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RUMEN METHANE PRODUCTION AND ITS REDUCTION USING NUTRITIONAL FACTORS

(review)

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Abstract

Methane is a powerful greenhouse gas with a higher global warming potential than carbon dioxide. Agriculture, especially animal husbandry, is considered the largest sector of anthropogenic methane production. Of farm animals, ruminants are the main producers of methane. Its world production and emissions are increasing due to abundant population of ruminants. The hydrogenotrophic scenario of methanogenesis from hydrogen and carbon dioxide, carried out by ruminal archaea, prevails. Over the past 50 years, numerous research papers have substantially improved our understanding of rumen fermentation and methanogenesis to develope strategies for assessing and reducing methan emission (K.A. Beauchemin et al., 2020). One of the proposed strategies is dietary intervention, i.e. improved dietes and the use of nutritional factors that affect the ruminal microbiota. The quality, feed preparation, the ratio of concentrated and roughage feeds affect methane emissions. Some feeds may increase propionate production or decrease acetate production by reducing the level of ruminal hydrogen converted to methane. Another strategy is the use of modifiers, the feed additives that directly or indirectly inhibit methanogenesis, and biocontrol manipulation using defaunization agents, bacteriocins, bacteriophages, and immunization aimed at reducing the counts of methanogens. The strategy may be also based on genetically or technologically improved productivity performance. With higher productivity, the relative methane emission per unit of meat or dairy product is reduced (M. Islam et al., 2019). Fat additives, organic acids, probiotics, ionophores, phytogenics can serve as strategies to reduce methane formation in ruminants (M. Wanapat et al., 2021; R.D. Marques et al., 2021; S.H. Kim et al., 2020). Feeding manipulation is a simplistic and pragmatic approach to improve animal productivity with a reduced CH_4 emission (M.D. Najmul et al., 2018). In the review, along with a description of methanogenesis, we also summaraized modern research data on the influence of various alimentary factors (i.e., special diets, phytogenic saponins, tannins, flavonoids and essential oils) on CH_4 emission. The type of diet, the quality of bulky and concentrated feeds, their chemical composition, ratio, pre-feeding preparation affect methane emission in ruminants. However, a promising approach to mitigate methane emissions is adding a small amount of grain to roughage and feeding high quality forages with less fiber and higher levels of soluble carbohydrates. Phytogenics made from various botanical parts of plants is a cheap and environmentally friendly agents to reduce greenhouse gas emissions. Phytogenics also positively affect animal resistance. There are few studies on the in vitro efficacy of flavonoids and other secondary plant metabolites as agents for reducing methane emissions. The data obtained are variable and depend on the type of herbal preparations, their characteristics and the diet fed to the animals. Further in vivo studies should establish the optimal dosages of phytogenics that provide a positive effect. The combination of various phytogenics seems to be relevant and promising. An integrated approach should provide high fragmentation activity, effective digestion and assimilation of feed nutrients.

Keywords: ruminants, greenhouse gases, methanogenesis, diet quality, diet composition, phytogenics, saponins, tannins, flavonoids, essential oils

The impact of greenhouse gas (GHG) emissions on climate change has become a global and publicly discussed environmental and health problem both in the world and in Russia. Agriculture is one of the largest sources of greenhouse gases, and wise use of the potential of the industry can limit the rate of global warming to 2 °C by the end of the century [1].

The efforts of the global community to prevent climate change are predominantly focused on reducing carbon dioxide (CO_2) emissions. At the same time, methane (CH_4), nitrous oxide (N_2O) and other CO_2-free greenhouse gases emitted during the production of crop and livestock products also contribute to global warming. Methane (CH_4) is one of the three major greenhouse gases (GHGs), in addition to carbon dioxide (CO_2) and nitrous oxide (N_2O), with a global warming potential 28 times greater than that of carbon dioxide (CO_2) [1-3]. Agriculture, due to the increase in land use and the reduction of CO_2 absorption spaces (forests, organic soils), is involved in increasing the production of carbon dioxide. Livestock and especially ruminants are the largest source of direct emissions; synthetic fertilizers also contribute heavily to direct emissions; livestock and fish farms account for 31% of greenhouse gases [4]. The livestock sector accounts for approximately 18% of global anthropogenic GHG emissions.

Among livestock, ruminants produce about 81% of greenhouse gases [5] due to massive methanogenesis by rumen microbes, which produce 90% of the total CH_4 emitted by ruminants [6]. Ruminants emit about 115 million tons of CH_4 annually, which is formed as a result of fermentation carried out in the rumen by a complex of bacteria, archaea, protozoa and fungi [7]. Globally, CH_4 emissions from dairy and beef production account for 30% and 35% of livestock emissions, respectively. Buffaloes and small ruminants contribute less, accounting for 8.7% and 6.7% of industry emissions, respectively [8]. Cows and other ruminants hold the record for methane emissions. In their multi-chambered stomachs, bacteria help digest food by synthesizing methane as a by-product. It is released into the atmosphere through belching, although a small part of it is also produced in the intestines. The digestive system of other farm animals differs from that of ruminants. Chickens and pigs emit less greenhouse gases, but their amount is many times greater than that produced by plants of nuts or peas. Fish reared in fresh water also serve as a source of greenhouse gases: excrement and unused food are deposited on the bottom of ponds, where there is almost no oxygen, that is, conditions ideal for the appearance of methane are created.

The harmful effect of methane on the state of the atmosphere is confirmed by the fact that with a conventionally accepted global warming potential (GWP) of carbon dioxide equal to 1, for methane GWP = 21, the half-life of methane is 11 years, and the duration of stay in the Earth atmosphere exceeds 100 years. It follows that methane as a greenhouse gas is no less dangerous than carbon dioxide.

Ruminants can produce between 250 and 500 liters of methane per day, and the contribution of cattle to global warming, which may occur in the next 50-100 years, is estimated at just under 2%. While emissions per unit of livestock production have decreased, global emissions have risen due to an increase in animal populations [8]. By 2050, total CH_4 emissions from ruminants are expected to increase significantly due to increased demand for milk and meat, given the growing world population [9]. This determines the importance of the problem of reducing CH_4 emissions in animal husbandry and the attention to environmental issues in general on the part of government structures [10].

It is possible to reduce the formation of methane in the digestive system of animals through the use of various feed additives, antibiotics and vaccines, as well as through the inclusion of high-quality roughage in the diets of cattle. In addition, a reduction in the volume and intensity of emissions can be achieved

through the use of modern methods to increase the productivity of animals. This strategy is very attractive as it increases farm profits at the same time [11].

Methane emissions from animals вdepends on the amount of feed consumed, the type of carbohydrates in the diet, the methods of preparing feed for feeding, feed additives of various nature that regulate the state of microbial processes. Management of these processes can reduce methane formation in the rumen of ruminants and, as a result, methane emissions into the atmosphere. The study of biochemical, microbiological and genetic aspects of methane formation in the rumen of ruminants is necessary for the use of nutritional factors to reduce CH_4 emissions in animal husbandry.

In recent years, the results of a huge number of studies have been published that have improved understanding of the complex processes of rumen fermentation and methanogenesis in ruminants, as well as ideas about means and methods for reducing methane production in ruminants [12].

The purpose of this review is to summarize current data on the effect of alimentary factors, in particular, the structure and composition of diets, phytogenics of various nature (saponins, tannins, flavonoids, and essential oils), on the formation of methane in ruminants.

Mechanisms of methane formation in ruminants. The microbial ecosystem of the rumen is very stable and optimized due to the natural selection of microorganisms, but not completely efficient. One reason for this is the loss of energy due to methane emissions [13]. For the host animal, the formation of CH_4 means a loss of 2 to 12% of the total energy intake that could be available for growth or production [14].

Carbohydrates are the main source of energy for ruminants. In the rumen, polysaccharides (mainly cellulose, hemicellulose and starch) are hydrolyzed to glucose and other hexoses and pentoses. Further, monosaccharides are metabolized into volatile fatty acids (VFA) and CO_2. Metabolic hydrogen is released during the conversion of monosaccharides to VFAs, restoring intracellular cofactors, and cofactors must be reoxidized to continue fermentation. This is largely due to hydrogenase activity and the formation of dihydrogen (H_2, that is, molecular hydrogen). Dihydrogen does not accumulate in the rumen because it is transferred from the fermenting consortium of bacteria, protozoa, and fungi to methanogenic archaea, which use H_2 to reduce CO_2 and other one-carbon compounds to CH_4 [15, 16].

Methanogens can be divided into three groups depending on the substrate used: methane derivatives: methylotrophic, hydrogenotrophic, and acetate (acetoclastic) [17, 18]. H_2 and CO_2 serve as the main substrates of methanogens, and hydrogenotrophic methanogenesis is considered to be the predominant route of CH_4 formation in the rumen [19]. There is a wide variety of methanogenic archaea in shape (cocci, spirilla, rods of various shapes), mobility (mobile and immobile) and other properties, but the general physiological characteristics of methanogens are the need for anaerobiosis and the use of energy, the formation of which is associated with methane bosynthesis, as its only source [20]. According to a meta-analysis of global data, 90% of rumen methanogens belong to the genera [21] *Methanobrevibacter* (63.2% of the methanogen population), *Methanomicrobium* (7.7% of the methanogen population), *Methanosphaera* (9.8%), "rumen cluster C", currently called *Thermoplasma* (7.4%), and *Methanobacterium* (1.2%). The production of methane from H_2 and CO_2 lowers the partial pressure of H_2, thus allowing the fermentation to continue. Without H_2 removal, H_2 accumulation will inhibit further reoxidation of reduced cofactors which, in turn, will consequently inhibit VFA production [16, 22]. In addition, the functional group of methanogens also uses formate (up to 18% of the total amount of methane in the rumen),

acetate, methanol, methylamines (mono-, di- and trimethylamine) and alcohol, but due to the biological characteristics of these microorganisms, this plays a small role in formation of this gas [23]. For example, *Methanosphaera stadtmanae* produces methane only through the reduction of methanol with the participation of hydrogen, having one of the most stringent energy exchanges of all methanogenic archaea [21].

The formation of methane consumes the maximum amount of hydrogen in the rumen. A smaller part of it is used for the production of propionate. A strong positive relationship between hydrogen and propionate concentrations indicates that elevated H_2 levels in the rumen may activate reactions that involve hydrogen in propionate production [24]. Propionate (an alternative hydrogen scavenger for CH_4) is the main glucose precursor in ruminants, so it is desirable to increase its levels in animals with a high demand for glucogen precursors [25]. Reductive acetogenesis (formation of acetate from CO_2 and H_2) is also desirable as a process for incorporating hydrogen into metabolism, since acetate serves as an energy source and building block in the synthesis of long chain fatty acids. However, reductive acetogenesis is thermodynamically inferior to methanogenesis in the normal rumen, but can be a useful hydrogen sink to enhance methanogenesis-inhibited rumen fermentation. Theoretically, the redirection of hydrogen from methanogenesis to fermentation end products that can be taken up and used by the host animal, as well as to the synthesis of microbial biomass, not only helps to reduce CH_4 emissions, but also has the potential to increase the productivity of the animal. However, this potential has not been consistently realized so far [26].

The rumen microbiota plays an important role in the production of biogenic methane. Information on how the hereditary factors of the host influence on the variability of the rumen microbiota and their combined effect on methane emissions are limited. Q. Zhang et al. [27], using a Bayesian model, in a sample of 750 dairy cows, the joint contribution of the host genotype and microbiota to the host's methane emission was estimated. The study showed that host genotype and microbiota accounted for 24% and 7% of variations in host methanogenesis activity, respectively. In addition, it appeared that certain host genes were significantly associated with the composition of the rumen microbiota [27].

Strategies to reduce methane emissions. According to various authors, methane emissions from dairy cows range from 151 to 497 g/day [28]. This value depends on climatic conditions [29], genotype [30], type of productivity, age [31], as well as the quality and composition of the diet [32, 33] and the provision of food needs of animals [34, 35]. Thus, lactating cows produce more CH_4 (354 g/day) than dry cows (269 g/day) and heifers (223 g/day). Dairy sheep emite 8.4 kg of CH_4 per animal per year. Holstein cows produce more CH_4 (299 g/day) than crossbreeds (264 g/day). Methane emissions from heifers grazing on fertilized pastures are higher (223 g/day) than their counterparts on uncultivated pastures (179 g/day). In beef cattle, average CH_4 emissions range from 161 to 323 g/day. Adult beef cows produce 240-396 g CH_4 daily, Suffolk sheep 22-25 g daily. Annual CH_4 emissions per bison are 72 kg [28]. In a 10-year follow-up in New Zealand, S.J. Rowe et al. [36] noted that sheep with low CH_4 emission had higher wool shearing, were leaner, and differed from their high CH_4 counterparts in fatty acid muscle tissue profile [36].

The development of strategies to reduce the release of methane in the body of ruminants during the fermentation process is of scientific and practical interest. The proposed approaches fall into several categories. For example, there are strategies that affect methanogenesis through nutritional factors. In particular, some

feeds increase the production of propionic acid or reduce the production of acetate, reducing the concentration of H_2, which can potentially serve as a source of methane. Feeding strategies also use so-called modifiers that change processes in the rumen, the substances that directly or indirectly inhibit methanogenesis or provide biological control (defaunation, bacteriocins, bacteriophages, and immunization) of the rumen biota, aimed at reducing the content of methanogens. Increasing animal productivity at the genetic level and by optimizing housing conditions for better use of nutrients in the body, which increases feeding efficiency and reduces gas emissions per unit of product (meat or milk). If the annual milk yield remains constant but comes from fewer cows, overall CH_4 emissions will be reduced.

A number of proposed strategies to reduce methane production in ruminants have been reviewed previously, including many that have been revised [2, 37, 38]. Reviews on methods for measuring methane emissions and their application [39-41], including in dairy cattle [42, 43] are of particular interest, as well as the study of methanogens and their role in methanogenesis [44].

Changing feeding patterns is a simplistic and pragmatic approach that can lead to higher animal performance and lower CH_4 emissions [4]. Changing diets is the most common example of such a strategy. Among the ways to control methanogenesis using nutritional factors, two main categories can be distinguished - improving the quality of food and changing the amount consumed per feeding, as well as the use of feed additives that either directly inhibit methanogens or change metabolic pathways, leading to a decrease in the production of substrate for methane synthesis.

Feed quality. Considerable attention is paid to the study of the effect of feed quality and diet structure on methane production in ruminants (Table 1). The rate of methane production in the rumen depends on the composition of the diet, the type of carbohydrates (cellulose or starch), proteins and lipids, which make the biggest impact on methanogenesis [21, 35], as well as physiological factors, e.g., the time of digestion in the rumen.

Feed quality is known to affect CH_4 production in the rumen [32, 45]. High-quality feed (e.g., young plants) can reduce CH_4 emission by altering the metabolic pathway, as this feed contains more easily fermentable carbohydrates and less neutral detergent fiber (NDF), which improves digestibility and increases the rate of passage of the feed through the gastrointestinal tract [46]. Feeding corn silage was reported to linearly decrease CH_4 output (21.7; 23.0; 21.0 and 20.1 g/kg DM) and CH_4 emissions as a share of total energy intake (6.3, 6.7, 6.3 and 6.0%) when using plants of later stages of maturity [47]. However, other authors have not noted differences in methane emissions when changing the stage of maturation of the grass used for haymaking [48]. Methane release during fermentation differs between grazing ruminants on natural and artificial pastures [49, 50] and also depends on the quality of grass stand [46]. Different feed types can also contribute differently to CH_4 emissions due to differences in chemical composition [51]. So, when replacing a fibrous concentrate with a starchy concentrate, methane production decreased by 22%, and when using the so-called protected starch, by 17%. Methane production was lower for legumes than for cereals (by 28%) and for silage compared to hay (by 20%) (51, 52). Legume feeds have lower CH_4 yields due to the presence of condensed tannins, low fiber content, high dry matter intake, and high transit rate [53]. Increasing consumption of alfalfa as a concentrate replacement can significantly reduce CH_4 emissions [54].

1. Methane emission in animals, as Influenced by feed quality and diets

Factor	Animsals, *n*	Method for methane measurement	Effect on methane peoduction	Reference
Feed quality (high, medium and low)	12 heifers (6 of Holstein breed and 6 of Charolais × Simmental breed, 12 months, BW 310 kg)	Sulfur hexafluoride (SF_6) tracer gas	The amount of methane per 1 kg of digested organic matter was highest on low-quality diets	[32]
Animal age, content of concentrates	45 heifers aged 9, 12 and 15 months, rations with different levels of concentrates (30, 40, 50%)	Sulfur hexafluoride (SF_6) tracer gas	Heifers aged 9, 12 and 15 months with an average weight of 267.7; 342.1 and 418.6 kg produced 105.2; 137.4 and 209.4 g CH_4/day. Average ratio of CH_4 to gross energy consumption 0.054; 0.064; 0.0667. With an increase in the level of concentrates, the release of methane decreased	[31]
Diet composition	40 Continental × British bulls (6 months, BW 252 kg)	Sulfur hexafluoride (SF_6) tracer gas	Methane production per day with high roughage or bulky feed (83.5% silage:11.5% grain) was 42% higher than high grain diets (41.8% silage:41.7% grain)	[150]
Feed quality (poor quality hay + protein supplements: .CF at 0.29% BW or DS at 0.41% of BW)	23 crossbred British bulls (BW 344 kg)	Open cycle gas quantification chamber (GreenFee emission monitoring system GEM; C-Lock Inc., Rapid City, SD)	Animals treated with .CF had higher CH_4 emissions (211 g/day) than those who received DS (197 g/day). With protein supplements, the emissions were higher than in the control (175 g/day). Methane emissions as a percentage of GE consumption were the lowest when animals consumed DS (7.66%), intermediate when .CF was consumed (8.46%), and the highest in control (10.53%)	[33]
Diet composition, animal genotype, age	Rumen contents of crossbred cows Limousin × Swiss (meat) and Limousin × Holstein (milk-meat)	in vitro	The first factor is diet: flaxseed reduced methane yield (by 6.5%), total gas production (by 3.6%), and methane/total gas ratio (by 2.7%) The second factor is the genotype: a lower methane output (by 15%) was noted in the Limousin × Swiss crossbreed cows compared to the Limousin × Holstein crossbreed cows. The third factor is age. In meat animals, methane emissions increased with age; in dairy and meat animals, the highest values were in young and old animals.	[30]
Feed quality	16 lambs received a diet of ryegrass (before flowering and at a late flowering phase)	Sulfur hexafluoride (SF_6) tracer gas	No difference observed	[48]

Continued Tabel 1

Feed quality and diet composition	9 heifers (BW 329 kg) zebu Brahman received one of three diets: hay GQ, hay LQ and low quality hay + molasses + urea (LQ + A)	Sulfur hexafluoride (SF_6) tracer gas	Methane emission (g/day) was the same in the LQ (110.4) and LQ + A (125.8) groups and lower than in the GQ group (181.5). The values of CH_4/kg of DM consumed were maximum in the LQ (31.0) and LQ + A (29.8) groups and lower in the GQ diet (23.0) (30% reduction in emissions compared to the LQ group)	{45]
Feed quality	Natural grassland and sorghum, natural grassland and alfalfa	Sulfur hexafluoride (SF_6) tracer gas	Methane emissions were lower in cows grazing on sorghum than those grazing on natural grass pastures; in cows on natural pastures and fed with alfalfa hay, methane emission was the same. Poor quality diets increase methane output	{49]
Feed quality and consumption	56 lactating dairy Holstein-Friesian cows, ration of grass silage, corn silage and compound feed (70:10:20). Animals are divided into 2 groups, with high (day 96 of lactation) and low (day 217 of lactation) consumption of DM	Open circuit gas quantification chamber	The total amount of methane released per day did not differ between the groups. Relative methane emissions (12.8±0.56 g/kg of milk) were lower (by 12%) with high feed intake and higher milk yield. Methane emissions increased as grass quality deteriorated, regardless of consumption level	[35]
Forage quality (cultivated and natural pastures)	11 Swiss lactating cows	Sulfur hexafluoride (SF_6) tracer gas	When grazing cows on cultivated pastures, methane emissions per unit of both consumed and digested organic matter were lower than when grazing on natural pastures	[50]
Feed quality	12 crossbred (Hu × Han) dry ewes (aged 3 years, BW 32 kg) received corn stover, alfalfa and concentrates (60:0:40, 60:15:25, or 60:30:10)	Respiratory chamber (open circuit)	Increasing the share of alfalfa in the diet reduced methane emission per day, including in relation to the consumed DM and OM	[54]
Feed quality	Different grazing systems consisted in changing the density of animals per ha (1 cow/ha and 2.5 cows/ha) that alters the quality of the grass stand	Sulfur hexafluoride (SF_6) tracer gas	Methane emissions per unit GE consumption (4.6%) was low for grazing animals	[46]

Continued Table 1

Consumption level	290-302 beef cattle (420 kg) and 1105-1251 beef cattle (430 kg), ad libitum/limited feeding	Automatic sampling systems	In CH_4 emissions from the feedlot, one peak was observed during the day with ad libitum feeding, and several peaks with limited feeding. Total emissions did not change	[34]
Diet nutritional value and climatic conditions	30 Simmental dry cows and Gelbfi beef cows (663 kg BW)	Sulfur hexafluoride (SF_6) tracer gas	The use of protein supplements in low-protein diets and prolonged exposure to cold reduced CH_4 emissions	[29]
Diet composition	8 lactating Holstein-Friesian cows, silage based rations or silage + hay	Respiratory chamber (open circuit)	Cows fed a diet based on silage and hay had higher daily methane emissions. There were no differences in methane emissions per 1 kg DM consumed or per 1 kg of milk	[52]
Diet composition	16 lactating cows	Respiratory chamber	Adding treated oilseeds as a source of fatty acids reduced methane production by an average of 13%	[62]
Consumption level and quality of ryegrass silage	56 Holstein-Friesian lactating cows	Respiratory chamber (open circuit)	Improving the quality of grass silage by harvesting feed at an earlier stage of plant growth significantly reduces intestinal CH_4 emissions regardless of DMC	[35]

N o t e. BW — bodyweight, DM — dry matter, CF — cotton flour, DS — dry stillage, GQ — good quality, LQ — low quality, A — additives, GE — gross energy, OM — organic matter, DMC — dry matter consumption.

Feed handling and storage also affect CH_4 emissions [55, 56]. For example, milling or granulating can reduce CH_4 emissions per kg of dry matter ingested, as their smaller particle size accelerates their degradation in the rumen. Methanogenesis is generally lower with ensiled feed [52] (presumably, because ensiled feed is already partially fermented during ensiling). Another study [35] showed that intestinal CH_4 emissions from dairy cows at different levels of feed intake depended on the nutritional value and chemical composition of grass silage. Feed based on young plants with less fiber and increased soluble carbohydrates has improved quality, and the addition of a small amount of grain to the forage also gives a favorable result.

The formation of methane in the rumen of ruminants also depends on the amount and composition of concentrates in the diet [54]. With fewer cell walls and easily fermentable carbohydrates (starch and sugar), concentrates promote propionic acid production, reducing CH_4 release [55]. It was noted that the reduction of CH_4 emissions occurred at the addition of concentrates to diets in amounts of 80 and 90%, while no effect was observed at their proportion equal to 35 or 60% [57]. Increasing the proportion of concentrates in the diet of ruminants is not a good strategy for reducing methane production, as diets high in concentrates are low in structural fiber and will compromise rumen function in the long term, leading to subacute or acute acidosis. Probably, it is necessary to select the optimal ratios of roughage and concentrates in the structure of the diet.

The composition of concentrates also influences rumen gas formation, as different ingredients have different carbohydrate compositions. Among non-structural components, sugar is more methanogenic than starch. All carbohydrate fractions contribute to the formation of CH_4, of which starch is the least (probably due to the formation of VFAs with a predominance of propionate). A large amount of starch in the diet reduces intestinal energy loss compared to diets dominated by roughage [58]. Starch fermentation promotes propionate production in the rumen by creating an alternative H_2 sink [59], lower rumen pH, inhibiting methanogen growth, reducing protozoa in the rumen, and limiting interspecific H_2 transfer between methanogens and protozoa. In addition, feeding starch, which can avoid rumen fermentation, potentially provides energy to host animals while ruminal methanogenesis is inhibited. Up to 30% of corn starch may not be fermented in the rumen and digested in the small intestine [60]. Data on the effect of protected starch on the reduction of methane emissions is still very limited, which requires further study of the problem. Sugar, on the other hand, is rapidly and completely degraded in the rumen, increasing butyrate production at the expense of propionate, thereby making sugar concentrates more methanogenic than starch [61]. Sugars increase butyric acid production at higher H_2 partial pressure and higher rumen pH, as confirmed by I.K. Hindrichsen and M. Kreuzer [61] who reported a 40% increase in CH_4 production with sucrose (compared to starch) at high rumen pH, while methane production decreased at low pH.

Replenishing protein deficiency with protein supplements [29] and adding processed oilseeds (as a source of fatty acids) [62] to diets can significantly reduce CH_4 emissions.

Thus, the type of diet of ruminants, the quality of bulky and concentrated feeds and their chemical composition, the ratio of roughage and concentrated feeds, and the preliminary preparation of feeds affect methane emissions into the atmosphere. A promising approach to reduce methane emissions is to add a small amount of grain to forage and feed high quality feed, feed with less fiber and a higher content of soluble carbohydrates.

Feed additives affecting methane production. *Fat supplements*

[63]. The mechanism of suppression of methanogenesis by fat is induced by reducing the fermentation of organic substances, the digestibility of fiber, as well as by direct inhibition of methanogens in the rumen [64]. Data on the methane production in ruminants when fed fat supplements are quite contradictory. For example, the additional inclusion of linseed oil in the diet of cattle contributed to an increase in the species diversity of the rumen microbiota, the number of bacteria of the phylum *Bacteroidetes* (64.2%), as well as a significant increase in representatives of the rumen archaea domain involved in methanogenesis [65].

Organic acids. It is likely that organic acids stimulate the production of propionic acid in the rumen by acting as hydrogen scavengers, thereby reducing the amount of CH_4 [66]. Malate, acrylate, oxaloacetate, and fumarate are carbohydrate fermentation intermediates that are converted to propionate or used in anabolism to synthesize amino acids or other molecules. They can react with hydrogen, which reduces the amount of hydrogen available to form methane [21]. Organic acid supplements have mainly been tested for effects on methane synthesis in vitro with conflicting results. The use of organic acids in diets to reduce gas formation in vivo requires further study. In addition, the use of organic acids may be limited by the risk of rumen acidification causing acidosis in animals.

Ionophores. Ionophores, which can change the movement of cations (in particular, calcium, potassium, sodium) through cell membranes, are classified as antibiotics and are synthesized by soil microorganisms. Among the inophores, monensin and lasalocide are most commonly used to reduce methane emissions. The mechanism of their influence on methanogenesis is associated with the impact on the number of protozoa and bacteria in the rumen. Ionophores act as antimicrobial agents that can disrupt the concentration gradient of calcium, potassium, hydrogen, and sodium ions across certain microbial membranes, initiating an inefficient ion cycle and providing a competitive advantage for some microorganisms at the expense of others. These compounds preferentially inhibit the growth of Gram-positive bacteria that produce lactate, acetate, butyrate, formate, and hydrogen as end products, thereby reducing the availability of hydrogen for methanogens [67]. Although ionophores can reduce methane production, they also appear to impair dry matter intake in both dairy cows and beef steers (68). It has also been shown that the effect of ionophores weakens over time due to the adaptation of protozoa and the development of resistance in succinate- and propionate-producing bacteria [21]. The temporary effect of ionophores and increasing public pressure to reduce the use of antimicrobial feed additives in agricultural production limit the long-term solution to CH_4 emissions with inonophores.

Probiotics. The effect of probiotics on the formation of gases in the rumen may be based, firstly, on an increase in the number of bacteria due to the separation of degraded carbohydrates between microbial cells and fermented products, and secondly, on a shift in the processes of hydrogen utilization from methanogenesis to reductive acetogenesis. Homoacetogenic bacteria produce acetate from CO_2 and H_2 and play an important role in the recycling of enzymatic hydrogen in the colon in monogastrics. For example, co-feeding of moringa extract and a live culture of yeast (*Saccharomyces cerevisiae*) in in vitro experiments performed with goat ruminal contents reduced methane production [69].

S.H. Kim et al. [70] indicate that most probiotics reduce CH^4 production by affecting the activity of ruminal microorganisms without adversely affecting animals. In addition, probiotics enhance rumen fermentation [70]. Other studies have shown that the effect of probiotics on gas exchange depends on their composition. Thus, in vitro results in ruminants showed that conventional and encapsulated probiotics from the group of lactic acid bacteria reduced the production of methane by 6.1 and 33.1%, respectively, compared with the control. In addition, the authors noted an increase in total gas formation by 15.7 and 23.3% when using

the same probiotics [71]. In the work of G. Guo et al. [72] lactic acid bacteria contributed not only to the reduction of CH_4 emission per unit of VFA yield, but also improved the quality of fermentation and digestibility of silage fiber. A decrease in the formation of methane in the rumen of dairy cows was noted when using a mixture of propionic acid and lactobacilli in the diet with a high content of starch and fiber in the diets [73]. However, the mechanism of inhibition of methane synthesis by lactic acid bacteria has not yet been fully studied, therefore, in the future, additional study of their effect on microorganisms is necessary. In a study on Holstein heifers, the use of the denitrifying ruminal bacterium Paenibacillus 79R4 (79R4) in the diet contributed to a decrease in the formation of methane in the rumen with intramuscular injection of nitrate and a decrease in nitrite toxicity (a decrease in the concentration of methemoglobin in blood plasma was noted) [74]. Feed additives containing *B. licheniformis* were effective in reducing methane emissions in sheep in vivo, with concomitant improvements in energy and protein utilization [75].

Summing up, we note that studies on the effectiveness of the use of probiotics to reduce the emission of methane and other gases are controversial, and in vivo experiments are few. Due to the availability and wide use of probiotics in animal husbandry, it is of interest to study their effectiveness and find the best products and their complexes to reduce methane formation.

Phytogenics. The term phytogenic feed additives or phytogenics was introduced in the 1980s by Delacon Biotechnik GmbH (Austria) and combines a wide group of natural substances obtained from herbs, spices and their extracts, for example, essential oils, saponins, tannins, flavonoids. Such supplements contain many active ingredients. In addition to improving the palatability and, as a result, increasing the attractiveness of the feed, they increase the enzymatic activity in the gastrointestinal tract of animals, the absorption of nutrients, exhibit antioxidant properties, improve the condition of the gastric mucosa and reproductive function [76].

We would like to dwell on this part of our review in more detail. Table 2 presents the results of in vivo studies on the use of saponins, tannins, flavonoids and essential oils to reduce methane emissions in ruminants.

Secondary products of phytobiocenoses. Plant secondary metabolites have long been considered toxic to animals and have been referred to as anti-nutritional factors [77, 78]. However, in the past few decades, interest in these components in animal nutrition has been growing due to their effect on parasite control, rumen fermentation, and methane synthesis [79].

Saponins and tannins. Recently, the potential impact of plant secondary metabolites (PSMs) in reducing methane production has been recognized. The effect of suppressing the release of this gas due to PSMs is associated mainly with the antimicrobial properties of PSMs [80]. Plants produce many secondary compounds, among which much attention has been given to condensed tannins [81, 82] and saponins [83]. The three main plant compounds effective in reducing methane emissions in vitro are condensed tannins, saponins, and essential oils [84].

Tannins are naturally occurring polyphenolic biomolecules found in the bark, wood, fruits, leaves, flowers, and roots of most plant species. Tannins are a subclass of plant polyphenols [78]. Several studies have evaluated the relationship between tannin-rich diets and CH_4 formation in ruminants both in vivo and in vitro [62, 85-87]. Tannins, depending on the chemical structure, can be divided into hydrolysable and condensed tannins [88, 89]. It should be noted that condensed tannins have been more studied with respect to their effect on methane production than hydrolysable tannins. Tannins have the ability to reduce methane synthesis in the rumen directly or indirectly by inhibiting the growth of methanogens or protozoan populations, respectively [78], which has been confirmed in in vitro studies [90, 91].

2. In vivo experiments to study the effect of saponins, tannins, flavonoids and essential oils on methane emission in ruminants

Factor	Animal species, breed	Diet	Effect on methane production	References
Condensed tannins from *Lotus pedunculatus*	Sheep aged 3-4 years and Friesian cows in the final stages of lactation	Pasture based on ryegrass, then alfalfa and then *Lotus pedunculatus*	Reducing methane emissions by the amount of CDM	[96]
Tannin extract (hydrolyzable tannins; *Castanea sativa* wood extract) and saponins (sarsaponin; *Yucca schidigera* extract)	Lambs	Hay: concentrates (1:1) and additionally wheat starch; tannins were added (1 and 2 g/kg DM or 2 and 30 mg/kg DM)	Methane emission increased at low tannin dose compared to control without additives	[119]
Concentrated tannin fodder (*Sericea lespedeza)*	24 female angora goats (BW 41.5 kg)	Pasture with *Sericea lespedeza* and cane fescue	Reducing methane emissions by 30% (g/day) and 50% (g/kg CDM)	[97]
Acacia mearnsii extract	Sheep (75 g fodder DM per kg metabolic body weight)	Partial replacement of ryegrass (*Lolium perenne*) with red clover (*Trifolium pratense*) or alfalfa (*Medicago sativa*) with the addition of 0 or 41 g of *Acacia mearnsii* extract containing 0.615 g/g KT per 1 kg DM	Reducing methane emissions by 15, 13 and 11% (kJ/MJ GE)	[98]
Condensed tannins from the plant *Lespedeza striata*	24 1 year old Boer × Spanish ($^{7}/_{8}$ Boer) cross-breeds goat kids	Sudanese sorghum + 33; 67 and 100 g tannins	Reducing the absolute emission of methane by 32.8, 47.3 and 58.4%	[99]
Foliage of two tannin-rich shrub legumes *Calliandra calothyrsus*	6 Swiss White Hill lambs	Replacement of $^{1}/_{3}$ or $^{2}/_{3}$ high quality herbaceous legume forage with shrub legume *Calliandra calothyrsus*	Reducing methane emissions by 24% per day per unit of feed and energy	[100]
Tannins extracted from the bark of black locust (*Acacia mearns*i, KT 60.3%)	60 lactating cows	Pasture with ryegrass, crushed triticale grain (5 kg/day), tannin (163 and 326 g/day with a decrease to 244 g/day by day 17)	Reduction of methane emissions by 14 and 29% in accordance with the dose (about 10 and 22% of CDM)	[102]

Continued Table 2

Pulp of pumpkin seeds (*Terminalia chebula*), garlic (*Allium sativum*) and their mixture	16 sheep (age 22 months, BW 29.96±1.69 kg)	Feed:concentrate (50:50) + phytonutrients (1% dietary DM, alone or mixed)	Reducing methane emissions up to 24% of digested DM and consumed DM	[103]
Yucca schidigera (YS) extract	Sheep for fattening	Grass silage:concentrate (70:30) + 120 mg YS extract/kg DM	Reducing methane emissions per body weight	[104]
Acacia mearnsii tannin extract	12 Holstein dairy cows	Pasture grass millet + 6 kg concentrates + 120 g tannin extract	Reducing methane emissions by 32%	[146]
Chestnut tannins or chestnut tannins + querbajo tannins	75 crossbred steers (BW 292±4.1 kg)	Alfalfa:barley (50:50) + chestnut tannins (0.25% DM) or chestnut tannins (0.125 or 0.75% DM) + querbacho tannins (0.125 or 0.75% DM)	No reduction in methane emissions was observed	[80]
Tea saponins alone and in combination with soybean oil	32 Huzhou weaned lambs (age 50 days, BW 14.2±1.38 kg)	60% Chinese wild rye (*Aneurolepidium chinese* Kitagawa) and 40% concentrate blend + saponins 3 g/day (or saponins 3 g/day + soybean oil 3% CDM)	Daily methane production decreased by 27.7% and 18.9% respectively	[117]
Tea saponins	12 Hu sheep (aged 7 months, BW 21.5±1.80 kg)	600 g/kg Chinese wild rye and 400 g/kg concentrate mix, 3 g/d tea saponins	Reduced CH_4 production in the rumen, effect similar to that of defaunization	[118]
Condensed tannin of tannic acacia species	Bapedi sheep (aged 1 year, BW 25±1.6 kg)	80% grass hay and 20% concentrates, refined condensed tannin (0, 30, 40, 50 g/kg DM)	Reducing methane emissions by 51-60%	[81]
Condensed tannins and saponins, obtained from *Enterolobium cyclocarpum* (Jacq.) Griseb. crushed pods mixed with foliage of *Gliricidia sepium* (Jacq.) Steud.	4 crossbred heifers (*Bos taurus* × *Bos indicus*) (aged 12 months, BW 218±18 kg)	79.9% hay of *Brachiaria brizantha* (Hochst. ex A. Rich.) and 20.1% balanced mix based on soybean meal, bran, cane molasses and minerals, 15, 30 and 45% DM based on dry and crushed leaves of *G. sepium* and pods of *E. cyclocarpum* in equal proportions	Methane emissions decreased by 0.16 times (calculated on DCP)	[120]
Dried leaves of *Leucaena leucocephala* (DLL)	4 crossbred heifers (*Bos taurus* × *Bos indicus*) (BW 310±9.6 kg)	Hay and concentrates + dried leaves of *Leucaena leucocephala* (DLL) (0, 12, 24 and 36% DM)	Reducing the formation of methane (on average by 25% (per 1 kg of DP)	[121]
Levcaena *Leucaena leucocephala* (Lam.) De Wit Cunningham variety, fresh	8 Lucerne heifers (aged 19±3 months, BW 218±18 kg)	100% chickweed *Cynodon plectostachyus* K. Schum) and 76% chickweed with 24% levcaena *Leucaena leucocephala* (Lam.) De Wit Cunningham variety fresh	No increase observed in methane emissions with an increase in productivity, which reduced methane emissions by 1 kg of production	[122]

Continued Table 2

Flour from the pods of *Samanea saman*	4 crossbred heifers (*Bos taurus* × *B. indicus*) (BW 261.5±1.29 kg)	Ground green grass feed, soy flour, wheat bran and sugar cane molasses, minerals with the addition of ground *S. saman* pods were 0, 10, 20 and 30% DM	At a dosage of ground S. saman pods of 30% DM, methane emissions per animal decreased by 50.9% (in absolute units) and by 56.9% (calculated per 1 kg CDM)	[123]
Tannins from tropical legumes *Desmanthus leptophyllus* and *D. bicornutus*	14 Droughtmaster bulls (aged 12 months, BW 296±5 kg)	Rhodes grass hay (*Chloris gayana*) with fresh desmanthus (0, 15, 31 and 22% DM)	Linear reduction of methane emissions without reduction of DM consumption	[82]
Mulberry leaf extracts and resveratrol from *Polygonum cuspidatum*	10 crossbred first-lamb ewes (Dorper × Han, BW 60.0±1.73 kg)	Basal diet without additives, supplemented with flavonoids from mulberry leaves (2 g/day per sheep) and supplemented with resveratrol (0.25 g/day per sheep)	Reducing the formation of CH_4 by 10.64% per 1 kg of CDM	[147]
Blend of essential oils containing coriander seed oil, eugenol and geranyl acetate	4 Holstein cows (BW 603±70 kg, day 296 of lactation) and 4 Belgian blue beef heifers (BW 484±111 kg)	Dairy cattle — grass silage (460 g/kg DM), corn silage (370 g/kg DM) and soybean meal (50 g/kg DM), concentrates, 0.2 g/day of a mixture of essential oils (120 g/kg DM); beef cattle — corn silage ad libitum and supplementary feeding with concentrates, 0.2 g/day of essential oils	After 6 weeks of supplementation in dairy cattle, CH_4 emissions decreased (g/day) by 15%, re-calculated for DM consumed by 14% ($p = 0.07$), in beef cattle, these indicators tended to increase by 10 and 11 % and decreased by 20% when calculated based on body weight	[142]
Coriander seed oil blend, geranyl acetate and eugenol	149 early lactating Holstein-Friesian cows	Grass feed, whole grain wheat, corn silage, 1 g of a mixture of essential oils with drinking water	Decreased methane production from 438 to 411 g/day	[143]
Essential oil blend (0.17 g/kg DM), lauric acid (65 g/kg DM), essential oil blend with lauric acid	8 cows (BW 610±59 kg)	Feed mix of 40% corn silage, 30% grass silage and 30% concentrates	The reduction in methane emissions was more pronounced when using a mixture of additives	[144]
A mixture of phytogenic supplements from dried and crushed leaves of *Populus deltoides* and *Eucalyptus citriodora* (50:50 by weight)	12 lactating Murra buffaloes (*Bubalus bubalis*) (BW 510.50±32.12 kg) at an early stage of lactation	Chopped young sorghum plants, wheat straw and mixtures of concentrates with phytogenic additives (15 g/kg DM)	Reducing the concentration of methane in exhaled air by 37.3%	[148]

N o t e. BW — bodyweight, DM — dry matter, CDM — consumed dry matter, GE — gross energy, DCP — digested crude protein, DP — digestible protein.

There are several possible hypotheses explaining the mechanisms of action of tannins on the reduction of methane formation in the animal body [89]. One of them suggests a direct effect of condensed tannins on the methanogenic archaea of the rumen due to the binding of protein adhesin or parts of the cell membrane, which disrupts the formation of the methanogen-protozoal complex, reduces the interspecies transfer of hydrogen, and inhibits the growth of methanogen. The high molecular weight and polyphenolic nature of tannins lead to the formation of complexes with microbial enzymes or cell walls. The activity shown can cause inhibition of cellulolytic or proteolytic bacteria or methanogens [92]. The mechanism of action of tannins strictly depends on their chemical structure, as well as on the type of bacteria [78]. Another possible explanation is indirect inhibition by reducing the availability of nutrients (carbohydrates, amino acids) for rumen microorganisms and the formation of tannin-protein complexes in the rumen [93], which reduces feed digestibility and disrupts the structure of the rumen microbiota. The latter theory suggests that the condensed tannins themselves act as hydrogen scavengers, reducing its availability for carbon dioxide reduction to methane [89]. Condensed tannins have been found to be more nutrient-binding than hydrolyzed tannins, mainly due to their higher degree of polymerization, making them more difficult to degrade in the rumen [91]. In another work, the same authors note that, on the contrary, hydrolyzed tannins had a greater ability to precipitate protein, which is associated with increased biological activity and a higher ability to suppress the formation of methane compared to condensed tannins [91].

An in vivo study in fistula sheep examined the direct inhibition of certain Gram-positive specialized ruminal fibrolytic bacteria (*Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Butyrivrio proteoclasticus*) by condensed tannins [94]. G.C. Waghorn and S.L. Woodward [95] report that condensed lotus tannins reduce methane production on a dry matter basis by about 15% in sheep and dairy cows. A similar effect has been noted in other studies [96]. Feeding goats with the perennial *Lespedeza cuneata*, which contains condensed tannins, showed a 57% reduction in methane production per kg of dry matter ingested compared to that observed in goats fed a mixture of *Digitaria ischaemum* and *Festuca arundinacea* [97]. It has been found that methanogenesis is reduced by 13% in sheep eating *Acacia mearnsii* with a tannin content of 41 g/kg dry matter [98]. A decrease in methanogenesis with the use of tannins from the plant *Lespedeza striata* in the diet of goats was noted by G. Animut et al. [99]. Tannin-containing *Callinada calothyrsus* and *Fleminga macrophylla* reduced methane production in lambs by 24% [100], but condensed tannin extract from *Schinopsis quebrachocolorado* [62] and tannin-containing sorghum silage [101] fed to cattle did not suppress methanogenesis. A decrease in methanogenesis in in vivo experiments on cows and sheep using tannin from various sources has been noted in a number of studies [102-104].

Saponins are natural detergents chemically defined as high molecular weight glycosides in which sugars are linked to a triterpene or steroidal aglycone moiety. As secondary plant metabolites, saponins have the ability to modulate rumen fermentation while reducing methane production and ammonia concentration [105]. Saponins mainly affect the population of protozoa [106-108], disrupting their cell membrane integrity [109, 110]. The symbiosis of protozoa with methanogenic bacteria in the rumen is well known and it has been suggested that selective suppression of protozoa may be a promising approach to reduce methane production. Plants rich in saponins have the potential to increase microbial protein flux from the rumen, increase feed efficiency, and reduce methanogenesis.

R. Wallace et al. [111] suggested that saponins can destroy protozoan cells by forming complexes with sterols on the membrane surface, which are then

destroyed and disintegrated. In addition, some saponins affect various types of membrane proteins, such as Ca^{2+} channel proteins and Na^{+}/K^{+} ATPase [112]. E. Ramos-Morales et al. [113] suggest that the effect of saponins on protozoa is temporary beause the bacteria can break down saponins into sapogenins, compounds that cannot affect protozoa.

Saponins have been shown to inhibit protozoa in vitro and also limit the availability of hydrogen for methanogenesis [114]. An in vitro study showed that liquid extracts of *Yucca schidigera* and *Quillaja saponaria* added in amounts from 2 to 6 ml/l of rumen fluid, reduce the number of protozoa in the rumen and can potentially change the ammonia content, propionate concentration and the ratio of acetate to propionate. In the same study, the effect of *Y. schidigera* was manifested in a decrease in the rate and formation and volume of methane, depending on the dose, by 42 and 32%, respectively, while in *Q. saponaria*, the effect of inhibition of methanogenesis was not manifested [115].

In an in vivo study, dietary extract of *Y. schidigera* (120 mg) reduced methane production in fattening sheep (104). In a study by L. Holtshausen et al. [116], cows received whole *Y. schidigera* plant powder (10 g/kg dry matter) or whole *Q. saponaria* plant powder (10 g/kg dry matter), both powders containing saponin. The authors stated that previous in vitro studies have shown a reduction in methane production at higher doses of saponins (15 g/kg DM or more), but these values were avoided in vivo to minimize the impact on feed digestibility [114]. Under natural conditions, no effect of the herbal supplement was found, and the authors concluded that the decrease in in vitro methane content was probably due to a decrease in digestibility and fermentation of the feed (116). Tea saponins, alone or in combination with fat supplements, have been shown to reduce methane emissions in sheep in vivo [117, 118].

Combinations of tannins and saponins in ruminant diets have proven to be effective in terms of methane emissions [119, 120]. In a number of in vivo experiments on ruminants, a decrease in methane emission in animals was also noted when saponins and tannins were included in the diet [121-123].

Flavonoids and essential oils. Flavonoids are C_6-C_3-C_6 polyphenols found in seeds and vegetables that exhibit anti-inflammatory, antioxidant, and antimicrobial properties [124]. Flavonoids are highly biologically active, reducing or preventing cellular damage caused by free radicals [125]. Flavonoids act on gram-positive microorganisms by inhibiting the functions of the cytoplasmic membrane, inhibiting the synthesis of the bacterial cell wall or nucleic acids. Flavonoids included in the diet of ruminants have been shown to increase productivity by increasing the production of propionate compared to acetate [126]. The influence of various flavonoids (flavones, myricetin, naringin, catechin, rutin, quercetin and kaempferol) at a concentration of 4.5% of the DM on the microbial activity of the rumen in vitro was evaluated [127]. The results showed that all flavonoids, except for naringin and quercetin, reduced the ability of the microbiota to degrade dry matter. Gas production decreased under the influence of flavone, myricetin and kaempferol, while naringin, rutin and quercetin markedly increased its production. Flavonoids significantly suppressed methane production. The total concentration of VFAs decreased in the presence of flavone, myricetin and kaempferol. All flavonoids, except for naringin and quercetin, significantly reduced the activity of carboxymethyl cellulase, cellulase, xylanase, and β-glucosidase, purine content, and microbial protein synthesis. Under the influence of flavones, myricetin, catechin, rutin and kaempferol, the microbial population of the rumen was reduced. The growth of the population of protozoa and methanogens was suppressed by naringin and quercetin. The results of this study showed that naringin

and quercetin at 4.5% DM are potentially suitable for suppressing methane production without any negative effect on microbial fermentation in the rumen.

A commercial citrus extract of a mixture of flavonoids reduced methane production, the abundance of hydrogenotrophic methanogenic archaea, while increasing propionate concentration and population of *Megasphaera elsdenii* in vitro [128]. In Holstein cows, when an extract of alfalfa flavonoids (60 mg/kg of body weight) was added to the diet, the ratio of valeric acid and the total amount of VFAs in the rumen increased, the composition of milk and the digestibility of nutrients improved, and there was a trend towards an increase in the ruminal population of *Butyrivibrio fibrisolvens* [129]. In an in vitro experiment, the flavonoid luteolin-7-glucoside was found to reduce methane [130]. Based on available data, flavonoids have the ability to reduce methane emissions, but further in vivo studies are needed.

In vitro experiments have examined the effect of the combination of garlic powder and bitter orange extract on methane production, rumen fermentation, and feed digestibility in various diet structures (ratios of roughage grasses to concentrates) [131]. The results showed a strong suppression of methane production in all variants. For a diet consisting only of grass, the effectiveness of the additive was 44.0%, when the diet was supplemented with concentrates in a ratio of 20:80, it was 69.2%. The use of flavonoids significantly increased the concentration of ammonia nitrogen and lowered pH, while the digestibility of organic matter and fiber did not decrease. When using these nutritional factors, regardless of diets, there was a change in rumen fermentation with less acetate and more propionate and butyrate, with an increase in total VFAs.

There is a known method for reducing the concentration of methane in the rumen of ruminants through the use of medicinal plants — wormwood herb (10.0 g/kg DM diet), elecampane rhizomes and roots (6.0 g/kg DM diet) [132]. It has also been proposed to orally administer a food composition containing flavanones from a citrus plant. The authors used compositions with different combinations of components: neohesperidin, poncirin, and naringin [133].

Essential oils are volatile plant-derived secondary metabolites with very strong antimicrobial properties that inhibit the growth and viability of most microorganisms in the rumen [134]. The mechanism of action of essential oils varies depending on their type [135]. All essential oils contain chemical components (terpenoids, phenols and phenols) and functional groups that have strong antimicrobial properties. Due to their lipophilic nature, essential oils have a high affinity for microbial cell membranes [136]. When essential oil is used, methanogenesis in the rumen is reduced, especially due to the reduction of microbial populations. Nevertheless, the mechanisms of the influence of essential oils on the processes of fermentation in the rumen of ruminants require more in-depth study.

The effect of plant secondary metabolites on methane production has been studied in vitro [137]. Nine concentrations of the following metabolites were compared: 8-hydroxyquinoline, α-terpineol, camphor, bornyl acetate, α-pinene, thymoquinone, and thymol. All compounds can alter rumen fermentation and reduce CH_4 production. The minimum concentrations that reduce the production of CH_4 were as follows: 8-hydroxyquinoline 8 mg/l, thymoquinone 120 mg/l, thymol 240 mg/l, α-terpineol + camphor + bornylacetate + α-pinene 480 mg/l. The authors attribute these effects to changes in the structure of the rumen bacterial community [137]. As shown by ion semiconductor sequencing, the influence of secondary plant metabolites was most pronounced in the predominance of the relative abundance of the families *Lachnospiraceae*, *Succinivibrionaceae*, *Prevotellaceae*, unclassified *Clostridiales* and *Ruminococcaceae*. CH_4 production correlated negatively with the relative abundance of *Succinivibrionaceae* and positively with

the relative abundance of *Ruminococcaceae*.

In other in vitro experiments, the effect of *Macleaya cordata* extract at six concentrations (0.01, 0.11, 0.21, 0.31, 0.41 and 0.51%) was studied when incubated for 12 and 24 h for methane formation [138]. Methane emission decreased depending on the dose of *Macleaya* extract after 3, 6, 9, and 12 h of incubation, but increased after 24 h. The addition of 0.11% *M. cordata* extract effectively reduced methane production without affecting in vitro digestion of DM.

Research by D. Petri et al. [139] showed in vitro that a substrate containing a mixture of medicinal plants (wormwood, chamomile, fumitory and mallow) had a strong antioxidant capacity in the rumen content and had the potential to reduce methane production. Thymol at a dose of 200 mg/l, when incubated in the cicatricial contents for 24 h, contributed to a decrease in methane formation, which the authors attribute to changes in the quantitative composition of bacteria, archaea, and protozoa [140].

The use of a mixture of essential oils, bioflavonoids and tannins in animal diets significantly reduced the total gas emission, which was noted for methane in an in vitro experiment after 16, 20 and 24 h of incubation. In addition, a decrease in the concentration of acetic acid and an increase in the concentration of propionic acid were observed in the rumen after 16 and 24 h. The group of animals receiving the mixture showed an increase in milk yield and DM consumption while maintaining the milk quality [141].

In general, it should be emphasized that the number of studies on the effect of flavonoids and other secondary plant metabolites on methane production in vivo is very limited. In addition to the examples above, the effectiveness of the methane synthesis suppression in the cattle rumen with dietary mixture of essential oils are reported [142-146]. Other in vivo experiments have examined sheep and buffalo gassing during fermentation as influendes by a mixture of phytogenic supplements in diets [147, 148]. Thus, the number of in vivo studies on the use of flavonoids and other secondary plant metabolites to reduce methane emissions is very limited. The results obtained are variable and depend on the type of metabolite, its characteristics and the diet of the animals. In addition to continuing research to assess the potential of phytogenics for animal husbandry practice, long-term observations are needed in connection with the possible adaptation of ruminal microorganisms to a bioactive metabolite, as well as identifying differences between its effects in vitro and in vivo. A promising way to reduce methane emissions into the atmosphere is the integrated use of various phytogenics in animal diets.

Summing up, we note several important, in our opinion, aspects. Although the efforts of geneticists, breeders, and animal nutrition specialists have significantly reduced methane emissions per unit of livestock production, the growing demand for food requires further reduction in both the intensity of emissions per unit of production and absolute emissions per animal. However, the available evidence on the effectiveness of various strategies to reduce methane emissions in ruminant species is conflicting [149]. Modern methods make it possible to more accurately assess the formation of greenhouse gases in the animal body, but remain expensive and technically complex, so their application is mainly limited to scientific research. The development of biomarkers for methane production is at a relatively early stage and should become a priority in the future. It also requires additional study of probiotics, phytogenics, other feed factors and their complexes as potential means of reducing methane emissions, taking into account the structure of diets, dosage, animal species and other factors. In addition, it is important to understand that researchers do not yet have sufficient information about the impact of strategies to reduce methane emissions into the atmosphere on productivity, animal health, the state of the antioxidant and hormonal systems, and the

structure of the rumen microbiome.

In summary, over the past 50 years, a significant amount of research has been carried out that has deepened the understanding of the complex processes of fermentation and methanogenesis in the rumen in ruminants and made it possible to gain an understanding of the means by which methane production can be reduced. However, sustainable strategies for dealing with the problem have not yet been adopted. As the results of studies show, the use of feed factors of various nature (ionophores, probiotics, plant secondary metabolites) can serve as a cheap and environmentally friendly strategy to reduce methane formation in ruminants with a positive effect on animal tolerance. A combination of various phytogenics seems to be an actual and promising approach. In numerous in vitro studies, the effectiveness of reducing methane emissions depends on many factors. Therefore, an integrated approach is needed to reduce gas formation in ruminants while maintaining the state of enzymatic processes, digestibility and assimilation of nutrients in feed rations.

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BEEF CATTLE EVALUATION BY FEEDING EFFICIENCY AND GROWTH ENERGY INDICATORS BASED ON BIOINFORMATIC AND GENOMIC TECHNOLOGIES

(review)

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Abstract

Beef cattle breeding is characterized by significantly higher feed costs per unit of output compared to other livestock industries. For most species of farm animals, breeding to improve the efficiency of feed use has been difficult until recently due to the complexity of the individual assessment of this indicator. The improvement of the trait occurred indirectly, through selection for an increase in the intensity of growth and a decrease in the fat content in carcasses. In 1960-1980, Förster-Technik GmbH (Germany) developed automatic feeding stations for individual fattening to account for data on energy costs for the growth and development of animals, which made it possible to derive the feed conversion rate (FCR), which remains one of the main parameters of feed efficiency (K.R. Koots et al., 1994). FCR as a trait is not important for genetic selection due to moderate heritability (A.A. Sermyagin et al., 2020; D.N. Crews et al., 2005). In this regard, and thanks to data from feedlots, in 1963 a new alternative concept for the FCR indicator, the predicted residual feed intake (RFI), was developed. RFI is an individual characteristic of an animal, which is determined by the results of test fattening (duration from 70 to 84 days), taking into account daily feed intake and live weight gain (R.M. Koch et al., 1963). The advantage of using RFI as a measure of feed efficiency in conjunction with FCR is that selection for a negative RFI will allow for reduced feed intake without compromising growth. In addition, the predicted residual feed intake does not depend on productivity, growth and body size, making it a trait that has a clear breeding value (G. Acetoze et al., 2015; J.A. Archer et al., 2000; G.E. Carstens et al., 2002). It has been established that RFI correlates with FCR (genetic correlation coefficients vary from 0.45 to 0.85), but RFI does not depend on average daily gain (ADG) and metabolic body weight (MWT) (B.W. Kennedy et al., 1993; P.F. Artur et al., 2001). The assertion that individuals of the same body weight require different amounts of feed to achieve the same performance provides the scientific basis for assessing RFI in beef cattle. Due to the fact that RFI is hereditarily determined (heritability coefficients vary from 0.08 to 0.49), a directed search for quantitative trait loci (QTL) is conducted using the GWAS (genome-wide association study) methodology. Since the 2000s, methods have been developed and implemented for assessing the breeding value of farm animals using information on a large number of SNPs (single nucleotide polymorphism), based on the principle of linear modeling. Linear models, depending on the approach to data structuring, are divided into rrBLUP (estimation of the effect of each marker), GBLUP (estimation of breeding value based on genomic relationship), and one of the most common modern one-step estimation method ssGBLUP (genomic breeding value estimation model that takes into account genomic relationship along with pedigree). BayesA and BayesB are applicable non-linear Bayesian models. Scientific studies using genome-wide association analysis have allowed the development of genomic selection programs and the identification of a number of SNPs associated with indicators of feed efficiency. Thus, seven positional candidate genes were found which were previously associated with the efficiency of feed use and growth energy in different types of farm animals, and were recently identified in Angus cattle. The analysis of foreign studies allows us to recommend the use of the described methods both in research work and for production purposes with the prospect of including these parameters in the criteria for genomic evaluation of beef cattle of different breeds bred in Russia.

Keywords: feeding efficiency, feed conversion, predicted residual feed intake, genomic technologies, genome-wide association search, GWAS, beef cattle, pig breeding

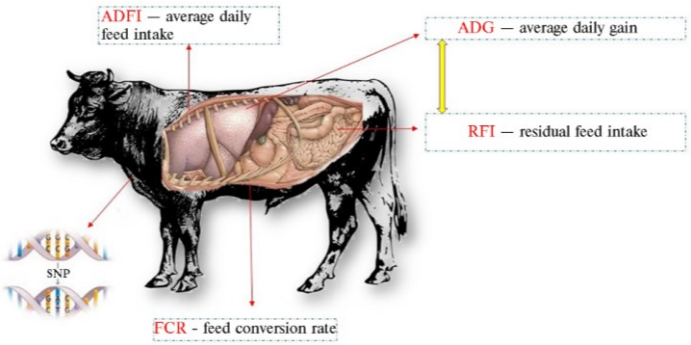
Improving feed conversion is a topical issue in livestock breeding programs, as increased feeding efficiency affects the economic efficiency of the industry as a whole. The problem is especially relevant for beef cattle breeding, where the cost of feed per unit of production is much higher than in other livestock industries. The feed cost per 1 kg weigh gain in beef cattle averages 6.0 kg/kg, while in pig and poultry farming it is 2.5 and 1.9 kg/kg, respectively. Despite significant progress in genetics and herd management technologies (animal feeding, keeping, health and welfare), feed costs account for 60-65% of the beef cost, and at some stages of beef production can exceed 80% of the total costs [1].

Although feed efficiency has improved significantly over the past 40 years, further progress is expected [2]. It is estimated that due to 10% improving feed efficiency, annual savings in the US beef industry could exceed $1 billion [3]. For most species of farm animals, breeding to improve feed efficiency has been difficult until recently, since this trait is difficult to evaluate individually. Basically, the improvement of the trait occurred indirectly, through selection for increased growth intensity and reduced fat content in carcasses. However, the effectiveness of such selection was relatively low. The problem was solved thanks to the development and implementation of automatic feeding stations that allow accurate individual accounting of feed intake [4].

The review analyzes methodological approaches to improving the efficiency of feed use in beef cattle breeding.

Automatic feed stations and feed efficiency indicators. Traditionally, evaluation of feed efficiency is based on feed conversion rate (FCR) as an economically significant indicator. FCR is the ratio of given feed weight (dry matter) over animal weight gain in a certain period of time. Animals with a low FCR value consume less feed per 1 kg weight gain, while animals with a higher FCR consume more. FCR strongly depends on the amount of feed consumed and the average daily weight gain of each animal. In 1960-1980, Förster-Technik GmbH (Germany) developed automated feedlots for individual fattening to study energy production and use in animals. Currently, there are several types of such systems in the market. These units provide various automation levels and were developed in stages [5]. At the first stage of automation ("mixing—portioning—pushing out" level), the built-in mobile equipment allowed filling the stationary mixer with silo from silo tanks. The advantage of this option is that a group of animals can be fed automatically several times a day, but filling the mixer stillremains time consuming. At the second stage of automation, stations were developed in which the mixer is automatically filled, then the feed undergo mixing—portioning—(pushing out). This type of station ensures that all animals are fed in groups several times a day, and the time when the farmer must fill the mixer and feed the animals is no longer fixed. The third stage provides for automation at the stages of unloading and transportation—filling the mixer—mixing—portioning—ejection. In systems of this type, fully automated feeding has so far been carried out only using tower or deep silos, but such systems are relatively expensive in design and power consumption.

Currently, approved and improved automated feeding technologies for cattle allow one to control feed quality and consumption, the growth rate, feeding behavior, and feed efficiency, which is especially important, since the development of animals can vary significantly. In meat cattle, different time and growth dynamics require cattle breeding not only for meat yield and quality, but also for feed conversion and predicted residual feed consumption (Fig.).



Feed efficiency indicators (ADFI, ADG, RFI, FCR) **as selective significant traits in improving the productive qualities of beef cattle.** Based on the phenotypic description by ADFI, ADG, RFI, FCR and the search for SNP (single nucleotide polymorphism), associations of genomic variants with these traits are searched for involvement in breeding programs.

FCR correlates with total energy intake, growth rate and body weight [6]. Genetic selection for feed conversion and average daily gain can be problematic as more attention is usually paid to traits with greater variability [7]. The genetic correlation to FCR is positive, that is, selection for feed conversion should lead to faster growth of beef cattle. Thereof, the animals will have a large final metabolic mass and will be more demanding on the keeping conditions. Selection for FCR alone is likely to be less effective in the long run. The consequence of selection for a decrease in feed conversion may be an increase in weight at weaning, upon reaching one year of age and an increase in costs due to an increase in the need of animals for nutrients [2, 8, 9]. Since the feed supply adequacy affects the profitability of the enterprise, it becomes essential to evaluate the efficiency of feed use which is pften based on indirect estimates [10, 11].

K.R. Koots et al. [12] reported a negative weighted genetic correlation between feed conversion rate, growth rate and animal size. The values of the correlation coefficients indicate that selection for a decrease in FCR increases the efficiency of feed use and leads to acceleration of animal growth and maturity. Although feed conversion is a moderately inherited trait [4[, it is not important as a parameter used to genetically improve feed efficiency rates [13].

An alternative feed efficiency parameter widely assessed in various farm animals, including beef cattle, is residual feed intake (RFI). RFI is an individual characteristic of an animal, which is determined during trial fattening for 70 to 84 days with daily allowance for feed intake and body weight gain. The RFI concept was introduced in 1963 by R.M. Koch et al. [14, 15] and has recently become the preferred parameter for measuring feed efficiency. This measure is unique because, unlike FCR, it separates feed intake into two different components, the feed intake for actual performance and predicted residual feed intake. RFI is a multifactorial and complex characteristic of beef cattle, the variability of this indicator is due to the interaction of many biological processes, which, in turn, are influenced by the physiological state and timely implementation of veterinary and preventive measures.

An advantage of RFI as a measure of feed efficiency in combination with FCR is that selection for a negative RFI will allow for reduced feed intake without compromising growth and physiological maturity of the animal [16, 17]. CRS with negative RFI values are more efficient than those with positive RFI. An economic analysis of genetic improvement schemes that include RFI testing of individuals has shown significant economic benefits compared to methods that do not include testing for this trait. In addition, negative RFI-based selection has the added benefit of reducing greenhouse gas emissions from cattle. Residual feed intake is considered an integral part of basic metabolic processes. The factor ensuring the profitability of livestock enterprises is the cost of feeding. Since the animal productivity indicator is not used when calculating the RFI value, this approach is considered

promising for planning this expenditure (11, 14, 16). Along with moderate heritability, RFI is independent of productivity, height, and body size, making it an ideal trait to involve in breeding for forage efficiency [18-20].

D.P. Berry et al. [11] reported on the impact of the genetic component to the RFI value. The authors point out that a reliable calculation of RFI requires additional estimates of the impact of genotype-environment interactions on variability in feed efficiency. In mathematical modeling, it is necessary to consider the genetic background quantitative traits (for example, meat quality) under the influence of the environment, as well as animal health indicators under specific growing conditions. Many studies have shown that metabolic body weight and feeding behavior [21], intestinal absorption of nutrients [22], mitochondrial function [23] and appetite regulation [24)] are genetically dependent. Since fattening qualities depends on cattle nutrition, health and the stage of rearing, the efficiency of feed use cannot be considered in isolation from the applied technological system. RFI is a moderately inherited trait [11], so it is important to evaluate the expected consequences when breeding for RFI. In addition, there is still insufficient information on the relationship between the RFI value and pastoral productivity, which is important for beef production. Publications on the relationship between RFI values in mother cows and their offspring, combined with moderate repeatability and heritability of the trait, suggest that selection for negative RFI as part of the multiple selection index is possible, cumulative and promising [1, 25].

In pig breeding, the efficiency of feed utilization serves as a useful trait in animal raising and breeding herd formation. In a review article, H. Gilbert et al. [26] summarized the results of a breeding experiment on residual feed intake (RFI) in nine generations of young Large White pigs. After nine generations, divergent selection for predicted residual feed intake resulted in very significant ($p < 0.001$) differences in RFI (–165 g/day vs. +300 g/day) and average daily feed intake (–270 g/day). Negative values were observed for growth rate (–12.8 g/day, $p < 0.05$) and carcass composition (fat thickness +0.9 mm, $p = 0.57$; lean meat content 2.64%, $p < 0.001$) with a marked decrease in the feed conversion ratio (–0.32 kg feed/kg body weigh gain, $p < 0.001$). Reduced pH limit and meat color ($p < 0.001$) and little effect on meat organoleptic quality were characteristic of RFI negative pigs. Changes in meat quality were associated with disorders of muscle metabolism. L. Fu et al. [27] conducted genome-wide association studies (GWAS) for feed efficiency parameters in Landrace pigs and found eight common QTL (quantitative trait loci) regions, of which three regions related to ADFI and RFI traits overlapped. Gene ontology analysis identified six candidate genes (*PRELID2*, *GPER1*, *PDX1*, *TEX2*, *PLCL2*, and *ICAM2*) corresponding to traits associated with feed efficiency. These genes are involved in the synthesis and breakdown of fats, lipid transport, and insulin metabolism.

Interrelation of forage use efficiency signs. Examination of feed efficiency found significant individual differences in feed intake that were both lower and higher vs. predicted from body weight and average daily weight gain. The assertion that individuals of the same body weight require quite different amounts of feed to achieve the same performance provides the basis for assessing RFI in beef cattle [27]. Breeding for RFI is prospective due to phenotypic independence of this index from the average daily weigh gain while the parameters used to calculate this index are hereditarily dependent with the heritability coefficients in different livestock populations from 0.08 to 0.49 [29-31].

Table 1 submeets data on the genetic relationship between feeding efficiency parameters and predicted residual feed intake in beef cattle. Residual feed intake correlates with FCR, genetic correlation coefficients vary from 0.45 to 0.85 [32]. This means that selection for improved RFI will lead to direct improvement

in FCR [33, 36, 37]. There is a positive correlation between RFI and feed intake, indicating that more effective cattle with lower RFI consume less feed. Residual feed intake is phenotypically independent from the average daily gain (ADG) and metabolic body weight (MWT) used in calculation. B.W. Kennedy et al. [7] argue the genotypic independane of such a relationship. P.F. Arthur et al. [6] showed that the genetic influence on weaning weight and weight at one year of age is divided into maternal, paternal, and individual effects of the animal. The genetic relationship between FCR and ADG is negative and moderate in most studies [6, 32]. Individuals selected for lower FCR will produce offspring with a high final body weight, resulting in high costs to maintain the breeding herd.

1. Genetic relationship (correlation coefficients) of fattening indicators with RFI in bulls of various breeds (r±SE)

Trait	Breed						
	Angus [6]	Charolais [19]	Hereford [17]	crossbred$_1$ [32]	crossbred$_2$ [33]	Wagyu [34, 35]	crossbred$_3$ [37]
Animal number n	1180	792	540	2284	1481	740	464
ADG, g	–0,04±0,08	–0,10±0,13	0,09±0,29	0,01	0,09±0,20	0,25±0,16	0,46±0,45
MWT, kg	–0,06±0,08		0,22±0,29	–0,17	–0,20±0,16	0,16±0,13	0,27±0,33
FCR, kg/kg	0,66±0,05	0,85±0,05	0,70±0,22	0,69	0,41±0,32	0,64±0,10	0,62±0,09
BW$_1$, kg	–0,45±0,22		0,34±0,34				
BW$_0$, kg	–0,26±0,14	0,32±0,10	0,15±0,28	0,81	0,43±0,15	0,19±0,15	
Feed intake, g	0,66±0,05	0,79±0,04	0,64±0,16			0,78±0,06	0,73±0,18

N o t e e. Crossbred$_1$ means hybrids of Charolais, Limousine, Simmental, Hereford and Angus breeds, crossbred$_2$ means hybrids of Brahmin cattle and Santa Gertrudis], crossbred$_3$ means hybrids of Alberta, Angus, Charolais; ADG — average daily gain, MWT — metabolic body weight, FCR — feed conversion rate, BW1 — initial body weight, BW0 — final body weight,

Currently, genetic selection aimes at improving the efficiency of feed use and profitability in beef cattle breeding. R.R. Westhuizen et al. [38] assessed genetic variation and covariance between weaning weight, reproductive performance, performance traits and feed efficiency. Metrics included residual feed intake (RFI), feed conversion rate (FCR), average daily body weight gain (ADG), weaning weight (WW), height at withers (SHD), scrotal circumference (SCR) and profitability during test feeding of young bychkov. Significant genetic correlations for WW were established with SHD (r_g = 0.50) and with ADG (r_g = 0.28). The heritability coefficient for FCR was 0.34, for RFI it was 0.31 with a genetic correlation between traits r_g = 0.75. The estimated genetic correlation of profitability (rand-value) with FCR and RFI was r_g = –0.92 and r_g = –0.59, respectively [38].

Traditional animal genetic improvement programs include selection for total body weight gain during the fattening period as the main trait and can reduce the profitability of fattening beef cattle [39]. This is because in bulls selected for weight gain alone, the average daily feed intake increases at the same time. Therefore, in order to achieve the desired profitability and genetic improvement of economically useful animal traits, breeding programs must invole a complex of indicators that characterize both the efficiency of feed use and fattening qualities.

Loci of quantitative traits. The intensive development of molecular genetic methods in the first quarter of the 21st century opens up new opportunities for accelerating progress in animal breeding, including increasing the efficiency of feed use.

DNA technology was first used in commercial beef farming in the 1990s. However, most economically useful traits in animals are polygenic in nature, that is, they are determined by the action of a large number of genes, called quantitative trait loci (QTL) [40]. Most QTLs have only minor effects on economic utility, and the likelihood of detecting their effect is highly dependent on sample size [41]. However, the use of some genes in animal breeding remains relevant. Thus, research is being conducted on the search for and implementation of DNA markers

calpastatin (CAST) and calpain (CLPN) associated with the tenderness of cattle meat into breeding programs.

In the early 2000s, approaches were proposed to ensure the introduction of genomic evaluation methods into animal breeding [42]. Subsequently, there were opportunities for the development of more advanced procedures for assessing the breeding value of animals using information on a large number of SNPs (single nucleotide polymorphism). This includes the linear models rrBLUP (assessment of the effect of each marker), GBLUP (estimation of breeding value by genome) and the one-step estimation method ssGBLUP (one of the best to date; allows ones to get an improved genomic estimate of breeding value, taking into account both genome and pedigree data), as well as BayesA and BayesB non-linear Bayes methods.

For the first time, genomic evaluation has been recognized as official for Holstein cattle in the United States since January 2009. From 2009 to 2013, the procedure was introduced in 12 additional countries. Currently, genomic selection is becoming a traditional practice in dairy cattle breeding both abroad [43] and in the Russian Federation [44]. The use of genomic assessment in dairy cattle breeding has made it possible to increase the accuracy of the prediction of the breeding value of young animals by 15-25% and begin their more intensive use, thereby reducing the interval between generations and accelerating progress in breeding.

In beef cattle breeding, the increase in the selection response due to the introduction of genomic selection is estimated at 29-58%, depending on the trait [45]. The lack of a phenotypic correlation between RFI and average daily body weight gains and the heritability of RFI has made this indicator the preferred measure for identifying biological mechanisms of feed efficiency for genomic breeding purposes [7, 31, 46].

For feed consumption and efficiency traits, accurate QTL mapping was performed using 2194 markers on 24 autosomes of beef cattle [47]. In the experiment, Angus and Charolais bulls from 20 populations were examined. A total of 4 QTLs exceeded the significance threshold for genome-wide associations at $p < 0.001$, 3 at $p < 0.01$, 17 at $p < 0.05$, and 30 could be considered suggestive. Nineteen chromosomes contained QTLs significant for RFI. The results of QTL for RFI obtained in many studies were generally similar, the positions were also similar but sometimes differed in significance. For FCR and DMI, fewer QTLs were found compared to RFI, 12 and 4, respectively. Some chromosomes contained QTLs for FCR but not for RFI, while all QTLs for DMI were on chromosomes where they were also found for RFI. The most significant QTL for RFI was located on BTA3 (82 cM; $p = 7.60 \times 10^{-5}$), for FCR on BTA24 (59 cM; $p = 0.0002$), and for DMI on BTA7 (54 cM; $p = 1.38 \times 10^{-5}$). The RFI QTLs with the closest mapping results to previous studies were on BTA1, BTA7, BTA18 and BTA19 [48]. The identified trait loci provide a starting point for identifying genes that influence feed intake and efficiency in marker-assissed breeding [49].

J.D. Nkrumah et al. [37] obtained information on the genotype of 20 hybrid bulls (Angus, Charolais and Alberta) with at least 400 offspring, 100 microsatellite markers and 355 SNPs. Traits analyzed included feedlot ADG, daily DMI, feed intake to body weight gain F:G (reciprocal of G:F gain efficiency) and RFI. Putative QTLs for ADG ($p < 0.05$) were found on BTA5 (130 cM), BTA6 (42 cM), BTA7 (84 cM), BTA11 (20 cM), BTA14 (74 cM), BTA16 (22 cM), BTA17 (9 cM), BTA18 (46 cM), BTA19 (53 cM) and BTA28 (23 cM). For DMI, putative QTLs that exceeded the threshold $p < 0.05$ were found on BTA1 (93 cM), BTA3 (123 cM), BTA15 (31 cM), BTA17 (81 cM), BTA18 (49 cM), BTA20 (56 cM) and BTA26 (69 cM). E.L. Sherman et al. [47] found associations of SNPs on BTA2, BTA5, BTA10, BTA20 and BTA29 with DMI, RFI and FCR. This

study shows that the detected SNPs can affect biological mechanisms of feed efficiency other than control of feed intake and weight gain. The resulting SNPs can be used in breeding [50].

The Animal QTLdb database (https://www.ani-malgenome.org/cgi-bin/QTLdb/SS/search) was created to accommodate publicly available QTL data for cattle, chickens and pigs. In 2007, tools were developed to map QTLs to various genome features such as bacterial artificial chromosome end sequences, SNPs, and oligonucleotide array elements. In addition to Animal QTLdb, a virtual comparison map (VCmap) is used to map QTLs across species, which was jointly developed by Iowa State University and the Medical College of Wisconsin (http://www.animalgenome.org/VCmap). The methodology for extracting and analyzing QTL data has been improved, and significant progress has been made over the past few years. The QTL database has now been expanded to include two more species (sheep and rainbow trout), providing additional opportunities for comparative mapping. The information provided is constantly updated. The number of publications that mention Animal QTLdb (1498 citations recorded as of August 2022) comfirms its popularity [51-53]. Obtaining more detailed information about changes in quantitative traits and improving the terminological apparatus of gene ontology increase the accuracy of QTL annotation. As sets of genes become available for microarray expression analysis and high-density SNP arrays for genome-wide association studies (GWAS) have been created, QTL analysis is no longer the only way to establish relationships between genes and traits, and involving SNPs in genome-wide association studies is nevitable. This concept requires a meta-analysis of large amounts of experimental data accumulated rapidly.

Genome-wide association study of feed efficiency. Considering the economic importance of developing and implementing genomic breeding programs aimed at improving feed efficiency and growth energy, research teams in various countries have started genome-wide association studies [54-57] using DNA chips of medium density (Bovine SNP50 BeadChip, Illumina Inc., USA) [58, 60] and high density (Bovine HD Bead Chip, Illumina Inc., USA) [59, 61]. The results allow researchers to clarify the methodological aspects of the development of genomic breeding programs for beef cattle and to identify a number of positional and functional SNPs associated with feed efficiency and fattening performance.

C.M. Seabury et al. [59] performed a GWAS analysis for average daily body weight gain (ADG), dry matter intake (DMI), metabolic body weight (MWT), and residual feed intake (RFI) using natural and imputed genotypes (Illumina 778K) for 3887 animals of three American beef cattle populations (Angus, Hereford, Simangus) and values of heritability coefficients based on genotypes for SNP markers. According to the calculated proportion of additive genetic variance, which can be explained by the effect of markers (PVE 1%), and the nominal p-value threshold ($p \leq 5e\text{-}05$), QTLs with medium or high effect were found in three populations for all studied traits. Identical or closely located (± 0.2 Mb) SNPs associated with ADG, DMI, MWT, and RFI scores co-supported the potential for pleiotropic QTLs or closely spaced mutations for several traits within and between analyzed populations. Marker-based heritability rates for all traits ranged from 0.18 to 0.60 in genomic analysis using the Illumina 778K HD chip and from 0.17 to 0.57 using the Illumina Bovine SNP50 chip. Identification of QTLs detected with the Illumina Bovine SNP50 chip has yielded mixed results. It is likely that the use of a low-density chip was not sufficient to detect significant QTLs in the populations studied, and appropriate breeding or screening programs should be based on the analysis of polymorphisms (imputed or directly detected) on higher-density chips.

M.H. Santana et al. [62] aimed to identify SNPs associated with DMI and RFI in Nellore cattle using medium density (Illumina® BovineSNP50 v2 BeadChip), high density (Illumina® BovineHD BeadChip) panels and their combination to search for possible candidate genes with known function. The authors identified three SNPs for DMI that exceeded the threshold of significance in the Bonferroni multiple comparison test, and two SNPs for RFI.

Using the Illumina 778K chip, seven QTLs with a significant effect were found in Angus cattle, distributed over seven autosomes. Most positional candidate genes located in or near the detected QTL (*XIRP2*, *HSPB8*, *TOX/TRNACGCA*, *DDB1*, *DAK*, *ADPRHL1*, *CDC-16*) have previously been associated with feed efficiency and growth vigor in other animal species (broilers , poultry, pigs) [63, 64] and obesity in humans [65, 66]. These genes are also involved in the resumption of the human cell cycle after the S-phase checkpoint [67-70]. Moreover, one positional candidate gene (*DAK*) is involved in the biosynthesis of riboflavin-4′,5′-phosphate [65] which acts as an electron acceptor in the oxidative metabolism of carbohydrates, amino acids and fatty acids and can donate electrons to the electron transport chain [69, 70]. Riboflavin is essential for energy production, which is required for growth and development of the body [65, 66, 69, 70].

2. Characterization of the main SNPs (single nucleotide polymorphism) of each chromosome in significant associations with RFI (residual feed intake) in beef cattle populations

Breed	Position (Chr_Mb)	$-\log_{10}$ p-value	Candidate gene	Functions	References
Angus	2_30	5.51	*XIRP2*	Feed efficiency and growth (cattle)	[63]
	17_58	4.77	*HSPB8*	Signs of obesity (human)	[64]
	14_27	4.56	*TOX/TRNAC-GCA*	Signs of obesity (human)	[65]
	29_40	4.55	*DDB1/DAK*	Cell cycle, nutritional efficiency and growth (human, chicken, duck, pigs)	[65, 66, 71]
	12_91	4.39	*ADPRHL1/CDC-16*	Feed efficiency and growth (chicken, pigs)	[67, 68, 72]
Hereford	6_113	6.51	*RAB28*	Signs of obesity (human)	[73]
	20_49	3.65	*STC2*	Role in obesity (human), growth suppression and development of bones, muscles (mouse)	[75]
	6_47	1.70	*NCAPG*	Associated with carcass and body weight (cattle)	[74]
	1_72	4.34	*DLG1*	Glucose uptake (human)	[73]

In Hereford cattle, genome-wide RFI analysis using the Illumina 778K DNA chip showed the presence of four high effect QTLs distributed over four autosomes (6_113 Mb, 19_54 Mb, 3_29 Mb, 1_72 Mb) (Table 2). Evaluation of positional candidate genes (*STC2*, *RAB28*, *DLG1*) revealed associations with human obesity, adipogenic differentiation, type I diabetes, and rheumatoid arthritis, and suppression of bone and muscle growth and development in mice. The *NCAPG* gene associated with the development of body weight in cattle was previously proposed by K. Setoguchi et al. [74] for molecular test systems as a candidate.

3. Major SNPs of each chromosome in significant associations with RFI, DMI, ADG, and MWT in a multi-breed beef cattle population

Trait	BTA	Position, bp	SNP	p-value	Candidate gene
RFI	1	121 176 492	rs109479784	8.27E–06	*SNORA70*
	2	28 511 594	rs379241952	9.69E–07	*B3GALT1*
	3	6 835 555	rs110523019	1.74E–07	*DDR2*
	4	89 834 757	rs42645457	6.12E–06	*GPR37*
	5	9 075 556	rs446215391	6.77E–07	*SYT1*
	12	54 262 083	rs382972340	8.21E–06	*U6*
	13	35 856 785	rs382536070	6.60E–06	*LYZL1*
	16	13 105 979	Chr16:13105979	8.38E–06	*RGS2*
	23	48 775 591	rs382491772	8.90E–06	*F13A1*

Continued Table 3

DMI	1	25 084 372	rs211318336	8.30E–06	*U2*
	2	112 157 337	rs109570141	1.96E–06	*U6atac*
	4	3 153 240	rs472695088	2.80E–06	*SNORA31*
	6	39 105 359	rs207689046	2.77E–25	*LCORL*
	10	31 282 009	rs109256612	7.06E–06	*DPH6*
	12	54 262 083	rs382972340	1.03E–06	*U6*
	13	19 004 111	rs384869645	5.56E–06	*PARD3*
	14	24 973 953	rs110092040	1.12E–08	*MOS*
	16	78 179 941	rs380573663	3.66E–06	*CRB1*
	20	4 791 751	rs43357086	4.33E–09	*5S_rRNA*
	22	30 879 104	rs211404023	3.71E–06	*5S_rRNA*
ADG	4	112 725 016	Chr4:112725016	3.79E–06	*CUL1*
	5	106 247 266	rs137822220	8.25E–07	*CCND2*
	6	39 113 335	rs110987922	3.28E–07	*LCORL*
	7	93 244 933	rs109901274	8.44E–08	*ARRDC3*
	14	25 006 125	rs134215421	4.82E–13	*PLAG1*
	20	4 916 731	rs42661323	3.65E–09	*STC2*
	24	15 100 338	rs111029508	5.47E–06	*snoU54*
	25	40 587 255	rs448890458	5.31E–06	*CARD11*
	28	45 058 986	rs469759962	9.68E–06	*TMEM72*
	29	41 512 334	rs137389740	3.74E–06	*SCGB1A1*
MWT	1	118 345 325	rs210255011	3.85E–06	*ERICH6*
	5	106 266 665	rs110358394	3.20E–07	*CCND2*
	6	39 111 019	Chr6:39111019	1.59E–04	*LCORL*
	7	93 244 933	rs109901274	9.61E–09	*ARRDC3*
	11	68 821 419	rs446606774	7.92E–07	*GALNT14*
	14	25 006 125	rs134215421	2.08E–28	*PLAG1*
	20	4 563 925	rs41934045	6.12E–21	*ERGIC1*
	21	21 679 784	rs209660822	8.25E–06	*AP3S2*
	26	8 545 128	rs133223744	3.27E–06	*A1CF*

N o t e. DMI is dry matter intake, RFI is predicted residual feed intake, ADG is average daily body weight gain, MWT is metabolic body weight. Compiled based on the data presented in the article by F. Zhang et al. (75).

F. Zhang et al. [75] showed 12 out of 16 significant SNPs for RFI, with 3 genes located within the SNP (Table 3). Genome-wide SNPs for DMI were located on 11 chromosomes, and the *LCORL* gene was identified in SNP rs207689046. Genome-wide SNPs on multiple chromosomes have also been found to be associated with ADG and MWT. Of the 12 SNPs that accounted for more than 0.30 of the phenotype variance for average daily gain, three SNPs are located near or linked to the gene, the rs110987922 is located at 121223 bp from the *LCORL* gene, the rs134215421 is located at 1166 bp and is linked to the *PLAG1* gene. In terms of metabolic body weight, 6 out of 10 genes were located within the SNP, and one gene was identified in the nearby region. SNP rs39111019 (BTA6, 118907 bp, linked to the *LCORL* gene) accounted for 5.80% of the phenotypic variance.

In general, the results of the GWAS analysis show that the vast majority of the identified patterns are population-dependent. Due to genetic (linkage disequilibrium) and phenotypic differences, it becomes necessary to conduct a GWAS for each breed or livestock population for which a genomic breeding program needs to be developed.

Thus, this review on the efficiency of feed use, including feed conversion, indicates the relevance of the topic in the countries that are leaders in meat animal husbandry. When evaluating this index, it is necessary to take into account a complex of technological, biological and economic factors, therefore, the mathematical apparatus, including models used in the practice of animal husbandry, becomes more complicated. Obviously, in Russia, the problem of feed efficiency must be given attention. The phenotypic and genotypic parameters considered in this review will be used in a set of criteria for the beef cattle genome assessment in Russia.

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PHYTOBIOTICS AS POTENTIAL REGULATORS OF THE GUT MICROBIOME COMPOSITION AND FUNCTIONAL ACTIVITY IN BROILER CHICKENS — A MINI-REVIEW

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Abstract

The efficiency of raising broiler chickens directly depends on the functional state of the gastrointestinal tract (N. Abdelli et al., 2021). The gut microbiome plays a key role in modulating the immune system and regulating digestive function. The relationship between diet and taxonomic profile is of particular interest to functional foods that have a positive effect on the microbiome (S. Khan et al., 2020). Metabolites synthesized by intestinal microorganisms serve as the main modulators of cross-communication between the host and the microbiome. Among these are short-chain fatty acids, tryptamine, conjugated linoleic acids, indole and its derivatives, as well as bile acids (S.A. Lee et al., 2017; S. Khan et al., 2020). Consequently, the microbiome is a fundamental link in maintaining productive interactions between the host and the intestine (S.A. Lee et al., 2017). Phytobiotics (FB) serve as a safe and effective alternative to feed antibiotics (M. Kikusato et al., 2021). The purpose of this review is to systematize information on the effectiveness of FB as potential regulators of the intestinal microbiome of broiler chickens. The beneficial functions of plant extracts mainly depend on their specific bioactive components (organic acids, polysaccharides, flavones), which can be synthesized as antimicrobial agents against pathogenic microorganisms (O.A. Bagno et al., 2018; J.J. Flees et al., 2021). It is known that the mechanism of action of FB consists in the destruction of the membrane of pathogenic microorganisms, modification of the cell surface with a change in virulence, stimulation of the immune system (S. Diaz-Sanchez et al., 2015). The contact of the microbiome and phytochemicals is a two-way process in which bacteria metabolize polyphenols into simpler metabolites, in turn, polyphenols affect the population of intestinal microorganisms, leading to a shift in metabolic activity (Y. Iqbal et al., 2020). FB control the growth and taxonomic composition of the intestinal microbiome, since phytochemicals, like prebiotics, have a positive effect on the state of the gastrointestinal tract even with minimal absorption in the small intestine (J. Martel et al., 2020). The feeding of phytochemicals is associated with high productivity indicators. It was found that the addition of the FB diet has a positive effect on the state of the metabolic activity of the organism and an increase in its adaptive potential, which is caused by the activation of the expression of certain genes (*IL6* and *BPIFB3*) in both infected and uninfected birds (G.Y. Laptev et al., 2021). Plant compounds can not only directly improve the health of broiler chickens, but also modulate the microbiota of the gastrointestinal tract and enhance the stimulating effect of productivity (O.A. Bagno et al., 2018). A review of the studies conducted on this topic demonstrates contradictory results. Therefore, studies of the dynamics of a multifactorial relationship between the environment, the host and the gut microbiome will allow for elucidating in-deep mechanisms of FB action on the intestinal ecosystem in broilers.

Keywords: phytobiotics, phytogenic compounds, broiler chickens, productivity, growth stimulants, microbiome

To meet the growing market demand for poultry meat, producers are forced to grow broiler chickens in conditions that provide the fastest possible increase in body weight, and therefore there is a need to use various growth promoters. However, consumers increasingly prefer natural products, which leads to

a restriction of the use of feed antibiotics. From 2018 to 2023, the global phytobiotics (PhB) market is projected to expand from approx. US$ 631.4 million to over US$ 962.5 million [1].

The productivity of broiler chickens is directly related to the functional state of the intestine, which is determined by the structure of the diet and the activity of the gastrointestinal tract (GIT), intestinal microbiome and the immune system associated with it [2]. The gut microbiome plays a key role in modulating the immune system, digesting nutrients, and regulating GIT function. Such effects are mediated by complex microbial interactions, qualitative and quantitative characteristics of metabolites produced by members of the microbial community or resulted from nutrient transformation [3]. The microbiota provides intestinal homeostasis, forms tolerance to infections and non-pathogenic stressors [4]. Metabolites synthesized by gut microorganisms serve as the main modulators of cross communication between the host and the microbiome. These include short-chain fatty acids, tryptamine, conjugated linoleic acids, indole and its derivatives, and bile acids [3]. Therefore, the microbiome is a fundamental link in the interaction between the intestine and other body systems of the bird, which ensures its high productivity [5]. Since broiler chickens are not tolerant to potentially pathogenic microorganisms such as *Escherichia coli*, *Salmonella* and *Clostridium perfringens*, the functional state of the gastrointestinal tract requires special attention [6].

Establishing a relationship between diet and the taxonomic profile of the gut microbiota has increased interest in functional foods that have a positive effect on the gut microbial community and, as a result, overall host health. One such product is PhB as a safe and effective alternative to feed antibiotics [1]. PhBs are mainly known for their transcription-modulating action and pronounced antioxidant, anti-inflammatory, and antimicrobial activity [7). Plant secondary metabolites have been shown to have properties comparable to antibiotic growth promoters that help maintain gut health and improve overall performance in broiler chickens [6).

PhBs have a stimulating effect due to chemical properties and palatability (i.e., PhBs change feed attractiveness and intake), antimicrobial activity, improved digestion and absorption of nutrients with enhanced intestinal functions, as well as direct and indirect anabolic effects on target tissues through endocrine and antioxidant defense systems [8]. The variable composition of PhB makes it difficult to establish the possible mechanisms of their effect on the body of broilers, in particular, on the ecosystem of the digestive tract [6]. It is the impact on the microbiome that mediates the impact on productivity, and, accordingly, on the efficiency of growing broilers [9].

The purpose of this review is to systematize information on the effectiveness of PhBs as potential regulators of the taxonomic composition and activity of the intestinal microbiome in broiler chickens.

Physicochemical properties of phytobiotics. Phytogenic additives affect the state of the gastrointestinal tract, which is primarily reflected in productivity indicators [7, 10].

PhBs are a heterogeneous group of plant-derived feed additives with a high content of bioactive compounds [7]. The raw materials for them are plant extracts or parts of plants (leaves, rhizomes, roots, flowers or bark, bulbs, stems, as well as fruits and seeds) with a maximum accumulation of biologically active substances [11]. Laf extracts have a higher biological activity. This is due to the fact that the composition of the leaves is characterized by an abundance of pharmacologically active components, in particular polyphenols [12].

Based on their chemical structure, PhBs are divided into six categories: phenolic compounds, alkaloids, nitrogen-containing compounds, organosulfur

compounds, phytosterols, and carotenoids [13]. In practice, bioactive extracts are also used, such as essential oils (EOs), pigments (mainly carotenoids, anthocyanins), alkaloids, glycosides, phenolic acids, phytosterols, flavonoids [11]. EOs are herbal essences obtained by distillation with water and/or steam (10), which are a mixture of chemical compounds — terpenes, terpenoids and polyphenols (phenylpropenes, flavonoids) [14]. EOs are valuable due to their antimicrobial, antiviral, antioxidant and antiparasitic properties [2]. The antioxidant activity of FB, especially phenolic acids and flavonoids, is determined mainly by the structure and delocalization of electrons above the aromatic nucleus [7].

One of the main advantages of PhBs is reduced or no toxicity, high availability, and ease of use in agricultural practice [15].

Mechanism of action of phytobiotics. It is assumed that PhBs have a direct and indirect effect on the microbiota of broiler chickens. The direct mechanism is to destroy the membrane of pathogenic microorganisms; cell surface modifications affecting virulence; stimulation of the immune system, in particular the activation of lymphocytes, macrophages and NK cells; protecting the intestinal mucosa from colonization by bacterial pathogens; encouraging the growth of beneficial bacteria such as *Lactobacilli* and *Bifidobacteria* [16].

The mechanism of the direct action of EOs has not been fully elucidated, however, there is evidence of a violation of the integrity of the cell membrane of microorganisms after interaction with EOs [17], which, due to their lipophilic characteristics, are able to diffuse through the lipid layer. Bacterial cell membranes, which provide a relatively stable internal environment for the life of bacteria, are responsible for the barrier and selective transport of metabolites, and also perform other important biological functions. i.e., maintaining hormonal homeostasis, enzymatic reactions (membrane proteins often have enzymatic activity and/or participate in its membrane regulation), cell recognition, and participation in signal transduction [18, 19]. For normal growth, bacteria need to maintain the morphological and functional integrity of the membrane. It is known that antimicrobial agents of various origins can influence cell metabolism, homeostasis, and morphology by targeting bacterial surface structures and modulating their permeability [20]. Membrane permeabilization leads to changes in ion influx, cytoplasmic coagulation, and structural damage to membrane-bound proteins, which determine antimicrobial effects [10, 21].

Oregano EO has pronounced antioxidant and antimicrobial properties, while the most bioactive compound in oregano EO is carvacrol. The action of carvacrol appears to be related to the hydroxyl group in its phenol ring and its hydrophobic nature; carvacrol interacts with the lipid bilayer of cytoplasmic membranes, disrupting their integrity and causing leakage of cellular contents - ions, adenosine triphosphate and nucleic acid [22].

There is a hypothesis that Gram-positive bacteria are more susceptible to the action of hydrophobic compounds such as EOs [23]. The reason for this is attributed to the presence of a cell wall in gram-positive bacteria, consisting of a thick layer of peptidoglycan, which is associated with hydrophobic proteins and teichoic acid, which facilitates the penetration of molecules that also have hydrophobic properties. Gram-negative bacteria have a more complex cell wall consisting of an outer membrane linked to an inner layer of peptidoglycan via lipoproteins. The outer membrane contains proteins and lipopolysaccharides (lipid A), which makes it more resistant to hydrophobic EOs [24]. Possible mechanisms of the effect of EO on bacterial reproduction are the destruction of the outer membrane or the double layer of phospholipids, changes in the composition of fatty acids, an increase in membrane fluidity with subsequent release of protons from the cell, impaired glucose uptake, and inhibition of enzyme activity [23].

The composition of EOs can include 100 chemical compounds, with the predominance of one or more of them forming the chemotype of EOs [25]. The biological function of essential oils is related to the activity of their main components, the structural configuration of the compounds present, their functional groups, and possible synergistic effects [26]. It has been established that combinations of EOs have a high antimicrobial effect due to the synergistic effect, when the combined effect of several compounds is greater than the sum of their effects separately [17].

The carbonyl groups of anethole and fenchone from fennel attach to cell membrane proteins and exert an antimicrobial effect by disrupting the structure of the microbial plasma membrane lipid layer, resulting in loss of cellular content [27].

The mediated mechanism of action of PhBs is due to their ability to modulate the functional activity of the microbiome, the change in which is associated with the state of the mucous membrane of the gastrointestinal tract (structure of microvilli, depth of crypts) [28]. It should be noted that the absorption of PhBs, including EO, in the small intestine is very low, approx. 2-15% [28, 29]. PhBs are mostly polyphenols. Due to low absorption, approx. 90% of phenolic compounds enter the colon unchanged [29]. More than 90% of the polyphenols entering broiler intestines are digested by representatives of the intestinal microbiota, but not digestive enzymes. Microbial degradation of polyphenols with the formation of intermediate products, including aglycones and aromatic acid metabolites, increases their bioavailability and enhances the effect of PhBs. PhBs modulate the microbiome to a greater extent in the caecum than in the ileum. This is due to the peculiarities of the composition of microbial associations, PhBs metabolism, and their absorption in different parts of the intestine [30].

The interaction of microbiota and phytochemicals is a two-way process. For example, bacteria degrade polyphenols, metabolizing them to simpler compounds, and polyphenols affect the composition of the population of intestinal microorganisms, leading to a shift in their metabolic activity [29]. PhBs are able to control the growth and composition of the gut microbiome, since phytochemicals, like prebiotics, have a positive effect on the state of the gastrointestinal tract even with minimal absorption in the small intestine [31].

Some PhBs lead to an increase in the production of short-chain fatty acids - lactate, acetate and butyrate, which indicates an increase in the metabolic breakdown of carbohydrates. Many PhBs themselves can be sources of organic acids and reduce the pH in the gastrointestinal tract, which inhibits the growth of pathogenic bacteria (particularly *Escherichia coli*) [32].

We noted above that morphological features (villus length, crypt depth and height) serve as indicators of intestinal health [29]. Elongation of the villi has a positive effect on digestion, improves the absorption of nutrients and promotes weight gain. Therefore, changes in gut morphology affect nutrient uptake and animal performance [29]. It has been shown that PhBs affect the functional state of the villi in the small intestine. An increase in villus size correlates with increased metabolite absorption. PhBs can lead to an increase in the height of the villi and the depth of the crypts in the intestines of broilers. In addition, one of the common effects of PhBs is a decrease in the abundance of E. coli, which also contributes to an increase in the surface area of the villi [33]. When the intestinal microbiota is modified by PhBs, the digestibility and absorption of nutrients increases [32].

Effect of phytobiotics on the microbiota of broiler chickens. Among PhBs, essential oils are more popular due to their pronounced antimicrobial properties (9). At the same time, each of the components that make up the essential oil has its own mechanism of action, which together leads to synergy [16]. The addition of EO to broiler diets improves growth performance, regulates

gut microbiome composition (Table), and significantly reduces the effects of exposure to pathogens such as *Salmonella* [34], *E. coli* [35], *Clostridium perfringens* [36] and *Eimeria* spp. [37].

Phytobiotics and their effect on the taxonomic profile of different parts of the intestine in broiler chickens

Producer plant	Active ingredient	Effect	References
	Ileum		
Capsicum annum L.	Capsaicin	↓ *Escherichia coli*, *Enterobacteriaceae*, gram-positive lactobacilli	(9)
Foeniculum vulgare Miller)	Anethole, limonene, fenchon, estragole, safrole, α-pinene, camphene, betapinene, sabeinene, β-myrcene, phellandrene, cisocymene, paracymol, γ-terpinene, camphor	↓ *E. coli*; ↑ *Lactobacillus* spp.	(27)
Coriandrum sativum L.	Linalool, α-pinene, γ-terpinene, geranyl acetate, camphor, geraniol	↓ *E. coli*; ↑ *Lactobacilli*	(46)
Pimpinella anisum L.	Trans-anethole, eugenol, anisaldehyde, polyacetylenes, methylchavicol, scopoletin, estragole, coumarins, umbelliphron, estrols and polyenes	↓ Colonization by pathogenic bacteria	(47)
Trachyspermum ammi L.	γ-Terpinene, thymol, p-cymol, β-pinene	↓ *E. coli*; ↑ *Lactobacilli*	(48)
Magnolia officinalis Rehder & E.H. Wilson	Magnolol, honokiol	↓ *Streptococcus* and unidentified *Cyanobacteria*; ↑ α-Diversity и β-diversity, abundance of *Firmicutes*, *Lactobacillus*	(43)
Lavandula angustifolia Miller	Myrcene, α-pinene, caryophyllene, linalool, α-terpineol, borneol, camphor, carvone, eukarvone, linalo-ol acetate, lavandulilacetate, geranyl cetate, neral	↓ *E. coli* and coliform bacteria; ↑ Probiotic bacteria, bacteriostatic effect	(49)
Thymus vulgaris L.	Thymol, carvacrol	↓ *E. coli* and the total number of gram-negative bacteria; ↑ Abundance of lactic acid bacteria	(50)
Allium cepa L.	Propyl thiosulfinate, propyl propyl panthiosulfonate	↓ *E. coli*; ↑ *Lactobacillus* и *Streptococcus*	(33, 45)
	Cecum		
Rumex nervosus L.	Gallic acid, catechin, chlorogenic acid and caffeine	↓ *E. coli*	(51)
Castanea sativa Miller and *Schinopsis lorentzii*	Tannins	↓ *Bacteroides*; ↑ Members of *Clostridiales* families *Ruminococcaceae* and *Lachnospiraceae*	(52)
Oríganum vulgáre L.	Carvacrol, thymol	↓ *Streptococcus*; ↑ *Enterococcus*, *Lactobacillus*; ↑ Production of acetic and butyric acids	(22, 40)
Eucalyptus globulus La Billardière	1,8-cineole (eucalyptol), α-pinene, -phellandrene, γ-terpinene, α-terpineol, cymene, limonene, and spatulenol	↓ *E. coli*; ↑ Abundance of lactic acid bacteria	(44)
Cinnamomum zeylanicum Jan Svatopluk Presl	Коричный альдегид, эвгенол, 3-фенил, 2-пропеналь	↓ *E. coli*; ↑ *Lactobacillus* и *Bifidobacterium*	(53, 54)
Melissa officinalis L.	Gallic acid, catechin, chlorogenic acid, caffeic acid, ellagic acid, epicatechin,	↑ Number of colonies of enterococci	(55)
Allium hookeri Thwaites	Rutin and quercetin	↓ *Eubacterium nodatum*, *Marvinbryantia*, *Oscillospira* и *Gelria*	(12)
	Small intestine		
Nelumbo nucifera Gaertner	Alkaloids and flavonoids	↓*Peptostreptococcaceae*; ↑ *Clostridiaceae* and *Bacteroidales* S24-7	(20)
Allium sativum L.	Alliin, diallyl sulfides, allicin	↓ *Escherichia coli*; ↑ *Lactobacillus*	(56)
	Jejunum		
Curcuma longa L.	Curcumin	↓ *E. coli*; ↑ Total number of aerobic mesophilic and lactic acid bacteriaй	(57)

Continued Table

Ileum and caecum			
Camellia sinensis L.	Catechins	↓ *E. coli*, *Lactobacillus*	(58)
Duodenum, jejunum and ileum/caecum			
Macleaya cordata Willdenow	Sanguinarine, chelerythrine	↓ *Corynebacterium*, *Brachybacterium*, *Dietzia*, proteobacteria; ↑ *Lactobacillus*, *Ruminococcaceae*, *Lachnospiraceae*, *Clostridium*, *Butyricicoccus*, *Faecalibacterium*, *Firmicutes*, the ratio *Firmicutes*/*Bacteroidetes*	(35, 41)

Note. Symbols ↓ and ↑ mean a corresponding decrease and increase in the indicator.

Lactobacilli metabolize polyphenols, producing energy substrates for cells. *Lactobacillus acidophilus* is able to convert plant glycosides to form aglycones [38). *L. delbrueckii* and *Eubacterium ramulus* can use these aglycones as nutrient substrates [29]. *Bacteroides ovatus*, *Veillonella* sp. and *Ruminococcus productus* further metabolize aglycones via ring opening, lactone cleavage, decarboxylation, dehydroxylation, demethylation, reduction and isomerization [39].

It has been shown that the addition of 1% oregano powder to the diet of broiler chickens leads to a selective change in certain groups of bacteria. In particular, the representation of *Streptococcus* decreases, *Enterococcus* increases, the ratio of *Lactobacillus* species changes. The production of short-chain fatty acids was 61% higher in the experimental groups compared to the control. As a result, these effects led to improved morphology of the intestinal mucosa, stimulation of the immune system and, in general, an increase in the productivity of broilers [40]. The antimicrobial activity of extracts of various oregano species has been demonstrated on gram-negative bacteria, including Salmonella typhimurium, *E. coli*, *Klebsiella pneumoniae*, *Yersinia enterocolitica*, and *Enterobacter cloacae*, as well as on Gram-positive bacteria, *Staphylococcus epidermidis*, *Listeria monocytogenes*, and *Bacillus subtilis* [17].

PhBs of *Macleaya cordata* extract contains sanguinarine and chelerythrin. These substances are included in the group of benzylisoquinoline alkaloids with antimicrobial and anti-inflammatory properties. In addition, sanguinarine is very similar in molecular structure to the benzylisoquinoline alkaloid berberine, which has a high clinical efficacy in the treatment of a number of diseases due to the modulation of the intestinal microbiota [35, 41]. *Macleaya cordata* extract leads to an increase in the presence of *Lactobacillus* in the foregut. Moreover, due to the mechanism of nutrient cross-use, lactate produced by *Lactobacillus* can be metabolized by anaerobic bacteria to form butyrate. Butyrate serves as an energy source for intestinal cells, which is accompanied by a pronounced anti-inflammatory effect [41]. It has been established that when sanguinarine is added to the diet of broilers, the taxonomic profile of the microbiota shifts towards *Ruminococcaceae*, *Clostridium*, *Lachnospiraceae*, *Butyricicoccus*, and *Faecalibacterium*, which produce short-chain fatty acids [35].

Berberine as a natural pentacyclic isoquinoline alkaloid isolated from the roots, rhizomes, stems, bark and leaves of *Rhizoma coptidis*, Cortex phellodendri, *Hydrastis canadensis* and *Berberis* has a strong antimicrobial, antioxidant, anti-inflammatory and immunomodulatory effect. Feeding this alkaloid to broilers leads to changes in the composition of the microbiota, in particular, to a decrease in the representation of the phylum *Firmicutes* and the genera *Lachnospiraceae*, *Lachnoclostridium*, *Clostridiales*, and *Intestinimonas*, with a simultaneous increase in the abundance of the phylum *Bacteroidetes* and the genera *Bacteroides* and *Lactobacillus* [42].

Magnolol and its isomer honokiol are the main phenolic compounds extracted from the root and bark of *Magnolia officinalis*. Magnolol and honokiol have

anti-inflammatory, antioxidant, antibacterial activity and are involved in the regulation of metabolism. The effect of these substances on the functional parameters of the microbiota of the ileum of broilers was established, in particular, an increase in the activity of metabolic pathways associated with the transformation of valine, aspartate, glutamate and dibasic acids was noted. In addition, the addition of magnolol enhanced the pathways associated with the biosynthesis of ansamycins. Rifamycins, which are part of ansamycins, exhibit antimicrobial activity against aerobic bacteria and *Salmonella* [43].

The constant feeding of citrus extract with a high content of flavone to broiler chickens leads to the modulation of the structure of the microbiota, in particular to an increase in the number of *Bifidobacterium* and activation of the expression of mRNA of the intestinal barrier genes (*ZO-1* and *Claudin*) in the ileum. Concomitantly, there was an increase in *Lactobacillus*, lactate, and short-chain fatty acids in the cecum, followed by a decrease in protein fermentation products (ammonia, p-cresol, indole, total amines, spermidine, methylamine, and tyramine) [32].

The antibacterial activity of eucalyptus (*Eucalyptus globulus*) is due to the biological action of 1,8-cineol, limonene and -pinene. It has been shown that the addition of eucalyptus EO to the diet helps to reduce the number of *E. coli* and increase the population of lactic acid bacteria in the contents of the caecum [44].

Broiler chickens are fed with *Allium onion* extracts as a feed additive. Onions contain organosulfur compounds. Among them, thiosulfinates have high biological activity. They are volatile and easily evaporate, which leads to a significant change in the final concentrations in the feed. Thiosulfinates in the presence of O_2 rapidly form highly stable thiosulfonates [45]. *Allium hookeri* contains allicin, which gives this onion its intense spicy flavor. Allicin is an organosulfur compound with characteristic antibacterial activity against various microorganisms, including *Staphylococcus* and *Pseudomonas*. This effect is due to a chemical reaction with thiol groups of enzymes that affect the metabolic activity of cysteine proteinases, putative factors of bacterial virulence [12].

As a source of PhBs, both whole plants and isolated active compounds (apigenin, quercetin, curcumin and resveratrol, terpenes eugenol, thymol, carvacrol, capsaicin, artemisinin and aldehydes cinnamaldehyde and vanillin) are studied. These molecules have not only antimicrobial (including antibacterial, antifungal, antiviral and antiprotozoal), but also anti-inflammatory, antioxidant, immunomodulatory properties and improve the morphology and functionality of the intestinal mucosa [59-61].

Thus, over the past 20 years, interest in the use of phytobiotics (PhBs) in animal husbandry has increased significantly. Phytobiotics are substances of plant origin with a high content of bioactive compounds, the main action of which is to modulate the intestinal microbiota and its metabolism. Due to potential bioactivity, PhBs may be used as the main agent in organic poultry farming. The beneficial functions of plant extracts depend on their specific components such as organic acids, polysaccharides and flavones, which can be synthesized as antimicrobial agents. It is important to understand the mechanism of action of each of the components, which, in turn, is determined by its physicochemical properties and mediates the effects of synergy or antagonism of these bioactive compounds. The available data confirm that the effectiveness of PhBs used as feed additives for broiler chickens is due not only to the modulation of the composition and metabolic activity of the symbiotic microflora in the gastrointestinal tract, but also to the improvement of its functional state even with minimal absorption of PhBs in the small intestine. A decrease in the proportion of pathogenic microorganisms in the intestines and an increase in the abundance of probiotics significantly

increases the productivity of broiler chickens. The mechanism of action of PhBs is determined, in particular, by their lipophilic structure and the ability to act on the cytoplasmic cell membranes. The effect of PhBs is more pronounced in the caecum, but not in the ileum. It is obvious that the interactions in the microbiota—host—environment during the use of dietary PhBs are complex, especially considering the diversity of these chemical compounds, their antagonistic and synergistic relationships. Therefore, further studies will elucidate the mechanisms of PhB action on the gut ecosystem of broilers.

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USING OF INFRARED HIGH-PERFORMANCE SPECTROMETRY DATA FOR GENOME-WIDE ASSOCIATIONS STUDY OF FATTY ACID COMPOSITION AND MILK COMPONENTS IN DAIRY CATTLE (*Bos taurus*)

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Abstract

Milk fat percentage is highly variabile and depends on environmental conditions which include feeding and farm technology, and on genetic factors such as breed and genotype features. The content of fatty acids (FA) is a biomarker for the physiological state of animals and a parameter of raw milk suitability for processing (yield of cheese, butter and cream). FA profile of mild in terms of C number, the chain length and saturation degree differs between individuals and at the population level. Therefore, the study of genetic and genomic variability of milk production traits to improve the efficiency of animal selection remains relevant. This study aimed at searching for genome-wide associations and polymorphisms in genes involved in milk fatty acid production. In the study, infrared spectrometry was used as an accurate and rapid method to analyze milk composition. Population variability of milk fatty acid profiles was studied using 36982 milk samples from Holsteinized Black-and-White and Holstein cows of 14 breeding herds from the Moscow region in 2017-2018. The heritability (h^2) and correlation (r_g) coefficients for cows' milk components were calculated using REML (residual maximum likelihood) method with BLUPF90 family software. SNPs were detected for a dataset of Holsteinized Black-and-White cows from an experimental herd (the breeding farm Ladozhsky, branch of Ernst Federal Research Center for Animal Husbandry, Krasnodar Territory, 2020-2021). Milk composition was determined using an infrared spectroscopy-based automatic MilkoScan 7 DC analyzer (FOSS, Denmark). A group of 144 cows subjected to phenotyping for fatty acids and milk components were individually genotyped (Bovine GGP 150K biochip, Neogen, USA). Plink 1.9 software was applied to control genotyping quality (110884 SNPs) and to perform GWAS (genome-wide association study) analysis and multidimensional scaling (MDS). Searching genes by identified significant polymorphisms was performed using the bovine genome assembly *Bos taurus* UMD 3.1.1 (https://www.ncbi.nlm.nih.gov/assembly/) and the Ensembl genome browser. QTL annotation was carried out using the Animal QTLdb database. In general, milk fatty acids showed a heritability level that ranged from low to moderate, varying from $h^2 = 0.018$ for polyunsaturated fatty acids to $h^2 = 0.125$ for medium-chain FAs, $h^2 = 0.155$ for long-chain FAs, $h^2 = 0.155$ for myristic acid, $h^2 = 0.176$ for monounsaturated FAs, and $h^2 = 0.196$ for oleic acid. Visualizing experimental cows' population structure by multidimensional scaling showed a moderate range of variability (PC1 = 7.82 %, PC2 = 4.65 %). For myristic and palmitic acids, common QTL clusters are identified on BTA5, BTA10, BTA14, BTA18, and BTA27. For stearic and oleic acids (as members of the long-chain FA family), similar location of QTLs is found on BTA9, BTA10, BTA11, BTA14, BTA17, BTA18, BTA19, BTA20, and BTA29. For short- and medium-chain FAs, there are associations revealed on BTA1, BTA5, BTA10, BTA11, BTA14, BTA18, BTA19, and BTA24. For long-chain FAs, QTLs are detected on BTA6, BTA7, BTA9, BTA10, BTA11, BTA17, BTA18, and BTA29. For short- and medium-chain FAs, saturated FAs, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$, the genes *CACNA1C*, *GCH1*, *ATG14*, *KCNH5*, *PRKCE*, *CTNNA2*, *CYHR1*, *VPS28*, *DGAT1*, *ZC3H3*, *RHPN1*, *TSNARE1* are identified which

form QTLs on BTA10, BTA11 and BTA14. Short- and medium-chain FAs, myristic and palmitic acids and saturated FAs show associations with polymorphisms in the *MED12L*, *EPHB1*, *GRIN2B*, *PRMT8*, *ERC1*, *PELI2*, *ARHGAP39*, *MROH1*, *MAF1*, *GSDMD*, and *LY6D* genes. For long-chain, monounsaturated fatty acids, stearic and oleic acids, there are significant associations with genes *RPS6KA2*, *CPQ*, *CPE*, *FTO*, *FAT3*, and *LUZP2* which may be valuable for genetic improvement of dairy cattle. Continued study of the inheritance of cows' milk fatty acids and other components is necessary to develop a strategy for breeding dairy cattle with a better fatty acid profile and milk composition.

Keywords: cow, fatty acids, milk components, heritability, GWAS, SNP, QTL, genes

Milk fat has the highest energy value and a wide range of biological activity. It is necessary for the absorption of various vitamins, tocopherols, phosphates and other important nutrients. In milk, milk fat is a suspension consisting of small fat globules ranging in size from 0.1 to 20 microns. According to its chemical composition, it is a derivative of the alcohol glycerol and fatty acids (FA), which account for 93-95% of the fat mass. The content of fatty acids in milk can vary significantly depending on the conditions of feeding animals, the season of the year, the stage of lactation and other factors. Fatty acids are divided into two categories, saturated and unsaturated FA. The latter, in turn, are divided into monounsaturated and polyunsaturated. The cow milk is 50.3-73.8% saturated FA and 49.3-6.2% unsaturated FA. Of the unsaturated fatty acids, milk contains the most monounsaturated fatty acids and the least polyunsaturated fatty acids [1].

Fatty acids are organic compounds that differ in the number of carbon atoms and position, as well as the number of double bonds they contain. Cow milk contains on average 3.6 to 4.8% fat by weight. Fatty acids enter milk both free and bound, in the form of glycerides or other lipids. Triacylglycerides, which are composed of glycerol and three fatty acids, account for 96 to 99% of milk fat, while free fatty acids account for only 0.1 to 0.4% [2].

The set of fatty acids differs depending on the breed, the season and the applied zootechnologies. The feeding conditions play an important role. The composition of milk fat changes during lactation. In the early period during lactation, the animal's body mainly uses C_{16} (palmitic) and C_{18} (stearic) fatty acids from the fat depot of tissues. During lactation, the proportion of newly synthesized (de novo) fatty acids ($C_{4:0}$-$C_{14:0}$) increases, while the proportion of fatty acids with 17 or more carbon atoms decreases [3].

Milk fat contains approx. 140 fatty acids, however, only 13 main FAs with an even number of carbon atoms ($C_{4:0}$-$C_{18:3}$) are found in an amount that is more than 1% each. The remaining acids (for example, $C_{10:1}$, $C_{12:1}$), present in amounts less than 1% and in the form of traces, belong to the so-called minor fatty acids [2-4]. In minor fatty acids, the proportion of milk lipids in triglycerides is 2.0-4.2% for butyric acid, 1.5-3.0% for caproic acid, 1.0-2.0% for caprylic acid, and 2.0-3.5%% for capric acid, 0.2-0.4% for decenoic acid, 2.0-4.0% for lauric acid, 0.6-1.5% for myristinoleic acid, 1.5-2.0% for palmitoleic acid, 3.0-5.5% for linoleic acid, up to 1.5% for linolenic acid, up to 0.3% for arachidic acid, and up to 0.1% for behenic acid. In the main fatty acids, this indicator for myristic acid is 8.0-13.0%, for palmitic acid 22.0-33.0%, for stearic acid 9.0-13.0%, for oleic acid 22.0-32.0 % [4-7].

Fatty acids act differently on the human body. Myristic acid has a negative effect on the cardiovascular system, causing diseases, while stearic acid does not have such an effect. The presence of fatty acids in the animal's body is due to a greater extent by their genetically determined synthesis than by intake with feed or mobilization from body fat tissues. The formation of $C_{6:0}$-$C_{16:0}$ fatty acids, according to the literature, is characterized by high heritability (h^2 = 0.41-0.43), and this increases the selection efficiency. Production of fatty acids important for human health ($C_{18:2}$ cis-9, 12) is characterized by relatively low heritability (h^2 = 0.17-0.33), but

since their production negatively correlates with the synthesis of short and medium-length fatty acids, then selection for this trait can also be successful [2].

It is known that compounds with a molecular weight of carbon $C_{18:0}$-$C_{18:1}$ affect fertility at an early stage of lactation, and the amount of $C_{18:1}$ cis-9 indirectly indicates the energy status of the cow and can be used for early prediction of ketosis. $C_{16:0}$ and $C_{17:1}$ cis-9 are a convenient tool for assessing methane production and feed conversion in cows (the lower the methane emission, the better the feed is digested) [8, 9].

To improve the efficiency of animal breeding and the search for informative DNA markers of productivity traits, it is of great interest to analyze the function of each of the components of milk in connection with one or another biological trait and to study the genetic and genomic variability of traits. Thus, research is underway to identify causal nucleotides (point mutations) for quantitative traits (QTL), which, along with many known non-coding polymorphic substitutions (SNPs), can increase the accuracy of detection of the corresponding mutations and the prediction of the breeding value of an animal. Work is underway to optimize the number of SNPs with a high degree of variability in causal variants, which is sufficient to construct a genomic matrix of relatedness, taking into account information on a large number of genotypes and improve the accuracy of estimates [10). So, causal SNPs, strongly associated with economically useful traits in dairy cattle were detected on chromosomes 5, 6, 9, 14, 15 and 20. The polymorphisms located close to or within the *DGAT1* (BTA14), *GHR* (BTA20), *ABCG2* (BTA6) genes had the highest genetic dispersion in turms of milk productivity [11-15].

Increasing the density of SNPs (reducing the distance between SNPs) will increase the likelihood of QTL detection and, to some extent, the accuracy of mapping. Genome-wide associations were used to analyze the composition of fat in the milk of Holstein and Jersey cows of Danish origin [16]. In addition to the standard genotyping procedure with high density chips (777K), this study used the KEGG PATHWAY Database (bioinformatics resource for genome analysis, https://www.genome.jp/kegg/pathway.html). The candidate gene *DGAT1* which very often appears in studies of the milk productivity in cattle was not defined as playing a significant role in the milk fat composition. This once again indicates the complexity of the inheritance of the trait. However, significant associations with the milk fatty acid composition were found for the *SCD* gene involved in the catalyzed conversion of $C_{10:0}$ to $C_{18:0}$ acids and the *ACSS3* gene involved in the activation and intracellular transport of fatty acids.

F. Kawaguchi [17] found 1993 polymorphisms in 23 genes in Japanese black cattle based on allelic differences between groups with high and low content of oleic acid $C_{18:1}$ using a genome-wide association study (GWAS). Among these 23 genes, based on the analysis of their function in the metabolism of fatty acids, three candidate genes were identified, the *CYB5R4*, *MED23*, and *VNN1* that affect the variability of the oleic fatty acid content.

In the Italian population of Simmentals and Holsteins, GWAS for milk fatty acids revealed significant signals on the BTA19 and BTA26 chromosomes. Further analysis identified not only some well-known genes (*FASN*, *SCD*, and *DGAT1*) of quantitative trait loci for milk FA components, but also other significant candidate genes that were associated with functional roles in lipid metabolism pathways. The identified mutations that are associated with the fatty acid profile are found in the *ECI2*, *PCYT2*, *DCXR*, *G6PC3*, *PYCR1*, *ALG12*, *CYP17A1*, *ACO2*, *PI4K2A*, *GOT1*, *GPT*, *NT5C2*, *PDE6G*, *POLR3H*, and *COX15* genes [18].

The discovery of quantitative trait loci and genes associated with milk fat composition can provide an insights into the complex metabolic networks that underlie changes in fatty acid synthesis and point to possible “points of impact” for

improving milk fat composition through breeding. C. Li et al. [19] performed a GWAS analysis for 22 milk fatty acids in 784 Holstein cows from a Chinese population. A total of 83 significant SNPs and 314 putative suggestive SNPs were found for 18 traits associated with milk fatty acid metabolism. Chromosome regions affecting the properties of milk FAs were mainly localized on BTA1, BTA2, BTA5, BTA6, BTA7, BTA9, BTA13, BTA14, BTA18, BTA19, BTA20, BTA21, BTA23, BTA26, and BTA27. Of these, 146 SNPs were associated with more than one trait in milk fatty acid metabolism; most traits were statistically significantly associated with several SNPs, especially $C_{18:0}$ (105 SNPs), C_{18} (93 SNPs), and C_{14} (84 SNPs) FAs. Several SNPs are found near or within the *DGAT1*, *SCD1* and *FASN* genes, which are known to affect milk composition in dairy cattle. In addition, 20 new highly significant candidate polymorphisms for $C_{10:0}$, $C_{12:0}$, $C_{14:0}$, $C_{14:1}$, indeces of C_{14}, $C_{18:0}$, $C_{18:1n9c}$, and index C_{18} were identified, including mutations in the *HTR1B*, *CPM*, *PRKG1*, *MINPP1*, *LIPJ*, *LIPK*, *EHHADH*, *MOGAT1*, *ECHS1*, *STAT1*, *SORBS1*, *NFKB2*, *AGPAT3*, *CHUK*, *OSBPL8*, *PRLR*, *IGF1R*, *ACSL3*, *GHR*, and *OXCT1* genes [19].

The fatty acid concentration is particularly relevant for milk chemical analysis, since the milk quality parameters, e.g., yield of cheese, butter and cream, largely depend on lipid metabolism. In studies by P. Gottardo [20] on 2977 Holstein, Brown Swiss and Simmental cows, it was found that the Holstein cows have the best ratio of saturated to unsaturated fatty acids in the total milk fat. Simmental cows are on an intermediate position followed by Brown Swiss cows [20].

In many countries, infrared spectrometry is a widely used method to detect and quantify fatty acids in milk. Metabolism of milk fatty acids is under influence of many factors. Therefore, estimation of variability and heritability indicators for such traits is necessary to choose the most effective breeding strategy. In Russia, FAs are relatively new trait for cow breeding and improving milk quality. In Western European countries, the fatty acid analysis quantifies saturated and unsaturated fatty acids. Besides, FA profile serves as an indicator of animal physiological state and numtrition level. In this regard, information obtained in herds at a population level and in experiments will clarify whether breeding for FA composition is prospective. Validation of genetic polymorphisms associated with the variability of the cow milk fatty acids along with other components, will provide new knowledge on the genomic architecture of milk productivity indicators.

Here, for the first time, we assessed the genetic variability of the milk fatty acid fractions in the Russian populations of Holsteinized Black-and-White and Holstein cows to involve these traits into breeding programs and genetic improvement of animals. In experiments, using infrared spectrometry of milk composition and high-performance genomic scanning, we created a database of cows' individual phenotypes and genotypes. Eventually, the quantitative trait loci and functional mutations that regulate the synthesis of milk lipids were revealed.

Our aim was to search for genome-wide associations and polymorphisms in the genes that determine the fatty acid composition of cow milk. To determine it, infrared spectrometry was used as one of the fastest and most accurate express methods for physicochemical analysis of milk composition.

Materials and methods. The results of interpopulation observations and studies in the experimental population were used to create the databases. At the first stage, in 14 breeding herds of Holsteinized Black-and-White and Holstein cattle from the Moscow region (n = 11529), fatty acid profiles were obtained using IR spectra. The milk samples (n = 36982 in total) were collected during 9 months of 2017-2018 in control milkings. Based on the milk composition, the population genetic parameters and variability of the content of the following fatty acids and components in milk: myristic ($C_{14:0}$), palmitic (C16:0), stearic ($C_{18:0}$), oleic

($C_{18:1}$) acids, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), short-chain fatty acids (SCFA), medium-chain fatty acids (MCFA), long-chain fatty acids (LCFA) and trans-isomers FA (TIFA), mass proportions of fat (MPF), protein (MPP), casein (MPC), lactose (MPL), dry matter (DM), DSMR (dry skimmed milk residue), traces of acetone and beta-hydroxybutyrate (BHB), urea concentrations, freezing points and acidity.

To calculate the heritability of the milk fatty acid composition and genetic correlations, the mixed model equation was used:

$$y = \mu + HFMTD_j + Age_k + Lact_l + sire_m + e_{jklm},$$

where y is the studied milk indicator of daughter cows; μ is the mean population constant for a sample of 14 herds; HFMTDj is the effect of the farm, month and date of control milking; Age$_k$ is the age of the 1st calving; Lact$_l$ is the effect of the last completed lactation No.; sire$_m$ is the effect of the father bull; e$_{jklm}$ is random error (unallocated variant). The residual maximum likelihood (REML) method based on maximization of the variance value likelihood through a multiple iteration procedure using the BLUPF90 family programs [21, 22] was applied in calculation for 14 herds.

Primaryly, the milk fatty acid profiles in the experimental herd of Holsteinized black-and-white cows were obtained at the PZ Ladozhsky (branch of the Ernst FRC VIZh, Krasnodar Territory, 2020-2021). The test group consisted of 144 cows previously phenotyped for milk FA spectra and milk components and genotyped using a Bovine GGP 150K biochip (Neogen, USA). DNA isolation from fragments pinched off ears of the cows, SNP genotyping and analysis of milk samples were performed according to standard protocols at the scientific facility Animal Biotechnology (OSIS BioTechZh) of the Ernst FRC VIZh.

In the herds and in the test group, the milk fatty acids and other milk componens indicated hereinabove were determined as recommended (an automatic MilkoScan™ 7 DC analyzer based on Fourier Transform InfraRed analysis, FOSS, Denmark). Animal productivity was assessed individually in 5 to 12 control milkings, on average 8.9 per animal. Milk samples were collected into 50 ml cups with the preservative (Microtabs, USA) during milking in the morning, afternoon and evening.

Genotyping quality control (110884 SNPs), analysis of genome-wide association studies (GWAS), and multidimensional scaling (MDS) were performed (Plink 1.9 software) [23]. As a result, genotypes representing 143 genomic SNP profiles with a genotyping level from 99.3 to 99.7% were selecte among the test cows.

The search for genes by identified significant polymorphisms based on the GWAS data was carried out using the assembly of the bovine genome version UMD 3.1 (Ensembl browser, https://www.ensembl.org/index.html). To determin quantitative trait loci on animal chromosomes, annotation of genes was performed using the international database Animal QTLdb [24].

The Data Analysis package in the MS Excel 2013 environment was used to calculate the mean values (M), standard errors of means (±SEM), and standard deviations (SD). The degree of trait variability was assessed by the coefficient of phenotypic variation (C_{vp}). To calculate heritability (h^2), we used the ratio of genetic variance to the sum of genetic and residual variance.

Results. Fatty acids are highly variable components of milk. In cows, milk FA quantitative profiles are influenced by both environmental conditions (eg feeding, housing) and genetic factors (breed, ancestors, and genotype). The ratio of fatty acids depending on the number of carbon atoms, the length of the chain and the degree of saturation differs both between individuals and at the population level. Understanding the mechanisms of fatty acid synthesis is important to determine

associated quantitative trait loci (QTL). We assessed the genetic variability of the milk fatty acid content in populations of the Holsteinized Black-and-White and Holstein cows on the example of several herds of the Moscow region in order to clarify the prospects of these traits for selection (Table 1).

The heritability of the milk fatty acid quantitative composition varied from low values for polyunsaturated fatty acids ($h^2 = 0.018$) to moderate values for medium ($h^2 = 0.125$), long-chain ($h^2 = 0.155$) and myristic acid ($h^2 = 0.155$), monounsaturated fatty acids ($h^2 = 0.176$) and oleic acid ($h^2 = 0.196$).

1. Phenotypic and genetic parameters of milk fatty acid composition in cows (*Bos taurus*) from 14 breeding herds of Holsteinized Black-and-White and Holstein cows (control milkings, Moscow Province, 2017-2018)

Fatty acid, fatty acid group	g/100 g milk		C_{vp}, %	h^2
	M±SEM	SD		
Myristic	0.680±0.001	0.148	21.8	0.155
Palmitic	1.845±0.003	0.633	34.3	0.071
Stearic	0.585±0.001	0.195	33.4	0.125
Oleic	0.875±0.001	0.281	32.2	0.196
Saturated	3.600±0.004	0.861	23.9	0.083
Monounsaturated	1.002±0.001	0.213	23.0	0.176
Polyunsaturated	0.005±0.000	0.011	238.8	0.018
Short chain	0.538±0.001	0.113	21.1	0.114
Medium chain	2.206±0.003	0.652	29.6	0.125
Long chain	1.486±0.002	0.463	31.2	0.155

N o t e. The total number of milk samples $n = 36982$. C_{vp} is the coefficient of phenotypic variation, h^2 is the coefficient of heritability.

Genetic correlations between milk yield, fat mass fraction (MFA) and fatty acid composition of milk are submitted in Table 2 (if $r_g > 0.050$, the obtained coefficients are significant at $p < 0.001$). It was found that there was practically no genetic relationship between daily milk yield and MFF ($r_g = –0.032$), while a closer negative correlation occurred with the content of trans-FA isomers ($r_g = –0.129$), myristic acid ($r_g = –0.110$) and MCFA ($r_g = –0.106$). The relationship between MFF and the content of various fatty acids ranged from 0.393 for oleic to 0.955 for SFA, for TIFA ($r_g = –0.286$) and more desirable PUFA ($r_g = –0.465$) the values were negative. The content of myristic and palmitic saturated fatty acids negatively correlated with the amount of unsaturated oleic (r_g from –0.160 to –0.427), MUFA (r_g from –0.072 to –0.337), PUFA (r_g from –0.554 to –0.584) and with the content of acids from more complex high molecular weight groups LCFA (r_g from –0.030 to –0.325). The relationship between the amounts of fatty acids $C_{14:0}$, $C_{16:0}$, on the one hand, and TIFA, on the other hand, showed that with an increase in the content of saturated fatty acids in milk, trace amounts of isomers blocking the synthesis of milk fat decreased (r_g from –0.469 to –0.637, respectively).

2. Genetic correlations (r_g) between daily milk yield, milk fat mass fraction and quantitative fatty acid composition in 14 breeding herds of Holsteinized Black-and-White and Holstein cows (*Bos taurus*) (control milkings, $n = 11529$, Moscow Province, 2017-2018)

Trait	1	2	3	4	5	6	7	8	9	10	11	12
MFF	–0.032											
$C_{14:0}$	–0.110	0.634										
$C_{16:0}$	–0.101	0.801	0.874									
$C_{18:0}$	–0.023	0.615	–0.122	0.300								
$C_{18:1}$	0.053	0.393	–0.427	–0.160	0.764							
SFA	–0.031	0.955	0.781	0.905	0.485	0.150						
MUFA	–0.008	0.463	–0.337	–0.072	0.767	0.983	0.211					
PUFA	0.075	–0.465	–0.554	–0.584	–0.037	0.113	–0.569	0.097				
SCFA	0.045	0.815	0.551	0.608	0.482	0.264	0.852	0.255	–0.455			
MCFA	–0.106	0.684	0.980	0.920	–0.033	–0.384	0.818	–0.293	–0.596	0.554		
LCFA	0.053	0.499	–0.325	–0.030	0.831	0.979	0.276	0.970	0.038	0.350	–0.276	

Continued Table 2

TIFA	–0.129	–0.286	–0.637	–0.469	0.282	0.384	–0.383	0.355	0.469	–0.300	–0.643	0.396

N o t e. The total number of milk samples n = 36982; 1 — daily milk yield, 2 — mass fraction of milk fat (MFF), 3 — $C_{14:0}$, 4 $C_{16:0}$, 5 — $C_{18:0}$, 6 — $C_{18:1}$, 7 — saturated fatty acids (SFA), 8 — monounsaturated fatty acids (MUFA), 9 — polyunsaturated fatty acids (PUFA), 10 — short chain fatty acids (SCFA), 11 — medium chain fatty acids (MCFA), 12 — long chain fatty acids (LCFA). TIFA — trans isomer of fatty acids. If $r_g > 0{,}050$, correlations are statistically significant at $p < 0.001$.

The amount of PUFA was negatively correlated with the amount of SCFA (r_g = –0.455) and SCFA (r_g = –0.596), but the relationship between the production of PUFA and TIFA was the highest and most positive (r_g = 0.469), which, in combination with the heritability coefficient, indicates a complex selection the process of increasing these indicators in the milk of cows. However, it should be noted that the problem of improving the ratio of saturated and unsaturated fatty acids in milk in favor of the latter remains unresolved. An increase in the proportion of oleic acid, MUFA, PUFA, LCFA in milk during the selection of animals simultaneously leads to a change in the fatty acid composition of trans-isomers, fatty acids with a short and medium length of carbon chain.

Summarizing the obtained data (see Tables 1, 2), we can conclude that the revealed correlations and indicators of genetic variability of fatty acids in cow's milk are promising for further GWAS analysis in order to adjust cattle breeding programs.

The results of the analysis of milk samples obtained in the control milkings of cows from the experimental group are shown in Table 3. It was found that 50.5% of the daily milking was in the morning; the rest was distributed approximately equally between daytime and evening milkings, 23.5 and 26.0 %, respectively. There was a clear inverse linear relationship between the amount of daily milk yield and the component composition of milk. With a smaller volume of milk for lunch milking of 6.1 kg, the percentage of fat fraction (up to 4.15%, including fatty acids) and dry matter (13.33%) increased.

For milk protein and casein, lactose, DSMR, BHB and urea, no significant quantitative differences were found when sampling at different times. The values of the molar mass of acetone detected in trace amounts were higher in the morning and evening milk samples (0.047 and 0.040 mmol/l, respectively), while the freezing point was lower ($–536.5\times10^{-3}$ °C and $–537.2\times10^{-3}$ °C, respectively). The coefficient of phenotypic variation (C_{vp}) regardless of the time (morning, lunch, evening) of milk sampling (20.0-24.2%) was higher for the mass fraction of fat than for other selectively significant traits of milk quality. Based on phenotypic variability, it can be assumed that the potential efficiency of selection for fatty acids will be higher for palmitic (C_{vp} = 22.0-25.0%), stearic (C_{vp} = 24.6-32.1%) acids, long-chain fatty acids (C_{vp} = 20.2-27.8%), short-chain fatty acids (C_{vp} = 23.0-27.5%), as well as the sum of saturated fatty acids (C_{vp} = 21.5-25.8%).

The repeatability (r) between adjacent control milkings (morning and evening) according to the studied indicators of milk composition was quite high, the exception was traces of metabolites — acetone, BHB and urea (r = 0.565-0.630) and the freezing point of milk (r = 0.505). Relatively moderate values were obtained for oleic acid (r = 0.625) and polyunsaturated fatty acids (r = 0.590). In general, it can be concluded that it is quite advisable to control the component composition of cows' milk either by an average sample or separately in the morning and evening, while accounting for the volume of milk produced from a cow should be equal to the number of milkings per day. We believe that when analyzing genome-wide associations, there will be no significant shift in the identified QTLs when using data obtained with a 2-fold control of productivity per day, that is, such control is sufficient.

3. An extended analysis of the cows' milk composition depending on the time of sampling (n = 144, Holsteinized Black-and-White experimental herd, PZ Ladozhsky, Krasnodar Territory, 2020-2021)

Trait	Control milking												repetability		
	morning				afternoon				evening						
	M	±SEM	SD	C_{vp}, %	*M*	±SEM	SD	C_{vp}, %	*M*	±SEM	SD	C_{vp}, %	m/a	a/e	m/e
Milk yield,kg	13.0	0.1	3.6	27.3	6.1	0.1	1.8	30.1	6.70	0.10	1.90	27.8	0.750	0.724	0.762
MFF, %	3.22	0.03	0.78	24.2	4.15	0.03	0.80	19.3	3.81	0.03	0.76	20.0	0.596	0.740	0.652
MFP (actual), %	3.24	0.02	0.43	13.3	3.25	0.02	0.42	12.8	3.26	0.02	0.44	13.6	0.869	0.915	0.867
MFP (raw), %	3.44	0.02	0.44	12.7	3.46	0.02	0.42	12.2	3.46	0.02	0.45	12.9	0.868	0.914	0.866
MFC, %	2.71	0.01	0.36	13.4	2.76	0.01	0.35	12.8	2.75	0.01	0.38	13.7	0.868	0.914	0.862
MFL, %	4.78	0.01	0.20	4.2	4.80	0.01	0.20	4.1	4.79	0.01	0.20	4.1	0.765	0.855	0.751
DM, %	12.33	0.04	1.11	9.0	13.33	0.04	1.09	8.1	12.97	0.04	1.12	8.7	0.734	0.835	0.764
DSMR, %	9.06	0.02	0.49	5.4	9.11	0.02	0.49	5.4	9.10	0.02	0.52	5.8	0.835	0.886	0.826
Acetone, mmol/l	0.047	0.002	0.049	105.1	0.036	0.002	0.040	111.5	0.040	0.002	0.056	139.0	0.608	0.681	0.569
Acetone, logarithm	–1.758	0.028	0.794	45.2	–1.930	0.030	0.817	42.3	–1.827	0.027	0.775	42.4	0.581	0.623	0.630
BHB, mmol/l	0.017	0.001	0.024	137.7	0.017	0.001	0.024	136.7	0.017	0.001	0.027	156.5	0.631	0.717	0.570
BHB, logarithm	–2.279	0.026	0.739	32.4	–2.282	0.027	0.738	32.3	–2.291	0.026	0.733	32.0	0.644	0.685	0.600
Urea, mg · 100 ml^{-1}	41.3	0.2	4.9	12.0	42.1	0.2	4.8	11.5	39.9	0.2	4.8	12.1	0.617	0.746	0.565
Freezing point, ×10^{-3} °C	–536.5	0.3	8.1	1.5	–538.5	0.4	9.5	1.8	–537.2	0.3	9.6	1.8	0.561	0.669	0.505
Acidity, pH	6.57	0.00	0.06	0.9	6.56	0.00	0.06	0.9	6.56	0.00	0.06	0.9	0.647	0.758	0.658
$C_{14:0}$, g/100 g	0.307	0.003	0.074	24.1	0.374	0.003	0.082	21.8	0.357	0.003	0.084	23.5	0.685	0.797	0.711
$C_{16:0}$, g/100g	0.794	0.007	0.198	25.0	0.969	0.008	0.225	23.2	0.923	0.008	0.220	23.8	0.684	0.806	0.718
$C_{18:0}$, g/100 g	0.295	0.003	0.095	32.1	0.383	0.003	0.094	24.6	0.352	0.003	0.091	25.8	0.658	0.775	0.709
$C_{18:1}$, g/100 g	1.016	0.009	0.247	24.3	1.327	0.009	0.247	18.6	1.194	0.008	0.231	19.3	0.577	0.692	0.625
LCFA, rg/100 g	1.243	0.012	0.345	27.8	1.664	0.012	0.336	20.2	1.494	0.011	0.315	21.1	0.590	0.705	0.633
MCAF, g/100 g	1.241	0.011	0.316	25.4	1.489	0.013	0.339	22.8	1.432	0.012	0.345	24.1	0.712	0.825	0.735
SCFA, g/100 g	0.437	0.004	0.120	27.5	0.576	0.005	0.133	23.0	0.524	0.004	0.125	23.8	0.609	0.758	0.637
MUFA, g/100 g	0.931	0.008	0.229	24.6	1.223	0.009	0.232	19.0	1.103	0.008	0.215	19.5	0.577	0.687	0.633
PUFA, g/100 g	0.124	0.001	0.025	20.3	0.150	0.001	0.026	17.3	0.135	0.001	0.024	18.0	0.545	0.656	0.590
SFA, g/100 g	2.139	0.019	0.551	25.8	2.730	0.022	0.587	21.5	2.542	0.020	0.571	22.5	0.636	0.775	0.676

N o t e. The total number of milk samples n = 2340. MFF — mass fraction of milk fat, MFP — mass fraction of protein, MFC — mass fraction of caseine, MFL — mass fraction of lactose, DM — dry matter, DSMR — dry skimmed milk residue, BHB — beta-hydroxybutyrat, LCFA — long chain fatty acids, MCFA — medium chain fatty acids, SCFA — short chain fatty acids, MUFA — monounsaturated fatty acids, PUFA — polyunsaturated fatty acids, SFA — saturated fatty acids; m/a — orning/afternoon repeatability, a/e — afternoon/evening repeatability, m/e — moning/evening repeatability.

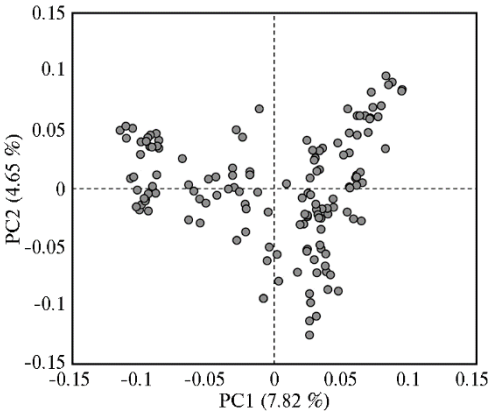

Fig. 1. Analysis of the genetic structure of a group of cows using the multidimensional scaling method (n = 144, an experimental herd of Holsteinized Black-and-White cows, PZ Ladozhsky, Krasnodar Territory, 2020-2021).

We also determined the genetic structure of the experimental animal group using the multivariate scaling (MDS) method (Fig. 1). Since the herd is represented by Holsteinized black-and-white dairy cattle, a moderate range of variability was observed for the components of variability (PC1 = 7.82%, PC2 = 4.65%). Currently, the experimental herd is consolidated due to individual selection of parental pairs (cows and Holstein bulls) in order to obtain as genetically homogeneous individuals as possible to study the inheritance of quantitative traits, including the component composition of milk. In our opinion, this will make it possible to more accurately assess the genotype of cows based on the phenotyping of economically useful qualities of each animal. We used the results of the MDS analysis of the sample as covariants along the PC1/PC2 axes to correct the effect of genetic variability on the population structure of the experimental herd and reduce the likelihood of obtaining false positive GWAS values of associations with direct phenotypic data on a number of milk components and the content of fatty acids in it.

Previously, in one of the studies, we detected 32 ($p < 0.001$-0.00001) causal SNP mutations associated with the evaluation of the breeding value of bulls by the content of fatty acids in the milk of daughter cows (the most significant were on the chromosomes BTA1, BTA5, BTA6, BTA10, BTA11, BTA14 , BTA19, BTA22 and BTA26) [25]. The genes *CHST11*, *ACO2*, *PPARGC1A*, *NRXN1*, *LPIN1*, *ASIC2*, *PCDH15*, *PRKG1* were directly associated with the synthesis of C_{14}, C_{16}, C_{18} fatty acids, conjugated linoleic acid, with an index of saturated and unsaturated fatty acids. In addition, genes located in QTL were found that are associated with animal fertility indicators, linear measurements of the udder and limbs (*NCAM2*, *FGD4*, *KCNIP4*, *SFXN1*, *NBAS*, *PGR*, *MON1B*, *GPLD1*, *PRKG1*). An analysis of the international database NCBI (https://www.ncbi.nlm.nih.gov/) on identified polymorphisms showed that the identified genes often exhibit a pleiotropic effect. This once again confirms the complex nature of the heritability of the quantitative composition of fatty acids and at the same time indicates the possibility of using the content of fatty acids in milk to control the health and fertility of dairy cows [25].

To further search for genome-wide associations with the quantitative composition of milk FAs, we correlated the data of GWAS analysis with the results of direct phenotyping of cows of the experimental herd for this trait and identified SNPs that are associated with the own productivity of daughters of sires assessed on populations from the Moscow region (14 herds). For indicators of the breeding value of these bulls-fathers by FA, the first results of the search for associations were previously obtained.

It has been established that all the studied traits of milk productivity of cows were characterized by the polygenic nature of inheritance and the multiple action of genes involved in the control of indicators of the quantitative composition of milk fatty acids with different selection significance. Thus, for the daily milk yield, we found quantitative trait loci (QTL) on the BTA1, BTA4, BTA7, BTA9, BTA14, BTA15, BTA18, and BTA25 chromosomes (Fig. 2). The variability in the mass

fraction of fat (MFF) in the GWAS analysis served as an indicator of the reliability of phenotyping of the other studied traits: the spectra of essential fatty acids and MFA were determined in the same samples, so the identified associations can be considered significant. On the BTA14 chromosome, we found clusters associated with the percentage of milk fat, which contain the milk fat candidate gene *DGAT1* (SNP mutation ARS-BFGL-NGS-4939) and a number of other genes linked to it. QTLs were also detected by the mass fraction of fat on chromosomes BTA5, BTA10, BTA11, and BTA19.

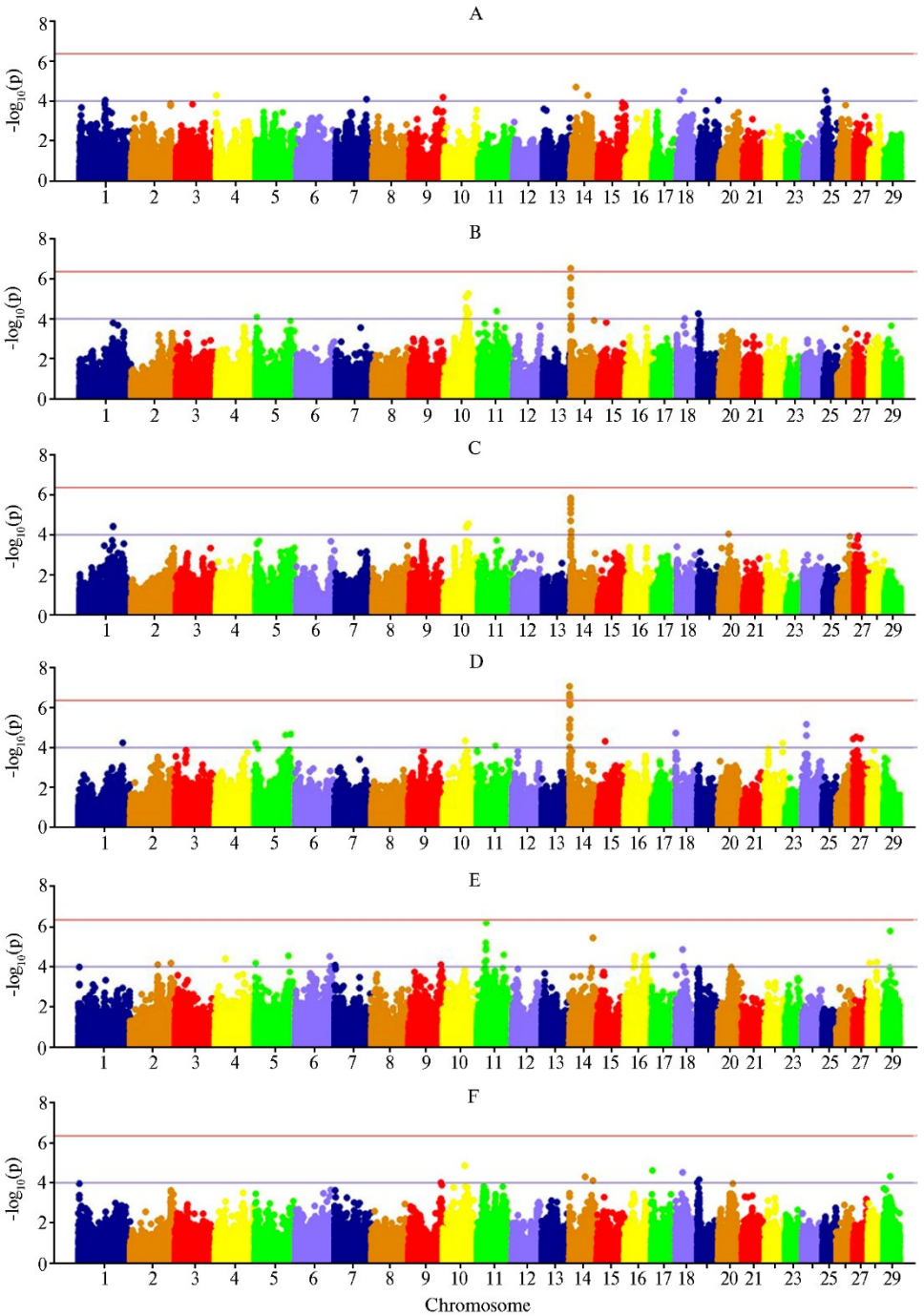

Fig. 2. GWAS analysis for daily milk yield (A)**, mass fraction of milk fat** (B) **and essential fatty acids,**

myristic (C)**, palmitic** (D)**, stearic** (E) **and oleic** (F) **based on direct phenotypic indicators** (n = 144, an experimental herd of Holsteinized black-and-white cows, Ladozhsky PZ, Krasnodar Territory, 2020-2021).

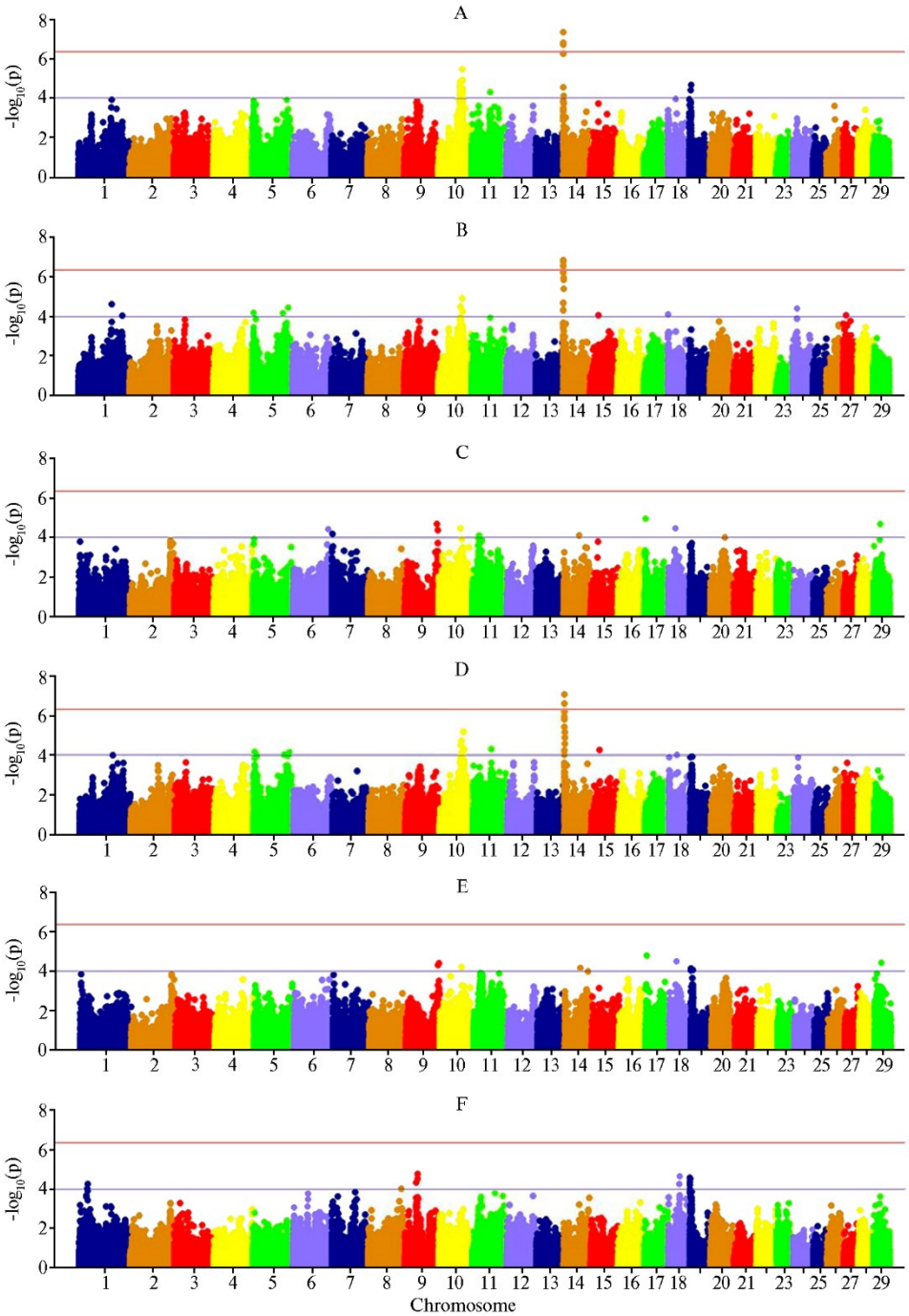

Fig. 3. GWAS analysis for short chain fatty acids (A)**, medium chain fatty acids** (B)**, long chain fatty acids** (C)**, saturated fatty acids** (D)**, monounsaturated fatty acids** (E) **and polyunsaturated fatty acids** (E) **based on direct phenotypic indicators** (n = 144, an experimental herd of Holsteinized black-and-white cows, Ladozhsky PZ, Krasnodar Territory, 2020-2021).

For myristic and palmitic FAs, we identified common clusters for BTA5, BTA10, BTA14, BTA18, and BTA27, which was largely consistent with the association profile for MFF. Stearic and oleic FAs, as long-chain FAs, showed similar

localization on the BTA9, BTA10, BTA11, BTA14, BTA17, BTA18, BTA19, BTA20, and BTA29 chromosomes (see Fig. 2). For short- and medium-chain FAs, associations were detected on the BTA1, BTA5, BTA10, BTA11, BTA14, BTA18, BTA19, and BTA24 chromosomes. In this regard, SCFA and MCFA turned out to be more similar to myristic and palmitic acids, the content of which in milk showed close genetic correlations: for SCFA, r_g = 0.551 and r_g = 0.608, respectively, for MCFA, r_g = 0.920 and r_g = 0.980. For long-chain fatty acids, QTLs were detected for BTA6, BTA7, BTA9, BTA10, BTA11, BTA17, BTA18, and BTA29 (see Fig. 2), which generally agreed with the data for stearic and oleic acids, which have a similar association profile, and with the identified genetic relationship between these traits (r_g = 0.831 for stearic acid, r_g = 0.979 for oleic acid).

The group of saturated and unsaturated fatty acids showed different patterns of QTL localization according to the identified associations, which, in our opinion, is mainly associated with the features of their synthesis and metabolic pathways (Fig. 3). Mono- and polyunsaturated fatty acids (as the most significant in terms of the nutritional value of milk) showed total QTLs for BTA1, BTA2, BTA9, BTA11, BTA18 and BTA19 (see Fig. 3). The presence of different loci that control the formation and secretion of milk fatty acids and their location on different chromosomes can also be associated with the pressure of artificial selection.

4. Annotations of identified significant polymorphisms ($p < 0.0001$) **on bovine chromosomes** (BTA) (n = 144, an experimental herd of Holsteinized Black-and-White cows, PZ Ladozhsky, Krasnodar Territory, 2020-2021)

Gene	Traits	BTA	Position, bp	
			start	end
MED12L	MCFA, $C_{14:0}$	1	117,548,538	117,917,463
EPHB1	MCFA	1	135,191,077	135,518,801
GRIN2B	MCFA, SFA, $C_{16:0}$	5	96,408,804	96,761,516
PRMT8	MCFA, SFA, $C_{16:0}$	5	106,812,249	106,812,249
ERC1	MCFA, $C_{16:0}$	5	108,308,618	108,549,124
CACNA1C	SFA, $C_{16:0}$	5	109,152,548	109,417,890
ARFGEF3	TIFA	9	77,035,587	77,158,234
RPS6KA2	LCFA, MUFA	9	102,918,982	103,074,109
GCH1	MFF, LCFA, MCFA, SCFA, SFA, $C_{14:0}$, $C_{18:1}$	10	67,576,390	67,631,089
ATG14	MFF, MCFA, SCFA, SFA	10	680,734,07	68,110,299
PELI2	SCFA	10	68,778,347	68,974,093
KCNH5	MFF, LCFA, MCFA, SCFA, SFA, $C_{14:0}$, $C_{18:1}$	10	75,235,434	75,637,242
PRKCE	MFF, $C_{18:0}$	11	27,935,104	28,472,632
CTNNA2	MFF	11	54,723,190	55,906,462
ARHGAP39	SCFA	14	1,563,866	1,600,378
CYHR1	MFF, MCFA, SCFA, SFA, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$	14	1,663,923	1,677,519
VPS28	MFF, MCFA, SCFA, SFA, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$	14	1,693,641	1,698,490
DGAT1	MFF, MCFA, SCFA, SFA, $C_{14:0}$, $C_{16:0}$	14	1,795,351	1,804,562
MROH1	MCFA, SCFA, SFA, $C_{14:0}$, $C_{16:0}$	14	1,844,664	1,894,424
MAF1	MCFA, SCFA, SFA	14	1,921,784	1,924,818
GSDMD	MCFA, $C_{16:0}$	14	2,341,290	2,346,302
ZC3H3	MFF, MCFA, SCFA, SFA, $C_{14:0}$, $C_{16:0}$	14	2,354,390	2,418,557
RHPN1	$C_{14:0}$	14	2,462,544	2,471,434
LY6D	MCFA, $C_{16:0}$	14	2,801,383	2,803,020
TSNARE1	MFF, MCFA, SCFA, SFA, $C_{14:0}$, $C_{16:0}$	14	3,054,763	3,171,546
CPQ	$C_{18:0}$, $C_{18:1}$	14	69,287,302	69,893,052
CPE	LCFA, MUFA	17	546,398	697,915
CDH13	Daily milk yield	18	9,512,739	10,162,782
AKTIP	Daily milk yield	18	21,926,577	21,937,955
FTO	MUFA, $C_{18:0}$, $C_{18:1}$	18	22,118,201	22,541,532
ABCC1	Daily milk yield	25	14,469,282	14,570,639
TNKS	TIFA, $C_{16:0}$	27	24,632,930	24,789,416
FAT3	LCFA, MUFA, $C_{18:0}$, $C_{18:1}$	29	1,965,869	2,605,125
LUZP2	LCFA, MUFA, $C_{18:0}$, $C_{18:1}$	29	20,259,769	20,557,376

Note. MFF — mass fraction of milk fat, LCFA — long chain fatty acids, MCFA — medium chain fatty acids, SCFA — short chain fatty acids, MUFA — monounsaturated fatty acids, PUFA — polyunsaturated fatty acids, SFA — saturated fatty acids, TIFA — trans isomer of fatty acids.

No significant QTLs were detected for trans-isomers FA, except for those detected for BTA1, BTA6, BTA18, BTA22, and BTA27, which is probably due to

the small dispersion in this parameter (data not shown in the figures).

We annotated the identified polymorphisms in the genes associated with the daily milk yield, fat mass fraction and fatty acid composition of milk in cows from the experimental group (Table 4). The comparison was carried out using the international database Animal QTLdb (https://animalgenome.org/cgi-bin/QTLdb/BT/index).

When mapping loci of quantitative traits for daily milk yield, we revealed the presence of three highly significant associations with polymorphisms in the *CDH13* and *AKTIP* (BTA18) and *ABCC1* (BTA25) genes (see Table 4). They are also associated with milk cholesterol content, animal fertility, long-term use, and somatic cell count in milk [24]. The *CACNA1C*, *GCH1*, *ATG14*, *KCNH5*, *PRKCE*, *CTNNA2*, *CYHR1*, *VPS28*, *DGAT1*, *ZC3H3*, *RHPN1*, *TSNARE1* genes which form QTLs on chromosomes BTA10, BTA11, and BTA14 have been identified for MFF, short-, medium-chain, saturated fatty acids, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$. It should be noted that all of the listed genes had a pleiotropic effect on a number of fatty acids. Annotation revealed genes associated with energy metabolism, which determines resistance to ketosis [26], content of conjugated linoleic acid in milk, percentage and yield of milk fat and protein, cholesterol content in milk, content of palmitic and palmitoleic fatty acids, milk yield per lactation, reproductive qualities of animals. Using GWAS analysis, we identified the diacylglycerol-O-acyltransferase 1 gene, which can serve as a marker of polymorphism in the study of milk fat indicators and allows us to indirectly assess the accuracy of the results obtained [14, 27]. We identified 70 different QTLs, predominantly associated with the fatty acid profile in cow's milk, casein content, animal energy status, calcium, potassium and phosphorus content in milk [24, 28].

Short- and medium-chain fatty acids, myristic and palmitic acids, saturated fatty acids had an association with polymorphisms in the *MED12L*, *EPHB1*, *GRIN2B*, *PRMT8*, *ERC1*, *PELI2*, *ARHGAP39*, *MROH1*, *MAF1*, *GSDMD*, *LY6D* genes (see Table 4), which were are associated with the number of successful inseminations, ease of calving, pregnancy rate of bull daughters, percentage of fat and protein in milk, palmitic FA, attachment and depth of the udder of cows, mastitis and the number of somatic cells in milk [24].

For long-chain, monounsaturated fatty acids, stearic and oleic acids, the annotation revealed the following selectable genes: *RPS6KA2*, *CPQ*, *CPE*, *FTO*, *FAT3*, *LUZP2* (see Table 4). Their polymorphisms are also associated with variability in the predisposition of cows to mastitis, linear measurements of the animal's exterior (limbs and udder), fertility, milk fat yield, and the number of somatic cells in milk (29). Trans FAs, despite their low variability compared to other fractions of milk fatty acids, in our study showed an association with polymorphisms in the *ARFGEF3* and *TNKS* genes, which are known to be associated with cow milk yield (30).

Thus, based on the study of the genetic and phenotypic variability of milk composition at the population level in 14 herds of dairy cattle (Moscow Province) and in the experimental herd of the Holsteinized Black-and-White cows (Krasnodar Territory), selection constants and a number of significant associations have been established between the identified gene polymorphisms and formation of milk fatty acids. Using a population genetic analysis based on the ratio of intergroup and general group variance, the highest heritability for oleic acid ($h^2 = 0.196$), monounsaturated fatty acids ($h^2 = 0.176$), long- and medium-chain fatty acids ($h^2 = 0.125$-0.55), stearic acid ($h^2 = 0.125$) was shown. These features can be recommended when evaluating sires for the quality of offspring. Studies conducted on a group of cows genotyped and phenotyped for the expanded component composition of milk have provided new data on the localization of QTL fatty acid composition in the genomes of animals of Russian origin. As a result of annotation in the genes

CACNA1C, *ARFGEF3*, *RPS6KA2*, *GCH1*, *ATG14*, *PELI2*, *KCNH5*, *PRKCE*, *CTNNA2*, *ARHGAP39*, *CYHR1*, *VPS28*, *DGAT1*, *MROH1*, *MAF1*, *GSDMD*, *ZC3H3*, *RHPN1*, *LY6D*, *TSNARE1*, *CPQ*, *CPE*, *FTO*, *TNKS*, *FAT3*, and *LUZP2* polymorphisms were found to be significantly associated with the variability of fatty acid content in milk. Saturated fatty acids compared to unsaturated fatty acids showed more variability in GWAS, probably due to stronger selection pressure. Further study of the genetic mechanisms of inheritance of the fatty acid composition of milk will make it possible to develop the basis for a selection strategy for this trait.

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STUDY OF THE GENETIC DIVERSITY OF DOMESTIC AND WILD REINDEER (*Rangifer tarandus* L., 1758) POPULATIONS USING NUCLEAR AND MITOCHONDRIAL GENOMIC MARKERS

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Abstract

The reindeer (*Rangifer tarandus* L., 1758) is an important biological species that plays a key role in the supporting livelihood of the peoples of the Far North of Russia. Due to climate change and anthropological impacts, this species may be endangered, therefore, in the modern world, the study and conservation of the genetic diversity of the reindeer is relevant. In this work, for the first time, the genetic variability responsible for the differentiation of domestic and wild forms of the reindeer was studied using an integrated molecular genetic approach, which consisted in the analysis of nuclear and mitochondrial genomes. Our aim was to evaluate the genetic diversity, genetic structure, and phylogenetic relationships of domestic and wild populations of reindeer bred in the Russian Federation based on complete mitochondrial DNA *CytB* gene sequences and microsatellite loci polymorphism. The research was carried out in 2022. Cuts from reindeer antlers served as material. The sample included wild reindeer from the tundra population (WLD), as well as domestic reindeer from Nenets (NEN), Chukchi (CHU), Even (EVN) and Evenk breeds comprising the Krasnoyarsk (EVK_KRA) and Yakut (EVK_YAK) populations. For the study of mtDNA, 123 unrelated individuals were selected. Microsatellite analysis was performed in 213 individuals of domestic breeds and 119 representatives of the wild population. The complete sequences of the *CytB* gene were determined using next generation sequencing (NGS) on a miSeq sequencer (Illumina, Inc., USA). Polymorphism of nine STR loci (NVHRT21, NVHRT24, NVHRT76, RT1, RT6, RT7, RT9, RT27, RT30) was investigated by fragment analysis using an ABI3130xl genetic analyzer (Applied Biosystems, USA). To assess the genetic diversity of each group of reindeer, indicators of mitochondrial variability (number of polymorphic sites S, average number of nucleotide differences K, number of haplotypes H, haplotype diversity H_D, nucleotide diversity π) and microsatellite variability (rarefied allelic richness A_R, observed heterozygosity H_O, unbiased expected heterozygosity uH_E, unbiased inbreeding coefficient F_{IS}) were calculated. The degree of genetic differentiation between groups was assessed based on pairwise F_{ST} and JostD values. Statistical processing of the raw data was performed using the programs MEGA 7.0.26, PopART 1.7, PartitionFinder 2, Arlequin 3.5.2.2, MrBayes 3.2.7, FigTree 1.4.3, DnaSP 6.12.01, SplitsTree 4.14.5, STRUCTURE 2.3.4 and R packages diveRsity, pophelper, adegenet and ggplot2. Analysis of mtDNA *CytB* gene sequences showed that all studied populations were characterized by high haplotype diversity, H_D = 0.519 (CHU)-0.997 (WLD), and nucleotide diversity, π = 0.00238 (CHU)-0.00626 (WLD). Based on the mtDNA analysis no clear genetic structure was revealed in the studied reindeer populations. Analysis of microsatellite variability showed that values of allelic richness ranged from 6.188 in CHU to 8.76 in WLD. In all six populations, observed heterozygosity ranged from 0.566 (CHU) to 0.687 (EVK_YAK) and 0.693 (WLD). All studied reindeer groups were characterized by a deficit of heterozygotes, as indicated by positive values of the fixation index, F_{IS} = 0.11 (EVK_YAK)-

0.262 (EVK_KRA). Network analysis showed the differentiation of the Chukotka breed from the rest groups, as evidenced by the highest F_{ST} and JostD values, which varied from 0.203 and 0.488 for EVK_KRA to 0.212 and 0.564 for EVN, respectively. Based on both nuclear and mitochondrial markers, wild reindeer populations showed higher genetic diversity compared to domestic populations. It may be assumed that selection work with domestic reindeer breeds led to the creation of unique populations that differ from the original wild relatives. However, both domestic and wild reindeer populations, which were studied in this work, were characterized by high genetic variability.

Keywords: reindeer, *Rangifer tarandus*, genetic diversity, phylogenetic assessment, mitochondrial DNA, microsatellite loci

The reindeer (*Rangifer tarandus* L., 1758) is a bioresource that is important for maintaining ecological balance via the influence on vegetation and as a livelihood for many indigenous peoples of the Arctic North. The reindeer was probably essential for human migration and colonization of the Eurasian Arctic and Subarctic after the last ice age. Recently, the reindeer has also been involved to create protected areas [1, 2]. Unlike most other livestock species whose wild forms are extinct (e.g., cattle, horses), endangered (e.g., donkeys, llamas, alpacas), or geographically restricted (e.g., sheep, goats), wild reindeer populations are still widespread in Northern Eurasia and North America (caribou). Almost 50% of approximatelly 3,000,000 deer in the Old World are wild animals, and in many areas wild and domestic herds coexist closely [2, 3]. This provides a unique opportunity to analyze the interaction between domestic and wild populations.

Reindeer are mainly distributed in the Arctic region of the Northern Hemisphere, including Norway, Finland, Sweden, Russia, Greenland, the United States, Mongolia, China and Canada. Fossils found indicate that during the Pleistocene *Rangifer* lived south of the ice sheet in both Eurasia and North America, as well as in Beringia, covering the Bering Land Bridge, Alaska and a large part of Siberia.

Based on morphological and historical data, populations of modern reindeer are classified into three ecological groups: forest (sedentary deer), tundra (migratory deer), and high arctic island deer [4]. These ecological groups include nine subspecies, of which seven [5] have survived to date. Domestic reindeer in the Russian Federation belong to four approved breeds, the Nenets, Even, Evenk and Chukchi [6].

The reindeer, like other Holarctic species, may become an endangered species due to climate change and human impact. Thereof, evaluation of phylogenetic structure at the species level is important to conserve genetic diversity [5] which allows species to adapt to environment and develop local adaptations [7]. The issue of studying the genetic diversity of reindeer populations is the subject of many works based on the use of nuclear and mitochondrial markers.

J.-C. Zhai et al. [13] characterized the genetic diversity of eight populations of reindeer from the Greater Khingan mountains using 11 microsatellite loci. The authors revealed a deficit of heterozygotes in all populations and a low degree of genetic differentiation. T.E. Deniskova et al. [14] assessed the genetic diversity of 15 populations of the Nenets breed using 14 microsatellite loci. Later, in 2020, V.R. Kharzinova et al. [15] studied the population structure of 528 domesticated reindeer of four breeds from the Russian Federation. In the same year, Yu. Stolpovsky et al. [16] studied 397 individuals of domestic and wild reindeer bred in various climatic zones of Russia. Analysis of microsatellite loci showed that 70% of the allelic diversity occurres in the wild reindeer populations.

In 2018, V.R. Kharzinova et al. [17] performed the first genotyping of reindeer of four Russian breeds using the BovineHD BeadChip and submitted a complete characterization of the genetic diversity of these breeds, as well as their ecotypes from four federal districts of the Far North of Russia. The Chukchi breed

and the Yakut intrabreed ecotype Khargin had low genetic diversity. Thereof, the preservation and increase of genetic variability in these groups is a priority [17].

As markers of the mitochondrial genome, two highly variable mtDNA regions, the *CytB* gene and the control region (D-loop), are used in the reindeer population studies. In 2018, C.D. Wilkerson et al. [19] based on the analysis of mtDNA D-loop and *CytB* gene sequences, identified 4 haplogroups (A, B, C and D) and 32 haplotypes in woodland caribou on the island of Newfoundland. Island caribou were characterized by a fairly high genetic diversity ($H_D = 0.894$ and $\pi = 0.00216$), with the exception of deer from the Avalon Peninsula, in which only three haplotypes were identified with a relatively low degree of haplotype ($H_D = 0.569$) and nucleotide ($\pi = 0.00052$) diversity. Phylogenetic analysis allowed the authors to trace the direction of the post-glacial recolonization of the island by reindeer [19].

Currently, an integrated approach is gaining popularity when several types of molecular genetic markers are used for a more accurate analysis to obtain complete information about the genetic diversity of animals. In 2012, F. Barbanera et al. [11] successfully investigated the poaching of the Cypriot moufflon (*Ovis orientalis ophion*) using 12 microsatellite loci as molecular markers together with the mitochondrial *CytB* gene. Later, in 2021, another poaching crime was uncovered in the Kabardino-Balkarian Republic. A. Rodionov et al. [12], using a complex approach based on 14 microsatellites and SNP genotyping (DNA chip), proved the fact of poaching of the Caucasian tur (*Capra caucasica*).

M.A. Cronin et al. [20] quantified genetic variation in 11 North American caribou herds using 18 microsatellite loci and *CytB* gene sequences. Such a comprehensive analysis confirmed the intraspecific classification of the reindeer into three ecotypes: living in the tundra on barren land, the mountain form and the forest form. Later, the same authors characterized the genetic diversity of domestic reindeer from Alaska, Siberia, and Scandinavia in comparison with wild caribou using 18 microsatellite loci and sequences of the mitochondrial *CytB* gene. The authors revealed differences in the frequencies of haplotypes and microsatellite loci in domestic reindeer and wild caribou. High genetic diversity for both markers was characteristic of wild deer {21].

In 2018, a research team from China [22] examined the genetic variation in a single Aolugui reindeer population using 10 microsatellite loci together with the *CytB* gene and revealed the varying degrees of inbreeding in the population. mtDNA polymorphism indicated a relatively low genetic diversity ($H_D = 0.468 \pm 0.091$, $\pi = 0.0017 \pm 0.001$), and five unique haplotypes were identified. The authors propose to form strategies for the conservation of the species and restoration of the population based on the data obtained [22].

A combination of several markers is now commonly used to quantify the genetic diversity of reindeer. Howevere, similar attempts to characterize Russian reindeer populations have not yet been made. In the presented work, we for the first time evaluated the genetic diversity of reindeer from the Russian Federation, revealed their phylogenetic relationships and assessed the degree of differentiation of the studied animals using an integrated approach based on the analysis of nuclear and mitochondrial genomes.

Our goal was to evaluate the genetic diversity, genetic structure, and phylogenetic relationships of domestic and wild Russian populations of reindeer based on complete mitochondrial DNA *CytB* gene sequences and microsatellite loci polymorphism.

Materials and methods. The research was carried out in 2022 using sections of antlers. The sampe included biomaterial collected from wild reindeer of the

tundra population (WLD), domestic reindeer of the Nenets (NEN) (Komi Republic), Chukchi (CHU) (Iultinsky district, Chukotka Autonomous Okrug), Even (EVN) (Neryungri district, Sakha Republic) breeds, as well as the Krasnoyarsk (EVK_KRA) (Krasnoyarsk Territory) and Yakut (EVK_YAK) (Aldan district, Republic of Sakha) populations of the Evenk breed. A total of 123 unrelated individuals were selected for mtDNA study. For microsatellite analysis, 213 domestic reindeer and 119 reindeer from wild populations were selected.

DNA was isolated using the DNA-Extran-2 kit (OOO Sintol, Russia) according to the manufacturer's standard protocol. The DNA concentration was measured (a Qubit 4.0 fluorometer, Invitrogen/Life Technologies, USA), and the absorption ratio $OD_{260/280}$ was assessed (a NanoDrop™ 8000 spectrophotometer, Thermo Fisher Scientific, Inc., USA). The DNA concentration ranged from 15 to 50 ng/μl with an $OD_{260/280}$ ratio of 1.8 or higher.

Next generation sequencing (NGS) was carried out in several staps. At the first stage (sample preparation), complete reindeer mitochondrial genomes were generated by amplification of six fragments of 2000 to 4500 bp in length with 120-780 bp overlapping region. The following primer pairs were used: F1 5'-TCCTCCCTTCTAGACTTAATCTGACT-3', R1 5'-CTCCTCCCACGACTAGTTGCAC-3'; F2 5′-ACTCCAACCTATTGCAGATGCCAT-3′, R2 5′-AAGGTTATTTCGACTGCATGTGCGGTTAC-3′; F3 5'-CTAACACTCAGATTAATTAGAGGACA-3', R3 5'-GTACTCCGCGGTTCATATTAATGAGAGG-3'; F4 5′-TGCTTGAGCAGGCATAGAAGGGAC-3′, R4 5′-TGGTGTGTCATTATGACTTGTTGTGCA-3′; F5 5′-GGAGGAATTACACTGGGATTAATAAG-3′, R5 5′-AATACCCTCTACTGCTATTGGCTTGA-3′; F6 5'-GGAACCGTAAAATTGATACAACTCCAA-3', R6 5'-GGGATTGCAAGCTTATATAGTTATGG-3'. Amplification was carried out in the following mode: 2 min at 96° °C (initial denaturation); 30 s at 96 °C, 30 s at 60 °C, 3 min at 72 °C (40 cycles), 10 min at 72 °C (final elongation) (an Applied Biosystems SimpliAmp thermal cycler, Thermo Fisher Scientific, Inc., USA). At the second stage, the libraries were prepared for sequencing using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, USA) according to the manufacturer's standard protocol. The samples were sequenced using paired end reads of 300 bp (a MiSeq instrument, Illumina, Inc., USA). The final stage was the processing of the obtained data.

From the complete deer mtDNA sequences, after alignment using the MUSCLE algorithm [23] in the MEGA 7.0.26 program [24], the *CytB* gene sequences were reassabmled. The sequence of the reindeer *CytB* gene from the NCBI database (https://www.ncbi.nlm.nih.gov/genbank/, GenBank accession number NC_007703.1) served as an outgroup. The median network was constructed using the PopART 1.7 program [25]. The best evolution models were determined in the PartitionFinder 2 program [26] using the adjusted Akaike information test (AICc) [27]. The evolutionary models HKY+I, HKY+I, and GTR+I for the first, second, and third nucleotides in the codon turned out to be optimal. F_{ST} analysis was performed using the Arlequin 3.5.2.2 program [28]. The Bayesian phylogenetic tree was built using MrBayes 3.2.7 software [29] followed by visualization in a graphical viewer FigTree 1.4.3 [30]. Monte Carlo search with Markov chains was performed using four chains with 10,000,000 steps, trees were selected every 500 generations (the first 25% of the selected trees were rejected using the burn-in algorithm).

The parameters of genetic diversity, i.e., the number of polymorphic sites (S), the average number of nucleotide differences (K), the number of haplotypes (H), haplotype diversity (H_D), nucleotide diversity (π), arithmetic mean errors (±SEM) were calculated using the DnaSP 6.12.01 program. The population expansion hypothesis was tested by calculating the Fu's Fu neutrality statistic and

the Tajima's D test in DnaSP 6.12.01 [31].

Polymorphism analysis of 9 microsatellites (NVHRT21, NVHRT24, NVHRT76, RT1, RT6, RT7, RT9, RT27, RT30) was performed as previously described [32]. The resulting DNA fragments were visualized by fragment analysis using Gene Mapper v. 4 (Applied Biosystems, USA). Analysis of population genetic parameters, including rarefied allelic diversity (A_R), observed (H_O) and unbiased expected (uH_E) heterozygosity, as well as unbiased inbreeding coefficient (F_{IS}) with a 95% confidence interval (CI), was performed using the diveRsity R package with subsequent visualization in the pophelper package [33]. The degree of genetic differentiation was assessed based on the matrices of pairwise values F_{ST} [34] and JostD [35]. To build phylogenetic trees using the Neighbor-Net algorithm, we used SplitsTree 4.14.5 software [36] and the diveRsity R package, followed by visualization in the pophelper package.

The genetic structure of the studied groups of reindeer was assessed using Principal Component Analysis (PCA) in the adegenet R package [37] and with visualization in the ggplot2 R package [38], as well as by clustering in STRUCTURE 2.3.4 program [39] using a mixed model (the number of assumed clusters K is from 1 to 10, the length of the burn-in period is 100,000, the Monte Carlo Markov chain model is 100,000). For each value of K, 10 iterations were performed. The STRUCTURE HARVESTER application [40] was used to determine the optimal number of clusters (ΔK) according to the method of G. Evanno et al. [41]. The source files were formed in the R 3.5.0 software environment (R Core Team) [42].

Results. The use of next generation sequencing (NGS) technology made it possible to obtain complete sequences of the mitochondrial *CytB* gene in the studied reindeer populations.

Analysis of the nucleotide sequences of the mitochondrial *CytB* gene. In the 1140 bp sequences of the mitochondrial *CytB* gene we obtained from 123 individuals, 40 haplotypes were identified. All animals were characterized by high genetic diversity ($H_D = 0.918 \pm 0.014$; $\pi = 0.00448 \pm 0.00023$) (Table 1). In wild reindeer populations, the highest haplotype ($H_D = 0.997 \pm 0.013$) and nucleotide ($\pi = 0.00626 \pm 0.00041$) diversity was observed compared to domestic populations ($H_D = 0.865 \pm 0.021$; $\pi = 0.00364 \pm 0.00022$).

1. Genetic diversity in populations of domestic and wild reindeer (*Rangifer tarandus* L., 1758) based on nucleotide sequences of the mitochondrial gene *CytB* (2022)

Population	*n*	S	K	H	$H_D \pm SEM$	$\pi \pm SEM$	Tajima's D	Fu's Fu
CHU	22	17	2.710	5	0.519±0.114	0.00238±0.00066	–1.53176 ns	1.780 ns
EVK_KRA	12	8	3.333	5	0.788±0.090	0.00292±0.00029	1.03140 ns	0.159 ns
EVK_YAK	14	15	4.396	7	0.846±0.074	0.00386±0.00055	–0.27767 ns	0.159 ns
EVN	21	12	3.200	10	0.848±0.059	0.00281±0.00029	–0.14393 ns	–2.304 ns
NEN	21	17	4.038	6	0.663±0.105	0.00354±0.00067	–0.53756 ns	2.014 ns
All domestic populations	90	35	4.153	21	0.865±0.021	0.00364±0.00022	–1.24481 ns	–4.365 ns
WLD	33	48	7.129	24	0.997±0.013	0.00626±0.00041	–1.45464 ns	–10.970
Total	123	61	5.098	40	0.918±0.014	0.00448±0.00023	–1.73284 ns	–19.784

N o t e. *n* — the number of samples, S — number of polymorphic sites, K — average number of nucleotide differences, H — number of haplotypes, H_D — haplotype diversity, π — nucleotide diversity, ns — $0.10 > P > 0.05$. CHU — Chukchi breed, EVK_KRA — Evenk Krasnoyarsk breed, EVK_YAK — Evenk Yakut breed, EVN — Evenk breed, NEN — Nenets breed, WLD — wild reindeer.

In the populations of domestic reindeer, the Even breed had the greatest haplotype diversity ($H_D = 0.848 \pm 0.059$). The highest nucleotide diversity and the highest average number of nucleotide differences were found in the Evenki Yakut population ($\pi = 0.00386 \pm 0.00055$, K = 4.396). The Chukchi breed was characterized by the least genetic diversity in all indicators ($H_D = 0.519 \pm 0.114$, $\pi = 0.00238 \pm 0.00066$, K = 2.710).

The obtained values of the Tajima's D (–1.24481 ns) and Fu's Fu (–4.365 ns) tests indicated a trend towards complete identity between home populations. According to these values, there is a limited difference in the number of polymorphic sites and the average number of pairwise nucleotide differences between the studied populations. That is, the studied domestic reindeer breeds are in genetic balance, which indicates the state of alleles and genotypes in the gene pool of their populations. This ensures adaptation to environmental changes caused primarily by anthropogenic factors. On the contrary, in wild deer, we found a high negative Fu's Fu (–10.970), indicating the flow of foreign genes due to spatial expansion, while a low D value (–1.45464 ns) indicated a stable state of the population.

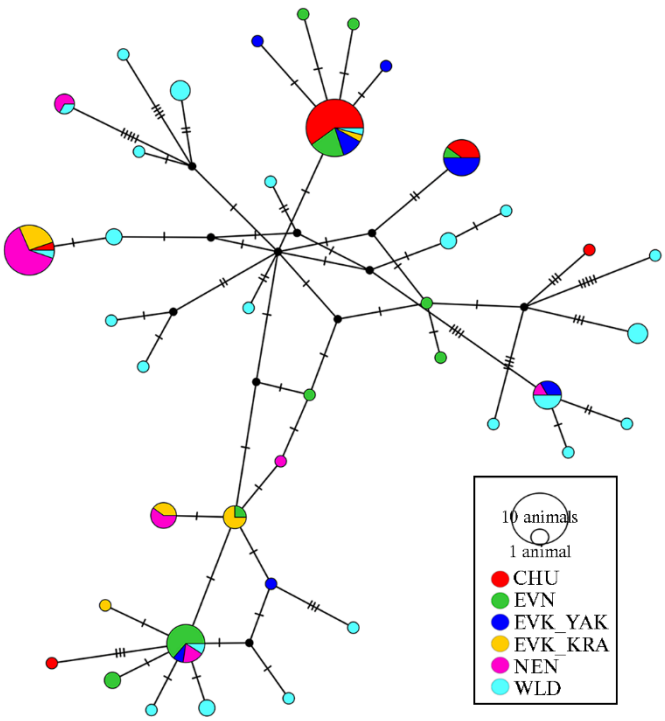

Fig. 1. The median network characterizing the relationships of haplotypes identified in domestic and wild reindeer (*Rangifer tarandus* L., 1758) based on the nucleotide sequences of the mitochondrial *CytB* gene: CHU — Chukchi breed (n = 22), EVK_KRA — Evenk Krasnoyarsk breed (n = 12), EVK_YAK — Evenk Yakut breed (n = 14), EVN — Even breed (n = 21), NEN — Nenets breed (n = 21), WLD — wild reindeer (n = 33). The diameter of the circle corresponds to the number of individuals of the corresponding haplotype. The number of transverse lines indicates the number of nucleotide substitutions. Black circles at network branching points represent hypothetical haplotypes (2022).

Among the reindeer of both wild and domesticated populations inhabiting the territory of the Russian Federation, we did not reveal a clear differentiation according to the maternal mtDNA marker.

Of all 40 haplotypes, only 8 were common, the remaining 32 haplotypes were found only in one representative from the studied deer sample (Fig. 1). Basically, the diversity of haplotypes was achieved due to populations of wild animals. Of 24 haplotypes identified in wild deer, only 5 were common with populations of domestic animals. Representatives of all 5 populations of domestic deer had one common haplotype with populations of wild reindeer.

In addition to the common haplotypes with wild deer, the domestic deer populations formed 3 common haplotypes. The Krasnoyarsk population of the Evenk breed had one common haplotype each with representatives of the Nenets and Even breeds, and one common haplotype was found in representatives of the

Chukchi, Even and Yakut populations of the Evenk breed.

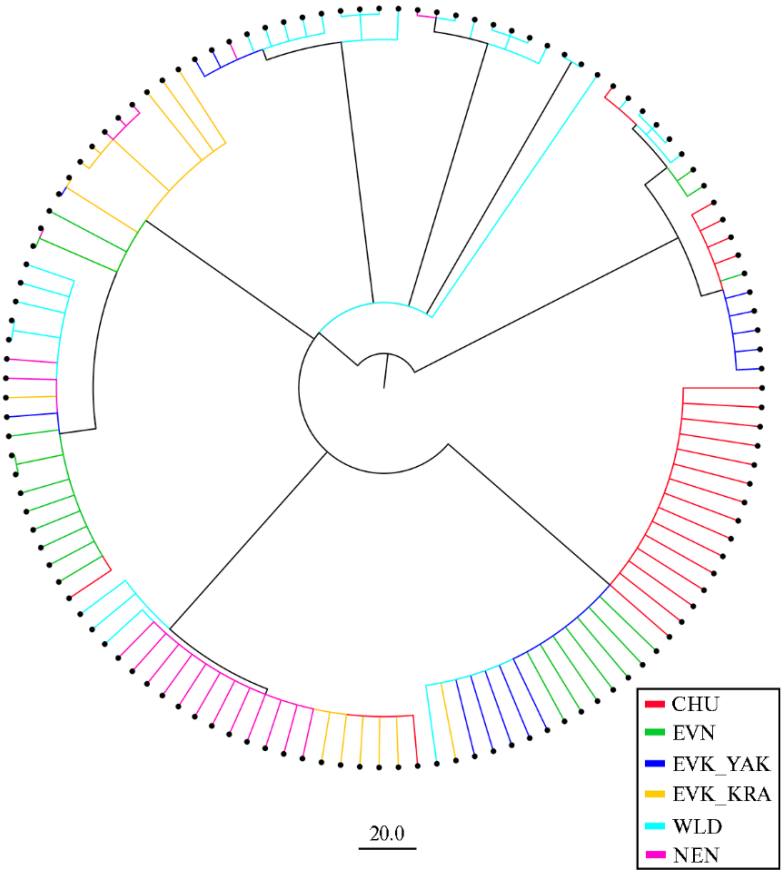

Fig. 2. Bayesian phylogenetic tree for the genetic relationships between domestic and wild reindeer (*Rangifer tarandus* L., 1758) based on the nucleotide sequences of the mitochondrial gene *CytB*: CHU — Chukchi breed (n = 22), EVK_KRA — Evenk Krasnoyarsk breed (n = 12), EVK_YAK — Evenk Yakut breed (n = 14), EVN — Even breed (n = 21), NEN — Nenets breed (n = 21), WLD — wild reindeer (n = 33) (2022).

Analysis of the Bayesian phylogenetic tree (Fig. 2) revealed a clear divergence of the studied groups of deer into two main clusters. One cluster included 17 haplotypes that occurred in CHU, EVN, and EVK_YAK. Individual representatives of these breeds also had haplotypes included in the second cluster. Only domestic reindeer EVK_KRA had haplotypes characteristic only for the second cluster. Wild deer carried haplotypes of both clusters. The Nenets breed was the most distant from CHU individuals, which was confirmed by the highest values of the criterion index F_{ST} = 0.32645 (Table 2, Fig. 3).

2. Pairwise F_{ST} genetic distances based on the nucleotide sequences of the mitochondrial *CytB* gene in populations of domestic and wild reindeer (*Rangifer tarandus* L., 1758) (2022)

Population	CHU	EVN	EVK_YAK	EVK_KRA	NEN	WLD
CHU	0					
EVN	0.16899	0				
EVK_YAK	0.03967	0.13174	0			
EVK_KRA	0.29011	0.08439	0.21856	0		
NEN	0.32645	0.21418	0.27135	0.00824	0	
WLD	0.12380	0.08769	0.05715	0.09139	0.12807	0

N o t e. CHU — Chukchi breed, EVK_KRA — Evenk Krasnoyarsk breed, EVK_YAK — Evenk Yakut breed, EVN — Even breed, NEN — Nenets breed, WLD — wild reindeer.

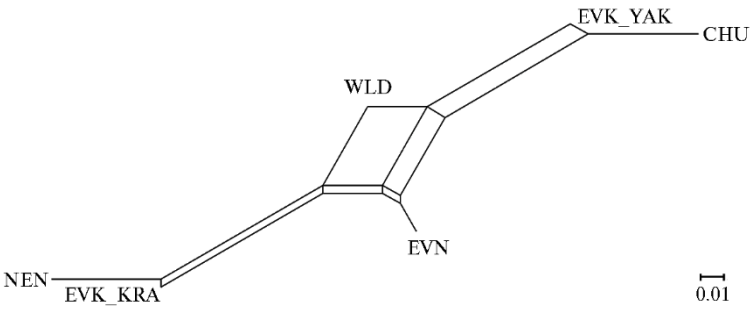

Fig. 3. Genetic relationships between populations of domestic and wild reindeer (*Rangifer tarandus* L., 1758) visualized as a Neighbor Net graph of the F_{ST} genetic distance matrix based on the nucleotide sequences of the mitochondrial *CytB* gene: CHU — Chukchi breed, EVK_KRA — Evenk Krasnoyarsk breed, EVK_YAK — Evenk Yakut breed, EVN — Even breed, NEN — Nenets breed, WLD — wild reindeer (2022).

We determined the closest genetic distances between EVK_KRA and NEN representatives whose fixation index was 0.00824.

We did not reveal a clear breed clustering of the studied groups of domestic deer. Most breed representatives carried similar mtDNA haplotypes, but some individuals had completely distant mitochondrial genotypes. This can be explained by the accidental mating of domestic and wild deer.

Microsatellite analysis. In this work, we used nine microsatellite loci to analyze 332 individuals of domestic and wild reindeer from the Russian Federation.

3. Characterization of genetic variability in populations of domestic and wild reindeer (*Rangifer tarandus* L., 1758) based on polymorphism of 9 microsatellite loci (2022)

Population	*n*	H_O	uH_E	uF_{IS} (95 % CI > 0)	A_R
EVN	33	0,655±0,041	0,746±0,022	0,129 [0,055; 0,203]	6,286±0,402
EVK_YAK	31	0,687±0,027	0,775±0,018	0,11 [0,042; 0,178]	7,014±0,389
EVK_KRA	15	0,576±0,072	0,767±0,031	0,262 [0,085; 0,439]	6,571±0,477
CHU	43	0,566±0,071	0,681±0,051	0,148 [–0,049; 0,345]	6,188±0,719
NEN	91	0,657±0,032	0,766±0,026	0,141 [0,08; 0,202]	7,036±0,441
WLD	119	0,693±0,036	0,841±0,018	0,177 [0,105; 0,249]	8,760±0,565

N o t e. *n* — number of samples, H_O — observed heterozygosity, uH_E — unbiased expected heterozygosity, uF_{IS} — unbiased inbreeding coefficient with 95% confidence interval, A_R — rarified allelic diversity. In parentheses, there are the range of uF_{IS} variability at a 95% confidence interval. CHU — Chukchi breed, EVK_KRA — Evenk Krasnoyarsk breed, EVK_YAK — Evenk Yakut breed, EVN — Even breed, NEN — Nenets breed, WLD — wild reindeer.

Analysis of genetic diversity (Table 3) showed that the population of wild reindeer was characterized by relatively high values of allelic diversity (A_R = 8.76) compared to the populations of domesticated reindeer. This parameter ranged from 6.188 in CHU to 7.036 in NEN. Similarly, the highest rates of observed and unbiased expected heterozygosity (H_O = 0.693; uH_E = 0.841) were in wild populations vs. domestic populations. The Chukchi breed was characterized by the lowest values of these indicators (H_O = 0.566; uH_E = 0.681). EVK_YAK among domestic reindeer was distinguished by the highest genetic diversity (H_O = 0.687; uH_E = 0.775). In all populations, there was heterozygote deficiences, as evidenced by the positive values of the coefficient of inbreeding uH_E, which ranged from 0.11 in EVK_YAK to 0.262 in EVK_KRA. The values of the confidence interval of the inbreeding coefficient in CHU were close to zero (–0.049, 0.345), which indicates the state of genetic balance in this deer population.

The genetic distances between the studied deer populations were estimated in pairs based on the values of the F_{ST} and JostD test (Table 4, Fig. 4). An analysis of the structure of the genetic network made it possible to identify two conditional

clusters (see Fig. 4). The first was represented by the EVN and EVK_YAK populations, which indicates their genetic relationship. This is confirmed by the lowest values of FST and JostD between them (0.045 and 0.089, respectively) (see Table 4). The second cluster was formed by the populations EVK_KRA, NEN, CHU, and WLD. In turn, the CHU population was the most distant from the others, which is explained by the geographical remoteness of its range.

4. Pairwise genetic distances FST and JostD based on polymorphism of 9 microsatellite loci for populations of domestic and wild reindeer (*Rangifer tarandus* L., 1758) (2022)

Population	EVN	EVK_YAK	EVK_KRA	CHU	NEN	WLD
EVN	0	0.089	0.219	0.538	0.198	0.194
EVK_YAK	0.045	0	0.171	0.564	0.130	0.180
EVK_KRA	0.085	0.068	0	0.488	0.141	0.150
CHU	0.212	0.207	0.203	0	0.364	0.408
NEN	0.099	0.068	0.066	0.175	0	0.178
WLD	0.055	0.051	0.039	0.147	0.053	0

N o t e. CHU — Chukchi breed, EVK_KRA — Evenk Krasnoyarsk breed, EVK_YAK — Evenk Yakut breed, EVN — Even breed, NEN — Nenets breed, WLD — wild reindeer.

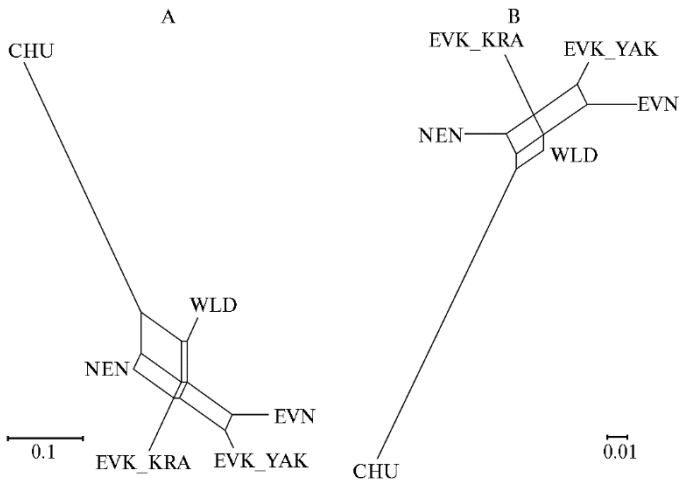

Fig. 4. Genetic relationships between populations of domestic and wild reindeer (*Rangifer tarandus* L., 1758) visualized as a Neighbor Net graph based on the matrix of values of genetic distances JostD (A) **and FST** (B) **for polymorphism for 9 microsatellite loci:** CHU — Chukchi breed, EVK_KRA — Evenk Krasnoyarsk breed, EVK_YAK — Evenk Yakut breed, EVN — Even breed, NEN — Nenets breed, WLD — wild reindeer (2022).

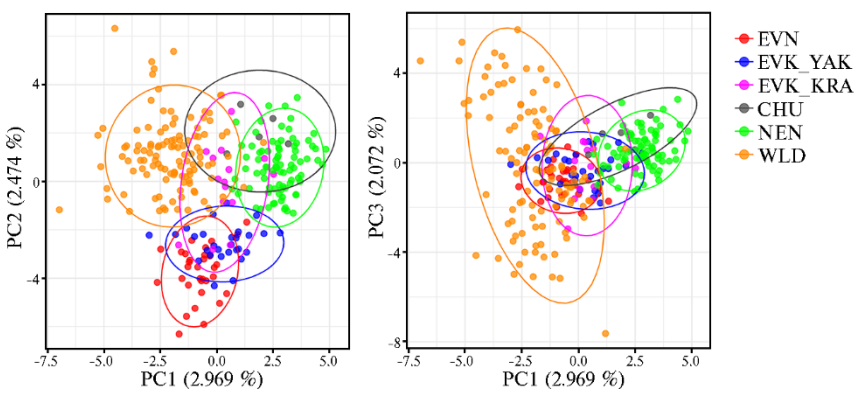

Fig. 5. Principal component analysis of relationships of domestic and wild reindeer (*Rangifer tarandus* L., 1758) based on polymorphism *of* 9 microsatellite loci*:*** CHU — Chukchi breed (n = 22), EVK_KRA — Evenk Krasnoyarsk breed (n = 12), EVK_YAK — Evenk Yakut breed (n = 14), EVN — Even breed (n = 21), NEN — Nenets breed (n = 21), WLD — wild reindeer (n = 33) (2022).

To determine the population structure of the studied groups of deer, we used PCA analysis (Fig. 5) and cluster analysis (Fig. 6).

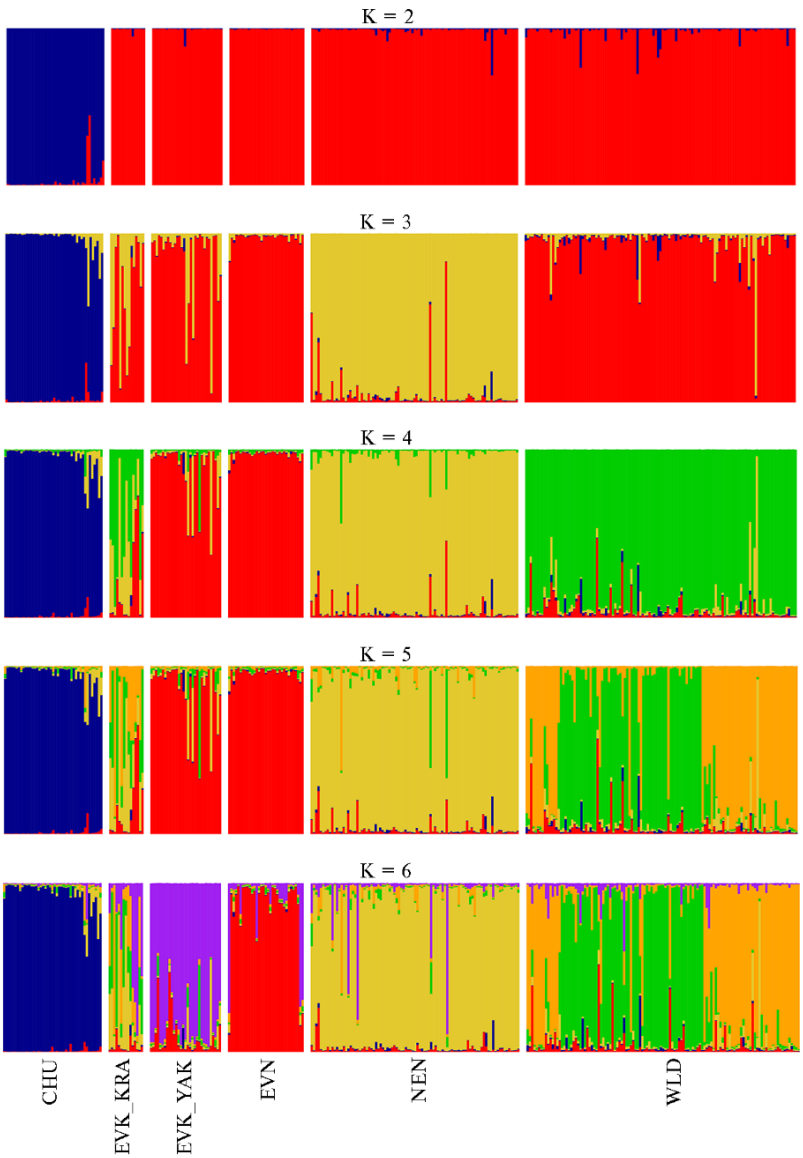

Fig. 6. Cluster analysis of populations of domestic and wild reindeer (*Rangifer tarandus* L., 1758) based on the polymorphism of 9 microsatellite loci (the STRUCTURE 2.3.4 program for a different number of clusters, K = 2, K = 3, K = 4, K = 5, K = 6): CHU — Chukchi breed (n = 22), EVK_KRA — Evenk Krasnoyarsk breed (n = 12), EVK_YAK — Evenk Yakut breed (n = 14), EVN — Even breed (n = 21), NEN — Nenets breed (n = 21), WLD — wild reindeer (n = 33) (2022).

The results of X-ray diffraction analysis demonstrated genetic differentiation between breeds and combined deer into clusters corresponding to similar ones on the phylogenetic tree (see Fig. 4). All studied reindeer populations showed a convergent nature of the genetic composition, forming intersecting clusters. The contribution to the total genetic variability attributable to the first, second, and third principal components was 2.969, 2.474 and 2.072%, respectively. Genetic differentiations for the first two main components and for the first and third main components were similar to each other. PC1 separated CHU and NEN from wild populations. Individuals of EVK_YAK, together with EVN, separated from other

animals by PC2. All studied animals were assigned to the PC3 axis.

Despite the fact that the algorithm based on the value of ΔK revealed the optimal number of clusters for the entire analyzed sample of reindeer, equal to 2 (K = 2, ΔK = 136.79), K = 4, K = 5 and K = 6 also proved to be effective for cluster analysis.

At K = 2, two main genetic pools were identified: the first pool consisted of three breeds, the Even, Evenk, and Nenets, as well as the mebers of the wild population, the second pool was formed only by individuals of the Chukchi breed. At K = 3, we found a clear separation of NEN from other populations. At K = 4, separation of EVK_KRA and wild individuals occurred. At K = 5, there was a separation of wild deer into two main groups with elements of genetic impurities of domestic breeds observed. Also, at K = 5, the WLD population was divided, which can presumably be associated with the large areas of habitat of the selected wild individuals. EVN was separated from EVK_YAK only at K = 6. It should be noted that EVK_KRA demonstrated complete mixing with representatives of all populations except CHU.

For the indigenous peoples of the Arctic North of Russia, the reindeer plays an important biological role, as it is a source of food, clothing and shelter, as well as a means of transportation [1]. In the world, the study of the genetic diversity of reindeer is carried out using a combination of several markers [20-22]. However, to date, the Russian populations of reindeer have been characterized by only one type of marker. In our work, to study domestic and wild reindeer, we used for the first time an integrated approach based on the analysis of the mtDNA *CytB* gene polymorphism and microsatellite loci. Haplotype and nucleotide diversity in the Russian reindeer populations (H_D = 0.519-0.997; π = 0.002-0.006) was comparable to values obtained in previous studies of Russian and Norwegian deer (H_D = 0.570-0.978; π = 0.002-0.019) [1], Aolugui populations from China (H_D = 0.468; π = 0.0017) (22), as well as Canadian reindeer (H_D = 0.890; $\underline{\pi}$ = 0.0022) [1], which indicates the adequacy of our alculating methodology.

The obtained values of observed heterozygosity (uH_E = 0.681-0.841) were close to the data obtained in other Russian populations of reindeer, e.g., H_E = 0.670 [14], H_E = 0.62-0.73 [16], H_E = 0.699 [15], H_E = 0.6491-0.7608 [13] and in deer populations from China, e.g., H_E = 0.650 [22]. Allelic diversity A^R = 6.188-8.760 was also coparable with the results of other researchers, e.g., A_R = 5.730-7.070 [14], A_R = 3.700-7.400 [21]. An analysis of the structure of the genetic network showed the differentiation of the Chukchi deer from the rest populations, which was demonstrated by V.R. Kharzinova et al. [17] who studied the genetic diversity of reindeer using single nucleotide sequence analysis. In the studies of other researchers, as well as in the present work, wild deer populations were characterized by a higher genetic diversity compared to domestic ones. The revealed pattern is most likely due to two factors, the breeding of domestic reindeer and migrations of the wild population which ensure a more intensive exchange of genetic material.

Thus, a combination of several markers revealed high genetic diversity in four breeds of domestic reindeer and the wild tundra reindeer population. In terms of genetic diversity, the sample of tundra wild reindeer exceeded the domestic population represented by individuals of the Nenets, Chukchi, Even breeds, as well as the Krasnoyarsk and Yakut populations of the Evenki breed. The phylogenetic analysis of the mitochondrial *CytB* gene nucleotide sequences did not reveal an isolated genetic structure among the reindeer populations. However, there is a clear divergence of the studied deer groups into two main clusters, which indicates the common origin of animals of the maternal line within one cluster. All statistical approaches that we used in the analysis of the genetic structure of the studied

reindeer by microsatellites (principal component analysis, phylogenetic and cluster analysis) revealed a clear genetic differentiation of domestic and wild reindeer. The results obtained are important both for improving reindeer selection and breeding, and as the basis for recommendations on nature management and protection of wild reindeer as the most important commercial resource traditional for the indigenous peoples of the Far North.

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EFFECT OF A COMPLEX FEED ADDITIVE ON THE COMPOSITION AND FUNCTION OF THE *Oryctolagus dominis* CAECUM MICROBIOME

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Abstract

There is a growing interest in the study of natural multicomponent feed additives to regulate gut microbiome composition and improve immune and physiological status of rabbits. In the present work, for the first time, it was bioinformatically found that a complex probiotic biological product affects the change in the predicted metabolic pathways of the rabbit intestinal microbiome. The aim of the work was to study the joint action of a complex containing minerals and a probiotic on physiological status, composition and functional potential of gut microbiome in rabbits. For the study (the vivarium of the FGBU VO SPKhFU of the Ministry of Health of Russia, St. Petersburg, 2021), ten Soviet chinchilla rabbits of 2.5 months of age (5.37-5.53 kg bw) were allocated to two groups of five rabbits each. Control group I received the recommended basal diet (BD, RAAS norms 2003), test group II was fed with the BD supplemented with a complex feed additive (30 mg per animal day^{-1}) consisting of the microelement preparation Silaccess at 5 mg/kg of bodyweigh (LLC TECHNOLOG 2D, Russia) and the probiotic strain *Bacillus subtilis* 1-85. On days 30 and 60, the animals were weighed before morning feeding, and blood was sampled to evaluate natural resistance parameters (bactericidal function, including lysozyme activity, and phagocytic activity of neutrophils). Chyme samples of the caecum for microbiome studies aseptically collected at the end of the experiment were immediately placed in sterile plastic tubes. Total DNA was isolated using the Genomic DNA Purification Kit (Thermo Fisher Scientific, Inc., USA). The bacterial community was assessed by NGS sequencing on a MiSeq automated sequencer (Illumina, Inc., USA) using primers to the V3-V4 region of the 16S rRNA gene which allows us to identify microorganisms to the species level: the forward primer 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′ and the reverse primer, 5′-GTCTCCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′. The reconstruction and prediction of the functional content of the metagenome was performed using the PICRUSt2 (v.2.3.0) software package (https://github.com/picrust/picrust2). Mathematical and statistical processing was carried out by the multivariate analysis of variance procedure using Microsoft Excel XP/2003, R-Studio (Version 1.1.453) (https://rstudio.com). In group II compared to control, the phagocytic index was higher ($p \le 0.05$) by 1.8 %, the phagocytic number by 32.3 % ($p \le 0.05$). NGS sequencing revealed the values of the Chao1, Shannon and Simpson biodiversity indices to be higher ($p \le 0.05$) in group II compared to group I. Taxonomic analysis of caecum microbial community disclosed 12 phyla of the kingdom *Bacteria* among which representatives of the phylum *Firmicutes* dominated (80.2±6.2 % in group I, 78.2±7.4 % in group II). In group II, there was

a 1.3-2.6-fold increase in the abundance of phyla *Verrucomicrobiota*, *Actinobacteriota*, *Patescibacteria*, *Proteobacteria*, *Desulfobacterota* and a 4.8-fold decrease in the abundance of the phylum *Campylobacterota* ($p \leq 0.05$). In the caecum of test rabbits, the genus *Bacillus* spp. increased 2.82 times compared to control ($p \leq 0.05$). *Staphylococcus sciuri* was found in group I (0.075±0.006 %) but not in group II. Data processing using the PICRUSt2 software tool (v.2.3.0) revealed 370 predictable metabolic pathways in the rabbit gut microbial community, 36 of which differed ($p \leq 0.05$) between the groups. In group II, the intestinal microbiome pathways related to the degradation of aromatic compounds and xenobiotics, protein, carbohydrate, and energy metabolism, alcohol biosynthesis, photorespiration, assimilation of formaldehyde, degradation of myo-, chiro- and scillo-inositol, cell wall synthesis and spore formation activated compared to group I ($p \leq 0.05$). The dominant proportion (15 pathways) of enhanced potential metabolic pathways was associated with the degradation of aromatic compounds and xenobiotics. Thus, a complex dietary additive based on the probiotic strain of *Bacillus subtilis* 1-85 and microelements has a multiple positive effect both on gut microorganisms (fewer pathogens, metabolic regulation) and on the macroorganism (higher values of immunity parameters, a better growth performance of Soviet chinchilla rabbits).

Keywords: probiotic, trace elements, resistance, domestic rabbits, microbiome, NGS, metabolic pathways

Rabbits (*Oryctolagus dominis*) are farm animals with a short pregnancy period, high fecundity, and good feed conversion [1, 2]. Rabbit meat is high in protein, low in fat, cholesterol, and sodium, and is easily digestible, which makes it a dietary choice [3]. In addition, rabbits are often used for experimental purposes as model animals.

In many countries, rabbit farming is becoming an important growing subsector [4]. The main factors hindering the development of rabbit breeding are viral and bacterial diseases, leading to mass mortality and significant economic losses [5].

The microbial populations inhabiting the gastrointestinal tract of animals form the microbiome, a complex ecosystem capable of autoregulating its homeostasis under favorable conditions. The mammalian gut microbiome plays an important role in digestive, metabolic, physiological and immunological processes, affects host susceptibility to many immune-mediated diseases and disorders (6), and affects productivity [7, 8]. This was also confirmed by the example of rabbits, whose economically valuable traits are also affected by the intestinal microbiota [8].

Due to the rapid development of sequencing technologies, several interesting studies have been carried out to analyze the microbial communities of the rabbit gut. In 2008 and 2012, the bacterial community of the caecum of rabbits was studied using high-throughput sequencing of the V3-V4 amplicon of the 16S rRNA gene [9, 10]. In 2018, changes in the microbiota of the rabbit by intestinal tract were described, with a focus on the microbiota of the caecum and faeces [11]. In 2019, the composition of the microbiota of the gallbladder of rabbits of different ages was studied [12], in 2020, the structure of the intestinal microbiota in commercial rabbits was studied in dynamics from weaning to the end of rearing [13]. More recently, the composition of the bacterial microbiota throughout the gastrointestinal tract in New Zealand rabbits has been characterized [5]. Despite the existence of microbial populations in the proximal and distal sections of the rabbit gastrointestinal tract [14], the caecum is the main organ where the most active enzymatic processes occur.

In modern conditions of rabbit breeding, factors such as overcrowding of a significant number of animals in a limited area, violation of the principles of the feeding system and keeping technology, changing diets, poor quality feed, uncontrolled use of antibiotics and other stresses, significantly increase the risk of infectious diseases. The weaning and rearing periods, when there is a transition from mother's milk to solid foods, stress from mother absence and regrouping, are critical [15]. This leads to enteritis and gastrointestinal infections, in particular those caused by the bacteria *Clostridia* spp. and *Escherichia coli*, *Lawsonia intracellulari*s, *Salmonella* spp. [5, 16]. One of the most dangerous disease of domestic rabbits is

epizootic enteropathy. It is a multifactorial gastrointestinal syndrome with a 30-95% mortality rate regardless of breed, with an increasing incidence in the post-weaning period [17).

Intestinal diseases often manifest as inflammation of the digestive system [18], which leads to a violation of the wall integrity [19]. Inflammation and damage to the intestines cause a redistribution of nutrients, which leads to a decrease in animal productivity and a significant increase in economic losses [20]. Studies using chromatographic methods have shown that a number of differential metabolites are involved in five metabolic pathways associated with intestinal inflammation (tryptophan metabolism, pyrimidine metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, lysine degradation, and bile secretion) [21]. In turn, an increase in the number of pathogenic and opportunistic bacteria (*Escherichia coli*, *Clostridium* spp., *Bacteroides* spp.) and a decrease in the presence of beneficial bacteria (*Lactobacillus casei*, *Bifidobacterium* spp. and *Lactobacillus* spp.) is associated with the release of pro-inflammatory signaling factors (cytokines, IL-6 and TNF-α), as well as increased secretion of immunoglobulin A (IgA). This process has been associated with decreased short-chain fatty acids, inhibition of intestinal ion and water absorption, and inflammation of the intestinal mucosa [22-24].

Strategies for regulating the microbiome and preventing digestive disorders include introducing feed additives such as probiotics into diets. During the last decade, several studies have been published on the effect of probiotics on the performance of rabbits [25-27]. Some authors have considered specific and non-specific immune responses to probiotic dietary supplements. Various hematological parameters were analyzed: total protein, immunoglobulins, leukocytes and lymphocytes [28, 29]. It has been established that probiotics have a positive effect on the composition of the microbiome, reducing the number of pathogens [30, 31].

Previously, in a study [32] based on bioinformatics processing of data from NGS sequencing of the 16S rRNA gene in the microbiome of the rumen of dairy cows we revealed that changes in the taxonomic structure of rumen microorganisms under the influence of the Cellobacterin+ probiotic were associated with metabolic shifts in the functional potential of microorganisms. In addition, our findings confirmed the role of this probiotic in maintaining metabolic homeostasis. In 2021, a genotype-dependent alteration of potential metabolic pathways of the gut microbiome based on 16S rRNA gene sequencing was demonstrated in rabbits, revealing potential biomarkers important for improving meat rabbit breeds [33].

However, experiments related to the evaluation of the effect of probiotics on the composition and potential metabolic pathways of the intestinal microbiome of rabbits have not been previously performed. That is, the mechanism by which microorganisms and probiotics interact at a metabolic level in the gut of these animals is unclear.

Dietary trace elements have a positive effect on various functions in rabbits, e.g., acid-base balance, nutrient metabolism and immunity. Iron deficiency in rabbits reduces animal activity, leads to loss of appetite, and deterioration of the skin and coat [34). Iodine is necessary for the proper functioning of the thyroid gland, while cobalt is directly related to the absorption of vitamins in fur-bearing animals [35, 36].

It is of interest to create and study the effectiveness of new complex feed additives that will make it possible to obtain environmentally friendly rabbit products of higher quality and reduce the risk of infectious diseases.

Here, we revelaled, based on bioinformatic methods, that a complex probiotic biological product affects the change in the predicted metabolic pathways of the rabbit intestinal microbiome.

This work aimed to investigate how a dietary complex containing minerals

and a probiotic affects physiological parameters of rabbits, gut microbiome composition and its functional potential.

Materials and methods. Experimetns were carried out in 2021 in the vivarium of the St. Petersburg Chemical and Pharmaceutical University (veterinary state registration certificate for the vivarium No. 78-0713/2). Feeding and keeping conditions corresponded to the guidelines for rabbits [37].

Ten Soviet chinchilla rabbits (2.5 months of age, 5.37-5.53 kg body weight) were divided into two groups (5 rabbits each). Animals of control group I received the basal diet (BD) as per recommended detailed norms of the Russian Academy of Agricultural Sciences (2003), experimental group II received BD with the addition of a complex feed additive (30 mg/animal daily). The complex feed additive contained the microelement preparation Silaccess (OOO TECHNOLOG 2D, Russia) [38], consisting of a mixture of mineral components of silicon (35-42.7%), iron (3.5-4.5%), copper (0 08-0.12%) and zinc (0.04-0.055%) in a stabilizing agent (GOST 12.1.007.-76. Moscow, 2007). The dosage of the Silaccess component was 5 mg/kg body weight. In addition, the supplement included the water-soluble probiotic Likvipro (OOO BIOTROF, Russia) at a ratio of 0.5 g/10 l of water. The introduction of the probiotic component was carried out around the clock into the drinking system using a Dosatron D25RE5 dispenser (Dosatron, France). Liquipro is based on the *Bacillus subtili*s 1-85 strain, the drug is produced in dry form in the form of a powder. The duration of the experiment was 75 days.

The growth rate of the animals was controlled by individual weighing on an empty stomach before morning feeding in 30 and 60 days (at 3.5 and 4.5 months of age) after the start of the experiment. The live weight of experimental rabbits was determined using an electronic balance for weighing animals Momert 6551 (MOMERT Co Ltd., Hungary) with an error of up to 10 g, the average daily gains for the noted periods were calculated using the generally accepted formula:

$$A = \frac{W1-W0}{t},$$

where A is the average daily gain in live weight, g; W_0 - live weight at the beginning of the experiment, kg; W_1 - live weight at the end of the experiment, kg; t is the time period, days.

The clinical and physiological state of the rabbits was assessed during daily examination. Attention was paid to the behavior, palatability of food, the condition of the coat.

Thirty days after the start of the experiment and at the end of the experiment (60 days), blood was taken from the rabbits on an empty stomach from the tail vein into two types of vacuum tubes (with the anticoagulant heparin and with a coagulation activator). Determined indicators of natural resistance (bactericidal activity, including lysozyme, phagocytic activity of neutrophils). The phagocytic activity of pseudoeosinoyls was assessed by counting phagocytic cells from 100 neutrophils. When determining the bactericidal activity of blood serum, the method of I.M. Karput [39]. Lysozyme activity in blood serum was determined by the nephelometric method according to V.G. Dorofeichuk [40]. Clinical (hematological) blood analysis was performed using an ABXMICRO 60-OT18 apparatus (Roche, France).

Chyme samples for microbiome studies were manually collected from the caeca at the end of the experiment under aseptic conditions as possible and immediately placed in sterile plastic tubes. All samples were frozen at –20 °C and sent in dry ice to the molecular genetic laboratory of the OOO BIOTROF company for DNA extraction.

Total DNA was isolated from the samples using the Genomic DNA Purification Kit (Thermo Fisher Scientific, Inc., USA) according to the attached

instructions. The method is based on selective detergent-mediated precipitation of DNA from a substrate using solutions for cell wall lysis and DNA precipitation, 1.2 M sodium chloride, and chloroform.

The bacterial community was assessed by NGS sequencing, which makes it possible to identify microorganisms to the species level, on a MiSeq automatic sequencer (Illumina, Inc., USA) using primers for the V3-V4 region of the 16S rRNA gene: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' (forward primer), 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3' (reverse primer). PCR conditions were as follows: 3 min at 95 °C; 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C (elongation) (25 cycles); 5 min at 72 °C (final elongation). Sequencing was performed using Nextera® XT IndexKit library preparation reagents (Illumina, Inc., USA), Agencourt AMPure XP purification PCR products (Beckman Coulter, Inc., USA), and MiSeq® ReagentKit v2 sequencing reagents. (500 cycle) (Illumina, Inc., USA). The maximum length of the resulting sequences was 2×250 bp.

Automatic bioinformatic analysis was performed using QIIME2 ver. 2020.8 (https://docs.qiime2.org/2020.8/). After importing sequences in the .fastq format from the sequencing instrument and creating the necessary mapping files containing the metadata of the studied files, paired read lines were aligned. Next, the sequences were filtered for quality using the default settings. Noise sequences were filtered using the DADA2 package built into QIIME2, which includes information about the quality of sequences in the error model (filtering of chimeric sequences, artifacts, adapters), which makes the algorithm resistant to a sequence of lower quality. In this case, the maximum length of the pruning sequence was used, equal to 250 bp (https://benjjneb.git-hub.io/dada2/tutorial.html). To construct de novo phylogeny, multiple sequence alignments were performed using the MAFFT software package, followed by a masked alignment to remove positions that differed significantly. For taxonomy assignment, the QIIME2 software was used, which assigns sequences a taxonomic identification based on ASV data (using BLAST, RDP, RTAX, mothur and uclust methods) using the Silva 138.1 16s rRNA database (https://www.arb-silva.de /documentation/release-138.1/).

Based on the obtained table of operational taxonomic units (OTU; operational taxonomic unit, OTU), using plugins of the QIIME2 package, biodiversity indices were calculated, and a graph of the dependence of the number of OTUs on the number of reads was plotted. In the statistical analysis of diversity indices, their additional transformation was not carried out.

The reconstruction and prediction of the functional content of the metagenome, gene families, and enzymes was carried out using the PICRUSt2 (v.2.3.0) software package (https://github.com/picrust/picrust2) [41]. We worked with the program according to the recommended scenario of actions, all settings were used by default. The OTUs of each sample were arranged according to their content, from largest to smallest, and the values were converted using the logarithmic transformation of Log2. The MetaCyc database (https://metacyc.org/) was used to analyze metabolic pathways and enzymes. The predicted profiles of MetaCyc metabolic pathways were assessed by the abundance of ASV (Amplicon Sequence Variants) [42]. Data visualization and calculation of statistical indicators were performed using the Phantasus v1.11.0 web application (https://artyomovlab.wustl.edu/phantasus/), which, in addition to the main visualization and filtering methods, supports R-based methods such as like k-means clustering, principal component analysis, or differential expression analysis with the limma package.

Mathematical and statistical processing of the obtained results was carried out by the method of multifactor analysis of variance (multifactor ANalysis Of VAriance, ANOVA) in Microsoft Excel XP/2003, R-Studio (Version 1.1.453)

(https://rstudio.com). Data are presented as means (*M*) and standard errors of means (±SEM). Significance of differences was assessed by Student's *t*-test, differences were considered statistically significant at $p \leq 0.05$. Means were compared using Tukey's Significantly Significant Difference (HSD) test and the TukeyHSD function in the R Stats Package.

Results. Clinical blood test parameters are extremely sensitive to physiological influences, and therefore the blood profile is a fairly accurate reflection of such influences [43-45]. In animals of the control and experimental groups, the content of hemoglobin and erythrocytes corresponded to age norms, only some minor deviations were noted: the number of erythrocytes and hemoglobin in the II experimental group after 60 days of the experiment (Table 1). In addition, the average content of hemoglobin in erythrocytes in both groups exceeded the norm. In both groups, age-related changes in blood parameters were observed at different stages of development of the rabbit organism. Thus, in 60 days, the amount of hemoglobin in test group II increased by 8.2% ($p \leq 0.05$) compared to the beginning of the experiment, in control group I by 4.45% ($p \leq 0.05$).There were no statistically significant differences in these indicators between the control and experimental groups ($p > 0.05$) (see Table 1).

1. Blood clinical analysis in Soviet chinchilla rabbits (*Oryctolagus dominis*) fed a complex feed additive based on the probiotic *Bacillus subtilis* 1-85 strain and trace elements ($n = 5$, M±SEM; the vivarium expeeriment, St. Petersburg, 2021)

Parameter	Days of experiment	Group I (control)	Group II	Norm
Erythrocytes, $\times 10^{12}$/l	1	7.01±0.43	7.24±0.31	5.2-7.8
	30	7.24±0.59	7.42±0.77	
	60	7.28±0.33	8.20±0.93	
Hemoglobin, mmol/l	1	143.80±8.80	152.20±4.09	100.5-160.0
	30	143.80±4.30	152.00±7.31	
	60	150.20±6.02	164.60±9.24	
The average content of hemoglobin in erythrocytes, pg/l	1	19.72±1.80	21.06±0.71	9.3-15.3
	30	20.70±0.79	21.32±0.73	
	60	20.44±0.58	22.14±2.34	

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

Regarding the fact that at an early age of rabbits their gastrointestinal tract is not sufficiently prepared for the digestion of solid feed, and the body during the growing period is exposed to stress factors that affect the immune system, we studied the indicators of natural resistance in experimental animals.

Based on the indicators that indirectly reflect the levels of innate immune mediators in the blood (lysozyme content, bactericidal characteristics of blood, phagocytic activity, phagocytic index, phagocytic number), natural resistance in rabbits treated with a complex feed additive was higher than control ($p \leq 0, 05$) (Table 2). Thus, the phagocytic index (the average number of phagocytosed microorganisms per one active leukocyte), reflecting the intensity of phagocytosis, was higher in group II compared to group I by 1.8 ($p \leq 0.05$), the phagocytic number (the ratio of the number of phagocytized leukocyte bacteria to the total number of counted leukocytes) was higher by 32.3% ($p \leq 0.05$).

An increase in natural resistance in rabbits treated with a complex feed additive is natural. It has long been known that blood contains antimicrobial components that provide rapid responses to infection [46]. Innate responses to microbial infections in mammals are mediated by signaling molecules, including Toll-like receptors, cytosolic kinases, nuclear factor (NF)-kB, and transcription factors [47]. Pathogen entry induces rapid expression of several genes encoding antimicrobial proteins and peptides [48, 49]. In turn, there are molecular mechanisms that provide a cross-relationship between the microbiome and the expression of host genes, primarily immunity genes [50]. The introduction of a probiotic strain

of bacteria in combination with trace elements into the intestines of rabbits could affect the expression of host genes associated with immunity. An increase in the content of total protein and globulins in the blood serum of rabbits under the influence of a probiotic has been previously demonstrated [28]. Data obtained by A.F. Mohamed et al. [51] indicate that the introduction of a probiotic strain of Lactobacillus acidophilus into the diet did not have a significant effect on the globulin content, but led to an increase in the number of leukocytes and the amount of total protein in the blood serum. It has been suggested that micronutrients play an important role in various physiological processes and are critical for the proper functioning of the immune system [52].

2. Natural resistance parameters in Soviet chinchilla rabbits (*Oryctolagus dominis*) fed a complex feed additive based on the probiotic *Bacillus subtilis* 1-85 strain and trace elements (day 60, $n = 5$, $M \pm$SEM; the vivarium expeeriment, St. Petersburg, 2021)

Parameter	Group I (control)	Group II
Lysozyme, %	36.18±1.04	46.29±1.36*
Bactericidal activity of blood serum, %	38.45±1.09	46.68±1.27*
Phagocytic activity, %	43.45±1.19	63.27±1.33*
Phagocytic index	5.33±0.10	7.13±0.15**
Phagocytic number	2.88±0.10	3.81±0.11*
Phagocytic capacity, thousand microbial bodies	73.12±1.53	75.89±1.21

N o t e. For a description of the groups, see the Materials and methods section.
* and ** Differences between the test and control groups based on Student's *t*-test are statistically significant at $p \le 0.05$ and $p \le 0.01$, respectively.

3. Daily weigh gain in Soviet chinchilla rabbits (*Oryctolagus dominis*) fed a complex feed additive based on the probiotic *Bacillus subtilis* 1-85 strain and trace elements ($n = 5$, $M \pm$SEM; the vivarium expeeriment, St. Petersburg, 2021)

Group	Days of experiment	Daily weigh gain, g
I (control)	1-30	5.53±0.32
	31-60	5.21±0.15
II	1-30	6.08±0.49
	31-60	5.90±0.21

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

Weighing data allow us to assume that the studied complex feed additive based on microelements and probiotics does not have a negative effect on bodyweight gain in rabbits (Table 3). However, it should be noted that the number of animals in the experimental groups did not provide a sufficient level of evidence, since the groups were formed according to the main goal of our study - to analyze the composition and functions of the microbiome.

Previously, the positive effect of probiotics and trace elements on the performance of rabbits was noted. A.A.A. Abdel-Wareth et al. [25] studied a complex preparation from a mixture of fenugreek seeds and a probiotic (AmPhi-Bact, American Pharmaceutical Innovations Company, LLC, USA) containing cultures of *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Bifidobacterium bifidum*, metabolites of *Bacillus subtilis* and *Aspergillus niger*, on 45-day-old New Zealand white rabbits for 6 weeks. The authors noted an improvement in feed conversion, a higher digestibility of crude protein and an increase in meat productivity in the groups treated with the drug. M. Lopez-Alonso et al. [53] opined that trace elements coordinate many biological processes and are therefore essential for the maintenance of animal productivity.

At the next stage, we studied the composition of the chyme microbiome from rabbit cecum by NGS sequencing. A total of 57.56 sequenced 16S rRNA gene sequences were generated (with median reads of 9.59 at min = 7.69 and max = 12.86). When comparing the control and experimental groups for the Chao1, Shannon and Simpson indices, it turned out that the values had statistically

significant differences ($p \leq 0.05$) (Fig. 1). In the trest group, the α-biodiversity indices were higher. That is, a complex feed additive, which included a probiotic strain and a mixture of trace elements, had a positive effect on increasing the species diversity of the microbiome in the intestines of rabbits. These results are consistent with other reports [54, 55].

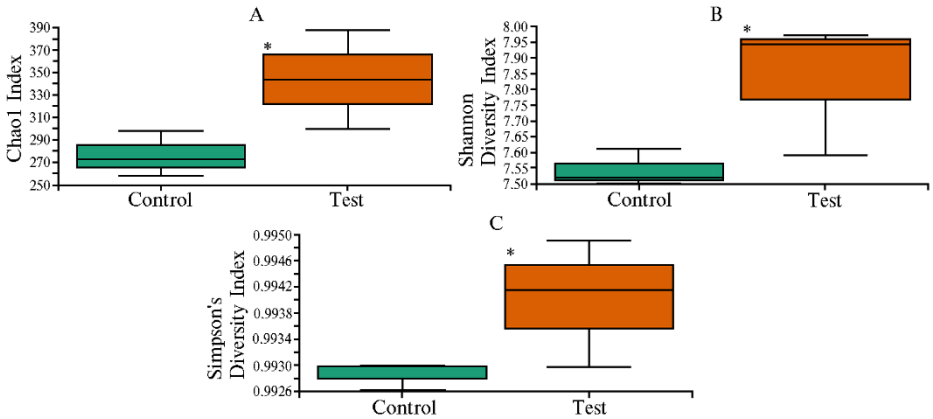

Fig. 1. Absolute values of α-biodiversity indices Chao1 (A)**, Shannon** (B)**, and Simpson** (C) **divericity indexes for the caecal microbiome of Soviet chinchilla rabbits (*Oryctolagus dominis*) fed a complex feed additive based on the probiotic *Bacillus subtilis* 1-85 strain and trace elements** (n = 5, M±SEM; the vivarium expeeriment, St. Petersburg, 2021). Calculated using Qiime2 ver. 2020.11. For a description of the groups, see the Materials and methods section.
* Differences between the test and control groups based on Student's t-test are statistically significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.

When studying the composition of microorganisms of the blind processes of the intestines of rabbits, 12 phyla of the kingdom Bacteria were identified (Fig. 2), among which representatives of the phylum *Firmicutes* dominated in number (80.2±6.2% in group, I.78.2±7.4% in group II), which indicates their ecological and functional importance of the phylum in the digestive tract. The main function of *Firmicutes* is the ability to degrade complex polysaccharides with subsequent formation of short-chain fatty acids [56]. Based on previous studies, the dominance of representatives of this phylum in the contents of the blind processes is quite typical for rabbits [11].

In our experiment, the phylum *Bacteroidetes* was the second most common in the intestines of rabbits (13.3±1.2% in group I, 12.3±1.8% in group II). Bacteroidetes have previously been shown [9, 57] to stimulate the development of gut-associated immune tissue in the digestive system. Results similar to ours were also obtained in wild rabbits, as well as in domestic Rex rabbits [58, 59]. On the whole, the quantitative representation of phyla in the intestines of rabbits corresponds to that of other monogastric herbivores [60, 61].

In the experimental group, compared with the control group, there was an increase in abundance of the phyla *Verrucomicrobiota*, *Actinobacteriota*, *Patescibacteria*, *Proteobacteria*, *Desulfobacterota* by 1.3-2.6 times and a decrease in the representation of the phylum *Campylobacterota* by 4.8 times ($p \leq 0.05$). In all likelihood, these data indicate a positive effect of the complex feed additive on the composition of the microbiome. For example, members of the phylum *Verrucomicrobiota*, such as *Akkermansia muciniphila*, have probiotic activity by modulating gut mucus thickness and enhancing intestinal barrier integrity [62]. Bacteria of the phylum *Actinobacteriota* produce antimicrobial substances active against pathogens [63]. Among the bacteria of the phylum *Campylobacterota*, which was represented by the only genus *Campylobacter* (Fig. 3), there are pathogenic forms associated

with rabbit proliferative enteropathy [64].

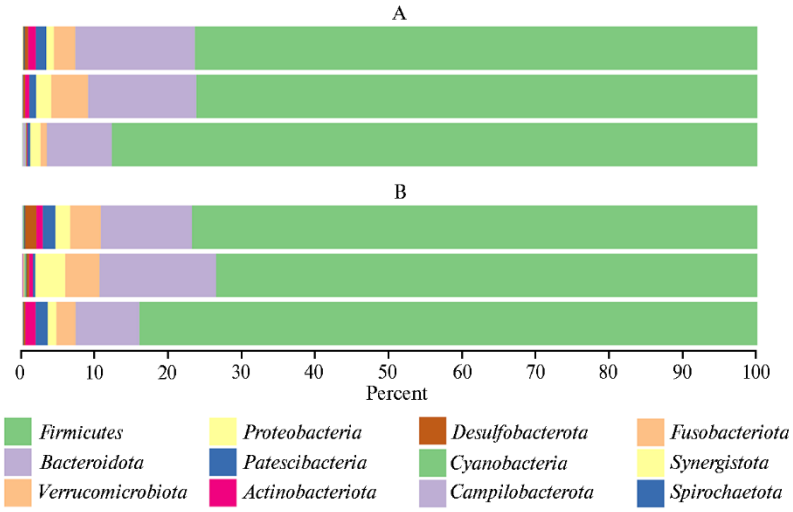

Fig. 2. The composition of caecal microbiome (at the bacterial phylum level) of Soviet chinchilla rabbits (*Oryctolagus dominis*) fed a complex feed additive based on the probiotic *Bacillus subtilis* 1-85 strain and trace elements (day 60, Next-Generation Sequencing of 16S rRNA gene amplicons; $n = 5$, $M \pm$SEM; the vivarium expeeriment, St. Petersburg, 2021). For a description of the groups, see the Materials and methods section.

In the caecum of rabbits from group II, an increase in the number of bacteria of the genus *Bacillus* spp. by 2.82 times compared to the control ($p \leq 0.05$) (see Fig. 3), which may indicate the survival and increase in the number of probiotic microorganism introduced as part of a complex feed additive. This is an important finding, since introduced strains of microorganisms may have different ability to survive in the aggressive environment of the host intestine [65]. Previously, RAPD-PCR proved the survival of probiotic bacteria *Bacillus clausii* in the gastrointestinal tract for 12 days [66].

It is important to note that in the caecum of the intestines of rabbits, we have identified a significant number of genera, including uncultivated ones, belonging to the cellulolytic bacteria of the families *Thermoanaerobacteraceae*, *Ruminococcaceae*, *Clostridiaceae*, *Eubacteriaceae*, *Lachnospiraceae*, which is consistent with the data obtained by C. Вдuerl et al. [67]. Cellulosolytic bacteria are the most relevant microorganisms of the phylum *Firmicutes*, contributing to the breakdown of fiber in plant foods. S. Combes et al. [68] found that bacteria of the genus *Ruminococcus* dominate in healthy rabbits, and when a disease occurs, the number of these microorganisms decreases. E. Cotozzolo et al. [5] also showed that the abundance of members of the families *Ruminococcaceae* and *Lachnospiraceae* serve as important indicators of intestinal health in rabbits. According to the authors, a higher abundance of Lachnospiraceae is characteristic of healthy animals, which is associated with the stimulation of caecotrophic behavior. Cecotrophy is an important feature of the order *Lagomorpha*, which makes it possible to increase the digestibility of low-nutrient plant feeds [69].

The fact is that in the process of digestion of rabbits, two types of feces are formed: solid, poor in nutrients, and soft, consisting of protein, vitamins, inorganic salts and containing a significant number of microorganisms. The latter are called cecotrophs and are eaten by *Lagomorpha* in the process of cecotrophy. Therefore, there is an assumption that cecotrophy is a phenomenon that ensures the formation of the correct composition of the intestinal microbiome. However, in our study, no significant difference was found in the content of genera of

cellulolytic microorganisms between the control and experimental groups ($p > 0.05$).

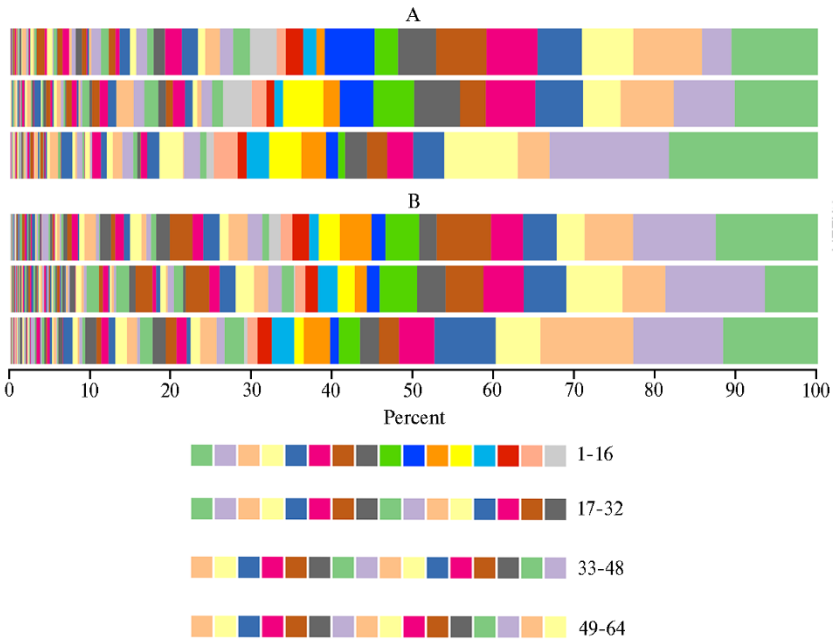

Fig. 3. The composition of caecal microbiome (at the bacterial genera levels) of Soviet chinchilla rabbits (*Oryctolagus dominis*) fed a complex feed additive based on the probiotic *Bacillus subtilis* 1-85 strain and trace elements: 1 — f. *Lachnospiraceae* g. unclassified, 2 — g. *Clostridia* UCG-014, 3 — g. *Oscillospiraceae* NK4A214, 4 — g. *Ruminococcus*, 5 — g. *Monoglobus*, 6 — g. *Oscillospirales* UCG-010, 7 — g. *Clostridia* vadinBB60, 8 — g. *Bacteroides*, 9 — g. *Akkermansia*, 10 — g. *Rikenellaceae* dgA-11 gut, 11 — f. *Bacilli* RF39 g. unclassified, 12 — g. *Lachnospiraceae* NK4A136, 13 — g. *Christensenellaceae* R-7, 14 — g. *Eubacterium coprostanoligenes*, 15 — g. *Oscillospiraceae* V9D2013, 16 — f. *Lachnospiraceae* g. unclassified, 17 — g. *Ruminococcaceae* Incertae Sedis, 18 — f. *Oscillospiraceae* g. unclassified, 19 — f. *Eubacteriaceae* g. unclassified, 20 — g. *Eubacterium siraeum*, 21 — f. *Barnesiellaceae* g. unclassified, 22 — g. *Alistipes*, 23 — g. *Rikenellaceae* RC9 gut, 24 — g. Candidatus *Saccharimonas*, 25 — g. *Subdoligranulum*, 26 — g. *Oscillospiraceae* UCG-005, 27 — g. *Tyzzerella*, 28 — g. *Campylobacter*, 29 — f. *Ruminococcaceae* g. unclassified, 30 — g. *Ruminiclostridium*, 31 — g. *Vibrionimonas*, 32 — g. *Muribaculaceae*, 33 — g. *Desulfovibrio*, 34 — g. *Colidextribacter*, 35 — f. *Anaerovoracaceae* g. unclassified, 36 — g. *Pelomonas*, 37 — g. *Papillibacter*, 38 — f. *Atopobiaceae* g. unclassified, 39 — g. *Staphylococcus*, 40 — f. *Acidaminococcaceae* g. unclassified, 41 — f. *Peptococcaceae* g. unclassified, 42 — g. *Pseudomonas*, 43 — g. *Eubacterium nodatum*, 44 — g. *Anaerovoracaceae* XIII AD3011, 45 — f. *Sutterellaceae* g. unclassified, 46 — g. *Stenotrophomonas*, 47 — g. *Oscillospiraceae* UCG-007, 48 — g. *Ruminococcaceae* CAG-352, 49 — g. *Prevotella*, 50 — g. *Lachnospiraceae* NK4B4, 51 — g. *Izemoplasmatales*, 52 — f. *Eggerthellaceae* g. unclassified, 53 — g. *Ruminococcaceae*, 54 — g. *Defluviitaleaceae* UCG-011, 55 — g. *Ruminococcaceae* UCG-001, 56 — g. *Holdemania*, 57 — g. Oscillibacter, 58 — f. *Erysipelatoclostridiaceae* g. unclassified, 59 — g. *Escherichia-Shigella*, 60 — g. *Odoribacter*, 61 — f. *Xanthobacteraceae* g. unclassified, 62 — g. *Parabacteroides*, 63 — g. *Lachnospiraceae* FCS020, 64 — g. *Parasutterella* (day 60, Next-Generation Sequencing of 16S rRNA gene amplicons; $n = 3$, $M \pm$SEM; the vivarium experiment, St. Petersburg, 2021). For a description of the groups, see the Materials and methods section.

Nevertheless, the positive effect of the complex feed additive on the composition of the microbiome was manifested in a decrease in the number of a number of opportunistic and pathogenic microorganisms in the digestive system of rabbits. Thus, the species *Staphylococcus sciuri* [70] ($0.075 \pm 0.006\%$) was present in the intestines of animals from the control group, while it was not present in the experimental group. Rabbit staphylococcosis is a dangerous disease that leads to pododermatitis, subcutaneous abscesses, mastitis, abscesses of internal organs, mainly lungs, liver, uterus. Arthritis, periodontitis, sinusitis and otitis media have also been described [71]. Despite the great importance of the species *Staphylococ-*

cus aureus in causing staphylococcosis in rabbits, the clinical significance of *Staphylococcus sciuri* seems to be increasing as the bacterium has been associated with various infections such as endocarditis, peritonitis, septic shock, urinary tract infection, endophthalmitis, inflammatory diseases of the reproductive systems [72]. The absence of *S. sciuri* in the experimental group indicates the positive effect of the feed additive, which included a probiotic strain and a mixture of trace elements, which probably act in synergy.

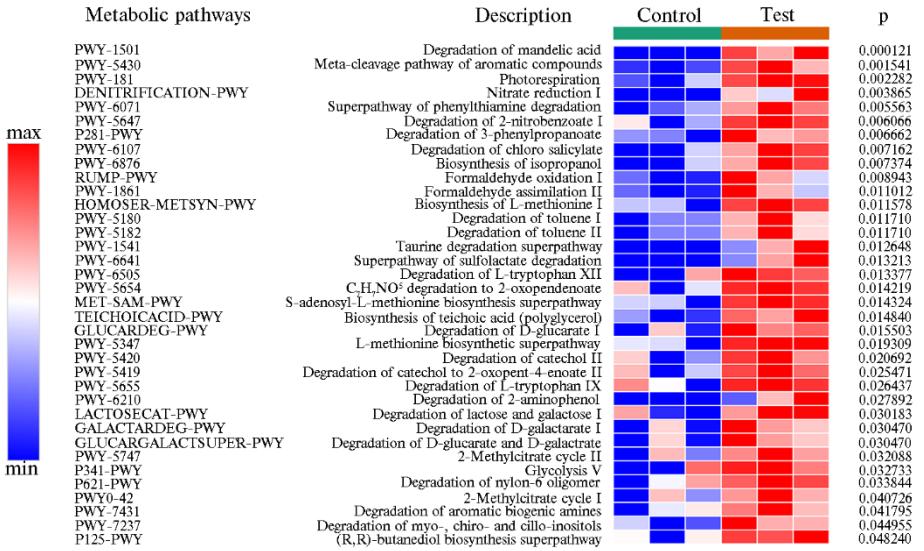

Fig. 4. Functional annotation of potential methabolic pathways in caecal microbiome of Soviet chinchilla rabbits (*Oryctolagus dominis*) fed a complex feed additive based on the probiotic *Bacillus subtilis* 1-85 strain and trace elements (day 60, Next-Generation Sequencing of 16S rRNA gene amplicons; $n = 3$, $M \pm$SEM; the vivarium expeerim, St. Petersburg, 2021). The data were obtained using the PICRUSt2 software package (v.2.3.0) (https://github.com/picrust/picrust2). The scale reflects the intensity of potential metabolic pathways of the microbiome: blue is the lowest (minimum) intensity, red is the highest (maximum).For a description of the groups, see the Materials and methods section.

As a result of the analysis carried out using the PICRUSt2 (v.2.3.0) software package, we found 370 predicted metabolic pathways in the intestinal microbial community of rabbits of the studied groups. Statistically significant differences between the experimental groups ($p \leq 0.05$) were revealed in 36 pathways (Fig. 4). In the intestinal microbiome of rabbits from group II, compared to group I, there was up to 4-fold activation ($p \leq 0.05$) of 8 pathways related to protein metabolism (biosynthesis and conversion of amino acids, nitrogenous compounds), 4 pathways related to carbohydrate metabolism (breakdown of various sugars), 3 to energy metabolism (methylcitrate cycle and glycolysis), 2 to the biosynthesis of alcohols, 1 to photorespiration, 1 to the assimilation of formaldehyde, 1 to the degradation of myo-, chiro- and scillo-inositol, 1 to the synthesis of the cell wall and spore formation (teichic acid biosynthesis pathway). Interestingly, the dominant number of potential metabolic pathways (15 pathways) was associated with the degradation of aromatic compounds and xenobiotics, including the degradation of toxicants such as catechin, formaldehyde, 3-phenylpropanoate, and nylon-6 oligomer [73, 74]. An increase in the potential for degradation of xenobiotics in the intestines of rabbits from the experimental group could be associated with an increase in the number of bacteria *Bacillus* spp. *Bacillus* spp. have long been considered as potential biodestructors and bioremediators capable of decomposing various toxic substances due to the synthesis of various enzymes [75, 76].

An increase in the degradation pathway of myo-, chiro-, and scyllo-inositol

(PWY-7237) in the second experimental group compared to the control ($p \leq 0.05$) could also be associated with an increase in the abundance of *Bacillus* spp. Previous studies have identified in *Bacillus subtilis* 1-85 a number of genes required for myo-inositol catabolism, including the *iolABCDEFGHIJ* and *iolRS* operons [77], as well as the *iolT* gene [78]. As for the enhancement of potential pathways involved in protein metabolism in the experimental group, it is of interest to activate three pathways at once (HOMOSER-METSYN-PWY, MET-SAM-PWY, and PWY-5347) associated with the synthesis of L-methionine. This echoes the conclusions of foreign researchers [79], who suggested that methionine and threonine are produced by microorganisms of the caecum and enter the body of rabbits in the process of caecotrophy of soft feces.

It is worth noting that in growing rabbits, an excess of dietary protein can lead to a higher incidence of mucoid enteropathy [80, 81]. Current recommendations for feeding rabbits tend to reduce the amount of protein in the diet and increase the fiber content in order to prevent digestive disorders [82]. Nevertheless, industrial rabbit breeding is interested in growing highly productive animals. According to G.G. Partridge et al. [83], there is a positive relationship between rabbit weight and protein requirements. Therefore, the possibility of reducing the protein content in the diet while maximizing the efficiency of synthesis and assimilation of amino acids in the digestive system as a result of the use of feed additives seems to be extremely relevant.

Previously, a study [33] was conducted to identify differences in gut microbiota functionality in two commercial rabbit breeds, Elco and Ira, based on 16S rRNA gene sequencing. An increase in the functional potential of the gut microbiome associated with bacterial chemotaxis, the conversion of pentose phosphate, fructose, mannose, and branched chain amino acids was revealed in Elco rabbits compared to Ira rabbits. The effect of feed additives on the metabolic potential of rabbits has not been studied before. However, similar work was carried out on cattle [32], pigs [84], and poultry [85]. On the example of Hyline Brown laying hens, it was shown [85] that a dietary probiotic based on *Bacillus subtilis* DSM 32324, *Bacillus subtilis* DSM 32325 and *Bacillus amyloliquifaciens* DSM 25840 provided an increase in the activity of pathways associated with the metabolism of vitamin B_6, retinol, phosphonates and phosphinates, tyrosine, biosynthesis of phenylpropanoids, monobactams, pantothenates and CoA, RNA degradation.

Thus, a complex feed additive which includes the strain *Bacillus subtilis* 1-85 and a mixture of mineral components of Si, Fe, Cu contributed to a change in the blood levels of innate immune mediators of the Soviet chinchilla rabbits. The content of lysozyme and other blood bactericidal parameters, phagocytic activity, phagocytic index, phagocytic number in rabbits of test group II fed a complex feed additive were higher than in control group I ($p \leq 0.05$). The NGS sequencing revealed higher values of the α-biodiversity indices Chao1, Shannon and Simpson ($p \leq 0.05$) in the test group vs. control. In the composition of the microbiome of the caecum of the intestines, 12 phyla of the kingdom *Bacteria* were found, among which *Firmicutes* dominated ($80.2 \pm 6.2\%$ in the control group, $78.2 \pm 7.4\%$ in the test group). In the tes group, the abundance of *Verrucomicrobiota*, *Actinobacteriota*, *Patescibacteria*, *Proteobacteria*, and *Desulfobacterota* increased and the representation of *Campylobacterota* decreased. In test rabbits, the number of the genus *Bacillus* increased by 2.82 times compared to the control group ($p \leq 0.05$), which probably indicates colonization of the intestinal chyme by a strain of a probiotic microorganism introduced into the diet as part of a complex feed additive. *Staphylococcus sciuri* was found in the intestines of control animals ($0.075 \pm 0.006\%$). In the rabbit gut microbial community, we revealed 370 predicted metabolic pathways, 36 of which showed differences between the experimental groups ($p \leq 0.05$).

In the intestinal microbiome of rabbits from the test group, compared to control, there was activation ($p \leq 0.05$) of pathways related to the degradation of aromatic compounds and xenobiotics, to the protein, carbohydrate, energy metabolism, alcohol biosynthesis, photorespiration, formaldehyde assimilation, and degradation of myo-, chiro- and scillo-inositol, cell wall synthesis and spore formation. The dominant number of enhanced potential metabolic pathways is associated with the degradation of aromatic compounds and xenobiotics. It seems interesting to further study other aspects of the beneficial effects of the introduced bacterial strain and the microelement complex on the host, in particular, the assessment of productive indicators and the development of technologies for introducing the presented feed additive into the digestive tract.

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GENOME-WIDE ASSOCIATION STUDIES OF GROWTH DYNAMICS IN QUAILS *Coturnix coturnix*

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Abstract

Identification and mapping genes that determine economically important traits in farm animals, including poultry, is a key task of genomic selection aimed at improving the efficiency of animal husbandry. In recent years, genome-wide association studies have identified many important candidate genes in various farm animals. Of poultry species, a significant proportion of studies on the search and identification of quantitative trait loci (QTL) was carried out on chickens. On quails, such studies are relatively few primarily due to the lack of commercial chips, which makes it difficult to search for SNPs and identify genes associated with valuable traits. To date, there is little information on the quantitative traits loci reliably associated with productivity performance of quails. The meat productivity of quails, e.g., bodyweight and growth rate, depend on feeding and keeping conditions and is genetically determined by a set of QTL. This report submits the results of a genome-wide association study of the growth rate of F_2 quails in a model resource population. We aimed to identify the QTL in the quail genome, to analyze the association of the found mutations with body weight parameters, and to characterize allelic variants in the studied F_2 quail population. The F_2 model resource population was obtained by crossing two breeds, the Japanese quails with a slow growth and the Texas quails with a fast growth. After filtering the data of GBS genotyping of the obtained F_2 individuals (n = 232), the 92686 SNPs were selected for further analysis. We used PLINK 1.9 software (https://www.cog-genomics.org/plink/) options geno 0.1, mind 0.2, and maf 0.05 to analyze the associations of whole genome genotyping data with the bodyweight which reflects growth and development of birds. High variability of bodyweight was found to be typical of the created resource population. In 1-day-old quails, this indicator varied from 5 to 11 g and averaged 9±0.1 g. At the age of 2, 4, 6, and 8 weeks, the bodyweight reached 69±1, 157±2, 219±2 and 252±2 g, respectively. GWAS identified 149 SNPs that were associated with bodyweight at a high statistical significance ($p < 0.00001$).These SNPs are located on chromosomes 1, 2, 3, 5, 6, 8, 11, 14, 15, 20, 24, 25 and 26. On chromosomes 1, 2, 3, 5, 11 and 26, there were blocks of 2-9 SNPs linked to the same gene. Seven candidate genes (*PCDH9*, *SMAD9*, *PAN4*, *EGFR*, *WDPCP*, *MDGA2*, and *PEPD*) were identified that were associated ($p < 0.00001$) with bodyweight of quails at 8 weeks of age. We intend to further study the detected SNPs as genetic markers in breeding quails for an increased bodyweight. Additionally, associations of these SNPs with other parameters of growth intensity and productivity performance in quails will be under consideration.

Keywords: *Coturnix japonica*, quail, QTL, SNP, GBS, GWAS, bodyweight, growth dynamics

Poultry meat and eggs, which today constitute a significant share of the total livestock production in the world [1, 2], are mainly produced using species belonging to the *Phasianidae* family. Quail products are in special demand, which is explained by the high nutritional value and taste of quail eggs and meat, as well as their precocity [3-5]. The market demand for these products contributed to the creation of large quail farms and the intensive development of the industry, as a result, quail eggs and meat have become everyday products. The profitability and competitiveness of quail farming are due to several reasons. One of the determining factors is the use of breeds and lines of poultry, which are characterized by important breeding traits, which include high egg and meat productivity, resistance to industrial stresses, infectious diseases, and precocity. The creation of highly productive breeds, lines and crosses is impossible without effective breeding work to search for and identify valuable genotypes using modern methods and approaches based on the study of the molecular genetic mechanisms of the formation and manifestation of selectively significant traits.

Thousands of quantitative trait loci (QTL) have now been identified in farm animals for a significant number of economically useful traits [6]. Of the numerous poultry species, a significant proportion of the research on the search and identification of QTLs has been carried out on chickens [7]. The chicken QTL database (Chicken QTLdb, https://www.animalgenome.org/cgi-bin/QTLdb/GG/index) contains information on 16656 QTLs of 370 different traits. Most of these QTLs have been identified using both microsatellites [8, 9] and SNPs identified by genotyping of single point mutations [10] or genome-wide analysis [11, 12] as genetic markers.

In contrast to chickens, studies in quails on the identification of QTLs associated with important breeding traits are relatively few in number. To date, the number of such publications is relatively small. A number of works reported on the identification of QTLs using microsatellites, studied the relationship of these genetic markers with growth [13-15], development [16], meat quality [17], and egg productivity [18] indicators. However, it should be noted that when QTL is detected using microsatellites, certain difficulties arise with the identification of the corresponding genes. To solve the problem, the search for SNPs associated with selectively significant traits is carried out, but such studies on quails are limited by the lack of appropriate commercial chips to detect SNPs. In recent years, the number of works on whole genome genotyping has been growing [19]. Using this approach, SNPs were identified and genes associated with productivity indicators [20], behavior [21, 22], plumage color [23, 24], and egg quality [25] in quails were identified.

In this paper, we present the results of genome-wide associative studies of growth rates in F_2 quails of the model resource population in comparison with the dynamics of changes in the body weight of birds aged from 1 day to 8 weeks. The novelty of our approach lies in the creation of a model resource population of quail, which is characterized by a high degree of variability of the studied growth indicators due to the use of maternal and paternal breeds bred in Russia, contrasting in the studied indicators (fast-growing and slow-growing breeds). New SNPs were identified and genes identified with high reliability ($p < 0.00001$) associated with growth rates. The detected SNPs can be further studied as genetic markers in breeding programs to increase the weight of quails, as well as improve other indicators of meat productivity.

The purpose of the work is to identify loci of quantitative traits in the quail genome and analyze the association of the found mutations with live weight, as

well as to characterize allelic variants in F_2 of the obtained model resource population of quails.

Materials and methods. The studies were carried out on the basis of the physiological yard of the Ernst Federal Research Center VIZh in 2021-2022 on 116 females and 116 males of F_2 quails of the model resource population, which were obtained through interbreeding of Japanese and Texas quails. Initially, interbreeding F_1 crossbreeds with bloodlines of 50% Japanese quail and 50% Texas quail were obtained, then the F_1 crossbreeds were crossed with each other. The F_2 progeny was used as a model resource population for molecular genetic studies and assessment of growth dynamics.

Parental quails (original breeds, F_1) were kept in separated multitiered quail cages, at least 250 cm^2 per bird, in groups of 5-6 birds (1 male, 4-5 females) to produce offspring. F_2 quails of the model resource population were kept in multitiered quail cages (no more than 25 birds per tier, stocking density of at least 162 cm^2 per bird). All cages were equipped with a nipple drinker system (at the rate of 1 nipple per 10 birds), mounted feeders (feeding front of at least 3 cm per bitd) and a manure removal system, in connection with which the birds had free access to clean water and a full-scale commercial compound feed. For young quails aged from 1 day to 6 weeks, a compound feed with a nutritional value of 11.92 (2850) MJ/kg (Kcal/kg) and a crude protein content of 285.00 g/kg was used. From the age of 7 weeks, quails were fed with transferred to feed for productive quails with an exchange energy of 12.13 (2900) MJ/kg (Kcal/kg) and a crude protein content of 180.00 g/kg. The temperature in the premice where the birds were kept was maintained at 18 to 25 °C. In brooders, where young animals were kept from hatching to the age of 4 weeks, the temperature varied from 35 to 23 °C as the individuals matured. Humidity in the premice did not exceed 70%.

The quails in the F_2 model resource population ($n = 232$) was weighed (an OHAUS Pioneer PA413C scale, OHAUS, USA) at the age of 1 day, 2, 4, 6, and 8 weeks.

DNA was isolated from tissue samples and quail feather pulp using the DNA-Extran 2 kit (OOO NPF Sintol, Russia) according to the protocol recommended by the manufacturer. The concentration of the obtained DNA was determined on a Qubit 2.0 fluorimeter (Invitrogen/Life Technologies, USA), the purity was assessed spectrophotometrically (NanoDrop 8000, Thermo Fisher Scientific, USA) (DNA with an $OD_{260/280}$ ratio of at least 1.8 was used for subsequent analysis), DNA quality also by gel electrophoresis in 1% agarose gel.

Whole genome genotyping of F_2 quail model resource population was performed using the genotype by sequencing (GBS) method [26]. The matrix was obtained that contained genomic DNA sequences of 232 F_2 individuals. Adapters were removed and the fastq file was demultiplexed (separation by sample; the cutadapt program, version 3.4, https://pypi.org/project/cutadapt/). Quality control of fastq files was performed (fastqc program; version 0.9.11, https://github.com/s-andrews/FastQC). For alignment, the reference genome was used (the bowtie2 package, version 2.4.4, https://github.com/BenLangmead/bowtie2). Variant invocation (SNP) and annotation were carried out (the bcftools package, version 1.13, https://samtools.gith-ub.io/bcftools/bcftools.html). The converted data were loaded into the R package [27] for further calculations. The genome of Japanese quail (Coturnix japonica 2.1, GCF_001577835.2) was used as a reference. From 115906 SNPs, after quality control and filtering of genotyping data (the PLINK 1.9 software package; http://zzz.bwh.harvard.edu/plink/), 92686 SNPs were selected for

GWAS (whole-genome associated study) analysis. SNPs were selected based on the quality of genotyping for all SNPs for each individual not lower than 90%, the frequency of occurrence of minor alleles, and the deviation of the frequency of SNP genotypes from the Hardy-Weinberg distribution in the aggregate of tested samples with a p-value $< 10^{-5}$.

Principal component analysis (PCA, https://www.datacamp.com/tutorial/pca-analysis-r) was performed and visualized (the R ggplot2 package, https://github.com/tidyverse/ggplot2). The data files were prepared in the R4.0 software environment.

Regression analysis using PLINK 1.9 was used to identify associations of SNPs with quail body weight. The reliability of the SNP influence and the identification of significant regions in the quail genome was assessed using the Bonferroni null hypothesis test at a threshold $p < 1\times10^{-5}$. The data were visualized in the qqman package using the R language [27]. The search for candidate genes located in the region of identified SNPs was performed using the Genome assembly Coturnix japonica 2.1 genomic resource (https://www.ncbi.nlm.nih.gov/data-hub/genome/GCF_001577835.2/, accessed 10.07.2022).

Statistical indicators were calculated by multivariate analysis of variance in Microsoft Excel 2013 using arithmetic mean (*M*) and standard error of mean (±SEM), minimum (min), maximum (max), median (*Me*), Pearson's test of agreement ($\chi2$), homozygosity coefficient according to Robertson (Ca), level of polymorphism according to Robertson at 2 alleles (Na).

Results. Obtained F_2 resource population using breeds that are contrasting in genotype and phenotype as the initial parental forms makes it possible to obtain populations of individuals on a relatively small number of birds, characterized by a significantly large range of variability in phenotypic traits. In purebred individuals of the same breed or line, as a rule, the variability decreased due to directed selection for a limited number of traits, which, in the case of using such a bird in molecular genetic studies for the identification of QTL, requires the collection and analysis of phenotypic data on a large sample of individuals. Works on the creation and use of F_2 model resource populations for molecular genetic studies are widely carried out on various types of poultry, e.g., chickens [28], turkeys [29], and quails [19-21]. In quails, lines of the Japanese quail contrasting in the studied trait were mainly used as the initial parental forms [19-21]. In our study, the model resource population derived from interbreeding Japanese and Texas quails. The Japanese quail is an egg breed characterized by a relatively low growth rate, the Texas quail is a meat breed with a high rate of body weight gain.

The body weight of 1-day-old F_2 quails of the model resource ranged from 5 to 11 g and averaged 9±0.1 g. At the age of 2, 4, 6, and 8 weeks, this indicator increased 8-fold, 17-fold, 24-fold, and 28-fold, compared to that for 1-day-old quails (Table 1).

1. Growth dynamics of F_2 the model resource population of quails (Japanese quail × Texas quail) (n = 232, the physiological yard of the Ernst Federal Research Center VIZh, 2021-2022)

Возраст	Body weight, g						
	absolute					gain	
	M	error	max	min	*Me*	absolute	daily
1 сут	9	0.1	11	5	9		
2 нед	69	0.9	117	32	69	61	9
4 нед	157	1.5	214	103	159	88	13
6 нед	219	1.9	322	159	217	62	9
8 нед	252	2.2	354	163	248	33	5

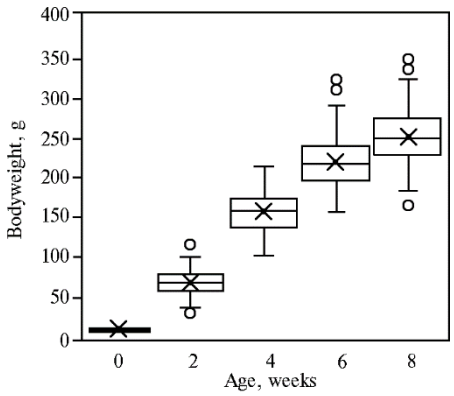

Fig. 1. Bodyweight of quails (Japanese quail × Texas quail) of the F_2 model resource population from 0 to 8 weeks of age (n = 232, the physiological yard of the Ernst Federal Research Center VIZh, 2021-2022).

It should be noted the high variability and significant spread in live weight in birds in the F_2 population. Figure 1 shows a diagram showing the distribution of quails in the study sample according to this indicator in different age periods. It can be seen that the range of variability was 25-40% for the maximum value and 27-54% for the minimum. We revealed the absence of a significant shift in the median in terms of live weight in F_2 quails over the age periods of observation, which indicates a relatively uniform distribution of the frequencies of occurrence of the maximum and minimum values of the trait in the studied population.

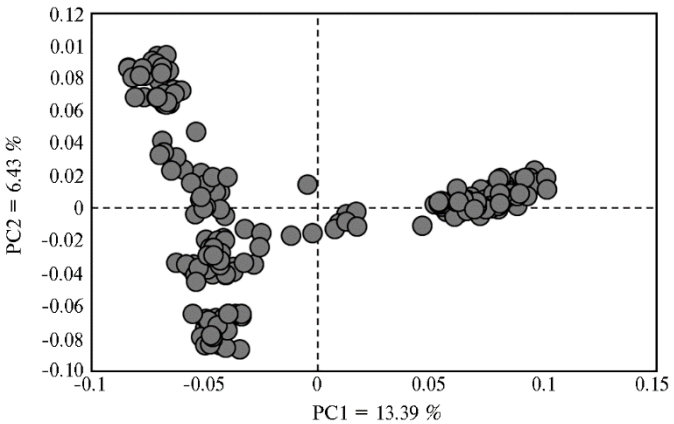

Fig. 2. Genotype by sequencing (GBS)-based PCA analysis of whole genome genotyping data for individuals from the F_2 model resource population of quails (Japanese quail × Texas quail) (n = 232, the physiological yard of the Ernst Federal Research Center VIZh, 2021-2022).

The F_2 heterogeneity of the model resource population of quails in terms of live weight was confirmed by Principal Component Analysis (PCA) based on nucleotide sequence data obtained from individual genome-wide analysis of individuals using the GBS method (Fig. 2). The first component is responsible for 13.39%, the second for 6.43% of genetic differences in birds in the sample under study. According to Figure 2, the studied population was represented by five clusters forming three branches diverging in space from a single point. The heterogeneity of the sample may be due to the structure of the population, consisting of families where there is a greater genetic similarity between individuals belonging to the same group than the average for the population.

To test the assumption about the genomic conditionality of productivity indicators in F_2 quails (in particular, live weight), we conducted a genome-wide association analysis (GWAS). GWAS analysis makes it possible to identify many genomic variants associated with the productive traits of poultry. As a result of the analysis, we identified 149 SNPs with high reliability ($p < 0.00001$) on chromosomes 1, 2, 3, 5, 6, 8, 11, 14, 15, 20, 24, 25, 26, on the chromosomes 1, 2, 3, 5, 11, 26, blocks of 2-9 SNPs related to one gene were detected. The ratio of mutations found in the study with SNPs that are highly associated with body weight,

and SNPs that are characterized by the mechanism of influence, is graphically shown in Figure 3.

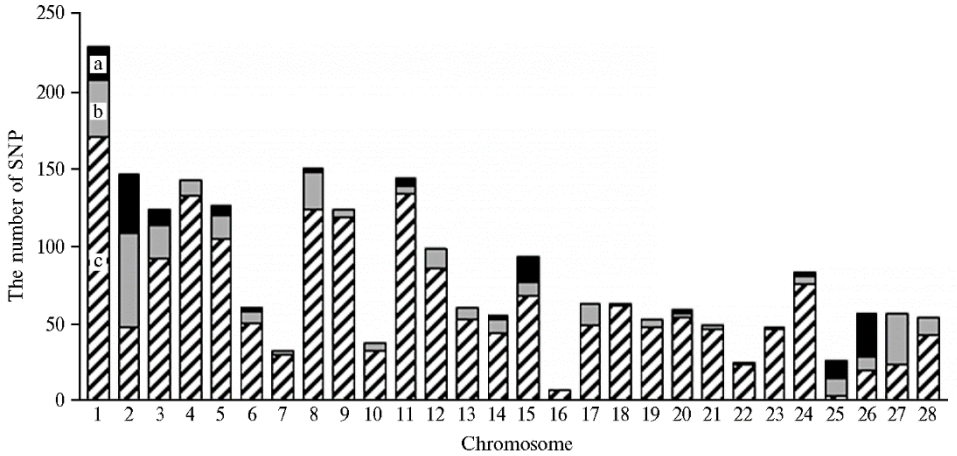

Fig. 3. SNPs associated with bodyweight parameters in quails (Japanese quail × Texas quail) of the F_2 model resource population from 0 to 8 weeks of age: a — characterized SNPs (located near functional genes) with association reliability higher than 1×10^{-5}, b — uncharacterized SNPs (not located near functional genes) with association reliability higher than 1×10^{-5}, c — SNPs with association reliability lower than 1×10^{-5} (n = 232, the physiological yard of the Ernst Federal Research Center VIZh, 2021-2022).

2. SNP and canidate genes associated with bodyweight parameters in 8-week old quails (Japanese quail × Texas quail) of the F_2 model resource population (n = 232, the physiological yard of the Ernst Federal Research Center VIZh, 2021-2022)

Chromosome	The number of SNPs	SNPposition, bp	p	Gene
1	3	142,741,390	7.25×10^{-8}	*PCDH9*
		154,153,381	1.04×10^{-5}	*SMAD9*
		157,425,751	1.35×10^{-5}	*PAN4*
2	2	73,896,881	1.23×10^{-9}	*EGFR*
		7,3896,952	3.67×10^{-8}	
3	1	1,993,165	6.77×10^{-10}	*WDPCP*
5	2	51,581,638	6.71×10^{-5}	*MDGA2*
		51,581,710	6.71×10^{-5}	
1	1	9,015,455	8.51×10^{-5}	*PEPD*

We used SNP loci ($p < 0.00001$) identified in the genome of quails at the age of 8 weeks and associated with the body weight indicator to identify positional and functional candidate genes associated with some mechanism of influence on processes in the body (Table 2). As a result, seven functional candidate genes for chromosomes 1 (*PCDH9*, *SMAD9*, *PAN4*), 2 (*EGFR*), 3 (*WDPCP*), 5 (*MDGA2*) and 11 (*PEPD*).

According to publications, in quails, the *EGFR* gene is responsible for the regulation of granulosa cell proliferation and influences the development of follicles [24]. Other mutations have been reported for other animals and birds as orthologous groups of genes [30], defined bioinformatically using a combination of protein sequence similarity and local synteny data.

We calculated the frequencies of occurrence, the degree of polymorphism, and genetic balance for the identified genes in the studied population of F_2 quails at the age of 8 weeks (Table 3).

According to the results obtained, for all detected point mutations in the genes, there were polymorphic variants of genotypes with a variation in the degree of homozygosity (according to Robertson) from 0.509 to 0.893. Deviations from genetic balance were found for the PAN4 (7.650), EGFR_1 (5.603), EGFR_2 (4.115), and WDPCP (20.819) genes.

3. Frequency of occurance of valuable selectable markers in quails (Japanese quail × Texas quail) of the F_2 model resource population (n = 232, the physiological yard of the Ernst Federal Research Center VIZh, 2021-2022)

Chromosome	Gene	Genotypr frequency, M±SEM			Allele frequency		χ^2	Ca	Na
		11, O and E	12, O and E	22, O and E	1	2			
1	*SMAD9*	0.178±0.019 0.188	0.511±0.024 0.491	0.311±0.022 0.321	0.434	0.566	0.120	0.509	1.966
	PSDH9	0.018±0.006 0.026	0.283±0.021 0.269	0.699±0.022 0.706	0.160	0.840	0.455	0.731	1.367
	PAN4	0.023±0.007 0.075	0.502±0.024 0.398	0.474±0.024 0.526	0.275	0.725	7.650	0.602	1.662
2	*EGFR_1*	0 0.026	0.320±0.021 0.269	0.680±0.022 0.706	0.160	0.840	5.603	0.731	1.367
	EGFR_2	0 0.019	0.274±0.020 0.236	0.726±0.021 0.745	0.137	0.863	4.115	0.764	1.310
3	*WDPCP*	0.065±0.003 0.021	0.159±0.021 0.248	0.776±0.021 0.731	0.145	0.855	20.189	0.752	1.329
5	*MDGA*	0 0.011	0.205±0.019 0.184	0.795±0.019 0.805	0.103	0.897	2.314	0.816	1.226
11	*PEPD_2*	0.009±0.003 0.003	0.095±0.015 0.107	0.896±0.015 0.889	0.057	0.943	2.545	0.893	1.120

N o t e. For the frequency of genotypes and alleles, coding options are given (11 means alleles 1 and 1, 12 means alleles 1 and 2, 22 means alleles 2 and 2); $\chi 2$ — Pearson's goodness-of-fit criterion, Ca — Robertson's homozygosity coefficient, Na — Robertson's polymorphism level at 2 alleles, O and E — observed and expected frequencies, respectively.

An analysis of open information sources showed that to date a small number of works have been published regarding the search and identification of QTLs that are associated with growth rates in quails. M.I. Haqani et al. [19] determined the QTL associated with body weight in quails by examining the age dynamics of this indicator in 277 F_2 individuals derived from crossing lines of Japanese quail with high and normal body weight. Body weight was determined weekly from hatching to 16 weeks of age. As a result, 125 SNPs associated with body weight were identified. On chromosomes 1 and 3, 4 QTLs associated with the body weight of birds aged 4 to 16 weeks were identified. No statistically significant QTLs were found in the early age period (up to 3 weeks) [19)]. S. Vollmar et al. [20] identified QTLs associated with phosphorus, calcium, feed conversion, and body weight gain on 920 F_2 Japanese quails, which were calculated from body weight data at 10 and 15 days of age. A total of 3986 SNPs were selected for analysis. As a result, the authors identified 12 significant SNPs and 4 candidate genes that were associated with the studied traits [20]. J. Recoquillay et al. [21] obtained an F_2 model resource population of Japanese quail by crossing two lines contrasting in behavior. Along with behavioral reactions, productive traits were studied, in particular, body weight at the age of 17 and 65 days and egg production. Based on genome-wide genotyping, 22 QTLs associated with productive traits were revealed. The identified SNPs were found on chromosomes 1, 3, 5, 8, 10, and 18 [21].

In our studies, one of the parental forms during the creation of the resource population was also the Japanese quail. At the same time, the use of Texas quail as a contrast breed made it possible to obtain an F_2 population with a significant range of variability in body weight. The novelty of our study lies in the identification of SNPs and candidate genes that were not previously described in open information sources and are associated with the body weight index in quails during different age periods. Significant SNPs located close to functional genes ($p < 0.00001$) associated with the body weight index of quails aged 8 weeks were localized on 13 of the 28 counted chromosomes, including chromosomes 1, 3, 5 and 8, which is similar to the data obtained by other researchers [19, 21]. The largest number of such SNPs were localized on chromosomes 1, 2, 3, 15, and 27 (see Fig. 3).

Thus, in the obtained F_2 model resource population of quails (Japanese

quail × Texas quail), the genome-wide DNA sequencing revealed 149 SNPs associated ($p < 0.00001$) with body weight on chromosomes 1, 2, 3, 5, 6, 8, 11, 14, 15, 20, 24, 25, and 26. Blocks of 2-9 SNPs linked to one gene were detected on chromosomes 1, 2, 3, 5, 11, and 26. In the regions where the QTLs are located, there are several positional and functional marker candidate genes involved in the determination of traits that are different from the body weight studied in this work. In particular, seven candidate genes (*PCDH9*, *SMAD9*, *PAN4*, *EGFR*, *WDPCP*, *MDGA2*, and *PEPD*) are identified that are significantly ($p < 0.00001$) associated with body weight of quails at 8 weeks age. The data we obtained is the basis for continuing research of the associations of the identified mutations with other selectively significant indicators of productivity.

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INFLUENCE OF ANTIBIOTICS, GLYPHOSATE AND A *Bacillus* sp. STRAIN ON PRODUCTIVITY PERFORMANCE AND GENE EXPRESSION IN CROSS ROSS 308 BROILER CHICKENS (*Gallus gallus* L.)

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Abstract

The combination of antibiotics and pesticide residues can compromise the therapeutic and production benefits of antibiotics in the poultry industry. These effects may be reflected in changes of gene expression. The present work, for the first time, shows that the stimulation of poultry meat productivity with veterinary antibiotics enrofloxacin and colistin is probably associated with the induced expression of *MYOG* gene which is known to promote the development and differentiation of muscles, genes of antimicrobial (*Gal9*, *Gal10*) and antiviral (*IRF7*) protection, and pro-inflammatory genes *IL6*, *IL8* and *PTGS2*. In addition, it was shown for the first time that glyphosate suppresses the expression of antimicrobial and antiviral genes in broilers of the Ross 308 cross. The aim of the study was to evaluate the change in the expression spectrum of key genes in broiler fed antibiotics, glyphosate and a biodestructor strain. The experiments were carried out on broilers of the Ross 308 cross from 1 to 35 days of age (the vivarium of BIOTROF$^+$ LLC, 2022). The broilers were divided into 4 groups of 40 birds each. Group I (control) was fed a diet without additives, group II received a diet with the addition of veterinary antibiotics enrofloxacin and colistin; group III experienced dietary antibiotics and glyphosate; group IV received dietary antibiotics, glyphosate and a strain of the microorganism-biodestructor *Bacillus* sp. GL-8. Glyphosate content was measured by ELISA using a STAT FAX 303+ analyzer (Awareness Technology, LLC, USA) and a Glyphosate ELISA Microtiter Plate test system (Abraxis, USA). Reverse transcription quantitative PCR was performed to evaluate gene expression of the caecum and pectoral muscle tissues. Total RNA was isolated from samples using the Aurum™ Total RNA mini kit (Bio-Rad, Hercules, USA). Specific primers were selected for immunity genes *IL6* (interleukin 6), *IL8* (interleukin 8), *IRF7* (interferon regulatory factor7), *PTGS2* (prostaglandin-endoperoxide synthase), *AvBD9* (*Gal9*) (β-defensin 9), *AvBD10* (*Gal10*) (β-биотро,bjnhdefensin 10). For productivity genes, *LGF-I* (insulin-like growth factor 1), *MYOG* (myogenin), *MYOZ2* (myosenin) and *GSTA3* associated with resistance to toxic and medicinal substances were tested. Amplification reactions were carried out using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, USA)

using a DTlight amplifier (DNA-Technology, Russia). The body weight of broilers was assessed at 7, 14, 21, 28 and 35 days of age. Mathematical and statistical data processing was performed using multivariate analysis of variance in Microsoft Excel XP/2003, R-Studio (Version 1.1.453) (https://rstudio.com). The results showed a 4.8-23.3 %-stimulated productivity ($p \leq 0.05$) of broilers from 14 days of life until the end of the experiment due to dietary antibiotics (group II vs. group I). At the end of the experiment, a negative effect of glyphosate on broiler productivity occurred (group III vs. group II, $p \leq 0.05$). In broilers of groups II and IV, the expression of *MYOG* gene was 2.0 and 2.1 times higher than in group I ($p \leq 0.05$). In the group fed glyphosate combined with antibiotics without a biodestructor strain added (group III), no activation of the *MYOG* gene expression occurred compared to group I ($p > 0.05$), which indicates a negative effect of glyphosate on the expression of productivity genes. Glyphosate (group III) also acted as a suppressor of the antimicrobial and antiviral genes *Gal9*, *Gal10* and *IRF7* as compared to group II ($p \leq 0.05$). The dietary biodestructor strain co-fed with glyphosate and antibiotics (group IV) provided an increase in *Gal9* expression compared to group III ($p \leq 0.05$). There was a tendency for a sharp increase in the expression of pro-inflammatory genes *IL6*, *IL8* and *PTGS2* (by 4.6, 11.2 and 6.6 times, respectively) in group II fed antibiotics vs. control group I ($p \leq 0.05$). Our findings once again confirms the effect of antibiotics on immune processes. For *GSTA3* gene associated with resistance to toxic and medicinal substances, it was shown that the introduction of antibiotics into feeds had some stimulating effect on the level of *GSTA3* gene expression in the caeca tissues of broilers (group II vs. group I, $p \leq 0.05$). Thus, the mechanism providing positive effects of antibiotics on productivity performance is probably partly due to the fact that they act as inducers of a set of important genes. Glyphosates fed in an amount corresponding to 1MPC reduced the stimulating effect of antibiotics. Glyphosates act, among other things, through the disruption of the activity of some key bird genes. The positive dynamics of the expression of various genes, including those involved in antimicrobial and antiviral defense, under the action of a biodestructor strain indicates the prospects for using probiotics as a means of smoothing out physiological imbalances caused by drugs and food contamination with toxic substances.

Keywords: mycotoxins, antibiotic, glyphosate, broilers, gene expression

Antibiotics play an important role in the fight against infectious diseases and are also used to stimulate the growth of poultry [1-3]. Metaphylactic administration of antibiotics, such as enrofloxacin, to chicks during the first few days of life, and sometimes during further rearing, is considered common practice among many poultry meat producers [4, 5]. Antibiotics can adversely affect the defense mechanisms of birds, which are determined by the functioning of the main organs of the immune system [6]. There is evidence that although enrofloxacin inhibits humoral immune mechanisms [7], it may promote cellular immune response in chickens [5].

Interestingly, the mechanism of growth stimulation of farm animals and poultry under the influence of antibiotics is still not clear. All hypotheses are reduced mainly to the modulation of the composition of the microbiota against their background [8]. H. Eyssen et al. [9] hypothesized that antibiotics stimulate chick growth through their antibacterial action against gram-positive micro-organisms that interfere with nutrient absorption. According to another hypothesis [10], a decrease in the population of lactobacilli in animals treated with antibiotics reduces the activity of bile salt hydrolase, which increases the relative abundance of conjugated bile salts, promotes lipid metabolism and energy synthesis. As a result, the weight gain of animals increases.

However, the use of antibiotics tends to compromise the immune system [11]. It has been shown in pigs [12] and poultry [13] that dietary antibiotics can interfere with gene expression.

In addition to antibiotics, many other factors affect broiler immunity, health and productivity [14]. For example, bird feeds, especially those based on genetically modified soybeans, contain a significant amount of glyphosate herbicide residues [15, 16], which can have a negative effect on the body [17, 18].

The digestive system serves as a protective barrier against exposure to pesticides and pathogens [19, 20]. Lymphoid tissues in the gastrointestinal tract of birds are well developed [21, 22] and are involved in the activation of immune responses [23-25]. It is important to note that the study of the effect of glyphosates

on the expression of genes in farm animals and birds has not been previously carried out.

The widespread use of antibiotics and the presence of glyphosates in feed can jeopardize the therapeutic and production effects of the use of antibacterial drugs. Glyphosate exposure has previously been shown to increase the tolerance of *Escherichia coli* and *Salmonella enterica* serovar *Typhimurium* to kanamycin and cephalosporin [26]. However, the combined effects of antibiotics and glyphosates have not been previously studied in animal models.

The search for agents that positively affect the bird gut microbiota by stimulating protective mechanisms and reducing the need for prophylactic and therapeutic use of antibiotics has been going on for many years. Beneficial microorganism strains undoubtedly rank first among such agents [27-29] and are widely used in poultry nutrition [30]. It is not uncommon for beneficial bacteria to be used concomitantly with antibiotics to prevent side effects of the latter [31]. The effect of microorganisms on immunity [32] and expression of host genes [33] has been proven. Strains of microorganisms-biodestructors were used for prophylaxis in cases of feed contamination with mycotoxins [34] and glyphosates [35]. In this regard, it is advisable to investigate whether the introduction of microorganism strains into diets can be a tool to smooth out the immunosuppression that has arisen against the background of antibiotics.

This paper is the first to report that the stimulation of the meat productivity of Ross 308 cross broiler chickens under the influence of the veterinary antibiotics enrofloxacin and colistin is probably associated with an induced expression of the *MYOG* gene mRNA which promotes the development and differentiation of muscles, antimicrobial genes (*Gal9*, *Gal10*), antiviral (*IRF7*) protection, and pro-inflammatory genes *IL6*, *IL8* and *PTGS2*. In addition, it has been shown for the first time that glyphosate suppresses the expression of antimicrobial and antiviral genes in broiler chickens.

Our goal was to evaluate the productivity and changes in the expression of genes associated with immunity, productivity, and resistance to toxic and medicinal substances in broiler chickens under the influence of antibiotics, including against the background of fodder contamination with glyphosate and the introduction of Bacillus sp. into the diet.

Materials and methods. The experiments were carried out in 2022 in the vivarium of OOO BIOTROF+ on the Ross 308 cross broilers (*Gallus gallus* L.) from 1 to 35 days of age; the requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental or other Scientific Purposes (ETS No. 123, Strasbourg, 1986) [36] were complied with. Feeding and keeping conditions corresponded to recommendations for cross-country [37]. From day 1 to day 28 of growth, PK 5 compound feed was used, from day 29 to day 35 PK 6 compound feed was used.

The birds were divided into 4 groups of 40 birds each. In intact group I (control), broilers received a diet without the introduction of antibiotics, glyphosate, and a microorganism strain. In group II, a diet was fed with the addition of veterinary antibiotics enrofloxacin and colistin in the form of Enroflon K (OOO VIK — animal health, Russia) at a dosage of 1 ml/l of water from day 1 to day 5 of growth and florfenicol (OOO Agrovetzashchita S.-P. NVTs, Russia) from day 17 to day 20 at a dosage of 1 ml/l of water. In group III, the diet was added with Enroflon K according to the scheme described above, as well as glyphosate in the amount of 20 mg/kg of feed, which corresponded to 1 MPC for feed [38]. In group IV, the diet was added with enrofloxacin, colistin, florfenicol, glyphosate, and the strain *Bacillus* sp. GL-8. The bacterial preparation was used at a concentration of 106 cells/kg of feed.

To analyze the ability to biodegrade glyphosate in vitro, 11 strains of Bacillus sp. incubated with glyphosate in the form of the herbicide Tornado, BP (ZAO Firma Avgust, Russia) containing glyphosate N-(phos-phonomethyl)-glycine (isopropylamine salt) (360 g/l) for 2 days. The drug was added to the medium in an amount of 20 μl, which corresponded to 144 mg/l of pure glyphosate. The strains were cultured in a semi-synthetic nutrient medium (molasses 2%; NaCl 0.02%; K_2HPO_4 0.2%; $MgSO_4 \cdot 7H_2O$ 0.05%; $CaCO_3$ 0.01%) in glass flasks with cotton stoppers on a shaker at 230 rpm and a temperature of 32±1.2 °C without additional aeration. The concentration of bacteria at the beginning of growth in all variants was 1.0×10^4 cells/ml, the duration of cultivation was 2 days. The concentration of bacteria at the end of cultivation ranged from $1.9\times10^7\pm7.9\times10^5$ to $8.7\times10^8\pm6.3\times10^6$ cells/ml. The decrease in the content of mycotoxins in the nutrient medium with the inoculated culture of live bacterial cells compared to the control was conditionally considered as the biodegradation of glyphosate.

The strain *Bacillus* sp. GL-8 isolated from the intestines of broilers was obtained from the collection of OOO BIOTROF+. The strain was aerobic immobile spore-forming rods 1.2-1.5 μm wide and 2-5 μm long. It formed elliptical spores of a central location. To obtain preliminary conclusions that the *Bacillus* sp. GL-8 does not have virulence factors and etiological significance in the development of infectious processes; its hemolytic activity was determined. It was established after 24 hours when viewing colonies grown on 5% blood agar.

In a production experiment, glyphosate was used as part of the preparation Agrokiller (ZAO Firma Avgust, Russia) containing 500 g/l of glyphosate (isopropylamine salt). For this, a working solution was prepared from the Agrokiller preparation, which was applied by spraying feed, 5 ml of working solution per 1 kg feed, to a final content of pure glyphosate in the feed of 20 mg/kg. Mixing was carried out mechanically in compliance with personnel safety requirements. Feed intake by broilers averaged 150 g/day, i.e., broilers of the experimental groups received glyphosate daily in the amount of 3 mg/bird. After the introduction of glyphosate, its concentration in the feed was monitored by enzyme immunoassay (ELISA). The diet of broilers practically did not contain background amounts of glyphosate, which indicates the purity of the experiment.

To analyze the content of glyphosates by ELISA in feed and nutrient media, a STAT FAX 303+ strip enzyme immunoassay analyzer (Awareness Technology Co LLC, USA) and a Glyphosate ELISA test system, Microtiter Plate (Abraxis, USA) were used. The test is based on a direct competitive ELISA reaction between glyphosate, which is present in the sample, and a glyphosate labeled enzyme to bind rabbit anti-glyphosate antibodies and goat anti-rabbit immunoglobulins immobilized in microwells. After the enzyme immunoassay, the intensity of the color signal of the solution in the wells was inversely proportional to the concentration of glyphosate present in the samples.

To determine the expression of genes at the end of the experiment, tissue samples of caecum and pectoral muscles were taken. The samples were stabilized with the RNAlater reagent (Thermo Fisher Scientific, Inc., USA) and immediately sent to the OOO BIOTROF+ for RNA isolation.

Gene expression analysis was performed using quantitative PCR. To obtain RNA, tissues were mixed with liquid nitrogen and homogenized. Total RNA was isolated using the Aurum™ Total RNA mini kit (Bio-Rad, USA) following the manufacturer's instructions. The reverse transcription reaction was performed to obtain cDNA from an RNA template using iScriptTM Reverse Transcription Supermix (Bio-Rad, USA) [39]. The following specific primers were selected using the NCBI toolkit (https://www.ncbi.nlm.nih.gov) for expression analysis:

Gene, protein	Primers (5′→3′)
IL6, interleukin 6	F: AGGACGAGATGTGCAAGAAGTTC R: TTGGGCAGGTTGAGGTTGTT
IL8, interleukin 8	F: GGAAGAGAGGTGTGCTTGGA R: TAACATGAGGCACCGATGTG
IRF7, interferon regulatory factor 7	F: ATCCCTTGGAAGCACAACGCC R: CTGAGGCAACCGCGTAGACCTT
PTGS2, prostaglandin endoperoxide synthase 2	F: TCGAGATCACACTTGATTGACA R: TTTGTGCCTTGTGGGTCAG
AvBD9 (*Gal9*), β-defensin 9	F: AACACCGTCAGGCATCTTCACA R: CGTCTTCTTGGCTGTAAGCTGGA
AvBD10 (*Gal10*), β-defensin 10	F: GCTCTTCGCTGTTCTCCTCT R: CCAGAGATGGTGAAGGTG
LGF1, insulin-like growth factor 1	F: GCTGCCGGCCCAGAA R: ACGAACTGAAGAGCATCAACCA
MYOG , myogenin	F: GGAGAAGCGGAGGCTGAAG R: GCAGAGTGCTGCGTTTCAGA
MYOZ2, миозенин ()	F: CAACACTCAGCAACAGAGGC R: GTATGGGCTCTCCACGATTTCT
GSTA3 gene associated with resistance to toxic and drug substances	F: TACATCGCAGGGAAATACA R: GGAGAGAAAGGAAACACCA

Primers for amplification of the housekeeping gene encoding the ACTB beta actin protein were used as a reference control: F, 5'-CTGTGCCCATCT-ATGAAGGCTA-3', R, 5'-ATTTCTCTCTCGGCTGTGGTG-3' [40]. The reaction was carried out using a DTlight amplifier (DNK-Technology, Russia) and a SsoAdvancedTM Universal SYBR® Green Supermix kit (Bio-Rad, USA) according to the manufacturer's protocol [41]. Amplification mode and conditions were as follows: 5 min at 95 °C (preheating); 30 s at 95 °C, 30 s at 60 °C 30 s at 70 °C (40 cycles) [42]. Relative expression was assessed by the $2^{-\Delta\Delta CT}$ method [43]. The live weight of broilers was determined at the age of 7, 14, 21, 28 and 35 days [44].

Mathematical and statistical processing of the results was carried out by the method of multivariate analysis of variance (ANOVA) in Microsoft Excel XP/2003, R-Studio (Version 1.1.453) (https://rstudio.com). Results are presented as means (*M*) and standard errors of the means (±SEM). Significance of differences was established by Student's *t*-test, differences were considered statistically significant at $p \leq 0.05$. Means were compared using the Tukey Significantly Significant Difference (HSD) test and the TukeyHSD function in the R Stats Package (https://www.rdocumentation.org/packages/stats/versions/3.6.2/topics/TukeyHSD).

Results. In 6 out of 11 studied *Bacillus* strains we revealed the ability to biodegrade glyphosate in vitro, the most pronounced in the strain *Bacillus* sp. GL-8 compared to others (53.0±4.10%) (Table). This fact suggests the presence of *Bacillus* sp. GL-8 enzymes associated with the biodegradation of xenobiotics. In the study of GL-8 for hemolytic activity on blood agar, we did not observe zones of enlightenment around the colonies.

The data obtained may be of great practical importance for the use of *Bacillus* sp. GL-8 as a probiotic in poultry populations exposed to glyphosates. Many bacteria have been shown to be able to metabolize glyphosate to non-toxic compounds. Its biodegradation leads to the formation of metabolites, which are used as a source of carbon, nitrogen and phosphorus, elements necessary for the development of organisms [45].

Bacterial degradation of glyphosate occurs via two metabolic pathways. The first pathway is carried out with the participation of the enzyme glyphosatoxidoreductase, which breaks down the glyphosate molecule into two metabolites: glyoxylate, which enters the tricarboxylic acid cycle and forms carbon dioxide due to complete oxidation, and aminomethylphosphonic acid which is hydrolyzed by the enzyme carbon-phosphorus lyase (C-P-lyase) to phosphate and methylamine. The latter is converted into ammonia (a direct source of nitrogen) and formaldehyde, which enters the tetrahydrofolate cycle. The second degradation pathway

involves the enzyme C-P-lyase, which, due to its hydrolytic activity, forms phosphate and sarcosine. At the next stage, due to the activity of the enzyme sarcosine oxidase, sarcosine is converted into the amino acid glycine, which is used directly for metabolism and microbial biosynthesis, and formaldehyde, which is introduced into the tetrahydrofolate cycle [46]. It was shown that *Arthrobacter* sp. GLP-1, *Alcaligenes* sp. GL, *Pseudomonas pseudomallei* 22 and *Flavobacterium* sp. GD1 use glyphosate as a source of phosphorus [47]. Probiotic strains of microorganisms have long been used as biodegraders of toxic compounds in the gut [48, 49]. Nevertheless, it cannot be ruled out that in our experiment a certain proportion or the entire volume of glyphosate could be subjected to sorption rather than biodegradation. Therefore, more extensive and detailed studies are required for conclusions.

Glyphosate biodegradation under the influence of *Bacillus* sp. from the collection of OOO BIOTROF+ ($n = 3$, $M \pm$SEM; in vitro test, OOO BIOTROF+, St. Petersburg, 2022)

Strain	Biodegradation rate, %
Bacillus sp. GL-1	15.4±2.40
Bacillus sp. GL-2	0
Bacillus sp. GL-3	19.2±3.90
Bacillus sp. GL-4	6.3±0.52
Bacillus sp. GL-5	0
Bacillus sp. GL-6	0
Bacillus sp. GL-7	0
Bacillus sp. GL-8	53.0±4.10
Bacillus sp. GL-9	0
Bacillus sp. GL-10	13.9±2.30
Bacillus sp. GL-11	25.6±2.40

According to the analysis of the increase in the live weight of poultry, antibiotics stimulated ($p \leq 0.05$) productivity from day 14 of life until the end of the experiment by 4.8-23.3% (group II compared to group I) (Fig. 1). An increase in the live weight gain of broilers under the influence of antibiotics has long been known [8]. At the end of the experiment, against the background of antibiotics, a negative effect of glyphosate on the productivity of broilers (group III compared to group II) was manifested ($p \leq 0.05$). This also seems logical since, firstly, glyphosate can cause intracellular changes and cytotoxicity [18]. Glyphosates are known to affect mitochondrial activity and likely increase DNA damage [17]. Second, at the tissue and body levels, glyphosates can interfere with neurotransmitter function and likely act as endocrine disruptors [50]. Recent studies in mammalian models have shown changes in hormone levels [51], impaired puberty and reproduction [52]. Third, glyphosates can affect organisms through changes in microbial communities. The shikimate pathway is present in most bacteria, and in many bacteria, its key enzyme, enolpyruvylshikimate 3-phosphate synthase (EPSPS), is sensitive to glyphosate [53, 54]. Recently, glyphosates have been found to adversely affect intestinal bacterial communities in several model organisms as well as in vitro cultures [55-57].

Application of Bacillus sp. GL-8 in combination with antibiotics and glyphosate did not have a statistically significant effect on broiler productivity (see Fig. 1). This may be due to various reasons, in particular, the negative effect of antibiotics on survival and gene expression in the biodegrading microorganism strain.

In connection with the revealed differences in the productivity of broilers, we analyzed the expression of genes associated with the growth and formation of muscle fibers in response to the introduction of antibiotics, glyphosate, and a strain of a biodegrading microorganism into the diets. The most significant changes concerned the *MYOG* gene, which promotes muscle development and differentiation. Expression of mRNA of the *MYOG* gene was 2.0 and 2.1 times higher in groups

II and IV, respectively, compared to group I ($p \le 0.05$) (Fig. 2).

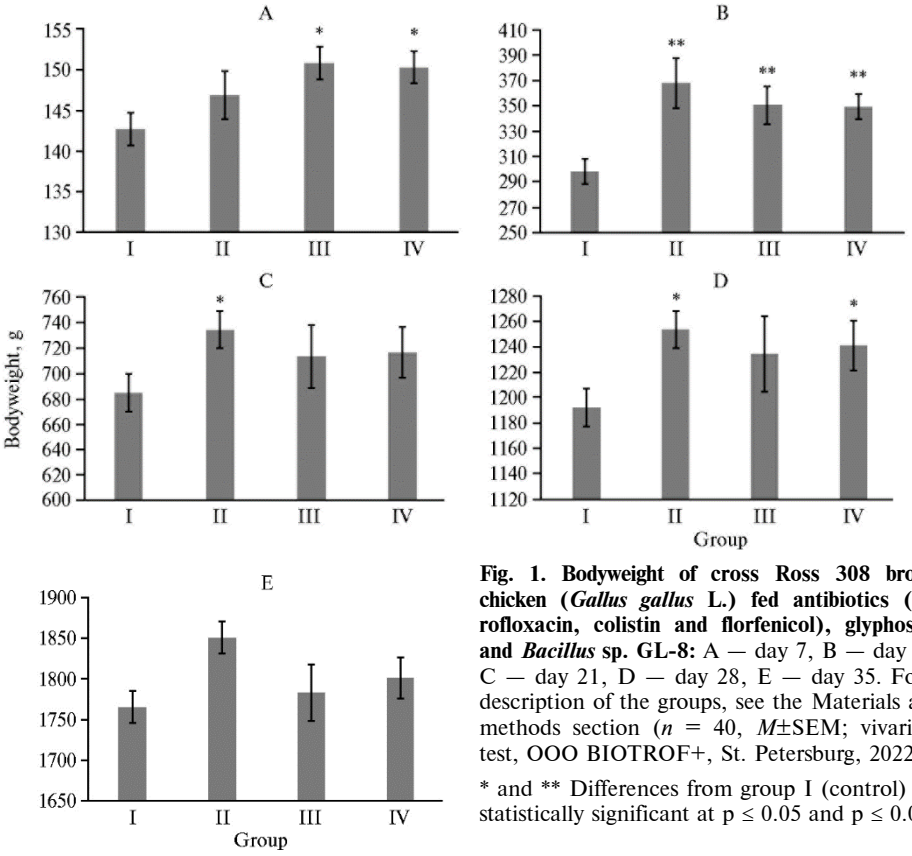

Fig. 1. Bodyweight of cross Ross 308 broiler chicken (*Gallus gallus* L.) fed antibiotics (enrofloxacin, colistin and florfenicol), glyphosate and *Bacillus* sp. GL-8: A — day 7, B — day 14, C — day 21, D — day 28, E — day 35. For a description of the groups, see the Materials and methods section (n = 40, M±SEM; vivarium test, OOO BIOTROF+, St. Petersburg, 2022).

* and ** Differences from group I (control) are statistically significant at $p \le 0.05$ and $p \le 0.01$.

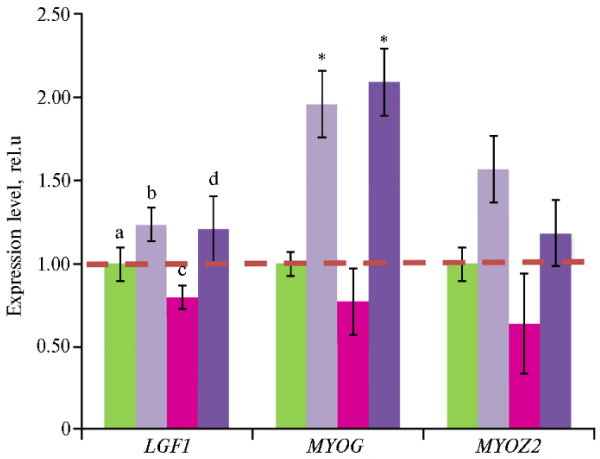

Fig. 2. mRNA expression levels of the genes *LGF1, MYOG* and *MYOZ* associated with growth and breast muscle formation in cross Ross 308 broiler chicken (*Gallus gallus* L.) fed antibiotics (enrofloxacin, colistin and florfenicol), glyphosate and *Bacillus* sp. GL-8: a — group I, b — group II, c — I group II, d — group IV; rel. u corresponds to the multiplicity of changes in expression compared with control group I, in which expression was taken as 1 (dashed red line corresponds to the the control expression). For a description of the groups, see the Materials and methods section (day 35, n = 3, M±SEM; vivarium test, OOO BIOTROF+, St. Petersburg, 2022).

* Differences from group I (control) are statistically significant at $p \le 0.05$.

The *MYOG* (myogenin) gene is a key regulatory transcription factor involved in muscle development during myogenesis [58]. There are also data on the role of *MYOG* after the completion of myogenesis. For example, a positive relationship has been reported between an increase in pectoral muscle mass and an increase in *MYOG* mRNA expression in 38-day-old broilers [59)]. Myogenin is known to play an important role in maintaining mitochondrial activity during exhausting exercise [60].

We believe that the increased expression of *MYOG* in our experiment

played a role in the increase in body weight of broilers in the variant with the antibiotic. Although *MYOG* functions are primarily associated with the induction of myogenesis, this gene also contributes to avian energy metabolism. Increased transcriptional activity of *MYOG* in experimental groups II and IV could be a factor contributing to the enhancement of mitochondrial function and increased energy accumulation. In group III, no activation of *MYOG* expression was noted ($p > 0.05$), which indicates a negative effect of glyphosate on the expression of bird productivity genes. In general, the data obtained indicated some smoothing of the negative effect of glyphosate during the introduction of a microorganism strain.

In the expression of the *LGF1* (insulin-like growth factor 1) and *MYOZ2* (myosenin) genes, we did not find any differences between the groups ($p > 0.05$).

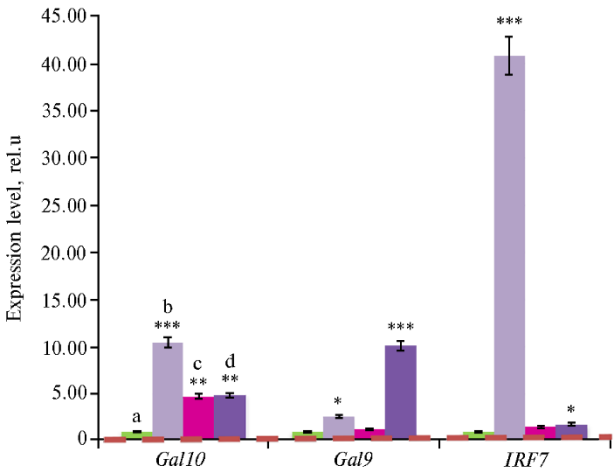

Fig. 3. mRNA expression levels of the genes of antimicrobial and antiviral defence *Gal9*, *Gal10* and *IRF7* in caecum of cross Ross 308 broiler chicken (*Gallus gallus* L.) fed antibiotics (enrofloxacin, colistin and florfenicol), glyphosate and *Bacillus* sp. GL-8: a — group I, b — group II, c — group III, d — group IV; rel. u corresponds to the multiplicity of changes in expression compared with control group I, in which expression was taken as 1 (dashed red line corresponds to the the control expression). For a description of the groups, see the Materials and methods section (day 35, $n = 3$, $M \pm$SEM; vivarium test, OOO BIOTROF+, St. Petersburg, 2022).

*, ** and *** Differences from group I (control) are statistically significant at $p \le 0.05$; $p \le 0.01$ и $p \le 0{,}001$.

With adding antibiotics in group II, the expression of antimicrobial and antiviral genes *Gal9*, *Gal10* and *IRF7* increased by 2.6, 10.5 and 40.8 times, respectively, compared to the control ($p \le 0.05$) (Fig. 3). *Gal9* (*AvBD9*) and *Gal10* (*AvBD10*) are genes associated with the synthesis of avian β-defensins [61]. Defensins promote adaptive immunity through the selective recruitment of monocytes, T-lymphocytes, immature dendritic and mast cells to infection sites [62, 63]. These compounds increase poultry resistance to many pathogens, including *Klebsiella pneumonia*, *Streptococcus bovis*, *Enterococcus faecalis*, *and Salmonella typhimurium* [64]. The *IRF7* gene, in turn, is associated with the synthesis of the regulatory factor interferon 7, a member of the family of regulatory interferon transcription factors [65]. Through its key role in immunity, *IRF7* has been implicated in increasing host resistance to many viruses through a variety of strategies [66]. We suggest that the expression of genes associated with antimicrobial and antiviral protection could be modulated both directly by antibiotics and by the luminal microbiota altered under their influence living in the caecum of broilers.

An increase in the expression of the described genes can also contribute to an increase in the live weight of broilers against the background of antibiotics due to a possible decrease in the pathogen load. Earlier T. Terada et al. [67] studied the effect of dietary antibiotics (penicillin and streptomycin) on gene expression in the caecum of broiler chickens. It was shown that on day 7 the expression of *AvBD1* and *AvBD2* decreased. However, on day 14, in the group treated with antibiotics, the expression of *TLR21* (toll-like receptor involved in antimicrobial protection) and antimicrobial peptide genes increased compared to the

control. In another study on 6-day-old chickens treated with enrofloxacin during the first 5 days of life, the antibiotic did not have a suppressive effect on the lymphocyte subpopulation [11].

In our experiment, glyphosate combined with antibiotics (group III) acted as a suppressor of the *Gal9*, *Gal10*, and *IRF7* expression compared to group II ($p \leq 0.05$). The decrease in the expression of the *Gal9* and *IRF7* genes in group III corresponded to the control without antibiotics ($p > 0.05$). The data obtained may indicate that glyphosate, present in feed even at the level of 1MPC (maximum permissible concentration), negatively affects the immune system, while reducing the therapeutic and zootechnical effects of antibiotics. This may partly explain the negative effect of glyphosate on broiler performance at the end of the experiment. Previously, similar data were obtained using the organochlorine pesticide dieldrin in rats [68]. Treatment of dopaminergic neuronal cells with dieldrin significantly reduced the expression of many genes, including antiviral response (*IFN*) genes [68].

The addition of *Bacillus* sp. GL-8 in feed against the background of glyphosate and antibiotics (group IV) led to increased expression of *Gal9* compared to group III (administration of glyphosate with antibiotics without a bacterial strain) ($p \leq 0.05$). Such results may indicate a certain prospect of reducing the negative impact of glyphosate on the mechanisms of antimicrobial and antiviral defense when using microorganisms with beneficial properties.

As for the antimicrobial and antiviral protection genes, there was a tendency to a sharp increase in the expression of pro-inflammatory genes *IL6*, *IL8* and *PTGS2* (4.6-fold, 11.2-fold, and 6.6-fold, respectively, in group II compared to control) ($p \leq 0.05$), which once again confirms the effect of antibiotics on immune processes (Fig. 4).

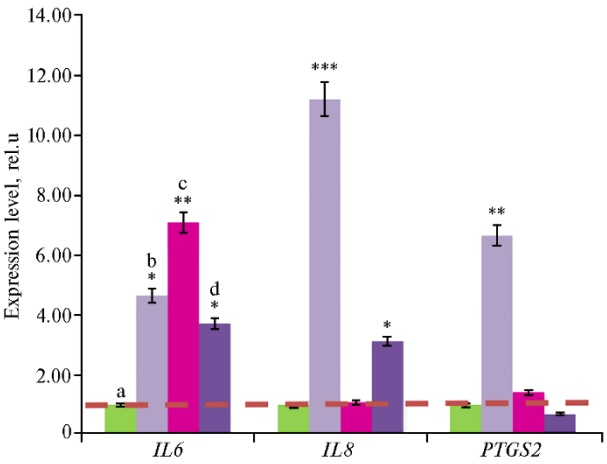

Fig. 4. mRNA expression levels of the proinflamatory genes *IL6, IL8* and *PTGS2* in caecum of cross Ross 308 broiler chicken (*Gallus gallus* L.) fed antibiotics (enrofloxacin, colistin and florfenicol), glyphosate and *Bacillus* sp. GL-8: a — group I, b — group II, c — I group II, d — group IV; rel. u corresponds to the multiplicity of changes in expression compared with control group I, in which expression was taken as 1 (dashed red line corresponds to the the control expression). For a description of the groups, see the Materials and methods section (day 35, $n = 3$, $M \pm SEM$; vivarium test, OOO BIOTROF+, St. Petersburg, 2022).
*, ** and *** Differences from group I (control) are statistically significant at $p \leq 0.05$; $p \leq 0.01$ и $p \leq 0{,}001$.

It is interesting that fluoroquinolones, which include enrofloxacin used in our experiment, affect the gene expression of many cytokines [69]. It has been noted that most fluoroquinolone derivatives superinduce the synthesis of interleukin 2 in vitro, but at the same time inhibit the synthesis of interleukin 1. Increased expression of pro-inflammatory genes when fed with an antibiotic can have various health consequences. On the one hand, interleukins (including *IL6*, *IL8*) are part of important innate protective immune responses, attracting additional leukocytes to the site of infection, which increase the resistance of epithelial cells [70, 71]. On the other hand, overproduction of pro-inflammatory cytokines is involved in the pathogenesis of a number of human diseases, including COVID-19 [72], and

is also associated with a decrease in the productivity of farm animals [73]. It has been proven [74, 75] that the administration of cytokine-based preparations to healthy animals provoked undesirable symptoms. Activation of pro-inflammatory cytokines is closely associated with *PTGS2* gene expression, since cytokines are able to induce it [76]. The *PTGS2* gene is associated with the synthesis of prostaglandin endoperoxide synthase (cyclooxygenase 2), which catalyzes the oxidative conversion of arachidonic acid to prostaglandin. Prostaglandin is subsequently metabolized to various biologically active metabolites such as prostacyclin and thromboxane A2, taking part in both local and systemic inflammatory responses [77].

In our experiment, the effect of glyphosate added to feed on pro-inflammatory genes manifested itself in different ways. Thus, the expression of *IL6* increased in group III compared to group II ($p \leq 0.05$), while the expression of *IL8* and *PTGS2* decreased ($p \leq 0.05$). The fact that pesticides in most cases serve as inducers of *IL6* expression has been reported in most previously published studies. For example, chronic exposure of rats to dichlorvosome (an organophosphorus insecticide) induces microglia activation with induction of NADPH oxidase and pro-inflammatory cytokines, including TNF-α, IL-1β, and IL6 [78]. Y. Zhang et al. [79] revealed an increase in malonic dialdehyde and IL6 in the muscles of rats exposed to omethoate, an insecticide widely used in developing countries. The authors concluded that omethoate may cause insulin resistance. In addition, a cross-sectional study has shown that farmers exposed to organophosphorus pesticides as a result of occupational activity are at risk of developing diabetes [79]. Cytokines produced by adipose tissue, such as TNF-α and IL6, control the secretion of C-reactive protein from the liver [80, 81]. Stimulation of this inflammatory mechanism appears to trigger insulin resistance in peripheral tissues [82].

The use of a microorganism-biodestructor had a positive effect on the expression of pro-inflammatory genes. Thus, *IL6* expression decreased in group IV compared to group III, while *IL8*, on the contrary, increased ($p \leq 0.05$). Indeed, some beneficial bacteria ferment dietary fiber to produce short-chain fatty acids such as acetate, propionate, and butyrate which are absorbed by intestinal cells and used as an energy source for their metabolism [83]. Short chain fatty acids, such as butyrate, have been shown to inhibit NO production and reduce the expression of cytokine genes such as *IL-1β*, *IL6*, *IFN-γ*, and *IL-10* [84].

The introduction of antibiotics into the diet of broilers had some stimulating effect on the expression of the *GSTA3* gene ($p \leq 0.05$) (Fig. 5). Interestingly, the addition of glyphosate to the diet against the background of antibiotics did not change the expression of this gene compared to that in group II ($p > 0.05$). The results obtained seem to be quite natural. The *GSTA3* gene is associated with the synthesis of glutathione-S-transferase, an enzyme responsible for the body's resistance to carcinogens, therapeutic drugs, environmental toxins, and oxidative stress products [85]. This enzyme catalyzes the nucleophilic scavenging of xenobiotics by glutathione, which neutralizes free radicals due to the high electron donating capacity of its sulfidryl (−SH) group and prevents damage to important cellular components, thereby participating in cellular defense against toxic substances. Glutathione S-transferase is abundant in the liver, gastrointestinal tract, lungs, and kidneys [85]. It is well known that coumarin, ethoxykin, aflatoxin B_1 and other compounds such as phenolic antioxidants and isothiocyanates act as inducers of xenobiotic metabolism enzymes [86-88]. Probably, in our experiment, antibiotics acted as inducers of the *GSTA3* gene, being substances foreign to the body.

The strain *Bacillus* sp. GL-8 had a positive effect on the expression of *GSTA3* (group IV compared to group III) ($p \leq 0.05$), it decreased to control values.

Previously, it was reported that the intestinal microbiota can influence the synthesis of enzymes that metabolize xenobiotics, in the large intestine and liver. The highest concentrations of enzymes that metabolize xenobiotics are observed in nonmicrobial animals [89]. Perhaps the effect of the introduction of the microorganism was associated with a decrease in the toxic load under the influence of antibiotics.

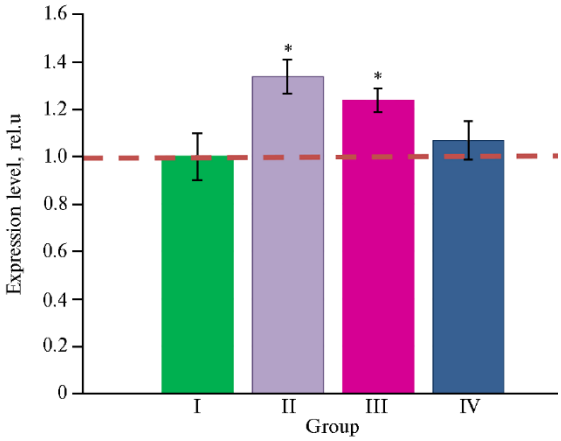

Fig. 5. mRNA expression levels of the *GSTA3* gene associated with esistance to toxic and drag subsyances in caecum of cross Ross 308 broiler chicken (*Gallus gallus* L.) fed antibiotics (enrofloxacin, colistin and florfenicol), glyphosate and *Bacillus* sp. GL-8. Rel. u corresponds to the multiplicity of changes in expression compared with control group I, in which expression was taken as 1 (dashed red line corresponds to the the control expression). For a description of the groups, see the Materials and methods section (day 35, $n = 3$, $M \pm$SEM; vivarium test, OOO BIOTROF+, St. Petersburg, 2022).
* Differences from group I (control) are statistically significant at $p \leq 0.05$.

Discussing the results obtained, it should be noted that information on the cellular and molecular processes by which antibiotics improve animal growth is limited, and the proposed hypotheses are reduced mainly to the possibility of microflora modulation. Understanding the biological mechanism of the action of antibiotics on the stimulation of the growth of farm animals and poultry is necessary to create effective alternatives to antibacterial drugs. Developed preparations should have similar stimulatory activity, but will avoid the problems of developing resistance to antimicrobial agents.

In the presented study, we revealed a positive effect of antibiotics on the performance of broilers, which corresponded to the level of expression of a number of genes, in particular, those associated with the development and differentiation of muscles. In all likelihood, the mechanism of the positive effect of antibiotics on productivity is partly due to the fact that they act as inducers of a number of important genes.

In practice, poultry is exposed not only to medicinal substances, but also to toxicants contained in feed, in particular pesticide residues. The health effects resulting from the synergistic action of antibiotics and pesticides are unpredictable. In our experiment, against the background of glyphosates applied in an amount corresponding to 1MPC, a decrease in the effect of productivity stimulation under the influence of antibiotics was observed in broilers. We have shown that glyphosate exposure occurs, among other things, through disruption of the activity of some key bird genes. The data obtained indicate the need to draw attention to the problem of the content of glyphosates in poultry feed and to clarify the limits of the MPC of glyphosates in feed.

The strain *Bacillus* sp. GL-8, which exhibits the properties of a biodestructor in vitro, did not contribute to a significant improvement in growth rates in broiler chickens with experimental fodder contamination with glyphosate. This indicates the need for selection of microorganisms, taking into account a complex of properties, including survival in the gastrointestinal tract, adhesion and other probiotic characteristics. Nevertheless, the observed positive changes in the transcription of a number of genes, including the genes of antimicrobial and antiviral protection, under the influence of a strain of a biodegrading microorganism indicate

the promise of using probiotics as a tool to mitigate the physiological imbalance against the background of the use of drugs and food contamination with toxic substances. In the future, it is of interest to accurately identify species and study other important probiotic properties and technological characteristics of the *Bacillus* sp. strain. GL-8, such as its resistance to antibiotics and drugs used to feed poultry.

This study presents the results of a complex multicomponent experiment in which we used three additives in the feed of broiler chickens with different effects and purposes (antibiotics, pesticide, *Bacillus* sp. strain). Of course, this complicates the interpretation of the results. For example, a bacterial strain could affect the productivity and expression of some genes in birds regardless of the administration of antibiotics, and antibiotics, in turn, could prevent colonization of the bird's intestines by this strain. In subsequent experiments, it is important to establish the exact effect of bacterial destructor strains on the productivity and expression of certain genes in birds, as well as the direct effect of antibiotics on the ability of destructor strains to colonize the intestines of birds. It is also of interest to study the colonization of the intestine by strains of probiotic microorganisms.

So, out of 11 studied strains of bacilli, *Bacillus* sp. GL-8 possesses the most pronounced ability to biodegrade glyphosate (53.0±4.10%). Antibiotics enrofloxacin, colistin and florfenicol stimulated the increase in body weight in Ross 308 cross broiler chickens from day 14 of life until the end of the experiment by 4.8-23.3%. By the end of the experiment, the negative effect of glyphosate on the productivity of broilers against the background of antibiotics was manifested. Expression of the *MYOG* gene mRNA which promotes muscle development and differentiation was 2.0 times and 2.1 times higher in broilers treated with antibiotics alone or in combination with *Bacillus* sp. GL-8, respectively, compared to control. When glyphosate was added to the feed against the background of an antibiotic without the introduction of a biodegrading microorganism strain, no changes in the expression of the *MYOG* gene were noted. With the introduction of antibiotics, the expression of antimicrobial (*Gal9*, *Gal10*) and antiviral (*IRF7*) protection genes increased by 2.6 times, 10.5 times and 40.8 times, respectively, compared to control. Glyphosate suppressed the expression of antimicrobial and antiviral genes. Dietary *Bacillus* sp. GL-8, when glyphosate and antibiotics were used, increased the expression of *Gal9*. Similar to antimicrobial and antiviral protection genes, the pro-inflammatory genes *IL6*, *IL8*, and *PTGS2* showed a tendency to a sharp increase in expression (by 4.6; 11.2 and 6.6 times, respectively) with the use of antibiotics. The introduction of antibiotics into the diet also had some stimulating effect on the expression of the *GSTA3* gene associated with resistance to toxic and medicinal substances.

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A NEW PRODUCER OF A RECOMBINANT AFLATOXIN-DEGRADING ENZYME OBTAINED VIA HETEROLOGOUS EXPRESSION IN *Pichia pastoris*

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Abstract

Contamination of food and feed with mycotoxins causes significant economic losses in the food and feed industry and poses a serious threat to the human health and animal life because of mutagenic, carcinogenic and other disruptive properties of these secondary metabolites of fungi. Enzymatic degradation of mycotoxins represents an efficient and environmentally safe alternative to the chemical decontamination of agricultural and food products. In this study, a synthetic *adtz* gene encoding ADTZ, an aflatoxin-degrading oxidase from *Armillaria tabescens*, was integrated into the genome of a *Pichia pastoris* GS115 strain under the control of a glyceraldehyde-3-phosphate dehydrogenase promoter. To amplify the *adtz* gene, oligonucleotide sequences were constructed with specific restriction sites HindIII and NotI added to the 5' end. The *adtz* gene-containing pPIG-ADTZ plasmid obtained with the use of the pPIG-1 vector was linearized by digestion with restriction endonuclease ApaI, followed by transforming the cells of *P. pastoris* recipient strain GS115 by electroporation. The transformed yeast cell were selected on YPD medium with an antibiotic. PCR amplification, restriction analysis and Sanger sequencing confirmed insertion of the target gene. As a result, 54 transformed clones containing the target gene were obtained, and the most productive clone secreting the recombinant ADTZ-14 (2.1 mg/ml of the total extracellular protein) was selected. Recombinant ADTZ represented a monomeric protein (78±3 kDa) possessing a high affinity to aflatoxin B_1 (AFB_1). Saving the functional properties of the recombinant protein was shown using experiments on assessment of its ability to degrade AFB_1 during short-time and prolonged incubation. The obtained protein was able to degrade AFB_1 by 14 % after a 2-h incubation at 40 °C; after 72 and 120 h of incubation at 30 °C, the content of AFB_1 in ADTZ-14 culture liquid (CL) reduced by 50 and 80 %, respectively, compared to content in CL of non-transformed control GS115. These data suggest a quite high biotechnological potential of a new recombinant ADTZ preparation in relation to the decontamination of agricultural products contaminated with AFB_1. Thus, the earlier developed expression system intended to increase the copy number of heterologous genes in *Pichia pastoris* was first used to obtain a recombinant protein able to degrade AFB_1. Using this approach, we transformed yeast cells with the pPIG-ADTZ plasmid and obtained 154 recombinant clones of *P. pastoris*, 77 % of which contained the target sequence of the *adtz* gene. Productivity of the best transformant (clone ADTZ-14) was 2.1 mg of protein per 1 ml of culture liquid, and about half of the pool of the extracellular proteins fell to the share of recombinant ADTZ able to degrade 80 % of AFB_1 incubated in cell-free culture broth at 30 °C and pH 7.0.

Keywords: aflatoxin B_1, mycotoxins, enzymatic degradation, ADTZ from *Armillaria tabescens*, synthetic *adtz* gene, recombinant proteins, heterologous expression, *Pichia pastoris*

Aflatoxins, a group of structurally similar secondary metabolites of the

fungal genus *Aspergillius* widely distributed in nature, are known as dangerous mycotoxins that contaminate feed and other agricultural products [1-4]. Currently, more than 20 aflatoxins (AF), their derivatives and closely related compounds have been identified [5]. Contamination of feeds for livestock and poultry with B- and G-type AF raises the most serious concern [6, 7]. These mycotoxins are derivatives of difuranocoumarin which have a bifuran group linked to the coumarin core and a cyclopentane (in B-type AF) or lactone ring (in G-type AF) [8, 9]. Due to the toxicity, carcinogenicity and mutagenicity of these compounds, and their resistance to heat treatments [10, 11], feed and other crop products contaminated with AF above the concentrations allowed by hygienic regulations are not suitable for direct use or further processing into food products. Globally, contamination with these mycotoxins, especially AFB_1 which surpasses all other AFs in hepatotoxicity and danger to warm-blooded animals [6, 7], causes serious economic damage to both agriculture and the food industry, and also create risks for human health [1, 3].

For decontamination, physical, chemical, and microbiological methods are used, which, however, have a number of well-known limitations [1, 12]. Therefore, there is a constant search for other effective, environmentally friendly means and methods of AF degradation and detoxification that do not affect the quality of agricultural products. From this point of view, an approach based on the ability of a number of fungi [13-15] and bacteria [16-19] to synthesize enzymes that transform AF to non-toxic or less toxic compounds seems to be very promising [20, 22]. The use of cell-free preparations containing such enzymes makes it possible to avoid problems that may arise when using the producers themselves (for example, deterioration of the organoleptic properties of processed products, a decrease in their nutritional value). In addition, enzyme preparations are technologically more convenient for feed processing [23] and, unlike those for the food industry, do not require expensive multi-stage purification of the target product.

It is known that some xylotrophic basidiomycetes of the genera *Pleurotus* [24, 25], *Phanerochaete*, and *Armillaria* [26-28] can be sources of enzymes for AF degrading and detoxifying. An enzyme with oxidase activity [28] called by the authors aflatoxin-detoxifizyme (ADTZ) was isolated from the mycelium of *Armillaria tabescens* using hydrophobic and metal chelate chromatography. It turned out that ADTZ can catalyze the opening and subsequent hydrolysis of the difuran ring [29], a structure associated with B-type AF toxicity. Further studies have shown that ADTZ is a 76 kDa monomeric protein with high affinity for AFB_1 [29]. Upon contact with ADTZ, the toxicity and mutagenicity of AFB_1 were significantly reduced [28, 30].

These data indicate the prospects for the development of detoxifying drugs containing ADTZ. However, their creation is primarily hampered by the lack of available technology for obtaining intracellular ADTZ from *A. tabescens* mycelium and, in part, by the fact that deep cultures of *A. tabescens* requires liquid media of complex composition, including very specific and expensive components [31], or a multi-stage fermentation procedure [28]. These obstacles could be overcome by using a heterologous expression system and creating an accessible producer of the recombinant ADTZ protein. However, there is still no suitable system for obtaining extracellular heterologous ADTZ in an amount sufficient for its use in decontamination of crop products. Nevertheless, a number of modern works [32, 33] report on the successful use of *Pichia pastoris* yeast cells as recipients for heterologous expression.

Previously, we adapted the expression system in *P. pastoris* by modifying the integration vector to increase the copy number of heterologous genes in the yeast chromosome (the integration vector and its preparation are patented) [34]. In the present study, this approach was used for the first time to create a new producer of the aflatoxin-degrading enzyme.

Our goal was to optimize and use this system for the heterologous expression of ADTZ in *Pichia pastoris* GS115 and to evaluate the ability of a cell-free culture liquid (CL) preparation of the resulting *P. pastoris* ADTZ-14 strain containing the extracellular recombinant ADTZ enzyme to degrade AFB_1.

Materials and methods. For the expression of the *adtz* gene encoding aflatoxin-detoxifizim, the yeast strain *Pichia pastoris* GS115 (syn. *Komagataella phaffii*) (Thermo Fisher Scientific, USA) was used. Yeast cells were cultured for 3 days at 30 °C in liquid YPD medium (glucose 20.0 g/l; yeast extract 10.0 g/l; meat peptone 20.0 g/l). For plasmid DNA, *Escherichia coli* XL1-Blue (Agilent, USA) was grown at 37 °C in Luria-Bertrani medium (tryptone 10 g/l; yeast extract 5 g/l; NaCl 5 g/l; pH 7.2-7.5). The pPIG-1 plasmid was used to express ADTZ [34]. The *adtz* gene encoding the aflatoxin degradation enzyme in *A. tabescens* (GenBank AY941095.1) was synthesized at ZAO Evrogen (Russia) according to the codon compositions in *P. pastoris*.

PCR mix for the *adtz* gene amplification (50 μl) contained 1× buffer with 3 mM $MgCl_2$ and 5 U Taq polymerase (NEB, UK), 0.2 μM ADTZ-fwd (5′-gaagcttctATGGCTACTACAACTG-3′) and ADTZ-rev (5′-cgcggccgcTTACAATCTTCTCTC-3′) oligonucleotides, and 0.1 ng DNA as a matrix. The reaction was carried out under the following conditions: 95 °C for 15 s, 62 °C for 15 s, and 72 °C for 120 s (25 cycles) (a T-100 amplifier, Bio-RAD, USA). The PCR products were evaluated electrophoretically (a 1% agarose gel, a Sub-Cell GT Cell, Bio-RAD, USA).

The amplification product, vector pPIG-1, was digested with HindIII and NotI restriction endonucleases according to the manufacturer's recommendations (NEB, UK).

The processed fragments were ligated with T4 DNA ligase (ZAO Evrogen, Russia), and *Escherichia coli* XL1-blue cells (Agilent, USA) were transformed with the 2 μl mixture by the heat shock method. Transformants were selected on Luria-Bertrani agar medium containing ampicillin (100 μg/ml). The pPIG-ADTZ plasmid was isolated from ampicillin-resistant transformants using the Plasmid Mini-prep kit (ZAO Evrogen, Russia). The presence of the target gene insert in the pPIG-ADTZ plasmid was confirmed by PCR amplification, restriction analysis as described above, and Sanger sequencing. Sequencing was performed in both directions from primers used for the gene amplification. Gene sequencing and synthesis of primers used for amplification were performed at OOO Sintol (Russia).

The pPIG-ADTZ plasmid was linearized by digestion with restriction endonuclease ApaI (NEB, UK) according to the manufacturer's protocol and transferred into *P. pastoris* GS115 by electroporation [35]. Transformants were selected on a YPD agar medium supplemented with 200 μg/ml antibiotic zeocin (Thermo Fisher Scientific, USA). DNA was isolated from antibiotic-resistant colonies [36] and the ADTZ insert was checked by PCR.

The recombinant ADTZ protein was produced by culturing the *P. pastoris* ADTZ-14 producer strain in 24-well plates (3 ml YNB liquid medium, 30 °C, aeration 200 rpm, 3 days). Every 24 hours, 40% glucose solution in 20 mM

potassium phosphate buffer (pH 6.0) was added to the wells to a final concentration of 2%.

The recombinant ADTZ in cell-free CL was detected by polyacrylamide gel electrophoresis with sodium dodecyl sulfate (DNS-PAGE, Mini-PROTEAN® Tetra, Bio-RAD, USA). Total protein concentration was measured by the Lowry method [37].

The degradation kinetics of AFB_1 and AFG_1 was studied in experiments with short-term incubation of *P. pastoris* ADTZ-14 cell-free CL. Commercial preparations of AFB_1 and AFG_1 (VNIIVSGE, Russia) were dissolved in 20 mM Na-phosphate buffer (pH 6.7) to a final concentration of 2.5 μg/ml each. The concentration was controlled using the molar extinction coefficients $\varepsilon = 21800$ and $\varepsilon = 17700$ (at $\lambda = 362$ nm) for AFB_1 and AFG_1, respectively. The CL of the *P. pastoris* ADTZ-14 transformant was incubated with toxins in the wells of a thermostated autosampler plate (30 or 40 °C). A 5 μl aliquots were taken from the reaction mixture every 30 min for 2.5 h and the AF content was determined by reverse-phase chromatography on a thermostated (30 °C) Kromasil Ethernity 5-C18 column (4.6×250 mm) (Akzo Nobel, Sweden) equipped with an appropriate guard column. An Agilent 1200 chromatographic system (Agilent Technologies, USA) with diode array detection was used. Chromatographic separation was performed in a water/acetonitrile gradient (from 40% to 68% acetonitrile in 20 min, detection at 360, 235, and 225 nm, slit width 8 nm). The degree of AFB_1 and AFG_1 degradation was assessed by the change in the area of the corresponding chromatographic peak. The CL of the untransformed strain *P. pastoris* GS115 was a control.

To assess the ability of the recombinant ADTZ enzyme to degrade AFB_1 during prolonged incubation, 1 ml of *P. pastoris* ADTZ-14 CL samples (2.1 mg total protein/ml) after pre-sterilization by filtration (membranes with a pore size of 0.22 μm, Millipore, USA) were added with 1.0 μg of AFB_1 (Sigma-Aldrich, USA) dissolved in a minimum volume of methanol. Samples (1 ml) of CL of non-transformed strain *P. pastoris* GS115 addedd with the same amount of AFB_1 were used as a control. Samples were incubated for 3 and 5 days at pH 7.0 and 30 °C. The post-incubztion AFB_1 concentration was measured using high-performance liquid chromatography (HPLC) on a thermostated (27 °C) Symmetry C18 column (5 μm, 150×4.6 mm) in isocratic elution mode (mobile phase methanol:water 60:40, 10 μl sample injected, $\lambda = 362$ nm) using a Waters 1525 Breeze system with a Waters UV 2487 detector (Waters Corp., USA) [12, 38]. Prior to HPLC analysis, CL samples were diluted 100-fold with the mobile phase. Toxin concentrations were measured in the linear detection range in the test and control samples, The concentrations were calculated from the calibration curve for the AFB_1 standard (Sigma-Aldrich, USA). The percentage of degradation was determined relative to the amount of toxin detected in the corresponding control sample.

Statistical processing of AFB_1 quantification data was performed using the STATISTICA 6.1 program (StatSoft, Inc., USA). Significance of differences at $p \leq 0.05$ was confirmed using Student's *t*-test for independent variables. The table and figures indicate the mean values (*M*) of two measurements for each of the three biological repetitions with standard deviations (±SD).

Results. The gene for the aflatoxin-degrading enzyme from *A. tabescens* was cloned by PCR using the developed oligonucleotides.

The size of the amplification product corresponding to the synthesized *adtz* gene was 2088 bp. Sequencing of the obtained product confirmed its identity with the sequence of *A. tabescens* (GenBank AY941095.1) (Fig. 1).

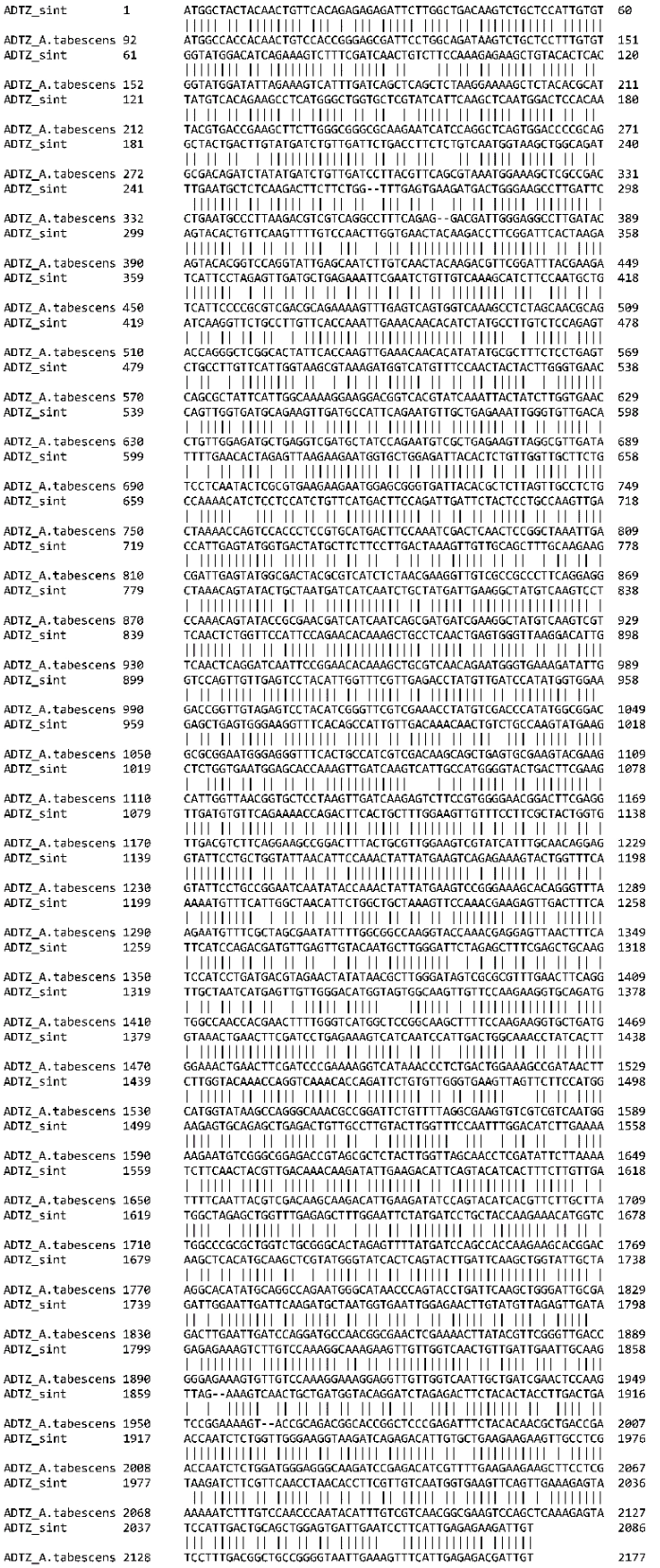


Fig. 1. Sequence alignment visualization of the codon-optimized *adtz* gene and the natural *adtz* gene

of *Armillaria tabescens* (GenBank AY941095.1, https://www.ncbi.nlm.nih.gov/genbank/). Optimization of the codon composition was carried out by ZAO Evrogen (Russia) using the codon frequency table for *Pichia pastoris* (https://www.kazusa.or.jp).

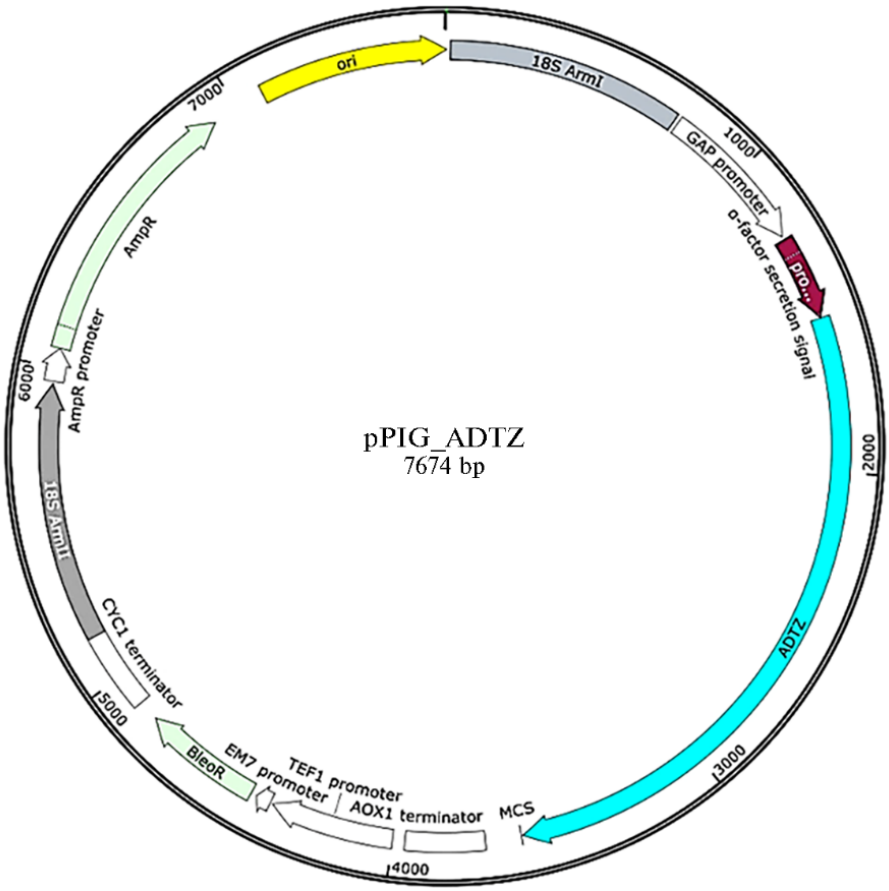
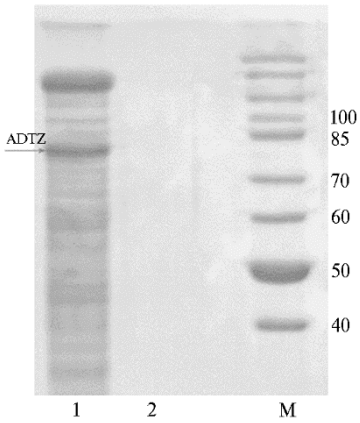


Fig. 2. A map of the pPIG-ADTZ plasmid obtained by cloning the sequence of the synthesized aflatoxin-detoxphyzyme (ADTZ) ***adtz*** **(*synthetic_ADTZ*) gene into the pPIG-1 vector.**

The resulting recombinant gene was integrated into the pPIG-1 vector by the restriction ligation method. Restriction analysis of the resulting new plasmid, after double digestion with HindIII and NotI, resulted in 5500 and 2100 bp products, confirming the correct integration of the target sequence into the pPIG-1 vector. The resulting recombinant plasmid (Fig. 2) was called pPIG-ADTZ.

Plasmid pPIG-ADTZ, linearized with restriction endonuclease ApaI, was electroporated into competent *P. pastoris* GS115 cells, and transformants were selected on YPD medium added with zeocin. Cloning resulted in 154 clonal colonies. Genomic DNA isolated from 70 randomly selected transformed clones was analyzed by PCR to identify the *adtz* gene insert. PCR analysis of the DNA of these transformants grown on the selective media showed that at least 54 clones contained the target *adtz* insert. Among them, the ADTZ-14 clone turned out to be the most productive and was used for further work. Already after 72 h of culture, expression of ADTZ in this clone led to the accumulation in the CL of the extracellular recombinant protein. The size of this protein according to the SDS-PAGE analysis was 78±3 kDa (Fig. 3), while in the CL of the nontransformed recipient *P. pastoris* GS115 we did not find proteins of a comparable size. In the CL of the transformed ADTZ-14 clone, the total protein concentration was 2.1 mg/ml.



Fid. 3. Electrophoregram of the culture liquid proteins of the strain *Pichia pastoris* ADTZ-14 (1) **transformed with the recombinant pPIG-ADTZ vector containing the synthesized aflatoxin-detoxifizyme (ADTZ) *adtz* gene, and the recipient *P. pastoris* GS115** (2). M — molecular weight marker PageRuler™ 26614 (Thermo Fisher Scientific, USA).

Comparison of the degradation kinetics of AFB_1 and AFG_1 in CL of *P. pastoris* ADTZ-14 showed that the recombinant enzyme is capable of destroying both toxins; however, its efficiency against AFB_1 turned out to be significantly higher than for AFG_1. Thus, under the action of CL of the producer of recombinant extracellular ADTZ, already after 2 h of incubation, the concentration of AFB_1 toxin decreased by approximately 14% compared to the initial level, while for AFG1 the decrease was only 4% (Fig. 4).

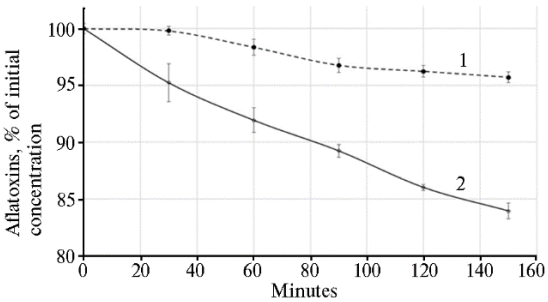


Fig. 4. Degradation kinetics of aflatoxins G_1 (1) **and B_1** (2) **in the cell-free culture fluid of the *Pichia pastoris* ADTZ-14 strain transformed with the pPIG-ADTZ recombinant vector conyaining the synthesized aflatoxin-detoxphyzyme** (ADTZ) ***adtz*** **gene at 40 °C and pH 6.7** (n = 3, M±SD).

The obtained results were consistent with the data of other authors who noted the high specificity of intracellular ADTZ from *A. tabescens* to AFV_1 [29]. In this regard, we studied the degradation activity of recombinant ADTZ with respect to the indicated toxin during its longer incubation with cell-free CL of the *P. pastoris* ADTZ-14 strain.

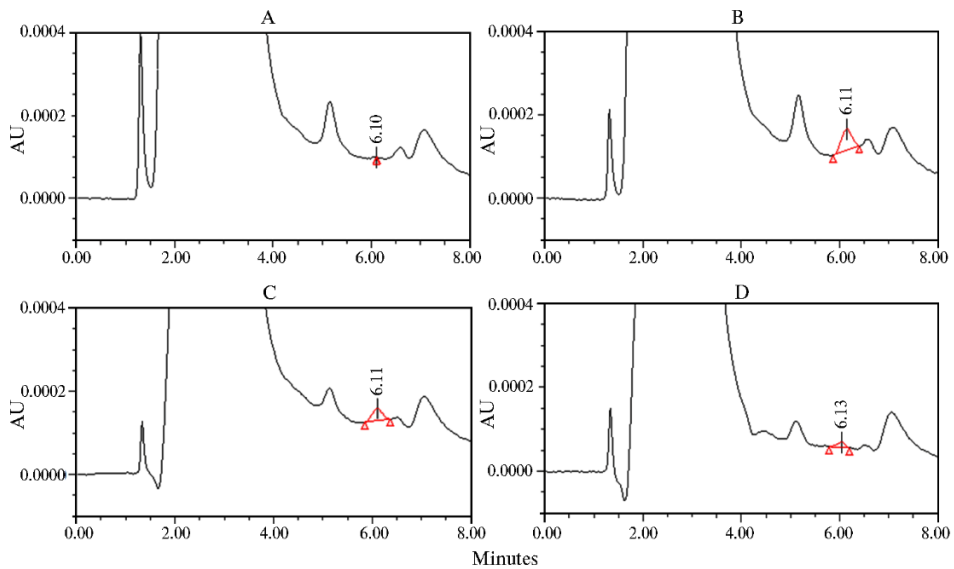


Fig. 5. Chromatograms of culture liquid (CL) **samples of Pichia pastoris** (incubation at 30 °C and pH 7.0).

A: CL of the non-transformed recipient strain GS115 (control).

B: CL GS115 + aflatoxin B_1 (APB^1, 1 μg/ml) after incubation (control sample). The peak on the chromatogram corresponds to 10 ng of AFB_1 in the injected sample.

C and D: CL of the *P. pastoris* ADTZ-14 strain transformed with the pPIG-ADTZ recom-

binant vector containing the synthesized aflatoxin-detoxiphyzyme (ADTZ) *adtz* gene, + AFV_1 (1 μg/ml) after 3 and 5 days of incubation, respectively.

In these experiments, it was found that after 3 days the concentration of the toxin added to the CL decreased by almost 2 times, and after 5 days the efficiency of its degradation reached 80% (Fig. 5, Table).

Dynamics of enzymatic degradation of aflatoxin B_1 (AFB_1) in the cell-free culture liquid (CL) **of the *Pichia pastoris* ADTZ-14 transformant straindecreting the recombinant aflatoxin-detoxphyzyme (ADTZ)** ($n = 6$, $M \pm SD$)

Strain	Incubation time				
	0 h	72 h		120 h	
	AFB_1, μg/ml	AFB_1, μg/ml	degradation, %	AFB_1, μg/ml	degradation, %
ADTZ-14	0.97±0.01	0.57±0.06	41.2[a]	0.19±0.04	80.4[b]
GS115 (control)	0.98±0.01	1.01±0.01	0.0[c]	0.90±0.05	0.1[c]

N o t e. The ADTZ-14 strain was obtained by transformation of the *P. pastoris* GS115 recipient with the pPIG-ADTZ recombinant vector with the synthesized aflatoxin-detoxphyzyme (ADTZ) *adtz* gene. Before incubation, AFB_1 was added to the culture liquid (CL) to a concentration of 1 μg/ml; for 0 h, the concentrations detected in the CL samples before incubation are indicated (opening from 96 to 99%).

[abc] Differences between percent degradation marked with different letters are statistically significant at $p \leq 0.05$.

The data we presented here indicate a rather high biotechnological potential of the new producer of recombinant ADTZ and expand the so far limited spectrum of recombinant enzymes of other xylothorophic fungi degrading AFB_1 obtained using the system of heterologous expression in *P. pastoris* [39].

It should also be noted that the ADTZ-14 producer was characterized by a rather high level of expression of extracellular proteins for *P. pastoris*. Probably, the use of a synthetic gene with optimized codons contributed to an increase in the productivity of yeast cells. A similar approach has been successfully used previously for the expression of bacterial α-amylase in *P. pastoris* [40]. However, according to recent data, the use of synthetic genes can lead to protein misfolding, degradation, and a decrease in its activity and stability. This may be the cause of partial degradation of secreted recombinant proteins [41], which, as noted above, we also observed in our experiments with electrophoretic analysis of the CL of the transformed clone ADTZ-14. Therefore, it is necessary to continue research on increasing the efficiency of AFB_1 degradation by recombinant ADTZ, additional testing of the effect of this recombinant enzyme on other aflatoxins, as well as experiments on the treatment of crop products contaminated with AFB_1 with the enzyme preparation. It is also possible that heterologous expression using other eukaryotes, e.g., filamentous fungi which are used for bioprocessing of feed to increase its nutritional value, will allow for obtaining new producers of highly active extracellular ADTZ. Such producers could be promising for the simultaneous decontamination of plant raw material contaminated with aflatoxin and increasing the availability of their nutritional components.

Thus, this article is the first report on the production of a recombinant protein capable of cleaving AFB_1 using an expression system developed by us earlier to increase the copy number of heterologous genes in *Pichia pastoris*. Yeast cells were transformed with the pPIG-ADTZ plasmid and 154 recombinant clones of *P. pastoris* were generated, 77% of which contained the target sequence of the ADTZ synthetic aflatoxin-detoxiphyzyme *adtz* gene. The protein yield of the most productive ADTZ-14 transformant was 2.1 mg/ml cell-free culture liquid, and in this case, about half of all extracellular protein pool was recombinant ADTZ. Incubation of AFB_1 with this recombinant ADTZ led to 80% degradation of the added toxin. The transformant strain *P. pastoris* ADTZ-14, secreting functional ADTZ, can be a producer of an accessible and sufficiently active recombinant enzyme for AFB_1 degradation. Based on *P. pastoris* ADTZ-14, preparations for the enzymatic degradation of AFB_1 can be developed in the future. Confirmation

of the decontamination potential of the recombinant enzyme will indicate the feasibility of optimizing biotechnology to increase the yield of the target product and develop its formulation.

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EFFECT OF EXTRACELLULAR VESICLES OF FOLLICULAR ORIGIN DURING in vitro MATURATION AND AGEING OF BOVINE OOCYTES ON EMBRYO DEVELOPMENT AFTER in vitro FERTILIZATION

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Abstract

Extracellular vesicles (EVs) isolated from ovarian follicular fluid (FF) are involved in vivo in the regulation of meiosis in female gametes. Recent studies suggested follicular EVs as potential regulators of oocyte quality capable to increase the efficiency of embryo production technologies in vitro (in vitro embryo production, IVP). In this work for the first time, we have analyzed embryo development competence of bovine oocytes after in vitro maturation (IVM) in the presence of EVs and ageing before in vitro fertilization. The aim of the study was to determine the effects of these conditions to oocyte ageing-related transformations during IVM in terms of their ability to develop blastocysts, as well as the quality of IVP embryos. The EVs were separated from FF by serial centrifugations and final ultracentrifugation at 100,000g. The isolated EV fraction contained 37.5 μg of total protein per ml of FF. Isolated vesicular fractions were analyzed using transmission electron microscopy that confirmed their enrichment in exosome-like EVs. For functional experiments, cumulus-oocyte complexes (COCs) were in vitro maturated in TC-199 medium containing 3 mg/ml of bovine serum albumin, 0.5 mM sodium pyruvate and 100 ng/ml EGF in the absence (control) or presence of EVs. Vesicles were added to IVM culture at the physiological concentration (EVs isolated from 1 ml of FF were added to 1 ml of IVM medium). After 24 hours of maturation, COCs were transferred to an ageing medium, cultured for further 12 hours, then in vitro fertilized and cultured for embryo development. At day 3 after in vitro fertilization, morphological evaluation of cleaved oocytes was carried out, and at day 7, the number of embryos developed to blastocyst stage and their quality were evaluated. The number of nuclei per embryo, calculated using the cytological method, served as an assessment of the quality of the embryo. In four independent experiments performed, the number of COCs in each group varied from 116 to 121. The cleavage rate of control oocytes was lower than that in the experimental EVs group (53.5 2.9 vs. 63.8 2.9 %, respectively, $p < 0.05$). In addition, EVs had a positive effect on embryo development up to the blastocyst stage after IVM and aging of the oocytes. In control, blastocyst rate was 17.3±1.6 %, and the presence of EVs during IVM increased this rate to 20.2±2.5 % ($p < 0.05$), whereas quality of produced embryos did not change. According to the reported data, EVs from follicular fluid added during IVM may increase the resistance of bovine oocytes to age transformations and, consequently, improve oocyte competence to embryo development after aging in vitro. Therefore, EVs can improve extracorporeal oocyte maturation and the efficiency of in vitro embryo production techniques in cattle.

Keywords: extracellular vesicles, bovine follicular fluid, bovine oocytes, in vitro maturation,

In vitro embryo production (IVP) biotechnology has broad prospects for research application and serves as the effective way to speed up genetic progress in breeding domestic animals, including cattle [1]. To date, a notable progress has been made in improving this technology, but the quality of embryos developed in vitro is still lower than in vivo [2, 3]. A limiting factors affecting the usefulness of IVP embryos is the quality of oocytes acquired during in vitro maturation (IVM) [4, 5]. However, IVM conditions still remain suboptimal and require detailing.

In standard practice, the modernization of maturation systems is aimed primarily at simulating the conditions that occur in vivo in ovarian follicles [5, 6]. A variety of molecular factors, e.g., hormones, steroids, growth factors, fatty acids, and various metabolites, are added to culture media to improve oocyte maturation [7]. Interest in thee substances is due to their presence in the follicular fluid (FF) of antral ovarian follicles, which, in turn, provides an optimal environment for oocyte growth, meiotic maturation, and acquisition of competence for future embryonic development [8]. The impact of these factors is manifested in ovarian follicles with a close bidirectional connection between the oocyte and the surrounding follicular cells [9, 10].

Recent basic research has identified secretory extracellular vesicles (EVs), including exosomes and microbubbles, as new participants in intercellular communication that are secreted by cells and can transport various regulatory factors to other cells capable of absorbing these EVs [11, 12]. In bovine ovarian follicles, EVs are present in FF [13]. They are involved in the transfer of various RNAs, proteins and lipids [14] and participate in the regulation of meiosis in the ovule and early development of the embryo [15, 16]. Such participants in intercellular communications are considered as potential regulators of oocyte quality and their competence for embryonic development under in vitro conditions and are actively studied in this aspect [17-19].

It should also be noted that the existing modern approaches to improving the efficiency of embryonic IVP technology take into account changes in the functional state of cumulus-oocyte complexes (COCs), mainly during oocyte maturation. Nevertheless, in different species, including *Bos taurus*, after the completion of the first division of meiosis in oocytes, aging processes are initiated that adversely affect the quality of mature ovules and their competence for further embryonic development [20, 21]. Under in vitro conditions, ovules isolated from different ovarian follicles and from different donor cows are heterogeneous in potential for development during in vitro maturation. In in vitro culture, some oocytes can mature, i.e., can reach the metaphase II stage of meiosis, much earlier than the period of in vitro fertilization, which leads to their earlier aging and a loss of quality compared to the rest of the population of maturing cells [22]. It is known that any delay in the oocyte fertilization can lead to low viability of embryos and to a weakening of fertility and a reduction in life expectancy of offspring if the offspring was born [20, 21, 23]. Cumulus somatic cells associated with the oocyte, which undergo apoptotic degeneration upon completion of the maturation of female gametes, can accelerate certain negative changes caused by aging [23]. The problem of rapid aging of oocytes which occurs primarily at the molecular cytoplasmic level is increasingly being addressed in the development of scientific approaches to modifying in vitro oocyte maturation systems [24-26]. However, the involvement of EVs in the regulation of aging has not yet been established.

In the present work, we for the first time investigated the competence for embryonic development in oocytes of cows (*Bos taurus*) when cultured in the presence of EVs during maturation and aging in vitro before in vitro fertilization.

The work aimed to study the influence of the tested conditions on age-related transformations in mature oocytes during IVM in terms of their further ability to develop to the blastocyst stage and also to assess the quality of the resulting embryos.

Materials and methods. In all experiments, except for specially indicated cases, reagents from Sigma-Aldrich (USA) were used.

To obtain EVs from FF, isolated post mortem cow ovaries were delivered to the laboratory on ice, freed from surrounding tissues, and repeatedly washed in sterile saline with 100 IU/ml penicillin and 50 rg/ml streptomycin (BioPharm-Garant, Russia). The saline was cooled to +4 °C. FF was aspirated from antral follicles of 3-6 mm in diameter, 2 ml of FF were centrifuged for 15 min at 300 g and room temperature. The supernatant, free from somatic cells, was transferred into new sterile tubes. At the next stage, FF was freed from apoptotic bodies (1-5 μm in size) and large microvesicles (200-1000 nm in size) by centrifugation for 15 min at 2000 and 12000 g, respectively. EVs were isolated from purified VF by ultracentrifugation (CS 150 NX centrifuge, Hitachi, Japan) for 90 min at 100,000 g; same mode. The precipitates were pooled, diluted in 100 rl of PBS, and stored at –80 °C until use, after taking two 5 μl aliquots of from the resulting volume for EVs quantification by protein concentration and for ultrastructural analysis of the particle preparation using transmission electron microscopy (TEM).

The protein concentration was measured on a Qubit 4 Fluorometer using a Qubit Protein Assay Kit (Thermo Fisher Scientific, USA) and a Qubit protein standard with a concentration of 0.125 to 5 mg/ml.

For a morphological study, 5 μl of the EVs suspension was mixed with 5 μl of a 2% glutaraldehyde for electron microscopy (EM) (Agar Scientific, Ltd., UK) and allowed for 1 h at room temperature. Suspensions of thus fixed EVs (2 μl) were applied to nickel EM grids (Agar Scientific, Ltd., UK) coated with formvar carbon film. The samples were incubated for 60 min in a humid chamber, and the grids were washed with distilled water (3 times 10 s) by applying a 10 μl drop to the surface and water removing by touching the grid edge with the filter paper. Next, negative contrasting staining was performed with a 2% aqueous solution of uranyl acetate (Electron Microscopy Science, USA). A 10 μl drop of uranyl acetate was applied to the grid with the EVs preparation, the procedure was repeated three times with an interval of 10 s, after the removal of the last drop, the grid was dried in air. The preparations were examined using a JEOL 1011 transmission electron microscope (JEOL, Ltd., Japan) and photographed (GATAN RIO 9 camera, DigitalMicrograph3 program, Gatan, Inc., USA). Based on the TEM analysis, the presence of EVs in the samples was established and their morphology was evaluated.

For IVP experiments, isolated post mortem cow ovaries were delivered from the meat processing plant to the laboratory in warm saline and dissected as described above. Cumulus-oocyte complexes (COC) were isolated from the ovaries by dissecting the walls of the follicles with a blade, washed in TC-199 medium containing 5% fetal bovine serum (FBS), heparin (10 μg/ml) and gentamicin (50 μg/ml). The morphology of the isolated COCs was examined. For further culture, we selected rounded oocytes with homogeneous cytoplasm, a pellucid zone of uniform width, which were surrounded by several compact layers of cumulus cells.

The COCs selected by quality for in vitro maturation were cultured in 4-well plates (Biomedical, Russia) in groups (approx. 30 oocytes) in 500 μl of TS-199 medium. The medium contained 3 mg/ml of bovine serum albumin (BSA), 0.5 mM sodium pyruvate, 100 ng/ml epidermal growth factor EGF (Thermo Fisher Scientific, USA), and 50 μg/ml gentamicin in the absence (control) or

presence of EVs. Vesicular protein was added to the maturation medium at a physiological concentration (per 1 ml, the number of EVs isolated from 1 ml of FF). Drops of medium were covered with 500 μl of light mineral oil and cultured in an incubator at 38.5 °C and 5% CO_2. After 24 h according to the IVM procedure, mature COCs were transferred to the aging medium and cultured for another 10 h. For prolonged culture, a medium of the same composition was used, but without EVs.

After a period of aging, COCs were transferred to BO-IVF medium (IVF Bioscience, UK) for in vitro fertilization. Active spermatozoa obtained by the swim-up method as described previously [26] were added to the oocyte wells (final concentration 1×10^6 spermatozoa/ml). In all experiments, frozen-thawed sperm of the same bull was used for fertilization. Oocyte fertilization in vitro was performed in 4-well plates (Biomedical, Russia) in 500 μl medium drops coated with 500 μl mineral oil. Germ cells were co-cultured for 15-16 h, then the oocytes were freed from cumulus cells and adhering spermatozoa. Putative zygotes were transferred to the embryo development medium (commercial medium BO-IVC, IVF Bioscience, UK), the drops were completely covered with mineral oil and cultured at 38.5 °C in an incubator with 5% CO_2, 5% O_2 and 90% N_2. In 3 s after fertilization, the medium was replaced with a fresh medium and the fragmented zygotes was morphologically assessed; on day 7, the number of embryos that developed to the blastocyst stage was estimated.

The quality of embryos was assessed cytologically by the total number of nuclei. The 7-day-old embryos were fixed with 4% paraformaldehyde solution in sodium phosphate buffer for 60 min at room temperature. After fixation, the embryos were permeabilized for 30 min in a 0.1% sodium citrate solution containing 0.5% Triton X-100, stained for 20 min with a DAPI solution (1 μg/ml) to localize the nuclei, transferred to a dry fat-free glass, and placed in Vectashield medium (Vector Laboratories, UK). For microphotography and evaluation of preparations, a motorized microscope Axio Imager M2 (Carl Zeiss, Germany) with a fluorescent attachment and ZEN 2 pro program (Carl Zeiss, Germany) were used.

Statistical processing was performed by the ANOVA method (the SigmaStat computer program, Systat Software, Inc., USA). Experimental results are submitted as mean values (M) and standard errors of means (±SEM). Tukey's test was used to assess the significance of differences between the compared means. Differences were considered statistically significant at $p < 0.05$.

Results. Extracorporeal maturation is an important step in the IVP technology. Nevertheless, the competence for embryonic development of oocytes maturing in vitro still remains significantly lower vs. in vivo maturation [4, 5]. The study of the nature and mechanisms of the influence of physiological factors, primarily of natural follicular origin, in the regulation of the quality of oocytes during their maturation in vitro can contribute to solving this problem [5-7].

Previously, many authors, as well as our own studies, have shown that the EVs isolated from FF which forms the natural environment of female germ cells, in the in vitro maturation medium increases ability of oocytes for embryonic development to the blastocyst stage after fertilization [27, 28]. In addition, EVs accelerates the development of embryos [17], and improves their quality, including lower apoptosis frequency in blastomeres [27-29]. A possible mechanism for such a positive effect could be an increase in the resistance of mature eggs to age-related changes that occur in the period that precedes the activation of oocytes by sperm and reduces the competence of fertilized oocytes to embryonic development [22].

To test this assumption, in the present work, isolated post mortem oocytes of cows were cultured in IVM medium in the absence (control) or in the presence of a physiological concentration of EVs of follicular origin (experimental group of

EVs). In addition, a comparative assessment was made of the effect of the studied conditions on age-related transformations in mature oocytes during their maturation in vitro in terms of the ability to reach pre-implantation stages of development after subsequent aging and in vitro fertilization. To study the changes associated with oocyte aging, we used the model of prolonged culture [20, 21]. According to the model, COCs after the in vitro maturation for 24 h and before in vitro fertilization were additionally cultured for another 10 h both in the control and test variants. For accuracy of our experiment, COCs both during maturation and further aging were cultured in TCM-199 medium supplemented with 3 mg/ml BSA, 0.5 mM sodium pyruvate and 100 ng/ml EGF, i.e., not containing gonadotropic hormones and serum.

In the experiment, we isolated EVs from 40 ovary follicles, characterized the preparations by the vesicular protein comcentration, and confirmed the presence of EVs in the samples using TEM. The methodology for isolating EVs from bovine follicular fluid was based on differential stepped centrifugation and ultracentrifugation at 100,000 g. As practice shows [17, 19], this methodology is effective in obtaining the fraction of EVs, which are mainly exosomes ranging in size from 30 to 150 nm. The described technique was first proposed for the isolation of EVs from the oviduct fluid [30, 31] and used by us in a partial modification [19].

Thus, we isolated 37.5 μg of vesicular protein per 1 ml of FF. The total pool collected from the entire FF volume was analyzed using TEM, which confirmed the presence of exosome-type vesicles in the sample. This allows EVs to be used in functional experiments. Figure 1 shows a typical view of vesicles from FF of bovine ovaries (follicle diameter 3-6 mm). Both single and aggregated vesicles are observed.

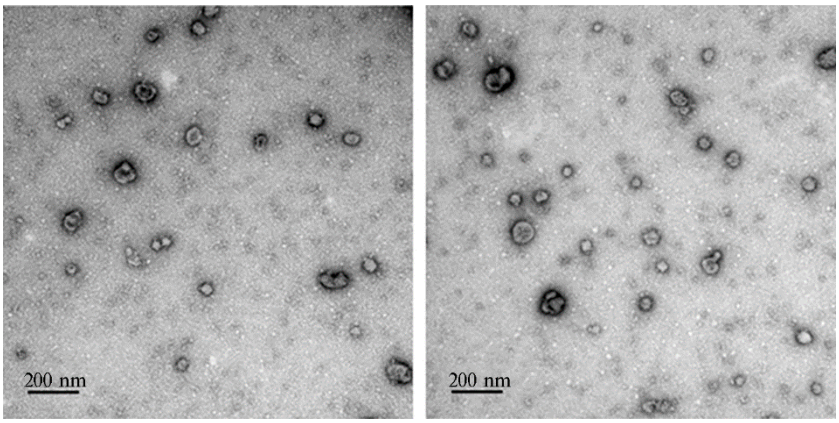

Fig. 1. Ultrastructure of extracellular vesicles (EVs) **from bovine (*Bos taurus*) ovarian follicular fluid** (transmission electron microscopy, JEOL 1011, JEOL, Ltd., Japan; GATAN RIO 9 camera, Gatan, Inc., USA; DigitalMicrograph3 program, Gatan, Inc., USA).

To evaluate the effect of EVs during the IVM period on oocyte competence for embryonic development after in vitro aging, we performed four independent experiments using vesicular protein from one isolation. Table 1 characterizes the developement of embryos when the IVP technology was applied. The proportion of fragmented zygotes (Fig. 2, A, D) on day 3 after in vitro fertilization in the control was lower vs. test cultures (53.5±2.9 vs. 63.8±2.9%, $p < 0.05$). For mature and aging in vitro oocytes, a positive effect of EVs on development to the blastocyst stage was found (Table 1, Fig. 2, B, E). In the control, the yield of blastocysts was 17.3±1.6% (see Table 1, Fig. 2, B). The presence of EVs in the maturation medium increased this indicator to 20.2±2.5% ($p < 0.05$) (see Table 1, Fig. 2, E). The trend persisted in the case of calculating this indicator from the

total number of embryos formed (41.8±1.2 vs. 32.3±1.9, $p < 0.05$).

1. Development of embryos obtained by IVP technology (in vitro production) **after maturation of cows (*Bos taurus*) oocytes in the absence** (control) **and in the presence of extracellular vesicles** (EVs) **from the follicular fluid and aging for 10 h** (*M*±SEM)

Group	Oocytes		Oocytes developed to to the blastocyst stage, %	
	total	cleaved after fertilization, %	from total	from cleaved
Control	116	53.5±2.9	17.3±1.6	32.3±1.9
Evs	121	63.8±2.9*	26.5±0.7*	41.8±1.2*

* Differences with control are statistically significant at $p < 0.05$.

2. Cytological analysis of blastocysts after in vitro fertilization of cow (*Bos taurus*) oocytes in vitro maturation in the absence (control) **and in the presence of extracellular vesicles** (EVs) **from the follicular fluid and aging for 10 hours** (*M*±SEM)

Group	Number		Average number of nuclei per blastocyst
	of experimrnts	of blastocysts	
Control	4	20	78,9±3,3
Evs	4	32	86,5±2,1

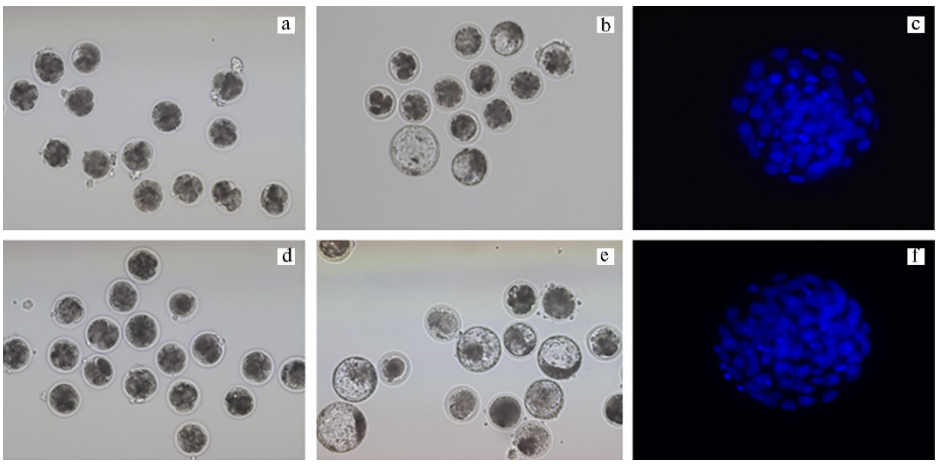

Fig. 2. Micrographs of bovine (*Bos taurus*) embryos after in vitro fertilization of oocytes matured in the absence (a-c) **or presence** (d-f) **of extracellular vesicles (EVs) from the follicular fluid and aging for 10 h:** a, d — fragmented zygotes (×100 magnification), b, e — embryos that have developed to the blastocyst stage (×100 magnification, Eclipse Ti-U microscope, Nikon, Japan); c, f — staining of nuclei in the blastocyst with DAPI (blue color; cytological preparation) (magnification ×400, microscope Axio Imager M2, Carl Zeiss, Germany).

The use of EVs did not significantly alter the quality of IVP embryos at the blastocyst stage, which was assessed by the number of nuclei on day 7 after fertilization (Table 2, see Fig. 2, C, E). According to cytological analysis, this indicator in the compared groups was the same and corresponded to the studied stage of development [32].

Oocyte aging is a complex biological process that can lead to a number of changes in the structure and function of mammalian oocytes, including DNA damage, decreased fertilization rate, disruption of mitochondrial structure, early apoptosis of oocytes, and decreased ability to develop embryos [22, 26, 33, 34]. Under in vitro conditions, changes in oocytes associated with aging can occur both during their maturation and during fertilization, which can adversely affect the development and quality of IVP embryos [25, 26, 34]. Nevertheless, despite the need to solve the problem of oocyte aging under in vitro conditions, there are very few studies aimed at finding regulators that reduce age-related transformations of oocytes. To date, the possibility of inhibition, at least partial, of the aging process when bovine oocytes are exposed to L-carnitine has been shown. The

introduction of this substance into the maturation medium reduces the level of oxidative stress and apoptosis in mature oocytes during their aging, and increases the yield of blastocysts from 20.9 (control) to 29.2% [25]. In addition, it has been found that the pituitary hormone prolactin (PRL) can specifically affect a mature oocyte and increase its resistance to aging processes, including those associated with the loss of oocyte competence for subsequent embryonic development [26]. When exposed to PRL, the proportion of oocytes that reached the blastocyst stage increased by 1.9 times compared to the control (15.2 vs. 8.2%, respectively). Here, we have demonstrated that EVs from bovine ovarian FF during maturation of oocytes can also increase their resistance to age-related changes and improve embryo development in vitro. However, unlike L-carnitine and PRL, the presence of EVs not only contributes to a similar increase in the yield of blastocysts (from 17.3 to 26.5%), but also improves the efficiency of fertilization, increasing the proportion of crushed oocytes from 53.5 to 63. 8 %.

Thus, the data obtained allow us to conclude that the use of extracellular vesicles (EVs) from the follicular fluid of cow ovaries in the in vitro maturation procedure increases the resistance of oocytes to age-related modification and, as a result, positively affects their competence for embryonic development after aging in vitro and fertilization. It is also clear that EVs when used during in vitro maturation of oocytes increase the efficiency of in vitro embryo production (IVP) biotechnology in cattle.

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THE INFLUENCE OF INDIVIDUAL FEATURES AND THE BREED OF DONOR HEIFERS ON THE EFFICIENCY OF OOCYTE RETRIEVAL BY OVUM PICK-UP

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Abstract

The oocytes' retrieval from lived cows (Ovum Pick-Up, OPU) is the most important element in the system of *in vitro* production (IVP) of cow embryos. In this regard, increasing the efficiency of OPU is a key factor for the widespread implementation of IVP technology in cattle breeding. The present work shows for the first time the possibility of obtaining IVP embryos from OPU oocytes obtained from a local breed of Yaroslavl cows. In Yaroslavl breed comparing to Simmental breed, the variability of the number of follicles was observed both between individual animals and between sessions in the same animals. We did not observe significant differences in the recovery rate and qualitative characteristics of the obtained cumulus-oocyte complexes, which allows using standardized protocols for donor heifers of both Simmental and Yaroslavl breeds. The aim of our work was to study the influence of individual features and breed characteristics of donor heifers on the efficiency of oocyte production by OPU. The studies were carried out in the laboratory of experimental embryology in 2020-2022 on mature clinically healthy heifers of the Simmental and Yaroslavl breeds aged of 17 to 36 months. Oocytes were obtained by transvaginal ultrasound-controlled puncture of follicles with an interval between sessions of 7 days according to the methodical guidelines using systems Bovine OPU (Minitube GmbH, Germany). In the first experiment, the efficiency of OPU was studied in individual donor heifers of the Simmental (n = 7; 50 sessions) and Yaroslavl breeds (n = 5; 25 sessions). In the second experiment, a comparative study of the OPU efficiency in donor heifers of the Simmental (n = 6; 12 sessions) and Yaroslavl breeds (n = 5; 25 sessions) was carried out using OPU parameters optimized for animals of the Simmental breed. The OPU efficiency was evaluated using the following criteria: the number of ultrasound-visible follicles, the number of retrieved cumulus-oocyte complexes (COCs), the degree of oocyte retrieval, the ratio of oocytes suitable for production of IVP embryos from the total number of derived oocytes. We observed high variability of the average number of ultrasound-visible follicles among individual donor heifers, both of Simmental (4.71-11.50 follicles; Cv = 47.9 %) and Yaroslavl breeds (5.80-9.80 follicles, Cv = 32.0 %), while the differences between some donors within breeds were highly significant ($p \leq 0.001$). Differences in the number of ultrasound-visible follicles led to differences in the number of derived COCs among individual donors, both of Simmental (2.33-5.17 COCs) and Yaroslavl breeds (3.60-6.00 COCs). Comparative studies of donor heifers of the Simmental and Yaroslavl breeds did not show significant differences in the average number of ultrasound-visible follicles (7.33±0.62 vs. 6.96±0.45), the number of obtained oocytes (4.17±0.69 vs. 3.36±0.41) and the number of suitable oocytes (3.08±0.60 vs. 2.52±0.29). Thus, a high variability in the average number of ultrasound-visible follicles and the number of obtained oocytes between individual donor heifers of the Simmental and Yaroslavl breeds was established. Considering the high positive correlation between the number of aspirated follicles and the number of retrieved oocytes (r = 0.97 and 0.72 for donor heifers of the Simmental and Yaroslavl breeds, respectively), it is advisable to select animals characterized by a large number of ultrasound-visible follicles to increase the efficiency of OPU. The absence of significant differences related to quantitative and qualitative characteristics of

the retrieved oocytes allows us to recommend the OPU parameters optimized for donor heifers of the Simmental breed to be applied in heifers of the Yaroslavl breed without a noticeable loss of efficiency.

Keywords: cattle, assisted reproductive technologies, OPU, in vitro embryo production

Currently, assisted reproductive technologies (ART) are the most important element in the genetic improvement of farm animals. The term ART describes procedures in which reproductive cycles, gametes or embryos are manipulated [1]. The relevance of the ART development is primarily due to the expected acceleration of selection and, as a result, genetic progress [2]. In addition, ARTs serve as an integral element in the development of genomic editing technologies [1-3].

In cattle, due to long generation interval and relatively low fecundity, ARTs are of particular relevance. ARTs used in cattle breeding include artificial insemination (AI), cryopreservation of semen and embryos, synchronization of the sexual cycle, multiple ovulation and embryo transfer (MOET), ovum pick-up (OPU) and in vitro production of embryos (IVP), semen sexing, embryo sexing, nuclear transfer (NT) [1].

The first widely used ART in cattle was the AI technology [4] which received mass adoption with the discovery of capacity for further viability of gametes after cryopreservation [5]. AI technology has enabled breeders and practitioners to better exploit the genetic potential of outstanding sires. For females, this problem was initially solved by the development and implementation of the MOET technology [6]. Classical MOET technology includes superovulation of genetically best donor cows and their artificial insemination followed by washing out of embryo, usually on days 6-7 after insemination. The resulting embryos are transplanted to recipients with a synchronized sexual cycle immediately after washing or after freezing-thawing. Embryos obtained by the MOET technology are defined as in vitro derived (IVD). Despite the advantages of MOET technology, the main factor limiting its widespread use, remains the need for hormonal treatment to induce superovulation. The lack of response to hormonal stimulation in some donors, the decrease in the effectiveness of superovulation with each subsequent hormonal treatment, the need for a break of several months between hormonal treatments, as well as high hormone costs significantly increase the cost of the resulting embryos.

An alternative to MOET was the development of IVP technology, the essence of which is to collect cow oocytes from the ovaries by aspiration of follicles, oocyte maturation, fertilization and in vitro development of embryos to the stages of late morula or blastocyst [7]. In combination with OPU [8], IVP technology is increasingly used in cattle breeding. The advantages of OPU/IVP procedure compared to MOET are evidenced by the progressive increase in the number of bovine embryos produced by IVP despite a decrease in the number of embryos derived from the classic MOET procedure. Thus, from 2000 to 2020, the number of IVD embryos worldwide decreased from 664,220 to 361,728, while the number of IVP embryos increased from 139,372 to 1,156,422 [9, 10].

An increase in the effectiveness of OPU was a decisive factor for the widespread of IVP technology. In this regard, studies have been carried out to identify factors affecting OPU effectiveness and optimization. The factors that affect the OPU performance are the type of aspiration needle used [11-13] and vacuum pressure [12, 14, 15], intervals between OPU sessions [16, 17], and whether hormonal synchronization of the sexual cycle [18-20] and superstimulation [21, 22] were applied. Individual features of donors, including breed [23, 24], age [25, 26], stage of the sexual cycle and individual response [27], climatic conditions [28, 29], nutritional factors, and operator experience also affect the OPU performance. The

potential of the resulting OPU oocytes to be fertilized and further developed is affected by the conditions of maturation, fertilization, and in vitro cultivation (30).

Previously, we determined the optimal type of aspiration needle, vacuum pressure [13] and the frequency of sessions in Simmental donor heifers [17]. However, the individual influence of donor heifers on the OPU performance has not been studied. In addition, it is necessary to evaluate the effectiveness of the methodology for conducting OPU, developed for donor heifers of the Simmental breed, on other cattle breeds.

In the presented work, for the first time, the possibility and efficiency of obtaining OPU oocytes in Yaroslavl cows is shown. In the Yaroslavl breed, compared to the Simmental breed, the variability in the number of follicles has been revealed both between animals and between sessions when the same animal is subjected to the procedure. We did not find statistically significant differences in the degree of isolation and qualitative parameters of cumulus-oocyte complexes (COC) which allows us to use standardized protocols for donor heifers of the Simmental and Yaroslavl breeds.

The purpose of this work was to investigate the influence of individual and breed traits of the Simmental and Yaroslavl donor heifers on the effectiveness of OPU oocyte production.

Materials and methods. The studies were carried out at the Ernst Federal Research Center for Animal Husbandry (Ernst FRC VIZh) in 2020-2022 on sexually mature clinically healthy Simmental and Yaroslavl heifers (*Bos taurus taurus*) aged from 17 to 36 months. Animals were fed diets balanced in terms of energy, nutrients and biologically active substances in accordance with the norms [31]. Oocytes were retrieved by transvaginal sonographic-assisted follicle puncture in accordance with the guidelines [32] using the Bovine OPU system (Minitube GmbH, Germany) completed with an ultrasound scanner ProSound 2 (Hitachi Aloka Medical, Ltd., Japan), 5 MHz ultrasonic sector probe (Aloka UST-9111-5, 5 MHz/90°/14 mm) with holder and vacuum pump.

In the first experiment, we studied the influence of the individual traits of the Simmental donor heifers ($n = 7$, 50 sessions) and Yaroslavl donor heifers ($n = 5$, 25 sessions) on the OPU effectiveness. Puncture of all ultrasound-visible follicles was performed with a 7-day interval using a 1.2×75 mm needle (18G×3″, long cut) at a vacuum pressure of 80 mmHg (Simmental breed) and 90 mmHg (Yaroslavl breed).

In the second experiment, the OPU effectiveness was evaluated depending on the breed characteristics of the Simmental donor heifers ($n = 6$, 12 sessions) and Yaroslavl donor heifers ($n = 5$, 25 sessions). All ultrasound-visible follicles were punctured within one time period using OPU parameters optimized for Simmental animals: aspiration needle 1.2×75 mm (18G×3″, long cut), vacuum pressure 90 mmHg, interval between sessions 7 days.

The criteria for evaluating the OPU effectiveness were the number of follicles visible by ultrasound, the number of retrieved oocytes in the COC, the degree of COC picking up, percentage of the COCs potentially suitable for IVP out of the total number of retrieved COCs. COCs were considered eligible if there were no obvious signs of cytoplasmic abnormalities in oocytes (e.g., degeneration, lysis, contraction, irregular shape), with the exception of mature COCs. Oocytes with homogeneous ooplasm, completely or partially devoid of cumulus cells, were considered suitable.

The resulting digital data were processed using the variation statistics methods in the Microsoft Excel program. In the experimental groups, the arithmetic mean values (M), standard deviations (σ), standard errors of the means ($\pm$SEM) were calculated. To assess the variability of the studied parameters, the coefficient of variation (Cv) was calculated. Statistical significance of differences

in arithmetic mean values was determined using Student's *t*-test. To compare two samples in terms of relative values, expressed as a percentage, Fisher's test with an angular transformation (φ-test) was used. The values were considered highly significant at $p \le 0.001$ and significant at $p \le 0.01$ and $p \le 0.05$.

Results. Significant differences were found in the number of ultrasound-visible follicles between individuals of both Simmental and Yaroslavl breeds (Table 1).

1. Number of ultrasound-visible follicles in donor heifers (*Bos taurus taurus*) Simmental and Yaroslavl breeds (the Ernst Federal Research Center for Animal Husbandry, Dubrovitsy, 2020-2022)

Breed	ID	Number of sessions	Number of follicles (*M*±SEM)	*Cv*, %
Simmental	3501	7	11.14±1.40[1, 5]	33.3
	3507	12	11.50±1.55[2, 6]	46.8
	3579	6	10.33±2.51[4]	59.6
	7019	6	9.33±0.33[3, 7]	8.7
	2480	9	7.78±0.57[1]	22.1
	2547	3	5.67±0.88[5, 6, 7]	27.0
	2581	7	4.71±0.46[1, 2, 3, 4]	26.6
	On average	50	9.08±0.61	47.9
Yaroslavl	451	5	9.80±1.16[1, 2, 3, 4]	26.4
	461	5	6.00±0.89[1]	33.3
	1884	5	5.80±0.49[2]	18.9
	1885	5	6.60±0.51[3]	17.3
	1890	5	6.60±0.81[4]	27.5
	On average	25	6.96±0.45	32.0

N o t e. ID — identification number of the heifer.
Differences between Simmental donor heifers marked with the same superscript numbers are statistically significant at [1, 2, 3]$p \le 0.001$, [5, 6, 7]$p \le 0.01$, and [4]$p \le 0.05$, between Yaroslavl donors heifers at [1, 2, 3, 4]$p \le 0.05$.

The average values of this indicator in Simmental donor heifers ranged from 4.71 to 11.50 follicles ($Cv = 47.9\%$), the differences between some donors were highly significant ($p \le 0.001$). The *Cv* values for the number of ultrasound-visible follicles in individual donors in different sessions were 8.7-59.6%, while there was a tendency for greater variability in heifers with a higher average value of the number of ultrasound-visible follicles ($r = 0.45$) . In Yaroslavl heifers compared to the Simmental heifers, there was a lower variability in the number of ultrasound-visible follicles both between heifers (5.80-9.80 follicles, $Cv = 32.0\%$), and in individual heifer between sessions ($Cv = 17.3$-33.3%).

The degree of COC extraction varied for Simmental heifers from 41.2 to 51.4%, for Yaroslavl heifers from 36.4 to 75.9%. As is known that a higher vacuum pressure during follicle puncture increases the COC extraction [12, 13], therefore, a higher rate in the Yaroslavl heifers may be due to the use of a higher vacuum pressure (90 mmHg) compared to Simmental heifers (80 mmHg).

A high positive correlation was noted between the number of punctured follicles and the number of obtained COCs in both Simmental heifers ($r = 0.97$, $p \le 0.01$) and Yaroslavl heifers ($r = 0.72$, $p \le 0.05$). The number of COCs retrieved from one donor per session averaged from 2.33 to 5.17 for Simmental heifers (with individual differences between donors being statistically significant, $p \le 0.001$) and from 3.60 to 6.00 for the Yaroslavl heifers (Table 2).

2. Number of cumulus-oocyte complexes (COCs) **obtained from donor Simmental and Yaroslavl heifers (*Bos taurus taurus*)** (the Ernst Federal Research Center for Animal Husbandry, Dubrovitsy, 2020-2022)

Breed	ID	Number of sessions	Number of COCs (*M*±SEM)	Suitable COCs, %
Simmental	3501	7	5.00±0.76[1]	77.14
	3507	12	5.17±0.93[2]	69.35[1]
	3579	6	5.00±1.03	70.00[2]
	7019	6	4.33±0.56	92.31[1, 2, 3]
	2480	9	4.00±0.58	80.56
	2547	3	2.33±0.88	57.14[3]
	2581	7	2.57±0.30[1, 2]	83.33

Continued Table 2

Yaroslavl	451	5	6.00±1.67	83.33[3]
	461	5	3.60±0.75	72.22[2]
	1884	5	4.40±0.40	72.73[1]
	1885	5	4.20±0.73	95.24[1. 2. 3]
	1890	5	2.40±0.68	83.33

N o t e. ID — identification number of the heifer.
Differences between Simmental donor heifers marked with the same superscript numbers are statistically significant on the number of COCs at $^{1,\ 2}p \le 0.05$, on suitable COC proportion at $^{1}p \le 0.01$, $^{2,\ 3}p \le 0.05$; between Yaroslavl donor heifers on suitable COC proportion at $^{1}p \le 0.01$, $^{2,\ 3}p \le 0.05$.

In the first experiment, the number of obtained COCs was not compared between the two breeds studied, since the time periods of manipulations with Simmental heifers and the Yaroslavl heifers differed. In addition, different vacuum pressures were used to aspirate follicles, which, as shown in numerous studies, affects the degree of oocyte retrieval, and hence the number of COCs obtained [12, 13, 15].

A high individual variability in the number of ultrasound-visible follicles and the number of received COCs was noted in a number of studies [24, 27]. In aboriginal Podolsk cattle, the number of follicles varied from 2.9 to 9.3, the number of COCs varied from 0.5 to 6.8 [24]; in Black-and-White cattle, the number of COCs was 1.6-9.2 [27].

Comparative studies conducted in the second experiment did not show statistically significant differences in the average number of ultrasound-visible follicles in the donor Simmental and Yaroslavl heifers (7.33±0.62 and 6.96±0.45, respectively). The values of this indicator we obtained generally agree with the results of other authors. Thus, in highly productive dairy Holsteinized Black-and-White cows, the average number of visualized follicles per donor was 3.88 [33], 4.81 [12] and 6.10 [34]. On Podolsk cattle, on average, 5.00 follicles were found in one donor [24].

We also did not observe significant differences in the degree of COC extraction between the Simmental and Yaroslavl heifers, 67.05 and 59.20%, respectively, which is in line with the results of on Podolsk and Belarusian Black-and-White cattle, i.e., 38.3-65 .0% [24], 68.8% [33] and 48.4-80.0% [12].

The average number of cumulus-oocyte complexes per donor heifer was 4.17±0.69 for Simmental cattle and 3.36±0.41 for Yaroslavl cattle, which corresponds to or exceeds the figures established by other authors for Podolsk and Belarusian Black-and-White cattle, 2.67 [33], 3.17 [12], 3.40 [24], 4.80 [12] and 5.00 [2]. The average number of COCs reported by L.N. Rotar et al. [26] for Black-and-White Holsteinized cows was significantly higher and accounted to 11.3.

In our experiment, 85.33% of COCs from Simmental heifers and 81.55% of COCs from Yaroslavl heifers were suitable for embryo production in vitro. These figures in most cases exceeded those established by other researchers. The viable COCs in the Black-and-White Holsteinized cows reached 42.48% [26]. The authors attribute the relatively low quality of COCs to an increased load on the cows due to lactation. The proportion of suitable oocytes reached 79.30% in native Podolsk cattle [24], 67.26% in Aberdeen Angus cows [26], 71.4 and 83.9% the Gir and Nelore cows, respectively [26]. In the report of V.K. Pestis et al. [34], the share of suitable COCs with optimal technical OPU characteristics reached 70.6%. The presence of a relatively larger proportion of usable COCs in our experience may be due to different criteria of the COC quality evaluation. Thus, in most studies, COCs lacking cumulus cells (denuded oocytes) are considered unsuitable for obtaining embryos in vitro [26, 34-36]. In our studies, the main criteria for the quality of COCs were the color and homogeneity of the ooplasm, and therefore denuded oocytes with homogeneous ooplasm were considered conditionally

suitable for IVP procedure.

In our experiment, the number of COCs suitable for embryo production in vitro averaged 3.08±0.60 per donor for the Simmental breed and 2.52±0.29 for the Yaroslavl breed. L.N. Rotar et al. [26] received 11.3 viable COCs per a donor Aberdeen Angus cow and 4.8 COCs per a donor Black Pied Holsteiner cow. In aboriginal Podolsk cattle, an average of 2.70 viable COCs per donor were identified [24], in Aberdeen Angus heifers, an average of 6.8 viable COCs per donor were obtained [37]. In studies on Holsteinized Black-and-White cattle, the number of eligible COCs per donor was 2.03 [33], 2.38 [12], 3.4 [27] and 3.90 [34].

Thus, we revealed a high variability in the results of OPU procedure between donor heifers of both Simmental and Yaroslavl breeds assessed by the average number of ultrasound-visible follicles and the number of oocyte-cumulus complexes obtained. These individual differences between donors were statistically significant. Given the high positive correlation between the number of punctured follicles and extracted retrieved COCs, it is reasonable to select animals with a large number of ultrasound-visible follicles to improve the OPU performance. Comparison of the OPU effectiveness in the donor Simmental and Yaroslavl heifers did not reveal significant quantitative and qualitative differences between the COCs. Therefore, the technical (type of aspiration needle and vacuum pressure) and technological parameters (number of OPU sessions) optimized for Simmental donor heifers can be used for Yaroslavl heifers without any noticeable loss of performance.

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CHANGE OF CULTURE MEDIUM POSITIVELY INFLUENCES THE DEVELOPMENT AND QUALITY OF *in vitro* CATTLE EMBRYOS

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Abstract

Although the practice of producing in vitro embryos (IVP) has become routine in the world, the quality of IVP embryos is still lower than that of in vivo embryos, and the conditions for obtaining in vitro embryos still need to be specified. This original research article presents data on the development and quality parameters of IVP embryos in cattle (*Bos taurus*), depending on the volume of in vitro culture medium (IVC) and its refreshment protocols. Post mortem bovine oocytes were cultured in a maturation medium, fertilized in vitro with frozen-thawed sperm, and transferred to 500 or 100 μl of BO-IVC medium (IVF Bioscience, UK) for embryonic development. The effects of not refreshing the IVC medium during the 8-day embryonic development period (NMR), half-refreshed medium (HRM) and completely refreshed medium (CRM) were compared. For HRM or CRM, after 3 days of culture, half of the initial 500/100 μl volume was replaced with fresh medium or embryos were transferred to 500/100 μl fresh medium. On day 8 of NMR, HRM, and CRM culture, the number of embryos developed to the blastocyst stage (BL) was evaluated, and the BL quality was assessed as per the total number of nuclei and the apoptotic nuclei counted cytologically. The 8-day-old blastocysts were also cultured for 2 days to the stage of hatched BL (HBL). CRM has been shown to improve embryo production regardless of the volume of the IVC medium when compared to NMR culture. Blastocyst formation increased from 23.0±1.5 and 25.8±0.8 % for 500 μl and 100 μl, respectively, to 45.7±4.8 and 52.1±4.9 % ($p < 0.01$), while apoptosis decreased from 6.53±0.88 and 6.47±0.66 to 3.60±0.12 and 3.50±0.29 % ($p < 0.05$ and $p < 0.01$). The yield of hatched blastocysts increased from 14.9±1.5 and 11.6±3.3 to 25.2±3.9 and 40.8±3.2 % ($p < 0.05$ and $p < 0.001$) from the total number of fertilized oocytes. In a 100-μl volume, the number of nuclei in BL also increased from 175.8±13.5 to 224.3±6.7 ($p < 0.05$). Similarly, the HRM culture was more favorable for embryo development compared to the NMR culture. As in the CRM culture, the blastocyst formation increased up to 44.9±0.7 and 44.1±5.0 % ($p < 0.01$ and $p < 0.01$ 0.05), and the HBL yield increased up to 26.2±3.2 and 27.7±1.4 % ($p < 0.05$). In a 100-μl volume, the number of nuclei in BL increased up to 230.4±8.4, ($p < 0.05$) while the proportion of apoptotic nuclei decreased to 3.10±0.17 ($p < 0.01$). No significant differences were found between CRM and HRM cultures for all parameters studied. However, with CRM and a 100-μl volume, the rates of BL and HBL were the highest. Thus, in cattle, CRM and HRM cultures, as compared to NRM without medium change, have a positive effect on the development and quality of IVP embryos. The noted effect obviously depends on the volume of the IVC medium. For BO-IVC medium with a volume of 500 μl, both manipulations to refresh the medium are effective, for a volume of 100 μl, CRM is the best.

Keywords: in vitro embryonic development, culture medium, IVP, IVC, cattle

The development and practical application of assisted reproductive technologies for in vitro embryo production (IVP) and embryo transplantation into recipient animals makes it possible to increase the rate of genetic progress in the selection of commercial breeds and the efficiency of programs for the conservation of small and gene pool breeds [1]. In vitro embryos suitable for transplantation develop from oocytes matured and fertilized under artificial conditions. Oocytes

can be obtained both in vivo (most often by transvaginal follicle puncture) [2] and post mortem [3]. In both cases, the goal is to get more offspring from better and more unique mothers.

Every year, the use of in vitro embryos is becoming more and more common worldwide, however, in terms of quality, IVP embryos are still inferior to in vivo embryos [1, 4]. IVP embryos are less resistant to freezing [4], and their viability after transplantation to recipients is lower than in embryos in vivo [3, 5].

An important procedure in IVP technology on which embryo quality depends is culturing oocytes after fertilization to the pre-implantation stages of development. In cattle, fertilized oocytes are cultured for approx. 7 days. The derived embryos that reached the blastocyst stage (BL) are either frozen or transplanted into recipient animals. The conditions for in vitro embryonic development critically affect both quantitative (the proportion of embryos at the BL stage) and qualitative (BL viability) BL parameters which are indicators of the IVP effectiveness [3].

For the development of cattle embryos in vitro, nutrient media self-prepared in the laboratory and simple in composition are most often used [6]. They should include the SOF medium [7] which is a synthetic analogue of the oviduct fluid, as well as the CR1aa medium [8]. In order to improve embryonic development, along with bovine serum albumin (BSA), at the final stage of culture, it is recommended to add fetal bovine serum (FBS) to both solutions, since FBS enables the embryos overcome the block at the morula stage, increases the release of BL and improves their quality [9, 10].

With the increasing commercialization of IVP technology over the past decade, there has been a need to develop off-the-shelf commercial in vitro embryo culture (IVC) media. The peculiarity of these media is that they provide a high yield of high-quality embryos of pre-implantation stages of development in the absence of FBS, which can be a source of pathogens and complicate the exchange of embryonic materials. In addition, the use of such media simplifies the procedure for obtaining embryos and makes it possible to standardize the conditions for their culturing [1, 6].

There is ample evidence in the literature that the media in which embryos are cultured ultimately determine their developmental potential and quality [11-13]. However, the modes of incubation, in particular the conditions for renewal of the medium, can improve this effects. Changing the culture medium is a common practice during the IVC procedure that positively influenses the development of the embryos, providing them with the necessary nutrients and removing toxic metabolites, such as ammonia and oxygen free radicals accumulated in the culture medium [14-16]. During in vitro culture, embryos secrete into the medium paracrine factors, such as epidermal growth factor, platelet activating factor, insulin-like growth factor, as well as messenger RNA and microRNA, which play an important role in the regulation of embryonic development [17-19]. These molecules, according to recent studies, are packed in extracellular vesicles (EVs) that can be secreted or taken up by the embryos [20, 21]. Changing the culture medium during the IVC procedure removes these important factors as well as EVs, and, therefore, can reduce the potential for in vitro embryonic development.

In cattle, IVC medium is usually replaced every 2-3 days of culture [3, 15, 22]. However, there are many reports about the dubious need for such a procedure and the possibility to reach high rates of development and quality of IVP embryos without changing or with a partial change of medium [16, 23, 24]. The last two approaches have become particularly popular due to the use of incubators with a low oxygen atmosphere [25]. That is, at the stage of maturation and fertilization, oocytes are cultured at the natural O_2 level (20%), and from the stage of the

putative zygote, at a lower air concentration of O_2. As a result, the production of reactive oxygen species and oxidative stress in the cells decrease. There is an opinion that, despite many years of experiments and routine work on obtaining IVP embryos, optimal mode of changing the IVC medium for both cattle [16] and other animals [26] has not yet been established. This is especially true for recently appeared new commercial solutions for which information is either limited or not available.

In our work, the embryonic development of mature and in vitro fertilized oocytes occurred in the commercial BO-IVC medium (IVF Bioscience, UK) chosen due to its high efficiency in IVP of embryos in cattle [27, 28]. The communication we present is the first to demonstrate the influence of the studied conditions on the development of in vitro fertilized bovine oocytes to the blastocyst stage. In addition, we assessed the quality of derived blastocysts in terms of the number of nuclei, the frequency of apoptosis, and viability.

The aim of the study was to study the influence of the conditions of the BO-IVC change on the quantitative and qualitative characteristics of embryos during their development in vitro. The possible dependence of such an effect on the volume of the culture medium was also evaluated.

Materials and methods. In all experiments, except for indicated cases, reagents from Sigma-Aldrich (USA) were used.

To produce embryos, isolated post mortem cow ovaries were delivered to the laboratory, freed from adjacent tissues, and repeatedly washed in sterile saline containing penicillin (100 IU/ml) and streptomycin (50 μg/ml) (BioPharmGarant, Russia). Cumulus-oocyte complexes (COCs) were dissected with a blade from the ovaries and separated from the walls of the follicles. COCs were washed 3 times in TC-199 medium containing HEPES (25 mM), Na-pyruvate (0.5 mM), PBS (5%), heparin (10 *r*g/ml) and gentamicin (50 μg/ml). During washing, the morphology of the COCs was examined to select oocytes for further culture according to common criteria [29].

For maturation, COCs were incubated for 24 h in TS-199 medium containing HEPES (25 mM), Na-pyruvate (0.5 mM), follicle-stimulating and luteoinizing hormones (10 μg/ml), epidermal growth factor (10 ng/ml) (Thermo Fisher Scientific, USA), FBS (10%) and gentamicin (50 μg/ml).

After maturation, COCs were subjected to in vitro fertilization (IVF). They were washed once in BO-IVF medium (IVF Bioscience, UK) and placed in drops of the same medium 30 min before contact with spermatozoa. Oocytes were fertilized using a fraction of active spermatozoa obtained by the swim-up method [30]. The straw with frozen sperm was thawed, the content of the straw was transferred to the bottom of test tubes containing 1 ml of Sperm-TALP medium [31] and placed in an incubator (MCO-18AIC, Sanyo, Japan) for 50 min. After incubation, the 750 μl upper layer from the tube was transferred to another tube containing Sperm-TALP medium and centrifuged at 300 g for 7 min (a centrifuge 3-30KS, Sigma, Germany). The sediment of motile spermatozoa was added to the BO-IVF medium (IVF Bioscience, Great Britain) containing previously transferred mature COCs, the final concentration of spermatozoa was 1.5×10^6/ml. Germ cells were co-cultured for 15-16 h. Then in Fert-TALP medium the oocytes were carefully released from cumulus cells and adhering spermatozoa and placed in the medium for embryonic development.

Embryos were cultured in 4-well plates (Nunc, Denmark) in 500 or 100 μl drops of BO-IVC medium (IVF Bioscience, UK) under a layer of mineral oil in an incubator (MCO-MCO-50M-PE, Sanyo, Japan) at 38.5 °C and a gaseous atmosphere containing 6% CO_2, 5% O_2 and 89% N_2. Three variants of incubation were compared, i.e., without medium replacement (NMR), with completely

refreshed mediume (CRM), and with half-refreshed medium (HRM). For NMR, during the entire period (8 days) the embryos developed without refreshing culture medium. For CRM, after 3 days of IVC, the embryos were transferred for further development into drops of fresh medium. For HRM, after 3 days of incubation, half of the initial volume of media (500 or 100 μl) was removed and replaced with an equivalent volume of fresh media. In all variants, after 3 days of IVC, the proportion of fragmented zygotes was assessed morphologically, on day 8 of culture, the embryos that developed to the BL stage was counted, and their viability and quality were estimated.

For quality analysis, a portion of 8-day-old BLs was fixed for 60 min with a 4% solution of paraformaldehyde. After fixation, the embryos were permeabilized for 30 min in a 0.1% sodium citrate solution containing 0.5% Triton X-100. For detection of apoptotic nuclei by the TUNEL method, the In Situ Cell Death Detection Kit, fluorescein (Roche Diagnostics, Switzerland) was used. Nuclei were stained for 20 min in a DAPI solution (1 μg/ml), transferred to a glass slide with an adhesive Superfrost Plus coating (Thermo Fisher Scientific, USA), and placed in Vectashield medium (Vector Laboratories, UK). Cytological preparations were viewed and photographed (an Axio Imager M2 fluorescent microscope equipped with a filter for TUNEL, excitation at 445-470 nm, and DAPI, excitation at 365 nm; an Axiocam 506 digital camera with ZEN 2 pro software, Carl Zeiss, Germany). The total number of nuclei in the embryos and the number of TUNEL-positive nuclei were counted.

To assess the viability, the second portion of the 8-day-old BLs were cultured for 2 days in a BO-IVC medium containing 5% PBS until the hatching stage. The hatched BLs (HBLs) at the beginning and at the end of culturing were counted.

Statistical processing was performed by the ANOVA method using the SigmaStat program (Systat Software, Inc., USA). The experimental results are presented as mean values (*M*) and their standard errors (±SEM). Tukey's test was applied to assess the significance of differences between the compared means.

Results. The in vitro matured oocytes used to produce embryos had an equal competence for further development. In the two culture options used, the results of cleavage on day 3 after in vitro fertilization did not differ significantly, and the proportion of oocytes that cleaved after fertilization ranged from 67.6 to 74.0% (Table 1).

The experiment revealed the influence of the IVC medium replacement mode on the development of in vitro fertilized oocytes to the BL stage (Fig. 1, A-B, Table 1). For 500 μl BO-IVC drops without changing the medium for 8 days, the BL yield was the lowest and amounted to 23.0±1.5%. Partial or complete change of the medium after 3 days of embryo growth equally increased the indicator 1.9-fold ($p < 0.01$). With a decrease in the drop volume to 100 μl, the influence of medium replacement on the development of zygotes up to the BL stage did not change. The described effects remained unchahged when the percentage of development to BLs was calculated from the number of fragmented zygotes, that is, from the total number of embryos obtained after fertilization.

1. Development of in vitro fertilized cow (*Bos taurus*) oocytes to the blastocyst stage depending on the mode of IVC (in vitro culture) **medium replacement** ($n = 6$, *M*±SEM, day 8 of culture)

Option	Oocyte number	Oocytes cleaved after fertilization, %	Development to the blastocyst stage, %	
			of total oocytes	of cleaved oocytes
		500 μl drops		
No refreshed medium	148	67.6±1.9	23.0±1.5	34.1±2.4
Completely refreshed medium	166	69.9±2.6	45.7±4.8**	65.1±4.8***
Half-refreshed medium	138	74.0±2.9	44.9±0.7**	58.5±1.6**

Continued Table 1

		100 µl drops		
No refreshed medium	68	68.4±3.4	25.8±0.8	37.9±2.8
Completely refreshed medium	88	72.5±4.5	52.1±4.9**	71.5±2.3***
Half-refreshed medium	94	72.2±2.3	44.1±5.0*	60.9±5.7**

*, **, *** Differences vs. no refreshed medium are statistically significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

2. Cytological analysis of bovine (*Bos taurus*) blastocysts (day 8) **depending on the mode of IVC** (in vitro culture) **medium replacement**

Option	Number of experiments	Total number of blastocysts	Number of nuclei per blastocyst, *M*±SEM	
			total	apoptotic, %
		500 µl drops		
No refreshed medium	3	17	216,9±9,8	6,53±0,88
Completely refreshed medium	3	38	180,4±9,2[a]	3,60±0,12*
Half-refreshed medium	3	31	198,3±15,4	4,30±0,46
		100 µl drops		
No refreshed medium	3	9	175,8±13,5	6,47±0,66
Completely refreshed medium	3	23	224,3±6,7*[b]	3,50±0,29**
Half-refreshed medium	3	21	230,4±8,4*	3,10±0,17**

*, ** Differences vs. no refreshed medium are statistically significant at $p < 0.05$ and $p < 0.01$.
[ab] Differences between mean values for the same mode of medium refreshment labeled with different letters are statistically significant at $p < 0.05$.

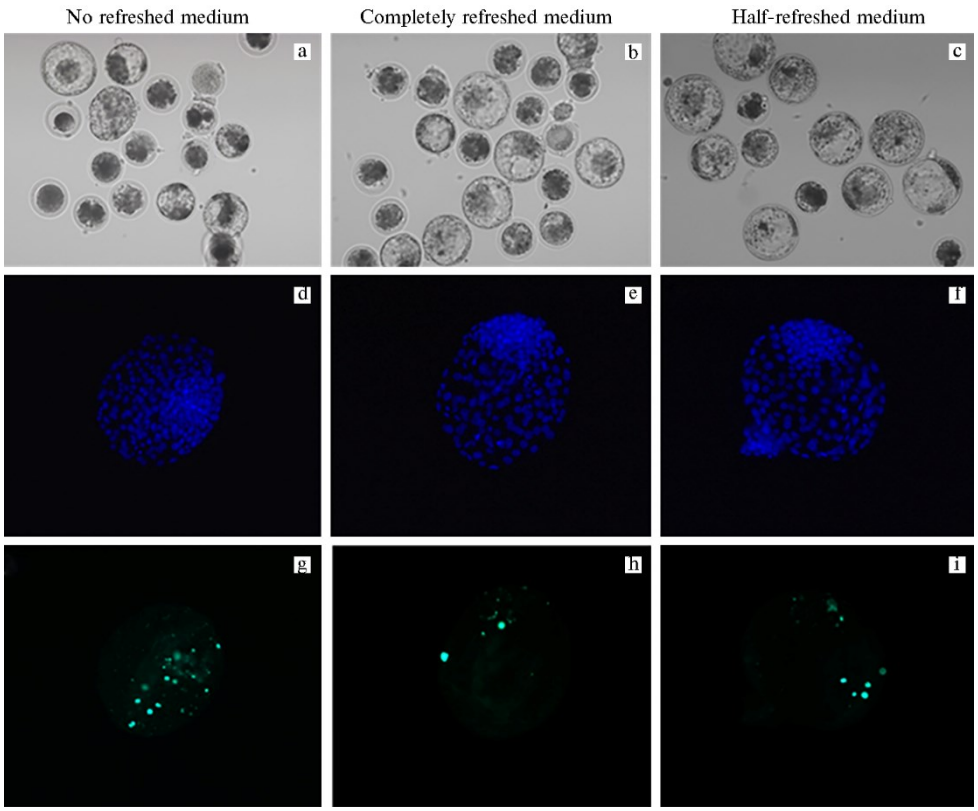

Fig. 1. Micrographs of bovine (*Bos taurus*) embryos after 8 days of in vitro culture under different modes of IVC (in vitro culture) **medium replacement:** a-c — embryos that have developed to the blastocyst stage (magnification 100×, microscope Eclipse Ti-U, Nikon, Japan); d-f — staining of nuclei in the blastocyst with DAPI (blue color; cytological preparation), g-i — staining of apoptotic nuclei in the blastocyst by TUNEL method (green color; cytological preparation) (magnification ×400, microscope Axio Imager M2, Carl Zeiss , Germany).

Cytological analysis testified to a rather high quality of the embryos that developed in vitro under the compared modes for the renewal of the IVC medium (Table 2). With a high average number of nuclei in BLs on day 8 (see Fig. 1, D-

F), no significant differences occurred between the modes if the embryos developed in 500 μl drops. On the contrary, with a decrease in the volume of IVC medium to 100 μl, CRM led to an increase in the analyzed parameter compared to NRM ($p < 0.05$), and also compared to CRM at 500 μl drops ($p < 0.05$) .

The tested modes (NRM, HRM, and CRM) also affect the incidence of apoptotic degeneration in BL. NRM mode + 100 μl drops negatively affected the proportion of apoptotic nuclei in BL (see Fig. 1, G), significantly increasing this indicator compared to both the CRM (Fig. 1, H) and HRM (see Fig. 1, I) ($p < 0.01$). As the volume of the IVC medium increased, the effect became less pronounced. With CRM, the analyzed parameter decreased compared to NRM ($p < 0.05$), but not to HRM. With a partial replasmant of the medium, we did not find significant differences, although in embryos there was a tendency to a decrease in the proportion of nuclei with apoptosis.

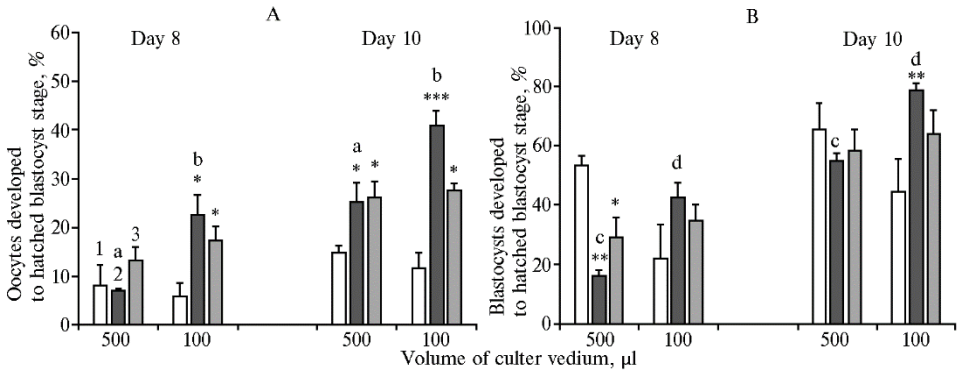

Fig. 2. Viability of bovine (*Bos taurus*) blastocysts after 8 and 10 days of in vitro culture under different modes of IVC (in vitro culture) **medium replacement:** 1, 2 and 3 — no refreshed medium, completely refreshed medium and half-refreshed medium, respectively ($n = 3$, $M \pm SEM$).
, **, ** Differences vs. no refreshed medium are statistically significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$.
[ab, cd] Differences between the means for one mode of medium replacement on day 8 and day 10 marked with different letters, are statistically significant at $p < 0.05$ and $p < 0.01$

It was found that the proportion of IVF oocytes that reached the HBL stage on day 10 of IVC, which serves as a criterion for the viability of IVP embryos, was higher in the CRM and HRV groups than in the NRM group (Fig. 2, A). In the latter case, this indicator for 500 and 100 μl drops did not differ and amounted to 14.9±1.5 and 11.6±3.3%, respectively. CRM and HRM cultures in 500 μl IVC medium drops increased the proportion of IVF oocytes that reached the HBL stage to 25.2±3.9 and 26.2±3.2% ($p < 0.05$), in 100 μl drops up to 40.8±3.2 ($p < 0.001$) and 27.7±1.4% ($p < 0.05$), respectively. That is, under the CRM mode, a decrease in the volume of IVC medium from 500 to 100 μl increased the viability of the embryos ($p < 0.05$), and the viability index here reached a level that was the highest among the tested cultures. It should be noted that when the proportion of IVF oocytes at the HBL stage was assessed on day 8 of IVC, a positive effect in CRM and HRM cultures vs. NRM occurred only in 100 μl drops ($p < 0.05$). In addition, in CRM culture, the viability index, as on day 10, was higher compared to that in 500 μl drops ($p < 0.05$).

The viability of embryos was additionally assessed by the proportion of HBLs to the total number of blastocysts (see Fig. 2, B). If culturing embryos for 10 days in 500 μl drops, the proportion of HBLs did not differ significantly between the modes of medium change and amounted to 65.6±8.7, 54.6±2.9 and 58.5±7.1% for the groups of NRM, CRM and HRV, respectively. On the contrary, with a decrease in the volume of the culture medium, this indicator was higher in CRM group than in NRM group (78.7±2.4 vs. 44.4±11.1%, $p < 0.01$), and

significantly ($p < 0.05$) exceeded the values in the same group for 500 μl drops. On day 8 in 500 μl drops, the HBL percentage of the total blastocyst number was higher in NRM group vs. CRM and HRM groups, 53.3±3.3 vs. 16.2±2.0% ($p < 0.01$) and 29.4±6.3% ($p < 0.05$). In 100 μl drops, the HBL percentage was the lowest among the compared modes, 22.2±11.1, 42 .6±4.9 and 35.0±5.0% for NRM, CRM and HRM, respectively.

Currently, the IVC media used in the IVP technology can provide a high yield of embryos suitable for freezing and transplantation into recipient animals. Nevertheless, the in vitro conditions that ensure the development of the embryo still require detailing [3, 6]. In particular, it remains unclear whether there is a need for an IVC medium refreshing procedure recommended every 48-72 h during embryo culture. On the one hand, the replacement of the medium provides the embryos with nutrients and removes harmful metabolites [14-16], on the other hand, this procedure leads to a stressful change in the microenvironment of the embryos and the removal of embryotropic factors necessary for embryo development [15, 16].

In the present work, embryos were cultured in 500 and 100 μl drops of commercial BO-IVC medium and assumed the embryo development to the blastocyst stage either without changing the culture medium or with complete or partial (50%) medium change. Our findings showed that the CRM mode vs. the NRM mode after 3 days of IVC provided a significant increase in BL yield (see Table 1), a decrease in the frequency of apoptosis (see Table 2), and an increase in the BL viability assessed as percentage of the total number of fertilized oocytes (see Fig. 1, A). These parameres did not depend on the IVC medium volume (500 or 100 μl drops), while the number of nuclei in BLs increased in 100 μl drops (see Table 2). A partial change of culture medium (HRM) was also more efficient in several indicators vs. culture without a change of medium. The blastocyst formation frequency (see Table 1) and viability (see Fig. 1, A) were higher similar to CRM group, and in 100 μl drops, an increase in the number of nuclei and a decrease in apoptotic nuclei in BLs (see Table 2) occurred. We did not find any significant differences between CRM and HRM in all the studied parameters; nevertheless, for CRM and 100 μl culture, there were the highest rates of BL development and the HBL number. In general, these results indicate a negative impact of the NRM culture on the development and quality of IVP embryos.

Our data are partly consistent with a recent study on rabbits, in which HRM was more effective in terms of BL development and quality (the number of nuclei) than in vitro culture without medium change. In addition, HRM and CRM modes similarly reduced the rate of apoptosis in embryos [26]. In the work of these authors, as in our study, the medium was changed once after 3 days of culturing. Nevertheless, in experiments on cattle, which are not so numerous, on the contrary, the CRM mode compared to NRM mode led to a decrease in the number of BL and HBL [23] and to deterioration in the quality and viability of embryos [16]. There are also reports of no differences between these cultures [24]. The fact that in these studies bovine embryos were cultured in SOF medium that was changed every 48 h IVC and the results of our own studies draw us to two assumptions. Firstly, such a frequent medium change can cause stress and CRM can negatively affect the development and quality of embryos [32], and, secondly, there is a need for detailed parameters of SOF medium refreshing.

Thus, complete (CRM) and partial (HRM) culture medium renewal positively affect the development and quality of in vitro produced (IVP) bovine embryos as compared to in vitro cultures without medium change (NRM). CRM provides significantly increased blastocyst production from 23.0±1.5 and 25.8±0.8% to 45.7±4.8 and 52.1±4.9% ($p < 0.01$) for 500 and 100 μl drops of the culture

medium. In addition, the apoptosis rate in embryos decreases from 6.53±0.88 and 6.47±0.66% to 3.60±0.12 and 3.50±0.29% ($p < 0.05$ and $p < 0.01$). In CRM culures, the yield of hatched blastocysts (HBLs) from the total number of fertilized oocytes increases from 14.9±1.5 and 11.6±3.3% to 25.2±3.9 and 40.8±3.2% ($p < 0.05$ and $p < 0.001$). All these changes occur regardless of the culture medium volume (data are presented for 500 and 100 μl, respectively). For 100 μl under CRM mode, the number of nuclei per blastocyst also increases (from 175.8±13.5 to 224.3±6.7, $p < 0.05$). For HRM mode, the frequency of blastocyst formation increaseы (up to 44.9±0.7 and 44.1±5.0%, $p < 0.01$ and $p < 0.05$), as does the proportion of HBL (up to 26.2±3.2 and 27.7±1.4%, $p < 0.05$) (data are presented for 500 and 100 μl, respectively). For 100 μl culture medium under HRM mode, the number of nuclei per blastocyst increases (up to 230.4±8.4, $p < 0.05$), while the proportion of apoptotic nuclei decreases (to 3.10±0.17%, $p < 0.01$). The revealed effects obviously depend on the BO-IVC medium (IVF Bioscience, UK) volume in IVC (in vitro culture). For 500 μl, both modes of medium renewal are effective, while for 100 μl, the CRM mode ensures the best results. The CRM mode and 100 μl culture medium provide the the highest development rates of blastocysts and the number of HBLs. Therefore, BO-IVC medium (IVF Bioscience, UK), if refreshed after 3 days of in vitro culture, can be recommended for in vitro production of cattle embryos.

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ANTIHYPOXIC AND ENERGY STIMULATING EFFECTS OF COBALT GLYCINATE DURING EMBRYOGENESIS OF QUAILS (*Coturnix japonica*)

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Abstract

Hypoxic manifestations, including those associated with certain periods of bird embryogenesis, lead to slowdown in development, and in severe cases, to multifaceted morphological and functional disorders in embryos. Numerous studies have confirmed the effectiveness of biostimulants with pronounced antioxidant properties, which can neutralize negative effects of hypoxia and provide conditions for a faster transition to aerobic glycolysis. These biostimulants include cobalt glycinate, synthesized at Scriabin Moscow State Academy of Veterinary Medicine and Biotechnology. The choice of the biostimulant components was due to the properties of each component separately and their hypothetical complementary effect. In the present work, it was found for the first time that cobalt glycinate has an antihypoxic effect and stimulates energy metabolism in quail embryos and 1-day-old quails. The purpose of the work is to investigate the effect of cobalt glycinate on energy metabolism and to provide a background for correction of adverse effects of hypoxia that occur during embryogenesis in quails under incubation. The experiment was carried out on hatching eggs from Japanese quail (*Coturnix japonica*) of the same age (Shepilovskaya Poultry Farm, Moscow Province, 2020). The eggs were sorted in two batches (experimental and control, 220 eggs each). The experimental eggs were sprayed once with 0.05 % cobalt glycinate solution (an aerosol dispenser HURRICANE 2792, Curtis Dyna-Fog, USA). The control batch was not treated. The eggs were incubated in IUV-F-15-31 type incubators (Energomera, Russia; the temperature range from 38.1 to 36.8 °C, a 10-15 mm ventilation flaps' opening). Key categories of incubation waste, hatchability rate of eggs, hatching, live weight of 1-day-old juveniles, body temperature, and the quality as per Pasgar and Optistart scaled criteria were assessed. Blood of 1-day-old juveniles was sampled by decapitation. Blood antioxidant activity (AOA), the content of lipid peroxidation products were measured using a Beckman DU-7 spectrophotometer (Beckman Coulter, Inc., USA). Concentrations of total blood proteins, lipids, glucose were measured using an automatic biochemical analyzer DIRUI CS-600B (Dirui Industrial Co., Ltd., China). The content of lactate and pyroracemic acid was analyzed by tandem chromatography-mass spectrometry (an Agilent 6410 Triple chromatograph, Agilent Technologies Inc., USA). The ATP content was determined by bioluminescent method (a luminometer and reagents from Lumtek, Russia), pH by direct potentiometry (an E-Lyte 5 blood electrolyte analyzer, High Technology Inc., USA). In the test group, the number of the main incubation wastes (blood rings and died-in-shell birds) was 1.82 and 2.28 times less, respectively, than in the control group, while the hatching rate increased by 8.64 % ($p < 0,05$) and hatchability by 7.97 % ($p < 0.05$). Treatment with an optimal dosage of cobalt glycinate prior to incubation contributed to a decrease in free-radical reactions and lipid peroxidation. The greatest differences (20 %) occurred in the concentration of oxodiene conjugates ($p < 0.05$). The reduced LPO intensity may be due to the stimulating effect of cobalt glycinate on the antioxidant system, which resulted in an increase in AOA by 12.9 % ($p < 0.01$) compared to control. The blood concentration of ATP in quails of the test group was 1.4 times higher ($p < 0.01$) than in the control group. The ATF level, along with an increase in glucose by 8.73 % ($p < 0.01$), pyroracemic acid by 12.5 % ($p < 0.05$), pH by 0.67 % and a decrease in the lactate by 16 %, were indicative of a more efficient use of energy substrates by the birds. The likelihood of development of an uncompensated acidosis decreased in the birds of the test group. Along with this, the stimulation of energy metabolism caused a statistically significant ($p < 0.01$) increase in body temperature measured rectally and under the wing, by 0.4 and 0.3 °C, respectively (39.1 and 37.5 °C vs. 38.7 and 37.2 °C). An increase in the blood concentration of total proteins by 3.88 % ($p < 0.01$) and an increase in live weight by 8.34 % ($p < 0.05$) should be especially noted. Therefore, under industrial conditions, the pre-incubation treatment of Japanese quail

eggs with 0.05 % solution of cobalt glycinate reduces the free radical level and, as a result, lipid peroxidation in 1-day-old quails. Additionally, cobalt glycinate stimulates energy metabolism, providing a faster transition of quails to aerobic glycolysis and reducing the likelihood of uncompensated acidosis. A higher concentration of ATP in 1-day-old individuals of the test group indicates both a better thermoregulatory function to ensure natural resistance and viability, and the absence of depleted energy metabolism during the previous periods of development, which determines the superiority in viability of embryos.

Keywords: hypoxia, embryogenesis, quail, antioxidant, cobalt glycinate, hatchability rate

Hypoxic changes, including those physiologically determined and associated with the peculiarities of the processes occurring in different periods of bird embryogenesis, lead to a slowdown in development, and in severe cases, to multifaceted morphofunctional disorders in embryos [1-3]. According to M.T. Tagirova and O.V. Tereshchenko [4], after the allantois closure or hatching, the transition of embryos to the use of oxygen is delayed even under standard conditions of chicken egg incubation in poultry farming, which practically does not occur during natural hatching. This is inevitably accompanied by excessive accumulation of lactate and, as a result, aggravation of acidosis with a growing risk of its uncompensated form [5]. An increase in the concentration of lactic acid due to the imperfection of its utilization systems in the embryo causes irreversible negative effects in all cells and tissues of the embryo [4]. The described processes adversely affect the viability of the embryos and lead to a significant increase in incubation waste products such as blood rings and addled egg [6, 7].

An acute state of oxygen deficiency represses biological oxidation, critically reducing the synthesis of ATP that leads to an acute hypoenergetic state. With this, the intensity of substrate phosphorylation increases many times, which hinders production of the required amount of macroergic compounds in birds [4, 8]. The formation and functionality of the adaptation mechanisms necessary to successfully overcome stressful conditions is reduced [9]. An energy-deficient state also causes insufficient thermogenesis, and, therefore, insufficient immunobiological activity and resistance [10]. Under the influence of hypoxic stress during critical periods of embryonic development, the future functionality of organs and tissues in birds decrease, adaptive capabilities and productive qualities are not fully realized, causing significant production losses and a decrease in the profitability of not only an individual poultry enterprise, but also the poultry farming as a whole.

Numerous studies have confirmed the effectiveness of various biostimulants with pronounced antioxidant properties, which can neutralize the negative effect of hypoxia and ensure a faster transition to aerobic glycolysis [11-13]. The most effective were those that had not only antioxidant activity, but also the ability to maintain the functionality of the mitochondrial respiratory chain (MRC) [14, 15].

These biostimulants include cobalt glycinate synthesized at the Moscow State Academy of Veterinary Medicine and Biotechnology — Scriabin MVA [16]. The choice of biostimulant components was based on the properties of each component separately [17-19]. Thus, glycine is able to maintain the functionality of MRC, maintaining the energy synthesis [19, 20]. Along with this, it exhibits antioxidant properties due to glutathione in its structure. A significant content of aminoacetic acid is characteristic of keratins and collagen which are necessary for the formation of bones, cartilage and skin. It is also involved in the synthesis of purine bases which are part of DNA, RNA, coenzymes NAD^+, $NADP^+$, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), macroergic compounds [19, 20]. Glycine belongs to the glycogenic amino acids and supports carbohydrate metabolism.

It should be noted that both components of the biostimulant can influence tissue trophism, primarily due to the participation in the synthesis of heme (glycine

as a participant in the first reaction and cobalt as an activator of a number of enzymes in this process) [19-22]. In addition, cobalt promotes the maturation of erythrocytes in the bone marrow by increasing the number of reticulocytes in the peripheral blood [18].

The important role of cobalt in the formation of nitrogenous bases, the formation of the primary structure of RNA and DNA, the synthesis of amino acids, and the intensity of carbohydrate and lipid metabolism has been reported [19, 20, 23]. It has also been proven that organic cobalt compounds are involved in reactions that suppress the synthesis of free radicals which are formed in excess during stress [24-29].

In general, the combination of antioxidant, membrane-protective, metabolism-stimulating properties of the biostimulator synthesized by us is of undoubted value, primarily for the intensive and full development of embryos under conditions of commercial incubation of poultry eggs. Glycine and cobalt have properties that cause a positive synergistic effect in leveling the negative consequences of hypoxia of various etiologies, and can become correctors of the conditions of energy-synthetic processes [15, 30].

In the present work, for the first time, it was found that cobalt glycinate has an antihypoxic and energy-stimulating effect on quail embryos and 1-day-old quails.

This work aims to reveal the effect of cobalt glycinate on energy metabolism to be further laid behind the procedure for correction of hypoxic processes that occur in quails during embryogenesis under commercial incubation.

Materials and methods. The experiment was carried out in 2020 at OOO Shepilovskaya Poultry Farm (Moscow Province, Serpukhov, Shepilovo village) on eggs of Japanese quail (*Coturnix japonica*) obtained from birds of the same age (63 days). The conditions for keeping the parent flock for all groups were the same, the birds were fed 2 times with barley-sorghum-soy type compound feed, balanced according to the existing standards of the RAAS. Experimental and control batches consisted of 220 eggs that were selected with regard to conditions and period of storage, the timing of laying and weight. The optimal biostimulant concentration has been identified in a series of previous experiments [16, 30, 31].

Before incubation, an experimental batch of eggs was once treated with a 0.05% solution of cobalt glycinate using a HURRICANE 2792 aerosol dispenser (Curtis Dyna-Fog, USA). The control batch was not treated (dry control). The eggs were placed in IUV-F-15-31 type incubators (Energomera, Russia; temperature range from 38.1 to 36.8 °C), adjusting the opening width of the ventilation dampers within 10-15 mm depending on the day of incubation.

The main categories of incubation waste, egg hatchability, hatched quail yield, body weight of 1-day-old chicks, and body temperature were assessed. In addition, 1-day-old birds were individually assessed according to the quality criteria of the Pasgar and Optistart scales.

Whole blood and serum were obtained from 1-day-old birds by decapitation. Antioxidant activity of blood plasma (AOA), products of lipid peroxidation, that is, alkadienes with isolated double bonds (IDB), diene conjugates (DC), triene conjugates (TC), oxodiene conjugates (ODC), Schiff bases (SB) were determined by colorimetric method. The optical density was measured on a Beckman DU-7 spectrophotometer (Beckman Coulter, Inc., USA), the concentration of total protein, lipids, glucose in blood serum on an automated biochemical analyzer DIRUI CS-600B (Dirui Industrial Co., Ltd., China) using commercial biochemical kits for veterinary medicine (ZAO DIAKON-DS, Russia). Concentrations of lactate and pyruvic acid (PVA) were determined by tandem chromatography-mass spectrometry using an Agilent 6410 Triple chromatograph (Agilent Technologies Inc., USA), the ATP concentration by bioluminescent method using a luminometer and a set of

reagents Lumtek (Russia), pH was measured by direct potentiometry on a blood electrolyte analyzer E-Lyte 5 (High Technology Inc., USA) [32].

Statistical processing of the experimental data was carried out in Microsoft Office Excel 2007. The mean values (*M*) and standard errors of the means (±SEM) were calculated. The statistical significance of differences was assessed by Student's *t*-test.

Results. Pre-incubation treatment of eggs with cobalt glycinate at an optimal concentration contributed to a decrease in the intensity of free-radical reactions and, as a result, in lipid peroxidation in quails from the experimental group. This creates the prerequisites for maintaining the integrity of cell structures, including mitochondria, which is necessary for biological oxidation that provides the embryos with the main pool of macroergic compounds during periods of embryogenesis not associated with hypoxia [27].

Antioxidant activity of blood plasma (AOA), products of lipid peroxidation, that is, alkadienes with isolated double bonds (IDB), diene conjugates (DC), triene conjugates (TC), oxodiene conjugates (ODC), Schiff bases (SB)

1. Lipid peroxidation and activity of antioxidant defense system in 1-day-old Japanese quails (*Coturnix japonica*) hatched after pre-incubation egg treatment with 0.05% cobalt glycinate solution (*M*±SEM, *n* = 5; OOO Shepilovskaya Poultry Farm, Moscow Province, Serpukhov, Shepilovo village, 2020)

Parameter	Group	
	control (without treatment)	experiment
AOA, %	49.60±1.43	56,00±1,00**
IDB, OD/ml	7.00±0.32	6,20±0,37
DC, OD/ml	2.30±0.03	2,16±0,04
TC, OD/ml	0.93±0.04	0,74±0,03
ODC, OD/ml	0.90±0.04	0,75±0,01*
SB, OD/ml	0.50±0.03	0,36±0,05*

N o t e. AOA — antioxidant activity of blood plasma, IDB — alkadienes with isolated double bonds, DC — diene conjugates, TC — triene conjugates, ODC — oxodiene conjugates, SB — Schiff bases; OD — optical density.
* and ** Differences vs. control are statistically significant at $p < 0.05$ and $p < 0.01$.

In 1-day-old quails from the experimental group, there was a decrease in lipid peroxidation vs. control (Table 1). The largest differences (20%) occurred in the concentration of ODC ($p < 0.05$). This confirms the assumption that it is possible to preserve the integrity of membrane phospholipids, including due to the prevention of the formation of hydroperoxides [33, 34]. A relatively high ODC value in the control quails indicated a more intense free radical processes [35] and modification of the membrane bilayer with an increase in the ionic permeability, which leads to lower ATP synthesis and disruption of cell functionality [33, 36-39].

In test birds, the concentration of Schiff bases decreased 1.38-fold vs. control ($p < 0.05$) that can be considered as a positive phenomenon. So, according to Yu.A. Vladimirov [33], this indicator characterizes the ability of endogenous aldehydes (fragments of the acidic components of phospholipids) to bind to the amino groups of proteins, which leads to the formation of intermolecular crosslinks that negatively affect the functionality of organelles as a whole [40]. In addition, a decrease in production of Schiff bases means that allergic reactions and autoimmune pathologies become less likely [41].

It should be noted that the reduced LPO intensity may have been associated with the stimulating effect of cobalt glycinate on the antioxidant system, which resulted in an increase in AOA by 12.9% ($p < 0.01$) compared to control. I.S. Lugovaya [42] proved that regression of excessive lipid peroxidation preserve the integrity of cell structures and the activity of enzymes, including those necessary for the formation of energy production which is generally consistent with our data.

2. Blood biochemical parameters of 1-day-old Japanese quails (*Coturnix japonica*) hatched after pre-incubation egg treatment with 0.05% cobalt glycinate solution (*M*±SEM, *n* = 5; OOO Shepilovskaya Poultry Farm, Moscow Province, Serpukhov, Shepilovo village, 2020)

Parameter	Group	
	control (without treatment)	experiment
ATP, μmol/l	2,89±0,21	4,01±0,14**
Glucose, mmol/l	9,62±0,16	10,54±0,15**
Pyruvic acid, mmol/l	0,24±0,01	0,27±0,01*
Lactate, mmol/l	1,09±0,04	0,94±0,05
pH	7,42±0,03	7,47±0,02
Total protein, г/л	27,20±0,14	28,30±0,21**
Total lipids, mmol/l	2,56±0,08	2,69±0,09

N o t e. ATP — adenosine triphosphoric acid.
* and ** Differences vs. control are statistically significant at $p < 0.05$ and $p < 0.01$.

The blood ATP concentration in the experimental quails was 1.4 times higher ($p < 0.01$) vs. control. The high ATP level, increased glucose (by 8.73%, $p < 0.01$), PVA (by 12.5%, $p < 0.05$), pH (by 0.67%) and a decreased lactate (by 16%) indicate a more efficient use of energy substrates in birds derived from eggs treated with cobalt glycinate due to a faster transition to aerobic glycolysis which is more beneficial in terms of energy supply (Table 2). In quails from the experimental group, the likelihood of developing an uncompensated form of acidosis decreased. Stimulation of energy metabolism also led to a statistically significant ($p < 0.01$) increase in temperature measured rectally and under the wing, by 0.4 and 0.3 °C, respectively (39.1 and 37.5 °C vs. 38.7 and 37.2 °C), which indicates better physiological state and natural resistance of young birds [43-45].

Note, there was an increase in the blood concentration of total protein by 3.88% ($p < 0.01$) with an increase in body weight by 8.34% ($p < 0.05$) (8.88±0.21 g in control, 9.62±0.19 g in the experimental birds, $n = 5$). That is, protein monomers were mainly used not for energy but for growth and development, which is necessary for economically important qualities of an individual in further ontogenesis.

Total protein, total lipids, and glucose in the control birds were close to the lower limits of the reference values [46], which indicates an overexpenditure of macroergic compounds in embryogenesis. Obviously, this was necessary to increase the efficiency of adaptation mechanisms and, at the same time, testified to the exhaustion of the body due to the impact of stressors caused by the conditions of commercial incubation.

3. Incubation biocontrol parameters (%) **1-day-old Japanese quails (*Coturnix japonica*) hatched after pre-incubation egg treatment with 0.05% cobalt glycinate solution** (*M*±SEM, *n* = 220; OOO Shepilovskaya Poultry Farm, Moscow Province, Serpukhov, Shepilovo village, 2020)

Group	Incubation waste					Egg hatchability		Hatched quails	
	1	2	3	4	5	total	to control	total	to control
Control	6,82±1,70	3,64±1,26	8,18±1,85	3,64±1,26	2,27±1,00	80,98±2,65		75,45±2,90	
Experimental	5,45±1,53	1,82±0,90	5,91±1,59	1,36±0,78	1,36±0,78	88,94±2,11*	+7,97	84,09±2,47*	+8,64

N o t e. 1 — unfertilized eggs (including falsely unfertilized), 2 — blood rings, 3 — dead embryos, 4 — addled eggs, 5 — weak embryos.
* Differences vs. control are statistically significant at $p < 0.05$.

According to Yu.S. Yermolova [47], the antioxidant balance and, as a result, optimized energy exchange improve embryonic viability and functional integrity of organs and tissues, which is consistent with our data (Table 3). Thus, in the experimental group, we found a significant decrease in all categories of incubation waste, especially those associated with hypoxia. In particular, the proportion of blood rings and addled eggs [15] was less than control by 1.82 and 2.28 times, respectively, with a significant increase in the number of hatched quails (by 8.64%, $p < 0.05$) and egg

hatchability (by 7.97%, $p < 0.05$). Besides, there is an increase in the quality of 1-day-old young birds, the scores on the Pasgar and Optistart scales are higher by 1.0 ($p < 0.05$) and 1.3 ($p < 0.01$), respectively. The data obtained are obviously largely due to the integrity of all cell membrane structures and, therefore, their functionality, which is necessary for tissue respiration of a growing embryo [48].

Thus, in Japanese quails, the treatment of eggs with a 0.05% solution of cobalt glycinate prior to commercial incubation optimizes metabolic processes, including due to the preservation of enzyme activity by reducing lipid peroxidation. This ensures a faster transition of quails to aerobic glycolysis and a decrease in the likelihood of uncompensated acidosis incidence. As a result, individuals from the experimental group were superior to the control birds in terms of embryonic viability. Along with this, cobalt glycinate at the optimal concentration has an energy-stimulating effect. A higher concentration of ATP in 1-day-old chicks of the experimental group indicates no depletion in energy metabolism during previous development. This provides better thermoregulation which characterizes natural resistance and biological responsiveness.

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CORRELATION INTERACTION OF TOTAL BILE ACIDS WITH BASIC BLOOD BIOCHEMICAL INDICATORS IN MINKS (*Mustela vison* Schreber, 1777)

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Abstract

Liver diseases of various origins and complications arising from the gradual destruction of the bile ducts cause the accumulation of bile acids in the liver, bile, and blood serum. This induces a pro-inflammatory response and an increased production of reactive oxygen species, leading to cytotoxic effects. Any decrease in extraction efficiency caused by impaired liver function leads to an increase in the level of total bile acids in the blood serum. Serum or plasma bile acid levels are sensitive measures of liver function in all species, reflecting both hepatic synthesis, secretion, and reabsorptive function. Thus, testing of blood serum can reveal functional abnormalities of liver function before the formation of more pronounced clinical signs. This high sensitivity is very important for making a clinical diagnosis. The novelty of the research lies in the study of the correlation between the bile acids and other biochemical parameters associated with the functional state of the liver, which was carried out for the first time. The aim of the work was to identify the correlation between standard biochemical parameters and the content of bile acids in the blood serum, as well as to evaluate the bile acids in the blood serum as a predictor of the state of the hepatobiliary system. The experiments were carried out in 2022 at the Mermeriny fur farm (village Mermeriny, Kalinin District, Tver Province). Palomino minks (*Mustela vison* Schreber, 1777) were chosen as model animals. Blood sampling from 100 females and 100 males at the age of 1 year was performed by cutting the tip of the tail. The main criterion for the selection of minks is the absence of clinical signs of liver pathologies. The content of total protein, albumin, total bilirubin, alkaline phosphatase, glucose, cholesterol, total bile acids, de Ritis coefficient was determined on a biochemical analyzer URIT 8021A VET (RIT Medical Electronic Group Co., Ltd., China). Total bile acids were detected using the BSBE Bile Acid Kit (BSBE, China). As part of the scientific research work, a correlation was calculated (Spearman's rank correlation coefficient and correlation-regression analysis) between the classical predictors of the hepatobiliary system (total protein, albumin, total bilirubin, de Ritis coefficient, alkaline phosphatase, glucose, cholesterol) and general bile acids as the main studied quantity. It was revealed that the measurement of total bile acids in blood is a promising way to identify pathologies of the hepatobiliary system, especially those accompanied by a violation of protein and fat metabolism. This statement is supported by the stable correlation of the blood level of bile acids with such indicators as alkaline phosphatase and the de Ritis coefficient revealed during the study. The relationship between these indicators is consistent with the biochemical properties of these compounds. Bile acids can stimulate the synthesis of alkaline phosphatase, and the cytotoxic or cytoprotective function of various representatives of the bile acid pool directly affects the level of alanine aminotransferase and the de Ritis coefficient. The coincidence of values obtained using two methods of statistical analysis of correlations at a high confidence level ($P > 95$ %) indicates the reliable nature of the identified relationships. When conducting these tests, it is necessary to take into account the heterogeneity of the results depending on the sex of the animals. In males, a moderate positive relationship between bile acids and concentrations of cholesterol and albumin was the most obvious, $0.2 \geq r \leq 0.5$ as per correlation and regression analysis method and $0.3 \geq r \leq 0.5$ as per Spearman's rank correlation coefficient technique. In females, there was a strong positive correlation between blood levels of bile acids and the total protein and bilirubin concentrations, $0.7 \geq r \leq 0.9$ for both calculation methods used.

Keywords: bile acids, minks, hepatobiliary system, liver, biochemical parameters, correlation

Bile acids are a group of steroid compounds derived from cholesterol [1]. They have a unique stereochemistry, hydroxyl groups and an aliphatic side chain with a terminal carboxyl residue [2]. These molecules have historically been described as lipid solubilizing agents and pancreatic enzyme activators, concomitant with their role in intestinal absorption [3]. While bile acids are endogenously toxic at elevated concentrations due to their amphipathic structure, some authors [4-7] point out that they also have endocrine and metabolic functions that include self-regulation of their synthesis, transport, and detoxification. Bile acids are involved in energy-producing reactions, in lipid and glucose homeostasis and affect the composition of the intestinal microbiota [8].

Changes in the metabolism and transport of bile acids lead to pathological state [7]. For example, an increased amount of these compounds in the enterohepatic circulation system can cause pathologies of the liver and intestines [9]. Conversely, deficiency results in nutrient malabsorption and fat-soluble vitamin deficiencies [10]. Therefore, a balanced metabolism of bile acids is important due to their significant role in homeostasis.

Liver diseases of various origins and severity, resulting from the gradual destruction of the bile ducts, lead to the accumulation of bile acids in the liver, bile and blood [11]. This process induces an inflammatory response and increased production of reactive oxygen species [12], leading to cytotoxic effects [13].

One of the functions of the liver is the removal of bile acids from the portal blood circulation, which is provided by bile acid transporters located on the sinusoidal membrane of hepatocytes [14]. The high extraction efficiency determines the low content of total bile acids in the peripheral blood compared to the portal blood. Any decrease in extraction efficiency caused by impaired liver function leads to an increase in the content of total bile acids in the blood serum [15, 16]. The amount of bile acids in serum or plasma is determined by hepatic synthesis, secretion, and reabsorption [16]. Therefore, blood serum testing makes it possible to detect liver dysfunction before the development of more pronounced clinical signs, which is very important for clinical diagnosis [16].

In various hepatobiliary disorders, elevated concentrations of bile acids in the blood serum were found, and therefore they are currently considered as one of the predictors of the state of the hepatobiliary system [17]. However, sensitivity, specificity and predictive value of such diagnostics is not fully understood. It has been proven that this method is able to detect hepatobiliary disorders, but it has not been compared with classical diagnostic methods (primarily with biochemical methods) [18].

Here, our findings for the first time established a correlation between the content of total bile acids and classical (biochemical) indicators of the state of the hepatobiliary system.

Our goal was to reveal the relationship between standard biochemical parameters and the content of bile acids in the blood serum and to evaluate these parameters as predictors of the hepatobiliary status.

Materials and methods. The experiments were carried out at the Mermeriny fur farm (Mermeriny village, Kalininsky District, Tver' Province, 2022). Palomino minks (*Mustela vison* Schreber, 1777) were chosen as model animals [19].

Blood of 1-year old 100 females and 100 males was takes from an incision at the tip of the tail into improvacuter vacuum test tubes for biochemical research (Guangzhou Improve Medical Instruments Co., Ltd., China) with blood clotting activator [20]. All asepsis and antiseptic measures were observed. The main criterion for selecting minks was the absence of clinical manifestations of liver pathologies.

Total protein, albumin, total bilirubin, alkaline phosphatase, glucose, cholesterol, total bile acids, aspartate aminotransferase and alanine aminotransferase (with further calculation of the de Ritis coefficient) was measured (a URIT 8021A VET biochemical analyzer, URIT Medical Electronic Group Co., Ltd., China). The concentration of total protein was determined by the biuret method using a color reaction with copper sulfate (AO LenReaktiv, Russia) in an alkaline medium. Albumins were quantified colorimetrically with bromcresol green (AO LenReaktiv, Russia). Total bilirubin was measured colorimetrically by diazo method according to Jendrashik-Cleggorn-Grof with sodium nitrate (AO LenReaktiv, Russia). The de Ritis coefficient was calculated as the ratio of the activity of serum aspartate aminotransferase and alanine aminotransferase [21]. Glucose content was assessed by the standard glucose oxidant method (glucose oxidase manufactured by OOO Biopreparat, Russia), cholesterol content by the Ilka method (Ilka reagent manufactured by AO LenReaktiv, Russia) [21].

Total bile acids were detected using a BSBE bile acid kit (BSBE, China). The method is based on the chemical properties of bile acids, that is, in the presence of ThioNAD, 3-α-hydroxysteroid dehydrogenase (3α-HSD) converts bile acids into 3-ketosteroids and Thio-NADH. The reaction is reversible and 3α-HSD can again convert 3-ketosteroids and Thio-NADH to bile acids and Thio-NAD [21, 22]. With an excess of NADH, an enzymatic cycle occurs, and the rate of Thio-NADH production was determined by a specific change in optical density at $\lambda = 405$ nm [21, 22].

The results obtained were considered random variables, for the processing of which stochastic modeling (correlation-regression analysis) was used [23].

Correlation-regression analysis was carried out using Pearson's formula [25] regarding the indicator of total bile acids as diagnostic criteria with an unknown correlation with respect to other indicators:

$$r_{xy} = \frac{\sum_{t=1}^{m}(x_t - \bar{x})(y_t - \bar{y})}{\sqrt{\sum_{t=1}^{m}(x_t - \bar{x})^2 \sum_{t=1}^{m}(y_t - \bar{y})^2}} = \frac{\mathrm{cov}(x,y)}{\sqrt{s_x^2 s_y^2}},$$

where $\bar{x}, \bar{y}$ are sample means xx^m, y^m, s_x^2, s_y^2 are sample variances, $r_{xy} \in [-1, 1]$.

Based on the results of the analysis, in order to confirm the conclusions about the presence, magnitude and strength of the correlation, the Spearman rank correlation coefficient was additionally calculated due to the small sample size and the deliberate nonparametric nature of the studied parameters [24].

The main studied value was the content of total bile acids, the correlation coefficients of other parameters were calculated vs. this criterion using the formula:

$$p = 1 - 6\frac{\sum d^2}{n^3 - n},$$

where d^2 are the squared differences between the ranks, N is the number of traits that participated in the ranking.

The results were processed using the Statistica 6.0 program (StatSoft, Inc., USA). Mean values of indicators (*M*), standard errors of means (±SEM) were calculated, the correlations, their magnitude and strength were determined. The lack of reliability calculation was due to the exploratory character of the survey and the absence of control groups.

Results. The choice of the Palomino breed was due to the species propensity of these minks to hepatopathy [19] and the maximum rate of enterohepatic circulation of bile acids among mammals. In the examined animals, protein, fat, carbohydrate and pigment metabolism, which serve as indicators of the functional state of the liver, were characterized as a variant of the physiological norm, that is, the indicators did not go beyond the reference values.

Blood biochemical parameters of Palomino minks (*Mustela vison* Schreber, 1777) without clinical manifestations of liver pathologies (M±SEM, the Mermeriny fur farm (Mermeriny village, Kalininsky District, Tver' Province, 2022)

Parameter	Males (n = 100)	Females (n = 100)	Reference values
Total protein, g/l	76.68±2.14	80.09±1.02	50-81
Albumins, g/l	34.75±0.71	33.32±0.56	20.0-50.0
De Ritis coefficient (AsAT/AlAT)	0.92	0.86	0.85-1.75
Alkaline phosphatase, IU/l	66.45±2.31	76.88±4.12	25.58-147.69
Glucose, mmol/l	2.87±0.11	2.99±0.17	6.5-12.1
Cholesterol, mmol/l	5.90±0.10	6.94±0.23	3.7-7.02
Total bilirubin, μmol/l	5.09±0.32	5.37±0.43	3.42-26.06
Total bile acids, μmol/l	4.63±1.02	5.56±1.19	2.00-7.00

N o t e. AsAT — aspartate aminotransferase, AlAT — alanine aminotransferase.

Figure 1 shows the results of the correlation-regression analysis (weak correlations were not accounted).

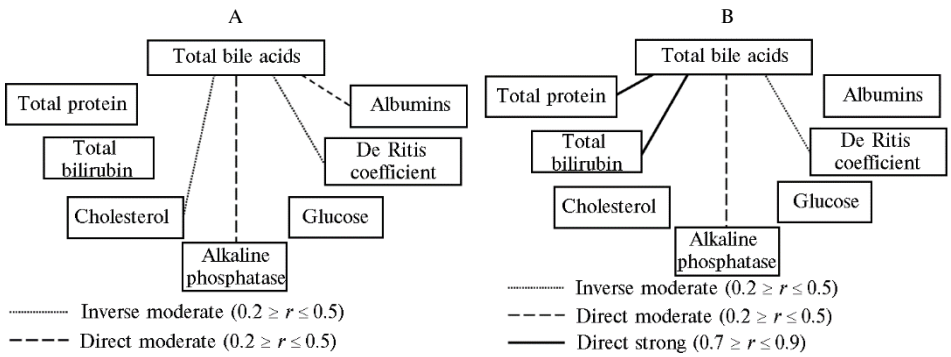

Fig. 1. Correlation-regression analysis of the relationship between the blood concentration of bile acids and the main blood biochemical parameters in Palomino mink (*Mustela vison* Schreber, 1777) males (A, n = 100) **and females** (B, n = 100) (P > 95%, the Mermeriny fur farm (Mermeriny village, Kalininsky District, Tver' Province, 2022).

In mink males, there was no correlation between the content of bile acids and the amount of total protein and bilirubin. (Fig. 1, A). This is due to the fact that the synthesis of proteins and bilirubin in the body is not associated with bile formation. Currently, it is believed that the concentration of bilirubin in the blood serum can be an individual constitutional indicator that goes beyond the reference values, which does not indicate pathological processes in the hepatobiliary system [26]. Thus, when studying the pathogenesis of hereditary constitutional hyperbilirubinemia, changes in the content of bile acids were not recorded [27].

A direct moderate correlation of the content of bile acids with albumins was due to the fact that the vast majority of them are synthesized by the liver, that is, there is a relationship between the protein-synthesizing and secretory functions of the organ. The direct moderate relationship with the activity of alkaline phosphatase and the inverse moderate relationship with the de Ritis coefficient can be explained by the biochemical properties of these compounds. Bile acids are able to stimulate the synthesis of alkaline phosphatase [28], and the cytotoxic or cytoprotective function of various members of the bile acid pool directly affects the content of alanine aminotransferase and the de Ritis coefficient. The amount of glucose in the blood serum, despite the fact that the main regulation of this indicator is carried out with the participation of the liver, depends on many neuroendocrine reactions, and therefore the lack of correlation with the content of bile acids is natural. The content of cholesterol as one of the precursors of bile acids had an inverse moderate correlation with their content.

The discrepancy in some correlation relationships between male and

female minks is explained by their physiological and hormonal differences (see Fig. 1). In particular, the high association with total protein and bilirubin, as well as the lack of association with albumin in females, were due to the estrus period [29, 30], during which the quantitative value of the first two indicators increased and the albumin content decreased. The lack of relationship with cholesterol content is explained by its predominant role in steroidogenesis in females (31).

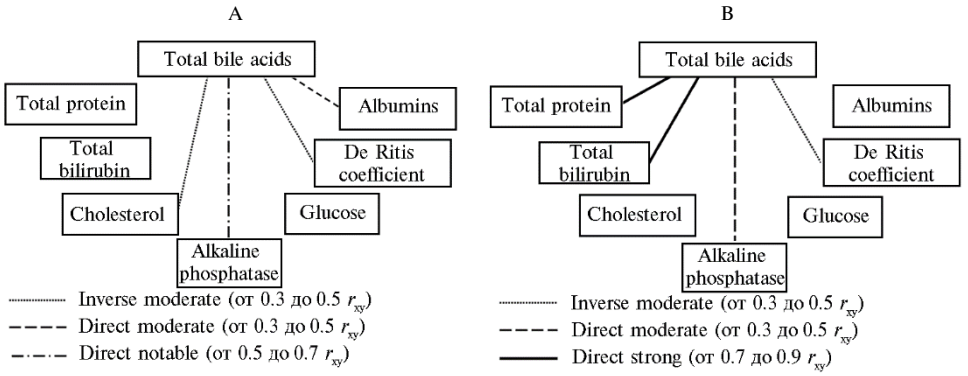

Fig. 2. Calculation of the Spearman rank correlation coefficient between the blood concentration of bile acids and the main blood biochemical parameters in Palomino mink (*Mustela vison* Schreber, 1777) males (A, $n = 100$) **and females** (B, $n = 100$) **minks (Mustela vison Schreber, 1777)** (P > 95%, the Mermeriny fur farm (Mermeriny village, Kalininsky District, Tver' Province, 2022).

To confirm the reliability of correlations, the Spearman rank correlation coefficient was additionally calculated (Fig. 2). The coincidence of the results of the correlation analysis performed by two methods indicates the reliable nature of the identified relationships. Theoretically, each factor that disrupts the enterohepatic circulation leads to a change in the content of bile acids in the blood serum. At that, we found a strong correlation between the amount of bile acids and the activity of alkaline phosphatase, as well as the de Ritis coefficient.

In the last few decades, the main predictor role of bile acids in blood serum has been associated not only with their total amount, but also with the qualitative composition of the pool, the components of which differ both in chemical activity and mechanisms of action from cytotoxicity to cytoprotection [17]. Nevertheless, the content of total bile acids remains an important prognostic and diagnostic criterion in the detection of hepatopathy of various origins.

Thus, the content of total bile acids in blood serum is a promising predictor of the hepatobiliary status, which can be used in diagnostics together with classical biochemical tests for total protein, albumin, total bilirubin, de Ritis coefficient, alkaline phosphatase, glucose, and cholesterol. Total bile acids may serve as indicators of hepatobiliary pathologies, especially those accompanied by a violation of protein and fat metabolism. This confirms the stable correlation relationship of this quantitative indicator with the activity of alkaline phosphatase and the de Ritis coefficient (for both sexes, a direct moderate correlation is $0.2 \geq r \leq 0.5$ according to correlation-regression analysis and $0.3 \geq r \leq 0.5$ when calculating the Spearman rank correlation coefficient. The coincidence of the values obtained using the two methods for statistical analysis of correlations at a high confidence level (P > 95%) indicates the significant character of the identified relationships. The character of some correlations varied depending on the sex of minks. Iin males, the most obvious relationship was traced between the blood bile acid concentration and cholesterol and albumins (direct moderate correlation $0.2 \geq r \leq 0.5$ according to correlation-regression analysis and $0.3 \geq r \leq 0.5$ when calculating the Spearman correlation coefficient). In females, there was correlation with total

protein and bilirubin level (direct high correlation $0.7 \geq r \leq 0.9$ for both calculation methods). We plan further studying the correlations between bile acids and biochemical parameters of metabolism in healthy animals and under various pathologies, given the predictor role of the bile acid pool qualitative composition.

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