeds [1-3]. To date, the infection is widespread in many countries in Europe and Asia, including the Russian Federation [4]. The causative agent of ASF is a DNA-containing arbovirus of the genus *Asfivirus* (family *Asfarviridae*), which has 10 serotypes identified in the hemadsorption delay test (HAD), and 24 genotypes identified by sequencing the variable C-terminus of the *B646L* gene, encoding the capsid protein vp72 of the pathogen [5-8]).

Russian scientists have studied in detail a number of ASF virus genotype II isolates from pigs and wild boars from various regions of Russia. Thus, isolates have been described that cause from hyperacute to subacute forms of the disease, with 100% lethality for infected animals, the death of which occurred in the period from 3 to 17 days after infection (d.a.i.) [9-12]. In addition to highly virulent isolates, those with reduced virulence have been described with a lethality up to 50% (isolate Lipetsk 12/16) and the period of death after infection within a wider range (5-30 d.a.i.) [13-15].

Similar results were reported abroad on identification of virus variants with reduced virulence, causing from acute to asymptomatic forms of the disease [16-22]. Special attention is paid to the discovery of non-hemadsorbing variants of the virus genotype II Lv17/WB/Rie1 (Latvia, 2017) [23], HeB/Q3/20 and HLJ/HRB1/20 (PRC, 2020) [19], and variants of genotype I HeN/ZZ-P1/21 and SD/DY-I/21 (PRC, 2021) [24].

Reports of ASF virus isolates with reduced virulence which were found in Russia and neighboring countries suggest the formation of endemic zones. This complicates the early diagnosis of the disease, posing additional problems in combating infection, and requires further study of the biological properties of the isolates [13, 25-27].

In this work, the biological features of the ASF virus isolate circulating in the Kaliningrad Province of the Russian Federation are established for the first time.

Our goal was to study properties of the ASFV/Kaliningrad 17/WB-13869 isolate.

Materials and methods. The work was carried out in 2018-2020 at the vivarium complex of the Federal Center for Animal Health Protection (ARRIAH) which provides for work with pathogens of pathogenicity groups 2-4. The test was carried out on pigs (*Sus scrofa domesticus* L.) from a pig farm free from major infectious diseases (Vladimir Province) in strict accordance with interstate standards for the maintenance and care of laboratory animals adopted by the Interstate Council for Standardization and Metrology and certification, as well as in accordance with the requirements of Directive 2010/63/EU of the European Parliament (EU Directive 2010/63/EU) and the Council of the European Union of 22.09.2010 on the protection of animals used for scientific purposes. Six Large White pigs weighing 15-20 kg, before the start of the experiment, were kept in quarantine for 7 days to assess their clinical condition.

Pigs Nos. 3-6 were infected with cultural material containing hemadsorbing ASF virus genotype II serotype 8 (isolate ASFV/Kaliningrad 17/WB-13869) from the tubular bone of a dead wild boar, discovered on November 7, 2017 in the Krasnoarmeyskoye village (Kaliningrad Province, Bagrationovsky District) (strain ASF/Kaliningrad-10/17, isolated and deposited by ARRIAH).

The virus-containing suspension was intramuscularly administered at a dose of 10 hemadsorption unins (HADU) per animal. Two uninfected pigs (Nos. 1, 2) were kept together with infected ones to assess possible contact infection. Biological tests and assessment of clinical signs and pathological changes were carried out in accordance with the ARRIAH methodological recommendations and guidelines [28, 29].

Clinical signs were visually monitored daily, the body temperature of each pig was measured rectally. The presence and severity of clinical signs and pathological changes were expressed quantitatively (sum of points for a number of indicators). Body temperature, fatness, behavior, appetite and water consumption, the state of the digestive and respiratory systems, skin and mucous membranes, nasal discharge and vomiting, and incubation period indicators were assessed. During the pathological autopsy, changes in organs were examined. Points were assigned on a scale of recorded sign severity from 1 to 3 (the most severe).

Blood samples (5.0 cm^3) were collected individually in test tubes with a coagulation activator until the death of pigs on days 0, 3, 6, 10, 13 and 19 after the start of the experiment. Blood samples were separated into clot and serum. On the day of animal death, pathanatomical autopsy of the spleen, kidney, liver, lung, submandibular and mesenteric lymph nodes was performed, one sample of each organ from each dead pig). The samples were examined immediately or stored at –70.0 °C.

Blood sera were used without pretreatment. Blood clots and organs were homogenized (an automatic homogenizer, QIAGEN GmbH, Germany) in sterile saline to a 10% suspension, then centrifuged at 400 g (Sigma Laborzentrifugen GmbH, Germany) for 2 min. DNA was extracted from the resulting supernatant.

To assess specific antibodies to the ASFV in blood serum, enzyme-linked immunosorbent assay tests INgezim PPA Compac (Ingenasa, Spain) and ID Screen, African Swine Fever Indirect, Screening Test (IDvet, France) were used in accordance with the manufacturers' instructions. Additionally, immunoperoxidase test (IPT) was used as guided by the ARRIAH methodological recommendations [30]. INgezim PPA Compac is a solid phase competitive enzyme-linked immunosorbent assay (SP-ELISA) based on the ASFV vp72 protein and monoclonal antibodies to it. ID Screen is an indirect version of SP-ELISA using the p32, p62 and p72 antigens of the ASFV. The essence of IPT is to identify specific antigen-antibody complexes resulted from the interaction of antigens of the virus replicating in an infected cell culture, the ASFV and the antibodies to ASFV.

DNA was isolated by phenol-chloroform extraction using the DNA-Sorb-B kit (Central Research Institute of Epidemiology of Rospotrebnadzor, Russia). RT-PCR was performed using the Test System for Detection of the African Swine Fever Virus Genome by Real-Time Polymerase Chain Reaction (ARRIAH, Russia) according to the manufacturer's instructions. The number of genome copies (g.c.n.) of the ASF virus in the blood and organs of animals was calculated accoding to recommendations [31]. All samples were examined once in one repetition.

Data processing and plotting were carried out using GraphPad Prism 8.0 software packages (https://www.graphpad.com/) and Microsoft Excel (https://www.microsoft.com/ru-ru/). Means (M) and standard errors of means (±SEM) were calculated.

Results. After the appearance of the first clinical signs, the disease lasted from 4 to 7 days in infected animals and from 2 to 14 days in contact animals (Table 1), which is typical for hyperacute, acute and subacute ASF [32, 33].

Throughout the entire observation period, clinical signs characteristic of ASF were recorded in the animals. Until death, contact pig No. 1 had no symptoms other than an increase in body temperature above the physiological norm (40.0 °C) and ataxia. In infected animals (Nos. 3-6) and in contact pig No. 2, clinical signs characteristic of ASF appeared. These signs were loss of appetite (up to complete refusal of feed and water), damage to the nervous system (from

asthenia to areflexia), respiratory system (from lung to severe dyspnea) and digestive system (registration of diarrhea from mild to dehydration), cyanotic zones (up to 15% of the skin surface), conjunctival hyperemia (from moderate to severe with serous-purulent exudate) (Fig. 1).

1. Assessment of clinical signs (score) **of the course of African swine fever in Large White pigs (*Sus scrofa domesticus* L.) infected with isolate ASFV/Kaliningrad 17/WB-13869** (tests in the vivarium of the Federal Center for Animal Health, 2018)

Group	Pig No.	Days after challenge																			
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Contact	1														3	6	†				
	2							4	4	5	4	8	8	6	7	11	14	17	19	20	21 †
Infected	3	ϟ				4	5	6	7	10	12	13	†								
	4	ϟ					6	4	5	9	11	14	†								
	5	ϟ						6	5	5	12	†									
	6	ϟ						5	5	8	9	15	†								

Note. ϟ — date of infection, † — date of death.

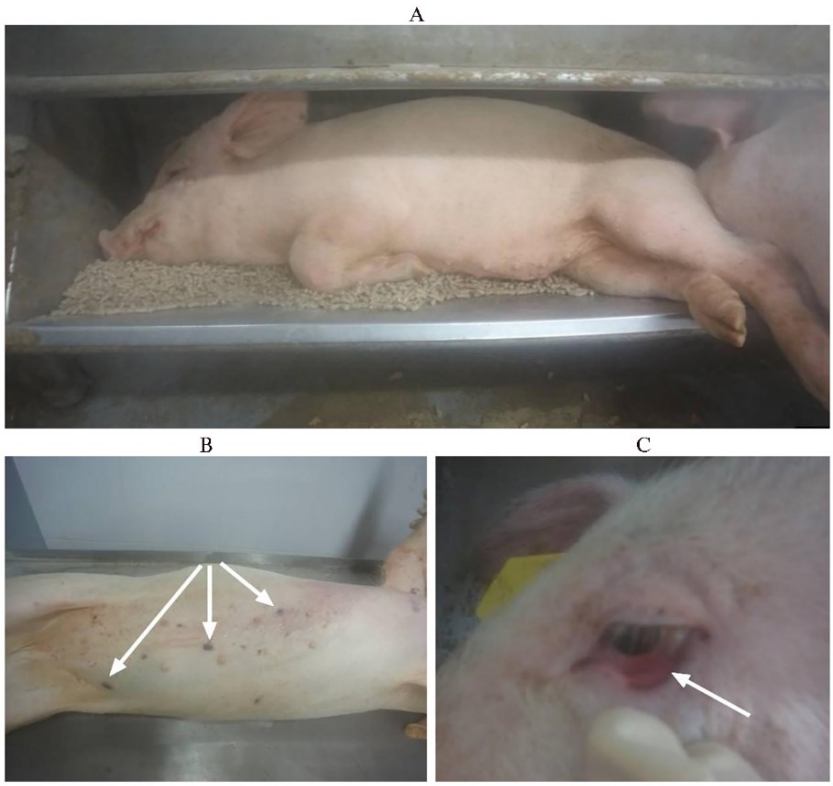

Fig. 1. Clinical signs of African swine fever in a Large White pigs (*Sus scrofa domesticus* L.) infected with isolate ASFV/Kaliningrad 17/WB-13869: A — ataxia, decreased response to external stimuli, B — foci of skin necrosis, C — hyperemia of the conjunctiva (tests in the vivarium of the Federal Center for Animal Health, 2018).

Thus, in total, pig No. 1 (superacute form) scored 6 points, infected pigs No. 3-6 (acute form) scored 12-15 points, and pig No. 2 (subacute form) scored 21 points.

An increase in body temperature (> 40.0 °C) was recorded in the infected animals starting from 4-6 days after the challenge, while in contact pigs No. 1 and No. 2 from 13 and 6 days, respectively (Fig. 2). In infected pigs Nos. 3, 4, viremia was recorded starting from day 3, in pigs Nos. 5, 6 from day 6. The maximum genomic load reached 7.8 g.c. per ml ($\log_{10}$).

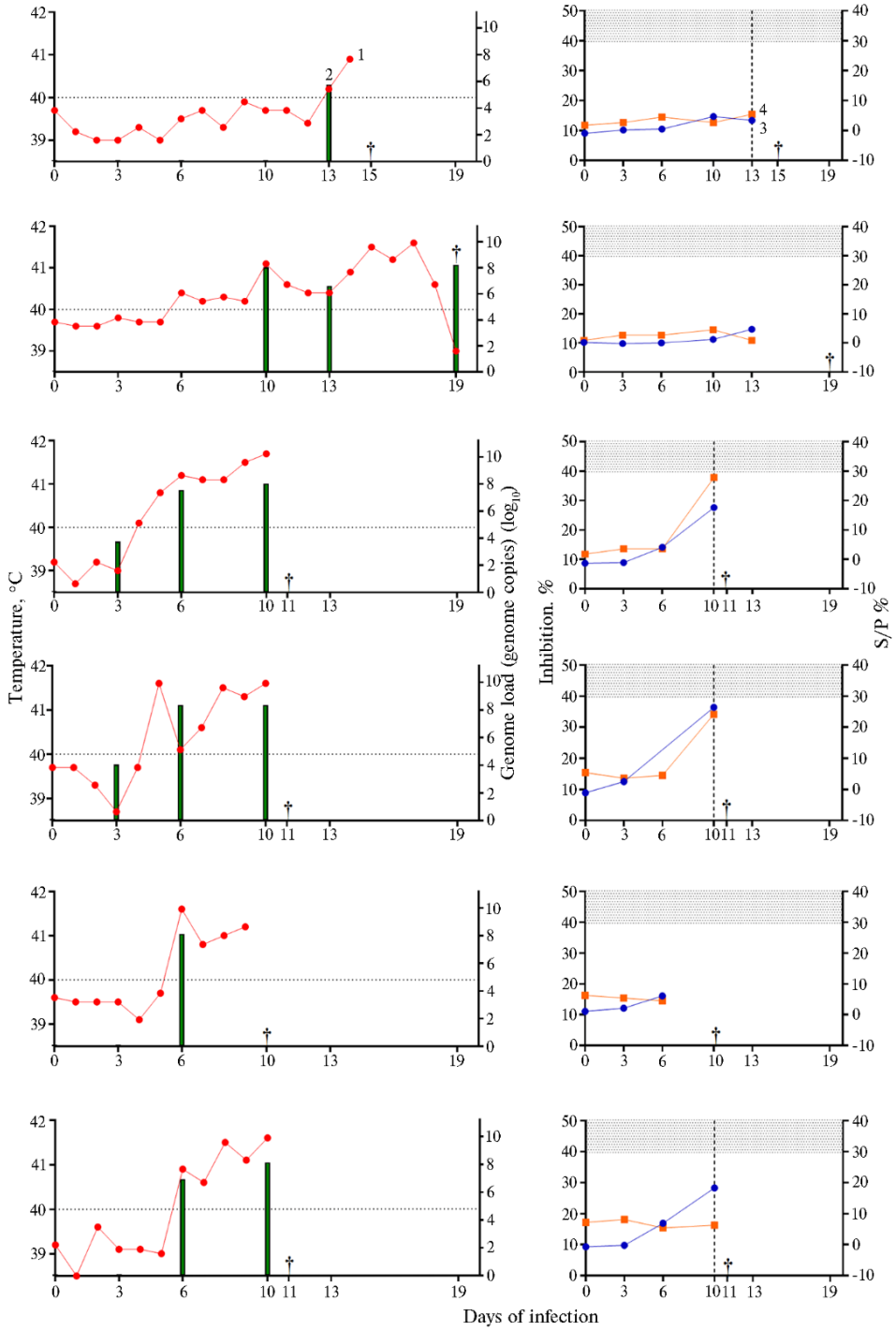

Fig. 2. Body themperature (1)**, genomic load** (2) **and blood sample assay by ELISA test** (3) **and immunoperoxidase test** (IPT) (4) **in Large White pigs (*Sus scrofa domesticus* L.) infected with the African swine fever virus isolate ASFV/Kaliningrad 17/WB-13869:** from top to bottom — pig No. 1, No. 2, No. 3, No. 4, No. 5, No. 6, respectively. The horizontal dotted line along the temperature axis marks the border of the physiological norm (40.0 °C); vertical dotted lines indicate positive results (IPM method); † — death dates (tests in the vivarium of the Federal Center for Animal Health, 2018).

Specific antibodies to the ASFV were not detected by TF-ELISA test. The degree of inhibition did not exceed 36.4% for the Ingezim PPA Compac

test system (samples with < 40% inhibition are considered negative). When using the ID Screen, African Swine Fever Indirect, Screening Test system, the maximum S/P% value (the ratio of the optical density of the test sample to the optical density of the positive control) was 27.8% (samples with an S/P < 30% are considered negative). Moreover, in simultaneous laboratory IPT assay, samples taken 1-2 days before the death of one contact (No. 1) and three infected animals (Nos. 3, 4 and 6) were positive for the specific antibodies.

2. Assessment of pathological changes in the Large White pigs (*Sus scrofa domesticus* L.) infected with the African swine fever virus isolate ASFV/Kaliningrad 17/WB-13869 (tests in the vivarium of the Federal Center for Animal Health, 2018)

Pig No.	Lungs			Heart		Spleen		Lymph nodes			Liver		Kidneys			Bladder	Transudate		Σ
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVIII	XIX	
1	-	-	1	2	-	1	1	1	–	–	–	–	1	–	1	–	–	–	8
2	2	2	1	2	1	3	2	3	2	2	2	2	2	2	2	2	2	1	35
3	1	1	1	1	1	2	2	2	1	–	1	1	1	1	1	1	1	–	19
4	2	2	1	1	2	2	2	2	2	1	–	–	2	2	2	1	2	1	27
5	1	1	1	1	1	2	2	1	1	–	1	-	1	–	1	1	1	–	16
6	2	3	1	2	1	1	1	1	–	1	1	1	1	1	1	1	1	–	20
Σ	8	9	6	9	6	11	10	10	6	4	5	4	8	6	8	6	7	2	125

N o t e. I — edema, II — pneumonia, III — hemorrhages under the pleura; IV — hemorrhagic diathesis, dystrophy, V — transudate in the pericardial cavity; VI — blood filling, VII — splenomegaly; VIII — submandibular lymph nodes, IX — mesenteric lymph nodes, X — inguinal lymph nodes; XI — hepatopathy, XII — bile ducts; XIII — hemorrhagic diathesis in the cortex and medulla, XIV — subcapsular hemorrhages, XV — hemorrhages in the renal pelvis; XVI — hemorrhagic diathesis in the mucous membrane, XVIII — chest cavity, XIX — abdominal cavity; Σ — sum of points. Dashes mean that the corresponding pathoanatomical changes were not identified.

After death, an autopsy of each animal was performed. Of the characteristic pathoanatomical changes, we noted, in particular, blood filling of the spleen (11 points), splenomegaly (10 points), enlargement of the submandibular lymph nodes (10 points), and pneumonia (9 points) (Table 2).

The same as for clinical signs, the severity of pathological changes depended on the form of the disease. Thus, the lowest score was in pig No. 1 (8 points), while the highest was in pig No. 2 (35 points).

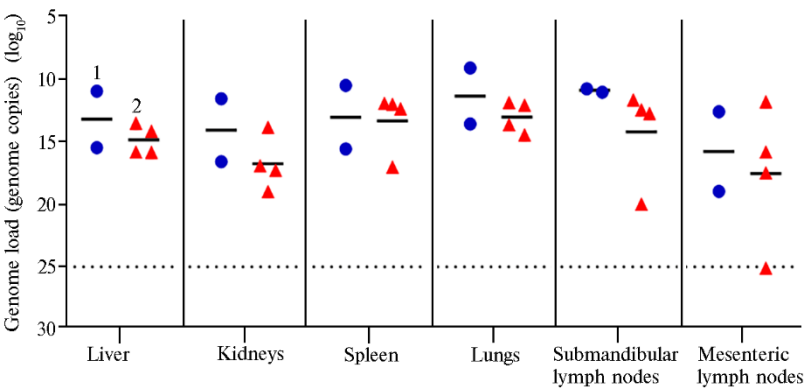

Fig. 3. The genomic load in the organs of dead Large White pigs (*Sus scrofa domesticus* L.) infected with the African swine fever virus isolate ASFV/Kaliningrad 17/WB-13869: 1 — contact animals, 2 — infected animals (tests in the vivarium of the Federal Center for Animal Health, 2018).

The ASFV genome was detected in all organs (Fig. 3) and the genomic load averaged 6.23±0.16 g.c. per ml ($\log_{10}$).

It should be noted that the identified biological properties of the isolate ASFV/Kaliningrad 17/WB-13869 are comparable with those of hemadsorbing isolates of the ASFV genotype II detected in the Russian Federation (Stavropol 01/08, Pskov-Yashkovo, ASFV/Primorsky 19/WB-6723 etc.) [22, 32, 34], the

Republic of Poland (Pol16/DP/OUT21) [17], the People's Republic of China (Pig/HLJ/18) [16], Georgia [2] and Armenia [1], which have 100% lethality for pigs. In our research, the time from infection with the studied isolate to an increase in body temperature above the physiological norm (40.0 °C) and the ASFV genome detection in the blood coincided with those for other highly virulent isolates and amounted to 4-6 days and 3-6 days, respectively. Other clinical signs of ASF were also noted, such as loss of appetite, damage to the nervous and digestive systems, appearance of cyanotic zones, and hyperemia of the conjunctiva [13, 16, 17, 32, 33].

In contrast to the ASFV genome that was recorded in the blood of infected pigs from the first assessment until the animal death, we found specific antibodies to the pathogen only when examining blood serum from three pigs infected intramuscularly and one contact pig using IPT. Comparable data were obtained by C. Gallardo et al. [17[. Using IPT, these authors detected specific antibodies to the ASFV in 83.3% of serum samples 7 d.a.i. with ASFV isolate Pol16/DP/OUT21 (Poland, 2016), while in SP-ELISA, only one contact pig at 16 d.a.i. was ASFV positive. In the work of A. Pershin et al. [13] specific antibodies were detected in 19.3% of blood serum from pigs infected with various ASFV isolates, 13 in total, sampled in 2013-2018 in the Russian Federation. The first positive results were noted from 14.14±2.00 d.a.i. However, A.R. Shotin et al. [22] did not detect specific antibodies to the ASFV whrn testig the ASFV/Primorsky 19/WB-6723 isolate (Primorsky Krai, 2019). The data obtained may be a consequence of the rather rapid death of animals, none of them lived for more than 7 days (for infected animals) and 14 days (for contact animals) from the moment when the first clinical signs of ASF appeared [35]. Another reason may be higher sensitivity of IPN compared to SP-ELISA [36-38].

The severity of pathological changes, as well as the severity of clinical signs, correlated with the duration of the disease. Characteristic lesions for ASF described by domestic and foreign researchers for ASFV isolates from China (Pig/HLJ/18) [16], Poland (Pol16/DP/OUT21) [17], Vladimir (Shikhobalovo 10/13) [9], Ryazan (Ryazan-Sapozhkovo/2016) [10], Novgorod (Novgorod-Okulovo) [11] regions, Primorsky Krai [22] and other regions of Russia, were observed in the lungs, heart, spleen and other organs.

Thus, the results of the studies allow us to characterize the isolate of the African swine fever virus (ASF) ASFV/Kalinin-grad 17/WB-1386 as highly virulent, capable of causing ASF in pigs, from hyperacute to subacute, with the death rate up to 100% of infected animals and those having a direct contact. Despite the data obtained, there is a possibility of survival of some animals infected, including with highly virulent isolates of the ASF virus, as well as the possibility of changing the biological properties of already circulating variants of the pathogen. This requires the additional use of direct (RT-PCR, virus isolation) and indirect (SP-ELISA, IPT) research methods to identify the ASF virus and/or its genome and specific antibodies to this infectious agent.

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