ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

AGRICULTURAL BIOLOGY

Since January, 1966

ANIMAL BIOLOGY

Vol. 55, Issue 6
November-December

2020 Moscow

A peer-reviewed academic journal for delivering current original research results and reviews on classic and modern biology of agricultural plants, animals and microorganisms
Covered in Scopus, Web of Science (BIOSIS Previews, Biological Abstracts, CAB Abstracts, Russian Science Citation Index), Agris

Science editors: E.V. Karaseva, L.M. Fedorova

Publisher: Agricultural Biology Editorial Office NPO

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

For citation: Agricultural Biology,
Сельскохозяйственная биология, Sel'skokhozyaistvennaya biologiya

ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)
ISSN 2412-0324 (English ed. Online)

SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA
[AGRICULTURAL BIOLOGY], 2020, Vol. 55, № 6

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AGRICULTURAL BIOLOGY,
2020, V. 55, № 6, pp. 1061-1072
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

Reviews, challenges

UDC 636.5:591.3:57.044

doi: 10.15389/agrobiology.2020.6.1061eng
doi: 10.15389/agrobiology.2020.6.1061rus

PRENATAL NUTRITION OF POULTRY AND ITS POSTNATAL EFFECTS
(review)

A.M. DOLGORUKOVA ✉, V.Yu. TITOV, V.I. FISININ, A.A. ZOTOV

Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, 10, ul. Ptitsegradskaya, Sergiev Posad, Moscow Province, 141311 Russia, e-mail anna.dolg@mail.ru (✉ corresponding author), vtitov43@yandex.ru, olga@vnitip.ru, inkub@vnitip.ru
ORCID:
Dolgorukova A.M. orcid.org/0000-0002-9958-8777
Titov V.Yu. orcid.org/0000-0002-2639-7435
Fisinin V.I. orcid.org/0000-0003-0081-6336
Zotov A.A. orcid.org/0000-0001-9761-3804
The authors declare no conflict of interests
Received December 12, 2019

Abstract

Fast growth rate in modern meat-type poultry is accompanied by several metabolic disorders resulting from the discrepancy between embryonic and postembryonic growth and development. Prenatal period of avian ontogenesis is characterized by abrupt physiologic and metabolic alterations and hence any disturbance at this stage can affect the hatch efficiency and subsequent postnatal growth and productivity (E.T. Moran, 2007; V.L. Christensen et al., 2004). The embryonic bird development can be supported by the in ovo nutrition using natural nutrients (amino acids, carbohydrates, vitamins) as well as growth stimulators and hormones; this approach can also prepare the poults for the intense postnatal growth (P.R. Ferket, 2016). From the nutrigenomic point of view, the nutrients and bioactive substances can affect gene expression (V.I. Fisininet al., 2006; L. Bordoniet al., 2019). The experiments with the in ovo nutrition proved that the injections of nutrients can affect physiological status of broiler embryos and hatched broiler chicks. E.g. the injections of carbohydrates enlarge the pool of energy available for the embryo and decreases the catabolism of proteins and lipids during the final stage of incubation, resulting in the increases in the weight of the hatched chicks and in postnatal growth rate, supported by better development of the gastrointestinal tract (R. Kornasioet al., 2011; R. Jhaet al., 2019). All amino acids are necessary for the developing embryo; the absence of any of the amino acids can disrupt protein synthesis and homeostasis in the embryo, resulting in poorer postnatal growth and development. A bulk of studies were published which demonstrated the positive effects of the in ovo injections of individual amino acids and their combinations on the postnatal growth rate (Y. Ohtaet al., 2001; T.M. Shafeyet al., 2014; L.L. Yu et al.,2018). Ca. 94% of total metabolizable energy in the embryo is generated via the oxidation of fatty acids. These oxidative processes, in turn, generate substantial amounts of free radicals which can result in vast cellular damage (P. Surai and V.I. Fisinin, 2013; A. Yigitet al., 2014). The administration of vitamins with antioxidative activity (like C or E) during the embryonic period positively affected the postnatal development of the immune system in chicks (S.A. Selimet al., 2012; S. Nowaczewskiet al., 2012). The administration of L-carnitine into the embryos was shown to enhance pre-hatch glucose utilization in the anaerobic conditions and postnatal growth in the chicks (T.M. Shafeyet al., 2010; A.M. Dolgorukova, 2017). The in ovo nutrition can therefore be an instrument of significant improvement of the hatchability of the eggs and subsequent growth efficiency in hatched chicks, resulting in explicit economic effect (E.D. Peebles, 2018). It should, however, be noted that this technique has not still found application in the commercial poultry production and that for wider knowledge on the stimulating effects of different nutrients on the development of avian embryo further research is required.

Keywords: embryonic development, broilers, prenatal period, in ovo feeding, amino acids, antioxidants, vitamin E, vitamin C, L-carnitine, growth rate

Poultry farming is one of the leading branches of agriculture for producing relatively cheap and biologically complete food products. The peculiarities of the reproduction of domesticated birds, in particular the fact that the efficiency of reproduction in birds is definitely higher than in mammals, led to the emergence of industrial poultry farming [1].

Over the past 50 years, significant progress has been made in improving the functional characteristics of poultry through genetic selection. Thus, from 1957 to 2005, the growth rate of broilers increased by about 400% (for 5-6 weeks of life). At the same time, a higher body weight is achieved in less time with a decrease in feed consumption per 1 kg of body weight gain [2, 3]. However, such rapid growth and development caused a number of adverse complications, including ascites, skeletal abnormalities, immunosuppression, as well as increased susceptibility to infectious diseases [4]. According to Buzała et al. [5], such metabolic complications can be caused by inconsistencies between the processes of growth and development during embryogenesis and in the postembryonic period [5]. In modern broiler breeds and crosses, the duration of embryonic and neonatal development reaches 50% of the period of productivity (35-42 days of life).

Genetic selection determines the genotype of poultry; however, how inherited genes are expressed depends on external conditions – nutrition and growing technology. According to modern concepts of nutrigenomics, nutrients and biologically active substances can influence gene expression [6, 7]. These factors have the greatest effect during periods of intensive cell division while embryogenesis, leading to permanent effects throughout the entire postnatal period [8, 9]. The nature of nutrients supplied to the chick during the prenatal period is formed by the so-called food (or epigenetic) imprinting, on which the further growth and development of the organism depend. During epigenetic imprinting, DNA methylation occurs in the regions of promoters of specific genes, which can modulate for a long time the adaptive response of the organism to various stimuli during critical periods of development [10]. In poultry, epigenetic programming can be conducted during two critical periods –in a young industrial stock during gametogenesis and during embryogenesis, when egg nutrients are consumed by the embryo through the amniotic fluid and yolk [11].

The embryonic development of poultry can be supported and the chicks can be better prepared for intensive growth by using *in ovo* nutrition with natural nutrients — amino acids, carbohydrates, vitamins, as well as growth stimulants and hormones. The purpose of this review is to analyze the data available in the literature on the effects of carbohydrates, amino acids, antioxidants, and vitamins, when administered *in ovo*, on the embryonic and post-embryonic development of poultry, on the possible mechanisms of these effects, as well as on the prospects for creating industrial technologies of prenatal nutrition.

Technology of prenatal *in ovo* nutrition. The last period of incubation is characterized by oral intake of amniotic fluid by the embryo, intensive resorption of the yolk, accumulation of glycogen stores in muscles and liver for their use during pecking and hatching, the onset of pulmonary respiration, retraction of the residual yolk into the abdominal cavity, and finally, after pecking, the chick comes out of the shell [12, 13]. During this period, sharp physiological and metabolic changes occur, and any arising disturbances (for example, delay in nutrient use, incubation temperature) affect hatching efficiency and subsequent productivity [14-17].

The experiments using *in ovo* nutrition technology have shown that injections of nutrients affect the physiological state of broiler and chick embryos after hatching. The nutrients and biologically active substances introduced in this way ultimately improve the nutritional status of the chicks, which leads to a greater growth potential [18-20]. The injection site mainly depends on the age of the embryo. Thus, Ebrahimi et al. [18] propose to introduce substrates into egg albumen to a depth of 12 mm before incubation, as well as at its initial stage. After 17 days of incubation, the injections are carried out into other parts of the egg —

the air chamber and amnion. For the introduction of exogenous sources of nutrients and biologically active substances *in ovo*, either insulin syringes are used (under laboratory conditions) [21] or installations that ensure the processing of a large batch of eggs. The needles used in such devices are designed to reach the amnion [22].

Features of carbohydrate metabolism and the use of their exogenous sources during embryogenesis. One of the main physiological processes during the hatching period is the maintenance of glucose homeostasis. The liver plays a central role in carbohydrate metabolism and the delivery of glucose to tissues during embryogenesis, performing glucose synthesis from non-carbohydrate precursors (gluconeogenesis), glycogen synthesis (glycogenesis), and glycogen breakdown (glycogenolysis) [23, 24]. Glycogen stores are consumed as embryos go through the hatching process [25]. The high growth rate of the embryo is associated with high energy consumption. The glycogen stores in the liver and muscles are unable to meet the metabolic needs of the embryo, especially on the last day of embryogenesis. Low glycogen content in the liver correlates with longer hatching and lower body weight at hatching [14]. For homeostatic regulation of the amount of glucose in the blood, the embryo is forced to generate energy using various metabolic processes, for example, gluconeogenesis using glycerol as a substrate, released after lipolysis, or an amino acid after proteolysis [13]. Proteolysis, during which protein degradation is conducted, negatively affects the development of embryos [26, 27]. Since there are no carbohydrates in the egg, glycogen stores will begin to replenish only when the hatched chick has full access to the feed [13].

The introduction of different types of carbohydrates *in ovo* is likely to increase the amount of available energy for the embryo and reduce the catabolism of proteins and lipids during internal hatching. Zhai et al. [22] showed that injections of carbohydrates (glucose, sucrose, maltose, and dextrins, 0.25 g of active ingredient per 1 ml of diluent) statistically significantly ($p \leq 0.001$) increased the body weight of chicks while hatching, which increased in direct proportion to the volume of the injected solution. The authors recommend injecting no more than 0.4 ml of sucrose and 0.7 ml of glucose, maltose, and dextrin, while maintaining 90% hatchability. Fructose, unlike other carbohydrates, reduced hatchability and the body weight of chicks. The authors did not explain the reason for this [22].

At the end of the incubation period, rapid growth and maturation of visceral organs occur [28, 29]. Over the last 6 days of incubation, the area of the absorption surface in the small intestine increases 5-fold, the number of enterocytes increases, goblet cells appear that produce acidic mucin, and the ability to digest and absorb develops [30]. The earlier the intestines reach functional maturity, the faster the chicks will be able to use the nutrients in the feed, effectively absorb minerals and vitamins, thereby supporting the development of the most important organs and systems (skeleton, immune system, pectoral muscle). The observed increase in the body weight of chicks receiving carbohydrates during embryonic development may be associated with improvement in the development of the gastrointestinal tract, which was confirmed in the study by Kornasio et al. [19]. In chicks which received a mixture of dextrin and hydroxymethyl butyrate during the embryonic period, the glycogen content in the liver and muscles increased and the proliferation of satellite cells of muscle tissue was activated. The effect of the solution, injected *in ovo*, was long-lasting and influenced the weight of the poultry at the end of the rearing period [19]. Salmanzadeh et al. [31] showed that chicks that received a mixture of glucose and magnesium during the embryonic period had a greater body weight at hatching and on the 42nd day of life compared with the control group; in addition, the slaughter yield and the yield of pectoral muscles increased [31]. Similar results were

obtained in the authors' experiments. In 1-day-old chicks of meat-type mini chicken of the B77 line, the relative mass of the glandular and muscular stomachs in the group receiving dextrin in the prenatal period was significantly higher than in the control intact group. The live weight of 21-day-old chicks from the experimental groups, injected *in ovo* with glucose and dextrin (0.5 ml of a 10% solution), exceeded that in the control group by 3.9-6.7%, respectively [20, 32]. Similar results were obtained for poultry of the meat production direction — Cornish chicks of the cross Smena 8 [33]. A similar effect was observed in the embryos of ducks: injection of glutamine and carbohydrates (sucrose and maltose) *in ovo* led to an increase in live weight at the end of rearing, improved the development of the intestines and pectoral muscles [34].

Bhanja et al. [35] showed that *in ovo* injections of glucose (50 mg per embryo) on day 18 of incubation affected the development of the digestive system and the biochemical profile of the blood of chicks. In 1-day-old chicks from the experimental group, the content of glucose and protein in the blood plasma, the weight of the liver, glandular and muscular stomachs, as well as the small intestine, increased. On day 10 of life, in chicks receiving glucose during the embryonic period, the concentration of glucose and uric acid in the plasma significantly decreased, and the weight of the spleen and small intestine increased [35].

The use of exogenous amino acids in embryogenesis. In poultry, the pectoral muscle tissues serve as the main source of amino acids for gluconeogenesis when there is a lack of energy, which can lead to its atrophy [25, 36, 37]. Under late access to the feed, the development and growth of skeletal muscles are delayed and lags behind until slaughter age [17].

Ohta et al. [38] conducted experiments to evaluate the effect of *in ovo* injections of a mixture of amino acids on their use by embryos. It was shown that in the group receiving amino acids *in ovo* on day 7 of incubation, on day 19, the content of amino acids in the embryo, yolk, albumen, allantoic fluid, and amnion fluid was significantly higher ($p < 0.05$) than in the control (distilled water); in comparison with the control group, the absolute and relative weight of 1-day-old chicks also increased [38].

These results were confirmed when studying the effects of introducing a mixture of amino acids (0.75 ml per embryo) on ducks: when hatching, the mass of chicks was 6.2 higher than in the control; in addition, an increase in the mass of lymphoid organs was noted [39, 40].

In a number of experiments, the effect of individual amino acids was studied. Thus, Coskun et al. [41] demonstrated a positive effect of the introduction of methionine (50 μl per egg) into the amnion of broiler embryos: the relative weight of chicks increased by 2.7% compared to the control. Tahmasebi et al. [42] demonstrated a positive effect of threonine (25 mg per egg) introduced into the amnion on day 14 of incubation, which led to an increase in the growth rate of chicks compared with the control group ($p \leq 0.05$), as well as improvement in the development of organs of the gastrointestinal tract.

Ohta et al. [43] suggested that the concentration of amino acids in eggs, for example, glycine and proline, was insufficient to support embryo development in the final phase of incubation. This is also confirmed by the studies [44], which showed statistically significant differences in body weight in 1-day-old and 3-week-old chicks, if glycine and proline were administered during the embryonic period, compared with the control group.

Tong and Barbul [45] state that arginine is an essential amino acid for embryos, which is mainly due to its role in protein synthesis. Arginine is involved in a number of metabolic pathways, taking part in the formation of various biologically active compounds, which also helps to maximize the development

potential of the embryo by stimulating the secretion of growth hormones. It is known that arginine serves as a substrate for the synthesis of nitric oxide, the rate of oxidation of which during embryonic development is associated with the growth rate of chicks after hatching [46, 47]. In chicks receiving arginine during the embryonic period, after hatching, the growth rate and enzymatic activity of the digestive glands increased, and the morphological development of the organs of the gastrointestinal tract improved [42, 48]. It was shown that the hatchability of eggs was higher in the group where the embryos were injected with arginine and lysine on day 18 of incubation; on day 42 of life, the body weight of the obtained chicks was higher compared to the control group [49]. In other experiments, *in vivo* injections of 0.6% arginine solution contributed to an increase in the concentration of albumin in the blood plasma, the deposition of protein in the pectoral muscles, and, as a consequence, an increase in the growth of pectoral muscles in chicks [50].

Similar effects were observed in other poultry species. After the injection of a 3% solution of arginine into the air chamber of quail embryos, the synchronism in the hatching of chicks increased, the live weight increased on days 7 and 42 of life, and the feed conversion improved compared to the control group [51]. In the experiments on turkeys, feeding *in ovo* with the solution containing 0.7% arginine contributed, on average, to a twofold increase in the activity of pancreatic digestive enzymes (saccharase, maltase, leucyl aminopeptidases) in the small intestine of embryos on day 25 [52]. On day 14 of life of chicks, the activity of these enzymes was 3 times higher than in the control [52].

As it was noted, the effect of *in ovo* feeding may be due to epigenetic mechanisms. It was shown that *in ovo* injections of L-arginine at different times of incubation of chick embryos increased the expression of genes for myoblast determination factors (MyoD) and myogenin in the pectoral muscles of embryos [53]. The authors of the study explain the observed effect by the fact that L-arginine is a precursor of NO [53]. The effect of nitric oxide on the stimulation of the processes of embryonic myogenesis by enhancing the expression of myogenic regulatory factors was shown during *in ovo* injections to chick embryos of NO synthase inhibitors — an enzyme involved in the formation of nitric oxide from L-arginine, or NO donors [54].

The epigenetic effect of exogenous amino acids was noted in the case of *in ovo* administration of sulfur-containing amino acids methionine and cysteine, which increased the expression of genes for insulin-like growth factor (IGF-1) and toll-like receptor 4 (TLR-4) [55].

Features of energy metabolism of poultry embryos and the use of antioxidants and vitamins in embryogenesis. The metabolism of poultry in the embryonic period has some peculiarities and differs from the metabolism in postnatal ontogenesis. This is due to the unique structure of the poultry's egg, in which almost the entire supply of energy sources is yolk triglycerides and partly proteins, while free carbohydrates are extremely small: 0.5% in the yolk and 0.2% in the albumen, of which 98% are glucose [56]. The successful development of the embryo in poultry depends on the delivery of a sufficient amount of lipids (in a certain ratio) from the yolk to the embryo and the metabolic ability of the embryo tissues to utilize them for growth and differentiation. It was estimated that 94% of the total metabolic energy of the embryo during development was generated as a result of fatty acid oxidation [28, 57].

The presence of a large amount of unsaturated fatty acids in the presence of oxygen is fraught with the occurrence of oxidative processes with the formation of oxygen radicals. They lead to damage to embryonic and germinal structures and

the accumulation of toxic compounds. Antioxidants counteract the negative effects of free radicals and thereby protect the embryo from damage [58, 59].

The positive effect of *in ovo* injections of vitamins E (10 mg) and C (3 mg) on the body weight of ducklings after hatching and on their subsequent growth rate was established. The experimental groups were characterized by improved feed conversion [60]. The study of the effect of vitamin E on productivity and immunological parameters of the blood of chicks after hatching revealed the effect of this compound on the development of the immune system in chicks during the rearing period. Injection of vitamin E at a dose of 30 mg resulted in increased resistance to avian influenza and infectious bronchitis. In the experimental groups, an increase in the titers of immunoglobulins IgG, IgM, and IgA was noted [61]. Nowaczewski et al. [62], considering the effect of vitamin C on the hatchability of chicken and duck eggs, found that its positive effect was manifested only in duck eggs. In chicken eggs, injections of vitamin C did not have a significant effect on improving hatchability. In duck eggs, the best hatching results were obtained in experimental groups, regardless of the dose and time of *in ovo* administration of ascorbic acid. On average, the difference in hatchability of duck eggs between the experimental and control groups was 32.5% [62]. Opposite results for chicken eggs were obtained by Zhu et al. [63]: when 11-day-old embryos were injected with ascorbic acid (3 mg/egg), both an improvement in hatchability and an increase in the growth rate of chicks up to 42 days of age were observed. Moreover, the same authors found an increase in the expression of the *IL-4* and *DNMT1* genes and a decrease in *IL-1β*, *Tet 2*, *Tet 3*, and *Gadd 45β* ($p < 0.05$) in the spleen tissues of 21-day-old chicks, which received ascorbic acid on day 11 of incubation [63]. Apparently, the effect of antioxidants depends on the characteristics of the fatty acid composition of the egg and the content of endogenous antioxidants.

During the last 2-3 days of incubation, due to the high energy intensity of the hatching process and the relatively low availability of oxygen, fatty acids cannot provide the embryo with all the necessary energy [13]. As a result, the embryo switches to anaerobic glucose catabolism, the intensity of which depends on the amount of glucose stored in the form of glycogen of liver, kidney and muscles and generated during gluconeogenesis from amino acids, glycerol, and lactate [23, 49].

One of the substances that stimulate the oxidation of fatty acids for energy production is L-carnitine, which belongs to the group of biologically active compounds and plays an important role in energy metabolism during embryogenesis, participating in the transfer of acyl groups of fatty acids from the yolk to the tissues of an embryo. Poultry embryos have a limited ability to synthesize L-carnitine during incubation. The studies on the effect of exogenous sources of L-carnitine gave ambivalent results. In some experiments, the introduction of L-carnitine before incubation at doses from 2 to 12 mg per egg did not significantly affect the postembryonic development of chicks [18]. Elevating the dose of L-carnitine increased the hatching time and decreased the hatchability; the authors do not provide an explanation of the mechanism that caused the decrease in hatchability [18]. In other studies, the use of L-carnitine *in ovo* did not decrease hatchability and did not increase the hatching period of chicks [64]. At the same time, there was an increase in the absolute and relative weight of chicks at hatching, the content of glycogen in the liver and pectoral muscle, and insulin-like growth factor in the blood plasma [64]. It was also shown that the injection of L-carnitine on day 14 of incubation significantly increased hatchability, increased the growth rate of chicks, and improved feed conversion [65]. Similar results were obtained in the authors' studies. As a result of *in ovo* injection of L-carnitine in an amount of 2-3 mg per egg, hatchability increased, in 1-day-old chicks, there was a statistically

significant increase in weight, a significant decrease in glucose concentration, and an increase in the activity of lactate dehydrogenase in the blood serum, which indicates increased utilization of glucose under anaerobic conditions during the hatching period [66, 67].

It should be noted that the dosage of L-carnitine used in the studies varies greatly. Apparently, a clear understanding of the rate of carnitine synthesis during embryogenesis, as well as control of its initial content and correction in the case of deviation from the norm, are required.

Thus, the prenatal period in the development of poultry is an extremely important and critical period. Egg feeding technology can help the chick successfully overcome this stage and fully realize its genetic growth potential. The increase in the growth rate of poultry is due to the ability of the intestines to better digest and assimilate food. This can be achieved by *in ovo* administration of compounds that stimulate the functional activity of the cells of the gastrointestinal tract. In the first week after hatching, metabolic and physiological changes occur in the organism of the chicks. Faster adaptation of the gastrointestinal tract to exogenous food is essential for growth and increased vitality. It is also important that prenatal feeding has an epigenetic effect, inducing the expression of genes, the products of which are involved in the main metabolic pathways and processes in tissues and organs and thereby affect productivity. Consequently, feeding in eggs can be a tool to significantly improve hatchability and the vitality of chicks.

Despite a lot of conducted research, the method of prenatal feeding has not yet found industrial application. The data on dosages of exogenous substances vary significantly, as well as the timing of their use. To select the optimal dose, it is necessary to control the carbohydrate, amino acid, and vitamin composition of the embryo. This, in turn, requires establishing the norms for the content of such compounds in the embryo and developing the technology for the rapid accurate determination and correction of the corresponding parameters in the hatching egg. To implement the discussed technology into practice, research is required using existing automated systems [68, 69].

Influence of *in ovo* administered substances on hatchability and the growth rate of chicks

Active substance	Incubation period, days/dose per embryo	Influence		Reference
		on hatchability	on growth	
Glucose, maltose, dextrin	18/25 mg	+	+	[22]
Dextrin, hydroxymethyl butyrate	18/0.7 ml, 0.4% solution	No data	+	[19]
Glucose, dextrin	17/0.5 ml 10 % solution	No influence	+	[32]
Glucose	18/50 mg	No influence	+	[35]
Mixture of amino acids	7/0.7 ml	–	+	[43]
Mixture of amino acids	7/53 mg	+	+	[38]
Methionine	16/50 μl	–	+	[41]
Threonine	14/25 mg	No data	+	[42]
Glycine + proline	14/5.8 ml	–	+	[44]
Arginine	17.5/0,6 mg	No data	+	[50]
Arginine	0/0.5 мл, 3% solution	+	+	[51]
Arginine	21/1,5 ml, 0.7% solution	No influence	+	[52]
Methionine + cysteine	17.5/6.3 mg	No influence	+	[55]
Ascorbic acid	11/3 mg	+	+	[63]
Ascorbic acid	17/3 mg	–	No data	[62]
Vitamin E	12/10 mg	+	+	[60]
Vitamin E	14/15-30 mg	+	+	[61]
Folic acid	11/100-150 μg	+	+	[18]
Carnitine	0/8 mg	–	No influence	[64]
Carnitine	18/25-100 μl	No influence	+	[65]
Carnitine	14/4-12 mg	+	+	[66]
Carnitine	17/3 mg	+	+	[22]

N o t e. "+" – improvement, "–" – deterioration, "no influence" — the indicator remained unchanged.

Moreover, the cost-effectiveness of such a technology is not clear.

Proceeding from the fact that its main significant indicators are hatchability and the growth rate of chicks, the authors briefly summarized the data available in the special literature on this topic (Table).

Thus, despite the fact that the overwhelming majority of researchers declare the positive effect of prenatal feeding on the growth rate of poultry, many questions remain unresolved and controversial. The effect on the viability of embryos after their exposure to exogenous substances is ambiguous; their dosage and delivery time to the embryo are not clear. Establishing the norms for the content of such exogenous substances in the egg and studying the physiological mechanisms of the response of embryos after exposure to them remains the most important task, without solving which it is impossible to successfully regulate the productive qualities of poultry at the embryonic stage.

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AGRICULTURAL BIOLOGY,
2020, V. 55, № 6, pp. 1073-1089
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 636.018:579.6

doi: 10.15389/agrobiology.2020.6.1073eng
doi: 10.15389/agrobiology.2020.6.1073rus

PROSPECTS OF ANTIQUORUM SUBSTANCES AS AN ALTERNATIVE TO ANTIBIOTIC THERAPY IN ANIMAL HUSBANDRY

(review)

K.S. KONDRASHOVA ✉, D.B. KOSYAN, K.N. ATLANDEROVA, S.V. LEBEDEV

Federal Research Centre of Biological Systems and Agrotechnologies RAS, 29, ul. 9 Yanvarya, Orenburg, 460000, e-mail christinakondrashova94@yandex.ru (corresponding author ✉), kosyan.diana@mail.ru, atlander-kn@mail.ru, lsv74@list.ru

ORCID:
Kondrashova K.S. orcid.org/0000-0003-4907-9656
Kosyan D.B. orcid.org/0000-0002-2621-108X
Atlanderova K.N. orcid.org/0000-0003-3977-4831
Lebedev S.V. orcid.org/0000-0001-9485-7010

The authors declare no conflict of interests

Acknowledgements:

Performed in accordance with the FRC BSAT RAS research plan for 2019-2021 within the framework of state order No. 0526-2019-0002

Received August 28, 2020

Abstract

Frequent and inappropriate use of antibiotics in animal husbandry threatens to expand the spectrum of antibiotic-resistant bacteria. Quorum sensing (QS) is one of the mechanisms responsible for this process. For its implementation, bacteria use autoinducers, the special signaling molecules for information exchange (A.A. Miller et al., 2011). The studies to give insight of this mechanism have shed light on the existence of substances that act as Quorum sensing inhibitors (quorum suppressors) (B. Remy et al., 2018), which made such studies even more relevant (J. Bzdreng et al., 2017). In our review, we have summarized the latest data on the search and development of the biologically active compounds that can become an alternative to antibiotic drugs used in animal husbandry. These include bacterial enzymes (AGL-lactonases, AGL-acylases, decarboxylases, and deaminases) that can degrade quorum sensing signal autoinducers (V.C. Kalia et al., 2011), as well as α-amylases, β-glucanases, lipases, and proteases involved in the destruction of biofilms (R. Sharma et al., 2001). The antimicrobial properties are also characteristic of animal enzymes acylase I (D. Paul et al., 2010), paraoxonase (J.F. Teiber et al., 2008), and lactonase, plant enzymes laccase (R. Al-Hussaini et al., 2009), alliinase, thiol-dependent enzyme and lactonase derived from garlic and medicinal plants (A. Adonizio et al., 2008), enzymes of marine organisms, particularly bromoperoxidase of the algae *Laminaria digitata*, alginate lyases from algae, invertebrates, and marine microorganisms, and halogenated furanones of *Delisea pulchra* (S.A. Borchardt et al., 2001; M. Manefield et al., 2000). In addition, we can distinguish antimicrobial digestive enzymes used as feed additives, e.g., phytase (O. Adeola et al., 2011), xylanase and lysozyme (G. Cheng et al., 2014). Studies of phytobiotics and essential oils as quorum sensing inhibitors are promising (V.I. Fisinin et al., 2018). Their inhibitory ability is shown due to the similarity of the chemical structures of some plant extracts to the structure of acyl-homoserine-lactone and inactivation of signaling molecules (R. Chevrot et al., 2006; F. Nazzaro et al., 2013). Another prospective alternative is the use of antimicrobial combinatins enabling a synergistic effect due to the variety of mechanisms of overcoming the recurrent bacterial communications and destroying persistent bacterial cells. These polypeptide cocktails may include the combination of antibiotics with natural compounds. The amtimicrobial efficacy has shown for combination of tobramycin and some plant extracts, partilularly cinnamaldehyde and baykalin hydrate against *Burkholderia cenocepacia* and *Pseudomonas aeruginos* (G. Brackman et al., 2011), a wide range of antibiotics, e.g., aminoglycosides (T.H. Jakobsen et al., 2012; M. Stenvang et al., 2016), quinolones (Q. Guo et al., 2016), polypeptide antibiotics (A. Furiga et al., 2016; Z.P. Bulman et al., 2017), cephalosporins and glycopeptides (D. Maura et al., 2017), and various quorum sensing inhibitors.

Keywords: quorum sensing, antibiotics, resistance, bacteria, plant extracts, enzymes

The discovery and use of antibiotics play an unprecedented role in solving many problems related to the prevention, control, and treatment of infectious diseases in animals [1]. In addition, the use of antibiotics in feed is an im-

portant factor in increasing its effectiveness, promoting growth, and improving the quality of animal products. However, despite all the advantages of using antibiotics, their excessive use has led to the emergence and increase in the number of resistant microorganisms [2, 3]. The use of antibiotics in agriculture not only causes resistance in animal microflora but also changes the composition and properties of microflora in natural habitats (soils, groundwater) in the direction of increasing the antibiotic resistance of the microbial community [4]. For this reason, in 1986, Sweden first introduced a ban on the use of certain antibiotics in animal feed [5]. In 2006, the countries of the European Union introduced a ban on antibiotics — the growth stimulants in accordance with the regulation of the European Parliament and the Council of the EU No. 1831/2003 of September 22, 2003. However, this caused negative consequences for livestock due to an increase in the incidence of infections. This led to the need not only to reuse antibiotics but also to increase the volume of their use [3, 6-8]. In the territory of Russia, there are no bans on the use of feed antibiotics, e.g., tetracyclines (biotin based on the producer of chlortetracycline), grisin, bacitracin (bihilicin), and tylosin are allowed. The only limitation is that antibiotics must be excluded from the diet 5 days to 3 weeks before slaughter [9]. The Russian government, in its order No. 604-r of March 30, 2019, approved an action plan for the implementation of the Strategies for preventing the spread of antimicrobial resistance.

Resistance in bacteria is controlled by a set of mechanisms to avoid exposure to antibiotics. It can be either congenital (the absence of a target for an antibiotic or its inaccessibility) [10] or acquired as a result of gene transfer from a neighboring organism [11-13], or it can arise due to an increased frequency of mutations [14-16], or manifest itself as an adaptive ecologically induced resistance [17]. Also, inactivation of an antibiotic can occur due to bacterial modification of the enzyme or with the participation of a degrading enzyme that changes the target of the antibiotic [18-22]. At the same time, bacteria can change the permeability of their cell wall for the outflow of antibiotics outside the cell using an efflux pump [23-25]. The clearance rate is usually higher than the drug penetration rate, thereby controlling the level of antibiotics in the cell [26, 27]. The American Society of Infectious Diseases has identified a group of microorganisms (*Staphylococcus aureus*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Enterobacter*) capable of "escaping" antibiotics with the described antibacterial mechanisms. These species represent a new paradigm of virulence, transmission, and antimicrobial resistance [28].

In addition, bacterial communities develop resistance through a process known as quorum sensing (QS), which will be considered as the main mechanism in this review. Its essence lies in the fact that microorganisms produce autoinducers, which act as intercellular signaling to control population density and coordinate its activities, including biofilm formation, virulence, reproduction, spore formation, and horizontal gene transfer [29]. Inside the biofilm, bacteria are approximately 1000 times more resistant to antibiotics than their planktonic precursors [8, 30].

The active substances that suppress QS are called quorum sensing inhibitors (QSIs). In contrast to the currently widely used antibiotics, these agents reduce the number of microbial infections by suppressing the induction of microbial QS, and, as a rule, they do not affect the growth of bacteria [31, 32]. Since QS induces various harmful traits, impairment of bacterial communication seems to be promising in many areas, especially in healthcare and agriculture [33, 34].

QS as a communication mechanism for bacteria. QS is a spe-

cial type of regulation of bacterial gene expression that functions under conditions of a critically high density of their population [35]. This molecular mechanism is required by microorganisms in order for them to collectively adapt their behavior in accordance with the density of the cell population and environmental conditions. This communication system allows bacteria to carry out processes that are costly and ineffective at low cell density, but become useful for the entire community at high cell density (virulence factor production, biofilm formation, and protease and siderophore synthesis) [36].

The QS system has been discovered and described in both gram-positive and gram-negative bacteria. In gram-positive microorganisms, autoinducing peptides (AIPs), autoinducer-2 (AI-2), and other signaling molecules such as quinolones, esters, and fatty acids that induce QS have been extensively studied. These peptides are species-specific and strain-specific and have been described in *Staphylococcus* spp., *Clostridium* spp., *Enterococcus* spp., and other strains [37].

In gram-negative bacteria such as *Pseudomonas* spp., *Acinetobacter* spp. and *Burkholderia* spp., another class of autoinducers, acyl-homoserine lactones (AHLs), has been described [38]. These compounds consist of a lactone ring and an aliphatic acyl chain of different lengths and with various modifications [38]. Most gram-negative bacteria combine several QS systems to integrate various signals or have a hierarchical system: for example, in *Pseudomonas aeruginosa*, it combines four QS systems (*las*, *rhl*, *iqs,* and *pqs*) acting in the network [39], while in the parallel hierarchical system *Vibrio harveyi*, three systems are integrated into one regulatory cascade [40].

Other types of signaling molecules have also been identified [41], including fatty acids used by *Xanthomonas* spp., *Burkholderia* spp., *Xylella* spp. [42], ketones in *Vibrio* spp., *Legionella* spp. [43], adrenaline, norepinephrine, AI-3 in enterohemorrhagic bacteria [44] and quinolones in *Pseudomonas aeruginosa* [45]. AI-2 (furanosyl borate diester) is used by both gram-negative and gram-positive bacteria [46].

Characterization of substances that suppress QS. The process that interferes with bacterial communication, known as quorum quenching (QQ), is of paramount importance to the problem of bacterial resistance. It was discovered as a natural phenomenon, first described in 2000, with the identification of the QQ enzyme capable of degrading AHL signals from *Erwinia carotovora* [47] during enzymatic hydrolysis.

In the QS system, the synthesis of signaling molecules plays a vital role in communication between cells [48]. Bacterial communication can be disrupted through several processes.

Suppressing the synthesis of signaling molecules by QSI is a direct way to disrupt QS. If no signaling molecules are produced, QS will not be felt. However, studies on inhibitors of signaling molecule synthesis are few and the data are very limited [49, 50].

The breakdown of signaling molecules is a more well-studied quenching process. It mainly involves enzymes produced by microorganisms or other organisms to destroy signaling molecules that perceive QS, which leads to a decrease in their concentration below the threshold value, as a result, pathogenic bacteria cannot express genes and produce pathogenic factors, losing the ability to infect the host [51 -54].

Inhibition of the conduction or binding of signaling molecules to receptors also plays an important role in reducing the pathogenicity of bacteria. Studies have shown that many organisms can secrete analogs of QS signals, compete with bacterial signal receptors, interfere with the regulation of the QS control system, and significantly reduce the pathogenicity of bacteria [55, 56].

Currently, all QSIs can be classified into several categories. According to their chemical structure, QSIs are classified into three groups: non-peptide small molecules, peptide and protein QSIs. Non-peptide QSIs include AHL analogs, ACP (acyl transfer protein) homologues, L/DS-adenosyl homocysteine and butyryl-S-adenosyl-L-methionine, peptide QSIs, mainly AIP homologues, and RNAIII inhibitory peptide (RIP) [57-59], interfering with the synthesis of QS signaling molecules or their binding to receptors. Protein QSIs include antibodies and enzymes [60], in particular, AHL acylase, lactonase, *Rhodococcus* oxidoreductase, and mammalian paraoxonase that destroy signaling molecules [61]. In addition, competing organisms are able to lyse signaling molecules for quenching QS [62]. For example, *Escherichia coli* is able to uptake AI-2, affecting QS in *Vibrio harveyi* [63].

QSI is divided into natural and synthetic. Among natural compounds, antagonist peptides designed to suppress gram-positive bacteria and QSIs aimed at QS of gram-negative bacteria and AI-2-mediated QS have been identified [64]. These include polyphenols isolated from tea or honey, ajoene from garlic, eugenol from cloves, and many compounds produced by marine organisms and fungi [65]. Among synthetic substances, fluorouracil (5-FU) and azithromycin can be distinguished [66, 67].

QSIs are likely to differ in mechanisms of action that are not always known [68]. Some molecules that inhibit QS, for example, azithromycin, are also considered antibiotics, since, starting at a certain concentration, they can inhibit bacterial growth [69]. Currently identified QQ enzymes mainly target AHL and AI-2-mediated QS: phosphotriesterase-like lactonases, lactonases, acylases, and oxidoreductases degrade AHL signals; the latter enzyme also targets AI-2 [70]. In this regard, a lot of research work has been done to find alternative approaches to prevent QS [71, 72].

Screening for natural antimicrobial agents. *Enzymes.* More than 2000 different enzymes are currently known. Enzymes are grouped into six classes: oxidoreductases, transferases, hydrolases, lyases (synthases), isomerases, and ligases [73].

There are several commercial hydrolase preparations effective against microbial biofilm: Spezyme GA300, Pandion, Resinase A2X, and Paradigm [74]. Substrates for hydrolases are peptidoglycans – components of the bacterial cell wall responsible for its rigidity. Degradation of the cell wall leads to cell lysis due to a violation of the internal osmotic pressure. Gram-negative bacteria are less sensitive to bacteriolytic enzymes than Gram-positive ones due to differences in the structure of the cell wall [73].

Proteases are enzymes that hydrolyze proteins; in particular, they include subtilisins, which are widely used to control biofilms under industrial conditions [75]. Lysostaphin is an endopeptidase that lyses the cell walls of staphylococci, including methicillin-resistant *Staphylococcus aureus* (MRSA), by cleaving pentaglycine cross-links of peptidoglycan [76]. Administration of lysostaphin in combination with oxacillin or vancomycin enhanced the antimicrobial effect [77].

Among the enzymes that hydrolyze polysaccharides, lysozyme, alginate lyase, dispersin B, and amylase have antimicrobial properties. Lysozyme immobilized in chitosan was effective in suppressing food spoilage by microorganisms [78]. Alpha-amylase hydrolyzes biofilms of *Staphylococcus aureus* [79]. The combination of proteases and amylases effectively removed *Pseudomonas fluorescens* biofilms [80].

Antimicrobial enzymes of bacteria. The enzymes that quench QS and are capable of degrading QS-signaling acylated homoserine-lactone au-

toinducers include AHL lactonases, AHL acylases, decarboxylases, and deaminases [62]. These enzymes are found in bacteria from different phyla — *Actinobacteria*, *Rhodococcus*, *Arthrobacter*, *Streptomyces*, *Firmicutes*, *Bacillus*, *Oceanobacillus*, *Anabaena*, *Cyanobacteria*, *Proteobacteria*, *Alteromonas*, *Comamonas*, *Halomonas*, *Hyphomonas*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Ralstonia*, and *Stappia* [81]. These bacteria have either AHL lactonases or AHL acylases; *Rhodococcus erythropolis* is the only known organism with two enzymes [82, 83]. Interestingly, *Bacillus thuringiensis* does not produce a QS signal, but produces AHL lactonase [84]. Microorganisms secreting bacteriolytic enzymes (for example, streptomycetes) usually express a complex of several enzymes with different specificities for cell wall degradation.

The use of lipase is considered an innovative and environmentally friendly approach for biofilm control due to the lytic and dispersing activity of this enzyme. Most of the lipases used in industry are of microbial origin. Lipases catalyze the hydrolysis of long-chain aliphatic acid esters. This enzyme is synthesized by eukarya, fungi, actinomycetes, yeast, bacteria, and archaea. Bacterial lipases are produced by representatives of the genera *Bacillus*, *Penicillium*, *Staphylococcus*, *Pseudomonas*, and *Aspergillus*. The properties of α-amylase, β-glucanase, lipase (EC 3.1.1.3), and protease, which destroy the flowing biofilms of *Pseudomonas fluorescens*, were also investigated. Four enzymes showed a moderate decrease in the number of colony-forming units in the biofilm [85, 86].

Antimicrobial enzymes of animals. Porcine kidney acylase I inactivated QS signals and prevented biofilm formation in *Pseudomonas putida* and *Aeromonas hydrophila* [87]. Mammalian paraoxonases have a hydrolytic effect on esters and lactones [88]. Mammalian lactonases differ from those isolated from bacteria in that the enzyme in mammals requires an active calcium ion [88]. Human epithelial cells are capable of inactivating the AHL autoinducer synthesized by *Pseudomonas aeruginosa* [89].

Pancreatic lipase catalyzes the synthesis of fatty acids in bacteria; therefore, it can serve as a potential antibacterial agent effective against many bacterial strains [86]. The mammalian enzymes paraoxonase and lactonase belong to the QSIs and can influence the development of infections caused by *Pseudomonas aeruginosa* [61].

Antimicrobial plant enzymes. Laccases, which are QSI enzymes, were found in extracts obtained from fruits, flowers, leaves, and bark of *Laurus nobilis, Combretum albiflorum,* and *Sonchus oleraceus;* analysis was performed using *Chromobacterium violaceum* [90]. Alliinase and a thiol-dependent enzyme isolated from garlic and medicinal plants act as a QSI for *Pseudomonas aeruginosa* [91]. Lactonase, which is present in clover, lotus, legumes, peas, sweet potatoes, and alfalfa, has shown the ability to degrade AHL in *Chromobacterium violaceum* CV12472 and CVO26 strains [92].

Enzymes of marine organisms. Algae, for example, *Laminaria digitata*, possess the enzyme haloperoxidase, which exhibits the ability to inhibit QS (QQ) through oxidation of the AHL signaling group [93]. Red algae *Delisea pulchra* contain halogenated furanones, which are structurally similar to bacterial AHL and can block receptors, interfering with the QS process [94, 95]. Alginate lyases (enzymes found in algae, invertebrates, and marine microorganisms) are used in combination with gentamicin against *Pseudomonas aeruginosa* in respiratory tract infections in patients with cystic fibrosis [96, 97].

Antimicrobial digestive enzymes. Digestive enzymes that supplement the diet to increase feed efficiency and stimulate nutrient absorption also affect the bacterial population in the digestive tract [98]. Several enzymes, such as phytases and carbohydrate-degrading enzymes, are marketed as feed additives

for monogastric animals [99]. Such additives increase the supply of nutrients to the intestinal flora, which allows it to better compete with pathogenic bacteria [98]. In broiler chickens, the addition of xylanase and lysozyme preparations to the diet minimized gastrointestinal damage by reducing the abundance of *Clostridium perfringens* in the ileum [100].

Limited information is available on the practical use of enzyme-based feed additives with antimicrobial properties. However, it is obvious that additives that inhibit QS are very promising and will be especially in demand in animal husbandry, given the current use of antibiotics in this industry. Unfortunately, the disadvantages of enzyme preparations – QS inhibitors – include the relatively high cost of their industrial production [101].

Plant extracts and essential oils (EOs). Plant substances known as phytobiotics are used in animal feeding as antioxidant, antimicrobial, anti-inflammatory, and antiparasitic agents [102, 103]. Many plants have beneficial multifunctional properties, and the bioactive substances obtained from them can have a beneficial effect on the animal's body. Plant extracts are generally considered safe, are effective against certain bacteria, are widely used in feed as growth stimulants and to protect animals, exhibiting antioxidant, antimicrobial, and immunostimulating effects [103, 104].

In pig breeding, the use of oregano, cinnamon, Mexican pepper, and thyme is recommended to suppress pathogenic microflora in the intestine [105-107], sangrovit and garlic extract containing allicin are able to increase live weight gain [108, 109], thyme, cloves, eugenol increase the productivity of pigs [110, 111]. The positive effect of phytogenic feed additives on the growth rates of poultry live weight has been reported [112].

Phytobiotic compounds are represented by phenols/polyphenols, alkaloids, terpenoids/EOs, and lectins/polypeptides [113]. Plant extracts have an *in vitro* antimicrobial effect at a minimum inhibitory concentration of 100-1000 μg/ml [114]. Some phytobiotics against pathogenic microorganisms exhibit QSI properties, since their chemical structure is similar to that of AHL [115]. In addition, gamma-aminobutyric acid, which is structurally similar to inducers of the *attKLM* operon, activates the expression of the AttM lactonase, which it calls, which, in turn, inactivates the QS signal [116]. The flavonoids kaempferol, naringenin, quercetin, and apigenin act as QSIs, inhibiting the HAI-1 or AI-2 QS-controlled bioluminescence autoinducers in *Vibrio harveyi* [117]. Catechins produced by tea plants can activate AHL-lactonase and suppress the transfer of the *Escherichia coli* conjugative R-plasmid, leading to its loss [118]. Furocoumarins and rosmarinic acid, present in grapefruit juice and sweet basil roots, disrupt biofilm formation in *Escherichia coli* and *Pseudomonas aeruginosa,* respectively [119]. Thymol is currently used in combination with vancomycin and ethylenediaminetetraacetic acid as an antimicrobial agent [120]. In addition, the combined action of the antibiotic tobramycin and some plant extracts (cinnamaldehyde and baicalin hydrate as QSI) was effective against *Burkholderia cenocepacia* and *Pseudomonas aeruginosa* [121-123]. The effect of herbal extracts *Artemisiae argyi*, *Cortex dictamni,* and *Solanum melongena on Pseudomonas aeruginosa* was studied [124]. It was also found that *Citrus sinensis* flavonoids were capable of inhibiting QS signals, which can significantly reduce the concentration of signaling molecules secreted by *Yersinia enterocolitica* and disrupt biofilm formation without affecting bacterial growth [125].

Quercus robur oak bark extract was widely used in animal husbandry, including for partial replacement of antibiotics. It inhibits the development of pathogenic microflora of the intestine of poultry on beef-extract agar due to anti-QS effects, which can be useful in the development of methods for controlling

bacterial infections [126].

Studies to assess the effectiveness of QSI in feeding poultry seem promising [127]. Seven components with anti-QS activity (in descending order) were found in the *Quercus cortex* extract: pyrogallol, propylresorcinol, coumarin, scopoletin, coniferyl alcohol, vanillin, antiarol [128]. The extract exhibits the most pronounced and stable anti-QS activity in the absence of obvious antibacterial substances in its composition [129]. This allows the use of QSIs isolated from oak bark as a feed additive for poultry, including in combination with other feed additives, among which probiotics and antibiotics in low doses can be distinguished [130]. It is also known that oak bark extract in the diet of cows increases the number of microorganisms that decompose cellulose and other polysaccharides, which stimulates the activity of various hydrolases in the rumen fluid [131].

The use of EOs is considered promising against epidemics caused by multidrug-resistant bacteria. EOs of lemon, white thyme, cinnamon, eucalyptus, and lemongrass have shown a high antibacterial effect against some resistant strains, in particular, representatives of the genera *Streptococcus*, *Candida*, and MRSA [132, 133]. A synergistic effect between EOs and antibiotics has been reported: the oils of *Mentha piperita*, *Thymus vulgaris*, and *Rosmarinus officinalis* in combination with ciprofloxacin exhibited a more pronounced antimicrobial effect [134]. Also, the anti-QS activity of essential oil or its components affects the expression of AI [135].

Analyzing the use of medicinal herbs and their extracts in animal husbandry, it should be noted that, due to their complex composition, their complex toxicological studies and safety assessment are difficult. It is necessary to identify biologically active components of additives based on plant raw materials and to quantify their effect on the efficiency of feed conversion, improvement of physiological parameters and the state of animal health. Currently, supplements in the market do not meet the principle of traceability and effectiveness. When used in large quantities (1-2%, sometimes up to 5% of the diet), they can negatively affect animals, in particular, digestion and absorption of nutrients. It is also important to consider the possible effects of phytogenic additives when combined with other feed additives. There is evidence of the adverse effects of the combined use of herbal preparations with enzymes [136] and with proteins, leading to their partial denaturation [100]. Although phytobiotics are a group of natural substances, more research is needed on their mechanisms of action, dietary compatibility, toxicity, and safety before they can be widely used in animal husbandry.

The combined effects of antimicrobial drugs. The combination of several drugs can provide a synergistic effect due to a variety of mechanisms required to overcome recurrent bacterial communication and kill persistent cells [73]. The composition of such multi-drug cocktails is not limited to antibiotics and may include combinations of antibiotics with natural compounds that have QQ properties and act as non-antibiotic adjuvants. The combined use enhances the antimicrobial effect and prevents the development of bacterial resistance [137], since the destruction of the biofilm makes bacteria more sensitive even to low doses of antibiotics. Combination of antibiotics and QSIs has been shown to be effective against resistant strains in staphylococcal infections, when the sensitivity of bacteria to commercial antibiotics was increased using the QS inhibitor — RNAIII-inhibiting peptide (RIP, YSPWTNF-NH_2) [138, 139]. QSIs such as furanone C30, patulin, penicillic acid, and garlic extract have been reported to increase the sensitivity of *Pseudomonas aeruginosa* to tobramycin and the phagocytic activity of leukocytes [8, 71]. Natural antimicrobial com-

pounds that can be used as adjuvants for antibiotics are of great interest to researchers [73].

Combination therapy with QQ in *Pseudomonas aeruginosa* infections has also been studied. The use of benzamide-benzimidazole inhibits the MvfR (PqsR) QS regulator, leads to a decrease in biofilm formation, and restores antibiotic susceptibility [140, 141]. Baicalin hydrate and hamamelitannin (respectively, AHL-oriented QSI and peptide QSI) enhance the destruction of biofilms in both gram-negative (*Pseudomonas aeruginosa* and *Burkholderia cepacia*) and gram-positive (*Staphylococcus aureus*) bacteria and show a synergistic effect *in vivo* and *in vitro* with tobramycin and clindamycin or vancomycin, respectively [121]. The effectiveness of a wide range of antibiotics, e.g., aminoglycosides [142, 143], quinolones [144], polypeptide antibiotics [145, 146], cephalosporins, and glycopeptides [141, 147], is enhanced by the addition of QSIs.

The results obtained show that QSIs are potential tools for increasing the sensitivity of microorganisms to antibiotics and, therefore, reducing the active doses of the latter. In addition, a similar trend and efficacy were noted for the combination of lactonase (QQ) and the antibiotic ciprofloxacin in experiments on mice [148]. The combination of antimicrobials and QQ has been shown to give promising results. Therefore, the use of QQ can be an effective strategy for reducing the applied doses of antibiotics, which is important for solving the problem of increasing resistance to them in farm animals.

Thus, substances acting as an alternative to antibiotics must meet a set of criteria: be non-toxic, have no side effects, be easily excreted from the body, do not stimulate bacterial resistance, persist stably in feed, do not decompose in the gastrointestinal tract, do not pollute the environment, do not affect the palatability, kill pathogenic microflora or suppress its growth, without affecting the normal flora, as well as improve the efficiency of nutrient assimilation of feed and the growth performance of animals. At present, there are no known compounds alternative to antibiotics that meet the listed requirements. Existing commercial enzyme preparations, as well as biofilm-inhibiting and quorum-suppressing enzyme preparations that are under development, are unstable and readily degraded in the digestive tract. In addition, the direct antimicrobial effect of antibiotics is higher than that of alternative compounds. Antibiotic drugs are made from one and relatively pure active substrate with high stability, their quality is ensured by long-term production practice. One recommendation is to use some of the natural antimicrobial compounds in combination with lower doses of antibiotics. Such combined use appears to be the most effective and fastest way to limit the adverse effects of antibiotic use and avoid the formation of bacterial resistance. This will minimize the economic losses caused by infections and maintain the high activity of antibiotics against pathogens if it is necessary to carry out effective antibiotic therapy.

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AGRICULTURAL BIOLOGY,
2020, V. 55, № 6, pp. 1090-1106
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 638.123:575.2

doi: 10.15389/agrobiology.2020.6.1090eng
doi: 10.15389/agrobiology.2020.6.1090rus

POLYMORPHIC STR MARKERS AS A TOOL FOR POPULATION-GENETIC STUDIES OF *Apis mellifera* L. HONEYBEES
(review)

O.Yu. FOMENKO ✉, M.S. FORNARA, A.V. DOTSEV

Ernst Federal Science Center for Animal Husbandry, 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail fomenych@rambler.ru (✉ corresponding author), margaretfornara@gmail.com, asnd@mail.ru
ORCID:
Fomenko O.Yu. orcid.org/0000-0001-7852-3790
Dotsev A.V. orcid.org/0000-0003-3418-2511
Fornara M.S. orcid.org/0000-0002-8844-177X
The authors declare no conflict of interests
Acknowledgements:
Supported financially by the Russian Ministry of Science and Higher Education within theme No. 0445-2019-0024
Received July 21, 2020

Abstract

The relevance of honeybee biology comprehensive study is increasing every year. Primarily, this is caused by the decline of honeybee populations which occurs all over the world including the Russian Federation. Historically, the Europe and Africa continents were the habitat of the only representative of genus *Apis*, the honeybee *Apis mellifera* L. from which a significant number of freely interbreeding races (subspecies) derived during evolution. Nowadays, due to human introduction of honeybees to other continents, *Apis mellifera* are found all around the world. The loss of unique gene pools and purebred status of native honeybee subspecies due to uncontrolled hybridization is a matter of great concern worldwide (P. de la Rúa et al., 2009). Therefore, evolutionary relationships and population genetics of *A. mellifera*, genetic control of domestic and imported breeding stock purity, breed authentication, genome-wide association mapping for traits of apicultural interest (e.g., queen performance, flight activity, honey and wax productivity, resistance to parasites, winter hardiness, royal jelly components, bee venom, etc.), and breeding value estimation are the key points of approach to biodiversity conservation in honeybees. The set of parameters characteristic of the population/line as a whole is the necessary base to preserve and maintain polymorphism as a component of population stability (N.I. Krivtsov et al., 2011). Genetic structure of breeding populations and relations between geographically isolated populations are relevant to characterize breed gene pool and optimize selection programs. The paper discusses general aspects of microsatellite structure, the main models of evolution (H. Fan et al., 2007) and putative mechanisms of origin in eukaryotic genomes (A.V. Omelchenko, 2013). Microsatellites are tandem repeats of short (2-6 bp) noncoding sequences that are dispersed throughout the nuclear genome (W.S. Sheppard et al., 2000). Microsatellites are located in both protein-coding and non-coding regions, including regulatory sequences (I. López-Flores et al., 2012). It is believed that microsatellites emerge and spread via formation of various non-canonical DNA structures that favor the slipping of replication forks (R.D. Wells, 1996). Microsatellite loci are a very convenient tool to analyze the genetic structure of populations, estimate genomic inbreeding and the level of heterozygosity, calculate genetic similarity coefficients, and determine the level of introgression. This paper overviews the use of STR markers for reconstruction of the honeybee evolutionary history. The principal research papers on population genetics of various *A. mellifera* subspecies from Europe, Asia, America, and Africa are considered. Special attention is paid to the Russian honeybee breeds and populations. To summarize, the STR markers due to the large number of alleles, the high frequency of mutational events and codominant type of inheritance continue to be extremely powerful tool for genomic mapping, verification of the genomic authenticity, and in genetic and evolutionary studies of populations.

Keywords: honey bee, microsatellite markers, STR markers, evolution, population genetics, gene pool, introgression

Honeybees (*Apis mellifera* L., *Hymenoptera*: *Apidae*) are the main pollinating insects on the planet, vital for the existence of many crops (rapeseed, sunflower, legumes), as well as for the preservation of natural plant biodiversity. From 1961 to 2007, agricultural dependence on pollinators in developed and developing

countries increased by 50% and 62%, respectively [1].

The relevance of a comprehensive study of the biology of the honeybee increases every year, which is caused by the emerging negative processes occurring in the populations of these insects, both in the Russian Federation and around the world. First, the pressure of biotic and abiotic environmental factors on bee colonies is increasing, resulting in a decrease in their number. For example, in Europe, the number of bee colonies has decreased by 16% in 20 years [2]. In the US, the annual loss of bee colonies is close to 50% [3]. The mass death of bee colonies, according to many researchers, in the short term can lead to irreparable consequences, up to the complete disappearance of beekeeping. Second, the import and reproduction of *Apis mellifera* subspecies, which are not scientifically justified, unsystematic and uncontrolled, lead to mass hybridization of bees and loss of purebredness [4]. With a high degree of confidence, these processes are associated with the mass collapse of bee colonies.

Bees of hybrid lines have less resistance to adverse environmental factors, such as abiotic stress factors [5, 6] and exposure to pesticides [7, 8], and are characterized by reduced immunity [9-11], which increases their susceptibility to parasites [12, 13] and pathogens [14, 15]. The result of interbreeding hybridization is a decrease in the adaptation of hybrid bee colonies to changing environmental conditions, which inevitably leads to an increasing death of bees. Ectoparasitic mites *Varroa destructor*, which causes varroosis [16], and microsporidia *Nosema ceranae*, which causes nosematosis type C [17], are recognized among the key factors causing the death of bee colonies in winter in Europe.

During hybridization and loss of purebredness, the gene pools of native bee subspecies are lost [18-20]. Currently, the dark forest bee *Apis mellifera mellifera* L., one of the unique subspecies of the honeybee, is recognized as endangered in Europe [21]. Thus, the issues of preserving the gene pool and native populations of the honeybee *A. mellifera* acquire worldwide significance.

Due to the frightening scale of interbreed hybridization, an important task of Russian beekeeping is to preserve the gene pools of populations of domestic breeds and subspecies of bees. Russia has some unique opportunities to preserve native honeybee populations [18, 22, 23]. The Central Russian honeybee breed *A. mellifera mellifera*, which is the most adapted to a long winter with low temperatures and resistant to some diseases, is of considerable interest and recommended for breeding in most of the country [24]. To restore the gene pool of the Central Russian breed, two large populations of the Russian honeybee that have been preserved in the Krasnoyarsk Territory and Bashkortostan can be used – the Yenisei and Burzyansk bees.

The purpose of this review is to consider some aspects of the molecular nature of microsatellite loci and the mechanisms of their evolution, as well as a retrospective analysis of the use of microsatellite markers in the population genetics of bees.

The evolution of the honeybee *A. mellifera* in its natural geographic range occurred in different climatic zones, which in the Old World led to the division of the species into 30 subspecies (according to some data, 31), of which only *A. m. mellifera* is adapted to exist in the climatic conditions of Northern Europe [25]. At the same time, Europe was the evolutionary cradle of the honeybee, which was reflected in the formation of 10 subspecies, which represent a significant part of the total genetic diversity of *A. mellifera* [26, 27].

Subspecies of the honeybee are divided into at least five evolutionary lines, the A (Africa), M (Western Europe), C (Eastern Europe), O (Middle East), and Y (North-East Africa) [28]. European subspecies are grouped into two evolutionary lines — M and C. The latter currently includes a large number of subspecies,

including two subspecies that are widely used in world commercial beekeeping – the Italian honeybee *A. mellifera ligustica* and the carnica *A. mellifera carnica*.

In their natural area, European honeybees are exposed to factors that are not related to beekeeping activities (the use of agrochemicals, destruction, and fragmentation of habitats), and those that are directly related to it (the import of parasites and pathogens, the targeted introduction of foreign queens) [29]. Of all the European subspecies of the honeybee, the subspecies *A. m. mellifera* is the most susceptible to the pressure of these factors, among which introgression plays the most important role [21, 30, 31]. The growing awareness of the importance of native subspecies as a valuable source of genetic material for the sustainable development of beekeeping has led to the creation of protected areas in Northern Europe to preserve the genetic integrity of the dark European honeybee [30-33].

It was previously thought that the dark forest bee was not in danger of passing away, as its demographics are supported by the activities of beekeepers. However, it has recently been shown that human activity is not able to compensate for the loss of honeybee biodiversity, and the conservation status of *A. m. mellifera* in Europe requires revision [34]. Over the past 200 years, the range of this subspecies in Eurasia has significantly narrowed due to the intensive reduction of forest areas, the introduction of various southern subspecies to their usual habitats, and the accompanying widespread of new invasive and infectious diseases (varroatosis, nosematosis type C, ascospherosis, etc.). In some European countries, the gene pool of the dark forest bee *A. m. mellifera* is recognized as gone. The disturbance of the native subspecies' areal continuity was caused by the introgression of southern subspecies in Western and Northern Europe, which is associated with the preferred breeding of bees of the evolutionary C branch. For example, in Europe, *A. m. mellifera* is partially, and in some cases completely, replaced by non-indigenous bees, for example, *A. m. ligustica* in Northern Europe [21] and *A. m. carnica* in Germany [35, 36]. In most of the Russian areal, *A. m. mellifera* has been replaced by subspecies *A. m. carpatica* and *A. m. caucasica* [37]. Soon, the gene pool of *A. m. mellifera* may be irretrievably lost due to the active introgression of genes of other evolutionary lines and a general decrease in the effective population of the subspecies. If a conservation strategy for this subspecies is not implemented, the collapse of bee colonies, family introgression, and population shifts will lead to the extinction of the dark forest bee, which has repeatedly happened to other species [38].

The study of the genetic structure and evolutionary relationships of *A. mellifera* populations in the territory of the Russian Federation, the determination of the purebredness of breeding stock of bees available and imported into the country, genetic certification, the identification of genomic associations with economically useful traits (in particular, with the egg production of queens, the flight activity of bees, honey and wax productivity, resistance to parasites, winter hardiness, components of royal jelly, bee venom), as well as the development of methods for assessing the breeding value of honeybees and the practical use of a genomic selection of *A. mellifera*, are of paramount importance in preserving the natural genetic diversity of domestic honeybee breeds.

When assessing the state of the breeding gene pool of bees and optimizing the selection of source material for breeding, it becomes mandatory to study the genetic structure of breeding farm populations and identify the evolutionary relationships between geographically isolated populations. To preserve the gene pool, the genetic certification of bees is necessary. It includes identification of their individual and/or group genetically determined parameters using morphological and/or molecular markers. To preserve and maintain polymorphism as a component of population stability, it is necessary to determine the parameters and group

characteristics of populations and lines [39].

It is generally assumed that populations of both eusocial and solitary representatives of the order *Hymenoptera* are characterized by an extremely low degree of polymorphism of allozyme loci [40-43]. In populations with relatively low allozyme variability, such as the majority of honeybee populations, population genetics and sociobiological analyses are difficult to perform [44]. In the search for adequate polymorphisms, much attention was paid to DNA markers, especially those associated with length variability, namely, minisatellites (DNA fingerprint) [45] and microsatellites (STR, short tandem repeats) [46]. Minisatellites are tandem repeats with a length of 15 bp or more, usually located in intergenic regions. Microsatellites consist of very short tandem repeats with a monomeric repetitive unit of 2-6 bp dispersed throughout the nuclear genome [47]. Microsatellites can be localized in both non-coding regions (including regulatory regions) and coding regions [48]. Microsatellites located inside the protein-coding regions are expected to be trinucleotide repeats, since otherwise the DNA reading frame is disrupted. The length of microsatellite clusters is on average from 20 to 60 bp (an exception is some hereditary human diseases, in which there is an expansion of triplet repeats).

Molecular mechanisms and characteristics of genome instability processes remain one of the most relevant issues in the biochemistry and molecular biology of nucleic acids. The factor of this kind of instability is, in particular, microsatellite DNA sequences. Due to the high rate of mutation processes in microsatellite sites (from 10^{-2} to 10^{-5} events per locus per generation), depending on the type of microsatellite [49], population-specific mutations accumulate quite quickly in them, which makes it possible to use information about the variability of microsatellite loci in the analysis of the population structure [50]. Studies of microsatellite regions of DNA have shown that the changes occurring in them are very diverse and depend on the types of repeats, alleles, species, and sex of living organisms, as well as the age of individuals [51].

Several models explain the variability of microsatellite loci [52-55]. Most of them fit into the so-called "stepwise mutational model", in which changes in the length of microsatellite loci occur sequentially by increasing or decreasing the repeat length by a single nucleotide. One of the main mechanisms that lead to the emergence and promote the expansion of microsatellites is the replicative fork slippage during the formation of thyroids [56]. The presence of complementary interactions between DNA/DNA duplexes of nucleotides in the regions of the DNA molecule flanking the microsatellite locus ensures the stability of these structures [57, 58]. Thus, the formation of a hairpin and a loop, and subsequent replication slippage of DNA chains during replication, are the key provisions of the “step-by-step” mutation model [59].

Computer modeling of the secondary structure of DNA molecules made it possible to establish a relationship between the number of monomeric units in microsatellite clusters and the ability of DNA molecules to form non-canonical secondary structures. It is shown that the appearance of non-canonical structures is also associated with the types of single nucleotide substitutions in microsatellite units and the types of microsatellite clusters [60-62]. There is evidence of the polarity of mutations within microsatellite DNA [63] and an increased frequency of single-nucleotide substitutions in the microsatellite-flanking regions of DNA [64]. Using the example of invertebrate dinucleotide microsatellites, it was shown that the frequency of mutations in the flanking regions may exceed the frequency of mutations in the microsatellite cluster itself [65].

Tandem repeats in general and microsatellite sequences, in particular, are considered to play an important role in the functioning of the genome at the

subcellular, biochemical, and molecular levels [66]. Currently, the most studied microsatellites are those of humans, some animals, and plants [67, 68]. At the same time, the dinucleotide microsatellite regions widely represented in the eukaryotic genome are the most interesting [69], which at the same time serve as the most evolutionarily conservative genetic markers of DNA.

Until about the mid-1990s, most information about population structure and relatedness in social insects was based on data on allozymes [70]. The first precedent for the use of DNA markers was to study the polymorphism of ribosomal RNA gene restriction sites in *Polistes* wasps [71], and since then the use of such markers has expanded rapidly. It is since microsatellite loci have some advantages: they are numerous, hypervariable, extremely informative, and widely represented throughout the genome. Microsatellite loci are a very convenient tool for analyzing the genetic structure of populations, assessing heterozygosity, the degree of inbreeding, determining the coefficients of genetic relation, calculating the genetic distances between populations and subspecies, and evaluating the inclusion of foreign genes of some species in the gene complexes of others. The first microsatellite loci in *Apis mellifera* were described in 1993 [72]. Microsatellite markers, due to inheritance from both parents, provide a more complete picture of population events, so they are actively used to assess introgressive hybridization as a result of mating drones and queens [32].

Thus, the analysis of polymorphism of microsatellite loci has become an important and popular method of population genetics studies of *A. mellifera* worldwide; to date, about 552 polymorphic genetic markers have been described [73]. Microsatellites are abundantly represented in the honeybee genome, which made it possible to create the first linkage map based on them for *A. mellifera* L. It was obtained mainly using the offspring of two hybrid queens (*A. m. ligustica* × *A. m. mellifera*). During the project implementation, 541 loci were mapped, including 474 microsatellite markers, and 24 linkage groups were identified. The average density of markers reached 7.5 cM, and the resolution was one marker for every 300 kbp of the genome [74]. In the honeybee genome, 60% of all microsatellites are located in the coding region, with 50% of the trinucleotide and 25% of the dinucleotide repeats located in the exons [75]. All these loci are polymorphic. Moreover, many of them are successfully amplified in three other species of the genus *Apis* — *A. cerana* (58%), *A. dorsata* (59%), and *A. florea* (38%). To obtain a statistically significant estimate of the structure of the honeybee population, as well as to assign individuals of unknown origin to particular populations based on the genetic distance between individuals and populations, it is sufficient to study the polymorphism of 10 microsatellite markers in 30-50 workers [76]. When using morphometric methods, processing of 200 to 750 workers is required to achieve the same degree of resolution [77].

To date, based on the data on the STR loci polymorphism level, introgression areas have been identified between bees of the subspecies *A. m. mellifera* and *A. m. ligustica* in the Alps, in Norway, and Switzerland [30], in Poland [31], and populations of Africanized honey bees in Central America [78]. The provinces of hybridization between the subspecies *A. m. ligustica* and *A. m. mellifera* in the territory of Northwestern Europe were determined [21]. The structure of honeybee populations in Spain was studied [79-81]. The phylogenetic analysis confirmed the data on the existence of evolutionary branches in *A. mellifera* corresponding to the geographical origin of its subspecies, previously obtained based on morphometric data and mtDNA analysis [82, 83]. The origin of honeybee populations in Europe [84, 85], the Middle East [86], and Africa [87] has been established. Methods of differentiation of bee populations and subspecies are proposed [88].

Studies involving the analysis of polymorphism of microsatellite markers

have been widely carried out not only to solve the problems of population genetics of the genus *Apis* but also to study other biological aspects, such as mating frequency [89], anarchy syndrome [90], and control of reproductive dominance [91].

Currently, there is a growing interest in studying the genetic structure of *A. mellifera* populations in developing countries as well. Thus, populations of the subspecies of the honeybee *A. m. jemenitica* native to Saudi Arabia [92] were studied using microsatellite markers A7, A24, A28, A88, A113, B124, Ap43, and Ap81 to determine the levels of introgression and hybridization with bees of subspecies actively imported into the country [93]. As a result, a slight deficit of heterozygotes in the subpopulations and a higher deficit of them in the general population of *A. m. jemenitica* (F_{IS} = 0.123, F_{ST} = 0.009, and F_{IT} = 0.13) were revealed. Introgression was bi-directional and more frequent in some regions than in others. At the same time, the structural analysis did not reveal different subpopulations among the samples of native bees. The high genetic diversity of local honeybees requires the urgent adoption of a program to preserve the integrity of the population.

Using the analysis of eight microsatellite markers, the polymorphism of three populations of the Iranian honeybee *A. m. meda* in the northwest of Iran was studied. Seven, five, and four polymorphic microsatellite markers were found in populations from Ardabil, Ardabil sharqi, and Ardabil gharbi provinces, respectively [94]. The total number of observed alleles is 42. Bees from the Ardabil sharqi province had the highest level of heterozygosity (0.563), and the lowest was determined for the population from the Ardabil gharbi province (0.438). In general, based on the F_{ST} assessment, the authors identified a low degree of genetic divergence between honeybee populations in Northwestern Iran.

Interesting results were obtained when studying the genetic characteristics of the population of the honeybee of the island of Rodriguez, located in the southwestern part of the Indian Ocean. In a study of 524 bee colonies from 20 different areas of the island using 18 microsatellite markers, all individuals were successfully genotyped at least 10 loci [95]. The number of observed alleles per locus ranged from three (for AP273) to 15 (for A029). Genetic diversity expressed as the representation of alleles varied between different sample collection sites from 4.75±1.58 to 5.09±1.38. Thus, the analysis of nuclear DNA showed that the honeybees on the island of Rodriguez represent a single genetically homogeneous population. It may be since the distances between settled families are extremely small to create genetic isolation (from 0.6 to 13.8 km). However, the level of genetic diversity in the studied population is comparable to that in the populations of *A. m. ligustica* and *A. m. carnica* in continental Europe. At the same time, the population of the Rodrigues Island bees, unlike the rest of the world, did not experience strong biological pressure caused by parasites and pathogens [96], which may explain the fact of its much higher heterozygosity compared to the populations of other island systems where *A. m. ligustica* was introduced [79, 97].

The genetic diversity of island populations was also studied by the example of the Balearic Islands, where 98 bee colonies from 22 areas of the archipelago were analyzed using eight polymorphic microsatellite loci – B124, A113, A7, A35, A24, A28, A88, and A8. At the same time, low variability was found, determined both based on the observed number of alleles and heterozygosity, which is expected for island populations [81]. Despite the low degree of genetic differentiation within the islands, there is a significant shortage of heterozygotes, indicating the existence of a subpopulation genetic structure. The honeybee populations of the Balearic Islands are divided into two clusters, the Gimnesias (the islands of Mallorca and Menorca) and Pitiusas (the islands of Ibiza and Formentera), which is consistent with the biogeographic hypothesis postulated for this archipelago. Phylogenetic

analysis confirmed the Iberian origin of the honeybees of the Balearic Islands, thus supporting the evolutionary scenario for *Apis mellifera* in the Mediterranean basin, according to which *A. m. Iberica* is a hybrid between the African subspecies *A. m. intermissa* and the dark European bee *A. m. mellifera* [83, 87].

When studying the genetic structure and diversity of 414 worker bees from eight Algerian populations using 14 polymorphic microsatellite loci, significant genetic diversity was found both in the number of alleles and in the degree of heterozygosity. The number of alleles in the studied loci varied from two (B24) to 22 (Ap43). Most of the populations were in the Hardy-Weinberg equilibrium. It was found that Algerian bees were represented by two subspecies – *A. m. intermissa* and *A. m. sahariensis* [98]. The conducted phylogenetic analysis placed them in a group separated from the evolutionary lines M, C, and O [99]. Data on the polymorphism of microsatellite loci in Algerian honeybee populations, as well as in reference populations studied earlier [82, 85, 98, 100], allowed clustering these populations, resulting in five groups depending on their origin: the lines M (France, Belgium), O (Armenia, Georgia), C (Greece, Italy), and A (Morocco, Guinea), as well as the Algerian group belonging to the African evolutionary branch A. At the same time, African honeybee populations are characterized by a high degree of polymorphism of microsatellite DNA loci, which was the result of pronounced migratory behavior and a tendency to swarming [83]. For some Algerian populations, a slight introgression of the M and C evolutionary lines was found.

Polymorphic STR loci are actively used in the study of the genetic structure of autochthonous honeybee populations in various regions of the Russian Federation: populations of hybrid bees of the Tomsk Region [101] and populations of *A. m. mellifera* of the Perm Region [102], the Republic of Bashkortostan [103], the Arkhangelsk and Vladimir Regions, the Krasnoyarsk Territory and the Republic of Tatarstan [104]; populations of *A. m. carpatica* of the Republic of Adygea [105]; populations of *A. m. caucasica* of the Orel Region and Krasnodar Territory [105], hybrid bees of the Novosibirsk Region [107].

To assess the variability of microsatellite loci A008, Ap049, AC117, AC216 in honeybees living in the Tomsk Region, four sample sets (Central Russian and Carpathian bees, hybrids of various origins) were formed based on previously conducted mtDNA study and morphometric analysis. In the studied loci, the samples of the Central Russian and Carpathian breeds differed in the observed allelic variants and the frequencies of their occurrence. At the same time, the spectrum of alleles identified for bees of the Central Russian breed was fully observed in hybrids based on the Central Russian and Carpathian breeds [101]. Based on the analysis of polymorphism of nine microsatellite loci of nuclear DNA among more than 300 DNA samples of bee families collected in the north of the Republic of Tatarstan, the Republic of Bashkortostan, and the Perm Territory, the population and genetic structure of the honeybee subspecies *A. m. mellifera* was studied. The results of molecular genetic analyses suggest the existence in the Urals of a fairly stable preserved population system of the dark forest bee, possibly the last in the world [102].

The results of the analysis of the genetic structure of the honeybee population in the southern part of Bashkortostan based on the polymorphism of five microsatellite loci of nuclear DNA (Ap243, 4A110, A8, A113, and A28) indicate that intensive interbreeding hybridization, which is indicated by the average F_{IS} value, has not yet led to the disappearance of the heterozygote deficit. The value of the degree of subdivision of subpopulations obtained by the authors suggested the presence of a border between the hybrid zone and the population of *A. m. mellifera* localized in the studied region [103]. The studied bee families were

differentiated into three groups. The Zil1 and Zil2 clusters likely correspond to the peripheral part of the *A. m. mellifera* population, but the question of its relationship with the Burzian population remains open. The Haib4 cluster can be attributed to the peripheral part of another local population of the Central Russian bee. The location of the hybrid interbreed zone reflects the other clusters.

To assess the variability of the allelic fund of STR markers during the formation of specialized honeybee lines of the Prioksky type of the Central Russian breed, microsatellite profiles were studied in six bee families of each of the two lines — Klever (selected due to pollination efficiency of meadow clover) and IV-ZT (selected due to winter hardiness) [104]. In a sample set of 88 individuals, the observed number of alleles per locus averaged 6.29±1.51 and 8.71±1.61, respectively. The high probability of using inbreeding in the breeding of Prioksky type bees is strongly evidenced by the lack of heterozygotes, which reached 24.5 and 10.8%, respectively. It was found that 85.7% of individuals of the Klever line and 86.8% of individuals of the IV-ZT line could be genetically assigned to their populations based on the analysis of microsatellites. As follows from the calculation of the R_{ST} fixation index (AMOVA), 23% of all variability is due to inter-population differences, 77% − to intra-population variability. It is convincingly shown that microsatellite analysis is fully applicable to the creation of specialized honeybee lines since the selection of such lines is always accompanied by a change in the allele-fund of microsatellites.

The information content of the test system developed for the analysis of seven microsatellite loci (A024, A88, A113, AP043, HB-C16-05, HB-THE-03, and HB-C16-01) was also studied. It was used to study the main parameters of the allelic fund of populations of honeybees of the gray mountain Caucasian (n = 70) and Central Russian (n = 65) breeds, as well as the Prioksky type of the Central Russian breed (n = 88) [105]. It was found that the average number of alleles per locus is 7.48±1.02, the number of effective alleles is 3.38±0.56, and the number of informative alleles is 3.62±0.71. Compared with the populations of the Central Russian and gray mountain Caucasian breeds that participated in the breeding of the Prioksky type, the latter revealed an increased genetic diversity of the allelic fund (9.57±1.88 versus 6.86±1.55 and 6.00±1.84). The introduction of alleles of the original breeds into the allelic fund of bees of the Prioksky type has been confirmed, the process of genetic consolidation of which, however, has not yet been completed. It was found that the share of inter-population differences accounts for 8% of the total allelic diversity.

The purebredness and differentiation of the main breeds of honeybees bred in the territory of the Russian Federation were evaluated based on the polymorphism of microsatellite markers of nuclear DNA, using multiplex analysis of eight loci − AO24, A88, A113, APO43, APxO1, HB-C16-05, HB-THE-03, and HB-C16-01 [106]. The high degree of isolation of the Carpathian bee breed was indicated by the presence of the largest number of private alleles. At the same time, there were no significant differences in the number of private alleles between the Central Russian and gray mountain Caucasian breeds. The analysis of STR markers demonstrated on average a high identity of individuals in the studied breeds (99%). The lowest degree of consolidation was characterized by the Carpathian breed (97.0%), and the most consolidated was the Central Russian breed (100%). The calculation of genetic distances showed that the gray mountain Caucasian and Carpathian honeybee breeds, which form a single cluster on the phylogenetic tree, are the closest to each other.

The comparison of the allelic fund in the Far Eastern honeybee population introduced to the Novosibirsk Region (n = 90) and in the populations of the Central Russian (n = 191, *A. m. mellifera*), gray mountain Caucasian (n = 113, *A. m. cau-*

casica), Carnica (*n* = 61, *A. m. carnica*), and Carpathian (*n* = 184, *A. m. carpatica*) breeds was performed using seven microsatellite loci [107]. The degree of genetic differentiation of the Novosibirsk population was estimated using the F_{ST}, R_{ST} (AMOVA) indices, and Nei genetic distances. As a result, it is shown that the Novosibirsk population of Far Eastern bees is characterized by a high degree of genetic diversity and, being a half-breed, is the closest in origin to the Carnica. Taking into account the origin of the Far Eastern bees from the Ukrainian steppe breed, the data obtained can be considered as an indirect confirmation of the close relationship of the Ukrainian steppe and Carnica breeds [107].

The analysis of microsatellite profiles for molecular genetic differentiation of the lines and families of the honeybee *A. m. caucasica* bred in the Sochi area revealed similar trends in the assessment of intra-and inter-family variability [106]. As an indication of the high heterogeneity of the first line, the observed excess of heterozygotes (F_{IS} = –0.048) can be considered. Representatives of this line were characterized by maximum inter-family (F_{ST} = 0.124) and minimum individual (F_{IT} = 0.052) variability. The 2nd-5th lines were characterized by relatively high individual variability (F_{IT} from 0.143 to 0.189) with the observed heterozygote deficiency (F_{IS} from 0.062 to 0.128), as well as significantly lower values of inter-family variability concerning the first line (F_{ST} from 0.095 to 0.104). The smallest inter-family differences (F_{ST} = 0.096 and F_{ST} = 0.095) were observed in the third and fourth lines among all the studied groups. The differentiation of the studied lines by morphometric features and STR markers revealed some differences in the structure of the family tree. The geographical distance of the lines from each other was reflected in a dendrogram based on the analysis using microsatellite markers.

The most important condition for the development and increase in the productivity of the beekeeping industry is the maintenance of the biodiversity of the honeybees. Regional populations can represent a significant reserve for its replenishment. Using seven microsatellites (A024, A88, A113, AP043, HB-C16-05, HB-THE-03, and HB-C16-01), the key characteristics of the allelic fund of the Primorsky population of the Far Eastern bee were determined and the level of its genetic differentiation was estimated [109]. The material was the worker bees of the Far Eastern population (DALN) (*n* = 143). In the pairwise comparison, the values D and F_{ST} were used. Forming comparison groups, purebred bees were selected based on the similarity coefficient Q. Its values averaged 98.0±0.1; 97.9±0.2; 98.1±0.1 and 95.8±0.4%, respectively, for the gray mountain Caucasian (SGK, *n* = 70), Central Russian (SR, *n* = 61), Carpathian (KARP, *n* = 55), and Carnica (CAR, *n* = 30) breeds. The relatively high genetic diversity characteristic of the comparison groups (12.43±2.71 alleles per locus for KARP, 11.29±2.49 alleles per locus for SR, and 10.00±2.07 alleles per locus for CAR) was comparable to that in the studied sample of DALN (11.14±1.30 alleles per locus). The effective number of alleles calculated for the DALN group exceeded the value typical for the other groups (4.94 alleles vs. 3.19-4.51 alleles). The deficiency of heterozygotes was the greatest in the population of Far Eastern bees (F_{IS} = 0.32); almost the same indicator was observed in the Central Russian breed (F_{IS} = 0.31). The DNA analysis data became the basis for assigning 96.5% of the DALN sample individuals to their population. The high degree of genetic consolidation of the Far Eastern breed can be an indicator of the almost complete absence of gene flow between this and the other studied breeds. Far Eastern bees form an independent branch on the family tree, which confirms their different origin compared to the other breeds in the sample. Based on the results obtained, the Far Eastern bee was included in the Russian State Register of Breeding Achievements in 2018 as an independent breed of honey bees (application No. 8356497, patent holder Chaika Far Eastern Federal Research Center for Agrobiotechnologies).

Microsatellite loci are also considered as a tool for studying the reproduction features of honeybees, in particular, polyandry. Polyandry is a specific phenomenon that provides an increase in genetic diversity. In an experiment to determine the degree of polyandry and the contribution of drones to genetic diversity, microsatellite profiles were compared at three loci (A008, Ap049, AC117) in hybrid and purebred bee families of *A. mellifera* (Central Russian and Carpathian breeds, Tomsk Region) [110]. It turned out that the share of alleles introduced into the bee family by the paternal line was 6.67-28.0%. At the same time, hybrid bee colonies were characterized by the greatest genetic diversity (a higher proportion of introduced alleles in the male line is shown – 25-28%).

So, the honeybee is a species that has a worldwide distribution (except for Antarctica) and is of the most important economic, agricultural, and environmental importance. However, in the previous few years, there has been a global decline in the total number of honeybee hives (from 21 to 15.5 million), which poses a threat not only to beekeeping but also to some crop production sectors, as well as to many natural ecosystems, the stability of which is supported by the participation of bees in the pollination of wild plants. The reasons for this decline are not fully understood but may be related to the loss of genetic diversity, the synergistic effects of parasite infestations (varroatosis and nosematosis), viral and bacterial infections, as well as the widespread use of pesticides in agriculture. Under these conditions, the determination of genetic diversity in honeybee populations using molecular methods is of primary importance. Microsatellites are represented by short tandem repeats (the size of the monomeric repetitive unit is from two to six base pairs), scattered throughout the nuclear DNA. They can be localized both in non-coding (including regulatory) regions and in the regions of the genome that encode proteins. Microsatellite loci are a very convenient tool for analyzing the genetic structure of honeybee populations, the degree of inbreeding and heterozygosity, calculating genetic relation coefficients, and determining the level of introgression. Using microsatellite markers, the evolutionary history of honeybee subspecies was revealed, the structure of a large number of their populations in the Old and New Worlds was studied, the inclusion of foreign genes of some subspecies in the gene complexes of others was evaluated, and methods for differentiating subspecies and populations were developed. A large number of alleles typical for microsatellite loci due to the high frequency of mutational events occurring in them, and the codominant type of inheritance make STR markers extremely powerful tools for genomic mapping, determining the reliability of origin, and conducting population genetic and evolutionary studies.

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AGRICULTURAL BIOLOGY,
2020, V. 55, № 6, pp. 1107-1125
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 636.4:636.087.7

doi: 10.15389/agrobiology.2020.6.1107eng
doi: 10.15389/agrobiology.2020.6.1107rus

USE OF ANTIOXIDANTS AS ADAPTOGENS FED TO PIGS (*Sus scrofa domesticus* Erxleben, 1777) (META-ANALYSIS)

A.A. SEMENOVA[1], T.G. KUZNETSOVA[1], V.V. NASONOVA[1], R.V. NEKRASOV[2], N.V. BOGOLYUBOVA[2], E.Yu. TSIS[2]

[1]*Gorbatov Federal Center for Food Systems RAS,* 26, ul. Talalikhina, Moscow, 109316 Russia, e-mail semmm@mail.ru, labsens@mail.ru, vvnas@mail.ru;
[2]*Ernst Federal Science Center for Animal Husbandry,* 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail nek_roman@mail.ru (✉ corresponding author), 652202@mail.ru, tsis-elen@yandex.ru
ORCID:
Semenova A.A. orcid.org/0000-0002-4372-6448
Kuznetsova T.G. orcid.org/0000-0002-5164-1807
Nasonova V.V. orcid.org/0000-0001-7625-3838
Nekrasov R.V. orcid.org/0000-0003-4242-2239
Bogolyubova N.V. orcid.org/0000-0002-0520-7022
Tsis E.Yu. orcid.org/0000-0003-1988-1189

The authors declare no conflict of interests
Acknowledgements:
Supported financially from Russian Science Foundation, project No. 19-16-00068
Received August 10, 2020

Abstract

Meat quality shaped by the affecting factors during an animal's lifetime is basically dependent on muscle tissue characteristics. Feeding disorders and stresses can cause myopathy, a destabilizing factor of farm animal meat quality. The muscle tissue injury preventing is of particular interest as it can improve lifetime meat quality formation. Dietary enrichment of farm animal nutrition with natural adaptogens and antioxidants offers potential to reduce myopathies of various etiologies. This paper is an overview of nutrition factors as protective agents under stress loads and myopathies in intensively growing pigs. Dietary adaptogens, e.g. selenium, tocopherol, quercetins, etc., inhibit peroxidation of lipids, generation of reactive oxygen species and are important for the control of glycolysis and oxidative stress. Most adaptogens are antioxidants, they have a beneficial effect on the cardiovascular system, including blood capillaries, prevent damage to cell membranes caused by free radicals and apoptosis. The beneficial effects of vitamin E-enriched diets (from 10 to 1000 mg/kg feed, approximately 200 mg/kg mainly) on porcine meat quality characteristics have been well studied in pig breeds and breed combinations during various periods of growing. However, no effect of dietary vitamin E on the growth rate of animals has also been reported. Feed enrichment with dietary tocopherol leads to its deposition in all tissues and organs, primarily in the blood, liver, heart, and in muscle and fat tissues. Vitamin E has a membrane-stabilizing effect, reduces oxidation of membrane lipids, increases the total amount of fatty acids in mitochondria, antioxidant capacity and muscle glycogen content. It has been shown that in pigs fed diets supplemented with vitamin E during fattening phase the vitamin E deposition level in meat is higher. This, in turn, improves meat taste and flavor, reduces the smell characteristic of reheated dishes, does not change the aldehyde profile of meat volatiles and reduces the accumulation of nitrogenous volatiles resulted from the breakdown of meat proteins during storage, including in a vacuum. Less attention is paid to administration of selenium as an adaptogen. It was shown that selenium combined with higher vitamin E level can neutralize the adverse consequences of hyperthermia in growing pigs and increase free fatty acid content in fat. The organic form of dietary selenium improves the antioxidant status of muscles in pigs. However, selenium has different effects on the oxidation of proteins and lipids during meat storage. In some studies, selenium reduced oxidation; in others, on the contrary, it was proved to be unable to inhibit the accumulation of products of oxidative damage. Two flavonoids quercetin and dihydroquercetin (Taxifolin) are well known for their antioxidant properties. The research articles are mainly deal with quercetin and dihydroquercetin bioavailability and deposition, the impact on antioxidant status and reproductive functions of sows, leveling transportation stress, and pork quality. Quercetin supplements have a pronounced effect at 25-50 mg/kg live weight, dihydroquercetin supplements at 1-3.5 mg/kg live weight. The flavonoids are effective when administered both during the fattening period and before slaughter or transportation. Despite the encouraging reports, little research has focused on the role of these flavonoids in the pork meat quality formation, so further study requires. Quercetin when fed up to 6 months at 2 % of the diet reduced damage to dystrophic skeletal muscle fibers in laboratory animals due to a decrease in reduced production of hydrogen peroxide in mitochondria. Adaptogens and directed muscle tissue development

regulators are proposed as potentially key supplements ensuring meat quality under intensive animal husbandry, therefore, further search for and study of bioactive substances which can protect muscle tissues from damaging factors are required.

Keywords: pigs, stress, pork, myopathy, adaptogen, antioxidant, selenium, vitamin E, quercetin, dihydroquercetin

Muscle tissue, as the main tissue of the animal's body, is considered as the main component that determines the quality of meat formed during the lifetime [1]. In this regard, it is of particular interest to study the factors affecting the appearance of myopathic conditions in an animal, as well as conditions that contribute to the leveling of the manifestation of such physiological deviations.

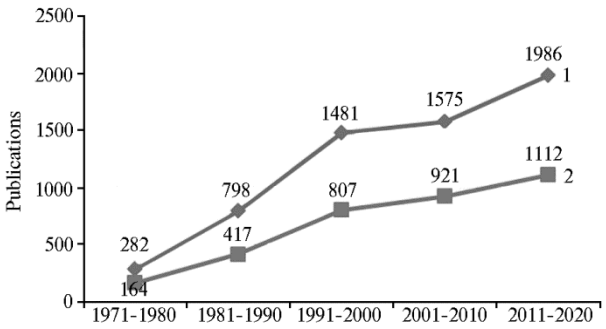

Fig. 1. Search results in ScienceDirect (https://www.sciencedirect.com) **for "myopathy", "stress"** (1), **and "food myopathy"** (2).

The initial information was collected in the ScienceDirect system using the main keywords: "myopathy", "food myopathy", "muscle fiber", "pig", "stress", "pork quality", and "pork PSE" (pale soft exudative). It was found that the number of scientific publications devoted to the study of the causes of the appearance and progression of myopathic states in humans, agricultural and wild animals and poultry, insects, fish, including ornamental ones, had been increasing since the beginning of the 1970s (Fig. 1). Thus, if in 1971-1980, the world published 282 works on the development of myopathies associated with the experienced stress and 164 works in the field of food myopathy, then in recent years (2011-2020), the search results revealed, respectively, 1986 and 1112 publications, that is, their number increased approximately 7-fold.

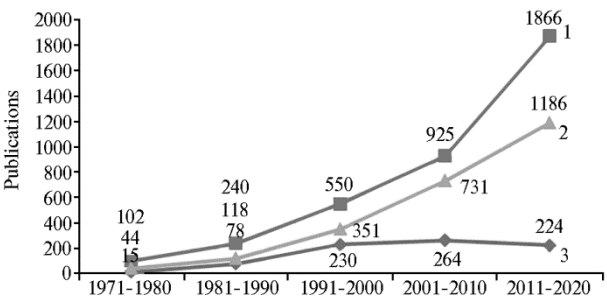

Fig. 2. Search results in ScienceDirect (https://www.sciencedirect.com) **for "pork quality, stress"** (1), **"muscle fiber, pork quality"** (2), **and "pork PSE"** (3, PSE — pale soft exudative).

Requirements for the consumer properties of pork have changed since the 1970s towards a decrease in the content of adipose tissue in carcasses and an increase in the mass fraction of muscle tissue, that is, the so-called leanness. The leanness of pork requires a certain quality of muscle tissue. In this regard, for half a century, there has been an exponential growth in the number of publications devoted to the study of the state of muscle fibers in terms of ensuring the quality of pork (Fig. 2).

It should be noted that until recently, when assessing the quality of pork, it was attributed to meat with a normal course of autolysis, or to the so-called PSE (pale soft exudative) meat, that is, having properties that make it dry and unattractive to the consumer. Interestingly, an analysis of the number of scientific publications devoted to this quality defect over the past 10 years has shown a tendency towards their reduction (see Fig. 2).

As it can be seen from Fig. 2, the maximum number of publications containing the words "pork" and "PSE" was in the 2001-2010 period. In the authors' opinion, the observed trend is explained not by the stabilization of the quality of pork, but rather by a change in the ideas of most specialists regarding the mechanisms and methods of ensuring its formation. This is evidenced by the increase in the number of publications devoted to the study of the quality of pork in relation to both the characteristics of muscle fibers and the influence of stress.

The development of methods for preventing myopathic conditions of various etiologies is based mainly on analyzing the possibility of using natural adaptogens and antioxidants in feeding productive animals. It is known that many drugs of natural (plant or animal) and synthetic origin have a stimulating effect on the nervous system and the organism as a whole. Such substances, possessing specific immune-stimulating and anabolic effects, stimulate the humoral response by sensitizing B-lymphocytes (synthesis of immunoglobulins), as well as T-lymphocytes (thymus-dependent cells), which provide a cellular immune response [2]. Vitamins (in particular, tocopherols), as well as microelements (primarily selenium), are the most common substances in pig breeding with antioxidant and adaptogen properties. The study of natural adaptogens – bioflavonoids contained in plants is one of the topical areas of research, therefore, to collect information, the authors searched for publications using additional keywords “vitamin E”, “tocopherol”, “selenium”, “dihydroquercetin”, and “quercetin”. For the analysis, the publications were selected that i) contained in the title or in the annotation (or in both), and in keywords (or in keywords) at least one of the specified main and additional keywords; ii) were original articles prepared by the authors based on the results of their own research on growing pigs; iii) included information on doses of vitamin E, selenium supplements and quercetins (dihydroquercetin), separately or in combination with each other, or in combination with other food components); iv) presented the data obtained after the slaughter of animals; v) dated no earlier than 1995.

The analyzed data set included the following information, presented in the form of tables: information regarding animals that were selected for feeding experiments (initial weight, age, pig breeds, breed combinations, etc.); the dose of adaptogen in the diet; the duration of feeding the adaptogen (adaptogens); achieved effects.

Vitamin E. The inclusion of adaptogens (selenium, tocopherol, and quercetins) in the diet allows controlling glycolysis and oxidative stress by inhibiting the formation of lipid peroxidation products and reactive oxygen species. Most adaptogens are antioxidants, they help to strengthen the cardiovascular system, including the capillary one, prevent the destruction of cells by free radicals, protecting them from apoptosis, and support the normal functioning of tissues and organs.

The use of vitamin E and its effect on the quality of meat has been well studied in growing pigs of different breeds and breed combinations. Out of 28 publications suitable for meta-analysis, 27 were devoted to the study of the role of vitamin E in pork production and one to the development of food myopathy in freshwater fish *Danio rerio* of the family Cyprinidae with vitamin E deficiency. The results were obtained in this study that are important for understanding the role of vitamin E in the development of food myopathies. In particular, it was shown that a lack of vitamin E in the diet caused sluggish behavior associated with multi-focal multiphase degenerative skeletal muscle myopathy. The manifestations of myopathy ranged from disseminated acute necrosis to advanced fibrosis. The addition of vitamin E to the diet of fish made it possible to maintain a 2-fold increase in the content of ascorbic acid ($p < 0.001$) in the muscles with a 3-fold decrease in the amount of malonic aldehyde ($p < 0.001$), which confirmed the high antioxidant status of the muscles. On the

contrary, vitamin E deficiency caused an increase in oxidative stress and a secondary decrease in the content of ascorbic acid, which led to serious damage to muscle tissue and impaired muscle function [2].

In the studies on growing pigs and pigs on final feeding, different doses of vitamin E were compared, ranging from 10 to 1000 mg/kg of feed. The most commonly used dosage was 200 mg/kg of feed [4-12]. The authors compared different durations of feeding vitamin E, from the minimum (1 day before slaughter) to 70 days, as well as during the period of change in the weight of pigs from the initial one to the required one for sending to slaughter (from 105 to 135 kg). The study of the effect of vitamin E on the state of animals before slaughter and the quality of meat showed that tocopherol, even 1 day before slaughter, increased muscle glycogen stores by 10%, and also increased the moisture-binding capacity (MBC), especially when using a feed additive in combination with moderate physical activity in animals [13]. However, these findings were challenged in 2004 in a large-scale experiment ($n = 92$), in which it was found that supplementing the pig diet with vitamin E 5 days before slaughter did not increase its concentration in the muscles, did not contribute to the improvement of MBC and the color of meat. The authors concluded that under stress, the level of which is lower than in usual procedures associated with slaughter, the short-term introduction of additives to the diet does not seem to affect the quality of meat [14].

Overall, the meta-analysis revealed 16 expected effects from the use of vitamin E as a feed additive (Table 1). In four studies with a fairly large amount of experimental data, the authors came to the conclusion that vitamin E in the amount of 140-220 mg/kg of feed did not affect the growth parameters of pigs even with prolonged feeding, including in combination with vitamin C [14-17]. Such a conclusion, obviously, should be considered as ambiguous and not final or as corresponding only to the studied range of doses of tocopherol in feed: vitamin E is a biologically active and necessary supplement, its deficiency can cause food myopathies, and, consequently, reduce the growth rate of animals. In one work [17], it was noted that vitamin E in the amount of 220 mg/kg of feed reduced ($p < 0.36$) its effectiveness. At the same time, even the long-term introduction of tocopherol into the diet in the amount of 140 mg/kg of feed did not affect the slaughter indicators – the yield of slaughter products to the live weight of pigs [15], as well as the qualitative characteristics of pork carcasses [10]. In another study [17], long-term administration of vitamin E resulted in an increase in the mass fraction of muscle tissue in a carcass.

In 14 studies, the authors analyzed the accumulation of vitamin E in organs and tissues of pigs. The results clearly indicated that the introduction of tocopherol into the diet of pigs promoted its accumulation in all tissues, primarily in the blood, liver, heart, as well as in muscle and adipose tissues. The maximum increase in the amount of vitamin E in muscle tissue was achieved in 28 days at a dose of 200 mg/kg of feed. There was no further increase in the content of vitamin E in muscles. It was also reported that synthetic tocopherol (a mixture of eight compounds not found in nature) was able to replace natural tocopherol in the organism of animals [4, 7, 9, 14-16, 18-25].

The results of the analyzed studies also allowed concluding that vitamin E stabilizes the state of membranes, reduces membrane lipid oxidation, and increases the total amount of fatty acids in mitochondria [8, 19, 26].

The effect of vitamin E on the condition of muscle tissue of pigs has been little studied. The search revealed only two studies [13, 23], the results of which show that vitamin E increases the antioxidant capacity and glycogen content in muscles.

1. Meta-analysis of the data on the use of vitamin E as an adaptogen in pig production (ScienceDirect, 1995-2019)

Intended effect	Number of animals in experiments	Use of vitamin E		Conclusion	References
		doses, mg/kg of feed	period of time		
Growth indicators of pigs	246	140-220	From 25 to 110 kg live weight	Does not affect even with prolonged feeding and in combination with vitamin C	[10, 15-17]
Efficiency of assimilation of feed	48	220	From 54 to 113 kg live weight	Decreases ($p < 0.36$)	[17]
Slaughter parameters	Not reported	140	From 25 to 105 kg live weight	Does not affect even with prolonged feeding and in combination with vitamin C	[15]
Qualitative characteristics of carcasses	198	200-220	From 54 to 113 kg live weight	Does not affect. An increase in the mass fraction of muscle tissue in the carcass is possible	[10, 17]
Content of vitamin E in the blood, organs, muscle, and adipose tissue of the animal	154	100-1000	From 5 to 70 days	Increases depending on the dose with prolonged use. The maximum increase in muscle tissue on the 28^{th} day	[4, 7, 9, 14-16, 18, 20-25]
Condition of cell membranes and mitochondria	Not reported	200-1000	From 46 days	Stabilizes the state of membranes, reduces membrane lipid oxidation, and increases the total amount of fatty acids in mitochondria	[4, 8, 19, 26]
Muscle tissue condition	56	500	From day 2 to the end of fattening	Increases antioxidant capacity, glycogen content in muscles. Vitamin E deficiency causes muscle tissue damage (degenerative myopathy)	[2, 13, 23]
Severity of the effects of transportation stress	288	200	28 days	Does not affect, as well as does not reduce the content of creatine kinase and cortisol in the blood serum, the expression of heat shock proteins in muscle tissue, damage to the intestinal epithelium	[12]
Accumulation of intramuscular fat and total chemical composition of muscle tissue	282	100-220	From 38 days; from 54 to 135 kg live weight	Overall, apparently, does not affect, although there are data that it increases the marbling score	[5, 17, 27]
Fatty acid composition of intramuscular fat	224	100-325	28 days and more	The data are contradictory: either does not affect or increases the content of polyunsaturated fatty acids	[5, 28]
pH, moisture-binding capacity, and the share of pale soft exudative meat	1060	80-1000	From 2 to 46 days; from 54 to 110 kg live weight and until the slaughter	There is no unambiguous understanding of the effect of vitamin E on pH, moisture-binding capacity, and pale soft exudative meat appearance	[5, 8, 11-14, 16, 17, 22, 25, 26, 28]
Accumulation of oxidative spoilage products	974	10-700	From 28 to 150 days, from 25 to 135 kg live weight	Secondary oxidation products (thiobarbituric value or hexanal): reduces the dynamics of their accumulation in meat; in some cases, vitamin E had no effect on the thiobarbituric value (two works out of 11). The peroxide number decreases (did not decrease in one case out of three)	[2, 5, 7, 10, 11, 15, 16, 20, 21, 25, 27, 29, 30]

Continued Table 1

General organoleptic score	84	100-500	During the fattening period until the slaughter	Does not affect	[5, 23]
Meat color	928	10-1000	From 5 to 70 days; from 51 to 113 kg live weight	There is no unambiguous understanding of the effect on color in general: if it improves it or does not affect. Redness: improves the indicator, in some cases has no effect; increases the redness of products with nitrite. Lightness: a significant positive effect if the meat is stored	[5, 7, 10, 11, 14, 17, 18, 21-23, 25, 27, 30]
Meat taste and smell	48	10-210	During the fattening period until the slaughter	It has a positive effect on smell and taste, enhances their intensity. Reduces the severity of the smell of reheated dishes. Does not affect the aldehyde profile of volatiles and reduces the accumulation of nitrogenous volatiles when stored under vacuum	[9, 16, 22]
Softness of meat	48	200-500	During the fattening period until the slaughter	The data is inconsistent: does not affect or increases	[16, 23]

The authors studied the possibility of mitigating the effects of transportation stress in pigs by adding vitamin E to their diet 28 days before transportation. However, the expected effect was not achieved. It turned out that vitamin E did not affect the clinical manifestations of this pathology, as well as did not reduce the content of creatine kinase and cortisol in the blood serum, the expression of heat shock proteins in muscle tissue, and damage to the intestinal epithelium [12].

The effect of vitamin E on intramuscular fat accumulation and the overall chemical composition of muscle tissue was examined. In general, it was concluded that there was no relationship between these factors, despite the data on an increase in the assessment of pork marbling [5, 17, 27]. The latter could be due to a change in color perception, and not to the accumulation of fat in the *longissimus dorsi* muscle.

The data obtained when assessing the effect of vitamin E on the fatty acid composition of intramuscular fat are contradictory: both the absence of a relationship [5] and an increase in the proportion of polyunsaturated fatty acids in the muscle tissue of pigs with the introduction of vitamin E into the diet were noted [28].

Twelve studies considered the possibility of reducing the proportion of PSE pork, increasing the pH and MBC of meat [5, 8, 11-14, 16, 17, 22, 25, 26, 28]. It was reported that in high doses (1000 mg/kg) vitamin E could stabilize cell membranes and regulate excess Ca^{2+} release, preventing the formation of meat with PSE defect and, at the same time, improving the MBC of muscle tissue [26]. Although an increase in MBC with different doses of vitamin E in the diet of animals was noted in a number of studies, it was not possible to draw an unambiguous conclusion, despite full-scale studies on a large sample of animals and with a significant duration of experiments. The opinions were divided approximately equally: in one part of the publications, it was concluded that there was no effect of vitamin E on pH and MBC, while in the other works, the introduction of vitamin E into the diet of pigs could reduce the proportion of PSE meat. As a reason, it can be assumed that, even with experimental slaughter, it is extremely difficult to create the same stress loads for all animals, as well as to take into account their individual characteristics of perceiving stressful situations.

In 13 works, the data on the dynamics of accumulating oxidative spoilage products during storage of both meat and finished meat products from animals in the diet of which vitamin E was introduced in an amount of 10 to 700 mg/kg of feed are presented [2, 5, 7, 10, 11, 15, 16, 20, 21, 25, 27, 29, 30]. The results of these studies testified to the inhibition of developing oxidative processes in meat and meat products with prolonged (from 28 days) use of tocopherol during the fattening period. Only under certain conditions (possibly, depending on the nutrient composition of the feed), such an effect was not achieved.

Two studies [5, 23] studied the effect of vitamin E in the diet of pigs on the overall organoleptic assessment of the resulting pork. The results showed no effect with long-term use during the fattening period at doses of 100-500 mg/kg of feed.

The possibility of stabilizing and improving the color and color characteristics of pork (L — lightness, a — redness, b — yellowness) was considered in 13 studies [5, 7, 10, 11, 14, 17, 18, 21-23, 25, 27, 30]. The results did not provide an unambiguous answer as to whether vitamin E affects the color of the pork. At least, it is clear that an increase in the amount of tocopherol in the diet and in meat did not lead to a deterioration in color indices even at the highest dosages of 1000 mg/kg feed. With regard to instrumentally measured color indicators (redness and lightness), the meta-analysis revealed their improvement in the case of meat sold in packaged form; in addition, vitamin E in meat raw materials

effectively increases the redness of products made with nitrite.

The effect of vitamin E on taste and smell was identified as positive based on the results of three studies [9, 16, 22]. When vitamin E was used as an additive during the fattening period, its content in meat increased, which enhanced the intensity of taste and aroma, in addition, the smell of reheated dishes, which is considered undesirable, was less pronounced. The data that vitamin E does not change the aldehyde profile of volatile substances in meat and reduces the accumulation of nitrogenous volatiles formed as a result of the breakdown of protein substances during storage, including in a vacuum, can be considered as positive.

The study of the effect of vitamin E on the softness of meat [16, 23], as in the case of the effect on MBC, gave conflicting results (either does not affect or increases). These data are consistent with the results for MBC.

Thus, judging by the activity of research, most scientists continue to consider vitamin E as an important nutrient that can ensure the achievement of high consumer characteristics of slaughter products in pig breeding, be used to stabilize color, taste, aroma, and also to effectively suppress oxidative processes during production and storage of meat products.

S e l e n i u m. A search for publications on the use of selenium in pig feeding (from 1999 to 2020) identified 20 papers suitable for meta-analysis, that is, the research on selenium is conducted less intensively than on vitamin E. The total number (11) of tested hypotheses regarding the possible effects of selenium is also less (Table 2).

Selenium was fed in the inorganic (sodium selenite) and organic (Se-enriched yeast and selenomethionine) forms at a wide dosage range from 0.045 to 50 mg/kg of feed, as well as together with vitamin E and with the simultaneous introduction of fats into the feed, rich in unsaturated fatty acids. The most commonly used doses were 0.2-0.3 mg/kg of feed. The duration of the experiments reached 65 days. The mass of animals in the experiment was from an initial weight of 10 kg to a final weight of 160 kg.

The effect of introducing selenium into the diet of pigs on slaughter parameters was assessed in two studies [31, 32] on a total sample of 479 individuals. At high doses of selenium (up to 30 mg/kg of feed) and prolonged feeding (in one work from the initial weight of animals 20 kg to the final 105 kg, in another work for 30 days), no effect of both inorganic and organic selenium was found, except for selenomethionine. In animals that received it as a source of selenium, the yield of carcasses during slaughter was higher. Under similar experimental conditions, it was found that selenium (in organic and inorganic forms, as well as in combination with vitamin E) did not affect the qualitative characteristics of pork carcasses [31, 33].

Seven studies were devoted to the effect of selenium in feed on its content in the blood, organs, and muscle tissue of animals. The experiments were conducted on a representative sample of animals (n = 603) and at different dosages (0.045-50 mg/kg). The animals were selected according to their initial live weight (from 20 to 60 kg) or age (30 to 40 days), feeding of additional selenium was continued until slaughter.

The accumulation of selenium in tissues is faster in the case of organic selenium, especially selenomethionine, and promotes the accumulation of vitamin E. The most effective daily dose of selenium was found to be 0.4 mg/kg of feed. Interestingly, with such a daily intake, a young healthy organism stops storing selenium after 28 days [31, 32, 34-38].

Three studies [36, 39, 40] evaluated the effect of selenium on the condition

of the muscle tissue of animals. The results of long-term experiments (26 days or more) made it possible to conclude that there was no such effect, including when selenium was combined with vitamin E. No changes in the microstructure of meat were revealed (regardless of the source of selenium). Nevertheless, during the maturation of meat, selenium (Se-yeast, selenium with vitamin E) intensified destructive changes on day 8, which is probably associated with a higher activity of tissue enzymes.

The possibility of leveling the effects of transportation stress in pigs due to the additional introduction of selenium into the diet was studied in one work [41] on 36 animals at a dose of 0.24 to 1 mg/kg and against the background of vitamin E consumption (17-100 mg/kg). The results clearly showed that selenium reduced the effects of hyperthermia in growing pigs.

The study of the fatty acid composition of intramuscular fat in animals that were given selenium against the background of basic and additional intake of vitamin E revealed an increase in the content of free fatty acids in fat under the influence of organic selenium. The effect is enhanced by vitamin E which increases the $C_{18:1}$ content and decreases the $C_{18:0}$ [38]. Eight large-scale studies in terms of the number of animals (n = 689) and duration of studies were devoted to the effect of selenium on pH, MBC, and the proportion of PSE meat. The results allow making an unambiguous conclusion that organic selenium increases MBC and pH [31, 32, 35, 38, 39, 42-44]. At the same time, it is unclear how to explain such a positive effect and why it does not agree with the data of studies on the state of muscle tissue.

The effect of additional selenium on oxidative processes during lifetime, as well as during storage of meat and meat products, is most studied. The unambiguous conclusion is that organic selenium increases the antioxidant status of muscles. However, the introduction of selenium into the diet had different effects on the oxidation of proteins and lipids during the storage of meat. The research results are ambiguous: in some studies, the data were obtained that allow concluding that oxidation decreases under the influence of selenium, in others, on the contrary, it was shown that selenium was not able to inhibit the accumulation of oxidative spoilage products [32, 34-36, 38, 39, 42, 44-48].

Two publications [33, 49] conclude that selenium and vitamin E do not affect the overall organoleptic assessment of pork. However, with regard to meat color, the results differed, which was explained by the form of administering adaptogens, as well as, possibly, the choice of the dosage and subjects of study. Thus, inorganic selenium did not affect or worsen the color indices, while organic selenium, on the contrary, either did not affect or improved the color indices. Only one study attempted to evaluate the effect of the combined introduction of selenium and vitamin E into the diet [50], in which such an effect was considered insignificant.

In general, in the authors' opinion, insufficient attention is paid to selenium as an adaptogen, important for the lifetime formation of meat quality.

Quercetin and dihydroquercetin. Quercetin and dihydroquercetin (taxifolin) are well-known and well-studied flavonoids. They are not toxic, have pronounced antioxidant and antimicrobial properties, as a result of which they are used both in the production of livestock products and during their processing and storage.

The number of publications on the required topics suitable for meta-analysis appeared to be limited, i.e., 23 articles (the sample was supplemented by Russian sources previously known to the authors of this meta-analysis), relating to the period from 1999 to the present (Table 3) and covering the results of the studies of the bioavailability of these flavonoids, their accumulation in animal tissues, the effect on the reproductive functions of sows, the antioxidant status of animals, a decrease in the effects of transportation stress, and meat quality.

2. Мета-анализ данных по применению селена в качестве адаптогена в свиноводстве (ScienceDirect, 1999-2019 годы)

Intended effect	Number of animals in experiments	Use of selenium		Conclusion	References
		doses, mg/kg of feed	period of time		
Slaughter parameters	479	0.05-30	From 20 to 105 kg; 30 days	No effect when using both inorganic and organic selenium (except selenomethionine, which increases carcass yield)	[31, 32]
Qualitative characteristics of carcasses	399	0.05-30	From 20 to 105 kg, 28 и 49 days	There is no effect when selenium is introduced into the diet in organic and inorganic forms, as well as in combination with vitamin E	[31, 33]
The content of selenium in the blood, organs, muscle tissue of the animal	603	0.045-50	From 20-60 kg until the slaughter; 30-40 days	The accumulation of selenium in animal tissues occurs faster when feeding with organic selenium, especially selenomethionine, and contributes to the accumulation of vitamin E. The most effective concentration is 0.4 mg/kg of feed	[31, 32, 34-37, 42]
Muscle tissue condition	Not reported	0.2-0.4	From 26 days; from 75 to 160 kg	Histological studies did not reveal the effect of selenium, including with vitamin E, on the condition of the pig's muscles (except that when the meat ripens, selenium as Se-yeast or selenium with vitamin E enhances destructive changes on day 8)	[36, 39, 40]
Severity of the effects of transportation stress	36	0.24-1 (with vitamin E, 17-100 mg/kg)	14 days	Reducing the effects of hyperthermia in growing pigs	[44]
Fatty acid composition of intramuscular fat	Not reported (meat was tested)	0.2-0.4 (with vitamin E)	Not reported (meat was tested)	Organic selenium helps to increase the content of free fatty acids in fat (the effect is enhanced by vitamin E). Se increases the content of $C_{18:1}$ and decreases the $C_{18:0}$ level.	[42]
pH, moisture-binding capacity, and the proportion of pale soft exudative meat	689	0.05-30	26-65 days; from 20-30 to 105-130 kg	Organic selenium raises moisture-binding capacity and pH	[31, 32, 35, 38, 42-44]
Accumulation of oxidative spoilage products	438	0.045-3	26-65 days	Organic selenium increases the antioxidant status of muscles, however, its effect on the oxidation of proteins and lipids during the storage of meat is not unambiguous, i.e., it reduces or does not affect it	[32, 34-36, 38, 39, 42, 44-47]
Total organoleptic assessment	107	Se (with vitamin E)	28-49 days	Does not affect	[33, 49]
Color of meat	380	0.045-0.5	30-65 days; from 20 to 105 kg	There is no clear understanding. Inorganic selenium does not affect or degrade color indicators, and organic selenium does not affect or improve color indicators	[48]
Taste and smell of meat	Not reported (meat was tested)	1 (with vitamin, 100 mg/kg feed)	Not reported (meat was tested))	Has an insignificant effect	[50]

3. Meta-analysis of data on the use of quercetin (QC) and dihydroquercetin (DHQ) as adaptogen in pig breeding (ScienceDirect, 1998-2019)

Intended effect	Number of animals in experiments	Use		Conclusions	References
		doses	period of time		
Growth indicators	10	DHQ (Ecostimul-2), 1 mg/kg of live weight per day);	DHQ during thermal stress; QC for 28 days	The survival rate and average daily weight gain of animals increases	[64, 66, 71]
	340	QC, 25 mg/kg of feed			
Qualitative characteristics of carcasses	Not reported (meat was tested)	DHQ	Form 72 kg to the slaughter (45 days)	DHQ will not affect the morphological composition of carcasses	[67]
The content of QC in the blood, organs, muscle, and adipose tissue of the animal	10	QC (aglycone, quercetin-3-O-glucoside, rutin – quercetin-3-O-glucoramnoside), up to 65 g/day	Up to 10 days	Bioavailable. The bioavailability of QC depends on its type and the diet of pigs; it is higher for glucoside. The highest content of QC and its metabolites is in the liver, small intestine, kidneys, blood plasma; low in the brain, heart, and spleen. No tendency to accumulate (no difference between single and repeated use)	[51-55]
State of the muscle tissue (the results of studies on mice)	Not reported (meat was tested)	QC, 0.2% of the diet	From 14 days to 6 months	Long-term addition of QC reduces damage in dystrophic skeletal muscle, prevents muscle loss, and inhibits the development of muscle fiber atrophy by reducing the formation of hydrogen peroxide in mitochondria	[72, 73]
State of mitochondria	Not reported (meat was tested)	QC, 0.2% of the diet	14 days	Reduces the production of hydrogen peroxide in mitochondria	[73]
The severity of the effects of transportation stress	510	QC, 25 mg/kg of feed; 1.25-40 μg/ml (in cell culture)	28 days from 72 to 100 kg)	QC better than vitamin E mitigates the negative effect of transportation, affects the proliferation of epithelial cells, protects against oxidative stress, positively affects intestinal integrity, reduces the production of reactive oxygen species in the intestine and intestinal inflammation during transportation stress	[62, 68, 71]
pH, moisture-binding capacity, and the proportion of pale soft exudative meat	376	QC, 2.5-25.0 mg/kg of feed; DHQ, 3.5 and 7.5 mg/kg of live weight per day	28-45 days; from 60 to 110 kg; 4 h before transportation to the slaughter	The effect of QC and DHQ is ambiguous: they increase or do not change pH and moisture-binding capacity, storage losses decrease or do not change. It is assumed that QC and DHQ slow down the rate of autolysis, but the final pH does not depend on the diet of pigs.	[67-70]
Accumulation of oxidative spoilage products	816	QC, 25-900 mg/kg of feed, 10 mg/kg of live weight per day; DHQ, 1.0-7.5 mg/kg of live weight per day	17-45 days; 24 h	QC protects tissues and organs from oxidative stress, increases the accumulation of vitamin E. The effect on the antioxidant status of weaned piglets is controversial. Increases and stabilizes the antioxidant status of blood plasma, in serum, muscles, and liver reduces thiobarbituric and peroxide values (less primary and secondary lipid oxidation products during storage both in muscles and in fat). The effect is more obvious during long-term storage	[56, 61-66, 68, 70, 71]
Meat color	340	QC, 25 mg/kg of feed	From 74 kg to the slaughter weigh	QC has a positive effect on the color of meat 24 hours after slaughter, but the effect is considered insignificant	[68, 70]

The bioavailability of quercetin from quercetin glycosides is determined by a complex interdependence between the chemical forms of flavonoids and the composition of the diet (types of feed) [51, 52].

The accumulation of quercetin in pig tissues as a result of adding a flavonoid to the feed has been studied in sufficient detail in many studies. Conjugated quercetin is the main metabolite of quercetin detected in blood plasma 24 hours after the intake of this supplement into the organism [52]. The highest content of quercetin and its metabolites is noted in the organs responsible for the excretion of metabolic products – in the liver and kidneys (5.87 and 2.51 nmol/g of tissue, respectively), significantly lower – in the brain, heart, and spleen. At the same time, in the blood plasma and in the heart of pigs, quercetin accumulates more slowly and in smaller quantities than in rats, while its accumulation in the kidneys, brain, and spleen does not differ in these animals. The content of quercetin in the blood plasma of pigs after 3 days of consuming high doses of quercetin (up to 500 mg/kg) did not exceed 1.25 mmol/l [53, 54]. Moreover, no differences were found between the results of long-term and repeated use of this flavonoid and a single use [55]. Thus, it has been proven that feeding quercetin is safe for animals due to the absence of its accumulation in tissues [53, 55].

Out of the dietary factors, fat in the diet (depending on its content) influenced the bioavailability of quercetin [52]. At the same time, the combination of vitamin E with quercetin leads to the best positive effect [56].

Scientists have been interested in studying the effect of quercetin on the reproductive function of pigs for more than 10 years [57-60]. It was found that quercetin did not affect the growth of granulosa cells, but (depending on the dose) inhibited the production of progesterone and modifies the production of 17β estradiol. A negative effect of quercetin on the physiological status of the ovaries is possible. In addition, the flavonoid interferes with the angiogenic process by inhibiting the production of vascular endothelial growth factor, as well as through changes in the oxidation-reduction status [57]. Exogenous flavonoids reduced the content of reactive oxygen species in oocytes, but at high concentrations (50 μg/ml) turned out to be toxic to oocytes [58]. Quercetin exhibits an inhibitory effect on the main functions of the ovaries, does not prevent or mitigate the effects of benzene on reproductive processes, and enhances the effect of benzene on the release of progesterone [60].

Quercetin did not affect the activity of glutathione peroxidase, glutathione reductase, and glutamate-cysteine ligase in the mucous membrane of the small intestine and liver of piglets after weaning, while the activity of hepatic glutathione transferase significantly increased on the 5th day after weaning when quercetin was taken at doses of 100, 300, and 900 mg/kg. Evaluating the content of malonic aldehyde in blood plasma, liver, and small intestine shows that the data on the effect of quercetin on the kinetics of glutathione in weaned piglets are contradictory, and the issue requires further study [61]. Quercetin helps to protect pigs' intestinal enterocytes from oxidative stress [62]. It was found that when the feed was contaminated with mycotoxins in animals receiving quercetin (alone or in combination with vitamins and selenium), the oxidative status was partially restored [63].

The use of dihydroquercetin in feeding piglets blocks the process of lipid peroxidation during the entire period of rearing and fattening, especially when exposed to high temperatures. The acid, peroxide number, and the content of malondialdehyde in the blood serum of piglets decrease with the use of dihydroquercetin, and the antioxidant defense of the organism is enhanced, which is expressed in an increase in the antioxidant activity of blood plasma. The

improvement of liver functions is noted. Taken together, this has a positive effect on the daily gain and safety of pigs [64-67].

Quercetins reduce the effect of transportation stress, contributing to a decrease in the serum concentration of endotoxin, the amount of reactive oxygen species and malondialdehyde in the intestinal tissues; in the jejunum of pigs, an increase in the height of the villi is noted with a simultaneous decrease in the expression of inflammatory cytokines [68].

The influence of quercetin and dihydroquercetin on the quality of meat has been studied in sufficient detail (in relation to various technological and consumer characteristics at different stages of production – from fresh carcasses to frozen meat with long-term storage). The addition of quercetin to the diet of piglets during the fattening period and even 4 hours before slaughter slowed down the rate of pH decrease in muscle tissue. However, the final pH value at 24 hours after slaughter was independent of diet. An increase in the MBC of meat and a decrease in weight loss during storage in retail conditions for 12 days were noted [69]. Adaptogens (dihydroquercetin at a dose of 3.5 mg/kg of live weight and rose petals at a dose of 0.255 mg/kg of live weight) contributed to a decrease in the accumulation of primary and secondary lipid oxidation products during storage both in muscles and in fat. A slight positive effect was noted for the pH and color of pork [67, 70]. The effective doses for pigs were established, the 25-50 mg/kg of animal weight for quercetinthe [55, 58], 1 mg/kg of animal weight [64-66] and 3.5 mg/kg of live weight [67, 70] for dihydroquercetin, as well as the fact that flavonoids were effective for feeding both during the growth of animals [55, 65] and right before slaughter and transportation [68, 69, 71].

The analysis of the published data shows that the role of quercetin and dihydroquercetin in the formation of meat quality still needs to be studied. In the last few years, there have been reports that long-term consumption of quercetin (up to 6 months, 0.2% in the diet) is able to reduce damage to muscle fibers in dystrophic skeletal muscle in laboratory animals [72]. In addition, its feeding prevents muscle loss and the development of muscle fiber atrophy by reducing the formation of hydrogen peroxide in mitochondria, even in cases where the atrophy is caused by injury to the nervous tissue of the muscles [73].

Thus, despite the small number of sources on quercetin and dihydroquercetin, the meta-analysis allows highlighting promising areas of research to identify the effect of quercetin (dihydroquercetin) on the lifetime formation of pork quality. Among them, there is the study of the role of these additives in preventing pork quality defects caused by myopathy (see Table 3).

Summing up the analysis of publications on the effect of antioxidants of various natures under stresses affecting the microstructure of muscle tissue, it should be noted that the use of such adaptogens in industrial animal breeding can become an important element of intensive technologies that ensure the lifetime formation of meat quality. An expert assessment of the degree of knowledge of the intended effects of each of the three adaptogens is presented in Table 4.

4. Expert assessment of the coverage of studies of the potential for using adaptogens in pig breeding (ScienceDirect, 1995-2019)

Intended effect of the adaptogen	Vitamin E	Selenium	Quercetin (dihydroquercetin)
Influence on the indicators of pig growth	4+	1+	3+
Influence on the efficiency of using feed	4+	1+	1+
Influence on slaughter parameters	4+	4+	1+
Impact on qualitative characteristics of pig carcasses	4+	3+	3+
Influence on the content of adaptogen in blood, organs, Muscle, and adipose tissue of the animal	4+	4+	3+
Influence on the state of cell membranes and mitochondria	4+	1+	3+
Influence on the condition of muscle tissue	3+	2+	2+

Continued Table 4

Influence on the severity of the effects of transportation stress in pigs	4+	4+	4+
Influence on intramuscular fat accumulation and the total chemical composition of muscle tissue	3+	1+	1+
Influence on the fatty acid composition of intramuscular fat	2+	3+	1+
Effect on pH, moisture-binding capacity, and the proportion of pale soft exudative meat	2+	3+	2+
Influence on the accumulation of oxidative spoilage products	4+	2+	2+
Influence on the overall organoleptic assessment	3+	3+	*
Influence on meat color	2+	2+	3+
Influence on the taste and smell of meat	3+	3+	1+
Influence on the softness of meat	2+	1+	1+

N o t e. 4+ — large-scale study, unambiguous conclusions, the likelihood of new knowledge is very low; 3+ — studied in a significant amount, the conclusions are agreed, but there is a possibility of obtaining new knowledge; 2+ — insufficiently studied or no unambiguous conclusions, the probability of obtaining new knowledge is high; 1+ — studied very little or not studied at all, requires further study.

Thus, the conducted meta-analysis led to the following conclusions. It is required to continue the study of two key aspects of the possible use of adaptogens in the production of pork − the effect of antioxidants on increasing the pH and MBC of meat, as well as on the stabilization of color and inhibition of oxidative processes. It should be noted that histological studies are rarely included in the programs of scientific work. This is probably due to the lack of highly qualified histology specialists. The accumulation of data on the effect of adaptogens on the functional and technological characteristics of pork indicates their relationship with the lifetime state of muscle tissue and the importance of studies to minimize the risk of myopathy in conditions of intensive pork production.

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AGRICULTURAL BIOLOGY,
2020, V. 55, № 6, pp. 1126-1138
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

Breeding and reproduction

UDC 636.4:636.082.2:612.311

doi: 10.15389/agrobiology.2020.6.1126eng
doi: 10.15389/agrobiology.2020.6.1126rus

FEEDING BEHAVIOR AS THE NEW BREEDING TRAITS IN PIGS

A.A. SERMYAGIN ✉, A.A. BELOUS, E.A. TREBUNSKIH, N.A. ZINOVIEVA

Ernst Federal Science Center for Animal Husbandry, 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail alex_sermyagin85@mail.ru (✉ corresponding author), belousa663@gmail.com, terramio7@mail.ru, n_zinovieva@mail.ru
ORCID:
Sermyagin A.A. orcid.org/0000-0002-1799-6014
Belous A.A. orcid.org/0000-0001-7533-4281
Trebunskih E.A. orcid.org/0000-0002-5208-3376
Zinovieva N.A. orcid.org/0000-0003-4017-6863
The authors declare no conflict of interests
Acknowledgements:
Supported by the RFBR, project No. 19-316-90008 and the Russian Ministry of Science and Higher Education
Received September 1, 2020

Abstract

Pig selection by fattening, meat and reproductive qualities as the main breeding criteria has been implemented in breeding practice long time ago. However, the used productivity traits do not involve a number of important economic indicators, in particular feed efficiency and behavioral characteristics of animals. The selection response for such traits is expected to give an additional increase in the accuracy of the breeding value of young animals when used at large nucleus farms. Currently, the transition to mass testing of animals at automatic feeding stations is the most accurate method for evaluating feed conversion rate and related indicators of feeding behavior. In the presented work, opportunity to use values of residual feed consumption to increase individual's selection effectiveness with direct consideration of parameters of fodder behavior has been determined on the Russian population of pigs of the duroc breed. With the same growth intensity, there are animals, which use fodder energy differently. These differences genetically cause about 20% of variability, which confirms the significance of the indicator in tandem selection of pigs for simultaneous improvement of fodder behavior and feed conversion characteristics. This work aimed to study the genetic features of feeding behavior and growth traits of Duroc boars in relation to the residual feed intake (RFI) for use in the breeding process. The studies were carried out at Nucleus Farm TopGene for a population of 800 animals breeds of Duroc labeled with electronic chips. Individual records for feed intake were collected using automatic feeding stations. Additionally, parameters of feeding behavior, average daily gain (ADG), and feed conversion rate (FCR) were recorded. To eliminate the influence of growing factors on the studied traits, a regression analysis was performed to correct feed conversion rate, as well as the calculation of genetic and paratypical variances. The RFI values were obtained based on the difference between the actual and predicted average daily feed intake, considering the average metabolic weight, and the body weight gain of animals according to the multiple linear regression equation. The average values and heritability of the main breeding traits were as follows: feed conversion rate 2.20 kg/kg ($h^2 = 0.214$, for the adjusted value), average daily feed intake 2.51 kg ($h^2 = 0.221$), number of visits per day 7.9 units ($h^2 = 0.494$), feed intake per visit 0.37 kg ($h^2 = 0.284$), time spent in feeding per visit 11.3 min ($h^2 = 0.168$), feeding rate 35.4 g/min ($h^2 = 0.269$). For RFI, the ratio of genetic variation was $h^2 = 0.215$. According to the ratio of RFI and ADG, the groups of Duroc boars were selected for desirable negative or low RFI values of –254.9 and –276.2 g vs. +266.8 and +353.9 g for positive RFI. Individuals that showed high gains (1057 g per day) with reduced feed intake (2.34 kg/day) can serve as the basis for developing a specialized line of pigs (group I) capable of efficient using feed energy for body growth. Boars with positive RFI values significantly differed for FCR_{corr} (–0.15 and 0.24 kg/kg), back fat (–1.90 and –2.49 mm), muscle eye area (+4.57 and +6.10 cm^2); for feeding behavior, the differences were –2.8 and –8.0 minutes for time spent in feeding per day, +1.7 and +2.0 visits per day, and –2.7 and –4.2 minutes for time spent in feeding per visit. That is, the more frequent visits to feeding stations at less time spent in feeding per visit, the more efficient the use of feed. The estimation of breeding value showed the similar RFI pattern for the desired group of animals. The higher estimates for feed intake compensated the existing differences between the phenotype and genotype for the number of visits per day and feeding rate due to the identified genetic correlations with RFI, $r_g = 0.702$ and $r_g = 0.033$, respectively. Thus, the feeding behavior traits of pigs along with the residual feed intake (RFI) are genetically determined and can be used to improve pig populations for economically important and productive characteristics.

Keywords: pigs, Duroc breed, feeding behavior, feed conversion rate, RFI, fattening productivity, heritability, breeding value

Intensive pig breeding for a limited number of traits, practiced in recent decades, leads to the achievement of the so-called breeding plateau when the selection process cannot provide further genetic progress in improving the traits. New strategies in animal breeding are required to solve this problem. The authors consider the search and evaluation of breeding indicators related to economically significant traits directly or indirectly as one of the promising approaches [1]. The integration of additional indicators into breeding programs will improve the accuracy of the assessment of the breeding value of the animal by the economically important characteristics associated with them and, as a result, accelerate the genetic progress in breeding. The development of automated computerized systems (feed stations, or feedlots) made it possible to account for indicators of feeding behavior of pigs [2], such as time spent at the feeding station per day (time in the feeding station per test day, TPD), the amount of feed consumed per day (average daily feed intake, ADFI), the number of visits to the feeding station per day (NVD), the average duration of one visit (time in the feeding station per visit, TPV = TPD/NVD), average feed intake per visit (feed intake per visit, FPV), feed consumption rate (FR = DFI/TPD) [3]. These indicators are considered as additional features for inclusion in pig breeding programs and can become one of the elements of the management system in pig breeding.

The analysis of indicators of feeding behavior revealed the presence of breed-specific features [4], as a result of which Fernández et al. [5] suggested that an increase in feed efficiency could be achieved by developing specific feeding strategies for pig breeds based on the genetic conditioning of these traits. It was found that the feeding behavior was characterized by a moderate degree of heritability. The h^2 values depending on the breed (Yorkshire, Landrace, Duroc) were 0.44-0.51 for NVD, 0.48-0.56 for TPD, 0.55-0.59 for FR, 0.49-0.57 for FPV, and 0.47-0.51 for TPV [6]. In the work of Kavlak et al. [7], the heritability coefficients of feeding behavior ranged from 0.17 to 0.47, with ADFI values highly correlated with production traits. Variations of the heritability coefficient of the daily feed consumption indicator in Landrace pigs of the Dutch breeding depending on the growing period were revealed, i.e., from $h^2 = 0.53$ on day 5 to $h^2 = 0.24$ on day 95 of the control growing [8].

The use of feeding behavior traits in breeding programs requires knowledge of the genetic relationships between indicators. In this regard, the correlations between the traits in pigs of different breeds were studied. For example, Do et al. [6] in a large-scale study conducted on Yorkshire, Landrace, and Duroc pigs of Danish breeding showed an improvement in feed conversion rate (FCR) with an increase in ADFI (r_g = 0.43-0.74) and NVD (r_g = 0.39-0.50) and a decrease in FCR with an increase in TPV (r_g from –0.35 to –0.43) and FPV (r_g from –0.27 to –0.40). An increase in ADFI and, as a result, in average daily gain (ADG) was found with an increase in FR [6]. It is consistent with the data of de Haer et al. [3] and Rauw et al. [9] who previously established that pigs that consumed feed quickly were characterized by higher feed efficiency, increased growth intensity, and accumulate more fat. Andretta et al. [10] showed that the feed intake rate and the number of feed station visits per day were most closely related to productive qualities. In addition, the amount of feed consumed per day and the rate of feed consumption are negatively correlated with feed efficiency [10]. In recent studies of Carcò et al. [11], performed on hybrid young boars, the traits of feeding behavior were highly correlated with fattening productivity and indicators of carcass quality. Thus, ADFI was positively correlated with ADG, TPD was negatively correlated with ADG

and positively correlated with FCR, FPV and ADG were directly correlated, FR had a strong association with ADG and ADFI.

The greatest interest is the use of indicators of feeding behavior to assess feed consumption efficiency. The traditional assessment is based on the determination of feed conversion rate as the ratio of the consumed feed (or dry matter consumed) to the increase in live weight over a certain time. In the authors' previous studies, the association of some feeding behavior traits with FCR was shown [12, 13]. However, when using this indicator in breeding, it is necessary to take into account its strong correlation with the amount of feed consumed and the average daily increase in live weight of the animal. In other words, two animals may have the same FCR values, but differ greatly in feed consumption and live weight gain. On the contrary, the same animal with different feed intake will be characterized by different FCR values, although the hereditary basis does not change.

An alternative indicator for assessing the feed consumption efficiency, which is widely used in different types of farm animals, including pigs, is the residual feed intake (RFI), or deviations from the predicted feed intake. In pigs, RFI can be calculated as the residual value of the feed consumption model equation, which includes the traits of growth rate (average daily gain) and fat depth as independent variables, and possibly the metabolic bodyweight of the animal [14-16]. In other words, this indicator can be defined as the difference between the actual feed intake and the expected feed requirements due to the need to maintain body weight and increase growth. Unlike FCR, the RFI indicator does not depend on the average daily weight gain; therefore, it serves to more accurately assess the feed consumption efficiency since it is based on the energy needs of the animal.

It was found that animals of different lines and selected for low RFI (LRFI) values had desirable indicators for fertility and lactation activity of sows, but worse values for fatness and a negative energy balance during lactation [17]. According to Colpoys et al. [18], young boars with LRFI were characterized by reduced behavioral reactivity when exposed to various stress factors, which allows better utilization of feed energy. For the preliminary selection of young animals by RFI, it is proposed to use the IGF-1 hormone as a physiological marker [19]. It was reported that direct selection by RFI was accompanied by changes in other traits, with the highest correlation between RFI and the content of IGF-1 in the blood, i.e., IGF-1 is genetically associated with fattening efficiency [19]. It is also shown that the diet, which differs from that used in the breeding of pigs with LRFI (high energy content and low fiber content), does not allow the maximum realization of the genetic potential of species according to RFI [20].

In this work, the possibility of using the residual feed consumption indicator to increase the selection efficiency with direct consideration of the feeding behavior parameters of species was established for the first time in the Russian population of Duroc pigs. With the same intensity of pig growing, some animals use feed energy in different ways. These differences genetically determine about 20% of the variability, which confirms the indicator significance in the tandem selection of pigs for the simultaneous improvement of the feed behavior and feed conversion characteristics.

The work objective was to study the genetic relationship of the feeding behavior traits with the feed consumption efficiency, estimated by the residual feed consumption indicator.

Methods. The studies were carried out based on the genetic and selection center Top Gen (Voronezh Region, Verkhnyaya Khava, 2017-2019) on 800 Duroc boars (*Sus scrofa*) labeled with electronic chips. The animals at the beginning of fattening aged 78 days, at the end 156 days. Boars were kept in groups of 15

animals in machines with slotted floors (floor area of 1.30 m^2/animal) at 18 °C, had unlimited access to feed and water. Individual feed consumption accounting was carried out using automatic feed stations MLP-RAP (Schauer Agrotronic AG, Switzerland) and GENSTAR (Cooperl, France).

The diets were the same for all groups of boars and varied depending on the fattening period, SK-52 in the first, SK-6 in the second, and SK-7 in the third (final) period. Composition of diets: SK-52 — dry matter (80%), metabolic energy (13.14%), crude protein (16.70%), crude fat (4.38%), crude fiber (4.39%), lysine (1.11%), methionine + cysteine (0.67%), calcium (0.55%), phosphorus (0.52%); SK-6 — dry matter (80%), metabolic energy (13.02%), crude protein (14.59%), crude fat (3.57%), crude fiber (4.12%), lysine (0.95%), methionine + cysteine (0.58%), calcium (0.55%), phosphorus (0.48%); SK-7 — dry matter (80%), metabolic energy (12.61%), crude protein (13.10%), crude fat (2.17%), crude fiber (4.49%), lysine (0.83 %), methionine + cysteine (0.51%), calcium (0.51%), and phosphorus (0.49%).

According to the results of quality control of individual and group (for all selection) parameters of feeding behavior for the normality of distribution (the minimum number of control records during the test is not less than 14, but not more than 144), 766 animals were selected for analysis with a total number of observations during the fattening periods of 49,577 with an average value of 64.7 records per 1 animal. The parameters of ADG (g), ADFI (g/day), TPD (min/day), NVD (units), FPV (g), FR (g/h), TPV (min), FCR (kg/kg), and RFI (g), BF, BF100 (fat depth above the 6-7 vertebra, absolute and reduced to a live weight of 100 kg, mm); LD, LD100 (muscle eye area, absolute and reduced to a live weight of 100 kg, cm) were studied.

The FCR values were calculated for each animal as the ratio of the amount of feed consumed to the live weight gain over the entire growing period. Taking into account the differences in the length of the growing period between groups, the following regression equation was used to obtain comparable feed conversion values, which was obtained using the STATISTICA 10 program:

$$FCR_{corr} = -4.2361 + 0.0890x_1 + 0.0922x_2 - 0.0841x_3 + 0.0057x_4, \quad (1)$$

where x_1 is the period of fattening at the automatic feeding station, day; x_2 is live weight at the start of fattening, kg; x_3 is live weight at the end of fattening, kg; x_4 is the average daily weight gain, g.

The calculation of genetic and paratypic correlations was performed using the REMLF90 program [21, 22] according to the following equation of the mixed model:

$$y = \mu + YM + DFSM + Party\ (Batch) + Period + b_1BW_{start} + animal + e, \quad (2)$$

where μ is population constant; YM is the year and month of birth of the animal, fixed effect; DFSM is start date × feed station × week, the fixed effect of the animal start at the feed station; Party (Batch) is evaluation batch at the feed station of animal groups, fixed effect; Period is duration of the animal evaluation, fixed effect; b_1BW_{start} is the start live weight, regression effect; animal is animal effect, randomized; e is a residual variance of the model.

The heritability coefficient was calculated based on the ratio of the additive genetic variance to the total phenotypic variability of the trait according to the variational components of the analysis:

$$h^2 = \frac{\sigma_a^2}{\sigma_a^2 + \sigma_e^2}, \quad (3)$$

where σ^2_a is variance between groups of descendants, σ^2_e is variance within a group of descendants, or the residual variance.

The deviation from the predicted feed intake (RFI) was determined according to the approach by Cai et al. [23] and Ding et al. [24] based on multiple linear regression:

$$RFI = ADFI - (a + b_1 MWT^{0.75} + b_2 ADG),$$
$$RFI = ADFI - (888.00 + 40.33 \times MWT^{0.75} + 0.64 \times ADG) \quad (4)$$

where a is constant term of the linear equation; b_1 and b_2 are regression coefficients; $MWT^{0.75}$ is the average metabolic mass representing the active mass of the body tissues of the animal that need to be provided with energy $[(BW\_start + BW\_end)/2]^{0.75}$, kg; BW_start and BW_end are live weights at the beginning and the end of the test fattening, respectively; ADG is average daily gain, g.

Statistical processing was performed in Microsoft Excel. The average, minimum, and maximum values for the entire sample were obtained using descriptive statistics, and the variation coefficient was calculated using the formula:

$$Cv = \sigma / M \times 100, \quad (5)$$

where σ is standard deviation, M is mean value of the trait.

Results. The study of feeding behavior using automatic feeding stations is of interest for understanding the feeding efficiency and feed digestibility. In this research, the average live weight of boars before fattening was 35.7 kg, the fattening period was 78.1 days. The phenotypic variability (Cv) of feeding behavior and feed conversion within the closed pig population ranged from 14.5 to 40.5%, which indicates potential selection opportunities (Table 1). The variability of the actual feed conversion values was 25.4%, corrected value was 9.1%, while the average value of the indicator remained the same (2.20 kg/kg). For the RFI indicator, the variation coefficient was not calculated since the sum of the values was 0.

A comparative analysis of the authors' data with the results obtained by other authors showed the presence of breed-specific features. If the indicators of feed efficiency (average daily consumption and conversion) were relatively stable (there was a progressive decrease in feed conversion over the past 15 years, due to intensive selection and improvement of diets), then the indicators of feed behavior varied greatly (Table 2).

1. Characteristics of the phenotypic parameters of the studied selection of boars (*Sus scrofa*) of the Duroc breed (n = 766; genetic and selection center Top Gen, Voronezh Region, Verkhnyaya Khava, 2017-2019)

Indicator	M±SEM	SD	Min	Max	Cv, %
ADG	957±5	139	424	1508	14.5
ADFI	2.51±0.14	0.40	1.14	4.45	15.9
TPD	74.9±0.5	13.8	45.7	139.9	18.5
NVD	7.9±0.1	2.6	3.5	16.3	32.2
TPV	11.3±0.2	4.5	4.0	26.0	39.8
FR	35.4±0.3	8.5	16.6	74.5	24.1
FPV	0.372±0.005	0.151	0.145	0.799	40.5
FCR	2.20±0.02	0.56	0.40	5.70	25.4
FCR_{corr}	2.21±0.01	0.20	1.80	3.70	9.1
RFI	0.00±13.46	372	–1227	1964	–

N o t e. ADG — average daily live weight gain, g; ADFI — average daily feed intake, kg/day; TPD — average time spent at the feed station, min/day; NVD — number of feed station visits per day, units; FPV — amount of feed eaten per visit, kg; TPV — duration of food intake per visit, min; FR — feed consumption rate, g/min; FCR — feed conversion rate, kg/kg; FCR_{corr} — corrected feed conversion rate, kg/kg; RFI — deviation from the predicted feed consumption, g. Dash means that the indicator was not calculated.

In our study, the number of visits to feeding stations for Duroc boars was 1.4 times less than in pigs of the same breed [6], with approximately equal values of ADFI and FCR (the differences were 4.6-5.0%), while the duration of one visit was 1.3 times longer. In addition, Duroc pigs consumed 1.5 times more feed per visit, and FR was 15.6% higher compared to the same values set by Do et al. [6].

2. Feed consumption efficiency and feeding behavior of pigs (*Sus scrofa*) of different breeds, described in the literature, in comparison with the indicators obtained for the Duroc breed in this work (n = 766; genetic and selection center Top Gen, Voronezh Region, Verkhnyaya Khava, 2017-2019)

Indicator	Duroc (n = 766)	PIC L-26 × C-15 [25]		Large White [4]	Landrace [4]	Pietrain [4]	PIC C-22 [26]	Duroc (D) [6]	Landrace (L) [6]	Yorkshire (Y) [6]	Финальный гибрид (Y×D)×L [27]	Maxgro [28]
Sex	X	B	G	B	B	G	B and G	B and G	B and G	B and G	B and G	H
ADG, g	0.957±0.01	0.997	0.980	0.87±0.08	0.85±0.08	0.71±0.07	–	1.03±0.10	1.00±0.09	0.93±0.09	–	-
ADFI	2.51±0.14	3.19	2.88	2.21±0.19	2.28±0.20	1.70±0.14	–	2.40±0.38	2.38±0.38	2.15±0.35	–	2.73±0.32
NVD	7.90±0.09	11.8	11.8	–	–	–	5.6±0.61	11.07±5.25	8.81±4.36	18.19±10.88	13.12±3.99	4.29±0.90
FPV	0.37±0.06	0.302	0.272	–	–	–	–	0.25±0.09	0.31±0.11	0.15±0.01	–	0.64
TPV	11.3±0.2	9.5	8.9	–	–	–	11.3±1.1	8.58±3.40	9.36±3.67	4.44±2.36	5.35±1.61	14.44
FR	35.4±0.3	32.1	32.0	35.90±6.50	35.30±6.50	30.70±5.20	–	30.61±0.66	34.81±0.78	36.69±0.84	–	45.38±8.79
FCR	2.20±0.01	3.13	2.94	2.57±0.18	2.68±0.22	2.40±0.17	–	2.31±0.34	2.36±0.30	2.29±0.29	–	2.26±0.23

N o t e. B — barrows, G — gilts, H — hogs; ADG — average daily live weight gain, g; ADFI — average daily feed intake, kg/day; NVD — number of visits to feed station per day, units; FPV — amount of feed eaten per visit, kg; TPV — duration of feeding per visit, min; FR — feed consumption rate, g/min; FCR — feed conversion rate, kg/kg. Dash means that the indicator is not shown.

3. Genetic and paratypic correlations of feeding behavior and feed conversion in Duroc boars (*Sus scrofa*) (n = 766; genetic and selection center Top Gen, Voronezh Region, Verkhnyaya Khava, 2017-2019)

Indicator	ADFI	TPD	NVD	TPV	FR	FPV	FCR	FCR_{corr}	RFI
ADFI	0.221[c]	0.385	0.230	–0.001	0.375	0.327	0.372	–0.369	0.928
TPD	0.390	0.290[c]	0.148	0.582	–0.639	0.132	0.153	–0.202	0.355
NVD	0.641	0.536	0.494[c]	–0.597	0.030	–0.715	0.143	0.013	0.254
TPV	–0.307	0.286	–0.593	0.168[c]	–0.554	0.679	–0.063	–0.079	–0.016
FR	0.303	–0.760	–0.094	–0.501	0.269[c]	0.123	0.129	–0.086	0.358
FPV	–0.047	–0.465	–0.721	0.532	0.457	0.284[c]	0.007	–0.199	0.288
FCR	–0.062	0.454	0.147	0.115	–0.538	–0.462	0.058[c]	0.257	0.546
FCR_{corr}	–0.287	0.530	0.002	0.298	–0.772	–0.467	0.861	0.214[c]	–0.099
RFI	0.910	0.575	0.702	–0.281	0.033	–0.293	0.311	0.113	0.215[c]

Note. ADFI — average daily feed intake; TPD — time in the feeding station per day; NVD — number of visits to feed station per day; TPV — duration of food intake per visit; FR — feed consumption rate; FPV — amount of feed eaten per visit; FCR — feed conversion rate; FCR_{corr} — corrected feed conversion rate; RFI — residual feed intake (deviation from predicted feed intake). The heritability coefficients h^2 is along the diagonal marked with the upper index [c], paratypical correlations are above the diagonal, and genetic correlations are under the diagonal.

The authors found moderate heritability for TPV (h^2 = 0.168), FCR_{corr} (h^2 = 0.214), RFI (h^2 = 0.215), and ADFI (h^2 = 0.221) (Table 3). The values of the heritability coefficients for FR, FPV, and TPD were higher and ranged from 0.269 to 0.290. The lowest genetic variance was found for the actual FCR (h^2 = 0.058), while the highest was found for the NVD (h^2 = 0.494).

The analysis of genetic correlations showed that the more often the animals visited the feeding stations, the longer they were there (r = 0.536), while the average duration of one visit decreased (r = –0.593), as well as FR (r = –0.760). In other words, the more often the animals visited the station, the less food they consumed per visit (r = –0.721). FCR increased with increasing duration of time at the station (r values 0.454 and 0.530) and decreased with increasing FR (r values –0.538 and –0.772), which was also associated with the digestibility and quality of feed. The influence of paratypic (environmental) factors on feeding behavior was noticeable in the relationship between the number of visits to the feeding station and the duration of one visit: the more often the animal visited the feedlot, the less time it was there (r = –0.597) and ate less food per visit (r = –0.715), which is also due to behavioral characteristics.

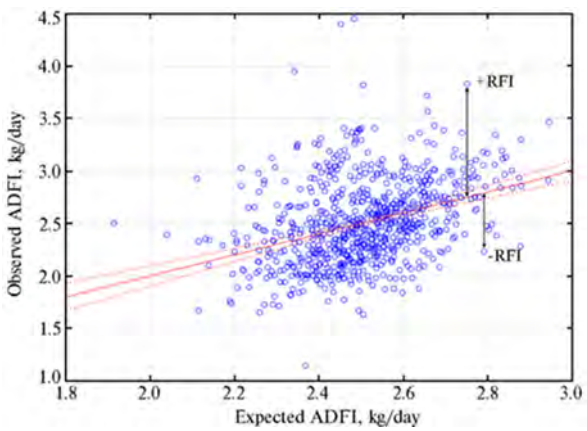

Fig. 1. Distribution of the initial and predicted values of the average daily feed intake (ADFI) by the Duroc boars (*Sus scrofa*) obtained by calculating the RFI based on multiple linear regression (genetic and center Top Gen, Voronezh Region, Verkhnyaya Khava, 2017-2019).

Figure 1 shows a graph of the initial and predicted ADFI values, the difference between which reflects the deviation from the predicted feed intake (RFI).

The effect of regression coefficients ($MWT^{0.75}$, ADG) on the value variable (ADFI) was significant at $p < 0.001$. Negative RFI values meant that the animals spent less feed energy on live weight gain and maintaining the body vital activity during the test period than predicted. On the contrary, a positive value of RFI indicated an excess of feed consumption or lower efficiency of its consumption for the needs of the body.

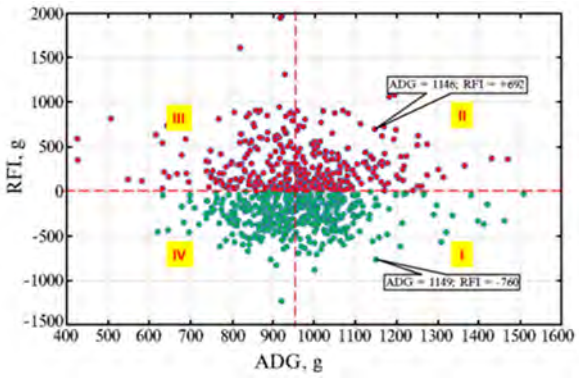

Fig. 2. Groups of boars (*Sus scrofa*) of the Duroc breed (I-IV) **distinguished by the ratio of phenotypic values of average daily gain** (ADG) **and deviations from the predicted feed consumption** (genetic and selection center Top Gen, Voronezh Region, Verkhnyaya Khava, 2017-2019).

Following the calculated values of RFI, ADFI, and ADG, groups of boars were identified with the most desirable RFI/ADG ratio (the groups I and IV with negative or low RFI values) and with the least desirable ratio (the groups II and III with positive or high RFI values) (Fig. 2).

The distribution of animals between groups I-IV was 25.3, 24.3, 22.1, and 28.3%, respectively. Therefore, group I, or about a quarter of the animals, could be selected as breeding replacement young that were successfully assessed on their productivity (high ADG values with reduced feed intake). Pigs of groups II, III, and partly IV with a low RFI value are recommended for use in commercial production (ranking).

4. Phenotypic indicators of fattening productivity and feeding behavior of Duroc boars (*Sus scrofa*) in groups, depending on the ratio of RFI (deviation from the predicted feed consumption) **and ADG** (average daily live weight gain) (genetic and selection center Top Gen, Voronezh Region, Verkhnyaya Khava, 2017-2019)

Indicator	Group I (n = 194)	Group II (n = 186)	Group III (n = 169)	Group IV (n = 217)
		Fattening productivity		
RFI	–254.9±12.7	266.8±17.1$^{a)***}$	353.9±26.3$^{b)***}$	–276.2±12.0
ADG	1057±7$^{b)***}$ $^{c)***}$	1069±7	844±8	864±5
ADFI	2.34±0.01$^{c)***}$	2.89±0.02$^{a)***}$	2.75±0.03$^{b)***}$	2.14±0.01
FCR	1.89±0.03	2.37±0.03$^{a)***}$	2.57±0.05$^{b)***}$	2.04±0.03$^{c)***}$
FCR_{corr}	2.12±0.01	2.10±0.01	2.36±0.02$^{b)***}$	2.27±0.01$^{c)***}$
BW_start	35.9±0.4$^{b)*}$	36.3±0.4	34.7±0.4	35.7±0.4
BW_end	114.8±0.7$^{b)***}$ $^{c)***}$	118.7±0.8$^{a)***}$	103.5±0.8	103.4±0.6
BF	17.43±0.54$^{c)**}$	19.92±0.67$^{a)**}$	19.33±0.81^{t}	15.24±0.47
LD	79.95±1.21$^{b)*}$ $^{c)***}$	84.20±1.64$^{a)*}$	75.38±1.40	73.85±0.91
BF100	15.25±0.52	16.60±0.70	18.72±0.80$^{b)***}$	14.80±0.48
LD100	68.36±1.08	66.52±1.32	72.10±1.42$^{b)*}$	71.48±0.87$^{c)***}$
Period	75.0±0.7	77.3±0.7$^{a)*}$	81.9±0.7$^{b)***}$	78.5±0.6$^{c)***}$
Age_end_off	153.6±0.7	156.3±0.7$^{a)**}$	158.8±0.8$^{b)***}$	156.3±0.6$^{c)**}$
BWG	78.9±0.6$^{b)*}$ $^{c)***}$	82.4±0.8$^{a)***}$	68.8±0.8	67.8±0.6
FI_all	149.0±2.8$^{c)**}$	195.0±2.8$^{a)***}$	175.6±3.5$^{b)***}$	137.4±2.2
		Feeding behavior		
TPD	72.3±0.8	75.1±0.9$^{a)*}$	80.3±1.2$^{b)***}$	72.9±0.9
NVD	8.9±0.2$^{a)***}$ $^{b)***}$	7.2±0.2	6.9±0.2	8.5±0.2
TPV	9.5±0.3	12.2±0.3$^{a)***}$	13.7±0.4$^{b)***}$	10.2±0.3
FR	33.8±0.4$^{c)***}$	41.2±0.7$^{a)***}$	36.8±0.7$^{b)***}$	30.9±0.4
FPV	0.304±0.010	0.464±0.011$^{a)***}$	0.456±0.011$^{b)***}$	0.289±0.007

Note. The distribution of animals into groups is illustrated in Fig. 2. RFI — deviation from the predicted feed consumption (residual feed intake), g; ADG — average daily live weight gain, g; ADFI — average daily feed intake, kg/day; FCR — feed conversion, kg/kg; FCR_{corr} — corrected feed conversion, kg/kg; BW_start, BW_end — live weight at the start and the end of fattening, kg; BF, BF100 — fat depth over the 6-7-th vertebra, absolute and reduced to a live weight of 100 kg, mm; LD, LD100 — the muscle eye area, absolute and reduced to a live weight of 100 kg, cm^2; Period — the duration of fattening at the station, days; Age_end_off — the age of the animal at the end of fattening, days; BWG — the increase in live weight during the fattening period, kg; FI_all — feed consumption during the testing period at the station, kg; TPD — the average time spent at the feed station, min/day; NVD — the number of visits to the feed station per day, units; TPV — the duration of the meal per visit, min; FR — the feed consumption rate, g/min; FPV — the amount of feed eaten per visit, kg. The number of animals counted according to the characteristics of BF, BF100, LD, and LD100 in groups I-IV is 122, 112, 93, and 158, respectively. *, **, *** Differences between groups ($^{a)}$ for I and II, $^{b)}$ for I and III, $^{c)}$ for I and IV) when comparing the average indicators are statistically significantat $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, $^{t}p < 0.1$ (trend).

Boars of group I had significantly more favorable economic values of

fattening productivity indicators compared to other groups (Table 4): for RFI +523.9 and +608.8 g (groups II and III, respectively, $p < 0.001$), for ADG +193 and +213 g (groups II and III, $p < 0.001$), for ADFI –0.41 and –0.55 kg/day (groups II and III, $p < 0.001$), for FCR –0.48, –0.68, and –0.15 kg/kg (groups II, III, and IV, $p < 0.001$), for FCR_{corr} –0.15 and –0.24 kg/kg (groups III and IV, $p < 0.001$), for BW_end +11.3 and +11.4 kg (groups III and IV, $p < 0.001$), for BF –1.90 and –2.49 mm (groups II and III, $p < 0.1$-0.01), for LD +4.57 and +6.10 cm^2 (groups III and IV, $p < 0.05$-0.001), for BWG +10.1 and +11.1 kg (groups III and IV, $p < 0.05$-0.001), and for FI_all –26.6 and –46.0 kg (groups II and III, $p < 0.001$). According to feeding behavior traits, the differences with other groups were –2.8 and –8.0 min/day for TPD (groups II and III, $p < 0.05$-0.001); +1.7 and +2.0 units for NVD (groups II and III, $p < 0.001$), and –2.7 and –4.2 min for TPV (groups II and III, $p < 0.001$). At the same time, the animals of group I were inferior to their herdmates from some groups in terms of BW_end (–3.9 kg, group II, $p < 0.001$), LD (–4.25 cm^2, group II, $p < 0.05$), BWG (–3.5 kg, group II, $p < 0.001$), FR (–3.0 and –7.4 g/min. groups II and III, $p < 0.001$), and FPV (–0.152 and –0.160 kg. groups II and III, $p < 0.001$).

The revealed differences between the groups indicated an optimal combination of qualitative characteristics of carcasses and economic indicators of cultivation in animals of group I. More frequent visits to feed stations with a shorter stay in it contributed to a more efficient consumption of the feed mixture. It is worth noting that the species of groups I and IV tended to minimize feed costs and did not significantly differ in terms of feeding behavior (except for the FR).

5. Genetic indicators of fattening productivity and feeding behavior of Duroc boars (*Sus scrofa*) in groups, depending on the ratio of RFI (deviation from the predicted feed consumption) **and ADG** (average daily live weight gain) (genetic and selection center Top Gen, Voronezh Region, Verkhnyaya Khava, 2017-2019)

Indicator	Group I (n = 194)	Group II (n = 186)	Group III (n = 169)	Group IV (n = 217)
Fattening productivity				
RFI	–28.9±4.1	+14.1±4.3[a)]***	+19.3±5.6[b)]***	–28.5±4.4
ADFI	–12.0±5.0[c)]***	+23.4±4.7[a)]***	+7.25±5.9[b)]***	–39.7±5.0
FCR	–0.029±0.002	–0.007±0.003[a)]***	+0.021±0.004[b)]***	+0.006±0.002[c)]***
FCR_{corr}	–0.018±0.002	–0.007±0.002[a)]***	+0.015±0.003[b)]***	+0.010±0.002[c)]***
Feeding behavior				
TPD	–1.79±0.25	+0.12±0.25[a)]***	+1.36±0.32[b)]***	–0.50±0.27[c)]***
NVD	–0.282±0.043	–0.001±0.041[a)]***	+0.068±0.045[b)]***	–0.252±0.046
TPV	–0.10±0.03	+0.03±0.04[a)]***	+0.07±0.04[b)]***	+0.16±0.03[c)]***
FR	+0.67±0.11[a)]* [b)]*** [c)]***	+0.29±0.11	–0.54±0.14	–0.38±0.11
FPV	+11.46±1.11[a)]*** [b)]*** [c)]***	+4.31±1.50	–3.70±1.62	+2.17±1.23

Note. ADG — average daily live weight gain, g; RFI — deviation from the predicted feed consumption (residual feed intake), g; ADFI — average daily feed intake, g/day; FCR — feed conversion rate, kg/kg; FCR_{corr} — feed conversion corrected for multiple regression, kg/kg; TPD — average time spent at the feed station, min/day; NVD — number of visits to the feed station per day, units; TPV — time of feed consumption per visit, min; FR — feed consumption rate, g/min; FPV — the amount of feed eaten per visit, kg.

*, **, *** Differences between groups ([a)] for I and II, [b)] for I and III, [c)] for I and IV) when comparing the average indicators are statistically significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Indicators of fattening productivity and feeding behavior in genetic terms (assessment of the breeding value of animals) had similar dynamics of change in groups (Table 5). The desired type of pigs according to RFI, ADFI, FCR, and FCR_{corr} was characterized by significantly more favorable values of fattening productivity and feed conversion. Feeding behavior traits showed a generally similar distribution, but since genetic correlations were taken into account, and the desired genotypes were evaluated by negative RFI values, a decrease of –0.283, –0.214, and +0.030 units was observed for NVD (groups II, III, and IV, $p < 0.001$), and significantly higher values were obtained for FR and FPV, by 0.38-1.21 g/min and 7.15-15.16 g (groups II, III, and IV, $p < 0.001$), respectively. Despite the

differences between the phenotypic and genetic expression of some feeding behavior traits, the selection of boars according to the breeding value of traits that determine the feed consumption efficiency will allow obtaining animals with economically justified consumption (ADFI) and conversion (FCR) of feed, the 2.1-2.4 kg/day and 1.9-2.1 kg/kg, respectively. Tandem selection (the time spent at the feed station, the increase in FR, and the amount of feed consumed per visit) will increase the feed efficiency.

The results of this study are generally consistent with the published data. The ADFI of Duroc boars in our experiments was slightly higher (2.51 kg/day) than that noted by Do et al. (2.40 kg/day) [6], but in PIC L-26 × C-15 and Maxgro animals [25, 28], this indicator had the maximum values (2.88 and 3.19 kg/day in pigs and castrates PIC L-26 × C-15, respectively, and 2.73 kg/day in Maxgro boars). The number of visits to the feeding station per day, in all probability, depends on the technical characteristics but our results (7.90 units) occupied an intermediate position between the values for castrates and Landrace pigs (8.81 units) and PIC C-22 hybrids (5.6 units) [6, 26]. The amount of feed consumed per visit in our studies was the highest (0.37 kg) after that observed in Maxgro boars (0.64 g) [28]. The feeding time was 11.3 min which generally exceeded the average values for purebred animals. Let us note that the FCR value itself in this study had one of the lowest values compared to its analogs (see Table 2). It did not depend on how often the animals could visit the feeding station (the maximum for Yorkshire and the minimum for Maxgro) and how much time they spent eating feed. We believe that the feed consumption efficiency depends not only on the behavioral reactions of the animal but also on the proportion between the growing period duration and an animal age at the start and the end of fattening. Achieving the highest intensity of average daily live weight gains contributes to the production of early-maturing animals with good economic indicators of feed consumption efficiency.

Thus, the justification of the joint use of the feeding behavior traits and the feed consumption efficiency by the Duroc boars to increase the effectiveness of breeding has been proved. The heritability (h^2) of the studied economically valuable indicators varied from 0.168 to 0.494, which confirms the potential possibility of ranking and selecting species based on productivity characteristics. In an isolated Russian pig population, a new selection parameter for individual selection by both phenotype and breeding value (by deviation from the predicted feed consumption RFI) was studied. This indicator is significantly associated with the fattening productivity traits, which will allow getting pork with more expressed meat qualities, optimal fat depth, and muscle eye area. Feed conversion efficiency will be 1.89-2.04 kg/kg, and the feeding behavior of animals will ensure a rational loading of feed stations. The obtained results will be used in the work of the selection center to increase the intensity of breeding Duroc pigs to reproduce high-value genotypes and increase the economically significant productivity.

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AGRICULTURAL BIOLOGY,
2020, V. 55, № 6, pp. 1139-1147
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 636.32/.38:636.082.26:636.061

doi: 10.15389/agrobiology.2020.6.1139eng
doi: 10.15389/agrobiology.2020.6.1139rus

IDENTIFICATION OF INTERSPECIFIC HYBRIDS ARGALI (*Ovis ammon*) AND DOMESTIC SHEEP (*Ovis aries*) OF DIFFERENT GENERATIONS BY EXTERIOR INDICATORS

B.S. IOLCHIEV ✉, N.A. VOLKOVA, V.A. BAGIROV, N.A. ZINOVIEVA

Ernst Federal Science Center for Animal Husbandry, 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail: baylar1@yandex.ru (✉ corresponding author), natavolkova@inbox.ru, vugarbagirov@mail.ru, n_zinovieva@mail.ru
ORCID:
Iolchiev B.S.orcid.org/0000-0001-5386-726
Bagirov V.A. orcid.org/0000-0001-8385-2433
Volkova N.A. orcid.org/0000-0001-7191-3550
Zinovieva N.A. orcid.org/0000-0003-4017-6863
The authors declare no conflict of interests
Acknowledgements:
Supported financially by Russian Science Foundation, grant No. 18-16-00079 and the Ministry of Science and Higher Education of the Russian Federation, theme no. AAAA-A18-118021590132-9.
Received August 17, 2020

Abstract

When creating new breeds and breeding forms, various breeding methods are used, including hybridization. For a long time in the Ernst Federal Science Center for Animal Husbandry, work is underway to use the genetic resources of wild species, in particular argali (*Ovis ammon*), to obtain interspecific hybrids with domestic sheep (*Ovis aries*) in the framework of creating new breeding forms and studying the biological characteristics of certain species of the genus *Ovis*. This raises the question of identifying the obtained interspecific hybrids. Along with conducting expensive studies on the genotyping of such animals, it is of interest to use informative phenotypic indicators characteristic of hybrid individuals. In this work, for the first time, comparative results of differentiation by exterior characteristics of interspecific hybrids of different generations from mating argali with sheep are presented. Romanov breed and original parental forms. The possibility of using exterior indicators for preliminary identification of hybrid individuals without expensive genomic studies was confirmed. Hybridization in the second and subsequent generations resulted in the splitting of hybrid individuals by genotype and phenotype. The work aimed at comparing morphometric parameters of the purebred Romanov sheep and their interspecific hybrids with argali and to reveal informative exterior indicators for identifying hybrid individuals. The lambs of Romanov breed (n = 20) and the interspecific hybrids $^1/_2$ Romanov sheep $^1/_2$ argali (F_1, n = 12), $^3/_8$ Romanov sheep $^5/_8$ argali (F_3, n = 17), and $^7/_{16}$ Romanov sheep $^9/_{16}$ argali (F_4, n = 18) were reared from birth under the conditions of vivarium (the Ernst Federal Science Center for Animal Husbandry, 2019-2020). The following measurements were recorded: height in withers, height at the sacrum, back height, oblique body length, body length, chest width, sacrum width, chest depth, metacarpal girth. Linear measurements were taken at the age of 6, 42 days, and 3 months using a measuring tape, tape measure, and a measuring compass. The animals were weighed on an electronic balance. To assess the development of animals on the basis of weight and linear measurements, the body indices were calculated: long-legged index, elongation index, overgrowth index, breast index, bone index, body mass index. The SPSS v.23 software was used for statistical analysis. As a factor influencing the linear measurements of lambs, the breed of individuals was chosen. Hybrid animals at the age of 3 months in comparison with purebred individuals of the Romanov breed had higher indicators of the long-legged index, which is typical for argali. The advantage of F_1, F_3 and F_4 hybrids over purebred animals for this indicator was 4, 8 and 2%, respectively. At the same time, hybrids F_1, F_3, and F_4 had a more compressed rectangular body shape, therefore, they were inferior to purebred lambs in terms of elongation index, respectively, by 18, 22 and 18% ($p < 0.05$). Differences were established between purebred and hybrid animals in linear and latitudinal measurements. The advantage of purebred sheep over F_1, F_3 and F_4 hybrids was 18, 20 and 14 % in oblique body length, 35, 33 and 20% ($p < 0.05$) in chest width, and 17, 19 and 7 % ($p < 0.05$) in chest depth. Hierarchical classification of hybrids and purebred animals according to the exterior characteristics, it showed that interspecific hybrids with $^1/_2$ and $^5/_8$ argali bloodlines were grouped in one cluster. Hybrids with $^9/_{16}$ bloodiness according to the argali and purebred Romanov sheep formed separate clusters. The results obtained confirm that the exterior parameters can be used for preliminary identification of hybrid individuals, in some cases reducing the cost of

expensive genomic studies.

Keywords: interspecific hybrids, *Ovis ammon*, argali, *Ovis aries*, Romanov breed, exterior

Various methods of genotypic and phenotypic analysis are used to analyze and assess the biological diversity of populations, as well as to identify breeds [1, 2]. Solving the problem of preserving the biodiversity of wild animals and the gene pool of farm animals requires an integrated approach using modern and classical methods [3].

Genetic variability makes it possible to expand the range of breeds through their introduction into a new natural and climatic zone and serves as the basis for the creation of new breeds, including those adapted to local conditions [4, 5].

To describe the breed characteristics of sheep, morphometric methods are used [6-8]. Morphological parameters, along with genetic ones, are used to differentiate populations and breeds [9, 10]. Currently, the characterization of the genetic variability of farm animals, including sheep, is carried out on the basis of the analysis of microsatellite markers [11].

Morphometric studies are important for characterizing the exterior, identifying differences between breeds [12], and assessing the physique of animals and economically useful traits [13-15]. Based on morphometric data, indices are calculated by which the body type can be determined [16]. Morphometric parameters are also used for indirect estimation of live weight and direction of animal productivity [17], selection and breeding by conformation [18]. The study of morphometric parameters over a long period allows one to characterize the structure of the breed and population, as well as the direction of selection for a certain period of time [19].

A number of studies have shown a close correlation relationship between some linear measurements of the exterior with live weight in sheep [20-22]. The live weight indicator depends on numerous factors, including breed, age, housing conditions, and feeding [23-26]. The number of lambs in a litter is negatively correlated with their live weight and affects preservation before weaning [27].

In this work, for the first time, the results of differentiation according to the exterior characteristics of interspecific hybrids of different generations from the mating of argali with sheep of the Romanov breed and the original parental forms are presented. The possibility of using exterior indicators for preliminary identification of hybrid individuals without costly genomic studies has been confirmed. During hybridization in the second and subsequent generations, the hybrids were split according to genotype and phenotype.

The aim of the work was a comparative characteristic of morphometric indicators of purebred sheep of the Romanov breed and their interspecific hybrids with argali to identify informative exterior indicators that identify hybrid individuals.

Methods. The studies were performed on purebred Romanov lambs ($n = 20$) and interspecific hybrids $^1/_2$ Romanov sheep $^1/_2$ argali (F_1, $n = 12$), $^3/_8$ Romanov sheep $^5/_8$ argali (F_3, $n = 17$), $^7/_{16}$ Romanov sheep $^9/_{16}$ argali (F_4, $n = 18$) (Ernst Federal Science Center for Animal Husbandry, 2019-2020). The sheep were kept in stalls and pastures. From May to October, the animals were released to artificial and natural pastures using an electric shepherd. In winter, the sheep were kept in shelters under a canopy on deep bedding. The winter daily ration included hay (2.0 kg), concentrates (0.35 kg), haylage (2.5 kg), table salt (15 g), in summer, the ration was pasture grass and table salt (15 g). During the lambing period, ewes with lambs were kept in groups depending on the age of the lambs (no more than 15 queens in a group with lambs up to 1 week of age). Then the ewe with the grown lamb was placed in a separate cage for 5-6 days. In those cases when the

ewe did not accept her lamb well, the lamb did not recognize the mother or was very weak, they were kept in an individual cage for a longer time. At the same time, the feeding of the lamb was closely monitored and, if necessary, it was put under the ewe every 2-3 hours.

The morphometry of animals was carried out according to the following measurements: height in withers (HW, cm), height at the sacrum (HS, cm), back height (BH, cm), oblique body length (OBL, cm), body length (BL, cm), chest width (CW, cm), sacrum width (SW, cm), chest depth (CD, cm), metacarpal girth (MG, cm). Linear measurements were taken at the age of 6, 42 days, and 3 months using a measuring tape, tape measure, and a measuring compass. The animals were weighed on an electronic balance. To assess the development of animals on the basis of weight and linear measurements, the body indices were calculated: leg length index, elongation index, overgrowth index, breast index, bone index, and body mass index.

The SPSS v.23 software (IBM, USA) was used for statistical analysis. The obtained data were processed by means of an analysis of variance. As a factor influencing the linear measurements of lambs, the breed of individuals was chosen. The arithmetic mean values (*M*) and standard errors of the means (±SEM) were calculated. To identify the statistical significance of differences in mean values, Student's *t*-test was used. Paired comparisons of each indicator were made depending on the factors taken into account. Physique indices were used for the hierarchical classification of the studied groups. Based on the data obtained, a dendrogram was constructed using the method of intergroup communication and Euclidean distances.

Results. Using analysis of variance of morphometric data, a statistically significant effect of the breed and type of animals on the height in withers, height and width at the sacrum, back height, metacarpal girth, body weight ($p < 0.001$), overgrowth ($p < 0.01$), elongation indices and body weight ($p < 0.05$) were established (Table 1).

1. Results of one-way analysis of variance of the influence of genotype on morphometric parameters in lambs of the Romanov breed (*Ovis aries*) and hybrids of different bloodlines from the mating of Romanov sheep with argali (*O. ammon*) (Ernst Federal Science Center for Animal Husbandry, 2019-2020)

Dependent variable	df	*F*	p
Height in withers, cm	3	5.27	0.006
Height at the sacrum, cm	3	5.66	0.004
Back height, cm	3	6.64	0.002
Width at the sacrum, cm	3	9.53	0.000
Metacarpal girth, cm	3	7.11	0.001
Oblique body length, cm	3	9.88	0.000
Chest depth, cm	3	3.58	0.030
Body weight, kg	3	4.00	0.020
Elongation index	3	3.18	0.030
Overgrowth index	3	5.60	0.003
Body mass index	3	2.77	0.050

N o t e. df — degree of freedom, *F* — F-test, p — significance level.

At the age of 6 days, hybrid animals were predominantly inferior to their purebred peers in terms of live weight and a number of linear measurements (Table 2). Significant differences were established between purebred animals of the Romanov breed and interspecific F_1 hybrids with $^1/_2$ argali blood lines. The advantage of purebred animals over F_1 hybrids in terms of body weight, HW, HS, BH, OBL, MG was 25, 8, 10, 10, 9, and 18%, respectively ($p < 0.05$). Differences in these indicators between purebred animals and hybrids of later generations F_3 and F_4 leveled off with an increase in bloodiness for the Romanov breed. The hybrids

exceeded their purebred counterparts in body length and sacrum width. The advantage of F_1, F_3, and F_4 hybrids over purebred animals in BL was 12, 17 and 16% ($p < 0.01$), in SW 35, 50, and 60% ($p < 0.01$).

2. Age dynamics of linear measurements and live weight of lambs of the Romanov breed (*Ovis aries*) (ROM) **and hybrids of different bloodlines from the mating of Romanov sheep with argali (*O. ammon*)** (ARG) (*M*±SEM, Ernst Federal Science Center for Animal Husbandry, 2019-2020)

Parameters	Genotype			
	ROM	1/2 ROM 1/2 ARG	3/8 ROM 5/8 ARG	7/16 ROM 9/16 ARG
		6-day-old		
Height in withers, cm	38.25±1.56	35.33±0.84	37.88±0.85*c	39.14±0.72*c
Height at the sacrum, cm	37.75±0.53*c	34.12±0.83	37.72±0.83*c	38.23±0.71*c
Back height, cm	37.75±0.15*c	33.97±0.82	37.88±0.83*c	38.52±0.71*c
Oblique body length, cm	28.00±1.95	25.45±1.05	28.83±1.06	28.18±0.90*c
Body length, cm	28.00+1.49	31.45+0.80	32.72+0.81*a	32.30+0.69*a
Chest depth, cm	13.00±0.69	12.50±0.37	12.85±0.37	13.66±0.32
Chest width, cm	6.50±0.79	6.75±0.43	7.50±0.43	7.92±0.37
Sacrum width, cm	5.50±0.68	7.43±0.37*a	8.25±0.37*a	8.77±0.31*ac
Metacarpal girth, cm	5.75±0.14*bcd	4.70±0.07	5.05±0.07*c	5.05±0.06*c
Body weight, kg	3.95±0.37	2.98±0.20	3.69±0.20	4.05±0.17*c
		42-day-old		
Height in withers, cm	44.90±1.02	43.50±1.18	43.75±1.40	47.78±0.97*cd
Height at the sacrum, cm	44.55±0.77	42.93±0.88	44.33±1.05	47.15±0.73*acd
Back height, cm	45.07±0.90	43.00±1.02	44.91±1.22	48.30±0.855*acd
Oblique body length, cm	38.72±0.70*bcd	31.87±0.79	30.83±0.95	33.32±0.66
Body length, cm	37.88±1.00	40.00±1.14	41.00±1.36	41.26±0.95*a
Chest depth, cm	19.03±2.47	15.62±2.83	15.33±3.37	17.80±2.35
Chest width, cm	13.22±2.91	8.56±3.32	8.83±3.96	10.56±2.76
Sacrum width, cm	10.35±0.55	9.62±0.63	10.75±0.75	11.07±0.52
Metacarpal girth, cm	5.50±0.25	5.18±0.29	5.33±0.35	5.67±0.24
Body weight, kg	9.06±0.51	7.82±0.58	7.58±0.69	9.04±0.48*cd

N o t e. Designation of the compared groups: a — Romanov breed; b — hybrids 7/16 ROM 9/16 ARG; c — hybrids 1/2 ROM 1/2 ARG; d — hybrids 3/8 ROM 5/8 ARG.
* Differences between the compared groups are statistically significant at $p < 0.05$.

At the age of 42 days, the advantage of purebred animals over hybrids of all generations was retained only in terms of body weight, OBL, CW, and CD (see Table 2). The differences between purebred sheep and F_1, F_3 and F_4 hybrids in body weight were 14, 16, and 0.2%, respectively ($p < 0.05$), in OBL 18, 20, and 14% ($p < 0.05$), in CW 35, 33, and 20% ($p < 0.05$), and in CD 17, 19, and 7% ($p < 0.05$). The hybrid animals, as at 6 days of age, overcome their purebred peers in body length. The excess in this indicator increased with an increase in argali bloodiness in F_1, F_3, and F_4 hybrids, amounting to 6, 8, and 9%, respectively ($p < 0.05$).

The differences between purebred and hybrid animals in terms of BH, HW, and HS varied depending on the bloodiness of the hybrids in the original parental forms. If the F_1 and F_3 hybrids were inferior in these linear measurements to purebred animals, then the F_4 hybrids, on the contrary, surpassed them.

The variability of differences in linear measurements between purebred animals and hybrids of different generations was also noted at the age of 3 months (Fig. 1). Purebred animals excelled F_1 and F_3 hybrids in all measurements, but were inferior to F_4 individuals in these indicators, except for OBL and body weight.

Body indices were calculated to assess the development of purebred and hybrid animals. At the age of 6 days, the hybrid animals had higher indices of breast, body weight, and bone structure and were inferior in terms of elongation and overgrowth indices as compared to purebred peers (Table 3). The established advantage of purebred animals over F_1, F_3, and F_4 hybrids in the breast index, extension, and overgrowth indices remained at the age of 42 days and 3 months. The opposite tendency was observed in the indices of bone density and body

weight: the differences revealed at the age of 6 days were further leveled.

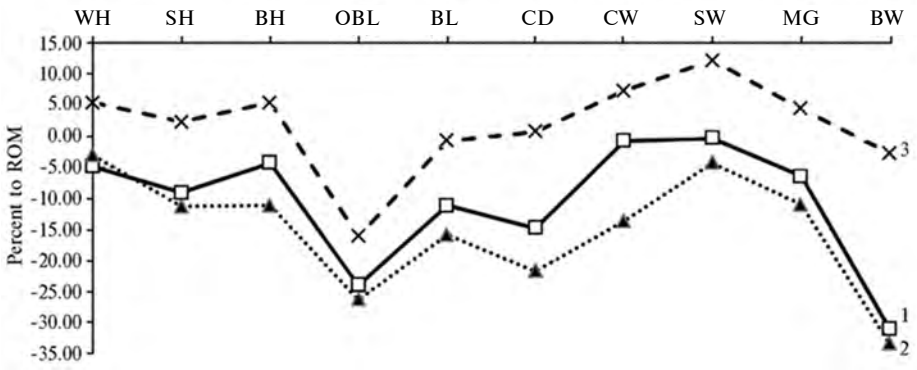

Fig. 1. Exterior profile of interspecific 3-month-old hybrids of different generations from the mating of argali *(Ovis ammon)* (ARG) with Romanov sheep (*O. aries*) (ROM) as compared to peers of the Romanov breed: 1 — 1/2 ROM 1/2 ARG, 2 — 3/8 ROM 5/8 ARG, 3 — 7/16 ROM 9/16 ARG; WH — height in withers, cm; SH — height at the sacrum, cm; BH — back height, cm; OBL — oblique body length, cm; BL — body length, cm; CW — chest width, cm; SW — sacrum width, cm; CD — chest depth, cm; MG — metacarpal girth, cm; BW — body weight, kg (Ernst Federal Science Center for Animal Husbandry, 2019-2020).

3. Age dynamics of body indices in lambs of the Romanov breed (*Ovis aries*) (ROM) and hybrids from the mating of argali *(Ovis ammon)* (ARG) with Romanov sheep (*M*±SEM, Ernst Federal Science Center for Animal Husbandry, 2019-2020)

Indices	Genotype			
	ROM	× ROM 1/2 ARG	3/8 ROM 5/8 ARG	7/16 ROM 9/16 ARG
		6-day-old		
Chest index	51.85±1.91	53.88±2.44	58.48±2.60*a	56.99±1.99
Elongation index	80.34±2.02*cb	73.19±2.57	75.74±2.75	71.86±2.10
Overgrowth index	100.63±0.71*cb	96.92±0.91	99.29±0.97	96.96±0.74
Long-legged index	65.28±0.88	64.27±1.13	66.09±1.21	64.99±0.92
Bone index	10.99±1.23	13.50±1.57	13.30±1.68	12.92±1.28
Body mass index	7.77±0.62	8.49±0.80	9.52±0.85	10.26±0.65*a
		42-day-old		
Chest index	58.90±2.43	55.55±3.05	58.40±3.60	59.13±2.33
Elongation index	87.21±1.25*cbd	74.05±1.59	70.73±1.85	71.08±1.19
Overgrowth index	100.09±1.05	99.10±1.31	101.05±1.56	99.45±1.00
Long-legged index	65.01±1.53	63.90±1.92	65.95±2.27	63.09±1.47
Bone index	12.04±0.37	11.98±0.47	12.27±0.56	11.85±0.36
Body mass index	20.28±1.17	18.29±1.47	17.50±1.74	19.64±1.12
		3-month-old		
Chest index	53.87±1.90	62.47±2.70	58.82±2.32	56.90±2.33
Elongation index	93.46±2.04	75.94±2.88	72.00±2.46	75.38±2.49
Overgrowth index	102.11±0.95	98.49±1.34	94.00±3.30	99.65±1.16
Long-legged index	58.32±1.24	62.20±1.76	66.00±1.95*a	59.85±1.52
Bone index	12.13±0.20	11.82±0.28	11.00±0.69	11.87±0.24
Body mass index	29.83±2.49	24.80±3.52	18.29±8.66	30.98±3.05

Note. Designation of the compared groups: a — Romanov breed; b — hybrids 7/16 ROM 9/16 ARG; c — hybrids 1/2 ROM 1/2 ARG; d — hybrids 3/8 ROM 5/8 ARG.
* Differences between the compared groups are statistically significant at $p < 0.05$.

It should also be noted that hybrid animals, in comparison with purebred individuals, were characterized by a higher leg length index, which is typical for argali. The advantage of F_1, F_3, and F_4 hybrids over purebred animals at the age of 3 months for this indicator reached 6, 12 ($p < 0.05$), and 3%, respectively.

A hierarchical classification was carried out on the basis of the conformation characteristics of purebred Romanov breed sheep and their interspecific hybrids with argali of different generations. It was found that interspecific hybrids with 1/2 and 5/8 argali bloodlines were grouped into one cluster. Hybrids with 9/16 bloodiness and purebred animals of the Romanov breed formed separate clusters (Fig. 2).

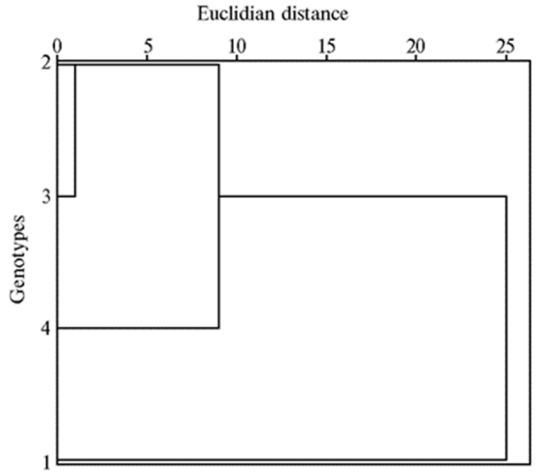

Fig. 2. Hierarchical analysis of interspecific hybrids of different generations from the mating of argali (*Ovis ammon*) (ARG) with sheep of the Romanov breed (*O. aries*) (ROM) and of the Romanov lambs: 1 — ROM, 2 — $^{3}/_{8}$ ROM $^{5}/_{8}$ ARG, 3 — $^{1}/_{2}$ ROM $^{1}/_{2}$ ARG, 4 — $^{7}/_{16}$ ROM $^{9}/_{16}$ ARG (Ernst Federal Science Center for Animal Husbandry, 2019-2020).

The research shows that the genotype is one of the main biotic factors affecting the conformation of sheep. The effect of this factor on body weight and linear measurements is statistically significant at $p < 0.001$. Particular attention is paid to morphometric indicators when studying the phenotype of animals, which is a combination of genotype-specific properties inherent in an individual at a certain stage of development. Morphological differences based on the general body type, morphometric parameters, or unusual anatomical forms are used to identify, compare, and classify species and groups, identify unidentified taxa, unknown hybrids, and identify mutations that lead to changes [28]. A morphological description is the most important component of the characteristics of the breed and breeding form [29] and can be used for their identification and classification [30].

For a long time, the main feature of the classification of breeds of farm animals, especially sheep, was the color of wool [31]. Eastham et al. [32] used morphometric indicators in their studies to identify several species of falcons and their hybrids. Hybrids and original species were grouped into separate clusters. In this study, the hybrid animals were also distinguished by their physique indices into separate clusters, the distances between which were determined by the genotype of the hybrids.

Our findings correspond to suggestions of using exterior indicators to identify breeds and genotypes during breeding [6-8].

Thus, the interspecific hybrids of the argali with the sheep of the Romanov breed inherited some of the conformational features of the argali in terms of conformation and differ from the original parent breed. Compared to purebred animals, they are characterized by a more compact constitution, have a more compressed rectangular body shape. This is expressed in a decrease in hybrid animals in comparison with purebred peers in the indices of the oblique body length and the elongation index, depending on bloodiness, by 14-20 and 18-22%, respectively ($p < 0.05$). Our findings confirm that exterior indicators can be used for preliminary identification of hybrid individuals, reducing in some cases the cost of expensive genomic studies.

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AGRICULTURAL BIOLOGY,
2020, V. 55, № 6, pp. 1148-1158
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 636.5:591.16:611.013.11:57.04

doi: 10.15389/agrobiology.2020.6.1148eng
doi: 10.15389/agrobiology.2020.6.1148rus

EFFICIENCY OF USING A COMBINATION OF MONO- AND DISACCHARIDES IN A DILUENT FOR FREEZING ROOSTER SEMEN

Yu.I. SILYUKOVA ✉, O.I. STANISHEVSKAYA, N.V. PLESHANOV, A.A. KUROCHKIN

All-Russian Research Institute for Farm Animal Genetics and Breeding — Branch of Ernst Federal Science Center for Animal Husbandry, 55A, Moskovskoe sh., pos. Tyarlevo, St. Petersburg—Pushkin, 196625 Russia, e-mail svadim33@mail.ru (✉ corresponding author), olgastan@list.ru, Klaus-90@list.ru, kurochkin.anton66@gmail.com
ORCID:
Silyukova Yu.L. orcid.org/0000-0003-1905-6373
Stanishevskaya O.I. orcid.org/0000-0001-9504-3916
Pleshanov N.V. orcid.org/0000-0002-4634-7515
Kurochkin A.A. orcid.org/0000-0003-3171-8499
The authors declare no conflict of interests
Acknowledgements:
Research conducted on the topic AAAA-A18-118021590132-9.
Received September 10, 2020

Abstract

Different combination of saccharides can provide better semen protection during freezing/thawing cycle. Until now, the disaccharide maltose has not been used as a component of the medium for cryopreservation roosters' semen. Since maltose is not involved in carbohydrate metabolism of spermatozoa, there is an assumption about its role in strengthening the structure of the glycocalyx, which is a progressive evolutionary cellular structure that regulates specific cellular adaptations to a certain temperature, chemical and other paratypical effects. In this work, in order to increase the fertility of frozen/thawed semen, we tested a combination of saccharides in the diluent for cryopreservation of roosters' semen. For the first time, maltose has been proven to be effective in combination with fructose in a diluent to increase the fertility of frozen/thawed roosters' semen. The aim of the study was to determine the optimal concentration of the test component of maltose based on the diluent of the Leningrad Cryoprotective Medium (LCM) (1984) for freezing the semen of roosters and determining the time of maintaining the functional usefulness of frozen/thawed cock semen in the genital tract of the hen. The experiment was carried out in the Center for Collective Use Genetic collection of rare and endangered chicken breeds (the Russian Research Institute of Farm Animal Genetics and Breeding, 2020) on a breed of Russian white chickens (♂n = 10, ♀n = 30) at the age of 46-50 weeks. Three variants of media for cryopreservation of roosters' semen with different ratios of saccharides were evaluated. In each group, the Mal-10 (fructose 0.72%, maltose 0.166%), Mal-20 (fructose 0.64%, maltose 0.326%) and LCM-control (fructose 0.8%, maltose 0%), there were 10 hens for insemination. The results show not only an increase in the total percentage of fertilized eggs when using frozen/thawed semen in the Mal-10 (92.6%) and Mal-20 (86.3%) groups compared to the LCM-control group (74.7%), but also an increase in the duration of the functional usefulness of spermatozoa in the genital tract of hen within 5 days at the level of native sperm. Counting the points of interaction of spermatozoa with the perivitelline membrane of the yolk 5 days after the last insemination showed that the functional ability of spermatozoa is much higher when using experimental media containing maltose, 67.0 pcs/cm^2 for Mal-10 and 110.7 pcs/cm^2 for Mal-20 vs. 40.1 pcs/cm^2 in maltose-free LCM-control. The longest duration of the functional usefulness of spermatozoa was noted in the Mal-20 group. Even on day 15 after the last insemination, the fertilization capacity of frozen/thawed semen was recorded at the level of 20 %. A regression equation was drawn up for the relationship of egg fertilization with the points of interaction of spermatozoa with the perivitelline membrane of the yolk. To obtain an egg fertilization level of ≥ 80%, the functional usefulness of frozen-thawed spermatozoa (expressed through the number of points of interaction with the perivitelline membrane of the yolk) should be ≥ 60 pcs/cm^2. This was achieved by using an experimental diluent with 0.326% maltose. The results obtained open up the possibility of using cryopreserved semen not only in preserving the gene pool, but also in breeding programs.

Keywords: roosters, semen, fertility, cryopreservation, media, maltose, perivitelline membrane

Cryopreservation of avian semen is an important and widely used method of long-term storage of male reproductive cells, but its effectiveness in poultry

farming is not as high as in dairy cattle breeding, where it is used for genetic improvement of breeds. The search for new concepts and protocols in the cryopreservation of rooster semen will make it possible to create cryobanks of genetic material not only as a repository of samples of rare and endangered breeds but also as working reserve and insurance funds of reproductive cells [1-3]. Cryopreservation causes adverse changes in sperm cells, which leads to damage and total cell death and reduces the quality of sperm. Thus, there is partially irreversible damage to the morphological structures of the cell, disruption of the structure of organelles, changes in biochemical processes and the supramembrane structure — a dense carbohydrate layer (glycocalyx) [4]. All the listed changes in the sperm cell and its epigenetic modifications associated with DNA methylation, as well as protamine defect and disruption of cellular parameters, including membrane integrity, DNA stability, and mitochondrial activity, become the reasons for a decrease in sperm motility and fertility during freezing/thawing [5, 6].

The retention of the functional capacity of avian sperm after thawing depends on species, breed characteristics, the composition of the freezing medium, including cryoprotectants, sperm balancing procedures used, and freezing and thawing protocols. In addition, the starting quality of fresh sperm is important for successful cryopreservation of sperm [7].

To obtain good results when using frozen-thawed rooster semen, damage caused by water crystallization during freezing must be prevented. The formation of intracellular ice crystals, especially the three-vector form of crystallization [8, 9], is one of the main causes of cell death. In addition, researchers are looking for ways to increase the fertility of cryopreserved semen not only through freezing and thawing (fast/slow) protocols [10] but also by selecting the optimal cryopreservation media. Such media contain antioxidants that neutralize the effect of accumulated active oxygen on cells (l-carnitine, ellagic acid, cysteamine, ergothioneine, serine, catalase) [11-14], as well as energy components (saccharides) [15] that support the functionality of sperm cells, in particular due to the strengthening of the glycocalyx structure [4]. The presence of components that perform a dehydration function (saccharides, hyaluronic acid) allows the intracellular osmotic pressure to be optimized [16-18]. The protocol for cryopreservation of reproductive cells implies the mandatory use of cryoprotective agents that have endo- and exocellular [19, 20] or combined effects. As a rule, such agents are used directly in the creation of cryoprotective media, as well as in combination with external cryogenic agents – glycerol, dimethyl sulfoxide, and dimethylacetamide [21, 22]. In most cases, when freezing, Lake (Lake, Stewart, 1978), Beltsville [13] and LCM (Russian Research Institute of Farm Animal Genetics and Breeding — VNIIGRZH, Inventor's Certificate No. 1130339 dated December 23, 1984, Russia) media are used to dilute native roosters' semen with the addition of the necessary components.

According to the literature, a different combination of non-reducing and reducing sugars can provide better protection of the semen during freezing/thawing [23]. The influence of a combination of monosaccharides (fructose, galactose, glucose, xylose) and disaccharides (lactose, trehalose, maltose, sucrose) in the composition of diluents for semen cryopreservation was evaluated on various animal species. At the same time, the effectiveness of maltose in the composition of a diluent for rooster semen has hardly been studied, although its potential positive effect has been shown in other animal species [24, 25].

Research on cryopreservation of rooster semen is mainly carried out on 28-32-week-old livestock [10, 17, 26] and much less often on older birds, which is probably associated with a decrease in reproductive qualities and the effectiveness of cryopreservation. However, due to the tendency to an increase in the

period of productive use of chickens up to 80 weeks of life, it becomes necessary to conduct experiments on roosters at the age of 46 weeks.

In this work, for the first time, the effectiveness of the use of maltose in combination with fructose in the composition of a diluent has been proven to increase the fertilizing ability of frozen-thawed rooster semen.

The aim of the work was to determine the optimal concentration of maltose in the composition of the diluent for freezing the semen of roosters aged 44-50 weeks and to establish the time frame for maintaining the functional usefulness of the frozen-thawed semen in the genital tract of the hen.

Methods. Experiments were carried out in 2020 on birds (*Gallus gallus domesticus*) of the Russian White breed (♂n = 10, ♀n = 30) aged 44-50 weeks, kept in individual cages in accordance with the technology adopted at the Center for Collective Use, Genetic collection of rare and endangered chicken breeds (VNIIGRZH) (2020). Sperm was collected by qualified specialists, the ingress of contamination into the ejaculates was excluded or minimized. Sperm was obtained by the method of abdominal massage [27] twice a week (glass vials with a volume of 10 ml were used) for 4 weeks. For an individual assessment of the quality of each ejaculate, a CASA imaging system (Computer-Assisted Semen Analysis; Argus-CASA software, ArgusSoft LLC, Russia; Motic® BA310E microscope, ×200, Motic Instruments Inc., Canada) was used. The ejaculate volume (ml), sperm concentration (billion/ml) (Accuread Photometer, IMV Technologies, UK), their total and progressive motility (%), and the degree of agglutination (%) were determined. The evaluation was carried out in 5 replicates. To eliminate individual differences between individuals, semen samples after each assessment were pooled and divided into three aliquots according to the developed experimental design. The study was conducted in accordance with the principles of bioethics in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental or Other Scientific Purposes (ETS 123, 1986, Article 5, Chapter 2).

The composition of the LCM-control medium (Leningrad cryoprotective medium) for freezing sperm [28] was as follows (per 100 ml of distilled water): sodium glutamate (1.92 g), fructose (0.8 g), potassium acetate (0.5 g), polyvinylpyrrolidone (0.3 g), protamine sulfate (0.032 g). For experimental diluents Mal-10 and Mal-20, the composition was calculated with partial replacement of fructose by maltose (0.72 g fructose + 0.166 g maltose and 0.64 g fructose + 0.326 g maltose, respectively), the rest of the components remained unchanged.

The diluted semen samples were cooled from 18 to 5 °C for 40 minutes. Then, dimethyl-acetamide (DMA) (Sigma-Aldrich, USA) was added to each sample to a final concentration of 6%. After the addition of DMA, the samples were incubated at 5 °C for 1 min. The pellets were frozen by dropping the seed into liquid nitrogen. The initial position of the pipette with the semen was controlled by a hand-held digital temperature indicator with a sensor (AHLBORN® THERM 24201L, Ahlborn Mess- und Regelungstechnik GmbH, Germany), in the area where the pipette was placed, the temperature was –15...–20 °C, –135 °C on the surface of liquid nitrogen. The average rate of semen supply to liquid nitrogen was ~ 1.4 drops per second. The frozen semen was stored in liquid nitrogen in Dewar flasks for 30 days. The granules were thawed according to the fast protocol on a heated metal plate at 60 °C (equipment developed by VNIIGRZH, 1989).

Locomotor activity (total and progressive motility) of frozen-thawed spermatozoa was recorded in each extender using a CASA imaging system (Motic® BA310E, ×200). Each sample was evaluated twice.

Virgin hens aged 46-50 weeks were used for artificial insemination (in total n = 30, n = 10 in each experimental group). Hens were inseminated intravaginally

with single daily doses of 0.04-0.07 ml of frozen-thawed semen (at least 70-80 million progressively moving active spermatozoa) [29]: during the first 2 days, one insemination, then one insemination every 2 days. Insemination time — after 14^{00}, in total, five inseminations were performved. Eggs for incubation were collected daily for 9 days, starting from the second day after the first insemination. Eggs (n = 239) were incubated for 6 days to assess fertility.

The eggs collected in each experimental group on days 5, 10, and 15 after the last insemination were broken and the fertilization was assessed by the state of the blastodisc.

The dynamics of the functional activity of frozen-thawed spermatozoa in the genital tract of hens on days 5, 10, and 15 from the last insemination was determined by the method of Bakst et al. [30] by the number of holes in the vitelline membrane of the yolk (sample size n = 55) formed as a result of interaction with spermatozoa. The yolks were carefully separated from the egg white. A filter paper ring was placed on the yolk in the blastodisc region, then the membrane was cut out with curved medical scissors with a sharp end along the outer diameter of the ring. The separated membrane was grasped with tweezers and washed several times with 0.9% NaCl solution at 4-5 °C until the yolk was completely removed, then carefully placed on a glass slide. For better visualization, the preparation was stained according to the following protocol. On the vitelline membrane with a micropipette, 30 μL of an alcoholic 10% formalin solution was evenly applied. After 15-20 s, formalin was decanted and placed on the membrane with ~ 30-40 μl of Schiff's reagent using a micropipette with a tip wrapped in aluminum foil (to minimize light exposure to Schiff's reagent). After the vitelline membrane acquired a purple hue (~ 30 s), the excess of Schiff's reagent was washed with distilled water, the preparations were dried in air for 5 min before microscopy. The area inside the filter paper ring (S = 1 cm^2) was determined using an Axio Imager microscope (Carl Zeiss Microscopy GmbH, Germany) in a dark field at ×200 magnification; the number of holes in the vitelline membrane was counted.

For statistical data processing and regression analysis, the Microsoft Excel 2013 and Statistica 7.0 software applications (StatSoft, Inc., USA) were used. Data were presented as means (M) and standard errors of means (±SEM). The samples according to the assessment of the native semen corresponded to the normal Gaussian distribution with the parameters $\chi^2_{theoretic} = 12.6 > \chi^2_{empiric} = 6.5$ in terms of assessing sperm volume and sperm motility. The differences between the samples were assessed by Student's t-test and were considered significant at $p < 0.05$.

Results. The total motility of freshly obtained semen averaged from 79.43 to 83.93%, the proportion of spermatozoa with rectilinear translational movement was from 63.22 to 70.90% (Table 1). The volume of individual ejaculates on different days varied from 0.3 ml to 1.1 ml; the concentration of spermatozoa was at least 3.2 billion/ml, agglutination did not exceed 10%.

1. Native semen quality indicators in Russian White roosters (*Gallus gallus domesticus*) (n = 50, M±SEM, the Center for Collective Use, Genetic collection of rare and endangered chicken breeds, 2020)

Rooster No.	Ejaculate volume, ml	Total motility, %	Progressive motility, %
1	0.54±0.03	82.88±2.50	70.07±3.52
2	0.36±0.06	79.43±5.30	64.37±6.21
3	0.05±0.05	83.93±1.27	70.12±1.82
4	0.45±0.07	83.93±1.05	70.90±0.54
5	0.60±0.04	78.25±5.39	63.26±5.78
6	0.46±0.02	81.35±2.41	67.49±2.10
7	0.73±0.07	81.15±1.17	66.22±1.23
8	0.54±0.04	82.85±2.44	67.34±1.02
9	0.36±0.04	81.45±1.48	63.95±2.49
10	0.39±0.07	82.83±2.18	67.19±3.01

2. Quality indicators (min-max according to the dates of the experiment) **of frozen-thawed semen of Russian White roosters (*Gallus gallus domesticus*) and egg fertilization in the first 9 days of collection, depending on the composition of the medium for cryopreservation** (♂n = 10, ♀n = 30, the Center for Collective Use, Genetic collection of rare and endangered chicken breeds, 2020)

Diluent	Total motility, %	Progressive motility, %	Egg number	Egg fertilization, %
LCM-control	51-54	45-50	83	74,7
Mal-10	51-54	45-50	81	92,6
Mal-20	51-54	45-50	75	86,3

N o t e. For the composition of media for freezing semen, see the Methods section.

Differences in the functional state of frozen-thawed rooster semen between the control and experiment were assessed by artificial insemination of chickens with subsequent incubation of eggs. The fertility rates of eggs (Table 2) differed depending on the composition of the diluent for semen cryopreservation. In the Mal-10 and Mal-20 groups, when maltose was used as a component of the medium for cryopreservation, a significant (by 11.6-17.9%) increase in egg fertilization was noted as compared to the control ($p < 0.05$). However, it should be noted that when studying frozen-thawed semen by optical microscopy, no differences were revealed between the control and experimental groups in terms of motility and the proportion of spermatozoa with rectilinear translational movement.

The highest percentage of egg fertilization on the 5th day of insemination was observed in the Mal-20 group (100% versus 50% in the LCM-control group), on the 10th day in the Mal-10 and Mal-20 groups, the fertilization was 60 and 100%, respectively, against 40% in control. On the 15th day from the last insemination in the LCM-control and Mal-10 groups, no fertilized eggs were obtained, however, in the Mal-20 group, their share was 20% (Fig. 1).

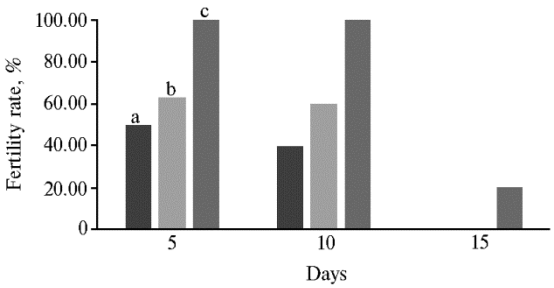

Fig. 1. Fertilization of eggs of Russian White hens (*Gallus gallus domesticus*) on days 5, 10, and 15 from the last insemination, depending on the composition of the medium for semen cryopreservation: a — LCM-control, b — Mal-10, c — Mal-20; for the composition of media for freezing semen, see the Methods section (n = 55, the Center for Collective Use, Genetic collection of rare and endangered chicken breeds, 2020).

Determination of the number of points of interaction of spermatozoa with the vitelline membrane of the yolk (Fig. 2) showed that the functional capacity of spermatozoa was significantly higher when using experimental media containing maltose.

Semen diluted with Mal-20 medium differed from other samples in increased viability in the genital tract of the hen: even on the 15th day after the last insemination, the number of points of interaction of spermatozoa with the vitelline membrane in the Mal-20 group was 45.8% higher than in control (Table 3).

To reveal the relationship between the number of points of interaction of spermatozoa with the vitelline membrane of the yolk and the fertilization of eggs, a regression equation was drawn up and the approximation coefficient was determined (Fig. 3). The result obtained in the Mal-20 group was confirmed by a positive linear relationship (the reliability of the regression equation was determined by the coefficient of determination $R^2 = 0.88$) of two signs on the interval of variability of the variable Y, where the variable X took values from 10 to 80. On the part of the curve where X took values > 80, the value of the variable Y was constant.

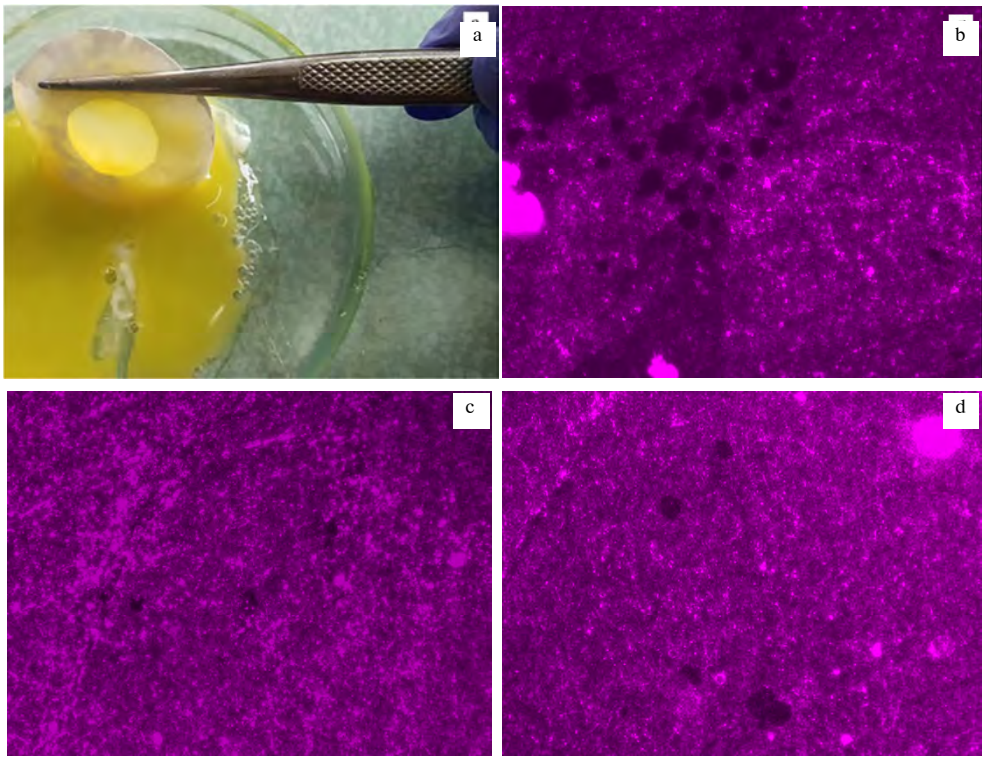

Fig. 2. Holes (dark points) **formed during the interaction of frozen-thawed spermatozoa of Russian White roosters (*Gallus gallus domesticus*) with the yolk vitelline membrane, depending on the composition of the medium for semen cryopreservation:** a — separation of the vitelline membrane; b — Mal-20 medium, c — Mal-10 medium, d — LCM-control (Axio Imager microscope, Carl Zeiss Microscopy GmbH, Germany, magnification ×200; staining with Schiff's reactive); for the composition of media for freezing semen, see the Methods section (the Center for Collective Use, Genetic collection of rare and endangered chicken breeds, 2020).

3. Interactions (number per cm^2) **of frozen-thawed spermatozoa of Russian White roosters (*Gallus gallus domesticus*) with the yolk vitelline membrane, depending on the composition of the medium for semen cryopreservation** ($n = 55$, $M \pm$SEM, the Center for Collective Use, Genetic collection of rare and endangered chicken breeds, 2020)

Diluent	Days from the last insemination		
	5	10	15
LCM-control	40.1±17.9[a]	36.4±15.2	26.6±6.1
Mal-10	67.0±5.5	67.0±18.7	27.8±5.8
Mal-20	110.7±15.8[b]	86.7±28.2	38.8±21.8

N o t e. For the composition of media for freezing semen, see the Methods section.
[a, b] Differences between the noted indicators are statically significant at $p < 0.05$.

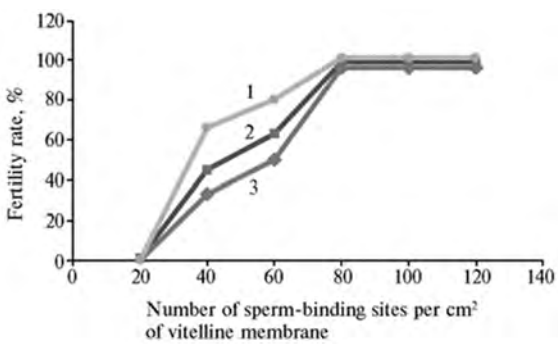

Fig. 3. Egg fertilization vs. the number of interaction points of frozen-thawed spermatozoa of roosters (*Gallus gallus domesticus*) with the yolk vitelline membrane, depending on the composition of the medium for semen cryopreservation: 1 — Mal-20 ($y = 1.585x - 17.5$, $R^2 = 0.8807$), 2 — Mal-10 ($y = 1.585x - 28.5$, $R^2 = 0.9913$), 3 — LCM-control ($y = 1.525x - 31.5$, $R^2 = 0{,}9701$); for the composition of media for freezing semen, see the Methods section (the Center for Collective Use, Genetic collection of rare and endangered chicken breeds, 2020).

Continuing the research of past years, we found that the decrease in the fertility of rooster semen in the freezing and thawing cycle was largely due to the high damage to the plasma membranes of spermatozoa [31]. Therefore, it is important not only to regulate the osmotic balance of the cell but also to strengthen the membrane itself by strengthening the structure of the glycocalyx. This problem can be solved due to the ability of disaccharides to attach to the heads of lipids and proteins on the outer surface of the cell membrane. Glycocalyx, reinforced with disaccharides dissolved in the medium for cryopreservation of rooster semen, stabilizes the sperm membranes and not only works as a cryo-resistant structure but also increases the period of preservation of the functional usefulness of spermatozoa in the genital tract of the hen. According to Tecle and Gagneux [32], the sperm glycocalyx mediates numerous functions of the female reproductive tract, including protection against innate and adaptive female immunity, and masks sperm proteins involved in fertilization. The glycocalyx of spermatozoa is modified during their movement in the genital tract of the hen and in crypts and represents the primary interface between the male gamete and the environment [33].

Saccharides (sucrose, lactose, trehalose, glucose, fructose) were used as energy sources and cryoprotectants as components of a semen diluent for cryopreservation of spermatozoa of various animals (dogs, Japanese black bears, goats, red jungle chicken) [34-37]. The study of the structure and properties of maltose disaccharide [38] gives grounds to predict its successful use in combination with a monosaccharide in the composition of a diluent for cryopreservation of rooster semen. The size of the maltose molecule does not allow crossing the cell membrane; in addition, this disaccharide has a low fermentation rate [39] and can enhance the “sugar coat” on the glycocalyx construct. An analysis of the available publications has shown that maltose has not yet been used as a component of a medium for cryopreservation of rooster semen. In cryopreservation of sturgeon semen, the use of maltose was equally successful with other disaccharides (lactose, trehalose, and lactulose) [40]. When added to a diluent for native rooster semen, maltose is slightly involved in the carbohydrate metabolism of spermatozoa [41]. Therefore, it can be assumed that it joins the glycocalyx, an evolutionarily progressive structure that provides the cell with the ability to specifically adapt to temperature, chemical, and other paratypical influences.

In our experiment, the task was not set to investigate the cellular mechanisms of the influence of the composition of experimental media on the viability of spermatozoa, but some hypotheses can be proposed. Maltose and fructose in the composition of Mal-10 and Mal-20 media in combination with DMA, in terms of their effect on the protective properties of the cell at low temperatures, represent a combination of three cryoprotectants of penetrating and non-penetrating action. DMA is a cryoprotectant of the amide group that promotes the formation of hydrogen bonds, which creates conditions for the formation of compounds that prevent the crystallization of water. Fructose, as an insignificant molecular unit, freely penetrates through the membrane into the cell plasma and functions both as an energy structure and as a component that reduces the osmotic load on the membrane during freezing/thawing. Maltose, according to our hypothesis, is fixed on the supra membrane shell (glycocalyx) of the cell, creating a stronger carbohydrate scaffold that protects the cell from cold shocks and prevents its destruction. This entire system of components allows minimizing the negative impact of the freezing and thawing processes on spermatozoa and maintaining their functional usefulness directly in the genital tract of the hen, since the integrity of the sperm glycocalyx becomes a critical factor when interacting with chicken gametes [4].

Thus, our experiments revealed an increase in the total fertility of chicken

eggs when inseminated with frozen-thawed semen, if maltose was used as a component of the medium for freezing rooster semen. With the addition of 0.166 and 0.326 g of maltose per 100 ml to the diluent, the fertilization of eggs was 92.6 and 86.3%, respectively, vs. 74.7% in the control. In addition, in the genital tract of the hen, spermatozoa retained their functional usefulness for 5 days, which is comparable to the indicators when using native semen for artificial insemination. Indicators of progressive motility, characterizing the integrity of the kinetic apparatus of spermatozoa, did not differ between the groups, but the functional usefulness of sperm, assessed by the number of interactions with the vitelline membrane, differed significantly. For acceptable fertilization of eggs (at least 80%), such functional usefulness of frozen-thawed spermatozoa is sufficient, at which the number of points of interaction with the vitelline membrane of the yolk is at least 60 pcs/cm^2. This was achieved using an experimental maltose diluent at a concentration of 0.326%. The results obtained open up prospects for the use of cryopreserved bird semen not only in programs for preserving the gene pool but also in breeding.

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AGRICULTURAL BIOLOGY,
2020, V. 55, № 6, pp. 1159-1170
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

Physiology, pathology

UDC 636.52/.58.087.3

doi: 10.15389/agrobiology.2020.6.1159eng
doi: 10.15389/agrobiology.2020.6.1159rus

THE PHYSIOLOGICAL ASPECTS OF THE SUPPLEMENTATION OF DIETS FOR BROILERS (*Gallus gallus* L.) WITH DIFFERENT VEGETABLE OILS

V.G. VERTIPRAKHOV ✉, I.A. EGOROV, E.N. ANDRIANOVA, A.A. GROZINA

Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, 10, ul. Ptitsegradskaya, Sergiev Posad, Moscow Province, 141311 Russia, e-mail Vertiprakhov63@mail.ru (✉ corresponding author), olga@vnitip.ru, andrianova@vnitip.ru, Alena_fisinina@mail.ru
ORCID:
Vertiprakhov V.G. orcid.org/0000-0002-3240-7636
Egorov I.A. orcid.org/0000-0001-9122-9553
Andrianova E.N. orcid.org/0000-0002-6769-6351
Grozina A.A. orcid.org/0000-0001-9654-7710
The authors declare no conflict of interests
Acknowledgements:
Supported financially from the Russian Science Foundation for the Project No. 16-16-04089-П "Study of physiological and microbiological aspects of digestion in meat chicken in embryonic and post-embryonic periods to develop diets which fully meet the genetic potential of poultry"
Received September 24, 2020

Abstract

The full-diet compound feeds with balanced contents of all limiting macro- and micronutrients are the essential key to the high productive performance in broilers (*Gallus gallus* L.). Fats are indispensable ingredients of animal diets necessary for energy supply and body structure, the source of essential polyunsaturated fatty acids (PUFAs), fat-soluble vitamins, and other bioactive compounds. This multi-functionality determines the physiological role of fats in nutrition. Vegetable oils (unlike animal one) contain a wide range of PUFAs playing an important biological role as a structural component of cell membranes. It is known that fatty acid profiles of individual vegetable oils do not fit the proportion of saturated, monounsaturated, and polyunsaturated fatty acids necessary for full support of the physiological requirements in human and animals. The optimization of the mixtures of different vegetable oils aimed at the improvement of fatty acid nutrition in human is at presented widely discussed; however, this aspect is often missed in the formulation of diets for poultry. In a previous study we presented the pioneer data on the correlation between the activities of the digestive enzymes in the intestine and blood in poultry was obtained. The aim of the study presented was the investigation of the effects of dietary lipid profile on the productive performance, digestibility of dietary nutrients, and biochemical blood indices in broilers. The trial was performed in 2019 in conditions of a vivarium on four treatments of broilers (cross Smena 8, 38 birds per treatment) from 1 to 35 days of age. The basal diets common for all treatments were supplemented with four different vegetable oils: sunflower oil (SFO, control treatment), soybean oil (SBO), flaxseed oil (FSO), and rapeseed oil (RSO) in doses 3.1 % of total diet from 1 to 21 days of age and 6.0 % from 22 to 35 days of age. The indices of the productive performance were recorded (live bodyweight weekly by individual weighing, mortality, average daily weight gains, feed consumption, feed conversion ratio FCR). At 30-35 days of age the balance trial was performed to determine the digestibility and retention rates of dietary nutrients; the biochemical blood indices and the activities of the digestive enzymes in pancreatic tissue were determined. The results evidenced that RSO significantly ($p < 0.05$) increased average live bodyweight at 14, 21, and 28 days of age in compare to control by 1.97; 10.51 and 2.85%, respectively; at 35 days of age this difference was 7.31 % while FCR was lower by 6.49 % in compare to control. RSO improved the digestibility of crude protein by 2.74 % and crude fat by 3.08 %; these improvements resulted in more intense growth in compare to control. It was found that dietary vegetable oils affected lipid profile and the activities of the digestive enzymes and alkaline phosphatase in blood serum thus indicating the modulation of lipid metabolism; the effects were specific and related to the fatty acid profiles of the oils.

Keywords: broiler chicks, sunflower oil, soybean oil, rapeseed oil, flaxseed oil, biochemical blood indices.

To ensure high productivity of broilers, full-diet compound feeds are required, balanced in all limiting nutrients. In poultry feeding, fats are one of the

important irreplaceable nutrients, energy and plastic material, a source of essential polyunsaturated acids, fat-soluble vitamins, and other biologically active compounds [1]. The physiological role of fats in nutrition is due to their diverse functions in the organism [2, 3]. Vegetable oils, unlike animal fats, contain a rich set of polyunsaturated fatty acids (PUFAs), the biological function of which is determined by their role as structural elements of biomembranes of cells. PUFAs are involved in the regulation of cell metabolism, normalization of blood pressure, and platelet aggregation [4]. They affect the metabolism of cholesterol, stimulating its oxidation and excretion from the body; have a normalizing effect on the walls of blood vessels; participate in the exchange of B vitamins; stimulate the defense mechanisms of an organism, increasing resistance to infectious diseases [4]. The cellular hormones prostaglandins are synthesized from PUFAs [4]. The biologically active components of vegetable oils, which ensure normal lipid metabolism, primarily include linoleic (ω-6) and linolenic (ω-3) PUFAs [5]. These fatty acids are not synthesized in the body of animals and humans, that is, they are irreplaceable (or essential) and must be supplied with food [6].

It is known that in vegetable oils, the fatty acid composition does not correspond to the ratio of saturated, unsaturated, and polyunsaturated fatty acids, which makes it possible to fully meet the physiological needs of animals and humans. It was found that rapeseed oil had a beneficial effect on the work of the heart in animals and humans [7]. Rapeseed oil (in comparison with other oils differing in the composition of unsaturated fatty acids) had a beneficial effect on high-density lipoprotein cholesterol, triglycerides, and blood pressure [8]. In sheep, the activity of trypsin and lipase in the biliary-pancreatic secretion increased with the addition of rapeseed and flaxseed oil to the diet, which indicates their positive effect on the digestion processes in ruminants [9]. When studying the effect of various vegetable oils (palm, rapeseed, sunflower, and flaxseed oils) on adult rats, the data were obtained that the composition of fatty acids could affect the rate of feed digestion, and then the lipid profile of serum [10]. This once again confirms that the lipid composition can modulate the state of digestion and absorption in the conditions of the gastrointestinal tract.

The issues regarding optimization of the formulation of vegetable oils (including the use of additives or based on a combination of different types of oil) in order to improve their physiological properties are widely discussed to ensure human nutrition, but when developing rations for feeding poultry, this factor is usually not taken into account. Knowing the mechanism of action of different vegetable oils on the metabolism and productivity of broiler chicks is necessary to improve rations for a more complete realization of the genetic potential of poultry productivity. Earlier, a comparative assessment of compound feed for broilers using unrefined sunflower, soybean, flaxseed, and rapeseed oils was not conducted.

In the study, for the first time, the authors revealed the effect of vegetable oils in the composition of feed for broiler chicks on the lipid profile of the blood, as well as the activity of digestive enzymes and alkaline phosphatase in the blood.

The aim of the work was to assess the impact of different lipid components of the diet on productivity, biochemical parameters of the blood of broiler chicks, and feed digestibility.

Methods. The experiments were conducted in the vivarium (Selection and Genetic Centre Zagorskoe, Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, Moscow Province, 2019) on four groups of broiler chicks (*Gallus gallus* L.) of the Smena 8 cross from 1 to 35 days of age. The groups (n = 38 in each) were formed by the analog method, in each group, the chicks received the main ration (nutritionally balanced compound feed

according to the standards of the All-Russian Scientific Research and Technological Poultry Institute — ARRTPI), supplemented with edible unrefined oils. i.e., sunflower, soybean, flaxseed, or rapeseed oil in groups I (control), II, III, and IV, respectively. The nutritional value of broiler rations by rearing periods during the reference period corresponded to the standards of the FSC ARRTPI RAS (Guidelines for optimizing compound feed formulas for agricultural poultry. Sergiev Posad, 2014), with regard to actual nutritional values of raw materials, determined in the Testing Center of FSC ARRTPI RAS in accordance with common methods.

The nutritional value of compound feeds, planting rates, light, temperature and humidity conditions, the feeding and drinking area throughout the entire experiment corresponded to the recommendations of the FSC ARRTPI RAS (Guidelines for optimizing compound feed formulas for agricultural poultry. Sergiev Posad, 2014). The broilers were kept in cages without separation by gender in compliance with the standards for stocking density, feeding and drinking area, duration and intensity of lighting. The poultry was fed ad libitum with dry compound feed, the feed was distributed by hand.

During the experiment, the main zootechnical indicators were taken into account: live weight of poultry at the age of 7, 14, 21, 28, and 35 days (individual weighing), livestock preservation, average daily live weight gain, consumption and costs of feed per 1 kg of live weight gain (Methodological guidelines for feeding agricultural poultry. Sergiev Posad, 2015). Physiological experiments to determine the digestibility and use of nutrients from the compound feed were conducted on poultry aged 30-35 days.

Blood samples were taken from the axillary vein prior to feeding. A freshly prepared sodium citrate solution was added to the test tubes, and the blood was centrifuged at 5000 rpm for 3 min. Biochemical blood analysis was performed on a Sinnowa BS-3000P flow-through semi-automatic analyzer (SINNOWA Medical Science & Technology Co., Ltd., China) using biochemical kits (DIAKON-VET, Russia). Blood plasma was tested for lipase activity on a Chem well 2900 (T) device (Awareness Technology, USA) with the required set of reagents (Human GmbH, Germany). Trypsin activity was assessed using a Sinnowa BS-3000P semi-automatic biochemical analyzer [11].

In the homogenate of the pancreas, the activity of amylase was measured by Smith and Roe procedure in the modification to determine the high activity of the enzyme [12], proteases — by hydrolysis of casein purified according to Hammerstein, with photometric control on KFK-3 (Zagorsk Optical-Mechanical plant, Russia) (wavelength 450 nm) [12], and lipases — using a semi-automatic biochemical analyzer SINNOWA BS-3000P with a kit of veterinary diagnostic reagents for determining the activity of lipase in the blood of animals (DIAKON-VET, Russia).

The obtained data were processed by the method of variation statistics. The results in the tables are presented as $M \pm SEM$, where M is the arithmetic mean, $\pm SEM$ is the standard error of mean. The significance of the differences was assessed by the Student's t-test at $p < 0.05$.

Results. The formulas for experimental compound feed for broilers are shown in Table 1.

The fatty acid composition and qualitative indicators of the used unrefined vegetable oils are presented in Table 2. Analysis of the composition of the used vegetable oils showed a relatively low content of saturated fatty acids, in particular, palmitic and stearic acids (see Table 2). The share of these two acids on average ranges from 2.95% in rapeseed oil to 14.84% in soybean oil.

1. Composition and nutritional value of experimental compound feeds for cross Smena 8 broiler chicks (*Gallus gallus* L.) of different ages (the vivarium of Selection and Genetic Centre Zagorskoe, Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, Moscow Province, 2019)

Ingredient, %, indicator	Chicks aged 1-21 days				Chicks aged 22-35 days			
	group				group			
	I (c)	II	III	IV	I (c)	II	III	IV
Composition								
Wheat	20.85	20.85	20.85	20.85	19.97	19.97	19.97	19.97
Corn	35.50	35.50	35.50	35.50	36.20	36.20	36.20	36.20
Soybean meal	26.00	26.00	26.00	26.00	25.00	25.00	25.00	25.00
Sunflower meal	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00
Fish flour	6.50	6.50	6.50	6.50	4.00	4.00	4.00	4.00
Sunflower oil	3.10	0	0	0	6.00	0	0	0
Soybean oil	0	3.10	0	0	0	6.00	0	0
Flaxseed oil	0	0	3.10	0	0	0	6.00	0
Rapeseed oil	0	0	0	3.10	0	0	0	6.00
Lysine monochlorohydrate	0.18	0.18	0.18	0.18	0.22	0.22	0.22	0.22
DL-methionine	0.24	0.24	0.24	0.24	0.23	0.23	0.23	0.23
Table salt	0.22	0.22	0.22	0.22	0.27	0.27	0.27	0.27
Monocalcium phosphate	0.30	0.30	0.30	0.30	0.60	0.60	0.60	0.60
Limestone flour	0.90	0.90	0.90	0.9	1.30	1.30	1.30	1.30
Premix	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21
Nutritional value								
Exchange energy:								
kcal/100 g of feed	304	305	304	304	320	320	319	319
MJ/kg	12.74	12.78	12.74	12.74	13.41	13.41	13.37	13.37
Crude protein	22.65	22.65	22.65	22.65	20.74	20.74	20.74	20.74
Crude fat	5.80	5.81	5.80	5.80	8.49	8.50	8.49	8.48
Linoleic acid	2.91	2.62	0.59	0.93	4.60	4.05	0.91	1.38
Crude fiber	4.30	4.30	4.30	4.30	4.21	4.21	4.21	4.21
Lysine (total)	1.36	1.36	1.36	1.36	1.25	1.25	1.25	1.25
Lysine (assimilable)	1.19	1.19	1.19	1.19	1.09	1.09	1.09	1.09
Methionine (total)	0.64	0.64	0.64	0.64	0.58	0.58	0.58	0.58
Methionine (assimilable)	0.58	0.58	0.58	0.58	0.53	0.53	0.53	0.53
Methionine + cystine (total)	0.98	0.98	0.98	0.98	0.90	0.90	0.90	0.90
Methionine + cystine (assimilable)	0.95	0.95	0.95	0.95	0.84	0.84	0.84	0.84
Threonine (total)	0.83	0.83	0.83	0.83	0.76	0.76	0.76	0.76
Threonine (assimilable)	0.70	0.70	0.70	0.70	0.64	0.64	0.64	0.64
Tryptophan (total)	0.27	0.27	0.27	0.27	0.25	0.25	0.25	0.25
Tryptophan (assimilable)	0.23	0.23	0.23	0.23	0.21	0.21	0.21	0.21
Calcium	0.82	0.82	0.82	0.82	0.90	0.90	0.90	0.90
Phosphorus (total)	0.64	0.64	0.64	0.64	0.63	0.63	0.63	0.63
Phosphorus (assimilable)	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41
Sodium	0.18	0.18	0.18	0.18	0.17	0.17	0.17	0.17
Chlorine	0.25	0.25	0.25	0.25	0.27	0.27	0.27	0.27

Note. Group I — control (c), sunflower oil, II — soybean oil, III — flaxseed oil, IV — rapeseed oil in the ration.

2. Basic fatty acids and qualitative indicators of vegetable oils used in experimental compound feeds for cross Smena 8 broiler chicks (*Gallus gallus* L.) (the vivarium of Selection and Genetic Centre Zagorskoe, Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, Moscow Province, 2019)

Fatty acid, %, indicator	Oil			
	sunflower	soybean	flaxseed	rapeseed
Saturated acids	13.25	15.50	12.28	4.58
including:				
myristic	0.08	0.14	–	0.17
pentadecanoic	0.31	0.40	0.27	0.34
palmitic	8.62	10.80	5.81	2.04
stearic	4.14	4.04	6.10	0.91
arachidic	0.10	0.12	0.10	1.12
Monounsaturated acids	26.69	26.56	21.40	66.32
including:				
myristoleic	–	–	–	0.20
palmitoleic	–	–	–	0.40
oleic	26.69	26.56	21.40	65.72
erucic	–	–	–	0.23

Continued Table 2

Polyunsaturated acids	59.60	57.94	66.32	24.84
including:				
linoleic	57.58	51.53	11.69	17.60
linolenic	0.28	4.54	54.32	6.60
eicosadienoic	0.40	0.25	0.20	0.10
arachidonic	1.34	1.62	0.11	0.54
Unsaturated to saturated acids	6.51	5.45	7.14	19.90
The ratio of palmitic and oleic acids	0.32	0.41	0.27	0.03
Acid value, mg KOH/g	12.44	17.25	6.07	5.31
Peroxide value J, %	0.17	0.22	0.15	0.14
Tocopherols, μg/g	750	627	620	1200

N o t e. The tests were performed in accordance with GOST 30418 (Vegetable oils. Method for determining fatty acid composition) at the Test Laboratory Center of All-Russian Scientific Research Institute of Poultry Processing Industry (Rzhavki, Moscow Province). Acid peak magnitude is indicated as a percentage of the total peak area of all fatty acids. Dashes indicate the absence of the corresponding fatty acid.

It should be noted that there are large differences in the content of polyunsaturated linoleic, linolenic acids, and monounsaturated oleic acid. Soybean and sunflower oils were 51.53 and 57.58% linoleic acid, respectively, while flaxseed and rapeseed oils were 11.69 and 17.60% linoleic acid. A high content of linolenic acid was characteristic of flaxseed oil, oleic acid — for rapeseed oil. By the amount of polyunsaturated essential linolenic acid, flaxseed oil exceeded soybean, sunflower, and rapeseed oils, respectively, by 49.78, 54.04, and 47.72%. In terms of linoleic acid content, rapeseed oil was inferior to soybean and sunflower oil (the levels were 33.93% and 39.98% lower), but exceeded flaxseed oil (the content was 5.91% higher). The tested batch of rapeseed oil practically did not contain erucic acid (0.23%).

The greatest excess of the amount of unsaturated fatty acids over the saturated ones was noted in rapeseed oil, the 19.90:1 vs. ratios below 8:1 in soybean, sunflower, and flaxseed oils (see Table 2). In rapeseed oil, the proportion between palmitic and oleic acids was the smallest, 0.03:1. In addition, in rapeseed oil, the acid and peroxide values were the smallest. In terms of the total number of tocopherols, rapeseed oil also exceeded soybean, sunflower, and flaxseed oils (by 91.4%, 60.0%, and 93.5%).

3. Productivity of cross Smena 8 broiler chicks (*Gallus gallus* L.) fed experimental compound feeds with different vegetable oils (M±SEM, (the vivarium of Selection and Genetic Centre Zagorskoe, Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, Moscow Province, 2019)

Indicator	Group (n = 35 in each)			
	I (c)	II	III	IV
Mortality rate, %	0	0	0	2,86
Live weight at different ages, g				
1 day	42.0±2.44	42.0±1.77	42.0±2.01	42.0±1.99
5 days	110.5±0.80	110.7±0.80	111.1±0.70	111.0±0.80
7 days	167.9±1.63	166.2±2.27	169.5±1.42	169.5±1.54
14 days	443.2±5.070	434.4±5.37	444.9±4.82	451.9±4.62
21 days	813.7±24.94	842.5±10.70	854.9±12.23	899.2±10.68*
28 days	1467.8±20.26	1453.5±17.52	1458.8±19.63	1509.6±22.44
35 days	2106.9±34.02	2169.6±32.48	2191.4±34.08	2235.5±38.78*
Average live weight on day 35	2110.79	2180.33	2222.56	2265.06
(difference with the control)		(+3.29 %)	(+5.3 %)	(+7.31 %)
including				
for male chickens	2247.47±36.40	2305.19±37.10*	2378.50±31.66	2432.36±46.67*
for female chickens	1974.11±34.48	2055.47±33.50	2066.67±30.18	2097.75±31.18*
Feed consumption:				
per chick, kg	3.663	3.669	3.623	3.636
per 1 kg weight gainkg, кг	1.773	1.724	1.686	1.658
difference with the control		–2.76 %	–4.91 %	–6.49 %
Daily weight gain, g	60.85	62.89	64.13	65.38

N o t e. Group I — control (c), sunflower oil, II — soybean oil, III — flaxseed oil, IV — rapeseed oil in the ration. The description of the rations is given in Table 1.

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

Thus, rapeseed oil differed from other vegetable oils used in the experiment by a low content of polyunsaturated and saturated acids and a high content of monounsaturated acids. It was found that the positive effect of the use of rapeseed oil in the diet is manifested in an increase in the content of unsaturated fatty acids in broiler meat [13]. This fatty acid composition of rapeseed oil, apparently, influenced the productivity of broiler chicks (Table 3).

The results of studying the effectiveness of the use of sunflower, soybean, flaxseed, and rapeseed oils in the rations of broilers (see Table 3) confirmed that the quality of experimental compound feeds with different sources of edible vegetable oils provided a favorable zootechnical background and, as a consequence, high productivity and safety of chicks during the entire accounting period. The average daily gain in live weight of 35-day-old broilers was in the range of 60.85-65.38 g with feed consumption per 1 kg of live weight gain 1.658-1.773 kg.

As is known, sunflower oil is mainly used in manufacturing compound feed [14, 15]. A compound feed with sunflower oil in our studies was fed to the chicks of the control group. In feed production, the practice of using soybean oil is widespread. Soybean oil surpasses sunflower oil in terms of metabolic energy content, serves as a good source of vitamin E, carotenoids, but contains less linoleic acid. Comparing the productivity of chicks from groups I (control) and II, it should be noted that in the initial period of rearing, chicks from group II were slightly inferior in terms of live weight to the control ones (by 2.6 and 0.97%, respectively, at 14 and 28 days of age) (the differences are insignificant). By the end of rearing, the average live weight of chicks in group II was 3.39% higher than the control, and the male chickens in terms of live weight significantly exceeded their counterparts from the control group (by 2.57%, $p < 0.05$). It was found that feed costs per 1 kg of live weight gain in chicks from group II decreased by 2.76% in comparison with the control. The obtained result confirms the justification of the widespread use of soybean oil in feed production, since it allows ensuring high productivity of poultry.

Unlike soybean and sunflower oil, the use of flaxseed and rapeseed oil in feed production is limited. This is due not only to the smaller volume of their industrial production but also to the possible presence of anti-nutritional factors. In the study, the authors explain the high growth rate of chicks that received flaxseed and rapeseed oil as part of compound feeds by the fact that freshly made high-quality edible oils were used in the experiments.

As it is seen from Table 3, 3.1% flaxseed oil in the composition of compound feeds for chicks from group III provided an increase in the live weight of individuals by 0.37 and 5.07% at 14 and 21 days of age as compared to the control. An increase in the dosage of flaxseed oil to 6.0% from 22 days of age did not negatively affect the growth rate of chicks: up to 28 days of age, their live weight gain did not differ from the control, and by the end of fattening it was 5.3% with a decrease in feed costs by 4.91% per 1 kg of live weight gain.

Rapeseed oil (group IV) contributed to a significant ($p < 0.05$) increase in this indicator at 14, 21, and 28 days of age by 1.97, 10.51, and 2.85%. By the end of rearing, a noticeable advantage of broilers from group IV in terms of average live weight in comparison with the control (a 7.31% excess) was manifested with an improvement in feed conversion (by 6.49%).

The data on the digestibility of nutrients by the groups are presented in Table 4. The obtained results indicate that the addition of soybean oil to feed increases the fat digestibility by 1.58% ($p < 0.05$), but the availability of methionine decreases by 2.8% ($p < 0.05$) when compared with sunflower oil. The use of flaxseed oil instead of sunflower oil increased the digestibility of feed protein by 2.4%

($p < 0.05$) and fat by 2.7% ($p < 0.05$). Replacing sunflower oil with rapeseed oil increased the digestibility of protein by 2.7% ($p < 0.05$), fat by 3.1% ($p < 0.05$), and the availability of lysine in the feed increased by 2.4% ($p < 0.05$).

4. Digestibility of experimental compound feeds and utilization of basic nutrients by the cross Smena 8 broiler chicks (*Gallus gallus* L.) (M±SEM, the vivarium of Selection and Genetic Centre Zagorskoe, Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, Moscow Province, 2019)

Indicator, %	Group (n = 35 in each)			
	I (c)	II	III	IV
Digestibility of				
dry matter	71.4±0.34	71.5±0.30	72.6±0.32	72.6±0.34
protein	90.4±0.42	90.6±0.40	92.7±0.37*	93.1±0.30*
fat	80.8±0.37	82.4±0.30*	83.5±0.32*	83.9±0.34*
fiber	28.5±0.27	29.4±0.22	28.6±0.25	28.8±0.27
Use of				
nitrogen	60.8±0.44	61.3±0.42	61.6±0.47	62.6±0.41
calcium	48.7±0.39	49.1±0.35	48.8±0.37	49.6±0.31
phosphorus	33.5±0.22	33.3±0.24	33.6±0.20	33.8±0.22
Availability of				
lysine	91.8±0.28	91.5±0.47	92.1±0.70	94.2±0.45*
methionine	93.9±0.20	91.1±0.49*	92.8±0.65	94.8±0.41

N o t e. Group I — control (c), sunflower oil, II — soybean oil, III — flaxseed oil, IV — rapeseed oil in the ration. The description of the rations is given in Table 1.
* Differences with the control are statistically significant at $p < 0.05$.

5. Blood biochemical parameters in 35-day-old cross Smena 8 broiler chicks (*Gallus gallus* L.) fed experimental compound feeds with different vegetable oils (n = 5, M±SEM, the vivarium of Selection and Genetic Centre Zagorskoe, Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, Moscow Province, 2019)

Parameter	Group			
	I (c)	II	III	IV
Trypsin activity, U/l	99±6.0	76±5.9*	72±4.1*	98±4.7
Lipase activity, U/l	7.4±0.13	8.5±0.31*	10.1±0.72*	8.7±0.23*
Triglycerides, mmol/l	1.1±0.01	0.9±0.02*	1.0±0.01*	0.8±0.02*
Cholesterol, mmol/l	2.6±0.11	2.6±0.18	2.3±0.09*	2.7±0.03
Total protein, g/l	30.0±0.9	32±0.5	31±0.9	33±0.2*
Alkaline phosphatase, u/l	2448±163.0	3265±502.0	2541±135.1	3309±248.1*
Phosphatase-protease index	24	43	35	34

N o t e. Group I — control (c), sunflower oil, II — soybean oil, III — flaxseed oil, IV — rapeseed oil in the ration. The description of the rations is given in Table 1.
* Differences with the control are statistically significant at $p < 0.05$.

Biochemical analysis (Table 5) revealed the unequal effect of the studied vegetable oils, differing in chemical composition, on the blood parameters of chicks. In terms of trypsin activity, broilers that received the addition of soybean and flaxseed oil lagged behind the control ones by 23.2 and 27.3%, respectively ($p < 0.05$), the increase in lipase activity upon replacing sunflower oil in groups II-IV was 14.9, 17.6, and 36.5% ($p < 0.05$), respectively, while the blood level of triglycerides decreased by 18.2% ($p < 0.05$) for soybean oil, by 27.3% ($p < 0.05$) for flaxseed oil, and by 9.1% ($p < 0.05$) for rapeseed oil. Flaxseed oil reduced blood cholesterol levels by 11.5% compared to the control ($p < 0.05$), rapeseed oil increased the activity of alkaline phosphatase by 35.2% ($p < 0.05$) and the amount of total protein by 10.0% ($p < 0.05$). The phosphatase-protease index (the ratio of the activity of alkaline phosphatase and trypsin) was optimal when sunflower oil was added to the feed and consistently increased for rapeseed, flaxseed, and soybean oil, which indicates intense metabolism in the liver.

The study of enzyme activity in the pancreatic tissue (Table 6) did not confirm the significance of differences between the groups due to the small sample size, but revealed a persistent tendency to an increase in lipase activity in broilers,

that received flaxseed and rapeseed oil in the rations. In group II, there was a decrease in enzymatic activity compared with control broilers, which may be associated with pancreatic hypertrophy when using soybean oil containing trypsin inhibitors. This is indicated by the mass of the pancreas (6.4% higher than the control value, $p > 0.05$).

6. Activity of pancreatic enzymes in the pancreas homogenates of cross Smena 8 broiler chicks (*Gallus gallus* L.) fed experimental compound feeds with different vegetable oils (n = 3, M±SEM, the vivarium of Selection and Genetic Centre Zagorskoe, Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, Moscow Province, 2019)

Parameter	Group			
	I (c)	II	III	IV
Pancreas weight, g	4.7±0.17	5.0±0.15	4.7±0.14	4.8±0.16
Amylase, mg/(g·min)	17600±150.0	16667±311.1*	17467±366.5	17533±283.4
Lipase, μmol/(l·min)	107940±4305.0	97116±7341.1	122740±5675.3	117584±8614.1
Proteases, mg/(g·min)	669±23.1	628±36.5	616±18.3	628±40.2

N o t e. Group I — control (c), sunflower oil, II — soybean oil, III — flaxseed oil, IV — rapeseed oil in the ration. The description of the rations is given in Table 1.
* Differences with the control are statistically significant at $p < 0.05$.

Thus, the biochemical parameters of the blood of broiler chicks reflect the state of metabolism when various vegetable oils are added to the feed. The most critical values are when using flaxseed and soybean oil. In this case, the phosphatase-protease index increases in broiler chicks. The reason for the low efficiency of fatty acid metabolism when using flaxseed oil, according to available reports [16], is an excess of linoleic acid, which distinguishes this oil from other oils in the experiment. There are data that flaxseed oil practically does not cause changes in adipose tissue, but promotes the accumulation of α-linolenic acid in the liver and blood of broilers [17]. It is known [18] that linoleic acid, coming mainly from plant sources, is used by various types of intestinal microbes to obtain conjugated linoleic acid, which has anti-inflammatory, antiadipogenic, antidiabetic, and anticarcinogenic properties. There is no consensus in the scientific literature on the ratio of ω-6:ω-3 fatty acids in oils. Thus, it is believed that for a healthy person the optimal ratio of ω-6 to ω-3 fatty acids in oils is 10:1 or 11:1 [19]. According to the results of the authors' experiment on poultry, the ratio of ω-6 and ω-3 fatty acids is 193:1 in sunflower oil, 11:1 in soybean oil, 5:1 in flaxseed oil, and 3:1 in rapeseed oil. The proportions in which these unsaturated acids enter the body with food significantly affect the further synthesized long-chain and more unsaturated fatty acid metabolites, which, under certain conditions, can cause an undesirable disruption of metabolic processes [20]. It is known that the addition of a mixture of soybean and flaxseed oils to broilers' diet has a positive effect on the content of ω-6 and ω-3 fatty acids in muscle fibers, improving the nutritional value of meat and having a beneficial effect on human health [21]. A mixture of palm and sunflower oil increases broiler carcass yield and reduces the content of muscle and abdominal fat [22]. The positive influence of the combination of vegetable oils on the quality of livestock products was noted. Thus, when a 3% mixture of vegetable oils (corn, palm, flaxseed, peanut and soybean) was added to the broilers' ration, an increase in the content of glucose, albumin, ω-6 and ω-3 fatty acids in the blood serum was noted, and also an increase in the color of muscle tissue in comparison with the option when only soybean oil was used [23]. At the same time, the age of poultry did not affect the absorption of fatty acids from the diet [24].

Rapeseed oil is rich in oleic acid, the content of which exceeds 50% [25]. There is evidence that the addition of peanut flour with a high content of oleic acid (10-12%) to the feed has a positive effect on the palatability of broiler meat

and reduces the cost of feed [26].

Our data are consistent with the studies of the effect of vegetable oils on the productivity and biochemical parameters of the blood of broiler chicks. In particular, various correlations were revealed between the composition of fatty acids in the feed and lipid profiles of blood serum (Pearson's *r* values are provided by the authors) [22]. It was established [27] that bile preparations can affect the lipid profile of the blood. This suggests that the composition of fatty acids can first affect the rate of food digestion and then the lipid profiles of blood serum. Therefore, the lipid composition of the diet can modulate digestion and absorption in the gastrointestinal tract. The data obtained by other authors give an idea of the presence of links between the lipid composition of vegetable oils and their functional differences [10, 23, 28, 29]. However, in our studies, it was first established that the activity of blood lipase with a change in the lipid component in the feed changes simultaneously with the activity of the enzyme in the pancreatic juice [30].

It is known that the addition of 3% rapeseed oil to the broiler diet increases the content of eicosapentaenoic acid and docosahexaenoic acid in phospholipids of the heart, which has a cardioprotective and antiarrhythmic effect on the heart muscle in animals and humans [7, 26]. With identical nutritional values, rapeseed oil significantly reduces the deposition of lipids in the liver, while soybean oil increases the amount of fat in the abdominal cavity [31].

It was found that the fatty acids prevailing in oil correlated with the parameters of meat in female chickens [32]; it was also suggested that the size of fat deposits could be changed depending on the fatty acid profile of the feed. In particular, a comparison of diets with the addition of beef fat, olive, sunflower, and flaxseed oils showed that when using supplements rich in PUFAs, broilers had fewer fat deposits than when enriching the diet with saturated or monounsaturated fatty acids [33]. On other species of farm animals, in particular, on sheep, it was shown that oil as a food additive could change the secretion of bile and pancreatic juice and the enzymatic activity of the pancreas, as well as affect the meat quality [9].

Thus, it should be noted that there is a relatively low content of saturated fatty acids (in particular, palmitic and stearic) in the vegetable oils, studied by the authors. There are large differences in the amount of linoleic, linolenic, and oleic fatty acids: a high content of linolenic acid is typical for flaxseed oil, oleic acid for rapeseed oil. The ratio of unsaturated and saturated fatty acids is also uneven: the highest is in rapeseed oil (20.8:1), the lowest is in soybean oil (5.4:1). It was established that soybean, flaxseed, and rapeseed oils in comparison with sunflower oil contributed to an increase in the live weight of chicks by 3.29, 5.3, and 7.31% with an improvement in feed conversion by 2.76, 4.91, and 6.49% due to improved metabolic processes, digestibility, and use of feed nutrients.

Thus, our experiments confirm that the effectiveness of vegetable oils depends on their fatty acid composition and the ability of digestive enzymes to adapt to the individual lipid components of the diet. This allows drawing the following conclusions. Compound feeds with the rapeseed oil containing 0.23% erucic acid contribute to an increase in the live weight of broiler chicks at 14, 21, and 28 days of age by 1.97, 10.51, and 2.85%, respectively. By the end of rearing, the advantage over the control in terms of average live weight in broilers from the group which received the rapeseed oil was 7.31% with an improvement in feed conversion by 6.49%. Rapeseed oil contributes to the high digestibility of protein and fat in feed (an increase in indicators by 2.74 and 3.08%, respectively) with better assimilation of lysine, which became the physiological basis for the intensive growth of broiler chicks of this group as compared to the control. The effect of dietary vegetable oils on the blood lipid profile, the activity of digestive enzymes and alkaline

phosphatase we revealed is an evidence of the modulation of metabolic processes when replacing the lipid component of the feed. The observed changes are specific for the type of oil and are determined by its fatty acid composition.

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AGRICULTURAL BIOLOGY,
2020, V. 55, № 6, pp. 1171-1181
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 615.9:546.77]:57.084.1

doi: 10.15389/agrobiology.2020.6.1171eng
doi: 10.15389/agrobiology.2020.6.1171rus

TOXIC EFFECTS OF ULTRA-DISPERSED FORMS OF METALS (Mo AND MoO_3) IN THE EXPERIMENT in vivo

E.V. SHEIDA[1, 2] ✉, E.A. RUSAKOVA[1, 2], O.Yu. SIPAYLOVA[3], E.A. SIZOVA[1, 2], S.V. LEBEDEV[1, 2]

[1]*Orenburg State University*, 13, prosp. Pobedy, Orenburg, 460018 Russia, e-mail elena-shejjda@mail.ru (✉ corresponding author), elenka_rs@mail.ru, sizova.178@yandex.ru, Lsv@list.ru;
[2]*Federal Research Centre of Biological Systems and Agrotechnologies RAS*, 29, ul. 9 Yanvarya, Orenburg, 460000;
[3]*Orenburg State Medical University,* 6, ul. Sovetskaya, Orenburg, 460000 Russia, e-mail osipaylova@mail.ru
ORCID:
Sheida E.V. orcid.org/0000-0002-2586-613X
Rusakova E.A. orcid.org/0000-0002-1622-1284
Sipaylova O.Yu. orcid.org/0000-0003-0243-6249
Sizova E.A. orcid.org/0000-0002-5125-5981
Lebedev S.V. orcid.org/0000-0001-9485-7010
The authors declare no conflict of interests
Acknowledgements:
Supported financially from the Russian Science Foundation, grant No. 20-16-00078
Received July 15, 2020

Abstract

Despite the increasing use of nanoparticles (NPs) in industry, there is a serious lack of information regarding their impact on human health and the environment. Thus, nanomaterials based on molybdenum attract attention due to their ultra-high specific surface area and unique optical, electronic, catalytic and mechanical properties. However, having a high penetrating ability, molybdenum can accumulate in excess in organs and tissues of the body, affecting their structural integrity and functional activity. In the present work, the hepatotropic effect of Mo and MoO_3 nanoparticles was first established in experimental rats based on an assessment of the degree of activation of the apoptosis marker, a decrease in the level of motor activity and suppression of the emotional state of animals. A decrease in the body weight of rats and liver weight was recorded as a result of a single intraperitoneal injection of NPs while an increase in brain weight occurred. Our goal was to investigate general effects of Mo and MoO_3 nanoparticles on the growth and development of the internal organs of rats, the peculiarities of their motor and emotional activities, and the hepatotropic effect of the nanoparticles based on the assessment of the Caspase 3 (Cleaved) expression in the cytoplasm and nuclei of liver cells. Biomedical studies were carried out with 30 white Wistar male rats weighing 110-180 g. Mo and MoO_3 NPs were produced by plasma-chemical synthesis. The experimental animals were divided into five groups (n = 6 each). For NPs administration, the rats of groups I and II were intraperitoneally once-injected with Mo at 1.0 and 25.0 mg/kg, respectively, and animals of experimental groups III and IV were once-injected with MoO_3 at 1.2 and 29.0 mg/kg, respectively. Animals of the control group were injected with isotonic sodium chloride solution (0.9 % NaCl) in an equivalent volume. The growth of the experimental individuals was monitored daily by individual weighing. At the end of the experiment (day 14), the rats were decapitated under Nembutal anesthesia. Anatomical dissection and weighing of internal organs (liver and brain) were carried out. The absolute and average daily gains were calculated, as well as the weight ratio of the studied organs to the body. To reveal the readiness of liver cells for programmed cell death, expression of caspase 3 (Biocare Medical, LLC, USA) in the cytoplasm and nuclei of hepatocytes was detected immunohistochemically on the stained sections. The open field test was used to assess the emotional, motor activity and behavior of experimental animals. The emotional factor was assessed by the degree of anxiety and fear (the number of fecal boluses), as well as grooming (the number of brushing, washing, and other care elements). A system "Infrared actimeter" with "Panel with holes" (ACT-01, Orchid Scientific & Innovative India Pvt. Ltd., India) was use to assess spontaneous locomotor activity (LMA) of animals. It was shown that Mo and MoO_3 NPs have a toxic effect on the normal functioning of some body systems. In particular, the body weight of rats and the weight of their liver decrease while the weight of the brain increases. It was found out that the maximum decrease in body weight occurs in animals that received Mo at a dose of 25.0 mg/kg and MoO_3 at 1.2 mg/kg. Mo NPs in both low and high doses provoked a significant decrease in liver weight (by 14.3 and 16.1 %) ($p \le 0.05$), and MoO_3 NPs at 1 mg/kg caused a 33.5 % decrease. The injections of Mo NPs at 1.0 mg/kg and 25 mg/kg, and MoO_3 NPs at 1.2 mg/kg led to a significant ($p \le 0.05$) increase in brain weigh (by 10.9, 3.85, and 5.49 %, respectively). This increase is possibly due to edema of the organ, which affects behavioral reactions and motor

activities in rats, indicating the neurotoxic effect of Mo and MoO_3 NPs. Its severity directly depends on time of exposure and particle dosage. There was a decrease in LMA of rats on days 1 and 7 after Mo administration, and the higher the dosage, the lower the activity. The level of locomotor activity decreased to the lowest level on day 14 after administration of 29.0 mg/kg MoO_3 NPs. The level of emotional activity was lower for all applied dosages of Mo and MoO_3 NPs, and the effect was maximum on days 1 and 7. Evaluation of immunohistochemical expression of activated caspase 3 as a marker of apoptosis in the test with cleaved caspase 3 antibodies revealed an increase in endogenous levels of a larger fragment (p17) of the caspase 3 proenzyme in hepatocytes of male Wistar rats upon administration of Mo and MoO_3 NPs. This confirms the hepatotropic properties of the Mo and MoO_3 NPs. The detected caspase 3 activation depended not only on the dosage and time after injection of NPs, but also on the lesions caused by the NPs. More severe liver lesions occurred when caspase 3 activation was lower compared to control.

Keywords: nanoparticles, rats, caspase 3, apoptosis, internal organs, brain, behavior, locomotor activity, emotionality

Recently, the number of experimental studies to assess the toxic effect of ultrafine particles has lagged far behind the intensive development of nanotechnology. Despite the increasing use of nanoparticles (NPs) in industry, there is a serious lack of information regarding their impact on human health and the environment [1-3]. The study of the biological action and toxic effects of nanosized particles of various origins on the cells and tissues of the body is becoming increasingly important [4-6]. *In vivo* and *in vitro* studies have shown the development of allergic reactions in the offspring of mice intranasally insulated with inhaled titanium dioxide (TiO_2), the adverse effect of NPs on spermatogenesis and histopathological changes in the testes, as well as changes in gene expression in the brain of the offspring of mice after subcutaneous injection of TiO_2 NPs to maternal individuals [4].

NPs of different sizes, such as silver (Ag; 15, 100 nm), molybdenum (MoO_3; 30, 150 nm), aluminum (Al; 30, 103 nm), iron oxide (Fe_3O_4; 30, 47 nm), and titanium dioxide (TiO_2; 40 nm) were also evaluated for potential toxicity by studying the morphological parameters of cells by light microscopy. It was shown that mitochondrial function was significantly reduced in cells exposed to 5-50 μg/ml of Ag NPs. However, Fe_3O_4, Al, MoO_3, and TiO_2 did not have a noticeable effect at lower doses (10-50 μg/ml), while a significant effect was observed at higher doses (100-250 μg/ml). According to microscopic results, cells exposed to NPs at higher doses became abnormal in size, shrinking and becoming irregular in shape. Significant depletion of the glutathione content, a decrease in the mitochondrial membrane potential, and an increase in the amount of reactive oxygen species have been shown, that is, Ag cytotoxicity (15, 100 nm) in liver cells is likely to be mediated by oxidative stress [5]. Also, a number of studies have established the toxic effect of NPs on the nervous system as a whole and on the brain of the offspring when they are transferred from the mother's body to the circulatory system and the fetal body [6, 7].

Molybdenum (Mo) is one of the most important chemical elements in a living organism. It is a part of xanthine oxidase, aldehyde oxidase, and sulfite oxidase [8, 9], participates in protein metabolism, sulfur exchange [10], as well as transport and excretion of iron [11]. Nanomaterials based on molybdenum have recently attracted attention due to their ultra-high specific surface area and unique optical, electronic, catalytic, and mechanical properties [12-14]. In a living organism, molybdenum NPs have a high penetrating ability and can accumulate in excess amounts, acting as antagonists of other vital elements, in particular copper, the deficiency of which affects the functional activity of the hematogenous system [15, 16]. At the same time, nanoscale Mo can provide protection against the effects of increased concentrations of heavy metals, such as Cd and Hg. It was suggested

that Na_2MoO_4 was capable of removing the acute toxicity of $CdCl_2$ in rats, and the protective mechanism of this metal was partially associated with increased induction of the synthesis of Cd-metallothionein in the liver [17]. The accumulation of Cd in sheep tissues decreased as the amount of Mo and sulfur in the diet increased [18, 19].

This work for the first time established the hepatotropic effect of Mo and MoO_3 NPs in experimental rats, which was expressed in an increase in the endogenous level of caspase 3, the apoptosis marker. A decrease in motor activity and suppression of the emotional state of animals was also noted, as well as a decrease in their bodyweight and liver weight and an increase in brain weight after a single intraperitoneal injection of Mo and MoO_3 NPs.

The goal of the research was to study the overall effect of Mo and MoO_3 NPs on the growth and development of the internal organs of rats, on the motor and emotional activity of animals, as well as to establish the hepatotropic effect of NPs based on the assessment of the expression of the marker of activated caspase 3 as an indicator of the development of apoptosis in liver cells.

Methods. A total of 90 male Wistar rats with body weight of 110-180 g were used in the biomedical studies according to the methodological recommendations (Assessment of the safety of nanomaterials; approved by order of the Federal Service for Supervision of Consumer Rights Protection and Human Welfare dated October 12, 2007 No. 280; https://www.rags.ru/stroyka/text/52003/#i396117), as well as guidelines [20]. Prior to the experiment, the animals were kept in the laboratory of biological tests and examinations of the Federal Research Center of Biological Systems and Agricultural Technologies RAS and fed a standard diet for laboratory animals (GOST R 50258-92) in accordance with the requirements of laboratory practice during preclinical studies in the Russian Federation (GOST 3 51000.3-96 and GOST 51000.4-96). The experiments were carried out within the framework of the requirements for the humane treatment of animals [21], with the confirmation of the ethics committee (Minutes No. 3).

The sources of trace elements were Mo and MoO_3 NPs obtained by plasma-chemical synthesis. Particle sizes were estimated based on measurements of the specific surface area using a Sorbi®-M device (META LLC, Russia). The microstructure of the powders was analyzed using a Philips CM-30 transmission electron microscope (Philips, Japan). To determine the phase composition, a Rigaku D/MAX-2200VL/PC diffractometer (Rigaku Corporation, Japan) was used, Cu Kα radiation. When obtaining lyosols, aqueous suspensions of Mo and MoO_3 NPs were treated with ultrasound on the dispersant UZDN-2T (NPP Akadempribor, Russia) at 35 kHz, 300/450 W, 10 μA for 30 min. The resulting lyosols of NPs were used for injections.

Experimental rats were divided into five groups (n = 18 each) and kept under the same conditions on a standard balanced diet for laboratory animals. The control and experimental groups were formed from individuals of the same age. The spread over the initial mass did not exceed 10%. Rats of experimental groups I and II were once intraperitoneally injected with Mo NPs at a dose of 1.0 and 25.0 mg/kg; animals of experimental groups III and IV were injected with MoO_3 NPs at a dose of 1.2 and 29.0 mg/kg. Animals of the control group were injected with isotonic sodium chloride solution (0.9% NaCl) in an equivalent volume during the experiment. The selected concentrations of ultrafine particles were within the maximum tolerated doses for the metal under study. On days 1, 7, and 14 of the experiment, the rats were decapitated under Nembutal anesthesia. After that,

anatomical cutting was performed to take liver samples for morphological analysis. The changes in the mass of internal organs (liver and brain) were taken into account on day 14 of the experiment.

The growth of experimental individuals was monitored daily by individual weighing in the morning before feeding (error ±2 g). The data obtained were used to take into account changes in the absolute body weight (BW) and calculate the ratio of the mass of the studied organs to BW.

Pieces of liver were fixed in 10% neutral formalin and embedded in HistoMix paraffin mixture (BioVitrum LLC, Russia). To reveal the readiness of liver cells for programmed cell death, the expression of the caspase 3 enzyme in the cytoplasm and nuclei of hepatocytes was immunohistochemically detected during the staining of the sections in accordance with the standard procedure recommended by the manufacturer of the kit (Biocare Medical, LLC., USA; antibodies to caspase 3). Immunopositive cells were counted per 1000 cells and expressed in % (light optical microscope MT 5300L, Meiji Techno Co., Ltd., Japan).

Behavioral tests were performed on days 1, 7, and 14 in the morning before feeding the animals. The open-field test was used to assess the emotional, motor activity, and behavior of experimental animals. The emotional factor was assessed by the degree of anxiety and fear (the number of fecal boluses), as well as grooming (the number of combing, washing, and other care elements).

Spontaneous locomotor activity (LMA) of the animals was assessed using an Infrared Actimeter system complete with a Perforated Panel system (ACT-01, Orchid Scientific & Innovative India Pvt. Ltd., India). Movement and curiosity were recorded using an infrared sensor system as the animals moved freely over a 16-hole panel. The rats crossed the holes or immersed in them, while the intersections and immersions were recorded by sensors by the refraction of rays in the X and Y planes.

The Statistica 10.0 software package (StatSoft, Inc., USA) was used for statistical data processing. Results are presented as arithmetic means (*M*) and their standard errors (±SEM). The significance of the differences between the compared indicators was determined by Student's *t*-test. Differences were considered statistically significant at $p \leq 0.05$.

Results. The Mo NPs used in the work contained no less than 99.7% Mo and 0.30% O_2, their size was 50.0±0.56 nm, and the specific surface area was 14.0 m^2/g. For MoO_3 NPs, the indices were as follows: 99.8% MoO_3 and 0.20% O_2, 92.0±0.54 nm, 12.0 m^2/g.

The introduction of Mo NPs at concentrations of 1.0 and 25.0 mg/kg (experimental groups I and II) led to a decrease in BW on day 14 of the study by 2.14% and 7.04%, respectively, compared to the control ($p \leq 0.05$). A similar trend occurred when exposed to MoO_3 NPs at doses of 1.2 and 29.0 mg/kg (groups III and IV), that is, BW decreased by 6.41 and 1.51% ($p \leq 0.05$) compared to the control (see Fig. 1, A). Weight of liver (WL) under the influence of Mo in experimental groups I and II significantly decreased by 14.3 and 16.1% ($p \leq 0.05$), under the influence of MoO_3 (group III), this decrease was maximum (by 33.5%) ($p \leq 0.05$), while in group IV, there was an increase in WL by 21.6% ($p \leq 0.05$) relative to the control group (see Fig. 1, B). The WL/BW ratio was maximum at a concentration of MoO_3 NPs of 29.0 mg/kg, which quantitatively was 23.4% more than in the control. A 25.0 mg/kg concentration of Mo NPs caused an insignificant decrease in WL/BW (by 9.80%). The maximum decrease in this indicator (by 29.0%) was established at 1.2 mg/kg MoO_3 NPs (see Fig. 1, C).

The concentration of Mo NPs 1.0 mg/kg led to the maximum (by 10.9% ($p \le 0.05$) increase in the weight of the brain (WB) of rats. In experimental groups II and IV, there was a uniform increase in WB by 3.85 and 5.49% ($p \le 0.05$), in contrast to group III where the WB decreased by 1.10% compared to the control (see Fig. 1, D). The ratio WL/WB was maximum at a concentration of Mo NPs of 1.0 mg/kg, which was 13.4% ($p \le 0.05$) more than in the control. The trend towards an increase in the WL/WB ratio persisted in groups II (by 11.7%), III (by 5.67%), and IV (by 7.11%) ($p \le 0.05$) (see Fig. 1, E).

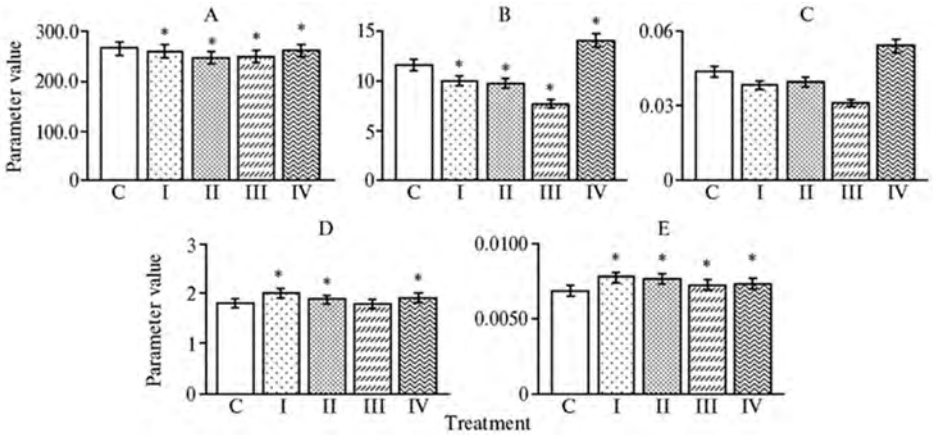

Fig. 1. Body weight, g (A)**, weight of liver, mg** (B)**, weight of liver to body weight ratio, mg/g** (C)**, weight of the brain, mg** (G)**, and weight of the brain to body weight ratio, mg/g** (E) **in male Wistar rats on day 14 after intraperitoneal injection of various forms of molybdenum NPs:** C — control, I — Mo, 1.0 mg/kg; II — Mo, 25.0 mg/kg; III — MoO_3, 1.2 mg/kg; IV — MoO_3, 29.0 mg/kg (n = 6, M±SEM).

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

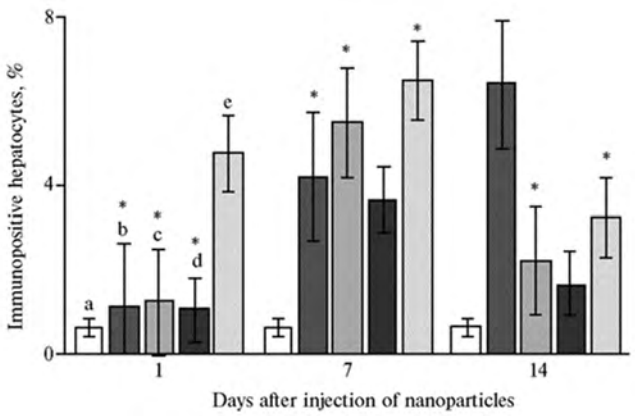

Fig. 2. Expression of the antigen of activated caspase 3 in liver cells of **male Wistar rats depending on time period from the intraperitoneal injection of various forms of molybdenum NPs:** a — control; b — Mo, 1.0 mg/kg; c — Mo, 25.0 mg/kg; d — MoO_3, 1.2 mg/kg; e — MoO_3, 29.0 mg/kg (n = 6, M±SEM).

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

Caspase 3 is one of the enzymes involved in apoptosis [22], which, in turn, acts as a fundamental and general biological mechanism responsible for maintaining the constancy of the cell number, cell formation and culling of defective cells. Expression of caspase 3 serves as a marker of apoptosis activation [23], which can be detected immunohistochemically using specific antibodies. In our experiments, an immunohistochemical study of caspase 3 activation revealed a dependence of the apoptosis marker expression on the administered dose of Mo and MoO_3 NPs, as well as on the time elapsed after injection (Fig. 2).

The counting of immuno-positive hepatocytes showed that intraperitoneal injection of Mo NPs at a dose of 1.0 and 25.0 mg/kg induced apoptotic changes in cells, especially on day 7 after injection and at an increased dose ($p \le 0.05$)

(Fig. 3, A). On day 14, the effect was opposite, i.e., the expression of the pro-apoptotic protein was the highest in the animals that received a smaller amount of NPs, despite the better morphofunctional state of the liver during this period (see Fig. 3, B) [24]. Apparently, this was influenced by the formation of a larger number of irreversibly damaged areas of the organ in rats, injected with a higher dose of Mo NPs (25.0 mg/kg).

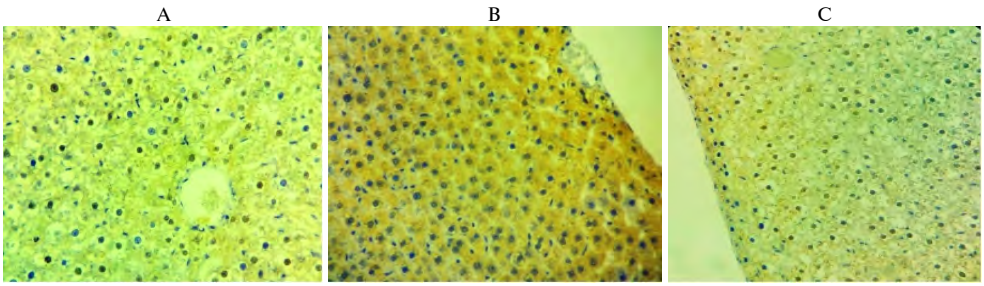

Fig. 3. Micrographs of hepatocytes in male Wistar rats: A — day 1 after injection of Mo (1.0 mg/kg), expression of activated caspase 3 in the nuclei and cytoplasm of hepatocytes (yellow-brown staining) along the periphery of the lobules under the capsule; B — day 7 after injection of Mo (25.0 mg/kg), expression of activated caspase 3 in the nuclei and cytoplasm of hepatocytes of the periportal zone; C — day 14 after injection of MoO_3 (29 mg/kg), weak expression of activated caspase 3 in the nuclei and cytoplasm of hepatocytes along the periphery of the lobules under the capsule (magnification ×400, light optical microscope MT 5300L, Meiji Techno Co., Ltd, Japan)..

An increase in the dose of MoO_3 NPs in group IV caused an increase in the expression of activated caspase 3 to a greater extent than in group III and control groups at all periods of the study, reaching a maximum value 7 days after administration. It can be noted that with an increase in necrobiotic processes in the liver tissue on day 14 in group IV, the degree of activation of the pro-apoptotic marker decreased and weakly stained immunopositive hepatocytes were mainly located along the periphery of the lobules under the capsule (see Fig. 3, C).

The changes in the motor activity of the experimental animals during the study in the "infrared actimeter" system corresponded to the data obtained in the "open field" test. LMA on days 1 and 7 of the experiment in the experimental groups decreased, that is, the higher the dosage of the introduced Mo, the lower the LMA was. On day 14, LMA in experimental groups I and II, on the contrary, was higher relative to the control. With the introduction of MoO_3 NPs, LMA in the experimental groups decreased in comparison with the control, and to a greater extent in the groups with the highest dosage and duration of exposure (on day 14).

The degree of anxiety and fear on day 1 of the study was the highest in animals of the control group and the lowest in experimental group I (by 86%) ($p \leq 0.05$). The grooming frequency in group II was 89% ($p \leq 0.05$) lower than in the control. On day 7 of the experiment, the indicator characterizing the emotional state decreased by 32% in group I and by 48% in group II compared to the control. The total indices of grooming were significantly lower in groups I and II than in the control (by 83 and 92%, respectively; $p \leq 0.05$). By the end of the experiment, the indicators in the groups receiving ultradispersed molybdenum particles were 22% lower compared to the control.

Upon injection of MoO_3 NPs, the degree of emotional activity (grooming, defecation) in the experimental groups of the animals on day 1 of the experiment decreased in groups III and IV by 52 and 69%, respectively ($p \leq 0.05$), compared to the control. On day 7, emotional activity was also suppressed, and, as on day 1 after application of NPs of MoO_3, a greater decrease was noted in animals that were injected with MoO_3 at a lower dosage. On day 14 after the injection of NPs,

emotional activity in both groups III and IV continued to decrease compared to the control (by 33 and 22%, respectively, $p \leq 0.05$).

Laboratory rats are the most common type of experimental animals for developing models of the consequences of acute and chronic intoxication. Currently, more than 100 separate outbred stocks and inbred lines of laboratory rats have been bred. Wistar rats, Bio Breeding Sprague-Dawley, C57BL, CFI, C3H are most often used in toxicological studies. In our studies of the toxic effect of ultrafine particles of molybdenum and its oxide, male Wistar rats were a bio-model. Previously, it was shown that a single intraperitoneal injection of Mo and MoO_3 NPs led to morphological changes in liver tissue in experimental animals of this line, the severity of which (from adaptive to necrobiotic) and reversibility depended on the dose and time elapsed after injection. An increase in the NP dose was accompanied by significant pathological changes, namely, the appearance of extensive areas of large vacuole hepatosis or foci of necrosis, or both, and the MoO_3 NPs exerted the most damaging effect on the liver tissue [24].

It can be assumed that the basis of pathomorphological changes in the liver against the background of the intake of Mo and MoO_3 NPs is their direct destructive effect on the vascular system of the animal organism and, as a consequence, the development of tissue hypoxia and necrobiotic changes. This assumption is based on the results of studies by Sherkhova et al. [25] who established the effect of an excess of Mo administered *per os* in the form of a salt ($Na_2MoO_4 \cdot 2H_2O$) on the vascular system of rats, including the liver. Despite the fact that in our studies, Mo was used in the nanoform, and not in the composition of the salt, the results obtained can be compared, since the possibility of a partial transition of NPs in the internal environment of the body to the ionic form is not excluded [26, 27].

In turn, destructive changes in cells can lead to the activation of the system of mononuclear phagocytes, which actively capture and accumulate nanosized particles [24], to the development of inflammation, and also, possibly, to the induction of apoptosis in the liver tissue, namely, to the activation of the proapoptotic protein caspase 3 [28] that we revealed in the liver of rats of the experimental groups.

Possessing a high penetrating power, NPs affect the organs and systems of the body, including the nervous one. When observing rats that were injected with NPs, the authors found signs of intoxication of the nervous system, in particular, inhibition of motor activity and an increase in brain mass. The results obtained indicate the absence of addiction in animals receiving NPs of molybdenum and its oxide. Previously, we have shown the toxic effect of ultrafine particles of iron, titanium, and titanium dioxide on the manifestation of cognitive functions in animals and the morphological structure of the brain, which confirms the data of other researchers on an increase in the absolute mass of the brain and changes in the emotional state of animals under the influence of NPs of trace elements metals [29-31].

Thus, the performed immunohistochemical analysis revealed an increase in the expression of the apoptosis marker, the enzyme caspase 3, in hepatocytes of male Wistar rats upon administration of Mo and MoO_3 NPs (NPs). The detected activation depended not only on the dose and time after injection but also on the degree of destructive changes in the organ during NPs administration. More severe liver damage was accompanied by a weaker activation of the proapoptotic enzyme compared to the control. A possible reason for this was the development of extensive necrobiotic processes in organ tissue without the initiation of apoptotic cell death. Mo and MoO_3 NPs had a toxic effect on the functioning of some

body systems. The maximum decrease in BW occurred in animals treated with Mo at a dose of 25.0 mg/kg and MoO_3 at a dose of 1.2 mg/kg. Both low and high doses of Mo NPs led to a significant decrease in WL (by 14.3 and 16.1%), and 1.2 mg/kg MoO_3 caused a 33.5% decrease. Mo at doses of 1.0 mg/kg, 25 mg/kg and MoO_3 at a dose of 1.2 mg/kg caused a significant increase in WB (by 10.9; 3.85 and 5.49%, respectively, $p \leq 0.05$). In turn, an increase in brain mass (possibly due to organ edema) led to changes in the behavioral reactions and LMA of rats, which indicated the neurotoxic effect of Mo and MoO_3 NPs, the severity of which directly depended on the time after administration and dosage of the particles. There was a decrease in LMA in rats on days 1 and 7 after Mo administration, and the activity was more strongly suppressed with increasing dosage (the higher the dosage, the lower the activity). The smallest values of indicators characterizing LMA were obtained on day 14 after the introduction of MoO_3 NPs at a dose of 29.0 mg/kg. It was shown that the emotional activity of rats decreased after the introduction of Mo and MoO_3 NPs in all studied dosages, with the greatest effect from the effect of NPs recorded on days 1 and 7 of the experiment.

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AGRICULTURAL BIOLOGY,
2020, V. 55, № 6, pp. 1182-1203
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

Functionality of foodstuffs

UDC 637.03:573.6.086.83.001.26

doi: 10.15389/agrobiology.2020.6.1182eng
doi: 10.15389/agrobiology.2020.6.1182rus

GENERATION OF BIOACTIVE PEPTIDES IN MEAT RAW MATERIALS EXPOSED TO LYSATES OF BACTERIAL STARTER CULTURES

I.M. CHERNUKHA[1], N.G. MASHENTSEVA[1] ✉, N.L. VOSTRIKOVA[1], L.I. KOVALEV[2], M.A. KOVALEVA[2], D.A. AFANASEV[3]

[1]*Gorbatov Federal Center for Food Systems RAS,* 26, ul. Talalikhina, Moscow, 109316 Russia, e-mail imcher@inbox.ru, natali-mng@yandex.ru (✉ corresponding author), n.vostrikova@@fncps.ru;
[2]*Federal Research Center for Biotechnology, Bakh Institute of Biochemistry RAS,* 33/2, Leninskii prosp., Moscow, 119071 Russia, e-mail kovalyov@inbi.ras.ru, m1968@mail.ru;
[3]*Moscow State University of Food Industry,* 11, Volokolamskoe sh., Moscow, 125080 Russia, e-mail dmitr.afanasjew2010@yandex.ru
ORCID:
Chernukha I.M. orcid.org/0000-0003-4298-0927
Mashentseva N.G. orcid.org/0000-0002-9287-0585
Vostrikova N.L. orcid.org/0000-0002-9395-705X
Kovaleva M.A. orcid.org/0000-0002-3486-2122
Kovalev L.I. orcid.org/0000-0001-6519-8247
Afanasev D.A. orcid.org/0000-0002-7463-4503

The authors declare no conflict of interests
Acknowledgements:
Proteomic study was performed using the equipment of the Center for Collective Use of the Federal Research Center of Biotechnology RAS (identifier RFMEFI62114X0002).
Supported financially by the grant of the Russian Scientific Foundation (project No. 16-16-10073П)
Received July 31, 20208

Abstract

Nowadays, preparations based on bacterial lysates are mainly applied in medicine. In food industry, bacterial lysates are still not widely used, in particular for manufacturing meat functional foodstuff. Though their potential for functional foodstuff production is predictable, the efficiency and specificity of action which depend on the characteristics of the strain and the method of cell disintegration require study. A set of peptidases identified in starter cultures, in particular endo-peptidases, aminopeptidases, dipeptidases, tripeptidases, and proline-specific peptidases stimulate interest in the lysates of these microorganisms for food biotechnology. In this work, we have shown that lysates of *Pediococcus pentosaceus* 28, *Staphylococcus carnosus* 108, *Lactobacillus curvatus* 1, *P. acidilactici* 38, *L. sakei* 103, *L. sakei* 105, *L. curvatus* 2, *L. acidophilus* AT-41 that we obtained by physical destruction of bacterial cells have the widest spectrum of enzymes and biologically active substances. Our goal was to determine the biochemical composition and enzymatic activity of the lysates of starting bacterial cultures and their role in the formation of biologically active peptides in raw meat. The bacterial suspensions were exposed either to lysozyme treatment followed by separation of the extract from the cell debris by centrifugation, or to ultrasonic treatment to compare two methods of cell destruction. The physical method was proved to be the most effective. For biochemical characterization, the proteolytic, lipolytic and collagenase activities of the lysates, and the concentration of organic acids, proteins, and free amino acids were measured. Enzymatic activities of the lysates were determined using API®ZYM tests. The *Lactobacillus curvatus* 2, *Lactobacillus acidophilus* AT-41, *Pediococcus acidilactici* 38 and *Staphylococcus carnosus* 108 lysates showed the widest range of intracellular enzymes, including leucine and valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and β-galactosidase. The proteolytic activity was the highest in *Staphylococcus carnosus* 108 (115.94 proteolytic capability PC units per mg protein), *Lactobacillus acidophilus* AT-41 (66.7 PC units per mg protein), *Lactobacillus curvatus* 1 (91.03 PC units per mg protein), and *Lactobacillus curvatus* 2 (72.20 PC units per mg protein) as compared to other strains. The level of malic, lactic and succinic acids in the lysates varied in the range of 0.002-0.02, 0.02-0.06, and 0.2-0.9 mg/100 g, respectively. The highest enrichment in free amino acids with 13 AA detected out of 17 AA studied was characteristic of *P. acidilactici* 38 lysate while only 7 AA were detected in the *L. sakei* 105 lysate. A comparison of 2D electrophoregrams of fermented raw meat showed both general effects on reducing total proteins and the lysate-specific effects toward various proteins, e.g., formation of protein conjugates and cleavage of target proteins, in particular actin skeletal muscle. Therefore, lysates of the studied starter cultures can serve as a source of various enzymes for practical use in the food industry, for example to improve the functional, technological and biocorrective characteristics of meat products.

Keywords: lysates, starting cultures, enzymatic lysis, biologically active peptides, two-dimensional electrophoresis, IEF-PAGE, MALDI-TOF, mass spectrometry

The modern human diet should include biologically active ingredients with known physicochemical characteristics, for which properties useful for maintaining and improving health have been identified and scientifically substantiated, and the daily physiological need has been established. Daily consumption of functional foods is seen as a way to reduce the risk of disease [1]. Numerous studies have focused on the production of bioactive peptides as nutraceuticals and functional food ingredients for their health benefits. These short peptides, exhibiting antihypertensive, antioxidant, mineral binding, immunomodulatory, and antimicrobial activities, are latent in the primary sequences of food proteins and are released during enzymatic proteolysis [2].

During microbial fermentation, bacteria synthesize vitamins and mineral compounds, with the participation of proteinases and peptidases, they form biologically active peptides and remove some non-nutritive substances. Microbial fermentation is considered as one of the main and economically most suitable processes for the production of biologically active peptides [3]. Lactic acid bacteria with complex proteolytic systems are successfully used as starter cultures in the production of a variety of fermented meat products. A deeper understanding of the functionality of the proteolytic system of starter cultures opens up future opportunities for obtaining new food compounds with potential health benefits [4]. Fermented foods combine a range of health benefits through antioxidant, antimicrobial, antimycotic, anti-inflammatory, antidiabetic, and antiatherosclerotic activities [5].

Recently, to obtain functional preparations and products, interest has been growing in lysates of cells of microorganisms subjected to mechanical, chemical, or enzymatic destruction. Cells are made up of water, inorganic ions, and carbon-containing (organic) molecules. Water is the most abundant molecule in cells, accounting for 70% of the total mass of cells. Inorganic ions of the cell, including sodium (Na^+), potassium (K^+), magnesium (Mg^{2+}), calcium (Ca^{2+}), phosphate (HPO_4^{2-}), chloride (Cl^-) and bicarbonate (HCO_3^-), make up to 1% of the cell mass. These ions are involved in cellular metabolism and play an important role in the functioning of cells. However, the uniqueness of a living cell is determined by organic molecules, most of which belong to one of four classes of compounds, i.e., carbohydrates, lipids, proteins, and nucleic acids. Proteins, nucleic acids, and most carbohydrates (polysaccharides) are macromolecules formed as a result of polymerization of low molecular weight precursors — amino acids, nucleotides, or simple sugars. Such macromolecules make up 80-90% of the dry weight of most cells. Lipids are also among the main components of the cell. The rest of the cell mass consists of many small organic molecules, including macromolecular precursors [6]. The cell wall of gram-positive bacteria is a complex structure formed by glycopolymers and proteins. It consists of layers of peptidoglycan (murein sac) surrounding the cytoplasmic membrane, stitched in the transverse direction with teichoic acids, and contains polysaccharides and proteins [7].

Bacteria serve as a source of various intra- and extracellular enzymes. Starter cultures synthesize proteases and peptidases, glycosidases, polysaccharide enzymes, malolactic enzymes, esterases, ureases, phenol oxidases, and lipases [8]. The proteolytic system of lactic acid bacteria used as starter cultures consists of proteinases (break down proteins into peptides), peptidases (break down the resulting peptides into smaller peptides and amino acids), and transport systems that are involved in the cellular uptake of small peptides and amino acids. A wide range of peptidases has been identified in lactic acid bacteria, in particular, endopeptidases, aminopeptidases, dipeptidases, tripeptidases, and proline-specific peptidases [9, 10]. The proteolytic systems of lactococci and lactobacilli are surprisingly similar

in components and mode of action; their proteolytic system consists of extracellular serine proteinase, transport systems specific for di-tripeptides and oligopeptides (> 3 residues), and many intracellular peptidases [11]. In addition, the importance of proteolytic and peptidolytic enzymes of lactic acid bacteria is that a number of strains, for example, *Lactobacillus helveticus* CP790, *L. rhamnosus* GG, *L. bulgaricus* SS1, and *L. lactis* subsp. *cremoris* FT4 are involved in the release of bioactive peptides [12].

Bacteria can produce both intermediate and final products of bacterial metabolism, e.g., lactic acid, hydrogen peroxide, and bacteriocins, as well as metabolites of small molecules (histamine, vitamins, short-chain fatty acids, polyunsaturated fatty acids, serpins, lactocepins, secreted proteins). Moreover, the metabolic potential of microbes varies greatly between species and even among strains of the same species [13].

Thus, from a chemical point of view, cell lysates are a mixture of short-chain peptides, free amino acids, organic acids, polysaccharides, vitamins of groups B, C, PP, folic acid, volatile fatty acids, peptidoglycan of cell walls, as well as various enzymes.

The study of the intracellular enzyme systems of bacteria requires cell disintegration [14, 15]. A comparison of some conventional methods (ultrasonic treatment, mechanical grinding with glass beads, freeze-thaw, chemical lysis with toluene solutions in acetone or ethanol) showed that ultrasonic treatment and grinding of *Bacillus subtilis*, *Pseudomonas putrefaciens*, and *Streptococcus durans* cells made it possible to obtain more protein in cell-free extracts than other methods [16].

Currently, preparations based on bacterial lysates are used mainly in medicine. Clinical studies have shown that oral bacterial lysates reduce the need for antibiotics and the risk of recurrent respiratory infections in children and adults [12]. The use of these drugs for the treatment of bronchial asthma has been described [17, 18]. It was reported about the regenerative effect of bacterial lysates of resident non-pathogenic microflora of spring water Comano (Comano Terme, Trento, Italy) in the culture of human skin fibroblasts in vitro. Bacterial strains isolated from this water were characterized by genomic sequencing. The collection included 182 isolates; bacterial lysates were obtained by autoclaving (121 °C, 20 min) [19]. Re-epithelialization of damaged tissues using a soluble fraction from the lysate of seven different probiotic strains belonging to the genera *Streptococcus*, *Lactobacillus,* and *Bifidobacterium* was shown using the HaCaT human keratinocyte line model *in vitro* [20]. An *in vitro* study revealed an increase in the content of hyaluronic acid in HaCaT cells treated with *L. plantarum* K8 lysates [21]. The use of microfluidized *Lactobacillus rhamnosus* lysates in the reconstructed Keraskin™ human epidermis improved the barrier function of the skin [22]. Lysates of *E. coli* and other pathogens are successfully used in the treatment of urinary tract infections in cases where there is a high resistance of bacteria to antimicrobial drugs [23]. Interventional studies using probiotics, prebiotics, and their hydrolyzed forms and bacterial lysates have shown a decrease in food sensitization and a positive effect in allergic diseases, including atopic dermatitis [24, 25]. Lysates of lactic acid bacteria are used to treat diseases of the gastrointestinal tract [26].

Cell-free gene expression systems are becoming an important platform for solving a wide range of problems in synthetic biology and biotechnology, including the production of reliable biosensors [27].

In food production, in particular, in the meat industry, bacterial lysates are still not widely used, although their prospects are predictable, which stimulates interest in this kind of research. For example, it was shown that under the influence

of the culture of the *L. plantarum* CRL 681 strain (originally isolated from meat products) in combination with its cell lysate, proteolysis of both sarcoplasmic and myofibrillar proteins occurred with the formation of various peptides of a hydrophobic nature [28]. With increased fermentation, the substrate is enriched with biologically active compounds that are produced by bacteria responsible for fermentation (conjugated linoleic acids lower blood pressure, exopolysaccharides exhibit prebiotic properties, bacteriocins exhibit antimicrobial effects, sphingolipids exhibit anticarcinogenic and antimicrobial properties) [29], in addition to bioactive peptides, exhibiting antioxidant, antimicrobial, opioid antagonistic, antiallergenic, and blood pressure lowering effects [30].

Earlier, the authors examined the effect of starter cultures on the formation of bioactive peptides in meat and meat products [31, 32]. In the development of these studies, in this work, the authors have shown that lysates of the studied starter cultures have a set of enzymatic activities, including high general and specific proteolytic activity (the presence of target proteins, the formation of protein conjugates) with the formation of spectra of low molecular weight peptides and can find a practical application for improving the functional, technological and biocorrective characteristics of meat products.

The aim of the work was to determine the biochemical composition and enzymatic activity of lysates of starting bacterial cultures and their role in the formation of biologically active peptides in raw meat.

Methods. The authors used the strains *Pediococcus pentosaceus* 28, *Staphylococcus carnosus* 108, *Lactobacillus curvatus* 1, *P. acidilactici* 38, *L. sakei* 103, *L. sakei* 105, *L. curvatus* 2, *L. acidophilus* AT-41(collection of the the Moscow State University of Food Productions).

To obtain the bacterial biomass of each strain, a cell suspension (10^9 CFU/ml) was introduced into a de Man, Rogosa, and Sharpe liquid nutrient medium (MRS) at the rate of 1 ml of suspension per 10 ml of medium. It was cultivated for 24 h at 37 °C [33]. The culture fluid was placed in two 40 ml centrifuge tubes. The cells were precipitated (4000 rpm, 4 °C, 15 min), the supernatant was decanted. The pellet was resuspended in 10 ml of 100 mM phosphate buffer (pH 7), the samples were combined, centrifuged (4000 rpm, 4 °C, 15 min), resuspended in 10 ml of 100 mM phosphate buffer (pH 7), and additionally centrifuged at the same conditions.

Lysates were obtained in two ways — by treating cells with lysozyme and by ultrasonic disintegration.

When using lysozyme, after removing the supernatant (second centrifugation), 100 mM phosphate buffer (pH = 7) was added to the biomass with the addition of lysozyme (2.5 mg/ml) and sucrose (20 mg/ml) to a cell suspension density of 10^9 CFU/ml (according to the McFarland turbidity standard). The biomass was carefully resuspended. In a glass test tube with a volume of 40 ml, 20 ml of the lysed suspension was taken and placed in a thermostat on a shaker (for uniform treatment with lysozyme) for 1 h at 30 °C. Then the samples were centrifuged (4000 rpm, 4 °C, 15 min) to separate cell debris from protoplasts. The supernatant was decanted, the pellet was resuspended in 10 ml of 100 mM phosphate buffer (pH 7) with sucrose (5 mg/ml) to create a hypotonic environment, leading to physical rupture of protoplasts. The samples were thoroughly mixed for 5 min, centrifuged (15,000 rpm, 8 min). The precipitate was separated from the supernatant (lysate).

For ultrasonic disintegration of cells after removing the supernatant (second centrifugation), 100 mM phosphate buffer (pH 7) was added to the biomass until the cell concentration in the suspension was 10^9 CFU/ml (according to McFarland turbidity standard). In a centrifuge tube with a volume of 40 ml,

20 ml of the lysing suspension was taken. The tube was placed in an ice bath and then processed on an ultrasonic disintegrator Soniprep 150 (MSE, UK) in the following mode: processing for 30 s, cooling for 30 s (six cycles with stirring every two cycles for uniform disintegration; operating wavelength 9 μm). Upon completion of ultrasonic disintegration, the mixture was thoroughly mixed for 5 min, centrifuged (15,000 rpm, 8 min), and the precipitate was separated from the supernatant (lysate).

The completeness of lysis was monitored by transmission electron microscopy (TEM) (JEM-1400, Jeol, Japan; operating voltage 80 kV, wavelength 500 nm). For this, the pellet obtained after centrifuging the lysing suspension and separating the supernatant (lysate) was resuspended in 0.5 ml of 100 mM phosphate buffer (pH 7), and preparations for TEM were prepared from aliquots of each suspension diluted 100 times with 100 mM phosphate buffer. The samples were applied to a support copper mesh coated with a formvar film and reinforced with carbon, dried for 15 min in air, viewed, and photographed; negatives were scanned and processed in a graphic editor.

The protein concentration in the lysate was measured by the Bradford method on a BioSpectrometer basic spectrophotometer (Eppendorf, Austria) based on the reaction with Coomassie Brilliant Blue R-250 (λ = 595 nm). Bovine serum albumin was used as a standard for constructing the calibration curve [34].

The proteolytic activity of the lysates was determined using the modified Anson method according to GOST 20264.2-88 "Enzyme preparations. Methods for determining proteolytic activity (with Amendment No. 1)" by the amount of tyrosine ptoduced during the hydrolysis of the substrate sodium caseinate (λ = 670 nm). When recalculating optical density (OD) values, a calibration curve was used for a series of standard solutions with a known tyrosine concentration. The values of proteolytic activity were expressed in units of proteolytic capacity in 1 mg of protein (PC units/mg protein).

The lipolytic activity of the lysates was measured by the modified method of Oto and Yamada [35] using an alkali to titrate fatty acids formed by lipase and olive oil as a substrate. The values of lipolytic activity were expressed in units of lipolytic capacity in 1 mg of protein (LC units/mg protein).

The collagenase activity of lysates [36] was assessed by a method based on determining the content of hydroxyproline in a mixture of native collagen formed as a result of hydrolysis, with the construction of a calibration curve for the relationship between the concentration of hydroxyproline in a buffer solution (in the range of 2-20 mmol/ml; OD_{555}). Collagenase activity values were expressed as a percentage of collagen dissolved (% cd).

To measure the enzymatic activities of the lysates, the API®ZYM test systems (BioMйrieux, France) were used, which allowed the determination of alkaline phosphatase, esterase, esterase lipase, lipase, leucine, valine and cysteine arylamidases, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucose-aminidase, α-mannosidase and α-fucosidase.

The amino acid composition of the lysates was investigated by a method based on the precipitation of proteins with trichloroacetic acid (TCA) followed by the extraction of free (unbound) amino acids. The isolated amino acids were derivatized with o-phthalic aldehyde (OPA) and 9-fluoromethyl chloroformate (FMOC). OPA was used to determine primary amino acids, FMOC − secondary, followed by high-performance liquid chromatography − diode-array detector (HPLC-DM) analysis and detection at wavelengths 338 and 262 nm, respectively (C18 PA column, 3.5μm×150 mm). The following reagents were used: deionized water obtained using a Milli-Q water purification system (Merck Millipore, USA),

acetonitrile for HPLC (≥ 99.9%) (Panreac, France), methanol for HPLC (Merck, USA), hydrochloric acid (≥ 37%), TCA (≥ 99.0%), FMOC (9-fluorenylmethylchloroformate, 10 mg/ml) (Sigma-Aldrich, USA), OPA (ortho-phthalic aldehyde, 10 mg/ml) (Sigma-Aldrich, USA). A mixture of D, L-amino acids (Merck, USA) was used as standards. Composition of solutions for gradient elution: eluent A — acetonitrile:methanol:water (45:45:10), eluent B — borate buffer (10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7$, pH 8.2). Amino acids were analyzed according to standard protocols on an Agilent 1260 Infinity LC liquid chromatograph with a diode array detector (Agilent Technologies, United States), column temperature in a thermostat 40 °C, operating pressure 1.6 MPa, eluent flow rate 1.5 ml/min, analysis time 25 min.

Organic acids in lysates were determined by a method based on the extraction of organic acids with an aqueous solution of TCA. Proteins from the extracts interfering with the determination of organic acids were precipitated by centrifugation; the samples were analyzed by HPLC on an Agilent 1260 Infinity LC liquid chromatograph with a UV detector (Agilent Technologies, United States) according to standard protocols. An anion-exchange HPLC column with a length of 50-150 mm and a diameter of 2.1-4.6 mm, a particle size of 1.8-5.0 μm, was used. The standards were solutions of organic acids with a basic substance content of at least 99.0% (Merck, USA). The acids were identified by the absolute retention time, the mass fraction was determined from the area of the chromatographic peak of the analyzed sample, comparing it with the peak of the reference sample with a known concentration. The reagents used for the determination and the conditions of the analysis are similar to the reagents and conditions established for the study of the amino acid composition.

The protein profiles of raw meat after processing were determined in the *longissimus dorsi* muscle of *Bos taurus*. Lysate was injected into a muscle tissue sample (5 ml per 50 g of raw material, ratio 1:10). The samples were kept in vacuum containers for 48 h at a temperature of +4±1 °C and, prior to analysis, were stored at –30 °C for 5 days. For the proteomic study of the processed meat raw material, 100 mg of the crushed sample was homogenized in 2 ml in a Teflon-glass system in a lysis solution (9 M urea, 5% mercaptoethanol, 2% Triton X-100, 2% ampholines, pH 3.5-10). The resulting homogenate was clarified by centrifugation at 800 rpm for 5 min, the supernatant fraction containing solubilized proteins (extract) was used for fractionation.

For proteomic analysis, proteins were separated by O'Farrell two-dimensional (2D) electrophoresis with isoelectric focusing in ampholine polyacrylamide gel (IEF-PAGE), as described previously [37, 38]. For visualization, proteins on 2D electropherograms were sequentially stained with Coomassie Brilliant Blue R-250 and silver nitrate [39]. The molecular weights of the protein fractions were determined using a set of highly purified recombinant proteins with molecular weights of 10-170 kDa PageRuler™ Prestained Protein Ladder (#SM0671 — 10 proteins, Fermentas, USA).

Computed densitometry used two-dimensional electropherograms in a wet state. Their complete digital images and/or images of individual fragments were obtained by scanning (Expression 1680, Epson, USA) [40] (resolution 300 dpi, 48 bit Color, saving the results in *.tiff format). The obtained digital images were processed in a graphics editor and the protein content was calculated using the ImageMaster 2D Platinum version 7 software package (GE Healthcare, Switzerland). When determining the amount of protein, at least three electropherograms with equal application were used. The deviation in optical density values was no more than ±1.5%.

To identify proteins, the cut fragments of 2D gel were homogenized and trypsinolized as previously described [41]. Sets of peptides were studied by MALDI-TOF MS and MS/MS mass spectrometry on an Ultraflex MALDI time-of-flight mass spectrometer (Bruker, Germany) with a UV laser (λ = 336 nm) in the positive ion mode in the mass range 500-8000 Da calibrated against known peaks of trypsin autolysis. Traditional bioinformatics technologies were used to decode mass spectra (peptide fingerprints). The mass spectra of tryptic peptides were analyzed using the Mascot program, the Peptide Fingerprint option (Matrix Science, USA) (0.01% accuracy of determining the MH+ mass) using the Protein NCBI database (https://www.ncbi.nlm.nih.gov/protein/). In a comparative analysis of the proteomic profiles of the presented samples, the authors used the information modules Proteins of bovine skeletal muscle (*Bos taurus*) from the Proteomics of Muscular Organs database (http://mp.inbi.ras.ru).

The quantitative data were statistically processed using the STATISTICA 14.0 software package (StatSoft, Inc., USA). All measurements were performed in 3 replicates. The results are presented as weighted arithmetic mean (*WAM*) with standard deviation ($\pm$SD). Statistical significance was calculated using the non-parametric Mann-Whitney U-test and the Kruskal-Wallis H-test. The critical level of significance of the null statistical hypothesis (p) was taken equal to 0.05.

Results. Peptides produced by starter cultures include ribosomally synthesized bacteriocins and protein hydrolysis products — bioactive peptides that can act as natural preservatives and nutraceuticals, respectively. Bioactive peptides are formed from substrate proteins under the action of intramembrane proteases and are extracellular protein residues that are not used by the proteolytic system of starter cultures for nitrogen assimilation and are released from the cell [11]. In some cases, cell lysis and the release of enzymes involved in proteolysis and generation of bioactive peptides are required [42].

The authors lysed the cell biomass of the starter cultures in two ways − enzymatic (treatment with lysozyme) and physical (ultrasonic disintegration of cells in suspension). In both cases, the cell debris was separated by centrifugation. The resulting supernatant was a lysis product of the cells of the starter cultures.

Electron microscopy. Microscopy showed that cell lysis by lysozyme was less effective than ultrasonic disintegration (Fig. 1). In this case, the cell wall is only slightly subject to destruction: both in the *Pediococcus pentosaceus* 28 strain and in the *Lactobacillus sakei* 103 strain, one can observe a violation of the integrity of the surface layers of the cell wall (the appearance of roughness), as well as an increase in its porosity. The treatment of cells with ultrasound gave a similar result, however, it is noticeable that the destruction occurs evenly over the cell surface, while when the cells are treated with lysozyme, it occurs locally.

Ultrasonic treatment of samples for TEM was first carried out in four cycles at an operating wavelength of 4 μm. Based on the results of microscopy, it was decided to increase the number of processing cycles to six at a wavelength of 9 microns. The obtained data are consistent with the results of the study by Tabatabaie and Mortazavi [43], where ultrasonic disintegration of cells of probiotic bacteria (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactococcus lactis* subsp. *cremoris*, and *Lactococus lactis* subsp. *lactis*) was carried out. Their TEM results confirmed the destruction of the bacterial cell wall after treatment. The authors noted that the nature of the damage depends on the duration of treatment. With the minimum time of exposure, microcracks and microvoids are formed on the cell surface, with a longer time − ruptures of the cell wall, its porosity increases. A further increase in the duration of disintegration leads to cell rupture and the release of intracellular contents into the working environment [43].

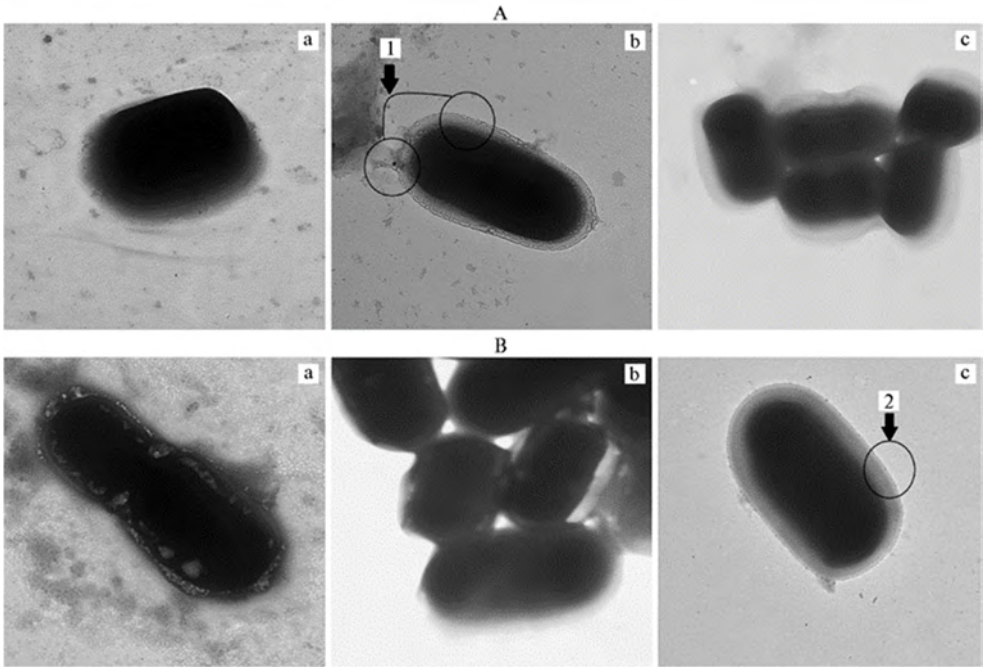

Fig. 1. Control of the completeness of lysis of *Pediococcus pentosaceus* 28 (A) **and *Lactobacillus sakei* 103 cells with different methods of obtaining lysates** (×30,000)**:** a — control sample (*P. pentosaceus* 28 and *L. sakei* 103 cells, no treatment); b — experimental sample (treatment of *P. pentosaceus* 28 and *L. sakei* 103 cells with lysozyme); c — prototype (ultrasonic disintegration of *P. pentosaceus* 28 and *L. sakei* 103 cells); 1 — local violation of the integrity of the surface layers of the cell wall (appearance of roughness), 2 — destruction occurs evenly over the cell surface (transmission electron microscopy, JEM-1400, Jeol, Japan).

Protein content and spectra of enzymatic activity of lysates. Based on the comparison of the two methods for obtaining lysates, we used ultrasonic treatment for these purposes. Table 1 shows the results of determining the protein concentration and proteolytic activity of the samples.

1. Protein concentration (μg/ml) **and proteolytic activity** (PC units/mg protein) **in lysates of starter cultures with different cell processing methods** (*WAM*±SD)

Strain	Protein concentration		Proteolytic activity (ultrasonic disintegration)
	lysozyme*	ultrasonic disintegration*	
Staphylococcus carnosus 108	0,24±0,012	0,99±0,006	114,88±2,162
Lactobacillus acidophilus AT-41	0,81±0,015[a]	1,94±0,050[e]	67,46±0,661
Lactobacillus curvatus 2	0,80±0,020[c]	1,79±0,026	72,19±0,717
Lactobacillus curvatus 1	0,34±0,006	1,28±0,038	91,07±0,905
Lactobacillus sakei 105	0,19±0,006[b]	1,00±0,041	55,77±1,137
Pediococcus acidilactici 38	0,47±0,021	1,27±0,035	42,17±1,478
Pediococcus pentosaceus 28	0,15±0,015[b, d]	0,80±0,036[f]	45,01±0,705
Lactobacillus sakei 103	0,23±0,021	0,86±0,017[f]	51,24±1,372

N o t e. PC — proteolytic capability. A pooled sample was used for analysis (three replicates).
[a-b, c-d, e-f] Differences between lysates for treatment option (column) are statistically significant at $p < 0.05$.
* Differences between treatment options are statistically significant at $p < 0.05$.

The protein content in the biomass samples of the starter cultures before lysis was low (up to 0.05 μg/μl), but it ranged from 0.17 to 0.82 μg/μl for lysozyme treatment and from 0.79 to 1.95 μg/μl for ultrasound. Consequently, the lysis of biomass with ultrasonic treatment is more intensive than with enzymatic treatment, which corresponds to the results of electron microscopy. The data obtained are consistent with the results of Mehmeti et al. [15] who also performed ultrasonic disintegration of cells of starter cultures. In their paper, the protein concentration in lysates of *Lactococcus lactis* NIZO 0900 cells averaged 1.25±0.02 μg/μl, *Pediococcus pentosaceus* OZF 1.32±0.01 μg/μl.

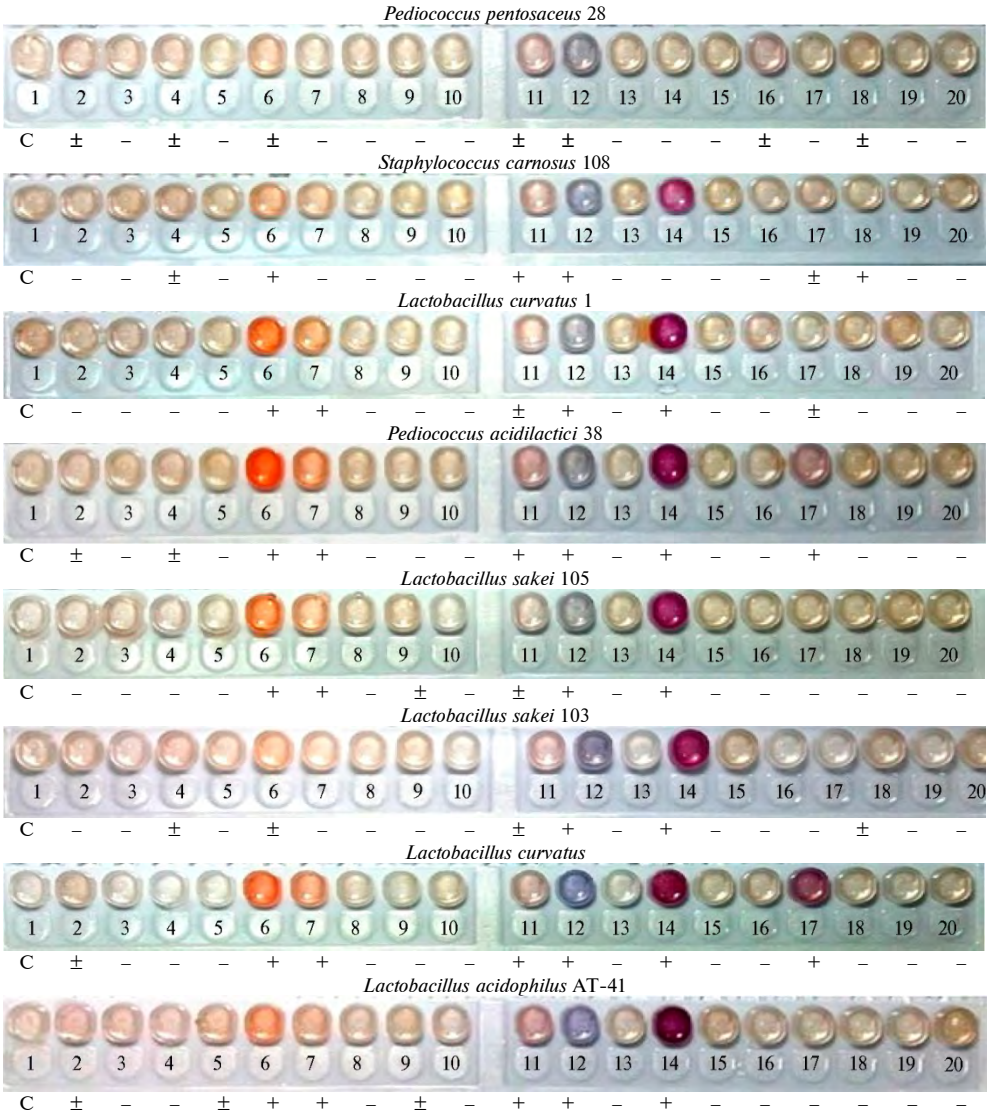

Fig. 2. Enzymatic activity of lysates of starter cultures after ultrasonic disintegration: 1 — control sample, 2 — alkaline phosphatase, 3 — esterase, 4 — esterase lipase, 5 — lipase, 6 — leucine arylamidase, 7 — valine arylamidase, 8 — cysteine arylamidase, 9 — trypsin, 10 — α-chymotrypsin, 11 — acid phosphatase, 12 — naphthol-AS-BI-phosphohydrolase, 13 — α-galactosidase, 14 — β-galactosidase, 15 — β-glucuronidase, 16 — α-glucosidase, 17 — β-glucosidase, 18 — N-acetyl-β-glucosaminidase, 19 — α-mannosidase, 20 — α-fucosidase; "+", "–", "±"— the presence, absence and weak manifestation of activity (API®ZYM test strips, BioMérieux, France).

The strains *Staphylococcus carnosus* 108, *Lactobacillus acidophilus* AT-41, *L. curvatus* 1, and *L. curvatus* 2 showed the greatest proteolytic activity. Lactic acid bacteria used as starter cultures are considered weakly proteolytic. Nevertheless, proteolysis is one of the main enzymatic reactions that occur in meat products under the influence of microorganisms. Lactic acid bacteria possess a complex proteolytic system, which consists of three components: proteases associated with the cell wall, which initiate protein breakdown into oligopeptides, peptide carriers, and intracellular peptidases, which decompose peptides into shorter peptides and free amino acids [44]. As a rule, published research results describe the proteolytic activity of lactobacilli directly in the culture medium (MRS medium, milk) [45]. In the paper of Donkor et al. [44], tripeptidase activity of lactobacilli in MRS medium was in the range of 200.0-3020.0 U/mg protein, dipeptidase activity within 50.0-

1100.0 U/mg protein. Parra et al. [46] evaluated the ability of whole cells, cell-free extracts, and cell lysates to accelerate proteolysis in curd suspensions. The obtained results allowed the suggestion that the developed model system of accelerated maturation based on cell lysates is indicative and can be used for rapid assessment of the contribution of strains to proteolysis during cheese maturation.

The ability of strains of lactic acid bacteria to produce bioactive peptides is based on the characteristics of the hydrolytic reactions of the proteins involved in them. The differences found in the proteinases of lactic acid bacteria explain the diversity of the resulting bioactive peptides, which is observed even when acting on the same protein matrix. The composition of bioactive peptides also depends on the substrate in which the hydrolytic enzymatic reaction took place [42].

To determine enzymatic activities in lysates, API®ZYM test systems were used with a visual assessment of the presence of activity by color reaction (Fig. 2) [47]. In the majority of producers, we found the enzymes naphthol-AS-BI-phosphohydrolase, leucine arylamidase, and β-galactosidase, characteristic of many lactic acid microorganisms (see Fig. 2). The obtained data are consistent with the results of other studies: the presence of β-glucosidase, β-galactosidase [48, 49], and leucine arylamidase [50] in lactic acid bacteria is known, which makes them useful starter cultures in food biotechnology.

Screening with API®ZYM showed that in the studied strains, lipolytic enzymes were either absent or contained in insignificant amounts. Using the modified method of Oto and Yamada, the authors did not reveal lipolytic activity in the lysates. In lactic acid bacteria, lipolytic activity is rarely found. Nevertheless, it has been shown that it occurs in *Lactobacillus plantarum* [51, 52]. This culture is one of the most valuable producers of many enzymes, including lipase and various esterases [53]. There are also mentions of lipase activity in the *Pediococcus acidilactici* culture, but in this species, lipase is synthesized in limited quantities and under certain conditions conducive to synthesis [54]. There are reports of lipolytic activity in strains of the species *L. helveticus*, *L. delbrueckii*, *L. bulgaricus*, *L. casei*, *L. plantarum*, and *L. acidophilus* [55].

In the lysates, no collagenase activity was detected, determined by oxyproline released as a result of hydrolysis of native collagen. Perhaps this is a consequence of the anticollagenase activity of strains. There is evidence in the literature that lactic acid microorganisms have such an activity, in particular, lipoteichoic acid, which inhibits collagen hydrolysis and activates its synthesis, can be distinguished among their metabolites [56-58].

Analysis of the biochemical composition of lysates. According to Table 2, the free amino acids in lysates are represented by the following ranges (mg/100 g of the sample): aspartic acid 0.07-0.23 (except for 1.5 mg/100 g in *L. sakei* 105), serine 0.16-0.31 (except for 8.95 mg/100 g for *L. sakei* 105), histidine 0.8-2.2, arginine 0.9-3.5, alanine 1.7-6.6, valine 0.18-0.70, phenylalanine 0.26-0.39, isoleucine 0.44-0.95, leucine 1.00-1.51, lysine 1.60-2.07, and proline 0.80-1.16. Cystine was found only in representatives of the genus *Pediococcus*, in the *P. pentosaceus* 28 and *P. acidilactici* 38 (0.010 and 0.424 mg/100 g, respectively). Methionine was also recorded in the *P. pentosaceus* 28 and *P. acidilactici* 38 lysates (0.293 and 0.782 mg/100 g), as well as in the *L. sakei* 103 strain (0.072 mg/100 g). Glutamic acid, glycine, threonine, and tyrosine were not detected in any sample. The widest range of free amino acids is presented in the lysate sample from *P. acidilactici* 38, the least – in the lysate sample from *L. sakei* 105. These data are consistent with those presented by Shaikhiev [59] with the results of amino acid analysis of total proteins in three strains of lactic acid bacteria in a culture medium.

2. Free amino acid concentration (mg/100 g of lysate) **in *Lactobacillus*, *Pediococcus*, and *Staphylococcus* strains after ultrasonic disintegration** (*WAM*±SD)

Amino acid	*L. acidophilus* AT-41	*P. pentosaceus* 28	*L. curvatus* 1	*P. acidilactici* 38	*L. curvatus* 2	*L. sakei* 103	*S. carnosus* 108	*L. sakei* 105
Aspartic acid	0.219±0.004	0.214±0.011	0.040±0.002	0.234±0.012	0.228±0.011	0.075±0.004	0.153±0.008	1.500±0.075
Glutamic acid	–	–	–	–	–	–	–	–
Serine	0.167±0.008	–	–	0.302±0.006	0.204±0.010	0.165±0.003	–	8.950±0.447
Histidine	0.898±0.045	1.093±0.055	1.298±0.065	2.166±0.108	1.074±0.054	1.106±0.055	0.874±0.044	–
Glycine	–	–	–	–	–	–	–	–
Threonine	–	–	–	–	–	–	–	–
Arginine	2.058±0.103	2.508±0.125	0.966±0.048	3.449±0.172	1.184±0.024	2.516±0.126	2.190±0.109	–
Alanine	3.260±0.163	3.821±0.191	1.799±0.090	5.139±0.257	6.598±0.330	3.882±0.194	3.235±0.162	–
Tyrosine	–	–	–	–	–	–	–	–
Cystine	–	0.010±0.001	–	0.424±0.021	–	–	–	–
Valine	0.470±0.024	0.460±0.023	0.692±0.035	0.600±0.030	0.527±0.026	0.447±0.022	0.442±0.022	0.185±0.009
Methionine	–	0.293±0.015	–	0.782±0.039	–	0.072±0.004	–	–
Phenylalanine	0.336±0.017	0.272±0.014	0.471±0.024	0.385±0.019	0.364±0.018	0.313±0.016	0.279±0.014	0.265±0.013
Isoleucine	0.452±0.023	0.448±0.022	0.943±0.047	0.930±0.047	0.515±0.026	0.565±0.028	0.575±0.029	0.795±0.040
Leucine	1.153±0.058	1.143±0.057	1.506±0.075	1.305±0.065	1.300±0.065	1.148±0.057	1.109±0.055	1.014±0.051
Lysine	1.505±0.075	1.465±0.073	0.646±0.032	1.659±0.083	2.033±0.102	2.040±0.102	1.652±0.083	0.710±0.036
Proline	1.112±0.056	0.897±0.045	1.160±0.058	0.887±0.044	0.986±0.049	0.966±0.048	0.994±0.050	–
Total amount	11.630±0.582	12.624±0.631	9.521±0.476	18.262±0.913	15.013±0.751	13.295±0.665	11.503±0.575	13.419±0.671

N o t e. A pooled sample was used for analysis (three replicates). Dashes indicate that the indicated amino acid is not detected in the lysate.

In the strains studied by Shaikhiev [59], the qualitative amino acid composition was identical (only 18 amino acids). At the same time, these strains of lactic acid bacteria practically did not differ in the content of leucine, threonine, phenylalanine, isoleucine, methionine, tryptophan, arginine, glutamic and aspartic acids, as well as proline in cultures.

3. Concentration of organic acids (mg/100 g sample) **in lysates of starter cultures after ultrasonic disintegration** (*WAM*±SD)

Strain	Malic	Lactic	Succinic	Total
Lactobacillus acidophilus AT-41	0.0038±0.0002	0.0333±0.0017	0.6915±0.0346	0.7286±0.0340
Pediococcus pentosaceus 28	0.0170±0.0009	0.0436±0.0022	0.5417±0.0271	0.6023±0.0301
Lactobacillus curvatus 1	0.0048±0.0002	0.0474±0.0024	0.0370±0.0019	0.0892±0.0045
Pediococcus acidilactici 38	0.0029±0.0001	0.0575±0.0029	0.2195±0.0110	0.2799±0.0140
Lactobacillus curvatus 2	0.1780±0.0089	0.0345±0.0017	0.5251±0.0263	0.7376±0.0370
Lactobacillus sakei 103	0.0194±0.0010	0.0375±0.0019	0.8692±0.0435	0.9261±0.0463
Staphylococcus carnosus 108	0.0076±0.0004	0.0439±0.0022	0.8223±0.0412	0.8738±0.0437
Lactobacillus sakei 105	0.0640±0.0032	0.0248±0.0012	0.0089±0.0004	0.0977±0.0049

N o t e. A pooled sample was used for analysis (three replicates).

On average, the content of malic acid in lysates (Table 3) varied within 0.002-0.02 mg/100 g, lactic acid within 0.02-0.06 mg/100 g, and succinic acid within 0.2-0.9 mg/100 g of the sample. An exception in terms of malic acid content was *L. curvatus* 2 lysate, where the content of this acid was several orders of magnitude higher than in other samples, the 0.178 mg/100 g. In *L. curvatus* 1 and *L. sakei* 105 lysates, the content of succinic acid, on the contrary, turned out to be several orders of magnitude less than in other samples, the 0.037 and 0.0089 mg/100 g, respectively. There are data in the literature on the study of the ability of lactic acid bacteria to produce organic acids. Thus, after 5 days of liquid fermentation, HPLC revealed acetate, citrate, formate, lactate, and succinate, with lactate and acetate dominating among the fermentation products in the cultures [44, 60].

Results of the proteomic study. In the studied lysates of starter cultures, a number of effects and specificity of the action on the muscle tissue of *Bos taurus* were revealed in comparison with the control (Fig. 3).

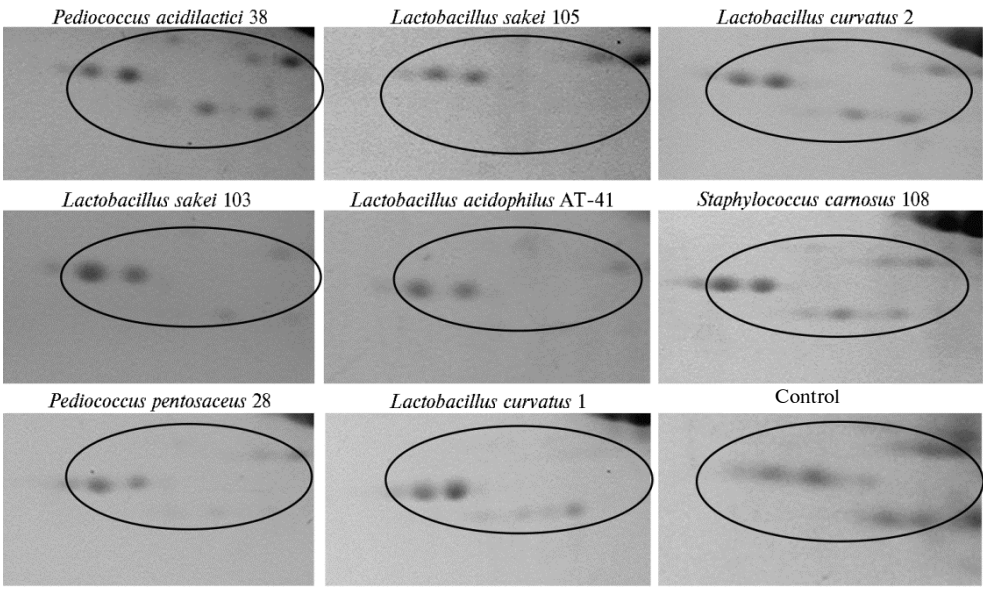

Fig. 3. Fragments of 2D electrophoretograms (IEF-PAGE) **of *Bos taurus longissimus dorsi* muscle tissue proteins after treatment with lysates of bacterial starter cultures.** The ovals enclose the zones of fractions of slow skeletal muscle troponins T. Staining with Coomassie Brilliant Blue R-250 (linear binding of the dye to the protein).

A comparative analysis of the obtained 2D electropherograms showed that when processing meat raw materials with the studied lysates of starting cultures, the amount of protein in the fractions decreased with a certain selectivity. In particular, such an effect was noted for some fractions of transcriptional variants of slow skeletal muscle troponins T (see Fig. 3) – products of the *TNNT1* gene. The position of these fractions and the results of their identification are presented in the information module Proteins of skeletal muscle of cows (*Bos taurus*) of the Proteomics of muscle organs database (http://mp.inbi.ras.ru) (Table 4).

4. Results of mass-spectrometric identification (MALDI-TOF MS and MS/MS) of **protein fractions of *Bos taurus longissimus dorsi* muscle tissue, in which changes appeared after treatment with lysates of starter cultures**

Protein (gene)	Number in Protein NCBI	S/M/C	Мм/pI	
			exp.	calculated
Musculoskeletal regulatory aggregate myosin light chain 2 (*MYL2*)	NP_001069115.1	157/12/82	240.0/5.10	19.0/4.91
Conjugated mixture of titin peptides (*TTN*)[b] (1)	DAA32835.1	59/8/< 1	160.0/6.60	3 713 421.0/6.07
Conjugated muscle peptides creatine phosphokinase (*CPK-M*)[a] (1)	NP_777198.2	216/7/23	200.0/7.40	42.9/6.63
Myoglobin conjugate (*MB*)[a] (1)	NP_776306.1	125/11/69	160.0/7.80	17.0/6.90
Fragment of L-lactate dehydrogenase A (*LDHA*)[a] (1) + Acetyl (Protein N-term)	NP_776524.1	76/24/66	27.0/7.50	36.0/8.12
Fragment of L-lactate dehydrogenase A (*LDHA*)[a] (1) + Acetyl (Protein N-term)	XP_005900750.1	127/9/30	26.5/7.50	36.0/8.12
Fragment of the light chain of myosin 6B (*MYL6B*)	NP_001069181.1	234/26/82	22.0/5.30	23.3/5.40
Musculoskeletal α-actin (*ACTA1*)	NP_001091.1	154/11/38	43.0/5.40	42.0/5.23
Fragment of a.a.s. 21-286 musculoskeletal α-actin (*ACTA1*)[a] (3) + Methyl (75H)	NP_776650.1	413/27/50	28.0/4.90	42.1/5.31
Fragment of a.a.s. 21-256 musculoskeletal α-actin (*ACTA1*)[a] (3) + Methyl (75H)	NP_776650.1	413/37/57	26.0/5.10	42.1/5.31
Fragment of a.a.s. 241-375 musculoskeletal α-actin (*ACTA1*)[a] (3)	NP_776650.1	189/18/	17.0/5.00	42.1/5.31

N o t e. a.a.s. — amino acid sequence. S/M/C: Score — an indicator of compliance, or "score" (Protein scores greater than 68 are significant, $p < 0.05$); Match peptides — number of matched peptides; Coverage — the percentage of coverage of the complete amino acid sequence of the protein by the identified peptides. Mm/pI (exp.) — molecular weight/isoelectric point based on the results of determination of 2D electrophoretic mobility, Mm/pI (calculated) — molecular weight/isoelectric point calculated on the basis of amino acid sequence data, taking into account the removal of the signal peptide using the ExPASy program Compute pI/Mw tool. [a] — msms (indication of confirmatory identification by tandem mass spectrometry, the number of tryptic peptides sequenced is indicated in parentheses). MALDI time-of-flight mass spectrometer Ultraflex (Bruker, Germany) with UV laser, positive ion mode in the mass range of 500-8000 Da.

Initially, in raw meat materials, five fractions are usually present, differing in pI and molecular weight. The higher molecular weight set contains three electrophoretic isoforms, and the lower molecular weight set contains two isoforms. Treatment with lysates of strains *Lactobacillus sakei* 105, *L. acidophilus* AT-41, and *Pediococcus pentosaceus* 28 led to the complete disappearance of low-molecular forms, and with lysates *L. sakei* 103 and *L. curvatus* 1 to a clear decrease in their number, that is, the studied lysates of starter cultures showed a selectivity of action on the actomyosin set of muscle tissue.

We did not find any specific changes in the samples treated with *Pediococcus acidilactici* 38, *L. sakei* 103, *L. acidophilus* AT-41, and *Staphylococcus carnosus* 108 lysates. The action of *L. curvatus* 2 lysate led to the formation of an atypical fraction with a molecular weight ~ of 240 kDa (Fig. 4, see Table 4), identified as an aggregate of the skeletal muscle regulatory light chain of myosin 2, which in monomeric form has a molecular weight of 19 kDa. This fraction on a 2D electrophoretogram is present in large quantities and forms a zone with characteristic mobility, but some of these molecules, under the influence of bacterial lysate, produce the oligomeric form, which consists of about 12 subunits. As a result of treatment with *L. curvatus* 2 lysate, a conjugate (~ 160 kDa) formed

by fragments of the titin protein appeared (see Fig. 4, Table 4). Titin itself has a molecular weight of about 4000 kDa and due to its size is not included in the PAGE plate. Tryptic peptides identified in the 160 kDa fraction are located in different regions of the protein molecule. Therefore, the 160 kDa fraction cannot be anything other than the resulting titin peptide conjugate. In general, it can be assumed that specific proteins and proteases are found in the lysate of the *L. curvatus* 2 strain that affect proteolysis and changes the protein folding.

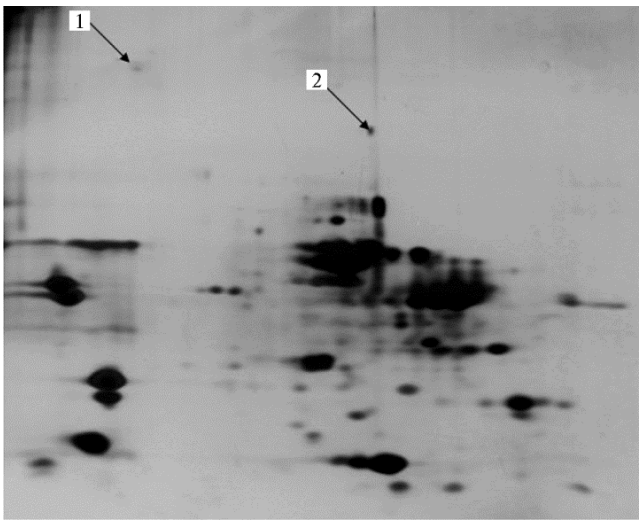

Fig. 4. 2D electrophoretogram (IEF-PAGE) **of *Bos taurus longissimus dorsi* muscle tissue proteins after treatment with a lysate of the *Lactobacillus curvatus* 2:** 1 — an aggregate of the skeletal muscle Myosin regulatory light chain 2 (*MYL2*), 2 — a conjugated mixture of titin peptides (TTN)[b] (1). Silver nitrate staining.

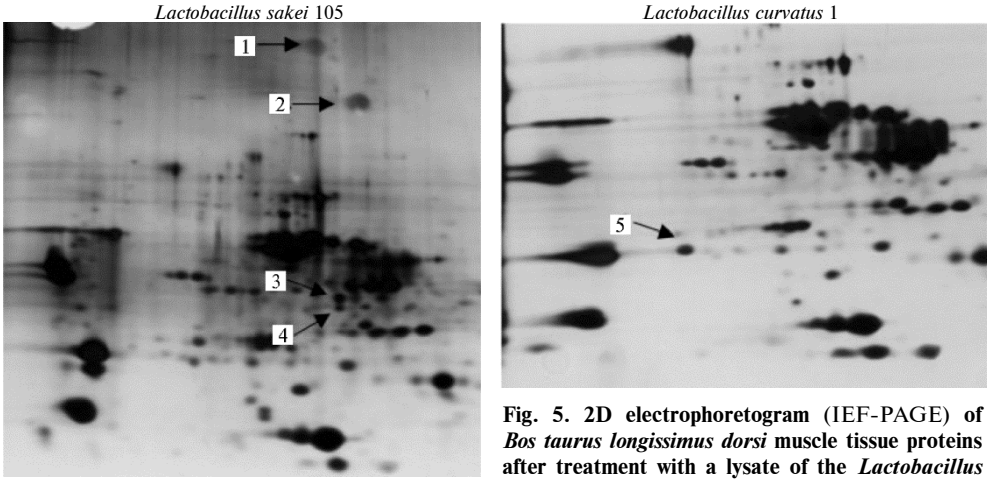

Fig. 5. 2D electrophoretogram (IEF-PAGE) **of *Bos taurus longissimus dorsi* muscle tissue proteins after treatment with a lysate of the *Lactobacillus sakei* 105 и *Lactobacillus curvatus* 1:** 1 — conjugated peptides of muscle creatine phosphokinase, 2 — myoglobin conjugate, 3 and 4 — fragment of L-lactate dehydrogenase A, 5 — fragment of the light chain of myosin 6B (high molecular weight oligomers/conjugates of proteins and fragments of L-lactate dehydrogenase A are indicated by arrows). Silver nitrate staining.

When treated with *L. sakei* 105 lysate, conjugates of muscle creatine phosphokinase and myoglobin were formed, while the monomers were preserved, and fragments of the A subunit of L-lactate dehydrogenase, acetylated at the N-terminus, appeared (Fig. 5, see Table 4). The action of *L. curvatus 1* lysate led to the appearance of a large fragment of the light chain of myosin 6B (see Fig. 5, Table 4), deaminated at several glutamine (Q) residues, which apparently served as a

protective mechanism (judging by the fact that when using other lysates, this fraction was completely destroyed).

Pronounced changes in the structure of actin of raw meat were observed when using lysates of *Staphylococcus carnosus* 108 and *Pediococcus pentosaceus* 28 (Fig. 6, see Table 4). When exposed to the enzyme complex from *Staphylococcus carnosus* 108, the actin fraction completely disappeared while preserving tropomyosins and myosin light chains, and treatment with *Pediococcus pentosaceus* 28 lysate led to the formation of three large actin fragments, two of which represented the N-terminal part of molecules of different lengths, methylated at the amino acid residue 75H. The lower molecular weight component contained a fragment of only the C-terminal part of the molecule, which indicates the presence of a specialized restriction enzyme in this species of microorganisms that recognizes a unique region of the actin amino acid sequence.

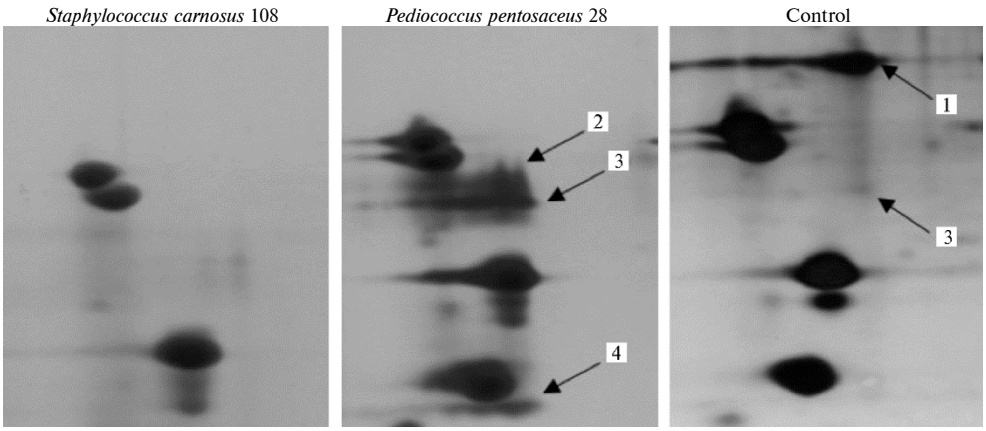

Fig. 6. Fragments of 2D electrophoretograms (IEF-PAGE) **of actin, tropomyosin areas and myosin light chains, illustrating the effect of lysates on the actin fraction:** 1 — musculoskeletal α-actin, 2 — fragment of the amino acid sequence (a.a.s.) 21-286 of musculoskeletal α-actin, 3 — fragment of a.a.s. 21-256 of musculoskeletal α-actin, 4 — fragment of a.a.s. 241-375 of musculoskeletal α-actin. Silver nitrate staining.

In general, it can be concluded that the authors have identified specific proteins − targets of enzyme preparations from different starter cultures.

Previously, we studied the effect of whole starter cultures on muscle tissue of cattle and meat products and found a number of changes in the protein composition [28], in particular, the formation of aggregates of myoglobin and troponin I, especially when using the culture of *Pediococcus pentosaceus* 31. Note that in an early study, the authors analyzed the changes that occur under the influence of living starter cultures, constantly producing sets of specific enzymes. In the presented study, enzymes released from cells during lysis using ultrasonic disintegration were used. Obviously, in preparations of cell lysates, these enzymes have a certain lifetime and a clearly lower concentration. In this case, the effect is naturally less pronounced, which was observed in our experiment, but the general mechanisms are confirmed.

In this work, under the influence of lysates of starter cultures, pronounced changes in proteins of meat raw materials occurred, including with the production of low molecular weight peptides that may have biological activity. During the fermentation of animal proteins by proteolytic enzymes secreted by *Lactobacillus helveticus*, *L. lactis* subsp. *cremoris* FT4, and *L. delbrueckii* subsp. *bulgaricus* SS1, biologically active peptides are formed, including antihypertensive peptides that inhibit angiotensin I-converting enzyme, opioid agonists, and peptide antagonists, as well as mineral binding, immunomodulatory, antibacterial, and antithrombotic

peptides. Angiotensin I-converting enzyme regulates blood pressure through the synthesis of the vasopressor angiotensin II from angiotensin I [44]. The appearance of bioactive peptides depends on the enzymatic activity of the culture; the factors affecting their production are specific for the strain. At the molecular level, the manifestation of the activity of the proteolytic system is influenced by the presence of proteins, amino acids, and carbon [61, 62]. In addition, the enzymatic activity depends on the growth phase of the microorganisms; activity persists during the exponential phase and in the initial period of the stationary phase, but decreases as the stationary phase progresses [63]; changes in enzymatic activity are also associated with the integrity of bacterial cells.

To date, the potential for obtaining bioactive peptides with the participation of starter cultures can be predicted in two ways: using genomic analysis of the components of the proteolytic system of starter cultures to identify various enzymatic activities or subelements and their strategic features [64] and based on an *in silico* approach using quantitative structure-activity relationship assessment methods to analyze the protein matrix that can release bioactive peptides [65]. To predict a large sample of starter culture strains, it is most effective to combine bioinformatics tools with experimental data [42].

The strains *Pediococcus pentosaceus* 28, *Staphylococcus carnosus* 108, *Lactobacillus curvatus* 1, *P. acidilactici* 38, *L. sakei* 103, *L. sakei* 105, *L. curvatus* 2, and *L. acidophilus* AT-41 that we selected to obtain lysates are active producers of many enzymes widely used in the food industry as starter cultures. The strains are selected in such a way that the results for lysates can be compared with those obtained earlier in terms of the effect of the corresponding live cultures on raw meat and finished meat products. For these strains, a complete scheme for obtaining bacterial lysates by the method of ultrasonic disintegration has been developed.

Thus, *Lactobacillus curvatus* 2, *L. acidophilus* AT-41, *Pediococcus acidilactici* 38, and *Staphylococcus carnosus* 108 lysates have the broadest set of intracellular enzymes, including leucine and valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and β-galactosidase. The lysates of *L. acidophilus* AT-41, *L. curvatus* 1, and *L. curvatus* 2 are characterized by the presence of alkaline phosphorylase. In the lysate of *L. acidophilus* AT-41, traces of lipase are found, as well as trypsin, in lysates of *Staphylococcus carnosus* 108 and *Pediococcus acidilactici* 38, traces of esterase lipase. All samples, except for *L. acidophilus* AT-41 lysate, contain β-glucosidase. The highest proteolytic activity was found in the lysates of *Staphylococcus carnosus* 108 (115.94 PC units/mg protein), *L. acidophilus* AT-41 (66.7 PC units/mg protein), *L. curvatus* 1 (91.03 PC units/mg protein) and *L. curvatus* 2 (72.20 PC units/mg protein). During the fermentation of raw meat with lysates of starter cultures, the formation of non-standard protein conjugates was revealed; for some cultures, specific target proteins were determined, in particular, musculoskeletal actin. The data obtained will be used in technologies for processing animal muscle tissue to increase the functionality of products. The continuation and addition of experimental studies should be genomic analysis of the components of the proteolytic system of lactic acid bacteria to identify various enzymatic activities.

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AGRICULTURAL BIOLOGY,
2020, V. 55, № 6, pp. 1204-1219
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

Probiotic additives

UDC 636.2.034:636.087.8:579.6:577.2

doi: 10.15389/agrobiology.2020.6.1204eng
doi: 10.15389/agrobiology.2020.6.1204rus

THE INFLUENCE OF A DIETARY *Enterococcus faecium* STRAIN-BASED ADDITIVE ON THE TAXONOMIC AND FUNCTIONAL CHARACTERISTICS OF THE RUMEN MICROBIOTA OF LACTATING COWS

E.A. YILDIRIM[1] ✉, G.Yu. LAPTEV[1], L.A. ILYINA[1], T.P. DUNYASHEV[1], D.G. TYURINA[1], V.A. FILIPPOVA[1], E.A. BRAZHNIK[1], N.V. TARLAVIN[1], A.V. DUBROVIN[1], N.I. NOVIKOVA[1], V.V. SOLDATOVA[1], S.YU. ZAITSEV[2]

[1]*JSC Biotrof+,* 19, korp. 1, Zagrebskii bulv., St. Petersburg, 192284 Russia, e-mail deniz@biotrof.ru (✉ corresponding author), ilina@biotrof.ru, bea@biotrof.ru, dumova@biotrof.ru, novikova@biotrof.ru, tiurina@biotrof.ru, tarlav1995@biotrof.ru, dubrovin@biotrof.ru; v.soldatova1@yandex.ru.
[2]*Ernst Federal Science Center for Animal Husbandry,* 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail s.y.zaitsev@mail.ru
ORCID:
Yildirim E.A. orcid.org/0000-0002-5846-4844
Laptev G.Yu. orcid.org/0000-0002-8795-6659
Ilyina L.A. orcid.org/0000-0003-2490-6942
Dunyashev T.P. orcid.org/0000-0002-3918-0948
Tyurina D.G. orcid.org/0000-0001-9001-2432
Filippova V.A. orcid.org/0000-0001-8789-9837
Brazhnik E.A. orcid.org/0000-0003-2178-9330
Tarlavin N.V. orcid.org/0000-0002-6474-9171
Dubrovin A.V. orcid.org/0000-0001-8424-4114
Novikova N.I. orcid.org/0000-0002-9647-4184
Soldatova V.V. orcid.org/0000-0002-4984-9609
Zaitsev S.Yu. orcid.org/0000-0003-1533-8680

The authors declare no conflict of interests
Acknowledgements:
Supported financially from Russian Foundation for Basic Research, grant No. 18-016-00207
Received August 10, 2020

Abstract

Today rations for dairy cows are designed to provide the highest growth rate and productivity in a short period of time. However, such intensive livestock farming affects, first of all, the health of animals, since metabolic pathways inherent in ruminants are disrupted. The use of 16S metagenomics approaches makes it possible to assess the genetic and metabolic diversity of the bovine microbiome, which allows identifying factors that can contribute to an increase in productivity and an improvement in the health of the host. In the feeding trial, dairy cows were fed with dietary probiotic Cellobacterin+ based on the *Enterococcus faecium* 1-35 strain (the winter-spring period of 2018, JSC PZ Plamya, Gatchinsky District, Leningrad Province). Two groups of ten Holsteinized black-and-white dairy cows (*Bos taurus taurus*) of the 2nd and 3rd lactation with an average annual milk yield of 7000-7500 kg were used. The basal diet was 10 kg compound feed, 2 kg yellow corn, 0.5 kg sunflower cake, 0.5 kg rapeseed cake, 1 kg hay, 25 kg grass silage, 1 kg beet molasses, and 0.2 kg MINVIT®-3 (Russia). In the morning, the test cows were fed with dietary Cellobacterin+ (OOO BIOTROF, St. Petersburg) at 40 g per cow. Cicatricial contents (10-50 g) were collected from three cows of each group at the end of the experiment. Fasting blood was taken for biochemical analysis from the tail vein with vacutainers. The blood was analyzed for total protein, total bilirubin, glucose, calcium, phosphorus, urea, reserve alkalinity, ketone bodies. The mass fraction of fat in milk was analyzed according to GOST 5867-90, protein according to GOST 23327-98, and the number of somatic cells according to GOST R 54761-2011. Total DNA from the studied samples was extracted using the Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) according to the attached instructions. Amplification for subsequent NGS sequencing was run (a Veriti Thermal Cycler, Life Technologies, Inc., USA) using the eubacterial primers (IDT) 343F (5′-CTCCTACGGRRSGCAGCAG-3′) and 806R (5′-GGACTANVGGGT-WTCTAAT-3′) flanking the V1V3 region of the 16S rRNA gene. Metagenomic sequencing (a MiSeq system, Illumina, Inc., USA) was performed with a MiSeq Reagent Kit v3 (Illumina, Inc., USA). Chimeric sequences were excluded from analysis using the USEARCH 7.0 program (http://drive5.com/usearch/). The processing of the obtained reads using the bioinformatics platform CLC Bio GW 7.0 (Qiagen, the Netherlands) included overlapping, quality filtering (QV > 15), and primer trimming. The taxonomic affiliation of microorganisms to genus was determined using the RDP Classifier program (http://rdp.cme.msu.edu/). Mathematical and statistical processing of the results was carried out using the software packages Microsoft Office Excel 2003, R-Studio (Version 1.1.453)

(https://rstudio.com). The mean values (*M*) and standard errors of the means (±SEM) were calculated. The results were deemed significant at $p < 0.05$. Analysis of microbial β-diversity of the samples by the principal component method was performed according to the Weighted UniFrac PCoA Emperor method using the QIIME software package. Reconstruction and prediction of the functional content of the metagenome, gene families, and enzymes was performed using the PICRUSt2 software package (v.2.3.0). MetaCyc database (https://metacyc.org/) was used to analyze metabolic pathways and enzymes. Feeding the probiotic had a significant effect ($p = 0.049$) on an increase in milk yield, as well as on a decrease ($p = 0.003$) in the somatic cell number in milk by 38,000/ml per cow. The NGS sequencing provided a complete taxonomic and functional characterization of the cicatricial microbiota, including uncultivated representatives. Significant differences were found between the groups for 13 bacterial genera. In particular, in the rumen of cows treated with the probiotic Cellobacterin+, compared to the control group, a lower proportion of the order *Clostridia* were found, namely the bacteria of the genera *Anaerofilum* sp. (2.3 times lower, $p \leq 0.05$) and *Anaerostipes* sp. (1.8 times lower, $p \leq 0.05$) that produce lactate in the rumen as the end product of glucose metabolism. A decrease occurred in the abundance of the genera *Campylobacter*, *Gemella*, *Mycoplasma*, *Shewanella* ($p \leq 0.05$), and *Fusobacterium* (including *F. necrophorum*) ($p \leq 0.001$) among which pathogens are often found. Changes in the taxonomic structure of rumen microbiota as influenced by the probiotic were also associated with metabolic changes. The predicted functional potential of seven metabolic pathways was enhanced in cows fed Cellobacterin+ compared to the control animals. Thus, when fed Cellobacterin+, there was a 3.5-fold increase ($p \leq 0.05$) in the predicted level of microbiome metabolic capabilities associated with the synthesis of glyoxylate from allantoin, and 2.3-fold increase ($p \leq 0.05$) in the biosynthesis of propionate from L-glutamate. These findings allow us to suggest an important role of the biological product Cellobacterin+ for maintaining the homeostasis of metabolic processes.

Keywords: biologicals, Cellobacterin+, lactating cows, rumen, 16S metagenomics, NGS sequencing, metabolism

Ruminants hold a specific place among other farm animals due to the unique features of the digestive system functioning. The rumen is inhabited by a large microbial community consisting of bacteria, archaea, and micromycetes, which allows the animal to use lignocellulosic material and convert non-protein nitrogen into microbial protein as a source of energy and amino acids [1, 2]. During polysaccharide fermentation, short-chain (so-called volatile) fatty acids are formed – acetate, butyrate, propionate, and others, which are absorbed through the rumen epithelium and are used by animals to maintain metabolism.

The rumen of ruminants is inhabited by a variety of traditionally non-culturable bacteria; therefore, it is often difficult to draw the right conclusions about their physiology and functions [3]. At the same time, these microorganisms can carry genes that determine a significant part of the microbiome metabolic diversity, play a decisive role in non-starchy polysaccharide and protein fermentation, synthesis of biologically active substances, engage in active intermicrobial interactions, and have a significant effect on the macroorganism. The advent of 16S metagenomics methods made it possible to establish DNA sequences for the whole population of microorganisms from natural sources and to obtain a complete taxonomic and functional characterization of cicatricial microbiota, regardless of the microorganism cultivation possibility [4]. High-throughput sequencing can assess the bovine microbiome genetic and metabolic diversity and identify factors that contribute to both ecological balance and host health [5].

Modern diets for dairy cows are designed to ensure the maximum growth rate and productivity in a short period [6, 7]. However, intensive technology affects primarily animal health since it disrupts the metabolic pathways inherent in ruminants. Because cicatricial microorganisms are practically the only enzyme source for plant feed digestion, as well as direct participants in metabolism, a violation of the rumen microbiocenosis composition can lead to many negative consequences. Conversely, a directed change in the rumen microbiota is accompanied by positive shifts in productivity, quality characteristics of milk, reproduction, and the duration of economic use, which can become one of the key factors in increasing dairy farming efficiency [8].

Due to the role of ruminants as producers of methane released into the atmosphere, researchers were focused mainly on methanogen microorganisms [9, 10]. However, at present, due to the physiology and nutrition problems of highly productive animals, there is a need to study the microbiome structure and metabolic pathways implemented by the cicatricial microbiota.

The regulating strategies for the microbiome composition include the nutritional intervention of feed additives (probiotics, prebiotics, phytobiotics, etc.) into the livestock diet [11-13]. The positive probiotic effects on the rumen microbiome are mainly associated with their positive effects on digestive processes, especially on cellulose digestion and microbial protein synthesis. *Saccharomyces cerevisiae* is the most popular yeast species used for rumen introduction [12]. As for probiotics based on bacteria, according to Fernández et al. [13], the use of genus *Lactobacillus* bacteria can become an alternative in the treatment and prevention of some diseases affecting ruminants. Thus, the calve nutritional intervention of *L. johnsonii* TP1.1, *L. reuteri* TP1.3B, *L. johnsonii* TP1.6, and *L. amylovorus* TP8.7 strains reduced the severity of diarrhea symptoms [13].

Although the positive effect of dietary supplements on the bovine rumen microbiome is well known, the need to study the properties of existing and new probiotics for animals remains high.

In this work, using PICRUSt2 and MetaCyc software, the fact was established of an increase in the predicted functional potential of some metabolic pathways in the cicatricial microbiota of cows fed dietary probiotic Cellobacterin+, containing *Enterococcus faecium* 1-35 strain.

The goal of the research was to assess the effect of the probiotic Cellobacterin+ on zootechnical indices, the cicatricial microbiome and its functional potential in dairy cows.

Methods. The experiment was carried out in the winter-spring period (JSC PZ Plamya, Leningrad Province, Gatchinsky District, 2018). Two groups (10 heads each) of Holsteinized Black-and-White dairy cows (*Bos taurus taurus*) of 2nd and 3rd lactation and 7000-7500 kg average annual milk yield were formed. The animals were kept in the same tie-stall barn.

The main diet included 10 kg of feed concentrate, 2 kg of yellow corn, 0.5 kg of sunflower cake, 0.5 kg of rapeseed cake, 1 kg of dry forage, 25 kg of grass silage, 1 kg of beet molasses, and 0.2 kg of MINVIT®-3 (AgroBalt Trade, Russia). The probiotic Cellobacterin+ (BIOTROF LLC, St. Petersburg), which included *Enterococcus faecium* 1-35 strain, was added at 40 g/head to the diet of the test group cows in the morning. Earlier, the dosage was tested on dairy cows [14]. The test duration was 60 days after a preparatory period of 15 days.

At the end of the experiment, samples of ruminal digesta (10-50 g) were aseptically taken manually from three cows of each group with a sterile probe. Simultaneously, fasting blood was taken for biochemical analysis from the tail vein using vacutainers. In the blood serum, total protein, total bilirubin, glucose, calcium, phosphorus, urea, reserve alkalinity, and ketone bodies were determined by standard techniques [15]. The mass fraction of fat in milk was analyzed according to GOST 5867-90, protein according to GOST 23327-98, and the counts of somatic cells according to GOST R 54761-2011.

Total DNA was isolated using the Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) according to the attached instructions. The method is based on selective detergent-mediated precipitation of DNA from a substrate using solutions for cell wall lysis and DNA precipitation, 1.2 M sodium chloride, and chloroform. Amplification (Veriti Thermal Cycler, Life Technologies, Inc., USA) for subsequent next-generation sequencing (NGS) was performed with eubacterial

primers (IDT) 343F (5′-CTCCTACGGRRSGCAGCAG-3′) and 806R (5′-GGACTACNVGGGGTWTC-3′) flanking the V1V3 site of the 16S rRNA gene. Amplification mode: 3 min at 95 °C (1 cycle); 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C (25 cycles); 5 min at 72 °C (1 cycle).

Metagenomic sequencing (MiSeq® system, Illumina, Inc., USA) was performed using MiSeq Reagent Kit v3 (Illumina, Inc., USA). The maximum length of the obtained sequences was 2×300 bp. Chimeric sequences were excluded from analysis using the USEARCH 7.0 program (http://drive5.com/usearch/). The processing of the obtained reads using the CLC Bio GW 7.0 bioinformatics platform (Qiagen, the Netherlands) included overlap testing, quality filtering (QV > 15), and primer trimming. The taxonomic affiliation of microorganisms to the genus was determined using the RDP Classifier program (http://rdp.cme.msu.edu/).

The α-biodiversity Chao1 index of the rumen microbiome was calculated [16]. Analysis of the microbial β-diversity by the method of principal components was carried out according to the Weighted UniFrac PCoA Emperor method using the QIIME software package [17]. Reconstruction and prediction of the metagenome functional content, gene families, and enzymes was carried out using the PICRUSt2 software package (v.2.3.0) [18]. The MetaCyc database (https://metacyc.org/) [19] was used to analyze metabolic pathways and enzymes. MetaCyc metabolic pathway profiles were assessed after normalization of the abundance of amplicon sequence variants using binary logarithm ($\log_2$) [18].

Mathematical and statistical processing of the results was carried out using the Microsoft Office Excel 2003 and R-Studio (Version 1.1.453) (https://rstudio.com) software packages. The mean values (M) and standard errors of the means (±SEM) were determined. Statistical analysis results were considered significant at $p < 0.05$.

Results. Dietary probiotic Cellobacterin+ did not have a statistically significant effect on fat and protein levels in milk, but there was a definite tendency of its positive effect on these indices (Table 1). However, feeding with Cellobacterin+ significantly influenced ($p = 0.049$) an increase in milk yield, as well as a decrease ($p = 0.003$) in the number of somatic cells in milk (by 38 thousand $\cdot$ ml^{-1} $\cdot$ head^{-1}). Previously, similar results were obtained on dairy cows by Spaniol et al. [20] who reported that the nutritional intervention of probiotics did not affect the milk biochemistry, but led to a decrease in somatic cells on day 15 of the experiment. In the work of Australian authors [21], the average daily milk yield of cows that consumed grass on pastures treated with probiotic bacterial strains was 1.21 l higher than that of control animals.

According to several studies based on the 16S rRNA gene characteristic, it was assumed that bovine mastitis with an increase in somatic cells in milk was the result of an imbalance between the normal biota of the mammary gland and pathogens [22]. Perhaps the decrease in milk somatic cells in the authors' test was associated with the modulation of the animal's immune system under the influence of a bacterium probiotic strain. Previously, it was demonstrated that through interaction with monocytes, macrophages, and dendritic cells, probiotics could modulate the balance of helper T-cells and thus influence the adaptive immune response [23, 24]. Other researchers [20] have shown that probiotic administration is associated with an increase in circulating cytokines (tumor necrosis factor, interleukin-4, and interferon) in the bovine blood.

Almost all of the studied biochemical parameters of the bovine blood were within the normal range or did not significantly exceed their limits (Table 2). The biochemical blood profiles of cows in the control and experimental groups did not

differ significantly.

1. Milk productivity of Holsteinized black-and-white dairy cows (*Bos taurus taurus*) fed dietary probiotic Cellobacterin+ (*M*±SEM, JSC PZ Plamya, Leningrad Province, Gatchinsky District, 2018)

Parameter	Control group (n = 10)	Treatment group (n = 10)	p values between groups
Average daily milk yield of natural milk, kg	31.7±1.50	33.3±1.60	0.049
The fat content of milk, %	3.68±0.150	3.97±0.200	0.260
The protein content of milk, %	2.88±0.170	3.14±0.140	0.250
Average daily milk yield of 4% fat, kg	29.2±1.20	33.0±1.40	0.048
Somatic cells, thousand · ml^{-1} · head^{-1}	163±8.5	125±6.9	0.003

N o t e. See the group description in the Methods section.

2. Blood biochemical parameters of Holsteinized black-and-white dairy cows (*Bos taurus taurus*) fed dietary probiotic Cellobacterin+ (*M*±SEM, JSC PZ Plamya, Leningrad Province, Gatchinsky District, 2018)

Parameter	Control group (n = 10)	Treatment group (n = 10)	p values between groups	Standard
Total protein, g/l	78.3±4.10	81.8±4.90	0.62	70-89
Albumin, % of total protein	50.7±2.90	40.9±2.00	0.07	38-50
Total bilirubin, mmol/l	2.2±0.20	2.33±0.110	0.6	0.17-5.13
Glucose, mmol/l	2.19±0.100	2.28±0.130	0.62	2.22-3.33
Calcium, mmol/l	2.28±0.140	2.37±0.190	0.73	2.6-3.5
Phosphorus, mmol/l	2.52±0.200	2.09±0.100	0.07	1.29-2.25
Alkali reserve, vol.% CO_2	57.8±2.30	55.0±2.30	0.45	46-56
Urea, mmol/l	4.4±0.30	3.77±0.150	0.16	3.3-6.7
Ketone bodies	–	–		–

N o t e. See the group description in the Methods section. Dashes indicate that no ketone bodies were detected.

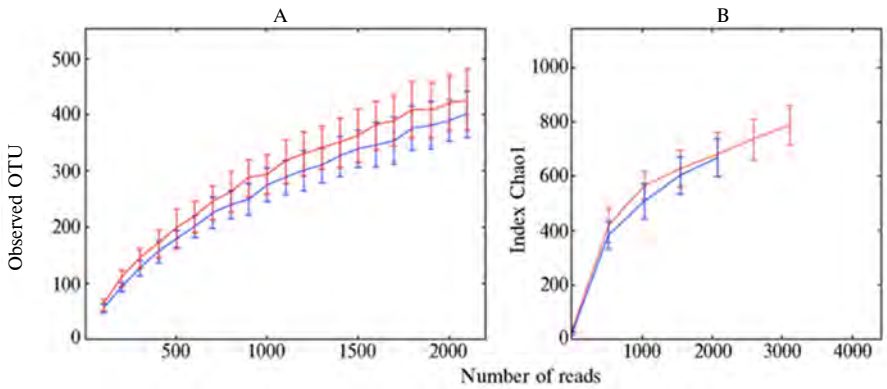

Fig. 1. α-Biodiversity of the rumen microbiome in Holsteinized Black-and-White dairy cows (*Bos taurus taurus*) in the control (blue graph) **and when fed dietary probiotic Cellobacterin+** (red graph): A — variation of operational taxonomic units (OTU), B — Index Chao1 (*M*±SEM, JSC PZ Plamya, Leningrad Province, Gatchinsky District, 2018).

Based on the NGS sequencing data, the parameters of the rumen microbiome α-biodiversity which was characterized by the abundance of operational taxonomic units (OTUs) within communities [25, 26] were calculated (Fig. 1). There were no significant differences in the number of OTUs and the Chao1 index between the test and control variants.

The results of the assessment of β-diversity, that is, a diversity between communities [25, 26], are presented as a three-dimensional graph of the PCoA Emperor (Fig. 2). The principal component PC1 described 67.97% of the data, PC2 described 15.96%, PC3 described 7.87%, i.e., in general, the method made it possible to characterize the changes in the microbiome, while retaining 91.8% of the information.

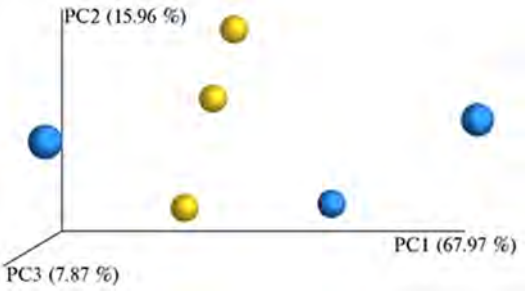

Fig. 2. Principal component analysis of β-diversity between rumen microbiomes in Holsteinized Black-and-White dairy cows (*Bos taurus taurus*) (one point corresponds to one animal) **in the control** (yellow balls) **and when fed dietary probiotic Cellobacterin+** (blue balls) (JSC PZ Plamya, Leningrad Province, Gatchinsky District, 2018).

Comparison of the cicatricial microbiota of cows from different groups using the method of principal components showed that the microbiomes of three cows from the control group were combined into one cluster, and the microbiomes of cows from the test group partially formed their cluster, which may indicate the probiotic effect on the microbiome structure. Nevertheless, clustering was more pronounced in the control than in the probiotic group, i.e., the shift along the axis of the PC1 component was less.

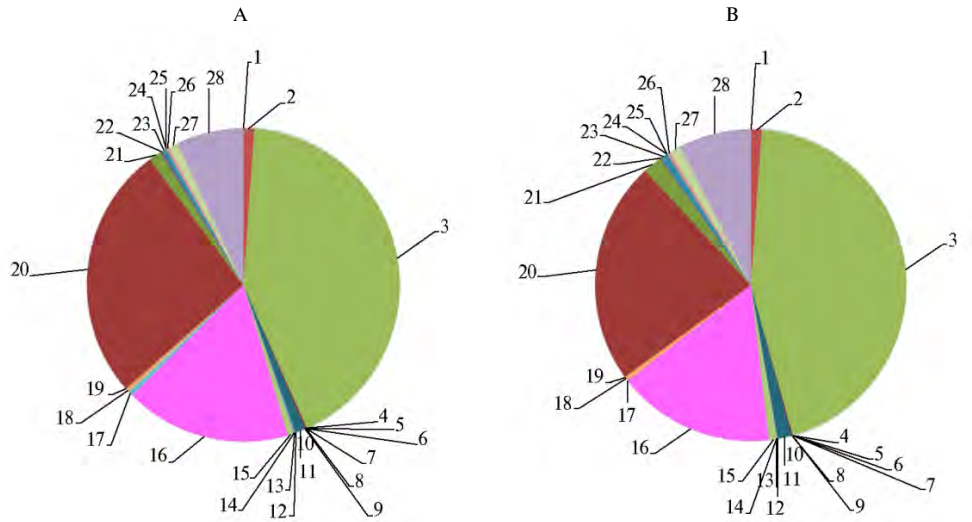

Fig. 3. Microorganisms (the phylum level) of the rumen of Holsteinized Black-and-White dairy cows (*Bos taurus taurus*) in the control (A) **and when fed dietary probiotic Cellobacterin+** (B) (data of NGS sequencing)**:** 1 — *Acidobacteria*, 2 — *Actinobacteria*, 3 — *Bacteroidetes*, 4 — *Caldiserica*, 5 — *Calditrhrix*, 6 — *Chlamydiae*, 7 — *Chlorobi*, 8 — *Chloroflexi*, 9 — *Chrysiogenetes*, 10 — *Crenarchaeota*, 11 — *Cyanobacteria*, 12 — *Deferribacteres*, 13 — *Elusimicrobia*, 14 — *Euryarchaeota*, 15 — *Fibrobacteres*, 16 — *Firmicutes*, 17 — *Fusobacteria*, 18 — *Nitrospirae*, 19 — *Planctomycetes*, 20 — *Proteobacteria*, 21 — *Spirochaetes*, 22 — *Synergistetes*, 23 — *Tenericutes*, 24 — *Thermi*, 25 — *Thermodesulfobacteria*, 26 — *Thermotogae*, 27 — *Verrucomicrobia*, 28 — unidentified (JSC PZ Plamya, Leningrad Province, Gatchinsky District, 2018).

According to estimates of taxonomic confinement of the microbiota in the rumen of the test cows, 27 phyla were found of which *Bacteroidetes* (42.2±2.9 to 44.5±3.1%), *Proteobacteria* (23.2±1.5 to 26.3±1.9%), and *Firmicutes* (16.3±0.9 to 17.2±1.2%) were dominant (Fig. 3). In the phylum *Bacteroidetes*, *Prevotella* bacteria prevailed (26.4±1.8 to 27.0±2.3%). Previously, the dominance of this genus of microorganisms in the rumen of ruminants has been repeatedly shown [27, 28]. Bacteria of the genus *Prevotella* play an important role in carbohydrate and nitrogen metabolism; succinate is one of the final products of their metabolism [29]. It was found that extracellular succinate in the rumen served as the main propionate precursor [30], the most important substrate for gluconeogenesis in ruminants [31]. As previously identified with the sheep rumen microbiome, most of the genus *Prevotella* bacteria are represented by uncultivated forms [32].

Bacteria in the rumen that did not belong to any known taxon from the

existing ones (according to the databases of the 16S RNA gene sequences) ranged from 6.9±0.5 to 7.5±0.8% (see Fig. 3).

No significant differences between the variants at the phylum level could be found (see Fig. 3). However, a detailed analysis of the rumen microbiome revealed significant differences between the groups for 13 genera of bacteria (Fig. 4).

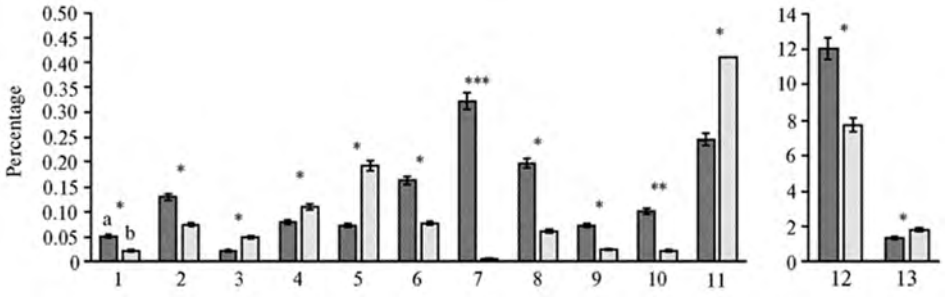

Fig. 4. Microorganisms (the genus level) of the rumen of Holsteinized Black-and-White dairy cows (*Bos taurus taurus*) in the control (a) **and when fed dietary probiotic Cellobacterin+** (b) (data of NGS sequencing)**:** 1 — *Anaerofilum*, 2 — *Anaerostipes*, 3 — *Anaerovibrio*, 4 — *Bdellovibrio*, 5 — *Bifidobacterium*, 6 — *Campylobacter*, 7 — *Fusobacterium*, 8 — *Gemella*, 9 — *Mycoplasma*, 10 — *Odoribacter*, 11 — *Pseudobutyrivibrio*, 12 — *Shewanella*, 13 — *Lachnospira* (*M*±SEM, JSC PZ Plamya, Leningrad Province, Gatchinsky District, 2018).

*, **, *** Differences between groups are statistically significant at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0{,}001$, respectively.

In particular, in the rumen of cows fed Cellobacterin+ as compared to the control group, we detected a lower proportion of representatives of the order *Clostridia*, bacteria of the *Anaerofilum* sp. (2.3 times lower, $p \leq 0.05$) and *Anaerostipes* sp. (1.8 times lower, $p \leq 0.05$). Representatives of these genera produce lactate as the final product of glucose metabolism [33, 34]. Our observations may indicate a positive role of the probiotic in the health control of cows, since, during high-concentration feeding in animals, dysbiotic disorders of the cicatricial microflora often occur with a shift in metabolism towards the lactate synthesis [35]. Lactate excess correlates with decreased rumen pH and lactate acidosis [35]. Associated with acidosis, as a resulting suppression of pH-sensitive producers of volatile fatty acids, such as *Selenomonas ruminantium* and *Megasphaera elsdenii* [36], the beneficial metabolite synthesis in the rumen decreases. Similarly, bacteria synthesizing cellulases decrease which leads to disruption of the feed non-starch polysaccharide digestion [35]. The obtained results are consistent with those of Goto et al. [37] who showed that the nutritional intervention of a multistrain bacterial probiotic for cows with induced subacute rumen acidosis caused a decrease in lactic acid in the rumen fluid.

In the rumen of animals treated with the probiotic, we found a decrease in the representativity of genera *Campylobacter*, *Gemella*, *Mycoplasma*, *Shewanella* ($p \leq 0.05$), and *Fusobacterium* ($p \leq 0.001$), among which pathogens are often found. Data on a decrease in the abundance of the genera *Campylobacter* and *Fusobacterium* in the test group animals are consistent with the above results on a decrease in somatic cells in milk, since it has been proven [38, 39] that these microorganisms are associated with mastitis in cattle. The 60.5-fold increase ($p \leq 0.001$) in abundance of *Fusobacterium* bacteria, represented mainly by *F. necrophorum*, observed in the control group cows, could be associated with an increase in the abundance of lactate-producing microorganisms in the rumen. The thing is, low acidity values are optimal for the *F. necrophorum* pathogen development for which lactic acid is the main nutrient substrate. *F. necrophorum* is an opportunistic pathogen causing necrotic rumen lesions (necrobacteriosis), laminitis, and liver

abscesses [40]. The presence of genus *Campylobacter* bacteria in milk can be dangerous to humans, as *C. jejuni* and *C. coli* can initiate gastrointestinal campylobacteriosis. *C. fetus* bacteria are associated with infertility and abortion in cattle [41].

It is known that the genus *Gemella* bacteria which decreased by 3.3 times ($p \leq 0.05$) as a result of the use of Cellobacterin+ are associated with respiratory tract infections and bacteremia [42]. Similarly, *Mycoplasma* representatives, in particular *M. bovis*, cause chronic bronchopneumonia with caseous and coagulative necrosis, as well as arthritis in cattle and calves [43]. Genus *Shewanella* (*S. haliotis* and *S. upenei*) bacteria were isolated from the lung tissue of people with respiratory infection and bacteremia [44]. An increase in the pulmonary pathogen pool in the rumen of control group animals may indicate an intercommunication between microbiomes with different localizations in the host organism and the existence of the rumen—respiratory tract axis, as well as the possible rumen microbiome interference during respiratory diseases. Previously, it was shown in rats that fecal transplantation of the microbiome induced changes in the lung microbiota [45].

The data obtained indicate the role of probiotic bacterial strains in not only the microbiota homeostasis but also the macroorganism health.

A decrease in the abundance of undesirable forms of microorganisms as a result of probiotic exposure could be associated with direct antagonism through the production of antimicrobial metabolites (bacteriocins, organic acids) [46], as well as with modulation of the indigenous microbiota composition and activity under the influence of a strain in the biological product. So, as a result of the Cellobacterin+ use, in the rumen, the number of *Bifidobacterium* and *Bdellovibrio* increased. *Bifidobacterium* is widely known in response to pronounced antimicrobial properties against a wide range of pathogens [47]. Representatives of the genus *Bdellovibrio*, i.e., *B. bacteriovorus*, are predatory microorganisms that control such pathogens as *Salmonella* sp. and *Escherichia coli* [48].

The obtained results demonstrating the modulating effect of the probiotic on the microflora, which was expressed in a decrease in the pathogenic forms and an increase in the abundance of microorganisms with antimicrobial activity, are consistent with the data obtained on calves [49]. The use of boluses based on *Pediococcus acidilactici*, *Enterococcus faecium*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum* helped to reduce diarrhea in animals.

An increase ($p \leq 0.05$) in bacteria of the families *Lachnospiraceae* (*Pseudobutyrivibrio* sp. and *Lachnospira* sp.) and *Selenomonadaceae* (*Anaerovibrio* sp.) in the rumen of cows from the test group could also make a positive contribution to the activation of metabolic processes. Genus *Pseudobutyrivibrio* bacteria were represented by the species *P. xylanivorans* which has a potent xylanolytic enzyme system with at least seven different xylan hydrolases (27-145 kDa) [50]. In this regard, it can ferment xylan polysaccharide in feed. The final product of its metabolism is volatile fatty acids, which are important for the metabolism, health, and productivity of animals, as well as bacteriocin-like inhibitory substances that are active against pathogens. Genus *Lachnospira* microorganisms were represented by the species *L. pectinoschiza* which shows a pronounced ability to ferment pectin by extracellular pectin methylesterase and Ca^{2+}-dependent exopolygalacturonate lyase [51]. The final product of the metabolism of bacteria of the genus *Lachnospira* is acetic acid as the main substrate for de novo lipid synthesis, in particular in the mammary glands of lactating cows.

The results of measuring the lipolysis rate with *Anaerovibrio* sp. pure cultures including *A. lipolytica* [52] showed that these bacteria played an important role in the ruminal digesta lipolytic activity. In this case, the fermentation products

include such important compounds as propionate, which is produced along the path of the dicarboxylic acid conversion to succinate. An increase in propionate biosynthesis can be associated with an increase in milk production in cows treated with the probiotic [30]. In addition, short-chain fatty acids produced by bacteria have other important properties. For example, they are involved in the epigenomic regulation of interactions between the microbiota and the host macroorganism [53]. It has long been known that epigenetic modifications can regulate gene expression, affecting its intensity and duration, without changes in the DNA sequence.

These study results are logical since we have previously described the mechanisms of the positive effect of Cellobacterin+ on the rumen and intestine microbiota [14]. These mechanisms are expressed in the ability of bacterial strains in a biological product to produce low molecular weight organic acids and other biologically active substances including antimicrobial factors. We have shown that the synthesis of xenobiotic biodegradation enzymes in the rumen results in the detoxification of feed mycotoxins with antimicrobial activity against the normal biota [14]. Consequently, the appearance of new metabolites in the rumen due to the introduction of a probiotic strain leads to changes in microorganisms.

Using the PICRUSt2 and MetaCyc software packages, the authors reconstructed and predicted the functional content of the metagenomic community of the bovine rumen. Changes in the taxonomic structure of rumen microorganisms under the influence of the biological product were associated with metabolic changes. The predicted functional potential of seven metabolic pathways was enhanced in cows fed Cellobacterin+ (Fig. 5).

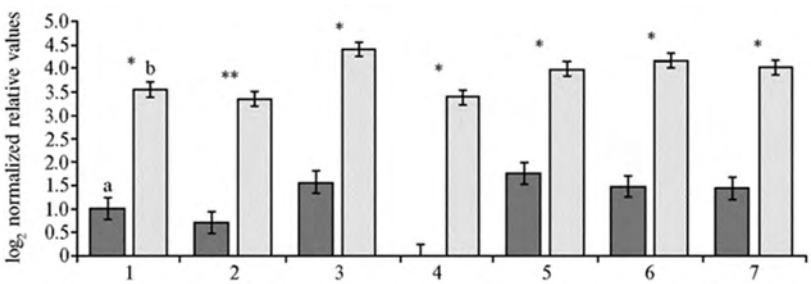

Fig. 5. Functional annotation of metabolic pathways in the rumen metagenomic community of Holsteinized Black-and-White dairy cows (*Bos taurus taurus*): 1 — glyoxylate synthesis, 2 — urea synthesis, 3 — γ-amino-N-butyrate synthesis, 4 — peptidoglycan synthesis, 5 — propionate synthesis, 6 — succinyl-CoA synthesis, 7 − succinate synthesis (PICRUSt2 and MetaCyc processing; *M*±SEM, JSC PZ Plamya, Leningrad Province, Gatchinsky District, 2018).
*, ** Differences between groups are statistically significant at $p \leq 0.05$ и $p \leq 0.01$, resprctively.

In particular, in cows from the test group, as compared to the control, the predicted metabolic capabilities of the microbiome increased 3.5 times ($p \leq 0.05$) that was associated with the synthesis of glyoxylate from allantoin by allantoinase (EC 3.5.2.5), allantoin amidohydrolase (EC 3.5.3.9), ureidoglycine aminohydrolase (EC 3.5.3.26), and ureidoglycolic lyase (EC 4.3.2.3). The initial substrate of the cycle is allantoin, a product of purine catabolism. Allantoin is rich in nitrogen, and many microorganisms can process it. The glyoxylate as a result of allantoin transformations serves as a substrate for the glyoxylate cycle (two-carbon acid cycle). The principal possibility of the glyoxylate cycle in the rumen is associated with the catalytic activity of key enzymes, i.e., isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2) [54]. These enzymes allow glucose synthesis which is deficient for physiologically hypoglycemic ruminants from acetic acid produced in high concentrations in the rumen. Compared to the tricarboxylic acid cycle, the pathway for the bicarboxylic acid oxidation is less energy-consuming and more

efficient, since it is a shorter cycle that can function as a tricarboxylic acid cycle excluding the rate-limiting reactions with isocitrate dehydrogenase and α-keto-glutarate dehydrogenase [54]. The ability of cattle gastrointestinal microorganisms to carry out the glyoxylate cycle can be considered as a factor contributing to metabolism intensification and an increase in productivity. Another reaction to the nutritional intervention of the probiotic Cellobacterin+ detected by bioinformatic data processing is the activation (4.8 times, $p \leq 0.01$) of the allantoin conversion through ureidoglycolate into urea. It is well known [55] that in ruminants, endogenous urea is partially recirculated in the body and used for the synthesis of a high-value microbial protein absorbing in the host small intestine.

In animals from the experimental group, there was a 2.8-fold activation ($p \leq 0.05$) of the potential of the rumen microflora associated with the synthesis of γ-amino-N-butyrate from L-ornithine previously studied in detail by Kurihara et al. [56]. γ-Amino-butyrate is the main inhibitory neurotransmitter in the mammal central nervous system, has an etiotropic effect on the health and growth rate of calves [57], and has a protective effect against neurotoxicant-induced cell death [58]. It is well known that genus *Bifidobacterium* bacteria actively produce γ-amino-N-butyrate from L-ornithine [59] the abundance of which increased in the current experiment in the rumen of animals fed dietary probiotic Cellobacterin+.

With the nutritional intervention of Cellobacterin+, the metabolic capabilities of the microbiome associated with the propionate biosynthesis from L-glutamate increased 2.3 times ($p \leq 0.05$). This pathway was first described for two members of the family *Veillonellaceae — Anaeromusa acidaminophila* and *Barkera propionica* [60, 61]. We detected bacteria of the family *Veillonellaceae* in the rumen of cows from the control and test groups; however, no significant differences in their content could be identified. Propionic acid being involved in gluconeogenesis becomes the main glucose source in the blood of ruminants [62]. Dietary Cellobacterin+ also activated synthesis of the important compounds, e.g., succinate through L-arginine, putrescine, and γ-amino-N-butyrate. Succinate is involved in the tricarboxylic acid cycle and serves as the main propionate precursor produced in the rumen [30].

Compared to the control group, the use of Cellobacterin+ increased 2.8 times ($p \leq 0.05$) the microbiome metabolic capabilities associated with the biosynthesis of succinyl-CoA from phenylacetate, which is a thioester of dicarboxylic succinic acid and coenzyme. The existence of a similar pathway in bacteria was reported as early as 1955 [63]. Ring dearomatization occurs through the conversion of phenylacetyl-CoA to 2-(1,2-epoxy-1,2-dihydrophenyl) acetyl-CoA with the participation of phenylacetyl-CoA 1,2-epoxidase (EC 1.14.13.149). Further, the reactive non-aromatic epoxide is isomerized to the seven-membered o-heterocyclic enol ether (2-oxepin-2(3H)-ylideneacetyl-CoA), as a result, the ring is cleaved. The rest of the pathway consists of β-oxidative steps leading to the formation of succinyl-CoA [64]. It is well known that succinyl-CoA is involved in many biochemical pathways, in particular, it serves as the Krebs cycle intermediate [65] and a precursor for the synthesis of α-aminolevulinic acid, a specific intermediate in the porphyrin synthesis.

An increase in the predicted metabolic capabilities of the microbiome associated with the synthesis of glyoxylate, γ-amino-N-butyrate, propionate, urea, peptidoglycan, succinyl-CoA, and succinate, identified in cows fed dietary Cellobacterin+, confirms the important role of the biological product for maintaining the metabolism homeostasis, health, and productivity of animals. This is a valuable scientific and practical conclusion since modern intensive livestock farming methods require the inclusion of a significant amount of starch in the diet, which puts

the animal at the risk of metabolic disorders, the occurrence of diseases, and a decrease in product longevity. The explanation of the data obtained on the increase in the potential of physiological and biochemical processes in the bovine rumen also requires an in-depth analysis of the complex interactions between microbiota and the macroorganism. Cellobacterin+ may also be useful for immunobiological effects in breast diseases, including mastitis, but further testing is needed to confirm this assumption.

Thus, biopreparations based on microorganisms effectively modulating the microbial community expand the list of tools for modifying the microbiome structure. In Russia, since the entry into force in 2020 of Law No. 280-FZ of August 3, 2018 "On Organic Products and Amendments to Certain Legislative Acts of the Russian Federation", interest in such natural supplements has sharply increased due to the restriction on the use of antibiotics (except for drugs permitted by the national, interstate, and international standards in the field of organic production in force in the Russian Federation).

So, the nutritional intervention of the probiotic Cellobacterin+ for dairy cows led to a significant ($p = 0.049$) increase in milk yield, as well as to a decrease in somatic cells in milk (by 38 thousand $\cdot ml^{-1} \cdot head^{-1}$, $p = 0.003$). According to the results of NGS sequencing, the biological product had a beneficial effect on the microbial community restoration. A detailed analysis of the rumen microbiome revealed significant differences in 13 bacterial genera. In particular, in the rumen of cows fed Cellobacterin+, there is a decrease in abundance of the genera *Anaerofilum* sp. (2.3-fold, $p \leq 0.05$) and *Anaerostipes* sp. (by 1.8-fold, $p \leq 0.05$), producing lactate as the final product of glucose metabolism, and taxa among which pathogens are often found, namely, *Campylobacter*, *Gemella*, *Mycoplasma*, *Shewanella* ($p \leq 0.05$) and *Fusobacterium* (including *F. necrophorum*) ($p \leq 0.001$). A decrease in the counts of somatic cells in milk was associated with a decrease in mastitis pathogens in the rumen. Based on bioinformatics data processing, the authors described in detail the metabolic changes in the cicatricial microbiota at the gene level as a result of the probiotic strain introduction and changes in the microbiome structure. The predicted functional potential of seven metabolic pathways was enhanced in cows fed with Cellobacterin+. It seems interesting to further study the beneficial effect of introduced bacteria on the host, in particular, the assessment of the viability, adhesive potential, and survival of the bacterial strain as part of a biological product in the digestive tract conditions.

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AGRICULTURAL BIOLOGY,
2020, V. 55, № 6, pp. 1220-1232
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 636.5.033:636.087.8:579.6:577.2

doi: 10.15389/agrobiology.2020.6.1220eng
doi: 10.15389/agrobiology.2020.6.1220rus

THE IMPACT OF VIRGINIAMICIN AND PROBIOTICS ON INTESTINAL MICROBIOME AND GROWTH PERFORMANCE TRAITS OF CHICKEN (*Gallus gallus* L.) BROILERS

D.G. TYURINA[1], G.Yu. LAPTEV[1], E.A. YILDIRIM[1] ✉, L.A. ILINA[1], V.A. FILIPPOVA[1], E.A. BRAZHNIK[1], N.V. TARLAVIN[1], E.P. GORFUNKEL[1], A.V. DUBROVIN[1], N.I. NOVIKOVA[1], T.P. DUNYASHEV[1], A.A. GROZINA[2]

[1]*JSC Biotrof+,* 19, korp. 1, Zagrebskii bulv., St. Petersburg, 192284 Russia, e-mail tirina@biotrof.ru, georg-laptev@rambler.ru, deniz@biotrof.ru (✉ corresponding author), ilina@biotrof.ru, filippova@biotrof.ru, bea@biotrof.ru, tarlav1995@biotrof.ru, elena@biotrof.ru, dubrowin.a.v@yandex.ru, novikova@biotrof.ru, timur@biotrof.ru;
[2]*Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS,* 10, ul. Ptitsegradskaya, Sergiev Posad, Moscow Province, 141311 Russia, e-mail Alena_fisinina@mail.ru
ORCID:
Tyurina D.G. orcid.org/0000-0001-9001-2432
Laptev G.Yu. orcid.org/0000-0002-8795-6659
Yildirim E.A. orcid.org/0000-0002-5846-5105
Ilina L.A. orcid.org/0000-0003-2789-4844
Filippova V.A. orcid.org/0000-0001-8789-9837
Brazhnik E.A. orcid.org/0000-0003-2178-9330
Tarlavin N.V. orcid.org/0000-0002-6474-9171
Gorfunkel E.P. orcid.org/0000-0002-6843-8733
Dubrovin A.V. orcid.org/0000-0001-8424-4114
Novikova N.I. orcid.org/0000-0002-9647-4184
Dunyashev T.P. orcid.org/0000-0002-3918-0948
Grozina A.A. orcid.org/0000-0001-9654-7710

The authors declare no conflict of interests
Received May 12, 2020

Abstract

Today, there is great interest in the development of environmentally friendly feed additives for poultry farming as a worthy alternative to antibiotics capable of positively modulating the microbiota to control pathogenic microorganisms. However, very few studies have been devoted to comparing the effects of probiotics and antibiotics on the structure of the gut microbiome in broilers. In this study, we compared the composition of the intestinal microbiota and zootechnical parameters in chickens of the Cobb 500 cross during the starter, growth and finishing periods when a probiotic (*Bacillus subtilis* in the composition of Cellobacterin®-T) or an antibiotic (Stafac® 110 based on virginiamycin) was added to the diet and showed that the *B. subtilis* strain accelerates the formation of intestinal microflora. The probiotic also reduces the number of microorganisms of the *Campylobacteriaceae* family which includes many types of gastroenteritis pathogens, and also increases the digestibility of fiber. T-RFLP analysis and qPCR method were used to assess changes in the intestinal microbiota of Cobb 500 broiler chickens fed a *Bacillus subtilis*-based dietary probiotic and virginiamycin-based dietary antibiotic Stafac® 110. On day 14, the total counts of cecal bacteria, as compared to control, were 9.1 times higher ($p \le 0.05$) in broilers fed Stafac® 110, and 54.2 times higher ($p \le 0.001$) when fed *B. subtilis* preparation. This indicates rapid microbial colonization of gastrointestinal tract of the chickens fed Stafac® 110 and *B. subtilis*. T-RFLP analysis revealed two dominant cecal phyla, *Firmicutes* and *Proteobacteria*, while phyla *Actinobacteria*, *Bacteroidetes*, and *Fusobacteria* were less abundant. The taxa are detected which ferment non-starch polysaccharides to produce short-chain fatty acids, inhibit the competing pathogens due to production of bacteriocins, and acidize the chyme as synthesize organic acids. Administration of the dietary antibiotic mostly positively influences the cecal microbiota, e.g., the cellulolytic bacteria and *Clostridia* forms involved in the synthesis of organic acids became more abundant ($p \le 0.05$). Similar beneficial effects, e.g., an increase in *Clostridia* counts ($p \le 0.05$) compared to control, occurred when the probiotic strain was administered. On day 14 of rearing, the dietary antibiotic and probiotic reduced abundance of *Campylobacteriaceae* family comprising gastroenteritis pathogens ($p \le 0.05$) when compared to control. An increase in bodyweight as compared to control (from 1845.8±20.9 to 1936.4±17.9 g, $p = 0.046$) occurred in 36-day-old chickens fed Stafac® 110 but not the probiotic strain but not the probiotic strain, despite recovery of gut microbiota in the chickens fed *B. subtilis*. A 7.1 % increase in fiber digestibility ($p = 0.0027$) occurred in broilers fed dietary probiotic and a 2.3 % increase ($p = 0.047$) in those fed the dietary antibiotic, which may be due to the action of cellulolytic microorganisms. Therefore, a dietary *B. subtilis*-based probiotic which promotes recovery of gut microbiota and increases fiber digestibility in feeds for broiler chickens can be an effective alternative to the virginiamycin-based antibiotic Stafac® 110.

Keywords: broiler chickens, Cobb 500, probiotic, *Bacillus subtilis*, Stafac® 110, T-RFLP analysis, microbiome, *Firmicutes*, *Proteobacteria*, *Clostridia*, *Campylobacteriaceae*

The widespread use of antibiotics in livestock and poultry farming leads to the emergence of pathogenic bacteria resistant to antimicrobial drugs, which seriously threatens the health of animals and humans [1, 2]. In 2016, the UN General Assembly recognized the use of antibiotics in livestock and poultry as one of the main causes of antimicrobial resistance in humans (United Nations meeting on antimicrobial resistance, 2016) [3]. In the European Union, the use of antibiotics was banned in 2006, in the United States, the Center for Veterinary Medicine, Food and Drug Administration (FDA) prepared an FDA Guidance for Industry in 2012, which recommends the use of antibiotics exclusively for therapeutic purposes for limited periods in case of outbreaks of infectious diseases [4]. In recent years, antibiotics have been widely used in the poultry industry in Russia for mass prevention of diseases and poultry growth stimulation, however, since 2020, the state has banned the use of antimicrobial drugs intended for veterinary use for non-medicinal purposes.

The microbiome of the gastrointestinal tract of poultry plays a vital role in the digestion and absorption of feed nutrients, the development of immunity, resistance to diseases, and the breakdown of toxins [5]. Disruption of the microbial community of the gastrointestinal tract can adversely affect the efficiency of feeding, productivity and health of poultry [6]. It has been proven [7, 8] that antibiotic therapy often causes a change in the structure of microbial consortia, provoking dysbacteriosis with subsequent physiological and metabolic disorders in the host's body. Disruption of the microbiota in broiler chickens is often associated with villous atrophy, decreased muscle thickness, and increased infiltration of T-lymphocytes in the intestinal mucosa [9].

In the last decade, interest in the development of environmentally friendly feed additives capable of positively modulating microbiota by controlling pathogenic microorganisms has been constantly growing [10-12]. The positive effects of probiotic strains of microorganisms and prebiotics in the prevention and treatment of gastrointestinal disorders in broiler chickens infected with *Clostridium perfringens* [13], *Campylobacter jejuni* [14], *Salmonella* sp. [15].

Antibiotic therapy and, in particular, uncontrolled intake of antibiotics negatively affect the composition of the human intestinal microbiota [15-17]. Thus, the negative effect of β-lactam therapy on the composition of the human microbiome has been proven [16]. The use of 16S rDNA and 16S rRNA sequencing showed that after 14 days of therapy, the microbial biodiversity collapsed. Similar data were obtained for some farm animals. For example, in cows that received penicillin (4.8 g per animal) and streptomycin (5.0 g per animal) for 14 days, disturbances occurred in the rumen microbiome [18]. In the rumen, after 3 days of antibiotic use the abundance of 45 high-level taxa decreased, after 14 days the abundance of 43 taxa.

For broiler chickens, similar information is limited. It was reported [19] that the abundance of *Lactobacillus* spp. in the ileum of chickens whose feed contained tylosin and a bacteriostatic was significantly lower than that of those who did not receive tylosin. Similar effects have been described in other studies [20-22]. However, very few studies have been devoted to comparing the effects of probiotics and antibiotics on the composition of the gut microbiome in broilers [23].

In the presented study, the authors showed that with the introduction of the *Bacillus subtilis* strain into the gastrointestinal tract of broiler chickens, the formation of the intestinal microflora occurred faster (starting from the 1st day of life) than when the antibiotic Stafac® 110, based on virginiamycin, was added to

the feed. The probiotic reduced the abundance of microorganisms of the *Campylobacteriaceae* family, including the *Campylobacter* genus, which includes many types of gastroenteritis causative agents, and also increased the digestibility of fiber and, therefore, can be an effective alternative to the feed antibiotic Stafac® 110.

The goal of the research was to compare the quantitative composition of the intestinal microbiota and zootechnical parameters in chickens in the starting, growth, and finishing periods when a probiotic or antibiotic was added to the diet.

Methods. Chickens of the Cobb 500 cross were randomly divided into three groups of 70 birds. The control group I received the basal diet, that is, complete mash feed, balanced according to the norms for the cross, including wheat, soybean and sunflower meal, soybean oil, fish meal and meat and bone meal, limestone, monocalcium phosphate, vitamin-mineral complex (fiber content in starter, growth and finishing periods is 4%). For poultry from group II, Stafac® 110 (Phibro Animal Health Corporation, USA) was added to the diet at a dosage of 180 g/t of feed. Stafac® 110 contains the active ingredient virginiamycin (11%) and excipients – carboxymethyl cellulose (4.4%), calcium carbonate (11%), mineral oil (0.2%), purified water-soluble granules (73.4%). In group III, the probiotic Cellobacterin®-T containing *Bacillus subtilis* (BIOTROF LLC, Russia) was added to the feed in the morning (10:00) (1 kg/t of compound feed according to the instructions for the preparation). The birds were kept in cage batteries of the R-15 type (Russia) (35 chickens per cage; the vivarium of All-Russian Research Veterinary Institute of Poultry, St. Petersburg, 2014). Chickens were provided with free access to feed and water. Technological conditions corresponded to the recommendations ("Resource-saving technology for the production of broiler meat: guidelines". Zagorsk, 1990).

The mortality of the livestock was recorded and the increase in live weight was assessed by individual weighing during the experiment (1-36 days of life). Physiological balance experiment to assess the digestibility and use of nutrients and minerals of the feed was carried out from days 28 to 36 ($n = 6$) according to the methodology of scientific and industrial research on feeding poultry of Federal State Budgetary Scientific Institution All-Russian Research and Technological Institute of Poultry (Sergiev Posad, 2013).

In each group, the contents of the ceca (5-10 g each) were taken postmortem from six chicks analogous in live weight to study the microbiota. At the age of 1 day, samples were taken 24 h after feeding, at days 7, 14, 21, and 36 samples were taken from individuals with a filled goiter. The collected samples were immediately placed in sterile centrifugal plastic tubes, frozen at –20 °C and delivered in dry ice to the molecular genetic laboratory of the research and production company BIOTROF LLC for DNA isolation.

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

T-RFLP (terminal restriction fragment length polymorphism) analysis was performed according to the method developed by the authors earlier [24].

For quantitative polymerase chain reaction (qPCR, thermal cycler DT Lite-4, NPO DNA-Tekhnologiya, Russia), a set of reagents for RT-PCR in the presence of an intercalating dye EVA Green (CJSC Syntol, Russia) was used according to the attached instructions. Universal primers were used to determine the total number of bacteria, the HDA1 5′-ACTCCTACGGGAGGCAGCAG-3′ and HDA2 5′-GTATTACCGCGGCTGCTGGCA-3′ [25]; amplification protocol: 3 min at 95 °C (1 cycle); 1 min at 95 °C, 1 min at 57.6 °C, 1min at 72 °C (40 cycles); 5 min at 72 °C (1 cycle).

The diversity of the bacterial community was assessed graphically using a heat map (the "pheatmap" package Version 1.0.12 for R, https://www.rdocumentation.org/packages/pheatmap/versions/1.0.12/topics/pheat-map) [26]. Hierarchical clustering by groups was carried out using the Ward-linkage clustering method on a matrix constructed from the squared Euclidean distances between objects [27, 28].

The software packages Microsoft Office Excel 2003, R-Studio (Version 1.1.453) (https://rstudio.com), and PAST (https://www.bytesin.com/software/PAST/) were used for mathematical and statistical data processing [29, 30]. Quantitative values were compared using Student's *t*-test. Statistical analysis results were considered significant at $p \leq 0.05$. Numerical data are presented as means (*M*) and their standard errors (±SEM).

Results. In our opinion, the changes caused by the anti- and probiotic in the structure of the microbiota of the cecum contents are of the greatest interest. It is in the cecum that the main processes of fermentation and digestion of complex substrates (cellulose, starches, other polysaccharides) occur, and the retention of feed here is the longest (12-20 h) [5]. For comparison with the antibiotic Stafac® 110, we chose Cellobacterin®-T, a feed additive with probiotic properties (TU 10.91.10-014-50932298-2019, registration number PVR-2-18.11/02763). It contains wheat bran (GOST 7169-2017), on which the microorganisms *Bacillus subtilis* are applied.

The results of determining the number of bacteria in the studied samples of the broiler cecum by qPCR are shown in Figure 1. Depending on the age and the treatment, it ranged from $2.4\times10^9\pm 4.7\times10^8$ to $1.4\times10^{11}\pm7.0\times10^9$ cells/g. This coincides with the known data reporting [31] that the number of bacteria in the ceca in 1-day-old chickens ranged from 10^8 to 10^{10} cells/g, reaching values from 10^9 to 10^{11} cells/g with age.

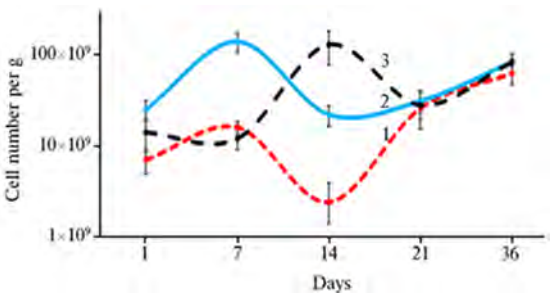

Fig. 1. Age dynamics of the total number of bacteria in the cecum of Cobb 500 cross broiler chickens fed the basal diet (BD, 1, control)**, BD supplemented with antibiotic Stafac® 110** (2) **or BD with the feed additive Cellobacterin®-T containing *Bacillus subtilis* with probiotic properties** (3) (n = 3, *M*±SEM, qPCR analysis; vivarium of the All-Russian Research Veterinary Institute of Poultry, St. Petersburg, 2014).

At the age of 14 days, the total number of bacteria in the cecal chyme of broilers fed Stafac® 110 was 9.1 times higher ($p \leq 0.05$) while in those fed the probiotic *B. subtilis* it was 54.2 times higher ($p \leq 0.001$) compared to the control (Fig. 1). However, under the influence of the antibiotic, a significant increase in the total abundance of bacteria in the blind processes compared to the control was noted already on days 1 and 7 ($p \leq 0.05$ and $p \leq 0.01$, respectively), while we did not find any differences during these periods for *B. subtilis*. The results obtained indicate rapid microbial colonization of the gastrointestinal tract of chickens from the experimental groups (especially when using an antibiotic), which is important during this period of life. Within 2 weeks after hatching, the immune system of the chickens is not yet fully developed, and they are most vulnerable to the negative impact of pathogenic microflora [32]. Thus, it is known [33] that from the first day of life, chicks begin to peck and swallow particles of litter seeded with microorganisms, including pathogenic ones (*Salmonella*, *Clostridium perfringens*, *Campylobacter jejuni*, and *Escherichia coli*).

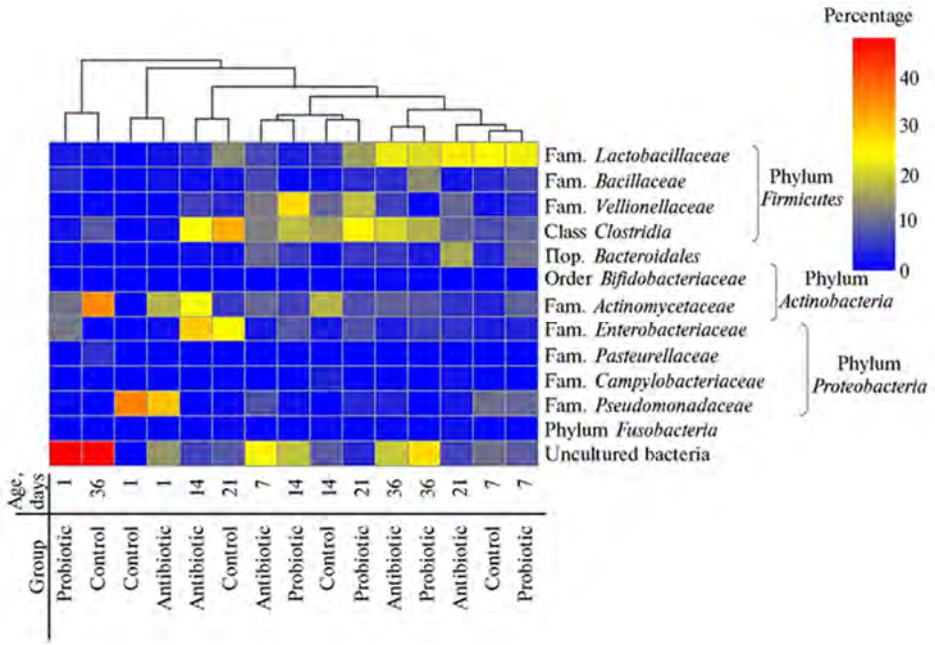

Fig. 2. Cluster analysis and heat map of the cecal bacterial community of Cobb 500 cross broiler chickens fed the basal diet (BD, control)**, BD supplemented with Stafac® 110** (antibiotic)**, or BD with the feed additive Cellobacterin®-T containing *Bacillus subtilis*** (probiotic) **when aged 1, 1, 7, 14, 21, and 36 days** (n = 3, M±SEM, qPCR analysis; vivarium of the All-Russian Research Veterinary Institute of Poultry, St. Petersburg, 2014).

Cluster analysis confirmed the conclusion about the rapid development of the microflora of the gastrointestinal tract of birds (Fig. 2). It can be seen that 1-day-old birds fed the probiotic were allocated to a separate cluster with the control group of adult broilers aged 36 days.

In the microflora of the cecal chyme of chickens, at the phylum level, two taxa dominated, the *Firmicutes* and *Proteobacteria* (see Fig. 2). The phyla *Actinobacteria*, *Bacteroidetes*, and *Fusobacteria* were less abundant. Earlier, other researchers reported [34] that the most common phylotypes of cecal microorganisms belonged to the phyla *Firmicutes* (44-55%), which is consistent with our findings, and *Bacteroidetes* (22-42%), and to the taxa *Actinobacteria*, *Chlorobi*, *Deferribacteres*, *Fusobacteria*, *Verrucomicrobia*, and *Proteobacteria* (the latter dominated in our experiment). The revealed fact of the dominance among the *Firmicutes* phylum of bacteria of the families *Lactobacillaceae*, *Bacillaceae*, *Vellionellaceae* and the class *Clostridia* suggests that the cecal microbiota plays an important role in the digestion of non-starchy polysaccharides associated with the synthesis of short-chain fatty acids, also through the exclusion of in lowering the pH of the chyme due to the synthesis of organic acids [35].

Control and test groups were distinguished into separate clusters on days 7 and 36 of growing. This indicates a more pronounced effect of the age of birds on the composition of microflora vs. the additives used.

Nevertheless, a detailed analysis of changes in the number of taxa showed an increase in the abundance of bacteria of the *Clostridia* class (among them there are forms involved in the breakdown of dietary fiber) when the antibiotic Stafac® 110 ($p \leq 0.05$) and the probiotic *B. subtilis* ($p \leq 0.05$) were used as compared to the control. A similar trend persisted throughout the entire rearing of chickens (excluding day 21). The greatest difference was noted on day 36 when the proportion of bacteria of the *Clostridia* class was 12.7% more ($p \leq 0.01$) in birds fed the antibiotic and 8.8% more ($p \leq 0.05$) in birds fed *B. subtilis* as compared to the

control. This is an important conclusion of great practical importance, since the digestion of cellulose in the intestines of birds is an exclusively microbiological process due to the absence of own cellulases in the macroorganism. In 2013, Stanley et al. [36] using pyrosequencing of the V3 region of the 16S rRNA gene found that an increase in the number of microbial groups in the gastrointestinal tract of birds, including cellulolytic bacteria *Clostridium islandicum* and *Ruminococcus* sp., was associated with an increase in productivity. Our results are consistent with the data of the metagenomic analysis of the cecal microbiota in 42-day-old Ross broilers, in which numerous enzymes that decompose polysaccharides and oligosaccharides have been identified in this intestine region [37].

The tendency of an increase in the number of other important representatives of *Firmicutes* — bacteria of the *Vellionellaceae* family was observed practically throughout the experiment when using Stafac® 110 ($p \leq 0.05$) and *B. subtilis* ($p \leq 0.05$) compared to the group without additives. This conclusion is also important, since it is known that, as a result of the activity of members of the *Vellionellaceae* family, the accumulation of short-chain fatty acids occurs in the cecum, which are further assimilated by the host [38]. It is known that in the cecum of birds, volatile fatty acids (VFAs) are absorbed across the epithelium via passive diffusion and are involved in various metabolic pathways [39]. Up to 95% of VFAs produced during microbial fermentation of carbohydrates [40, 41] are used by the host, providing up to 30% of the total energy requirement. Ruminants receive almost 100% of the required energy as a result of the activity of the rumen microbiome [42]. VFAs are used as a source of energy and carbon. In addition, they affect blood flow, stimulate the growth and proliferation of enterocytes, and regulate mucin production, influencing the intestinal immune response [39]. There is evidence that these compounds activate the immunity of a macroorganism by influencing the expression of Il1β, TNFa, chemokines, and immune barrier genes [43]. Earlier, when analyzing the microbial contents of the cecum of the intestine of birds, genes associated with butyrate production with the participation of 3-hydroxybutyryl-CoA dehydrogenase, phosphate butyryl transferase, and butyrate kinase were found [37]. In addition, the presence of acetate CoA transferase, which is responsible for the synthesis of acetate, and clusters of genes encoding beta, gamma, and delta subunits of methylmalonyl CoA decarboxylase, involved in the formation of propionate, was found [37]. Also, genes of 12 hydrogenases produced mainly by bacteria of the genus *Megamonas*, which belong to the *Vellionellaceae* family, have been identified in the cecum. The authors suggested that these hydrogenases can serve as hydrogen acceptors, promoting the formation of succinate [37].

Among the bacteria of the phylum *Proteobacteria,* the families *Enterobacteriaceae* (up to 28.8±1.8%) and *Pseudomonadaceae* (up to 35.4±2.4%) dominated. *Pseudomonas* sp. were previously also found in the gastrointestinal tract of birds [5]. A large representation of the *Pseudomonadaceae* family in the control and when using the antibiotic Stafac® 110 was noted in chickens on days 1 and 7 (at $p \leq 0.001$ and $p \leq 0.05$, respectively) compared to older birds. Many bacteria of the *Pseudomonadaceae* family are capable of hydrolyzing phytate and degrading starch, but it should be noted that the species *Pseudomonas aeruginosa* causes omphalitis, a dangerous disease that becomes a common cause of death in birds at 1-14 days of age. This species is resistant to sulfisoxazole, ceftiofur, penicillin, lincomycin, bacitracin, oxytetracycline, erythromycin, nalidixic acid, and tetracycline [44].

Among the phylum *Actinobacteria*, bacteria of the *Actinomicetaceae* family dominated (up to 35.3±3.1%). Earlier, a significant amount of metagenomic sequences encoding endoglucanases, usually synthesized by representatives of this

taxonomic group, which degrade polymers, in particular cellulose and xylan, were found in the contents of the gastrointestinal tract of chickens [37]. In our experiment, the representation of bacteria of the *Actinomicetaceae* family was higher than in the control, in chickens of 1-14 days of age when using an antibiotic ($p \leq 0.05$) and 1 and 7 days of age when replacing it with a probiotic ($p \leq 0.05$).

The identification and study of pathogenic bacteria in the microbiota of broiler chickens is important for the health of both poultry and humans. Attention should be paid to the fact that in the authors' experiment, bacteria of the *Campylobacteriaceae* family were detected in the cecum of the intestine in chickens. Gastrointestinal infections in humans caused by a member of this family, *Campylobacter*, are mainly associated with the consumption of poultry products [45]. The use of the antibiotic Stafac® 110 and the *B. subtilis* strain had a significant effect on the decrease in the abundance of these microorganisms in the intestine on day 14 of growing (the differences from the control were statistically significant at $p \leq 0.05$). In chickens, among the previously described taxa that can cause diseases in humans, one can distinguish *Campylobacter* (mainly *Campylobacter jejuni* and *C. coli*), *Salmonella enterica*, *Escherichia coli*, and *Clostridium perfringens* [46].

The results indicating the normalization of the microflora of poultry after the introduction of probiotic strains of bacteria into the diet have been repeatedly confirmed [44, 47, 48]. With regard to the human intestinal microbiota, a stable opinion has been formed that most of the known antibiotics suppress not only pathogenic but also commensal microflora. In our study and in the works of other researchers, the introduction of virginiamycin into the diet of birds had a positive effect on representatives of the intestinal normal flora. Thus, in 2012, an increase in the number of intestinal lactobacilli in broilers was demonstrated under the influence of virginiamycin [49]. Two years later, it was reported [47] that virginiamycin significantly reduced the number of *E. coli* in the intestines of broilers on day 42 of rearing and promoted an increase in the abundance of bacteria of the genus *Lactobacillus* compared to the control group.

1. Age dynamics of zootechnical indicators of Cobb 500 cross broiler chickens fed the basal diet (BD, control)**, BD supplemented with Stafac® 110** (antibiotic), **or BD with the feed additive Cellobacterin®-T containing *Bacillus subtilis*** (probiotic) ($n = 60$, $M \pm$SEM, vivarium of the All-Russian Research Veterinary Institute of Poultry, St. Petersburg, 2014)

Indicator	Group		
	control	Stafac® 110	*B. subtilis*
Mortality, %	2.9	0	0
Live weight, g:			
1 day,	45.1±0.4[a]	45.1±0.3[a]	45.1±0.3[a]
14 days	392.4±7.2[a]	412.7±6.7[a]	410.5±6.9[a]
21 days	786.5±10.4[a]	825.2±9.9[a]	820.54±10.0[a]
36 days			
average for livestock	1989.0	2089.9	2080.3
cockerels	2132.2±38.1[a]	2243.5±31.3[a]	2233.1±32.9[a]
chicken	1845.8±20.9[a]	1936.4±17.9[b]	1927.5±19.4[a]
Daily average live weight gain, g	55.5±2.5[a]	58.4±2.2[a]	58.2±3.4[a]
Feed consumption per 1 head for the entire period, kg	3.5±0.2[a]	3.6±0.2[a]	3.6±0.2[a]
Feed consumption per 1 kg of live weight gain, kg	1.8±0.1[a]	1.7±0.1[a]	1.7±0.2[a]

[a-b] Differences between values marked with different superscript letters are statistically significant at $p \leq 0.05$.

Comparison of zootechnical indicators (Table 1) revealed a significant ($p = 0.046$) increase in live weight in 36-day-old females fed with the antibiotic Stafac® 110. We did not observe such an effect in cockerels during the entire period of rearing. Previously, sex differences in the response to Stafac® 110 in broilers of the Cobb 500 cross at 36 days of age were also described, but a greater increase in body weight was characteristic of males while not observed in females

[50]. It has been shown that the main mechanism of the positive action of antibiotics is associated with the suppression of pathogenic microflora and, as a consequence, a decrease in the amount of toxic metabolites produced by it, especially the decomposition products of ammonia and bile [51], which was probably also observed in our experiment, as followed from the restoration of the composition of microflora. In addition, there is an opinion that the positive effect of antibiotics is associated with an increase in the availability of nutrients for the macroorganism in the intestine and an increase in the digestibility of dietary protein [51]. We also did not find significant differences between the groups in the live weight of hens up to 21 days of rearing.

It was found (see Table 1) that the antibiotic Stafac® 110 and the *B. subtilis* strain did not have a significant effect on feed consumption.

The obtained results agree with the known data. Thus, a group of scientists [52] studied the effectiveness of the probiotic Lacto G based on lactobacilli when introduced into the diet of broiler chickens against the background of artificial infection of birds with the causative agent of coccidiosis *Eimeria tenella*. The results obtained showed that, despite the decrease in pathogen infection with the use of the probiotic, there was no positive effect of the drug on the indicators of live weight gain and feed conversion.

2. Digestibility and nutrient utilization in 28-36-day-old Cobb 500 cross broiler chickens fed the basal diet (BD, control)**, BD supplemented with Stafac® 110** (antibiotic), **or BD with the feed additive Cellobacterin®-T containing *Bacillus subtilis*** (probiotic) (n = 6, M±SEM, vivarium of the All-Russian Research Veterinary Institute of Poultry, St. Petersburg, 2014)

Indicator	Group		
	control	Stafac® 110	*B. subtilis*
	Digestibility, %		
Protein	90.8±4.9[a]	91.9±5.3[a]	91.4±5.2[a]
Fats	80.1±3.8[a]	82.3±5.5[a]	81.7±4.6[a]
Fibre	11.5±0.6[a]	13.8±0.4[b]	18.6±0.5[c]
	Utilization, %		
Nitrogen	53.5±2.6[a]	55.2±3.2[a]	54.6±2.8[a]
Calcium	46.0±2.5[a]	46.9±2.8[a]	46.6±2.1[a]
Phosphorus	38.1±1.7[a]	39.5±2.1[a]	39.1±1.9[a]

[a-c] Differences between values marked with different superscript letters are statistically significant at $p \leq 0.05$.

In our tests (Table 2), the digestibility of fiber in the group with the introduction of the *B. subtilis* strain into the diet was 7.1% higher than in the control ($p = 0.0027$), of the antibiotic 2.3% higher than in the control ($p = 0.047$). This is probably due to the restoration of the intestinal microbiome structure in chickens from the experimental groups and an increase in the number of microorganisms exhibiting cellulolytic activity, for example, bacteria of the genus *Ruminococcus*, as well as cellulolytics of the genus *Clostridium* [53].

As follows from a detailed analysis of the microbial community of the birds' intestines, the cecum was mainly dominated by the microbiota which plays an important role in the digestion of non-starchy polysaccharides and participates in the synthesis of short-chain fatty acids and the displacement of pathogenic microflora using the synthesis of bacteriocins. It is obvious that the bird microbiome, which has such a pronounced effect on the functioning of the macroorganism, needs to be adjusted and maintained. To date, data have been obtained on both the positive and negative effects of antibiotic therapy on the composition of the gut microbiota of birds. Our study revealed a predominantly positive effect of the feed antibiotic Stafac® 110 on the structure of the microbiome due to an increase in the abundance of cellulolytics and bacteria involved in the synthesis of organic acids by the macroorganism in the most important metabolic processes. However, similar data on positive changes in the structure of the microbial

community were obtained during the introduction of the probiotic strain *B. subtilis*. These results may be of great practical interest due to current consumer protests and government restrictions on the use of antibiotics in poultry and livestock. So, by the order of the Government of Russia No. 604-r dated March 30, 2019, within the framework of the state Strategy for Preventing the Spread of Antimicrobial Resistance in the Russian Federation until 2030, from 2020 it is prohibited to use veterinary antimicrobial drugs for non-medicinal purposes. For violation of this prohibition, the introduction of administrative responsibility is expected. In addition, from 2020 the use of antimicrobials in the manufacture of feed should be regulated (with corresponding changes in the existing legislation).

In our opinion, replacing antibiotics in feed with probiotics in conditions of rejection of antibacterial agents is quite real, but it requires additional research to understand the molecular mechanisms of the positive effect of antibiotics and probiotics not only on the microflora of the large intestine but also on other parts of the intestine. It would be interesting in the future to compare the effect of feed and medicinal antibiotics on the structure of the microbiome of chickens, as well as to evaluate changes in the microflora of the gastrointestinal tract of adult birds (parent flocks and layers) under the influence of antibiotics or probiotics.

Thus, the obtained results indicate that the introduction of the *Bacillus subtilis* strain into the gastrointestinal tract of broiler chickens provides a faster formation of the intestinal microflora (already in day 1 of life) in comparison to the basal diet without additives, as well as with the introduction of an antibiotic Stafac® 110 based on virginiamycin. On day 14, both the antibiotic and probiotic strains decreased, as compared to the control, the abundance of microorganisms of the *Campylobacteriaceae* family among which causative agents of gastroenteritis can be found. Dietary antibiotic Stafac® 110 increased body weight in 36-day-old females, but not in males (despite the restoration of their microflora). The digestibility of cellulose during the introduction of the *B. subtilis* strain increased compered to the control and the dietary antibiotic, which may be associated with the activity of cellulolytic microorganisms. The antibiotic Stafac® 110 and the probiotic strain *B. subtilis* had no significant effect on feed consumption. Dietary probiotic *B. subtilis* strain of Cellobacterin®-T restores intestinal microflora in broilers and increase fiber digestibility. Therefore, the Cellobacterin®-T can be an effective alternative to the feed antibiotic Stafac® 110 based on virginiamycin.

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AGRICULTURAL BIOLOGY,
2020, V. 55, № 6, pp. 1233-1244
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

Unconventional feeds

UDC 636.087.6:636.52/.58:591.11

doi: 10.15389/agrobiology.2020.6.1233eng
doi: 10.15389/agrobiology.2020.6.1233rus

THE STANDARDIZED ILEAL DIGESTIBILITY OF AMINO ACIDS FROM PROTEIN CONCENTRATE BASED ON THE LARVAE OF COMMON GREEN BOTTLE FLY *Lucilia* spp. (*Diptera: Calliphoridae*) AND ITS EFFECTS ON THE MORPHOLOGICAL AND BIOCHEMICAL BLOOD INDICES IN BROILERS (*Gallus gallus L.*)

M.S. ZHURAVLEV[1] ✉, V.G. VERTIPRAKHOV[2] ✉, M.V. KOSHCHEYEVA[2], N.P. BURYAKOV[1], M.I. SMAHLIUK[3], A.I. ISTOMIN[3]

[1]*Timiryazev Russian State Agrarian University—Moscow Agrarian Academy,* 49, ul. Timiryazevskaya, Moscow, 127550 Russia, e-mail mikhail.sterh@gmail.com (✉ corresponding author), kormlenieskota@gmail.com;
[2]*Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS,* 10, ul. Ptitsegradskaya, Sergiev Posad, Moscow Province, 141311 Russia, e-mail Vertiprakhov63@mail.ru (✉ corresponding author), lkhasanova@mail.ru, vlk.733@mail.ru;
[3]*OOO «Zooprotein»,* 64 of. 701, Sovetskaya ul., Lipetsk, Lipetsk Province, 398001 Russia, e-mail ms@misma.pro, ais@misma.pro
ORCID:
Zhuravlev M.S. orcid.org/0000-0002-4586-2937
Vertiprakhov V.G. orcid.org/0000-0002-3240-7636
Koshcheyeva M.V. orcid.org/0000-0002-0744-1883
Buryakov N.P. orcid.org/0000-0002-6776-0835
Smahliuk M.I. orcid.org/0000-0003-3676-9746
Istomin A.I. orcid.org/0000-0003-0394-5280
The authors declare no conflict of interests
Acknowledgements:
Supported financially from the Russian Science Foundation for the Project No. 16-16-04089-П "Study of physiological and microbiological aspects of digestion in meat chicken in embryonic and post-embryonic periods to develop diets which fully meet the genetic potential of poultry"
Received September 24, 2020

Abstract

Bioconversion is an ecologically friendly and effective way of the utilization of organic wastes; it involves the use of these wastes as a substrate in the biotechnologies of different products. The larvae of the black soldier fly (*Hermeia illucens* L.) grown on the organic wastes have been earlier studied as a raw material for feed ingredients for poultry; recently the larvae of common green bottle fly (*Lucilia* spp.) draw the attention as a potential source of feed-grade protein for animals and poultry. The dried defatted biomass of the larvae contains crude protein (no less than 62 %), fat (10 %), lysine (no less than 4 %), methionine + cystine (2.0 %) and hence could be a promising protein source in diets for poultry. However, despite the apparent advantages of the bioconversion of organic wastes by insects into the dietary protein sources for poultry there is a scarcity of the data on the quality of these products and on their effects on poultry. Earlier research evidenced the efficiency of the supplementation of diets for growing turkeys with 5.0-7.5 % of dried full-fat *Lucilia* larvae; however, the ileal digestibility of amino acids in turkeys (necessary for the balancing of dietary amino acids) was not assessed. The study presented is a first attempt of the *in vivo* assessment of ileal digestibility of amino acids from *Lucilia* larvae protein concentrate (LLPC) in broilers (*Gallus gallus* L.). The aim of the study was the determination of apparent (AID) and standardized ileal digestibility (SID) of amino acids from LLPC within the experimental diet and its effects on the morphological and biochemical blood indices in broilers. AID and SID were determined in the vivarium of the All-Russian Research and Technological Institute of Poultry in 2019 on broilers (cross Smena 8, 18-42 days of age) with chronic ileal fistulae fed mono-protein diet contained LLPC, dextrose, fiber, and a premix of vitamins and minerals. SID was calculated with the endogenous losses of amino acids taken into account. SID of potentially limiting amino acids were as follows: lysine 82.9 %, methionine 86.6 %; threonine 80.4 %; arginine 89.5 %; isoleucine 80.0 %; leucine 81.9 %; valine 79.9 %; histidine 82.9 %, and phenylalanine 85.7 %. The beneficial effects of LLPC on the blood indices were found: the activity of alanine transaminase was significantly higher by 23.5 % in broilers fed LLPC in compare to control while the activity of aspartate transaminase was lower by 24.6 % ($p < 0.05$) indicating the prevalence of the anabolic processes over the catabolic. The significant increase in total protein concentration in serum (by 20.0 %, $p < 0.05$) and increase in hemoglobin concentration by 4.2 % in compare to control evidenced the activation of the metabolism. The use of SID (instead of AID)

allows for more accurate balancing of dietary contents of available amino acids, more adequate amino acid supply to poultry, and for the reduction of nitrogen emissions into the environment due to the optimization of dietary protein content.

Keywords: broilers, poultry farming, alternative protein sources, ileal digestibility, insects, zooprotein

A large volume of organic waste and by-products of food production creates problems of the natural environment pollution – water, air, and soil. Bioconversion is an ecologically friendly and effective way of the utilization of organic wastes; it involves the use of these wastes as a substrate in the biotechnologies of different products for various purposes. One of the promising directions is the growing of fly larvae of the order *Diptera* on such waste, from which it is possible to produce a feed protein supplement [1, 2] and bioactive substances [3]. Dipterans have some advantages in agriculture as waste processors: they produce much fewer greenhouse gases and ammonia than traditional farm animals [4], require less space for growing [5], feed conversion is more efficient [6], while they can convert unsuitable for animal and human nutrition by-products and wastes into high-protein raw materials and energy [7].

The black soldier fly *Hermetia illucens* L. is considered one of the most studied insects used for poultry feeding [8]. The use of dried and partially defatted fly larvae of this species as a source of protein for broilers [9], laying hens [10], and meat quails [11] is surveyed in this article. The use of live black soldier fly larvae in turkey feeding has also been investigated [12]. In addition to poultry farming, *H. illucens* larvae can be used in pig farming [13] and aquaculture [14, 15].

Currently, the investigations are in progress to search for and study other insects as promising sources of protein for animal feeding. In the work by Huis and Oonincx [16], the experiments on the replacement of fish meal in the diets of farm animals with insect protein were analyzed and it was concluded that a partial replacement was justified. A meta-analysis of 75 studies [17] showed that when feeding poultry, insect protein, in general, did not have a statistically significant adverse effect on the live weight gain, feed consumption and conversion but more than 10% of insect biomass in the diet led to a decrease in the average daily growth.

In addition to studying insects as a source of protein, regulatory issues and the use of insect products are discussed. For example, in the article by Sogari et al. [18], the issues of the regulatory framework of the European Union, North America, and some Asian countries on the use of insects in feed production and their consumer evaluation were considered. The authors concluded that these objects were still underutilized in the production of animal feed but it was expected that with the development of commercial insect growing, their use and evaluation by the end-user would increase. Such potentially useful insects in animal feeding include green bottle flies (*Lucilia* L., *Diptera: Calliphoridae*) [19].

The possibility of using dried full-fat larvae of the *Lucilia* genus for weaning piglets (1-2% of the diet) was studied in Russia [20]. Previously [21], 5-7.5% of dried full-fat *Lucilia* spp. larvae was proved to be effective in the diets for fattened turkeys. However, the assessment of the ileal digestibility of amino acids from these sources, which is necessary for rationing diets for amino acids, was not carried out. The digestibility of amino acids of a feed component is one of the key indicators that determine its quality [22]. The coefficient of standardized ileal digestibility allows the most accurate calculation of the percentage of amino acids digestibility in the intestine, taking into account their endogenous losses in the body and the use of ceca or the large intestine by microorganisms [23]. It should be noted that in general, despite all the advantages of growing insects on organic

waste to obtain raw materials for the production of feed, information about the quality characteristics of such sources of feed protein is still limited.

This paper presents the first data on the ileal digestibility of amino acids of protein concentrate from the larvae of *Lucilia* spp. flies, which was 79.3% for apparent digestibility, and 80.7% on average for standardized digestibility. The used supplement had a beneficial effect on the metabolism due to changing the ratio of aminotransferases, increasing the total blood protein and hemoglobin in the of poultry.

The objective of the paper was to evaluate the ileal digestibility of amino acids of protein concentrate from larvae of *Lucilia* spp. flies and their influence on the biochemical and morphological parameters of blood in broilers to optimize the regulations for the use of the larvae of common green bottle fly as a promising source of feed protein.

Methods. The protein concentrate has been produced by the Zoprotein group of companies (Lipetsk, Russia) from the larvae of *Lucilia* spp. flies. After hatching, the larvae were grown for 4 days on a substrate of food products with an expiring shelf life (meat and its processed products), before pupation, the larvae were separated from the substrate and dried for 1 hour at 110 °C. The obtained protein-lipid concentrate (43.8% crude protein, 23.5% crude fat) was defatted in a cold press for oilseeds, the obtained cake was crushed in a hammer mill to a loose powder state (62.70% crude protein, 13.46% crude fat, 4.09% lysine).

Experiments were performed on six 18-42-day old broilers (*Gallus gallus* L.) of cross Smena 8. Poultry were grown in a vivarium (All-Russian Research and Technological Poultry Institute RAS, Sergiev Posad, Moscow Porvince, December 2019—January 2020) according to the recommendations developed for the cross.

At the age of 15 days, all broilers were surgically fitted with ileal fistulae [25] in compliance with the requirements of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes [24]. The poultry had no feed 12-18 hours before surgery. All surgical manipulations were performed using painkillers (analgin and diphenhydramine), xylazal (0.2 ml) was used for immobilization. Broilers were fixed in the left side position on a special operating table, a 0.5% novocaine solution was used for conducting anesthesia and infiltration anesthesia (into the abdominal cavity along the incision line). Through an incision on the right side behind the last rib in the caudal direction at a distance of 4-5 cm slightly above the edge of the lateral process of the thorax, the caudal part of the ileum was removed and, by retreating cranially from the confluence of the ceca 1-2 cm, an incision of the intestine was made. The ceca were washed with a disinfectant solution and ligated to stop their activity completely. A seromucous purse suture was made at the caudal part of the intestine, and then, after a serous suture on top, the previous suture was immersed inside. A small hole was made in the abdominal wall, retreating 4-5 cm below and to the right of the cloaca, and the cranial segment of the ileum was sewn to the resulting hole with interrupted sutures, by forming an artificial anal orifice. After wound closing, a 1.5-2.0 cm long PVC tube was sewn into the hole with interrupted sutures. The post-surgery recovery period lasted 3-5 days.

Experiments on the digestibility of amino acids and the assessment of the physiological state of poultry were performed on broilers with ileal fistulae ($n = 6$), from which groups were formed ($n = 3$ in each). To obtain reliable results, feeding experiments were performed at least 3 times on each broiler, by replacing the groups according to the Latin square scheme for the following periods: 3 days for the control diet, 2 days for the transition period, 3 days for the test period

(feeding with a mono protein diet based on a concentrate from the larvae of *Lucilia* spp. flies).

The standard and test diets were composed in such a way that they had the same crude protein content (23.6%). The experimental feed was prepared in such a way that the only source of amino acids of the feed was protein concentrate from fly larvae in an amount corresponding to 23.6% of crude protein. Dextrose (Roquette Freres SA, France) was used as the main source of energy. The feed was balanced in calcium, phosphorus, vitamins, and trace elements according to the norms of Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS (2014), the necessary amount of fiber was provided by chitin protein concentrate from fly larvae and the addition of cellulose of the Arbocel trademark (J. Rettenmaier & Söhne GmbH + Co KG, Germany).

Blood (2-3 ml samples) was taken from the axillary vein in the morning before feeding the poultry. As an anticoagulant, a 3.8% solution of sodium citrate was used in a volume ratio of 1:10 with a blood sample. The sample was centrifuged at 3000 rpm for 5 min to separate the plasma from the formed elements. The resulting plasma was examined using a Sinnowa BS-3000P semi-automatic flow analyzer (SINNOWA Medical Science & Technology Co., Ltd., China) using biochemical kits (DIAKON-VET, Russia), the total protein, uric acid concentration, alanine-, aspartate aminotransferase, and trypsin activity were determined [26]. In feeding trials, the amount of feed consumed and the excreted brood was recorded. All the excrement during the 3-day trial period was collected, packed, stored at –20 °C in a laboratory freezer, after which it was lyophilized and analyzed for the amino acids.

The content of amino acids in the feed and chyme of the ileum was evaluated by ion-exchange chromatography with post-column derivatization with ninhydrin reagent and subsequent detection at $\lambda = 570$ nm (for proline $\lambda = 440$ nm). The analyses were performed using the YL 9100 HPLC System for high-performance liquid chromatography (Young Lin Instrument Co., Ltd., Korea).

The values of apparent (AID) and standardized (SID) ileal digestibility were calculated using the formulas:

$$AID = \frac{\text{AA consumed} - \text{AA in SI chyme}}{\text{AA consumed}} \times 100\ \%,$$

$$SID = \frac{\text{AA consumed} - (\text{AA in SI chyme} - \text{basal EL})}{\text{AA consumed}} \times 100\ \%,$$

where AA is amino acids, SI is small intestine, EL is endogenous losses.

To convert AID to SID, the average values of the main endogenous losses of amino acids [27] in the ileal chyme of broilers obtained on a nitrogen-free diet [9] (mg/kg of dry matter consumed) were used: 9234 for crude protein, 255 for lysine, 79 for methionine, 169 for cystine, 571 for threonine 82 for tryptophan, 216 for arginine, 390 for isoleucine, 381 for leucine, 449 for valine, 209 for histidine, 237 for phenylalanine, 280 for glycine, 1023 for serine, 580 for proline, 301 for alanine, 612 for aspartic acid, and 1037 for glutamic acid.

For statistical processing of the results, JMP Trial 14.1.0 software (SAS Institute, Inc., USA) was used. The mean values (M) and standard errors of means ($\pm$SEM) are presented. To compare the obtained values of protein concentrate digestibility with the traditionally used feed raw materials, the AminoDat 5.0 database (Evonik Industries AG, Germany) was used which represents the average values of the content of standardized ileal amino acid digestibility for a global selection.

Results. The experimental feed composition is shown in Table 1.

1. Experimental mono-protein diet for Smena 8 cross broiler chicks (*Gallus gallus* L.) based on *Lucilia* spp. fly larvae concentrate (vivarium of the Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, Sergiev Posad, Moscow Province, 2019)

Ingredient composition	Content, %
Protein concentrate from fly larvae	37.5
Dextrose	52.5
Cellulose (Arbocel)	1.5
Sunflower oil	1.5
Dicalcium phosphate	3.0
Sodium bicarbonate	1.5
Potassium chloride	1.0
Vitamin and mineral premix	1.5

The amino acid analysis shows that the control and experimental feed differed in the content of some amino acids (Table 2). In the experimental feed, the glutamic acid was 1.46% lower, arginine 0.43% lower, proline 0.37% lower, cystine 0.17% lower, and methionine 0.17% lower than in the control feed, although the proportion of each of these amino acids in the protein concentrate from the fly larvae was relatively high. However, the experimental feed was superior to the control feed in the levels of alanine (by 0.50%), phenylalanine (by 0.36%), and histidine (by 0.30%).

2. Amino acid composition (%) **of the pooled sample of feed for Smena 8 cross broiler chicks (*Gallus gallus* L.) and the protein concentrate from *Lucilia* spp. fly larvae** (vivarium of the Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, Sergiev Posad, Moscow Province, 2019)

Ingredient	Feed		Protein concentrate from fly larvae
	control	experimental	
Amino acid:			
aspartic acid	2.20	2.23	5.98
threonine	0.74	0.8	2.30
serin	0.91	0.86	2.47
glutamic acid	4.33	2.87	7.9
glycine	1.01	0.94	2.74
alanine	0.97	1.47	4.22
valine	1.07	1.16	3.26
isoleucine	0.93	0.89	2.48
leucine	1.54	1.37	3.76
tyrosine	0.73	1.42	4.21
phenylalanine	1.01	1.36	3.75
histidine	0.54	0.84	2.26
lysine	1.42	1.46	4.09
arginine	1.50	1.07	2.99
proline	1.29	0.92	2.57
cystine	0.36	0.19	0.51
methionine	0.75	0.58	1.48
Crude protein	23.61	23.63	62.79
Crude fiber	3.52	7.65	3.56

Table 3 shows coefficients of apparent and standardized ileal digestibility of amino acids in protein concentrate from the fly larvae.

3. Apparent ileal digestibility (AID) **and standardized ileal digestibility** (SID) **of amino acids in protein concentrate from *Lucilia* spp. fly larvae for Smena 8 cross broiler chicks (*Gallus gallus* L.)** (n = 6, M±SEM, vivarium of the Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, Sergiev Posad, Moscow Province, 2019)

Amino acids	AID, %	SID, %
Lysine	82.3±1.47	82.9±1.46
Methionine	86.1±1.30	86.6±1.29
Cystine	50.0±0.60	53.5±0.56
Threonine	77.9±1.63	80.4±1.58
Arginine	88.8±0.51	89.5±0.51

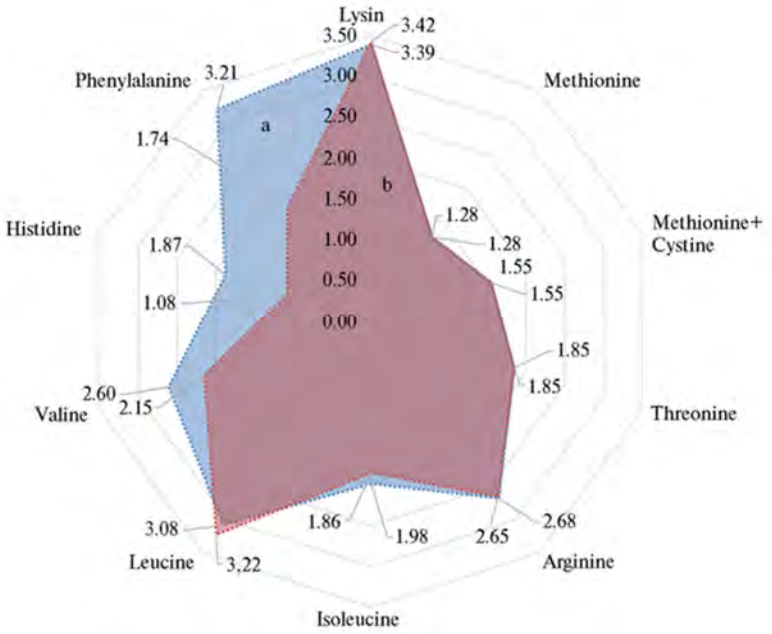
Continued Table 3

Isoleucine	78.4±1.31	80.0±1.28
Leucine	80.9±1.23	81.9±1.21
Valine	78.5±1.47	79.9±1.44
Histidine	82.0±0.85	82.9±0.84
Phenylalanine	85.1±0.35	85.7±0.35
Tyrosine	90.3±0.51	90.3±0.51
Glycine	65.4±3.51	66.4±3.46
Serin	80.9±1.91	85.0±1.82
Proline	79.3±1.27	81.6±1.23
Alanine	78.9±1.70	79.6±1.68
Aspartic acid	80.7±1.21	81.7±1.19
Glutamic acid	82.6±0.85	83.9±0.84

Given the indicators of apparent (AID) and standardized (SID) ileal digestibility, the amino acids of the protein concentrate from the fly larvae were absorbed on average by 79.3% and 80.7%, respectively. The obtained experimental data on the digestibility of amino acids in protein concentrate were compared to the average values of the SID content of amino acids in the global selection from the AminoDat 5.0 database (Evonik Industries AG, Germany). Among the essential amino acids, the lowest SID value was found in valine (79.9%), which is 0.9% higher than its digestibility from rapeseed meal (79.0%), and the highest in arginine (89.5%), which is similar to corn gluten (89.0%). Among the nonessential amino acids, cystine had the lowest SID value (53.5%), which, however, is higher than the digestibility of cystine from feather flour (48.0%), but slightly lower than for poultry meat and bone meal (56.0%). Tyrosine was best absorbed from the protein concentrate (90.3%), which is comparable to its absorption from fish meal.

The digestibility of the main limiting amino acids was quite high, i.e., 82.9% for lysine (higher than for corn gluten, 80.0%) and 86.6% for methionine (the digestibility corresponds to that of fish meal, 86-87%). By the amount of digestible essential amino acids, protein concentrate from the larvae of *Lucilia* spp. flies is close to salmon fish meal (comparison of amino acid profiles of essential amino acids is shown in the figure). In the global selection, salmon fish meal contains on average 3.42% of the digestible lysine (according to SID), 1.55% of methionine + cystine, 1.85% of threonine, 2.68% of arginine, which is consistent with the average content of the digestible amino acids in the protein concentrate from fly larvae in the protein concentrate is 0.12%, 0.45%, 0.79%, and 1.47% higher, respectively. In terms of digestible leucine content, the protein concentrate is inferior to the salmon fish meal by 0.14%.

The content of amino acids in the protein concentrate, taking into account their ileal digestibility, is presented in Table 4. It is worth noting that in the fish meal taken for comparison, according to a selection of 33 analyses, the proportion of crude protein is on average 54.71%, while in the protein concentrate from the larvae of *Lucilia* spp. flies it averaged 62.79%. Such quantity difference in crude protein is due not only to the higher digestibility of amino acids but also to the fact that the crude protein index in the protein concentrate from fly larvae includes protein nitrogen and a large amount of chitin and melanin nitrogen, which makes it difficult to use the classical nitrogen-to-protein conversion factor equal to 6.25. Janssen et al. [28] propose a nitrogen-to-protein conversion factor, taking into account chitin nitrogen and other nitrogen-containing compounds, for protein concentrates from fly larvae, equal to 5.60±0.39. In the protein concentrate from the larvae of *Lucilia* spp. flies used in the study, when using a refined coefficient, the content of crude protein adjusted for chitin is 56.26%, which is consistent with the content of amino acids in the fish meal compared to it, taking into account the digestibility of these feed ingredients.



The average content of SID amino acids in protein concentrate from *Lucilia* spp. fly larvae (a)**, used in the preparation of experimental feed for Smena 8 cross broiler chicks (*Gallus gallus* L.), and salmon fish meal** (b) (data from AminoDat 5.0, Evonik Industries AG, Germany).

4. Amino acid contents in protein concentrate from *Lucilia* spp. fly larvae with regard to standardized ileal digestibility (SID) **for Smena 8 cross broiler chicks (*Gallus gallus* L.)** (*M*±SEM, vivarium of the Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, Sergiev Posad, Moscow Province, 2019)

Amino acids	In total, %	With regard to SID, %
Lysine	4.09	3.39
Methionine	1.48	1.28
Methionine + cysteine	1.99	1.55
Threonine	2.3	1.85
Arginine	2.99	2.68
Isoleucine	2.48	1.98
Leucine	3.76	3.08
Valine	3.26	2.60
Histidine	2.26	1.87
Phenylalanine	3.75	3.21
Tyrosine	4.21	3.80
Glycine	2.74	1.82
Serin	2.47	2.10
Proline	2.57	2.10
Alanine	4.22	3.36
Aspartic acid	5.98	4.89
Glutamic acid	7.9	6.63

We also compared blood biochemical and morphological parameters of birds, reflecting the state of their metabolism, when using protein concentrate from the larvae of *Lucilia* spp. flies in the diet (Table 5). The results of the study show that the metabolism of broilers when the protein concentrate from fly larvae was fed changed towards increasing anabolic and reducing catabolic processes. This is evidenced by a decrease in the De Ritis ratio by almost 1.7 times compared to the control group. A 4.2% ($p \leq 0.05$) increase in the hemoglobin level indicates the intensity of oxidative processes in broilers fed the experimental feed. Thus, the use of protein concentrate from the larvae of *Lucilia* spp. flies in the diet for broilers has a positive effect on the metabolism due to a significant increase in the activity of alanine aminotransferase (by 23.5%), a decrease in the activity of aspartate

aminotransferase (by 24.6%), a 20.0% increase in the total protein, and a 4.2% increase in the hemoglobin level compared to the control group.

5. Blood biochemical and morphological parameters in Smena 8 cross broiler chicks (*Gallus gallus* L.) fed protein concentrate from *Lucilia* spp. fly larvae (n = 6, M±SEM, vivarium of the Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, Sergiev Posad, Moscow Province, 2019)

Indicator	Group	
	control	test
Total protein, g/l	25±0.4	30±0.4*
Uric acid, μM/l	79±4.4	90±3.4
Trypsin, units/l	59±2.4	65±3.3
Alanine aminotransferase, units/l	6.8±0.72	8.4±0.25*
Aspartate aminotransferase, units/l	338±15.9	255±18.6*
De Ritis ratio	50	30
Erythrocytes, $\times 10^{12}$/l	1.8±0.03	1.9±0.04
Hemoglobin, g/l	94±0.5	98±1.2*
Leucocytes, $\times 10^{9}$/l	31.2±1.00	30.9±1.00

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

The data obtained are consistent with other studies on the protein digestibility of fly larvae of other species. In dried full-fat larvae of the housefly *Musca domestica* L. (*Diptera: Muscidae*) grown on broiler brood [29], the percentage of apparent ileal digestibility of amino acids averaged 83.16% vs. 79.30% for *Lucillia* spp., and the AID values for lysine, methionine, threonine, valine, and arginine were 87.0, 88.0, 78.0, 81.0, and 88.0% vs. 82.3, 86.1, 77.9, 78.5, and 88.8%. In our test, the digestibility was lower, which is probably due to the different age of the poultry in the experiments, the biological characteristics of flies of different species, as well as the product form. Hall et al. [29] used another drying technology to produce full-fat biomass of larvae, i.e., 65 °C for 3 hours and then 40 minutes at 95 °C vs. 1 hour at 110 °C in this study. Heat treatment of insect larvae plays an important role in digestibility. Thus, a comparison of the digestibility of crude protein in vitro, depending on the method of preparation (cooking, frying in a pan, cooking in a vacuum, and cooking in an oven) of yellow mealworm beetle *Tenebrio Molitor* L. (*Coleoptera: Tenebrionidae*) [30] showed its improvement with any heat treatment, although there are also opposite effects [31], they were obtained when using higher temperatures of prolonged heating. It is shown that prolonged heat treatment increases the number of disulfide bonds in protein molecules and accelerates their oxidation, thereby changing the conformation of proteins and reducing the digestibility of peptide bonds for enzymes [31].

The protein digestibility of the black soldier fly *Hermetia illucens* L. (*Diptera: Stratiomyidae*) larvae has been compared in numerous publications. Thus, the average percentage of apparent ileal digestibility of amino acids from *H. illucens* larvae grown on grain waste and dried at a temperature of 60 °C for 20 hours without further defatting was 68% [32], which is significantly lower than the value obtained for *Lucillia* spp. and is not consistent with the results of another study [33], in which AID and SID were obtained for a defatted protein concentrate from *H. illucens* equal to 80.7 and 86.0%, respectively. In the work of Mwaniki et al. [34], the values of apparent ileal digestibility for some amino acids are close to those obtained by us, e.g., the AID of lysine, methionine, threonine, valine, arginine is 83.9, 85.3, 78.2, 85.0, and 88.7% for *H. illucens* and 82.3, 86.1, 77.9, 78.5, and 88.8% for *Lucillia* spp., respectively. The data of Mwaniki et al. [34] are also confirmed by the paper of Schiavone et al. [35] in which, when using defatted black soldier fly flour (65.5% crude protein) dried at 60 °C for 20 hours, the average apparent ileal digestibility of amino acids was 80%, and AID for lysine, methionine, threonine, valine, and arginine was 80, 78, 77, 91, and 80%, respectively. That is consistent with the results of this study.

Thus, it is found that the protein of *Lucilia* spp. larva is a valuable source of digestible amino acids. For the first time, the ileal digestibility of amino acids of protein concentrate from the *Lucilia* L. larvae was determined (the apparent and standardized average was 79.3 and 80.7%). The obtained data on the standardized ileal digestibility will be useful for innovative poultry feed formulations. The protein concentrates from fly larvae in the diet for broilers positively affected the metabolism by increasing the activity of alanine aminotransferase (by 23.5%, $p \leq 0.05$), reducing the activity of aspartate aminotransferase (by 24.6%, $p \leq 0.05$), and increasing ($p \leq 0.05$) the total blood protein level (by 20.0%) and hemoglobin (by 4.2%) compared to the control group.

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AGRICULTURAL BIOLOGY,
2020, V. 55, № 6, pp. 1245-1256
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 636.5:636.086.2/.3

doi: 10.15389/agrobiology.2020.6.1245eng
doi: 10.15389/agrobiology.2020.6.1245rus

EFFICIENCY AND PHYSIOLOGICAL SAFETY OF PEAS IN THE DIETS FOR HENS (*Gallus gallus* L.) OF THE PARENT FLOCK DURING THE LATE LAYING PERIOD

E.N. ANDRIANOVA[1] ✉, I.A. EGOROV[1], V.V. PRONIN[2]

[1]*Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS,* 10, ul. Ptitsegradskaya, Sergiev Posad, Moscow Province, 141311 Russia, e-mail andrianova@vnitip.ru (corresponding author ✉), olga@vnitip.ru, vnitip@vnitip.ru, alex.shevy@mail.ru;
[2]*Federal Center for Animal Health Control*, FGBU VNIIZZh, mkr. Yurievets, Vladimir, 600901 Russia, e-mail proninvv63@mail.ru
ORCID:
Andrianova E.N. orcid.org/0000-0002-6769-6351 Pronin V.V. orcid.org/0000-0002-6240-3062
Egorov I.A. orcid.org/0000-0001-9122-9553
The authors declare no conflict of interests
The work was performed in accordance with the task No. R&D AAAA-A17-117062660105-5.
Received July 13, 2020

Abstract

Feeds are accounted for ca. 70 % of the commercial production costs of eggs and poultry meat. The use of other legumes than soybeans (lupine, peas, horse beans, vetch) can lower the feed costs. However, the antinutritive factors of the legumes should be taken into account in the receipt formulation, especially in diets for pedigree flocks. Earlier we have proven physiological safety and effectiveness of the inclusion of 15 % of lupine in diets for layer parental flock. The substitution of peas for soybeans and lupine as the dietary protein source can further lower the costs of finisher diets for broilers and post-peak diets for layers where reduced protein levels are required. In the study presented we found efficient the substitution of peas (5 and 10 %) for soybean meal and sunflower cake in diets for parental flock of cage-housed layer chicken (cross SP 78, 30 birds per treatment) at the end of prolonged productive period (54-71 weeks of age). Control treatment 1 was fed the complete mash compound feed with soybeans as protein source; in diets for treatments 2, 3, and 4 the soybeans were increasingly substituted by peas (5, 10, and 15 % of total diet, respectively). After the artificial insemination 100 eggs from each treatment was taken since 70 weeks of age and incubated in DANKI incubator (Belgium) in constant regime. The temperature was set at 37.7 °C during 1-18 days of incubation and 37.2 °C during 18-21 days and controlled with the accuracy 0.1 °C; the relative humidity during these periods was 52-53 and 52-75 %, respectively. Mortality level, egg production, feed conversion ratios (per 10 eggs and per 1 kg of eggs laid), egg fertility and hatchability were recorded. The concentrations of vitamins and caroteniods in liver, chemical composition of liver, tibial concentrations of calcium and phosphorus were determined according to standard methods. The effects of the antinutritive factors were assessed via the histological study of the liver (at 71 weeks of age, $n = 12$); the samples were taken in 1 hour after the euthanasia from the similar liver part (lateral side of the right lobe) and fixed in 10 % neutral aqueous formalin. It was found that the inclusion of 5 and 10 % of peas into the diets of parental layer flock increased the intensity of lay by 2.38 and 4.97 % in compare to control, respectively, output of egg mass per hen housed by 3.78 and 12.23 %; the percentage of infertile eggs in these treatments was lower in compare to control. The histological study of liver revealed that 15 % of peas can launch the cytotoxic effect on the hepatic structures and induce the hepatic steatosis. This effect should be taken into account in the receipt formulations. It could be also concluded that peas can be included into the diets without preliminary thermal treatment; the antinutritive effects of pectin, trypsin inhibitors and proteases, tannins could be alleviated via the additional supplementation of the diets with exogenous enzymes, organic acids, and hepatoprotective agents.

Keywords: *Pisum sativum* L., peas, laying hens, egg production, hatch of chicks, hatchability of eggs, fertility of eggs, mortality, hepatic histology

Feeds are accounted for ca. 70% of the commercial production costs of eggs and poultry meat. To reduce the cost of diets, non-traditional sources of protein based on sunflower cake and legumes — soy, lupine, beans, and peas are

used [1-4]. Russia is one of the World's leading producers of peas (https://agro-vesti.net/lib/industries/beans/rossijskij-rynok-gorokha-tendentsii-i-prognozy.html), while the main consumer of peas is the food industry, and only a small part is used for feed purposes. The relative cheapness of peas in comparison with soy and its processed products serves as a prerequisite for increasing the use of peas in the production of protein feed in livestock breeding [5]. The use of peas in feed production is limited by a wide range of antinutritive factors in seeds — antigenic proteins, lectins, non-starchy polysaccharides, trypsin inhibitors, 1.5-2.5% of tannin [6-8]. The expansion of the prospects for the use of peas in poultry feeding is associated with the creation of varieties with a low content of antinutritive factors and the use of enzyme preparations and acidifiers based on organic acids [7, 9].

In general, all legumes are characterized by a high proportion of protein in the grain and enrich the diet with essential amino acids and minerals. The composition of the protein in legumes determines its high digestibility – 85-89%, which is 10% higher than the corresponding indicator for the protein in cereals [6]. Peas are a good source of the essential amino acid lysine [10, 11], methionine limits its biological value. Modern pea varieties selected in Russia contain 23-26% protein, its increased percentage is typical for varieties grown in the North Caucasus region (up to 28%) and in Eastern Siberia (up to 25.2%) [5]. The amount of starch in peas in Russia reaches 61%, especially in smooth-grain varieties. Pea seed envelopes make up 8-10% of the grain weight and contain 50% fiber, 20-25% hemicellulose, up to 17% soluble polysaccharides in the form of pectin [7]. The nutritional composition and quality of peas depend not only on the variety but also on the climatic conditions and the place of cultivation [12]. Due to the presence of trypsin inhibitors in pea seeds and the high content of pectin [7, 13, 14], the availability of amino acids is low (less than 80%), which reduces their actual digestibility.

Tannins are esters of aromatic acids (most often gallic acid) with carbohydrates. Tannin solutions can precipitate proteins due to their astringent effect. During acid hydrolysis or exposure to the tinase enzyme, tannins break down to form simpler compounds of phenolic and non-phenolic nature. As well as many phenolic compounds, tannins are antinutritive substances [6, 14, 15] that can negatively affect animals by disrupting mineral metabolism and reducing the digestibility of nutrients [6]. However, tannins are not accumulated in the body and eliminated after 3-7 hours. In addition, they have the property of precipitating toxic alkaloids and heavy metal salts. The harmful effect of tannins is reduced by adding synthetic methionine to the feed [14, 15].

The use of enzyme preparations [16-18], acidifiers [9] based on organic acids, and hepatoprotectors can reduce the negative impact of antinutritive factors and increase the share of peas in poultry feed without reducing its productivity [18, 19].

In general, the effectiveness of the use of mixed feeds with the inclusion of peas has been studied on broilers [9, 12, 15, 18]. A small part of the studies has dealt with the use of this culture in the feeding of commercial layers [6, 16, 17, 19, 20].

Currently, much attention is paid to extending the life of commercial layers and parental flock layers. With the age of laying hens, the energy and protein content of their diets should be decreased [10]. The authors believe that during this period, to reduce the cost of the diet, it is justified to use peas, the protein content of which is lower than in soy and its processed products, taking into account that the digestive system of an adult bird can ensure good absorption of nutrients from mixed feeds with peas. In the publications known to the authors on the study of the influence of peas in diets in different productive periods, the

age of laying hens was up to 50 weeks [17, 19, 21, 22]. There are practically no reports on the effects of mixed feed with peas at the later stages of the life of the parental flock layers.

Earlier, the authors have proven the physiological safety and effectiveness of the inclusion of 15% of lupine in diets for parental flock layers [1]. In this paper, the authors have shown for the first time the possibility of replacing peas with soy and sunflower processing products in mixed feeds for parental flock layers up to 71.14 weeks of age. It was found that the short-term inclusion of peas in feed for breeding poultry at a dose of 5-10% did not harm the liver, its micromorphology and allowed providing high livability, productivity, and yield of 1-day-old chicks, which are not inferior to those of poultry that received soy processing products. An increase in the proportion of peas to 10-15% in combination with prolonged feeding reduces the productivity of laying hens and increases the incidence of fatty liver syndrome.

The study aims to characterize the biological and productive effect of peas in the case of inclusion in the feed of parental flock layers as a potential alternative to soy and sunflower processing products.

Methods. The studies were performed on four treatments (with $n = 30$) of laying hens (*Gallus gallus* L.) of the cross SP 789 during a 4-month productive period from 376 to 498 days of age (vivarium of the Genetic and Selection Center Zagorskoye of the Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS — FSC ARRTPI RAS, Moscow Province, 2020). The peas used as a feed ingredient contained 21.3% protein, 1.5% fat, 5.8% fiber, 1.53% lysine, and 0.22% methionine (the analysis was performed in the testing center of Federal State Budget Scientific Institution Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS). The nutritional content of mixed feeds, stock density, temperature and humidity conditions, feeding and drinking space during the entire experiment corresponded to the recommendations (FSC ARRTPI RAS, Moscow, 2014). Laying hens from treatment 1 (control) received balanced crumbled complete mixed feeds without the inclusion of peas (the main diet), in treatments 2, 3, and 4, soy processing products in mixed feeds were replaced by 5, 10, and 15% of peas, respectively. The birds were kept in a cage battery KBL-4 (Pyatigorskselmash, Pyatigorsk). The feed was given manually.

At the age of 65.8 weeks, eggs were collected from artificially inseminated laying hens and incubated (100 eggs from each treatment, experimental hatchery, DANKI, Belgium). Insemination was carried out according to the standard practice using a medium for diluting the sperm of roosters (the insemination dose was 0.1 ml of sperm diluted in a ratio of 1:3) [23]. During the preliminary incubation period (1-18 days) the temperature was maintained at 37.7 °C (control with an accuracy of 0.1 °C using a sensor), in the hatching period (18-21 days) — 37.2 °C; relative humidity — 52-53% and 52-75%, respectively.

The main zootechnical indicators of poultry were recorded: live bodyweight at the beginning and end of the studies (by individual weighing), stock livability, egg production; feed consumption and costs per 1 bird, 10 eggs, and 1 kg of egg mass were calculated; elastic deformation of eggshell, incubation indicators (fertility rate, hatchability), the content of vitamins and carotenoids in the egg and liver, the chemical composition of eggs and liver, the content of calcium and phosphorus in the tibia were determined [1, 10].

At the end of the experiment, tissue samples were taken from the lateral side of the right lobe of the liver for histological studies ($n = 12$) in 1 hour after euthanasia and fixed in a 10% aqueous solution of neutral formalin. The authors

carried out the entry (TLP-720 tissue processor, Kreonika, Russia) and filling of the material (ESD-2800 station, Kreonika, Russia). Sections with a thickness of 5-8 rm were prepared on a rotary semi-automatic microtome RMD-3000 (Kreonika, Russia) and stained with hematoxylin and eosin (ALS-96 automatic linear stainer, Kreonika, Russia). The preparations were examined using a Micmed-6 microscope (LOMO, Russia), and an E31S PM video camera (Hangzhou Touptek Photonics Co., Ltd, China) and ToupView software (Hangzhou Touptek Photonics Co., Ltd, China) (magnification ×100 and ×400) were used for measuring the size of hepatocytes, trabeculae, and sinusoids, and for photo documentation. The measuring scale of the video camera was calibrated using the object micrometer of the transmitted light FLT (LOMO, Russia). The large and small diameters of hepatocytes and their nuclei were measured, the volumes of cells, cytoplasm, and nuclei, and the nuclear-cytoplasmic ratio were calculated [24, 25]. The volume of hepatocytes and nuclei was determined by the formula $V = \pi/6 \times L \times B^2$, where L — large diameter of cells (nuclei), μm; B — small diameter of cells (nuclei), μm [25]. The obtained digital data was subjected to biometric processing [26].

For the accounting and measurement indicators, the mean values (*M*) and standard errors of means (±SEM) were calculated. The significance of the differences was evaluated according to Student's *t*-criterion, the differences were considered significant at $p \leq 0.5$.

Results. In pea grain, the fraction of globulins (simple proteins, the proportion of which reaches 60%) is dominated by legumin [27]. It is insoluble in water but is well soluble in neutral salts. Legumin contains a high content of lysine, valine, glutamic acid, serine, threonine but a very small content of methionine and tryptophan. Consumed legumin can react with inorganic salts and organic acids in the intestine with transformation into substances that are not available for digestion. Tannins can also affect mineral metabolism and nutrient digestibility negatively [6] (1% of tannins reduces protein digestibility by 6%).

1. Productivity of parental flock layers (*Gallus gallus* L.) of SP cross with different amounts of peas in the diet (*M*±SEM, vivarium of the Genetic and Selection Center Zagorskoye of the Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, Moscow Province, 2020)

Parameter	Treatment (n = 30 each)			
	1 (control)	2 (5%)	3 (10%)	4 (15%)
Mortality, %	0	3.33	0	0
Live weight of hens, g:				
at the beginning of the experiment	1669.8±23.5	1733.0±24.4	1698.3±18.2	1674.8±18.0
at the end of the experiment	1720.83±30.8	1787.43±41.1	1804.48±50.2*	1740.43±32.4*
Feed consumed:				
per 1 bird/day, g	113.101	113.85	112.77	113.02
per 10 eggs, g	1.861	1.803	1.715	1.761
per 1 kg of egg mass, kg	2.866	2.780	2.546	2.634
Intensity of lay, %	60.77	63.15	65.74	64.18
Average egg weight, g	64.95	64.85	67.39	66.86
Yield of egg mass per laying hen, kg	4.815	4.997	5.404	5.235
Eggshell deformation, μm:				
at the age of 65.8 weeks	24.60±1.71	26.46±1.15	23.60±1.99	24.07±1.04
at the age of 71.0 weeks	27.96±1.83	27.62±1.73	26.76±1.30	27.05±1.53
Ca content in the eggshell, %:				
in chickens aged 53.0 weeks	36.61	36.61	36.61	36.61
in chickens aged 65.8 weeks	36.34	36.00	36.67	35.64
Content in the tibia, % (at the age of 71.14 weeks):				
ash	60.84	63.75	59.43	55.86
Ca	23.28	23.26	22.47	20.92
P	9.62	9.59	9.49	8.85

* Differences with the control group are statistically significant at $p \leq 0.5$.

As the results showed (Table 1), in our experiments, feeding peas (5, 10, and 15%) to laying hens for 4 months from 37.6 weeks of age did not affect the

productivity and mortality of the birds negatively. The mortality of birds was 0% for 10 and 15% of peas and, though reached 3.33% in the group fed 5% of peas, was not associated with the feed factor. Birds readily consumed mixed feed with peas, and the live bodyweight of laying hens of treatments 2, 3, and 4 at 71 weeks of age exceeded the control by 3.87, 4.86 (in this treatment, the difference is statistically significant at $p \leq 0.5$), and 1.14%, respectively. At the same time, per 10 eggs and 1 kg egg mass, laying hens from treatments 2, 3, and 4 consumed less feed, by 3.12, 7.85, and 5.37%, respectively, and by 3.0, 11.16, and 8.09%. In the treatments receiving peas, compared with the control, the intensity of egg production increased by 2.38, 4.97, and 3.41%, respectively and the yield of egg mass per laying hen by 3.78, 12.23, and 8.72%.

Despite the presence of trypsin inhibitors and tannins in peas, the inclusion of peas in the mixed feed for parental flock layers in the dosages studied by the authors did not affect the livability of poultry, which is consistent with the results of studies [19, 21] performed on both broiler chickens and commercial layers. The good livability of birds can be mediated by a stimulating effect of the peas on the synthesis of proteins associated with immunity. E.g., it was reported [28] that the inclusion of peas in mixed feed for broilers led to an increase in their blood of the fraction of β- and γ-globulins, which contributed to the improvement of poultry immunity.

Mineral metabolism is of great importance in the feeding of parental flock layers [29], which ensures the long-term maintenance of the good condition of the chicken bones, the quality of the eggshell, and the production of a standard hatching egg. The value of elastic deformation, which characterizes the quality of eggshells, at 65.9 weeks of age in treatments 3 and 4, receiving 10 and 15% of peas as part of mixed feeds, varied from 23.6 to 24.07 rm vs 24.6 rm in the control. At the age of 71 weeks, the index of elastic deformation of the eggshell decreased significantly compared to the control (26.76-27.62 rm vs 27.96 rm), while in treatment 3 (10% of peas in the diet), the indicator was higher than in treatments 2 and 4. The thickness of the eggshell in all treatments during the entire accounting period corresponded to the standard for the cross. By the end of the accounting period, the deposition of calcium and phosphorus in the tibia of laying hens from treatments 2 and 3 (5 and 10% of peas in the composition of mixed feeds) remained at the control level, and with an increase in the number of peas up to 15%, it decreased significantly (by 2.36 and 0.77%) (see Table 1).

As it is known, peas are inferior in amino acid composition [30] to soy, which can affect the quality of eggs and reduce their weight. In the authors' experiment, the inclusion of peas in the diet did not affect the weight of eggs negatively: on average, in treatment 2 (5% of peas) it remained at the control level, and in treatments III and IV (10 and 15% of peas) it exceeded the control by 3.76 and 2.94%.

The analysis of the chemical composition showed (Table 2) that the fat content of hatching eggs from laying hens of treatments 2, 3, and 4 exceeded the control by 1.41, 1.55, and 1.10% (differences are insignificant), and the protein content decreased slightly (by 1.30, 1.21, and 0.12%, differences are insignificant). In terms of the amount of ash, the authors also did not find substantial significant differences between the treatments.

It should be noted that the inclusion of peas in mixed feed in all treatments contributed to a significant increase in the deposition of carotenoids in eggs — by 1.45, 2.02, and 3.73 rg/g, respectively. At the same time, with 10-15% of peas in the diets, the content of vitamin A decreased by 2.13-3.85 μg/g, vitamin E — by 14.39-36.31 μg/g, vitamin B_2 — by 1.24-0.99 in the yolk and by 0.20-0.08 μg/g in

the albumen. A significant decrease in the content of vitamin E in eggs was observed in laying hens that received 15% of lupine in the mixed feed [1].

2. Chemical composition and vitamin content of the hatching eggs of 65.8-week-old parental flock layers (*Gallus gallus* L.) of SP cross with different amounts of peas in the diet (per dry matter; vivarium of the Genetic and Selection Center Zagorskoye of the Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, Moscow Province, 2020)

Parameter	Treatment (n = 30 each)			
	1 (control)	2 (5%)	3 (10%)	4 (15%)
Moisture, %	75.34	74.85	74.89	73.10
Protein, %	50.29	48.99	49.08	50.17
Fat, %	34.77	36.18	36.32	35.87
Ash, %	3.28	3.00	3.18	3.00
Vitamin A, μg/g	9.30	10.73	7.17	5.45
Vitamin E, μg/g	114.20	176.98	99.81	77.89
Vitamin B_2 in the yolk, μg/g	6.32	6.34	5.08	5.33
Vitamin B_2 in the albumen, μg/g	4.22	4.24	4.02	4.14
Carotenoids, μg/g	4.22	5.67	6.24	7.95

N o t e. Analysis of combined samples of 10 eggs from each treatment. According to the method used, the differences are considered significant if the difference in the protein content exceeds 1%, fat — 2%, fat-soluble vitamins — 20%, water-soluble vitamins — 15%.

3. Biocontrol of incubation of eggs of 65.8-week-old parental flock layers (*Gallus gallus* L.) of SP cross with different amounts of peas in the diet (vivarium of the Genetic and Selection Center Zagorskoye of the Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, Moscow Province, 2020)

Parameter	Treatment (100 eggs each)			
	1 (control)	2 (5%)	3 (10%)	4 (15%)
Fertility rate, %	83	88	91	86
Infertile eggs, %	17	12	9	14
Blood ring, %	3	4	4	2
Stunned embryos, %	0	0	2	0
Contaminated embryos, %	1	0	0	0
Breach embryos, %	5	4	0	10
Weak and cripple chicks, %	1	3	2	3
Hatchability, %	89.16	90.91	93.41	86.05
Hatch, %	74	89	85	74
Hatched:				
standard chicks	73	77	83	71
total chicks	74	80	85	74

The full development of the embryo at the beginning of the incubation period affects the quantitative and qualitative results of incubation [19, 23]. The fertility rate of eggs in laying hens of 65.8 weeks of age was 83-91% and was higher in comparison with the control in all chickens receiving peas (5, 8, and 3%, respectively) (Table 3). In laying hens that received 5 and 10% of peas as part of mixed feeds, the mortality during incubation was lower than in the control by 1 and 3%. However, in the late incubation period, a significant increase in the number of addle eggs in treatment 4 (15% of peas) was observed, which then manifested itself in a deterioration of incubation indicators — a decrease in the hatchability of chickens (86.05% vs. 89.16% in the control) and a decrease in the number of standard chickens by 2.74%. Therefore, the use of 5-10% of peas in the diets of laying hens is more effective, increases the hatching of chickens in comparison with the control by 5 and 11%, and allows getting more (by 5.48 and 13.7%) standard chickens.

Previously, similar results were noted for preserving the quality of hatching eggs and hatch of chickens [19], as well as for the inclusion of 20% of peas in combination with CELLOVIRIDIN G20X (Russia) (60-70 g/t of feed) in mixed feed for broiler chickens [17, 19]. It should be noted that the use of enzyme preparations makes it possible to replace soybean meal with peas in the diets of

laying hens effectively, and at the same time, the intensity of egg-laying and the quality of eggs remain high [21, 22, 31].

With long periods of poultry management, the decrease in egg production, and the livability of laying hens, the deterioration in the quality of hatching egg is often due to fatty liver syndrome in laying hens, which can be provoked by antinutritive factors of feed [6, 10]. The revealed increase in the accumulation of fat in the liver of laying hens from the treatments that consumed mixed feed with different amounts of peas in the diet (Table 4) served as the basis for a histological examination of this organ to determine the optimal dosage of peas in the diet.

4. Chemical composition (%) of liver in 65.8-week-old parental flock layers (*Gallus gallus* L.) of SP cross with different amounts of peas in the diet (n = 12, vivarium of the Genetic and Selection Center Zagorskoye of the Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, Moscow Province, 2020)

Parameter	Treatment			
	1 (control)	2 (5%)	3 (10%)	4 (15%)
Moisture content	69.40	64.04	65.92	69.98
Protein	52.34	49.66	46.20	46.93
Fat	25.49	40.71	33.08	25.53
Ash	3.88	3.68	3.90	3.88

N o t e. Analysis of combined liver samples of three laying hens. According to the method used, the differences are considered significant if the difference in the protein content exceeds 1%, fat — 2%.

5. Morphometric liver parameters of 65.8-week-old parental flock layers (*Gallus gallus* L.) of SP cross with different amounts of peas in the diet (n = 12, M±SEM, vivarium of the Genetic and Selection Center Zagorskoye of the Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, Moscow Province, 2020)

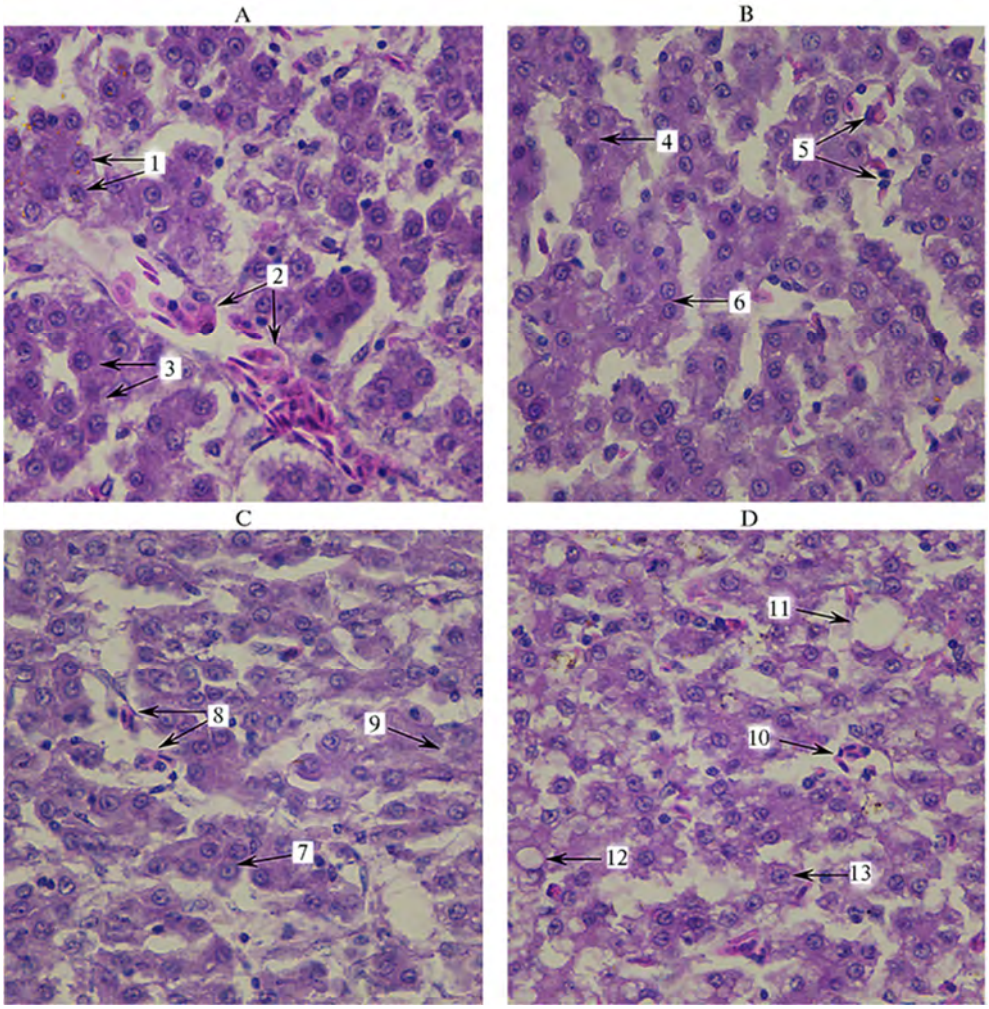
Parameter	Treatment			
	1 (control)	2 (5%)	3 (10%)	4 (15%)
Hepatocyte volume, μm^3	471.50±18.21	465.13±14.48	496.12±16.88	468.24±12.51
Nucleus volume, μm^3	41.44±3.11	39.18±3.42	39.47±3.18	41.66±4.56
Cytoplasmic volume, μm^3	430.99±23.40	426.71±22.75	457.63±29.87	427.87±31.14
Nucleus/cytoplasm ratio	0.09±0.01	0.09±0.01	0.09±0.01	0.10±0.01
Trabeculae, μm	11.27±1.43	14.33±1.56	13.48±1.71	17.92±1.98
Sinusoids, μm	8.42±1.07	10.57±1.11	9.88±1.17	8.84±1.19

Although during the morphometric study, the authors did not find substantially significant differences between the control and experimental treatments (Table 5), the number of peas in the diet affected the degree of observed destructive changes by 71.14 weeks of age. Histological examinations of the liver in laying hens showed the least liver damage in poultry from the control treatment. It was found that the liver histostructures had a typical structure, consisting of the stroma and parenchyma. The stroma is represented by a connective-tissue capsule and interlobular partitions. Mild connective-tissue septa extend from the capsule in places deep into the organ, the lobular structure is not expressed. Hepatocytes form hepatic tubules, which have a branching, sulcated appearance. In the lumen of the great veins and branches of the portal vein, the formed elements were found. The boundaries of hepatocytes are not expressed, the cells have a polygonal shape, the cytoplasm is colored unevenly, the nuclei are usually located in the center, sometimes eccentric, rounded in shape, contain 1-2 nucleoli (Fig., A).

The structure of the liver of chickens from treatment 2 was characterized by minor signs of fatty liver syndrome − the cytoplasm of some cells is foamy, the boundaries between the cells are not defined, the boundaries of the nuclei are clearly outlined, the nucleoli are clearly distinguishable in them, the sinusoid capillaries are slightly expanded, the tubule structure is preserved (see Fig., B).

The histostructure of the liver of chickens from treatment 3 indicates a

violation of the tubule structure, hepatocytes of various shapes (from oval to irregularly polygonal), the boundaries between them are not defined, the cytoplasm of the cells is cloudy, granular, signs of granular-protein dystrophy are expressed, the nuclei are usually located in the center of the cell, the nuclei are not detected in some hepatocytes, single erythrocytes are found in the sinusoid capillaries (see Fig., C).



Liver micromorphology of 71.14-week-old parental flock layers (*Gallus gallus* L.) of SP cross with different amounts of peas in the diet: A — treatment 1 (control, without peas, 1 — hepatocytes, 2 — central vein with erythrocytes, 3 — hepatic tubules); B — treatment 2 (5% peas, 4 − hepatocytes with foamy cytoplasm, 5 — sinusoid capillaries, 6 — hepatic tubules); C − treatment 3 (10% peas, 7 — hepatocytes, 8 — sinusoid capillaries with erythrocytes, 9 — liver beams); D — treatment 4 (15%, 10 — sinusoid capillaries with erythrocytes, 11 — large fat inclusions in the cytoplasm of the hepatocyte, 12 — small fat spheromes in the cytoplasm of the hepatocyte, 13 — hepatocytes, 14 — liver tubules). The preparations were stained with hematoxylin and eosin; light microscopy (a Micmed-6 microscope, LOMO, Russia; magnification ×400) (vivarium of the Genetic and Selection Center Zagorskoye of the Federal Scientific Center All-Russian Research and Technological Poultry Institute RAS, Moscow Province, 2020).

The histological pattern of the liver of chickens from treatment 4 indicates an expressed fatty liver syndrome. Single cells of the lymphoid series and single erythrocytes in the sinusoid capillaries are observed in the stroma and parenchyma. The tubule structure is not preserved, a significant number of large and small fat spheromes that shift the nuclei to the periphery are observed in the cytoplasm of

most cells, and such cells have a ring shape. In some cells, the boundary of the nuclei is not defined or they are absent (see Fig., D).

Changes in intestinal morphology (shortening of the villi, reduction of their surface area, and crypt depth) have been reported in broilers fed with peas compared to poultry fed with soy [32]. When 20% of raw or heat-treated seeds of grass pea (*Lathyrus sativus*) were added to the diet, changes in the size of the pancreas and the depth of the intestinal crypts were noted [33]. The authors have not found data on the effect of peas in the diet on the liver histostructure of poultry in the available literature. The excessive content of vegetable protein in treatments 3 and 4 leads to a deterioration in the metabolic function of the liver, which affects the content of lipids in it.

Certainly, the development of new varieties of peas [34, 35], the improvement of pea processing methods [36], and the use of enzyme preparations [9, 21] will make it possible to fully use this legume crop in poultry farming not only in feeding laying hens up to 50 weeks of age but also to include it in the feed of commercial and breeding poultry in later age periods.

Thus, this study has established the possibility of short-term inclusion of 5-10% of peas, containing 21.30% protein, 1.50% fat, 5.80% fiber, 1.53% lysine, 0.22% methionine, in the feed for breeding laying hens, to replace soybean and sunflower processing products. The inclusion of peas in the amount of 5 and 10% in the feed for parental flock layers in the late production period (53.7-71.14 weeks) increases the intensity of egg production by 2.38 and 4.97% compared to the control and increases the yield of egg mass per laying hen by 3.78 and 12.23% with a decrease in the number of infertile eggs. The results of histological examinations indicate that an increase in the number of peas in diets up to 10-15% can lead to cytotoxic effects, changes in the histostructure, and fatty liver syndrome. Nevertheless, zootechnical and incubation indicators allow arguing that to reduce the cost of mixed feed for parental flock layers, 5-10% of pea seeds can be included in the diets for a short time instead of processed soybean and sunflower products.

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AGRICULTURAL BIOLOGY,
2020, V. 55, № 6, pp. 1257-1267
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

Fodder crops and feeds

UDC 633.367:632.4(470.333)

doi: 10.15389/agrobiology.2020.6.1257eng
doi: 10.15389/agrobiology.2020.6.1257rus

DEVELOPMENT OF SCLEROTINIA IN NARROWLEAF (*Lupinus angustifolius* L.) AND WHITE (*Lupinus albus* L.) LUPIN SINGLE AND MIXED CROPS UNDER DIFFERENT WEATHER CONDITIONS IN BRYANSK REGION

L.I. PIMOKHOVA, G.L. YAGOVENKO, Zh.V. TSARAPNEVA, N.V. MISNIKOVA ✉

All-Russian Lupin Research Institute – Branch of the Federal Williams Research Center of Forage Production & Agroecology, 2, ul. Berezovaya, pos. Mitchurinskiy, Bryansk Province, 241524 Russia, e-mail lupin.fitopat@mail.ru, yagovenko@bk.ru, lupin_mail@mail.ru, lupin_nvmisnikova@mail.ru (✉ corresponding author)
ORCID:
Pimokhova L.I. orcid.org/0000-0002-9565-8176
Yagovenko G.L. orcid.org/0000-0003-3205-230X
Tsarapneva Zh.V. orcid.org/0000-0002-0311-5896
Misnikova N.V. orcid.org/0000-0001-5746-6539
The authors declare no conflict of interests
Acknowledgements:
Supported financially by the MSHE of the Russian Federation within the framework of the theme "To research species and population of the main forage crops' pathogens and to develop methodology for field infection load creation. To identify new sources with increased field resistance to diseases" (the RAS Program for Fundamental Research, AAA-A18-118072590033-1)
Received July 9, 2020

Abstract

White rot caused by ascomycetous fungus *Sclerotinia sclerotiorum* (Lib.) de Bary. is a widely spread disease of many cultivated and wild plants. In the Non Chernozem zone of Russia the epiphytotic development of white rot in white and narrow-leafed lupin crops began in 2008 and is related to climate change towards warming. The article presents the first report on dependence between weather conditions in the Non Chernozem zone and white rot infection of white and narrow-leafed lupines in single and mixed crops. High significant correlation coefficients have been obtained between air moisture and pods' white rot infection of the narrow-leafed lupin in June ($r = 0.95$, $p = 0.001$), and of the white lupin in June and July ($r = 0.90$, $p = 0.006$; $r = 0.81$, $p = 0.026$, respectively). High significant negative correlation coefficients between narrow-leafed and white lupin yields in single and mixed crops and *Sclerotinia* infection of pods have been revealed ($r = –0.92$, $p = 0.003$ and $r = –1.00$, $p = 0.002$, respectively; $r = –0.97$, $p = 0.000$ and $r = –0.88$, $p = 0.122$, respectively). The lupin species differed in susceptibility to the pathogen. This work aimed to evaluate the white rot development and harmfulness in white and narrow-leafed lupin crops depending on weather of the vegetation season and crop type under the conditions of Bryansk region. The narrow-leafed lupin var. Belozernyi 110 and the white lupin var. Dega were cultivated in single crops and in mixed crops with spring wheat var. Iren in 2008-2012, 2014, and 2016 in the North-East of Bryansk region (an experimental field of the All-Russian Lupin Scientific Research Institute). Climatic data were received from the meteorological station on the territory of the institute. Lupin plants were infected with white rot mycelium by the wet chamber method. Plant infection was evaluated during the vegetation period (stem and bud formation, flowering and pods' formation stages). The yield of each plot was weighted after the total threshing (a combine-harvester Sampo-500, Sampo Rosenlew, Finland). Intensive pathogen development on the tested lupin species in single and mixed crops was in rainy and warm June-August, at 66.2-80.3 % air moisture. Under rainy, warm and windy conditions white rot spread among plants in lupin crops both by ascospores and by mycelium particles. The first lesion focuses appeared on stems in low field sites and in dense crops. Depressive disease development occurred at air moisture from 54.1 to 60.3 %. White lupin plants were more susceptible to the disease, probably due to the morphological peculiarities of this species. Under favorable conditions for the disease, the incidence of pod infection averaged 15.3-34.8 % in white lupin single crops and 8.4-34.7 % in narrow-leafed lupin single crops. Herewith seed yield losses made 14.3-39.2 % and 3.0-34.7 %, respectively. Under dry conditions of 2010, the white lupin infection incidence was 0.3 % in mixed crops with no infection of the narrow-leafed lupin observed. The infection of the narrow-leafed and white lupines in their single crops made 0.1 and 1.3 %, respectively. The white rot was more harmful for lupin crops upon a combination of sufficient or excessive moisture and optimal temperature in the second part of vegetation. During this

period, the pods were formed on the main and lateral stems, and the plant foliation was maximum too. The latter creates favorable conditions for the pathogen inside the crops. Pod infection in lupines decreased significantly in mixed lupin-and-cereal crops, 1.4-1.6-fold for the white lupin, and 1.3-2.3-fold for the narrow-leafed lupin. Obviously, the lupin-and-cereal mixed crops create the conditions which are less favorable for the pathogen development and spread, therefore decrease infection of lupin plants and pods.

K Keywords: *Sclerotinia sclerotiorum*, crop type, air moisture, harmfulness, *Lupinus angustifolius* L., narrow-leafed lupin, *Lupinus albus* L., white lupin

Lupin is a valuable pulse crop that has high protein content in the grain and herbage and a beneficial effect on soil fertility. Currently, two types of lupin are widely cultivated in the Russian Federation – white (*Lupinus albus* L.) and narrow-leafed (*Lupinus angustifolius* L.). Modern varieties of these cultivars may provide a yield of 3-5 mt/ha of grain and up to 300-500 mt/ha of herbage under production conditions. At the same time, the protein content in the grain is 35-38%, exceeding the same indicator in peas and vetch by more than 10% [1-3]. Lupin protein is a source of crude protein in mixed feeds, which is especially important when there is a lack of high-quality animal feed and expensive imported soybean meal [4, 5].

Despite its valuable properties, lupin is not widely used in agricultural production. The phytosanitary situation in its crops is constantly changing due to many factors. One of them is the change in climatic conditions in the areas of lupin cultivation. According to available forecasts, by the end of the 21st century, the Earth's temperature may increase by 1.8-4.6 °C [6]. In Russia, only from 1990 to 2000, the increase in air temperature was 0.4 °C. According to the Russian Hydrometeorological Center, the most active warming occurs in the northern regions of the country, and the average winter temperature throughout Russia may increase by 2-5 °C. The increase in summer temperatures will be less expressed and will be 1-3 °C. In the next 20-30 years, the heat supply of the central and northern cultivated lands of the Central Federal district may reach or exceed the current indicators of the South of Russia [7]. In the Bryansk Region, from 1976 to 2016, the average annual air temperature increased by 2.1 °C, with fluctuations from 3.4 °C (1987) up to 7.4 °C (2016), with a long-term average of 6.2 °C, and since 1996, it has been increasing steadily [8].

Climate change affects all the functions of living organisms – survival, reproduction rate, spatial distribution, etc. [9-11]. The increase in air temperature in Russia is primarily manifested in warming in the autumn-winter (October-December) and winter-spring (January-May) periods. In recent years, it has led to an increase in the intensity of the development of endogenous diseases, the infectious matter of which persists in the soil, on plant residues, and wintering plants [12-14]. An increase in soil temperature in the spring-summer period stimulates the activity of soil fungi from the genera *Pythium*, *Rhizoctonia*, and *Sclerotinia*, reduces the latent period of their development, and increases the aggressiveness of pathogens [15-17]. As the temperature increases, the number of plant pathogens moving from south to north increases, which leads to an expansion of the areal of thermophilic fungal species [11].

Climate change is now becoming a major problem for all sectors of agriculture. Its effect on the host-pathogen system has been proven. Deviations of climatic parameters from long-term values over the past decades lead to epidemics of plant diseases all over the world. In the context of a changing climate, it is necessary to revise the strategy for combating plant diseases [18-20].

White rot is a widely spread disease of many cultivated and wild plants. The causative agent is the ascomycetous facultative fungus *Sclerotinia sclerotiorum* (Lib.) de Bary (class *Ascomycetes*, order *Helotiales*, family *Sclerotiniaceae*). White

rot affects crops in South and North America, Europe, China, Australia, and New Zealand. Especially many cases of its manifestation are registered in countries where crop rotations include soy (USA) [21-23]. Rot caused by *Sclerotinia sclerotiorum* is an economically significant disease of soybean (*Glycine max*) in the north-central United States and other temperate regions of the world. The occurrence and severity of stem rot caused by *S. sclerotiorum* in the field largely depend on environmental factors [24, 25].

Researchers from France, Germany, and Belgium report that *S. sclerotiorum* is the causative agent of stem and fruit rot and causes significant economic damage to crops of narrow-leafed and white lupin. Most of the seedlings that emerge from the affected seeds die during germination. Infection of plants through ascospores occurs on the internodes, in the sinuses of leaves or flowers; it requires drops of liquid or high humidity [26-28].

In the conditions of Belarus and Ukraine, this disease was detected in crops of yellow lupin [29, 30]. In Russia, in the main areas of lupin sowing, primarily in the central regions of the Non-Chernozem zone, economically significant lupin diseases until recently included fusariosis, anthracnose, ceratophorosis, gray rot, phomosis, bacteriosis, and viral overgrowth [31]. White rot on lupin did not occur or was very rare and did not cause significant lesions to lupin crops.

The mass development of white rot on the crops of narrow-leafed and white lupin in the conditions of the Non-Chernozem zone of Russia has begun in 2008. Crop losses of seeds of cultivated lupin species were significant. In the scientific literature, there are few papers devoted to *S. sclerotiorum* on narrow-leafed and white lupin in single-species crop and there are no studies dedicated to mixed crop with cereals. For the further development of lupin sowing in Russia, it is necessary to know about the harmfulness of the disease on narrow-leafed and white lupin in different phases of crop development and how meteorological conditions affect *S. sclerotiorum* during the growing season of the crop.

In this paper, the dependence between the weather conditions of the Non-Chernozem zone and the white rot of narrow-leafed and white lupin in single-species and mixed crops was revealed for the first time. It was found that at high humidity, the spread and development of the fungus *Sclerotinia sclerotiorum* on lupin occurred not only by ascospores but also by mycelium particles, and differences in the susceptibility of lupin species to the pathogen were revealed.

Our goal was to assess the development and harmfulness of white rot on white and narrow-leafed lupin crops, depending on the weather conditions of the growing season and the type of crop in the Bryansk Region.

Methods. The research has been carried out in 2008-2012, 2014, and 2016 in the North-East part of the Bryansk Province (an experimental field of the All-Russian Lupin Scientific Research Institute). Narrow-leafed lupin variety Belozernyi 110 and white lupin variety Dega were cultivated in single-species and mixed crops with spring wheat variety Iren. The plot area was 34 m^2, the repetition was 4-fold. The crop was sawn with an SN-16PM seeder (MZOK VIM, Russia) in the standard way. Data on climate indicators were provided by a weather station located in the territory of the Institute.

Parts of lupin plants (stems and pods) were infected with white rot mycelium by the wet chamber method [32] in 2009 and 2011. From the end of flowering to the beginning of pod formation, a fragment of mycelium was placed on a healthy part of the plant. The infection of lupin plants with white rot was evaluated during the growing season (the phases of stemming, budding, flowering, and pod formation). The sample consisted of 5 plants from the plot in 12-fold repetition. The seed yield from each of the 12 plots was determined by continuous threshing

of the crops with a Sampo-500 combine harvester (Sampo Rosenlew, Finland).

Statistical processing of the results was carried out by the method of variance analysis at a 95% confidence interval with the determination of the least significant difference between the variants according to Fischer *F*-test [33]. The mean values (*M*) and standard deviations of the mean values (±SD) were calculated. The correlation analysis method (STATISTICA 7.0, StatSoft, Inc., USA) was used to identify the relationship between air humidity, white rot lesion, and lupin yield. The tables show the least significant difference (LSD) for yield values, correlation rates (*r*), and significance level (p).

Results. Mass destruction of lupin pods by white rot, which led to serious losses of the seed yield and a decrease in their crop qualities, was first identified in the region in 2008 during a survey of the crops. To find out the cause of the occurrence and intensive development of the disease in lupin crops, meteorological indicators for June-August were analyzed. In general, the weather conditions during this growing season were warm and humid. The average daily air temperature exceeded the long-term average values by +0.9 °C, the amount of precipitation by 11 mm. The warmest and wettest conditions were in July. The amount of precipitation was 88.9 mm, which was 6.9 mm higher than the long-term average. The average daily temperature (19.3 °C) exceeded the long-term average by 1.1 °C. The average monthly humidity was also the highest, 78%. In June and August, the average monthly humidity was also high and amounted to 76.3 and 73.8%, respectively (Table 1).

Fig. 1. White rot (*Sclerotinia sclerotiorum*) on the stem of the white lupin (*Lupinus albus* L.) variety Dega in a single-species crop: a — formation of white cottony mycelium, b — formation of black matte sclerotia (the experimental field of the All-Russian Lupin Research Institute, Bryansk Province, 2012).

The first signs of white rot infection of lupin plants were found in a single-species crop at the end of June. They were observed on the surface near the root and higher up the stem (a white cottony coating of fungal mycelium and black sclerotia of various shapes). Then, the stems of neighboring plants were infected. The affected tissue was discolored, softened, acquired a brownish-green color, and then a wet rotting spot was formed, subsequently covering the entire stem and spreading up and down from the infection place. A cottony dense mycelium has developed on the surface and inside the affected stem (Fig. 1, a). Later, odd-shaped sclerotia of 0.5-2.0 cm formed on it, which were matte-black on the outside, and white on the inside (see Fig. 1, b). The stems and leafstalks became fragile and eventually broke, and the whole plant died and dried up.

Long rainy and warm period in July-August contributed to the intensive development and spread of white rot, which led to significant losses in the seed crop. At this time, pods were formed on the main and lateral branches, and the plants were as leafy as possible, which created favorable conditions for the active development of the pathogen inside the crop. First, the pods that were on the main stem were affected, since they were located lower than the side pods, and were less ventilated from moisture.

1. White rot infection and yield of green-ripened seeds of narrow-leafed lupin (*Lupinus angustifolius* L.) variety Belozernyi 110 and white lupin (*Lupinus albus* L.) variety Dega in a single-species and mixed crops depending on the air humidity (*M*±SD, the experimental field of the All-Russian Lupin Research Institute, Bryansk Province)

Average monthly humidity, %			Narrow-leafed lupin				White lupin			
			infection rate, %		seed yield, ha		infection rate, %		seed yield, ha	
June	July	August	2	2	1	2	1	2	1	2
					2008					
76.3	78.0	73.8	12.3	5.4	12.8±4.80	8,7±0,28	21,8	13,7	18,9±0,22	12,7±0,22
					2009					
72.1	76.6	73.7	10.7	8.4	13.7±0.28	7,1±0,22	15,3	9,7	21,7±0,35	14,3±0,22
					2010					
58.6	59.8	63.4	0.1	0	17.3±0.29	12,4±0,27	1,3	0,3	25,3±0,26	15,8±0,22
					2011					
72.7	79.4	73.1	8.4	6.7	16.8±0.23	8,2±0,20	21,7	15,4	19,2±0,20	13,9±0,24
					2012					
78.4	74.3	80.3	20.3	–	11.3±0.22	–	34,8	–	15,1±0,24	–
					2014					
60.3	52.4	54.1	0	–	16.7±0.29	–	0,7	–	24,7±0,26	–
					2016					
66.2	79.1	78.3	2.2	–	17.1±0.22	–	22,9	–	20,8±0,25	–
				LSD_{05}	2.69	0.38		LSD_{05}	0.38	0.34

N o t e. 1 — single-species crop, 2 — mixed crop with spring wheat variety Iren. Dashes indicate no mixed crops in the years of observation.

Fig. 2. White rot (*Sclerotinia sclerotiorum*) on pods of the white lupin (*Lupinus albus* L.) variety Dega (a) **and narrow-leafed lupin (*Lupinus angustifolius* L.) variety Belozernyi 110** (b). The formation of a white cottony mycelium and black sclerotia is shown (the experimental field of the All-Russian Lupin Research Institute, Bryansk Province, 2012).

The development of the disease on the pods began with their lower part, which was attached to the stem. By spreading, the pathogen could infect all the pods on the main stem. The affected pod tissue softened, first turning pale green, then brown. Over time, the pods were completely covered with the white cottony fungal mycelium, on which black sclerotia of various shapes and sizes appeared (Fig. 2).

From the affected pod leaves, the disease spread to the seeds, which were covered with a white coating of mycelium; subsequently, the mycelium turned into dark sclerotia. The affected pods became loose and fell to the ground along with the rotted seeds. If dead plants were present in the crop during this period, they were completely affected by white rot and died. According to Han et al. [25], a severe infection of *Canavalia gladiata* DC crops with white rot in South Korea in 2018 led to necrotic formations of stems and pods in contact with the soil.

It should be noted that in the conditions of the growing season of 2008, narrow-leafed lupin showed greater resistance to the disease in comparison with white lupin. In the green ripeness stage, the highest percentage of pod infection in single-species and mixed crops was observed on white lupin, 21.8% and 13.7%, respectively. The lesion of narrow-leafed lupin pods was significantly less, 12.3% in a single-species crop and 5.4% in a mixed crop (see Table 1). A similar pattern of lesion of narrow-leafed lupin and white lupin was observed in other years favorable for the disease development.

When examining lupin crops, the researchers found small fragments of

whitish mycelium on unaffected parts of plants that were close to the affected plant with a cottony fungal mycelium. To establish the possibility of spreading white rot by mycelium particles with the help of wind and rain, in 2009 and 2011, the artificial infection of white and narrow-leafed lupin with the pathogen mycelium was carried out in the field. In warm weather (18-22 °C) and sufficient moisture, the mycelium was introduced into the tissue of a growing pod or leaf of the plant with a dark spot formation, then a white mycelium and black sclerotia. The symptoms of the disease during artificial infection did not differ from the natural infection during this period on other crops. Consequently, the spread of white rot in lupin crops occurs not only with the help of ascospores but also with the transfer of mycelium particles from plant to plant by wind and rain. This increases the infection load in the crop, and under conditions favorable for the fungus (high humidity) leads to epiphytotic development of the disease and significant losses of seed yield. During the examination, the first foci of the disease on the stems of lupin plants were detected in low areas of the field and thickened crops, therefore, the disease in the crops has a focal nature.

Our long-term observations have shown that the development and spread of white rot in lupin crops are promoted by moderate, sometimes heavy rains and high humidity in the second half of the lupin growing season. It is confirmed by studies conducted in different regions of the world on other cultures. For example, Simic et al. [34] found that the grain yield and the manifestation of disease symptoms varied significantly depending on the amount of precipitation and temperature during the growing season of the sunflower. The authors note that the minimum grain yield (1.14 mt/ha) and the highest frequency of disease symptoms (24-67% of diseased plants) were observed in the wet year 2005, and the highest yield (3.87 mt/ha) and the lowest white rot lesion (3.2-5.8% of diseased plants) in 2007. According to the results of studies conducted in Vietnam [35], white rot is often found in the cool, humid winter and spring seasons on dwarf and climbing pods, peanuts, and sometimes on Chilli pepper plants. A severe lesion was observed in peanut crops in the spring of 2008 after a period of wet weather, in the phase of the beginning of flowering. The results of laboratory experiments with millet confirm that the duration of humidity and incubation temperature affects the germination of *Sclerotinia sclerotiorum* ascospores and the effectiveness of ascosporic infection. Ascospore germination is optimal when incubated at constant humidity at 21 °C. Lower germination was observed at 10 and 30 °C. Interrupting wet incubation delayed the manifestation of disease symptoms and inhibited plant infection [15].

In this study, favorable weather conditions for the development of white rot on lupin crops were in 2009, 2011, 2012, and 2016, when in June-August the relative humidity was 13.8-17.0% higher compared to 2010 and 2014, characterized by less precipitation and low relative humidity. The greatest infection of white rot was observed on crops of white lupin, but the disease caused the maximum lesion to both types of lupins in 2012. Wet and excessively wet conditions of the growing season developed in all the ten-day periods of June and August, while the average daily humidity was high and amounted to 78.4% and 80.3%, respectively. In July, the indicator slightly decreased, reaching 74.3%. The rate of fungus development was higher in single-species crops of narrow-leafed and white lupines. The lesion of pods in the green ripeness stage by white rot was the maximum and amounted to 20.3% and 34.8%, respectively. The seed yield this year was the lowest, 10.7 and 15.1 dt/ha. In comparison with the indicator obtained in 2010, the significant decrease in the yield of narrow-leafed and white lupines was 6.0 dt/ha (34.7%; LSD_{05} 2.69) and 10.2 dt/ha (40.3%; LSD_{05} 0.38), respectively.

The pods located on the main stem were more affected by lesions, especially in the white lupin. It may be due to differences in morphology. Plants of the narrow-leafed lupin in the green ripeness stage are less leafy than white, therefore, the crops are better ventilated, moisture evaporates from their surface faster, which reduces the intensity of the pathogen development. On white lupin plants, a larger number of leaves are formed by the green ripeness stage, which helps to create a denser crop, in which moisture persists longer, the illumination of the pods decreases, especially on the main stem, and favorable conditions for fungus development are created.

According to the results obtained by the authors, the development of white rot was determined by the humidity of the air, especially in July and August (see Table 1). Thus, in 2014, during these months, the humidity was 52.4% and 54.1%, and the disease development on the narrow-leafed lupin was not observed, and on the white lupin, it was insignificant (0.7%). With a small amount of precipitation and low relative humidity, the affected parts of the plants dried up, and the sclerotia formed on them fell off. Plants with affected stems died. In the dry years 2010 and 2014, the disease development on lupin occurred only near the root and in the middle part of the stem. The lowest lesion of white lupin in mixed crops (0.3%) was observed in the dry conditions of the growing season 2010. The lesion of narrow-leafed lupin in the same crop was absent. In a single-species crop, the lesion of narrow-leafed and white lupin plants was 0.1% and 1.3%, respectively.

2. Correlations between the studied variables in single-species and mixed crops of narrow-leafed lupin (*Lupinus angustifolius* L.) variety Belozernyi 110 and white lupin (*Lupinus albus* L.) variety Dega ((the experimental field of the All-Russian Lupin Research Institute, Bryansk Province, 2008-2012, 2014, 2016)

Variables	*r* and p values		
	June	July	August
Single-species crop			
White lupin			
Air humidity (%) × lesion of pods (%)	–0.94* (p = 0.002)	–0,71 (p = 0,071)	–0,83* (p = 0,021)
Yield (dt/ha) × lesion of pods (%)	0.90* (p = 0.006)	0,81* (p = 0,026)	0,92* (p = 0,003)
Air humidity (%) × lesion of pods (%)	–0.97* (p = 0.000)	–0,97* (p = 0,000)	–0,97* (p = 0,000)
Narrow-leafed lupin			
Air humidity (%) × lesion of pods (%)	–0.80* (p = 0.029)	0,38 (p = 0,399)	–0,56 (p = 0,190)
Yield (dt/ha) × lesion of pods (%)	0.95* (p = 0.001)	0,58 (p = 0,174)	0,71 (p = 0,072)
Air humidity (%) × lesion of pods (%)	–0.92* (p = 0.003)	–0,92* (p = 0,003)	–0,92* (p = 0,003)
Mixed crops with spring wheat			
White lupin			
Air humidity (%) × lesion of pods (%)	–0.89 (p = 0.105)	–0,86 (p = 0,144)	–0,94 (p = 0,060)
Yield (dt/ha) × lesion of pods (%)	0.95 (p = 0.050)	0,97* (p = 0,029)	0,91 (p = 0,089)
Air humidity (%) × lesion of pods (%)	0.88 (p = 0.122)	–0,88 (p = 0,122)	–0,88 (p = 0,122)
Narrow-leafed lupin			
Air humidity (%) × lesion of pods (%)	–0.93 (p = 0.074)	–0,93 (p = 0,075)	–0,88 (p = 0,116)
Yield (dt/ha) × lesion of pods (%)	0.90 (p = 0.096)	0,91 (p = 0,090)	0,85 (p = 0,147)
Air humidity (%) × lesion of pods (%)	–1.00* (p = 0.002)	–1,00* (p = 0,002)	–1,00* (p = 0,002)

* *r* values are statistically significant at $p < 0,05$.

Our observations have shown that the type of crop has a significant impact on the development and spread of the disease on lupin. In the conditions of the Bryansk Province, the greatest losses of the seed yield from white rot were observed

in the single-species crop of white lupin. With prolonged precipitation and high humidity in the second half of the growing season, pod lesions varied from 15.3% to 34.8%, which reduced the seed yield by 14.3-39.2% (LSD_{05} = 0.38 at 95% significance level). The correlations revealed a significantly high dependence of the lesion of white lupin plants in a single-species crop on humidity in June and July, the r = 0.90 (p = 0.006) and r = 0.81 (p = 0.026), respectively (Table 2).

The lesion of lupin pods under similar conditions ranged from 8.4% to 20.3%, and the yield loss from 3.0% to 34.7% (LSD_{05} = 2.69). In June, in the single crop of narrow-leafed lupin, a high reliable relationship between humidity and white rot lesion to plants (r = 0.95, p = 0.001) and an inverse reliable relationship between yield and pod lesion (r = –0.92, p = 0.003) were revealed.

Multi-year observations have shown that in a mixed crop with cereals, both lupins were less affected by white rot than in a single-species crop. Thus, in 2011, which was favorable for the disease development, in the mixed crop, the lesion of pods in narrow-leafed and white lupins was 6.7% and 15.4%, respectively, while in a single-species crop, 8.4% and 21.7% pods were affected. In the mixed crop in the years with high humidity (2008, 2009, 2011), the yield of narrow-leafed and white lupin seeds decreased 1.4-1.7 and 1.2 times, respectively (LSD_{05} = 0.38, LSD_{05} = 0.34) compared to the year with low humidity (2010). It can be assumed that in mixed crops with cereals, microclimatic conditions are created that are less favorable for the disease development, which reduces the lesion to plants and lupin pods.

Thus, the development and spread of white rot on crops of white and narrow-leafed lupin in the Non-Chernozem zone of Russia is largely determined by meteorological conditions. Intensive development of the pathogen in single-species crops and crops mixed with spring wheat occurs in case of rainy and warm weather in June-August and the air humidity of 66.2-80.3%. In years with less precipitation and low relative humidity, white rot lesion to lupin crops fell sharply or was absent. The species dependence of the susceptibility of lupin to the disease was revealed. The greatest white rot lesion was noted on white lupin. The lesion of white lupin pods in a single-species crop reached 15.3-34.8%, of narrow-leafed lupin pods 8.4-34.7%. In a single-species crop, high reliable negative correlation (r = –0.97, p = 0.000) was obtained between the yield of white lupin and its white rot lesion. In a mixed crop with cereals, the pod lesion decreased 1.4-1.6 times in white lupin and 1.3-2.3 times in narrow-leafed lupin. The specificity of mixed crops, where the second component is a cereal crop, allows suggestion on their deterrent role in the development of the studied fungal disease. To reduce the harmfulness of white rot on lupin, it is advisable to cultivate it in mixed crops with cereals.

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AGRICULTURAL BIOLOGY,
2020, V. 55, № 6, pp. 1268-1284
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

UDC 636.085.52:579.64:577.2

doi: 10.15389/agrobiology.2020.6.1268eng
doi: 10.15389/agrobiology.2020.6.1268rus

FERMENTATION PROCESSES IN ALFALFA HAYLAGE WITHOUT ADDITIVES AND WITH INTRODUCTION OF *Lactobacillus plantarum* STRAIN

Yu.A. POBEDNOV[1], A.A. MAMAEV[1], M.S. SHIROKORYAD[1], E.A. YILDIRIM[2] ✉, G.Yu. LAPTEV[2], L.A. ILYINA[2], E.A. BRAZHNIK[2], N.V. TARLAVIN[2]

[1]*Williams Federal Research Center for Fodder Production and Agroecology,* 1, Nauchnii Gorodok, Lobnya, Moscow Province, 141055 Russia, e-mail yurypobednow@yandex.ru, Anton.mamaev@inbox.ru, m-latysheva@list.ru;
[2]*JSC «Biotrof+»,* 19 korp. 1, Zagrebskii bulv., St. Petersburg, 192284 Russia, e-mail deniz@biotrof.ru (corresponding author ✉), laptev@biotrof.ru, ilina@biotrof.ru, bea@biotrof.ru, tarlav1995@biotrof.ru
ORCID:
Pobednov Yu.A. orcid.org/0000-0001-8701-009X
Mamaev A.A. orcid.org/0000-0002-7962-4048
Shirokoryad M.S. orcid.org/0000-0001-8333-752X
Yildirim E.A. orcid.org/0000-0002-5846-4844
Laptev G.Yu. orcid.org/0000-0002-8795-6659
Ilyina L.A. orcid.org/0000-0003-2490-6942
Brazhnik E.A. orcid.org/0000-0003-2178-9330
Tarlavin N.V. orcid.org/0000-0002-6474-9171
The authors declare no conflict of interests
Received July 3, 2020

Abstract

The optimal pH required for the functioning of proteases in alfalfa is lower than that of meadow clover or cereal grasses, and this culture is rich in protein and pectin which is not favorable for high-quality feed production. It is recommended to accelerate the acidification of the alfalfa being hayed by adding preparations of lactic acid bacteria. In the present work, for the first time in Russia, a diversity profile of haylage microbiota during fermentation was revealed using NGS sequencing. The work aimed to study the peculiarities of alfalfa fermentation during haylage with and without using Biotrof, a lactic acid bacteria-based preparation. The experiments were performed in 2018-2019. In the first experiment, the peculiarities of biochemical and microbiological processes during alfalfa haylaging were examined. Alfalfa *Medicago sativa* L. nothosubsp. *varia* (Martyn) Arcang cv. Pastbishnaya 88 was grown (experimental field, the Williams Federal Research Center for Forage Production and Agroecology, Moscow Province), cut for hay, dried in swaths for 7 hours to a dry matter content of 43.5 % and put into 0.5 l glass vessels for haylaging. The pH dynamics, ammonia, sugar and fermentation acid levels were measured on days 0, 4, 7, 14, 28, 60, and 90 of storage. The composition of the microbial community of the plant biomass and the alfalfa haylage was analyzed in dynamics using NGS sequencing according to a modified technique. In the second series of experiments, the effect of the preparation of lactic acid bacteria Biotrof (OOO Biotrof, Russia) based on *Lactobacillus plantarum* No. 60 on storability and biochemical parameters of the haylage from alfalfa cv. Pastbishnaya 88 biomass dried to a dry matter content of 47.6 and 51.3 %, was studied. The biomass was put into 0.5 l-containers equipped with devices for measuring evolved gases for two treatments, with no additives and upon introduction of the Biotrof preparation in the recommended dose (10^5 CFU/g green mass). It was shown that a short-term wilting of alfalfa biomass to the haylage moisture resulted in 0.03-0.04 % ammonia and 0.08 % butyric acid concertation followed by an increase to 0.08-0.09 and 0.13-0.14 %, respectively, when haylaging. During wilting and early fermentation, the sugar contents in the biomass increased noticeably. In addition, the wilted alfalfa accumulates at least 3.7 % of malic acid which, like sugar, can be fermented by lactic acid bacteria. Butyric acid producers, the bacteria of the *Clostridiaceae* family, were not detected during fermentation. During haylage storage, among the bacteria of the *Clostridia* class the typical rumen microorganisms were identified of the families *Eubacteriaceae*, *Lachnospiraceae*, *Peptostreptococcaceae*, and *Ruminococcaceae*. We have found a relationship between an increase in the abundance of bacteria of the genus *Ruminococcus* and an increase in the amount of malic acid ($r = 0.80$, $p \le 0.05$), and also between an increase in the amount of malic acid and an increase in the number of bacteria of the phylum *Bacteroides* in the haylage ($r = 0.84$, $p \le 0.05$). The accumulation of malic acid improved the fermentability of plant biomass, causing a rapid acidification of the feed to pH 4.4-4.3 due to the introduced preparation of lactic acid bacteria Biotrof. This method improved the biochemical parameters of the feed, contributing to a decrease in the butyric acid level, however, it did not lead to a noticeable improvement in the preservation of nutrients and an increase in the energy nutritional value of the dry matter of the

obtained haylage due to the favorable fermentation process in dried alfalfa biomass. Acceleration of the acidification of the dried mass with the Biotrof preparation did not have a significant effect on the reduction of ammonia formation during fermentation. *Staphylococcus arlettae*, *Salmonella subterranea*, *Streptococcus gordonii*, and *Enterococcus cecorum* capable of causing diseases in humans and animals, survived up to 4-14 days of storage in haylage without additives. In this regard, the stored haylage, if technological disturbances occur, may contain pathogens of farm animals, therefor, antimicrobial biologicals are required for conservation. Therefore, the main effect of the Biotrof application was reduced only to an improvement in the biochemical parameters of the feed without leading to a noticeable increase in its preservation.

Keywords: alfalfa, haylage, proteolysis, microbiota, biologicals, lactobacteria, acidification, feed quality, NGS sequencing, quantitative PCR

Alfalfa is a non-silage plant because it is low in sugar and has a high buffering capacity [1]. The biological characteristics of this species have a significant influence on the result of preservation. The optimal pH required for the functioning of proteases in alfalfa is lower than in meadow clover or cereal grasses [2]. Moreover, the main proteases, under the influence of which most of the protein contained in alfalfa is hydrolyzed to non-protein nitrogen, exhibit maximum activity at pH 4.0 [3], which is due to intense proteolysis in the silage mass even in the case of rapid creation of the necessary active acidity in it [4]. The presence of a large amount of protein and pectin in alfalfa does not contribute to obtaining high-quality silage. The content of the latter in the dry matter of leaves and stems of plants reaches 10-12 and 6-9%, respectively [5]. Even in alfalfa wilted to a dry matter content of 30-35%, there is a significant amount of water weakly bound to protein and pectin, which, against the background of slight acidification of the feed, contributes to the development of bacteria in it that carry out putrefactive fermentation. For this reason, alfalfa is more often used for haylage, wilting plants to a dry matter content of 45-50% [6]. However, due to the above-mentioned reasons, a certain amount of butyric acid can still accumulate in the feed [7]. To prevent this, it is recommended to accelerate the acidification of the alfalfa being hayed by adding preparations of lactic acid bacteria [8].

Since during the haylage making of alfalfa, along with the necessary wilting of plants, the degree of acidification plays a role, the methods aimed at improving the fermentability of the dried mass are important. According to the available data, it is possible to increase the sugar content in the dry matter of alfalfa and, therefore, to improve its fermentability due to short-term (4-8 h) wilting in swaths to the haylage moisture content [9]. However, the mechanism of this process has not yet been fully understood. The reasons for the increase in sugar content in alfalfa haylage making are not fully understood, although this phenomenon has been known for a long time. There is no ultimate clarity regarding the formation of butyric acid in alfalfa haylage making. Some authors argue that in this case, butyric acid does not accumulate at all, despite the presence of clostridium spores in the dried mass [10].

Some researchers believe that the safety of haylage is associated exclusively with the phenomenon of physiological dryness, which inhibits the development of putrefactive microflora [11, 12]. At present, in Russia, the studies of the microflora of preserved feed using molecular methods are carried out only in the laboratory of OOO BIOTROF (St. Petersburg). Abroad, the research works are devoted to the analysis of the microbiocenosis of silage [13, 14]. This may be due to the problem of obtaining pure DNA material of haylage microflora because of to the presence in the biomass of a large number of organic impurities (polysaccharides, various organic acids, degradation products of protein and fats, nucleases, etc.) [15], which can reduce the quality of DNA purification [16].

Earlier, for the first time in Russia, we optimized the procedure of DNA extraction for microbial community of haylage [17]. Using terminal restriction fragment length polymorphism and quantitative polymerase chain reaction (qPCR) analysis, we studied the microflora composition of alfalfa haylage, dried to a dry matter content of 55±1.9%, on day 30 of storage. The total number of bacteria in the haylage microflora was $1.1\times10^8\pm3.4\times10^6$ genomes/g. The number of phylotypes of microorganisms represented in the haylage was 58±3.9, the average Shannon index was 3.3±0.22, and Simpson's index was 0.96±0.05. These results demonstrated the complexity of organizing microbial communities in haylage.

In our opinion, the further study of the microbial ecosystem of haylage in dynamics is extremely interesting. Such an ecosystem is constantly changing and subject to anthropogenic interference, which makes it a unique and complex ecological niche.

In this work, for the first time in Russia, the diversity of the composition of microorganisms during the fermentation of haylage was revealed using next-generation sequencing (NGS). During the fermentation, no typical silage microorganisms, producers of butyric acid of the *Clostridiaceae* family, were found. Among the bacteria of the *Clostridia* class in the haylage during storage, typical rumen dwellers were identified, i.e., bacteria of the families *Eubacteriaceae*, *Lachnospiraceae*, *Peptostreptococcaceae*, and *Ruminococcaceae*. For the first time, a relationship was revealed between an increase in the abundance of bacteria of the genus *Ruminococcus* and an increase in the amount of malic acid, as well as between an increase in the level of malic acid and an increase in the number of bacteria of the phylum *Bacteroides*.

This work aimed to investigate the peculiarities of fermentation and the structure of the microbiome for convenient alfalfa haylaging and using the preparation Biotrof based on lactic acid bacteria.

Methods. The experiments were performed in 2018-2019. In the first experiment, the features of biochemical and microbiological processes during alfalfa haylaging were studied. The raw material used was variegated alfalfa *Medicago sativa* L. nothosubsp. *varia* (Martyn) Arcang of the cultivar Pastbishchnaya 88 (experimental field of the Federal Williams Research Center of Forage Production and Agroecology, Moscow Province). The crop was harvested in the budding phase. Before filling in 0.5 l glass bottles, alfalfa biomass was dried in swaths for 7 hours to a dry matter content of 43.5%. The dynamics of pH, the content of ammonia, sugar, and fermentation acids were assessed during the alfalfa haylage making in laboratory vessels in the usual way. The haylage was analyzed after 0, 4, 7, 14, 28, 60, and 90 days of storage. Simultaneously, the obtained feed samples were frozen at –25 °C for molecular studies.

The composition of the microbial community of the original alfalfa plant mass and haylage was analyzed using NGS sequencing with modifications [17]. Total DNA was extracted using Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) according to the attached instructions. When obtaining products for subsequent NGS sequencing, PCR was performed on a Veriti Thermal Cycler DNA amplifier (Life Technologies, Inc., USA) with eubacterial primers (IDT) 343F (5′-CTCCTACGGRRSGCAGCAG-3′) and 806R (5′-GGACTANVGGGTWTCTAAT-3′), flanking the V1V3 region of the 16S rRNA gene. Metagenomic sequencing (MiSeq system, Illumina, Inc., USA) was performed using the MiSeq Reagent Kit v3 (Illumina, Inc., USA). The maximum length of the obtained sequences was 2×300 bp. Chimeric sequences were excluded from analysis using the

USEARCH 7.0 program (http://drive5.com/usearch/). Processing of the obtained 2×300 bp reads was performed using the CLC Bio GW 7.0 bioinformatics platform (Qiagen N.V., the Netherlands) and included detection of overlapping sequences from forward and reverse primers for unambiguous sequence reading, quality filtering (QV > 15), and primer trimming. The taxonomic affiliation of microorganisms to genera was determined using the RDP Classifier program (https://sourceforge.net/projects/rdp-classifier/).

qPCR (real time PCR — RT-PCR) was performed using a DTlite-4 detecting amplifier (OOO NPO DNA-Tekhnologiya, Russia) with a set of reagents for RT-PCR and primers HDA1 (5′-ACTCCTACGGGAGGCAGCAG-3′) and HDA2 (5′-GTA-TTACCGCGGCTGCTGGCA-3′) in the presence of an intercalating dye EVA Green (ZAO Syntol, Russia). The amplification protocol was as follows: 3 min at 95 °C (1 cycle); 1 min at 95 °C, 1 min at 57.6 °C, 1 min at 72 °C (40 cycles); 5 min at 72 °C (1 cycle).

The diversity of the bacterial community was assessed graphically as a heatmap constructed using the "pheatmap" package Version: 1.0.12 for R (https://cran.r-project.org/web/packages/pheatmap/pheatmap.pdf). Hierarchical clustering by samples was performed according to Ward's method on a matrix built according to the Euclidean distances.

In the second series of experiments, we studied the effect of the Biotrof preparation (OOO Biotrof, Russia) based on *Lactobacillus plantarum* No. 60 on the safety and biochemical parameters of haylage from alfalfa cultivar Pastbishnaya 88 when wilted to a dry matter content of 47.6 and 51.3%. The haylage was prepared in laboratory 0.5 l containers equipped with devices to measure volumes of the emitted gases when haylaging without additives and with the introduction of the Biotrof preparation in the dose recommended by the manufacturer (105 CFU/g of green mass).

The dry matter content in the green biomass and the resulting feed was determined by drying the weighed portions at 105 °C to constant weight, the sugar concentrations were measured by the Bertrand method, ammonia according to Longi, pH with an I-500 potentiometer (Russia), organic acids (lactic, acetic, butyric, formic, propionic, succinic, malic, citric, tartaric, and oxalic) by capillary electrophoresis (KAPEL-105M, Lumex, Russia).

The mathematical and statistical processing of the results was performed by standard methods of analysis of variance in Microsoft Excel XP/2003, PAST (http://priede.bf.lu.lv/ftp/pub/TIS/datu_anali-ize/PAST/2.17c/download.html), and R-Studio (https://rstudio.com). The results are presented as the mean (M) and standard errors of the mean (±SEM). The differences were assessed using Student's t-test. The results were considered statistically significant at $p \leq 0.05$.

Results. It was found that even during the 7-hour wilting of alfalfa in swaths, up to 0.03% of ammonia and 0.08% of butyric acid accumulated in its dry matter (Table 1). The main reason for the accumulation of ammonia was protein hydrolysis under the influence of plant enzymes, followed by deamination of the formed amino acids.

It is important, however, to note that with brief wilting of alfalfa to haylage moisture, the accumulation of ammonia in its dry matter did not increase in comparison with its content in the dry matter of freshly cut alfalfa. This indicates that, along with the breakdown of protein during wilting of alfalfa, synthetic processes take place, in which ammonia is consumed for forming amides. The latter, as it is known, occurs under the condition that high intensity of respiration remains in the dried mass.

1. Biochemical parameters of haylage from alfalfa *Medicago sativa* L. cv. Pastbishchnaya 88 (wilted to 43.5 % dry matter) during storage (lab test)

Parameter	Storage						
	0 day	4 days	7 days	14 days	28 days	60 days	90 days
Content in dry matter, %:							
ammonia	0.03	0.05*	0.05	0.09*	0.08	0.09	0.09
sugar	4.52	5.24*	4.01*	5.55*	4.45*	2.28*	1.25*
organic acids							
lactic	0.09	0.76*	1.00	0.21*	0.61*	4.17*	6.09*
acetic	0.05	0.35*	0.13*	0.21*	0.19	0.34*	0.45*
butyric	0.08	0.13*	0.13	0.14	0.11	0.11	0.10
succinic	0.09	0.15*	0.15	0.20	0.19	0.24	0.34*
malic	3.72	3.90	3.19*	3.89*	3.12*	2.05*	2.03
citric	0.48	0.48	0.50	0.48	0.45	0.34	0.19*
pH	6.18	5.92*	5.93	5.95	5.87*	5.32*	4.93*

* Differences with the indicator in the previous time period is statistically significant at $p \leq 0.05$.

There are grounds to believe that a rather significant increase in the sugar content in the dry matter of the dried alfalfa biomass is associated with synthetic processes. This phenomenon is still explained by the hydrolysis of starch contained in plants under the influence of enzymes. However, this statement is not supported by the available experimental data. In particular, it was found that, during wilting, plants accumulated not maltose, as would be expected during starch hydrolysis, but sucrose, which is the main product of photosynthesis [18]. Based on the generally accepted point of view, the authors explain this by the fact that when starch decomposes, sucrose is formed not primarily, but by a secondary path, that is, as a result of its subsequent synthesis from glucose and fructose. Also, the available data show that such a process occurs only when alfalfa is dehydrated in the sun in swaths and is not initiated when plants wilt in the dark [9]. This is evidence that the sugar content during short-term wilting of alfalfa increases as a result of photosynthesis, which also takes place in the mowed mass for some time.

The initial and not entirely correct interpretation of the discussed issue, obviously, was formed on the basis of the obtained data. In particular, it was found that photosynthesis slowed down when plants lost more than 15-20% of moisture, while respiration proceeded intensively even with a higher degree of wilting of plants [19]. From this, it was quite possible to conclude that the slowed-down photosynthesis combined with intensive respiration cannot provide a noticeable increase in the sugar content in the dry matter of the wilted biomass.

However, it became known that with intense dehydration, plant growth first stops, then photosynthesis is inhibited, and only then the respiration of plant cells is suppressed [20]. It is the cessation of plant growth, which causes a delay in the outflow of the sucrose formed during photosynthesis to other organs, including the root system already torn away from them, that contributes to a noticeable increase in the sugar content in the vegetative mass. This phenomenon is of a protective nature and is of fundamental importance when growing plants in drought conditions. Despite the cessation of the growth of the vegetative mass, the root system (and above all its growth zones) is still in rather favorable conditions and uses the excess sugar formed in the leaves for its own enhanced growth, leading to the reclamation of deeper and, therefore, more waterlogged layers of soil.

As it was noted above, with intensive wilting on haylage, alfalfa still retains a high respiration rate due to the adaptive restructuring of the respiratory apparatus to work under conditions of dehydration. This, in particular, is indicated by the high accumulation of dried mass of malic acid in the dry matter, which allows plants to synthesize citric acid directly from malic acid [21]. The described mechanism makes it possible to reduce the dependence of plants on such a costly process as glycolysis, while simultaneously ensuring the work of the Krebs cycle in a shortened type. Meanwhile, alfalfa, wilted to the specified dry

matter content, already suffers from a lack of oxygen. This, in particular, is indicated by the accumulation of a certain amount of succinic acid in it. The appearance of the latter indicates its release from mitochondria and serves as an indicator of the development of progressive hypoxia in the mass [22]. During normal plant respiration, succinic acid is either not detected or is determined in trace amounts, since it is formed only in mitochondria, where it is instantly utilized. Obviously, the accumulation of a certain amount of butyric acid in the dried mass of alfalfa is also associated with the onset of hypoxia. There are reports in the literature [18] indicating the possibility of butyric acid synthesis in plants with a lack of oxygen due to a violation of fat metabolism. Malic acid, or malate, is formed as a result of the glycolytic decomposition of starch, which is very quickly synthesized in the light from sucrose formed during photosynthesis and is deposited in chloroplasts [23]. This explains the fact that when alfalfa is wilted in the dark, that is, in the absence of photosynthesis, neither sugar nor malic acid is formed in it [9].

An increase in the content of sugars, and, consequently, an improvement in the fermentability of alfalfa was observed in the first 4 days of its haylage making. As it is while wilting, this phenomenon was aimed at preserving the vital activity of plants in the conditions of the onset of anaerobiosis, but was conducted due to the use of their own reserve nutrients. According to the available data [24], the main source of sugar in this case is the hydrolysis of hemicelluloses, which occurs under the influence of plant enzymes.

From the obtained results, it follows that this process proceeded most vividly at the very beginning of alfalfa haylage making, when there was still no noticeable fermentation in it associated with the accumulation of organic acids. As the intensity of lactic acid fermentation increased at the final stages of haylage making, the sugar content decreased markedly.

Along with an increase in the content of monosaccharides, an important condition for improving the fermentability of alfalfa is the possibility of using malic acid by lactic acid bacteria, the accumulation of which in the dry matter of the wilted mass was only slightly inferior to the sugar content. Our studies have shown that, as in the case of monosaccharides, malic acid fermentation took place only at the later stages of haylage making, when the content of lactic acid in the feed increased markedly. From this, it can be concluded that the main condition for the fermentation of both malic acid and monosaccharides was the provision of developing intensive lactic acid fermentation in the feed. The same can be stated about citric acid found in plants.

According to the data we obtained, a significant ($p \leq 0.05$) increase in the ammonia content in dry matter of the haylage mass was noted only in the first 2 weeks. Then its amount stabilized and did not change during the entire subsequent storage period of the feed. In protein degradation during haylage and silage making form dried alfalfa mass, researchers usually distinguish two main stages [25]. At the first stage of fermentation of the biomass, plant enzymes are mainly active, causing the breakdown of protein to free amino acids. The main role in the deamination of amino acids, that is, in the accumulation of ammonia as such, is assigned to microbial enzymes.

Some authors note that a certain amount of ammonia can be formed as a result of the influence of plant enzymes on the protein [18]. They came to this conclusion on the basis of the results of experiments on ensiling meadow clover with toluene. The latter is known to inhibit the development of bacteria without having a noticeable negative effect on the activity of enzymes. It was found that in the absence of bacterial development in the feed, protein decomposition was accompanied by the accumulation of a certain amount of ammonia in the mass,

the nitrogen of which was up to 5.0% of the total feed nitrogen. It is not excluded, however, that in this case the addition of toluene, causing the death and lysis of bacterial cells, promoted the release of the enzymes contained in them into the external environment. In other words, both plant and microbial enzymes were involved in proteolysis.

In the first 4 days of haylage making, a significant ($p \leq 0.05$) increase was noted in the content of butyric acid in the dry matter of the feed, which increased 1.6 times compared to the accumulation of the original dried alfalfa in the dry matter. The fact that butyric acid was formed at the very beginning of the haylage making of alfalfa, when no noticeable fermentation was yet observed in it, indicates that, as while wilting, some amount of butyric acid can be formed in a purely biochemical way, i.e., without the participation of microbes.

Thus, Maevskii et al. [26], relying on the results obtained in the experiments on animals, note that under conditions of hypoxia, the amount of reduced nicotinamide adenine dinucleotide (NADH) in mitochondria noticeably increases, which leads to a decrease in the oxidation of NAD-dependent substrates. As a result, there is an excessive accumulation of acetyl-CoA, a product of β-oxidation of fats, the further oxidation of which is inhibited due to the fact that an increase in NADH causes a rapid restoration of oxaloacetate to malate. As a result, acetyl-CoA remains without a partner, which is necessary to enter the Krebs cycle, and instead of complete oxidation, it becomes a source of ketone bodies, fatty acids, and cholesterol formation. There are researchers who believe that, when making silage from dried alfalfa, along with proteolysis, the lipolysis process actively proceeds in it, the main cause of which is plant, rather than microbial, lipase [27].

For a more detailed study of ammonia and butyric acid formation during alfalfa haylage making, we determined the dynamics of the total number and species composition of microorganisms by the storage period of the feed.

qPCR analysis showed that the total number of bacteria in the haylage at all periods ranged from $1.5\times10^6\pm9.3\times10^5$ to $2.0\times10^7\pm1.1\times10^6$ cells/g (Table 2). That is, the alfalfa haylage at all stages of storage was quite significantly contaminated with microorganisms, which indicates a fairly active microbiological fermentation and contradicts the data on the absence of microbial fermentation processes in the haylage [11, 12]. Nevertheless, comparing the obtained data with our previous results on silage [17], it should be noted that the fermentation processes in haylage are less active. The authors have shown that the total content of bacteria in the silage ecosystem during the conservation of perennial grasses ranged from 5.2×10^7 to 99.4×10^9 genomes/g.

However, the composition of the haylage microflora sharply differed from the composition of epiphytic microorganisms of the original plant mass of alfalfa and changed in the course of successional changes occurring during storage (see Table 2). Probably, these changes occurred as a result of creating anaerobic conditions and changes in the biochemical composition of the feed. Thus, the composition of the haylage microflora was represented by 8 phyla of microorganisms (Fig.), while the structure of the epiphytic microflora included 18 phyla of bacteria.

Typical [28] microorganisms of the phylum *Acidobacteria* significantly dominated on the mowed alfalfa plant mass (62.6 ± 3.4 % at $p \leq 0.05$) (see Fig.). Despite their widespread prevalence in the environment, knowledge of the metabolism of these bacteria is in its infancy due to the almost complete impossibility of cultivation on nutrient media. The information on the presence of these bacteria in the epiphytic microflora of plants was obtained only after the appearance of methods for analyzing the sequences of 16S rRNA genes [29].

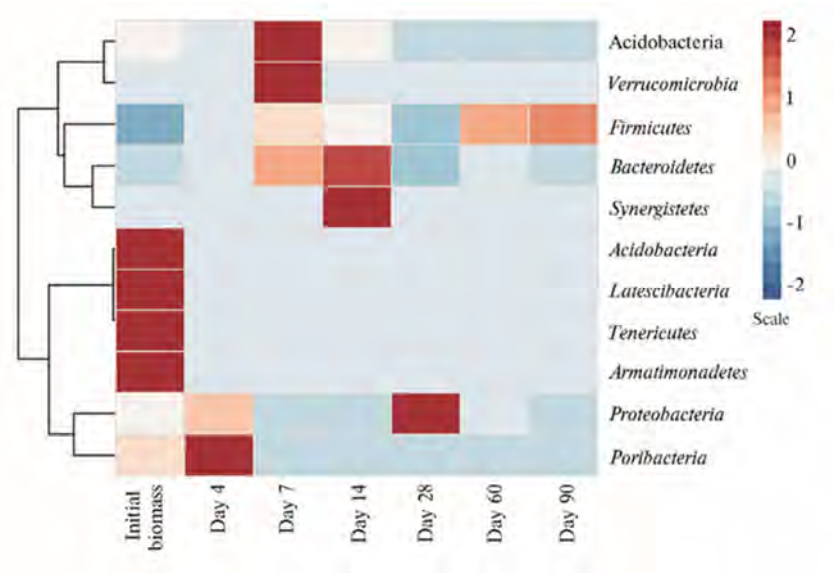
2. Microbial community composition in haylage from alfalfa *Medicago sativa* L. cv. Pastbishchnaya 88 (wilted to 43.5% dry matter) during storage (M±SEM, n = 3, lab test)

Taxon	Original biomass	Storage					
		4 days	7 days	14 days	28 days	60 days	90 days
		dPCR analysis, cells/g					
Total counts	$3.3\times10^8\pm1.4\times10^7$	$2.0\times10^7\pm1.1\times10^6$*	$9.0\times10^6\pm2.1\times10^5$*	$4.2\times10^6\pm5.6\times10^5$	$1.15\times10^7\pm9.9\times10^5$*	$1.5\times10^6\pm9.3\times10^5$*	$1.15\times10^7\pm8.9\times10^5$*
		NGS sequencing, %					
Unclassified bacteria	14.80±0.790	47.08±2.600*	50.00±3.100	53.85±3.500	35.96±1.900*	45.45±2.300*	50.91±3.900
Class *Acidobacteria*	62.56±3.400	3.64±0.210*	1.43±0.073*	0	1.12±0.059*	0	0
Class *Actinobacteria*	1.29±0.070	0.73±0.050*	4.29±0.340*	1.10±0.054*	0	0	0
Class *Aiphaproteobacteria*	2.76±0.150	0.73±0.048*	1.43±0.062	0	23.60±1.700*	0	0
Class *Anaerolineae*	0.02±0.001	0	0	0	0	0	0
Class *Aquificae*	0.02±0.002	0	0	0	0	0	0
Class *Armatimonadia*	0.02±0.002	0	0	0	0	0	0
Class *Bacilli*:	1.76±0.080	14.05±0.720*	7.14±0.360*	1.10±0.052*	7.87±0.420*	31.82±1.750*	45.45±2.600*
family *Bacillaceae*	0.37±0.020	2.74±0.150*	4.29±0.29*	1.10±0.049*	1.12±0.059	4.55±0.310*	0
family *Staphylococcaceae*	0	0	1.43±0.081*	0	0	0	0
family *Lactobacillaceae*	1.39±0.078	11.31±0.450*	4.29±0.330*	0	6.74±0.450*	27.27±1.600*	45.45±2.400*
Class *Bacteroidia*	1.16±0.063	3.47±0.190*	12.86±0.520	17.58±0.92*	0	4.55±0.380*	1.82±0.160*
Class *Betaproteodacteria*	1.45±0.081	0.18±0.009*	0	0	0	0	0
Class *Caldilineae*	0.02±0.002	0	0	0	0	0	0
Class *Caldisericia*	0.06±0.003	0	0	0	0	0	0
Class *Chthonomonadetes*	0.35±0.020	0	0	0	0	0	0
Class *Clostridia*	1.23±0.073	3.10±0.220*	15.71±0.800*	19.78±0.99*	0	9.09±0.560*	0
Class *Cytophagia*	0.31±0.030	0.18±0.007*	0	0	0	0	0
Class *Deinococci*	0.02±0.004	0	0	0	0	0	0
Class *Deltaproteobacteria*	0.06±0.004	0	0	0	0	4.55±0.290*	0
Class *Erysipelotrichia*	0.02±0.002	0	0	0	0	0	0
Class *Flavobacteria*	0.15±0.008	0.18±0.008	0	0	0	0	0

Continued Table 2

Class *Gammaproteobacteria*:	11.03±0.750	26,09±1,500*	0	2,20±0,190*	31,46±1,460*	4,55±0,300*	1,82±0,170*
order *Enterobacteriales*:	5.61±0.290	23,54±1,200*	0	1,10±0,150*	30,34±1,110*	0	0
Salmonella subterranea	0	0,36±0,019*	0	0	0	0	0
Class *Mollicutes*	0.04±0.005	0	0	0	0	0	0
Order *Selenomonadales* (class *Negativicutes*)	0.10±0.005	0,55±0,030*	4,29±0,360*	3,30±0,220*	0	0	0
Class *Opitutae*	0.02±0.003	0	0	0	0	0	0
Class *Phycisphaerae*	0.02±0.001	0	0	0	0	0	0
Class *Planctomycetia*	0.04±0.004	0	0	0	0	0	0
Class *Sphingobacteriia*	0.50±0.150	0	0	0	0	0	0
Class *Spirochaetia*	0.04±0.006	0	0	0	0	0	0
Class *Synergistia*	0.020±0.0021	0	0	1,10±0,130*	0	0	0
Class *Thermodesulfobacteria*	0.020±0.0026	0	0	0	0	0	0
Class *Thermomicrobia*	0.020±0.0015	0	0	0	0	0	0
Class *Verrucomicrobiae*	0.020±0.0023	0	1,43±0,160*	0	0	0	0

* Differences with the indicator in the previous time period is statistically significant at $p \leq 0.05$.



Heatmap analysis of bacterial community in the initial plant biomass and haylage of alfalfa *Medicago sativa* L. cv. Pastbishchnaya 88 (wilted to 43.5 % dry matter) during storage (lab test). Minor (< 0.06%) phyla *Fusobacteria*, *Spirochaetes*, *Chloroflexi*, *Aquificae*, *Planctomycetes*, *Caldiserica*, and *Thermodesulfobacteria* are not shown.

The abundance of the phylum *Acidobacteria* sharply decreased (to 3.64±0.21 at $p \leq 0.05$) already on day 4 of haylage storage, and after 28 days, these bacteria were almost completely eliminated. The obtained data are logical, since bacteria of the phylum *Acidobacteria* are oligotrophic [30], and, probably, the ecosystem of stored feed, rich in nutrients, is unfavorable for them. At the same time, representatives of the phyla *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* were the dominant bacteria during the storage of haylage, which repeats the trends observed during the development of silage succession [13, 31].

Nevertheless, even after 3 months of storage, only about half of the microorganisms contained in the feed were represented by lactic acid bacteria of the *Lactobacillaceae* family (see Table 2) of the phylum *Firmicutes*, the traditional inhabitants of the silage microflora [31]. Probably, the environmental conditions typical for haylage (pH, dry matter content, etc.) were not so favorable for the vital activity of these microorganisms. It is known that lactobacteria play a decisive role in the processes of microbial fermentation of preserved feeds. They transform the mono- and disaccharides of feed with the formation of lactate, which leads to acidification of the feed and the displacement of microflora, conducting fermentation processes undesirable for haylage [32].

As a feature of fermentation of alfalfa haylage, it should be noted that there is no relationship between the accumulation of butyric acid and the number of Clostridia (class *Clostridia*) in the dried mass ($p \leq 0.05$). Thus, if the maximum amount of butyric acid in the dry matter of the feed was formed already after 4 days of fermentation, then the greatest number of Clostridia was noted only after 14 days of storage. This indicates that a significant part of the butyric acid that appears at the very beginning of alfalfa haylage has a bio-chemical origin.

Filatov et al. [33] also did not find a relationship between the accumulation of butyric acid and the number of Clostridia. The authors noted a high number of butyric acid bacteria (about 2.0×10^5 CFU) in 1 g of haylage from dried alfalfa (47.5% dry matter) during the entire 90-day storage period of the feed. However, the resulting feed did not accumulate butyric acid. This indicates that

in alfalfa haylage, clostridial spores in most cases remain inactive during the entire storage period of the feed. At present, foreign researchers also share this opinion [10]. However, this is not always the case. The authors associate the differences in the formation of butyric acid with the varietal characteristics of alfalfa, the degree of wilting of plants, on which, in their opinion, the clostridial community largely depends, and, consequently, the time of occurrence and intensity of butyric acid fermentation, as well as with other factors.

Indeed, some members of the *Clostridia* class, belonging to the *Clostridiaceae* family, produce butyric acid during fermentation, including in preserved feeds. Some of them (*C. sporogenes*, *C. bifermentans*, *C. sphenoides*) have proteolytic properties, and therefore their presence in preserved feed is undesirable [32]. However, in the authors' experiment, representatives of the *Clostridiaceae* family were detected only in the initial plant raw material of alfalfa; during fermentation, they were completely replaced by representatives of another microflora. Interestingly, among the bacteria of the *Clostridia* class, such families as *Eubacteriaceae*, *Lachnospiraceae*, *Peptostreptococcaceae*, and *Ruminococcaceae* were found in haylage during storage. The main products of their metabolism are acetic, valeric, malic, and propionic acids, while butyric acid is formed in minor amounts, and some types of bacteria do not form it at all [34]. Interestingly, in the authors' experiment, when calculating Pearson's correlations, a significant relationship was found between an increase in the content of bacteria of the genus *Ruminococcus* and an increase in the amount of malic acid ($r = 0.8$ at $p \leq 0.05$). Bacteria of the families *Eubacteriaceae*, *Lachnospiraceae*, *Peptostreptococcaceae* and *Ruminococcaceae* are representatives of the normal flora of the intestinal and cicatricial microbiocenosis [35].

There is a quite interesting fact, which is worthy of discussion, that a significant relationship was also found between an increase in the number of bacteria in the phylum *Bacteroides* and an increase in the content of monosaccharides ($r = 0.76$ at $p \leq 0.05$), butyric ($r = 0.95$ at $p \leq 0.05$) and malic ($r = 0.4$ at $p \leq 0.05$) acids. This is quite expected, since it is known that the metabolism of starch with the release of a glucose molecule [36] is the main pathway of metabolism in these microorganisms. As a result, the bioavailability of glucose can be increased for microorganisms that produce butyric and malic acids.

Consequently, a feature of alfalfa haylage is that proteolytic forms of clostridia do not receive significant development in it, that is, the feed is characterized by a favorable direction of the fermentation process.

Nevertheless, on days 4 and 28 of storage in haylage, the number of bacteria of the *Enterobacteriaceae* family increased sharply, up to 23.5±1.2 and 30.3±1.11%, respectively ($p \leq 0.05$). They are undesirable for the fermentation process of feeds, because monosaccharides become the source of their vital activity, which makes them direct competitors of lactobacteria [32]. Among the genera of enterobacteria, during storage of haylage, both typical epiphytic bacteria (*Erwinia*, *Serratia*, *Pantoea*) and pathogenic forms (*Enterobacter*, *Escherichia*, *Shigella*, *Klebsiella*, *Salmonella*) were identified.

Interestingly, microorganisms *Staphylococcus arlettae*, *Salmonella subterranea*, *Streptococcus gordonii*, *Enterococcus cecorum*, capable of causing diseases in humans and animals, survived up to 4-14 days of storage in haylage. Thus, coagulase-negative staphylococci are becoming a common cause of bacteremia [37]. They are detected in the blood of people with cardiovascular diseases. Earlier, *Staphylococcus arlettae* was isolated from the skin and manure of poultry and goats [37]. *Salmonella subterranea* is a relatively new species found in 2004 in natural sources [38]. The survival of *Salmonella subterranea* in haylage at the initial stages of fermentation is understandable [38]. *Streptococcus gordonii* is a bacterium that is recognized as the

causative agent of bacterial endocarditis and human empyema [39]. In bovine cattle, bacteria from the genus *Streptococcus* are considered pathogens associated with subclinical mastitis, many diseases of the reproductive system (abortion, stillbirth, vulvitis, vaginitis, and metritis), valvular endocarditis, and septicemia [40]. *Enterococcus cecorum* is a causative agent of joint diseases; however, to date, its pathogenicity has been proven only for poultry [41]. Consequently, stored haylage, the making of which took place in violation of technological methods, may contain pathogens of agricultural animals, which determines the need for the use of biological products with antimicrobial activity for its preservation.

The number of unidentified bacteria in the experiments ranged from 35.9±1.9 to 50.9±3.9%. These bacteria belonged to the rank of objects for the cultivation of which there are currently no nutrient media; therefore, they became known only with the development of molecular-biological methods [42]. The high proportion of unclassified microorganisms, noted in the haylage during the entire storage period, also did not lead to any significant increase in nutrient losses and deterioration of the biochemical parameters of the obtained feed. Despite the presence of these microorganisms, the authors did not observe an increase in the accumulation of ammonia and butyric acid, as well as a decrease in sugar content, a decrease in the amount of which began only after 2 months of storage of haylage, that is, after activation of lactic acid fermentation in it. It can be concluded that the introduction of lactic acid bacteria during alfalfa haylage should not be accompanied by any significant effect.

To be convinced of this, we prepared haylage from dried alfalfa without preservatives and with the introduction of lactic acid bacteria in the form of the Biotrof starter culture. The introduction of Biotrof preparation, stimulating lactic acid fermentation, caused an increase in the breakdown of nutrients to gaseous products, as a result of which the volume of gases released during haylage making increased by 1.5-2.4 times ($p \leq 0.05$) (Table 3). This was due to an increase in the fermentation of mono-sugars, the content of which in the dry matter of the feed decreased by 3.1-3.7 times ($p \leq 0.05$). Stimulation of lactic acid fermentation led to rapid acidification of the feed to the limit excluding the development of butyric acid bacteria. As a result, the accumulation of butyric acid decreased by 1.3-2.8 times in comparison with its content in ordinary haylage. However, in this case, the accumulation of butyric acid in the dry matter of the feed was 0.11-0.15%, that is, it was as much as can be formed as a result of biochemical processes.

3. Gas emission and biochemical parameters of conventional haylage of alfalfa *Medicago sativa* L. cv. Pastbishchnaya 88 and haylage added with Biotrof preparation (M±SEM, n = 3, lab test)

Biotrof preparation	Emission, l/kg dry matter of green mass	pH	Percent per feed dry matter			
			ammonia	organic acids		monosugars
				lactic	butyric	
Wilted to 47.6% dry matter						
Not added	2.39±0.430	5.59±0.010	0.23±0.010	0.30±0.040	0.20±0.010	2.26±0.090
Added	5.73±0.120*	4.31±0.010*	0.27±0.010	15.48±0.320*	0.15±0.010*	0.73±0.040*
Wilted to 51.3 % dry matter						
Not added	3.04±0.220	5.45±0.090	0.17±0.010	2.86±0.100	0.31±0.040	4.63±0.260
Added	4.62±0.120*	4.25±0.010*	0.14±0.020	14.06±0.200*	0.11±0.030*	1.25±0.040*

* Differences with the indicator for haylage without additives is statistically significant at $p \leq 0.05$.

The acceleration of the acidification of the alfalfa haylage mass under the influence of the Biotrof preparation did not lead to a significant reduction in the accumulation of ammonia in the feed. That is, the main parameter that determines the degree of protein breakdown to ammonia during silage and haylage making of

alfalfa is the degree of wilting of plants. According to the available data [33], with an increase in the dry matter content in the silage mass of alfalfa from 21.1 to 31.5; 41.5 and 52.0%, the amount of ammonia nitrogen in relation to the total nitrogen of the feed is reduced from 18.6 to 8.8; 4.5 and 4.9%. Since the use of lactic acid bacteria preparations does not lead to a noticeable improvement in the preservation of nutrients in alfalfa haylage, improving only its biochemical parameters, it does not lead to a noticeable increase in the energy nutritional value of the dry matter of the obtained feed [43]. The haylage from alfalfa treated with preparations of lactic acid bacteria has a higher productive effect [44]. The authors explain this phenomenon by an increase in the total mass of the microflora of the rumen chyme, which can be a source of protein for animals.

Thus, with short-term wilting of alfalfa to haylage moisture and at the very beginning of its haylage making, there is a noticeable increase in the sugar content in the dry matter of the green mass. In addition, malic acid accumulates in large quantities in wilted plants, which, like mono-sugars, is fermented by lactic acid bacteria. High-throughput sequencing identified typical rumen inhabitants of the families *Eubacteriaceae*, *Lachnospiraceae*, *Peptostreptococcaceae*, and *Ruminococcaceae* among bacteria of the *Clostridia* class in haylage during storage. Interestingly, butyric acid producers, bacteria of the *Clostridiaceae* family, were not detected during fermentation. A significant relationship was shown between an increase in the content of bacteria of the genus *Ruminococcus* and an increase in the amount of malic acid, as well as between an increase in the content of malic acid and an increase in the number of bacteria of the phylum *Bacteroides*. The accumulation of malic acid led to an improvement in the fermentability of alfalfa being hayed, as a result of which, under the influence of the preparation of lactic acid bacteria Biotrof, it was quickly acidified to pH 4.4-4.3, which ensures the stability of the feed during storage. The expediency of this technique is due to the fact that in the haylage mass in large quantities (up to half of the total number of microorganisms) there are unclassified bacteria that do not grow on conventional nutrient media and have not been sufficiently studied. These bacteria do not lead to significant losses of nutrients during haylage making, however, they worsen the biochemical parameters of the feed, contributing to an increase in the accumulation of butyric acid in it. Therefore, the main effect of the use of lactic acid bacteria preparations in alfalfa haylage is reduced only to an improvement in the biochemical parameters of the feed, without leading to a noticeable improvement in its preservation. Also, *Staphylococcus arlettae*, *Salmonella subterranea*, *Streptococcus gordonii*, and *Enterococcus cecorum* were found in the haylage, which can cause diseases in humans and animals.

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