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Original Paper

Effect of azodicarbonamide on rats with a high-fat hypercaloric diet

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Azodicarbonamide is an important chemical widely used in the industry as a blowing agent in the foam industry and in the food industry as a flour bleaching agent and a dough conditioner. The discussion about its biosafety is ongoing, in a number of countries, the use of azodicarbonamide is limited or even prohibited since a slightly carcinogenic effect of its by-products has been found. Despite this, many manufacturers continue to use it as a food additive. In a laboratory experiment, the effect of various doses of azodicarbonamide on the organism of laboratory animals under the background of a high-fat diet was determined by changes in their body weight, the state and mass indices of internal organs, blood parameters, the functional state of the nervous system, and changes in the intestinal microbiota. Four groups were formed from laboratory male rats, which for 21 days were consuming: a high-fat diet with the addition of 4%, 1%, 0.25%, 0% azodicarbonamide. It has been determined that azodicarbonamide did not cause a change in the organ mass index, but the addition of 4% and 1% of the substance to the diet significantly reduced the intensity of animals' body weight gain. Both excess fat in the diet and different doses of azodicarbonamide mainly caused functional disorders of the parenchymal organs, as evidenced by changes in the activity of blood enzymes (Aspartate aminotransferase, Alanine aminotransferase, De Ritis ratio (AST/ALT), Alkaline phosphatase) and protein metabolism (Total protein, Globulins, Protein coefficient, Urea). Significant changes in the physical and orientation activity and emotional status of the animals were not observed. Low concentrations of azodicarbonamide (0.25% and 1% of the feed mass) caused a pronounced decrease in the number of normal enzymatic properties of *Escherichia coli* strains below the control group and the reference range, and a high concentration (4%) significantly reduced the number of *Lactobacillus* bacteria in comparison with the control one but did not exceed references.

Keywords: blowing agent, relative mass of the organs, biochemical indicators of blood, bodyweight increase, gut microbiota

1 Introduction

Azodicarbonamide is a chemical compound with the molecular formula $C_2H_4O_2N_4$. It is an odourless, yellow to orange-red crystalline powder. Azodicarbonamide is used as a blowing agent in the rubber and plastics industries (Sims & Jaafar, 1994). The compound is used to foam a wide range of polymers, including polyvinyl chloride, polyolefins, natural and synthetic rubbers (Stehr, 2016). The main use of azodicarbonamide is in the foam industry as an additive (Hanifarianty & Fathurrohman, 2022). Thermal decomposition of azodicarbonamide results in the release of nitrogen, carbon monoxide, carbon dioxide and ammonia gases, which are trapped in the polymer in the form of bubbles, forming a foamed product (Zauzi et al., 2019). Azodicarbonamide used in plastics, synthetic leather and other applications may be pure or modified. Pure azodicarbonamide typically reacts at around 200 °C (Jaafar & Sims, 1993). In plastics, leather and other industries, modified azodicarbonamide (average decomposition temperature 170 °C) contains additives that speed up the reaction or react at lower temperatures (Lee et al., 2018; Krutko et al., 2019). Although azodicarbonamide is widely used as a blowing agent in the manufacture of many thermoplastics and elastomers (PVC, PVA, synthetic threads), as well as artificial leather, etc., in the United Kingdom and Ireland was used to bleach wheat flour and increase elasticity dough from wheat flour in bakery production, its use in Europe as a blowing agent is prohibited for the manufacture of plastic products that come into direct

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contact with food (Joiner et al., 1963; Nestmann et al., 2005; Lee et al., 2018).

It is known that the food consumed by humans and animals consists of many substances that are beneficial, such as nutrients and dietary fibre, or undesirable, such as natural toxins, pesticide residues, mycotoxins, animal drugs, or other potential contaminants derived from as a result of production, storage or transportation. Azodicarbonamide as a food additive is known under the number E927. It is used to whiten and extend the shelf life of bread because it reacts with wet flour as an oxidizing agent, and the main reaction product is biurea, a bake-stable urea derivative. As a result, the bread becomes whiter by reacting with the cartotene naturally present in the flour. It also improves the strength of the flour, improves the ability of the dough to hold gas, and makes the bread more elastic. Secondary reaction products include semicarbazide and ethyl carbamate (Cañas et al., 1997; Dennis et al., 1997a, 1997b; Noonan et al., 2008). Azodicarbonamide is reported to be one of the fast-acting food oxidizers and it is also used for leavening baked goods, rice, chewing gum, flour and grains. Many scientific works are devoted to the detection and quantification of azodicarbonamide and its metabolites in food (Ye et al., 2011; Xing et al., 2012; Chen et al., 2016; Yasui et al., 2016; Wang & Chan, 2016; Che et al., 2017; Wang et al., 2018; Chen et al., 2021; Wang & Zhao, 2021; Zhang et al., 2021).

The Food and Drug Administration's guidelines on food and health safety for corporations permit the use of azodicarbonamide. Since the compound is allowed by the FDA as GRAS ("Generally Recognized as Safe"), any business can use it in their food and need not report its usage. In the United States, azodicarbonamide is allowed to be added to flour at levels up to 45 ppm. The United States ("21CFR172.806" Code of Federal Regulations. April 1, 2012) and Canada permit the use of azodicarbonamide at levels up to 45 ppm.

However, the widespread use of azodicarbonamide in food causes environmental and public health problems (Rice et al., 1997; Becalski et al., 2004, 2006; Nestmann et al., 2005; Zawadzki & Maksymowicz, 2007). In this regard, in Australia and several countries of the European Union, azodicarbonamide has been banned in the food industry. This was facilitated by the discovery of many facts about this substance effect on human health (Whitehead et al., 1987; Szilagyi et al., 2006). Studies have shown that azodicarbonamide is a respiratory sensitizer in the workplace and can cause respiratory diseases (Shopp et al., 1987; Arts & Kimber, 2017, 2018). Many studies report the ability of azodicarbonamide to cause pulmonary and skin sensitization (Ferris et al., 1977; Bonsall, 1984; Mewhinney et al., 1987; Suojalehto et al., 2018). Some cases of occupational asthma associated with exposure to azodicarbonamide are known, of which only a few cases have been confirmed by specific inhalation problems (Slovak, 1981; Valentino & Comai, 1985; Bechtold et al., 1989; Normand et al., 1989; Kim et al., 2004; Li et al., 2015).

According to literature data, it is known that a significant amount of azodicarbonamide is excreted with faeces, it also easily turns into biurea, which is subsequently excreted in the urine, and into semicarbazide, a metabolite of the veterinary drug nitrofurazone, which has a strong carcinogenic, teratogenic and mutagenic effect (Barnabás & Miklós, 2005; Tian et al., 2021). Semicarbazide is carcinogenic in mice but shows no or little mutagenicity in the Salmonella microsome test (Hirakawa et al., 2003). Semicarbazide, a by-product of the breakdown of azodicarbonamide, causes damage to immune cells in humans and DNA in animals (Tassignon et al., 1999, 2001; Vlastos et al., 2010). Oral administration of various doses of semicarbazide (40, 75, 140 mg/kg body weight per day) to rats during the juvenile period for 28 days had a pleiotropic effect (Maranghi et al., 2009). A number of studies have demonstrated the ability of azodicarbonamide to cause DNA damage through the formation of free radicals, which ultimately leads to cancer. A diet containing azodicarbonamide may alter neurobehavior in rats, but this is not associated with signs of oxidative stress in the brain or neurohistomorphological changes (Olofinnade et al., 2021). The effect of azodicarbonamide on the body's endocrine system has been repeatedly described (Gafford et al., 1971; Ferris et al., 1977; Maranghi et al., 2010).

Gerlach et al. (1989) state that no skin, eye or respiratory tract irritation has been observed in laboratory animals following short-term (4 weeks) use of non-protein bound azodicarbonamide. Hartwig (2018) would explain these results by arguing that animal inhalation studies may not be suitable for determining minimal alveolar concentration (MAC) values, as the human airway is likely to be more sensitive than the rodent airway. However, in laboratory animals, the induction of pyelonephritis with the formation of urinary cylinders and crystalline deposits in the renal tubules was found to be provoked by prolonged oral administration of high (>200 mg/kg body weight per day for one year) doses of azodicarbonamide. It has been reported (Hartwig, 2018) that azodicarbonamide caused mutagenic changes in bacteria (*Salmonella typhimurium* strains TA100 and TA1535), but no mutagenic effects were observed in mammalian cells (Abramsson-Zetterberg & Svensson, 2005). Also in mammals, negative results of indicator

tests for clastogenicity were observed, but chromosomal aberrations and polyploidy were induced. There was no induction of clastogenic or polyploid effects in mouse bone marrow *in vivo*. Also, azodicarbonamide did not cause mutations in *Drosophila*.

However, there is very little information in the literature on the toxicokinetic effects of azodicarbonamide. Its effect on the reproductive system, the induction of the formation of carcinogenic and mutagenic cells, and the effect on the intestinal microbiome are also not sufficiently studied (Fagny et al., 2002). There is also no data on the levels of azodicarbonamide and its by-products in ambient air, water, soil or precipitation and its effect on the macroorganism (Pereira et al., 2004; Gao & Ru, 2013). It has long been a fact that chemicals in foods, regardless of their origin, can be hazardous to the consumer (Cooper et al., 2007; Tian et al., 2021). Even when food additives are approved for use, their biosafety has often been determined in healthy adult animal models. Currently, the human diet is often unbalanced not only in terms of vitamin and mineral composition, but also in terms of basic nutrients (excess carbohydrates and fats, against the background of a lack of complete proteins), which causes metabolic disorders and the occurrence of a number of diseases (metabolic syndrome, diabetes mellitus, obesity, etc.). The presence of permitted food additives in food products can cause unforeseen effects against the background of metabolic disorders in the body of animals and humans. Therefore, the purpose of this study was to determine the effect of different doses of Azodicarbonamide on changes in body weight and internal organs, hematological parameters, the functional state of the nervous system and intestinal microbiota of model animals consuming a high-fat diet.

2 Material and methods

2.1 Etics

The choice of animals for the experiment, research protocols, and withdrawal of animals from the experiment was approved by the local ethical committee of Dnipro State Agrarian and Economic University (Dnipro, Ukraine). The maintenance, nutrition, care of animals and their withdrawal from the experiment were carried out in accordance with the principles set forth in the "European Convention for the Protection of Vertebrate Animals used for Experimental or other Scientific Purposes" (Strasbourg, France, March 18, 1986, ETS No. 123) and in Law of Ukraine "On protection of animals from cruel treatment" (Kyiv, February 21, 2006, No. 3447-IV).

2.2 Animals

For this experiment, 24 outbred laboratory male rats aged 1.5 months with an average weight of 200 ±10 g were randomly divided into four groups: three experimental and one control group of 6 animals each. Animals were kept in polycarbonate cages (three per cage) in a room with a controlled temperature (20–22 °C), a light regimen of 12 hours of light and 12 hours of darkness.

2.3 Diet

From the beginning of the experiment, for 20 days, all animals received a diet with an excess fat content (15% sunflower oil was added to the standard diet (75% grain mixture, 8% root crops, 2% meat and bone meal, 2% mineral-vitamin complex)). The control group of animals received a high-fat diet, while the experimental group received a high-fat diet supplemented with 0.25%, 1% and 4% azodicarbonamide. The animals had free access to food and water. During the experiment, the amount of food and water consumed by each group was taken into account.

2.4 Morphometric indicators

Daily observation and weighing of each animal was carried out, followed by calculation of the total increase in animal weight and daily gains in live weight.

2.5 Functional state of the nervous system

The orientation-physical activity and the emotional status of experimental animals were studied in the "open field" test. We used a setup consisting of a square area of 1 m^2, divided into 16 squares and bounded by an opaque wall of 20 cm high. The experiment was carried out in complete silence with intense illumination of the field itself. An experimental animal, taken from a cage in a previously darkened room, was placed in the centre of the field. The exposure time was 2 minutes. The animals were tested for four days (days 1–4) at the beginning of the experiment and four days at the end (days 16–20). The number of crossed squares (peripheral and central) was counted – for assessing physical activity; peripheral (with support on the wall) and central (without support on the wall) racks – for orientation activity; number of acts of grooming, defecation and urination – to assess emotional status (Lieshchova & Brygadyrenko, 2021).

2.6 Condition of internal organs

Animals were slaughtered on the 20th day of the experiment under anaesthesia (80 mg/kg of ketamine and 12 mg/kg of xylazine, intraperitoneally) by total bloodletting from the heart. After the autopsy, the condition of the internal organs was visually assessed for

the presence of pathological changes. The selection of organs (heart, liver, lungs, thymus, spleen, stomach, small intestine, blind, colon, rectum, kidneys, brain) was carried out with surgical instruments. The mass of internal organs was determined with an accuracy of 10 mg.

2.7 Blood study

Blood sampling was carried out during the euthanasia of rats, followed by biochemical and morphological analysis. After introducing rats into anaesthesia, blood was collected directly from the heart with a syringe into two test tubes. In the first tube, whole blood (1.0–1.5 ml) was collected to obtain serum and conduct further biochemical analyses. In the second one, 0.5–1.0 ml of blood was collected by adding an anticoagulant (potassium EDTA) for further automatically counting the number of blood cells and making blood smears to do a leukogram. Biochemical parameters were determined using a Miura 200 automatic analyser (I.S.E. Srl, Rome, Italy), and High Technology reagent kits (High Technology Inc, North Attleborough, MA, USA), PZ Cormay S.A. (Cormay Diagnostics, Lublin, Poland) and Spinreact S.A. (Spinreact, Girona, Spain). Red blood cell and white blood cell count in the rats' stabilized blood were determined using an automatic haematology analyser, BC-2800Vet (Mindray, Shenzhen, China). For leukogram, blood smears were prepared according to Pappenheim with their further staining according to Romanovsky-Giemsa (Lieshchova et al., 2018, 2019, 2020; Brygadyrenko et al., 2019).

2.8 Microbiological research

Faecal samples were taken from the rectum of laboratory rats that were kept for 20 days on a diet with the addition of azodicarbonamide (three experimental groups: 0.25%, 1% and 4% powder) and the control group (without the addition of azodicarbonamide) (n = 24). Under sterile conditions, 1 g of native faeces was ground in a mortar with 9 mL of saline (10^{-1}). Then serial 10-fold dilutions up to 10^{-10} in saline were made from the main dilution (1 : 10) and sown on selective-differential media (bifidum medium, lactobac agar, *Enterococcus* agar, Endo's medium, bismuth sulphite agar, Wilson & Blair medium, Baird-Parkeragar, Sabouraud dextrose agar, blood agar (HiMedia Laboratories Pvt. Ltd, India) for qualitative and quantitative assessment of microbiota. Anaerobic conditions were achieved in desiccators using GENbox anaerobic bags (Biomerieux, France). Control of anaerobic conditions was performed using Anaer Indikator (Biomerieux, France). The number of living microorganisms in Petri dishes was determined by counting the grown colonies in each of the parallel inoculations of one dilution after 2–4 days of incubation at a temperature of +37 °C and +24 °C. The arithmetic mean value of each dilution was determined and it was expressed in CFU/g (colony-forming units per 1 gram of mass of the material). Identification of microorganisms was carried out by taking into account the study morphology (after Gram-staining of smears), cultural and biochemical properties of isolated cultures according to Bergey's Manual of Systematic Bacteriology. The sterility of nutrient media was controlled in a thermostat, where Petri dishes and sample tubes with the corresponding media without inoculum were placed (Topsall, 1992; Bilan et al., 2019).

2.9 Statistical analysis

All the data were analysed using Statistica 8.0 program (StatSoft Inc., USA). Results in the tables are demonstrated as x ±SE (mean ±standard error). Differences between the control and experimental groups values were determined by using the Tukey test (with consideration of Bonferroni's correction), where the differences were considered significant at P <0.05.

3 Results and discussion

The addition of azodicarbonamide (Figure 1) did not lead to a significant change in the body weight of the animals in all three experimental groups.

Animal intake of azodicarbonamide did not stimulate an increase in feed and water intake (Table 1). Daily weight gain also remains unchanged (Table 1).

The relative organ mass of the animals did not change significantly with both low and high dietary concentrations of azodicarbonamide (Table 2).

Most of the biochemical parameters of experimental animals' blood did not change when azodicarbonamide was consumed with food (Table 3). De Ritis ratio (AST/ALT) significantly decreases with the use of 1% and 4% of the feed weight (from 1.80 ±0.51 to 1.13 ±0.15 and 0.53 ±0.11 relative units, respectively). This index was decreased below the physiological norm already when eating 0.25% azodicarbonamide by weight of the feed.

When using different concentrations of azodicarbonamide, there were also no changes in the cellular composition of the blood, hemoglobin content and hematocrit (Table 4).

Animals fed with 4% azodicarbonamide by weight of the daily diet reduced the level of physical activity (Table 5) from 6.83 ±1.57 at the beginning of the experiment to 2.79 ±0.81 after 20 days of the experiment. The same strong changes occurred when eating 1% azodicarbonamide by weight of the diet (decrease from 7.75 ±1.39 to 2.38 ±0.66). A significant decrease in orientational activity in animals from 4.54 ±0.64 to 2.29 ±0.45 and emotional status from 2.17 ±0.40 to 1.04 ±0.26 was noted when fed with 4% of the studied food supplement.

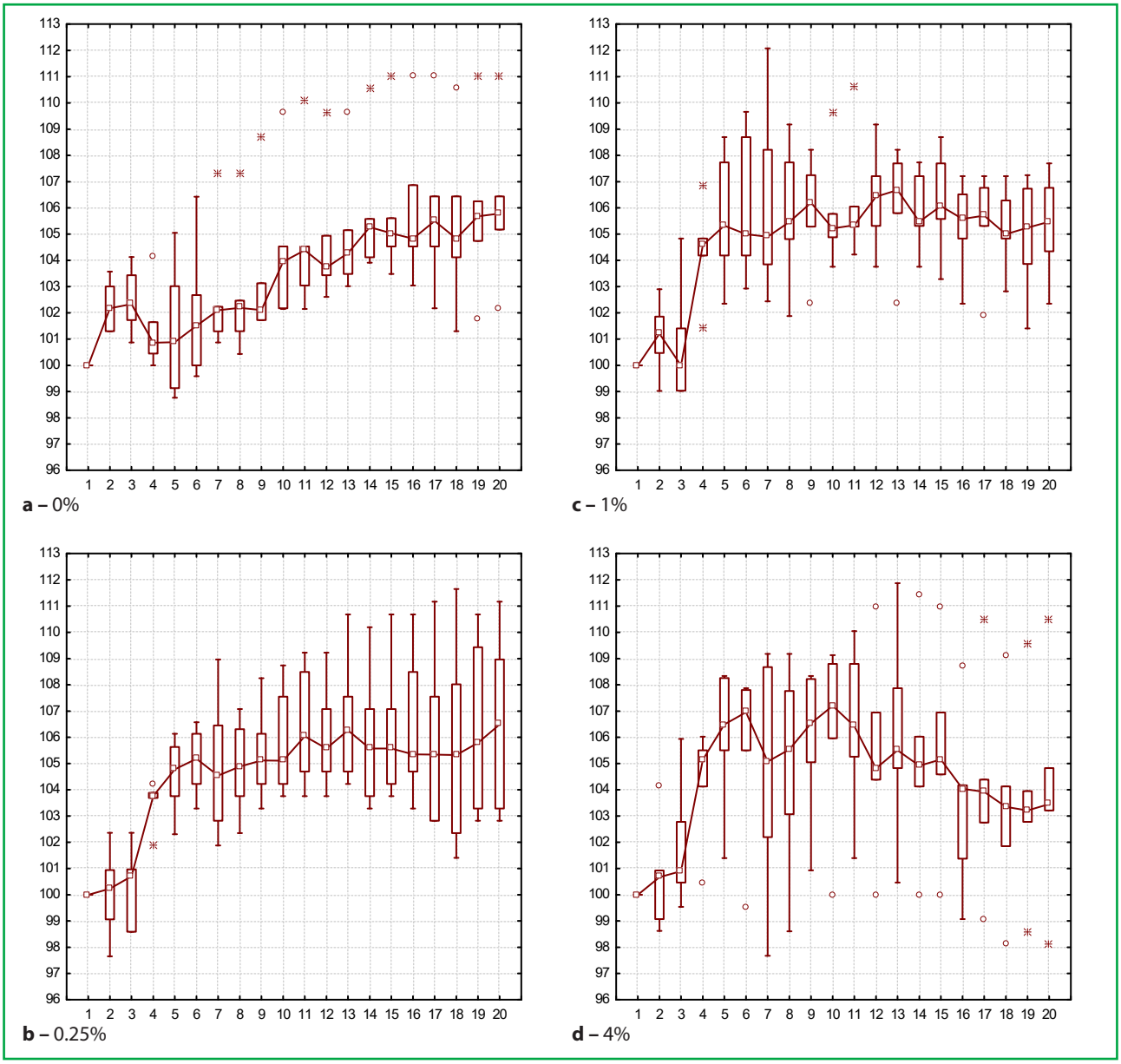

Figure 1 Change in the rats' body weight when azodicarbonamide was added to their diet
a – 0%, b – 0.25%, c – 1%, d – 4% of azodicarbonamide from the mass of food; the abscissa is the day of the experiment, the ordinate is the body weight of the animals (% relative to the initial body weight before the start of the experiment, taken as 100% for each of the experimental animals); small square – median, upper and lower borders of the rectangle – 25% and 75% quartiles, vertical line – minimum and maximum values, asterisk and circles – outliers; $n = 6$

Table 1 Change in the body weight and food consumption of young male rats under the influence of azodicarbonamide addition to their diet (x ±SE, $n = 6$, duration of experiment – 20 days)

Parameter	0%	0.25%	1%	4%
Food consumption by rats (g/day)	22.9	22.9	18.3	19.6
Water consumption by rats (g/day)	14.4	14.4	14.2	14.8
Body weight change (µg/day)	692 ±125[a]	692 ±150[a]	558 ±79[a]	433 ±177[a]
Body length (cm)	20.67 ±0.21[a]	20.58 ±0.42[a]	20.08 ±0.30[a]	20.17 ±0.53[a]

no significant differences were found within one line of the table according to the results of comparison using the Tukey test with Bonferroni correction

Table 2 Change in organs' relative mass (%) of male rats under the influence of azodicarbonamide supplementation to their diet (x ±SE, n = 6, duration of experiment – 20 days)

Organ	4%	1%	0.25%	0%	P
Heart	0.351 ±0.017	0.333 ±0.013	0.328 ±0.009	0.334 ±0.020	0.740
Liver	3.200 ±0.046	3.406 ±0.108	3.185 ±0.091	3.220 ±0.163	0.471
Lungs	0.714 ±0.061	0.778 ±0.030	0.804 ±0.025	0.700 ±0.047	0.291
Thymus	0.179 ±0.040	0.204 ±0.053	0.247 ±0.034	0.156 ±0.011	0.388
Spleen	0.272 ±0.019	0.293 ±0.017	0.317 ±0.027	0.305 ±0.019	0.491
Stomach	0.650 ±0.016	0.696 ±0.048	0.596 ±0.049	0.696 ±0.062	0.406
Small intestine	2.152 ±0.117	2.338 ±0.172	2.311 ±0.079	2.023 ±0.061	0.216
Cecum	0.441 ±0.040	0.437 ±0.055	0.444 ±0.080	0.574 ±0.087	0.437
Colon	0.283 ±0.043	0.301 ±0.022	0.295 ±0.019	0.315 ±0.027	0.893
Rectum	0.289 ±0.027	0.307 ±0.063	0.312 ±0.047	0.301 ±0.046	0.988
Right kidney	0.303 ±0.017	0.291 ±0.009	0.283 ±0.009	0.295 ±0.014	0.720
Left kidney	0.311 ±0.013	0.293 ±0.010	0.300 ±0.015	0.303 ±0.012	0.809
Brain	0.762 ±0.021	0.682 ±0.043	0.704 ±0.032	0.675 ±0.032	0.263

different letters indicate values that differed one from another reliably within one line of the table according to the results of comparison using the Tukey test with Bonferroni correction

Table 3 Change in blood biochemical parameters of male rats under the influence of azodicarbonamide (x ±SE, n = 6, duration of experiment – 20 days)

Parameters	4%	1%	0.25%	0%	P
Total protein (g/L)	80.2 ±3.5	82.0 ±3.2	77.3 ±3.7	81.2 ±4.2	0.818
Albumins (g/L)	41.7 ±2.4	44.8 ±1.6	41.0 ±2.0	43.3 ±3.2	0.670
Globulins (g/L)	38.5 ±2.4	37.2 ±2.1	36.3 ±2.1	37.8 ±1.6	0.893
Protein coefficient (U)	1.10 ±0.07	1.22 ±0.06	0.97 ±0.20	1.15 ±0.08	0.512
Urea (mmol/L)	2.92 ±0.29	2.43 ±0.27	2.47 ±0.13	2.72 ±0.28	0.500
Blood urea nitrogen (BUN) (mg/100 g)	5.57 ±0.57	4.63 ±0.50	4.73 ±0.25	5.20 ±0.54	0.505
Creatinine (μmol/L)	75.7 ±6.7	77.0 ±6.5	71.7 ±3.5	65.2 ±5.1	0.456
Aspartate aminotransferase (AST) (U/L)	83 ±16	169 ±33	107 ±13	200 ±63	0.147
Alanine aminotransferase (ALT) (U/L)	177 ±31	159 ±31	150 ±21	117 ±14	0.973
De Ritis ratio (AST/ALT) (U)	0.53 ±0.11^{a}	1.13 ±0.15^{b}	0.75 ±0.04^{ab}	1.80 ±0.51^{b}	0.018
Alkaline phosphatase (U/L)	273 ±79	293 ±61	199 ±55	265 ±71	0.775
Total bilirubin (μmol/L)	3.82 ±0.75	2.45 ±0.47	3.35 ±0.55	1.80 ±0.36	0.073
Glucose (mmol/L)	4.82 ±0.41	5.37 ±0.25	4.67 ±0.27	4.50 ±0.60	0.481
Total calcium (mmol/L)	2.52 ±0.20	2.80 ±0.09	2.47 ±0.16	2.45 ±0.13	0.335
Non-organic phosphorus (mmol/L)	3.22 ±0.55	2.97 ±0.35	2.33 ±0.19	3.05 ±0.32	0.382
Ca/P	0.90 ±0.14	1.02 ±0.12	1.10 ±0.12	0.85 ±0.12	0.487
Gamma-glutamyl transferase (GGT) (U/L)	11.5 ±1.2	10.0 ±1.2	8.8 ±1.6	10.2 ±1.4	0.694
Cholesterol (mmol/L)	1.817 ±0.128	1.850 ±0.138	1.667 ±0.067	1.783 ±0.087	0.663

different letters indicate values that differed one from another reliably within one line of the table according to the results of comparison using the Tukey test with Bonferroni correction

Table 4 Change in CBC and WBC differential of male rats under effect of azodicarbonamide addition (x ±SE, n = 6, duration of the experiment – 20 days)

Parameter	4%	1%	0.25%	0%	P
Hemoglobin (g/L)	113.3 ±2.7	118.7 ±3.3	121.2 ±3.4	115.5 ±4.5	0.430
Hematocrit (%)	23.3 ±1.3	24.8 ±0.5	23.3 ±0.8	24.0 ±1.8	0.788
Erythrocytes (10^{12}/L)	3.48 ±0.17	3.52 ±0.07	3.59 ±0.15	3.50 ±0.12	0.944
Erythrocyte sedimentation rate (ESR) (mm/h)	1.0 ±0.0	1.0 ±0.0	1.0 ±0.0	1.0 ±0.0	1.000
Thrombocytes (10^9/L)	336 ±38	416 ±14	363 ±45	334 ±54	0.474
WBC (10^9/L)	2.88 ±0.21	3.22 ±0.94	2.82 ±0.41	2.87 ±0.30	0.951
WBC differential					
Basophils (%)	0.00 ±0.00	0.50 ±0.22	0.50 ±0.34	0.50 ±0.34	0.467
Eosinophils (%)	0.17 ±0.17	0.83 ±0.31	0.83 ±0.40	0.67 ±0.33	0.409
Myelocytes (%)	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	1.000
Young neutrophils (%)	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	1.000
Band neutrophils (%)	0.17 ±0.17	0.50 ±0.22	0.33 ±0.33	0.33 ±0.21	0.813
Neutrophils with segmented nuclei (%)	23.7 ±3.6	26.0 ±2.7	24.8 ±2.2	24.2 ±2.4	0.939
Lymphocytes (%)	74.8 ±3.7	70.8 ±3.0	72.8 ±2.4	74.3 ±2.4	0.773
Monocytes (%)	1.2 ±0.3	1.3 ±0.3	0.7 ±0.2	1.2 ±0.3	0.427

no statistically significant changes were found between samples

Table 5 Change in behavioral characteristics of three rats groups during 2 minutes of the experiment, in which diet azodicarbonamide was added (x ±SE, n = 24, duration of the experiment was 20 days)

Feature	4%, 1th day	4%, 20th day	1%, 1th day	1%, 20th day	0.25%, 1th day	0.25%, 20th day	0%, 1th day	0%, 20th day
Number of visited peripheral squares	6.46 ±1.43	2.75 ±0.81	4.58 ±1.33	2.38 ±0.66	7.08 ±1.31	4.08 ±1.02	10.13 ±1.17	6.50 ±1.10
Number of visited central squares	0.375 ±0.198	0.042 ±0.042	0.167 ±0.130	0.000 ±0.000	0.083 ±0.083	0.000 ±0.000	0.083 ±0.083	0.000 ±0.000
Number of upright stands in peripheral squares	3.46 ±0.43	1.83 ±0.35	2.58 ±0.30	1.50 ±0.23	2.50 ±0.36	1.50 ±0.25	3.79 ±0.50	2.92 ±0.31
Number of upright stands in central squares	1.08 ±0.28	0.46 ±0.15	1.08 ±0.19	0.42 ±0.13	0.63 ±0.17	0.58 ±0.17	1.00 ±0.21	0.96 ±0.24
Number of grooming acts	1.00 ±0.21	0.54 ±0.19	0.50 ±0.15	0.75 ±0.18	0.71 ±0.19	0.75 ±0.15	0.54 ±0.15	0.83 ±0.18
Number of faecal bolus	1.08 ±0.28	0.42 ±0.13	1.08 ±0.26	0.54 ±0.16	0.83 ±0.20	0.25 ±0.11	1.58 ±0.32	1.00 ±0.23
Number of urinations	0.083 ±0.058	0.083 ±0.058	0.042 ±0.042	0.042 ±0.042	0.167 ±0.078	0.042 ±0.042	0.042 ±0.042	0.000 ±0.000
Physical activity	6.83 ±1.57^{a}	2.79 ±0.81^{b}	7.75 ±1.39^{ac}	2.38 ±0.66^{b}	7.17 ±1.34^{a}	4.08 ±1.02^{ab}	10.21 ±1.21^{c}	6.50 ±1.10^{a}
Orientational activity	4.54 ±0.64^{a}	2.29 ±0.45^{b}	3.67 ±0.35^{ab}	1.92 ±0.28^{b}	3.13 ±0.45^{ab}	2.08 ±0.37^{b}	4.79 ±0.61^{a}	3.88 ±0.45^{ab}
Emotional status	2.17 ±0.40^{a}	1.04 ±0.26^{b}	1.63 ±0.31^{ab}	1.33 ±0.24^{ab}	1.71 ±0.31^{ab}	1.04 ±0.15^{b}	2.17 ±0.36^{a}	1.83 ±0.29^{ab}

physical activity – the number of visited squares of the open field, for orientational activity – the number of standings upright, for emotional status – the number of grooming actions, defecation and urination; there were no significant differences between the groups for most of the parameters studied; differences in the number of visited peripheral squares are indicated by different Latin letters (P <0.05) according to the Tukey test results with Bonferroni correction

It was established that representatives of the obligate microflora (about 90%) prevailed in the rats' feces samples in both the control and experimental groups (about 90%), and the presence of concomitant (facultative) and transient microflora was defined. Microbiological studies of hemolytic *Escherichia coli* and enterobacteria of the genus *Citrobacter* spp. were negative.

Under the influence of a high concentration of azodicarbonamide (4% of the feed mass), the number of bacteria of the genus *Lactobacillus* in the intestinal contents of animals was significantly decreased from 7.66 ±0.40 in the control group to 5.99 ±0.37 in the group of animals with the maximum concentration of the test substance (Table 6). Nevertheless, the number of *Lactobacillus* was still within the physiological norm. Also, a pronounced decrease in the number of normal enzymatic properties strains of *Escherichia coli* below the physiological norm was found even at the minimum and average concentrations of azodicarbonamide (0.25% and 1.00%). The remaining groups of intestinal microorganisms did not significantly change the number under the influence of azodicarbonamide (Table 6).

It should be noted that the decrease in representatives of the genus *Lactobacillus* and normal enzymatic properties of *Escherichia coli* strains contributed to a slight increase in the number of representatives of the facultative (associated) microflora in the experimental groups: lactose-negative strains *E. coli*, *Enterobacter* spp., *Klebsiella* spp., *Proteus* spp., *Enterococcus* spp., *Clostridium* spp., *Candida* spp.

In this study, we evaluated the effects of various doses of the azodicarbonamide dietary supplement on the body of white laboratory rats with a diet that contained excess fat during a 20-day experiment on indicators of food and water intake, changes in body weight and organs, biochemical and morphological parameters of blood, functional state nervous system and changes in the composition of the gut microbiome.

The body weight of experimental animals and its changes under the influence of various factors is an important indicator that is used to determine the toxicity of various drugs and food additives (Lieshchova et al., 2018, 2020; Lieshchova & Brygadyrenko, 2021). In laboratory animals, one of the most important indicators that can be used to determine well-being and health is the amount of food and water consumed. Weight loss may be an indicator of adverse effects of test substances (Lieshchova et al., 2019). The results of an azodicarbonamide toxicity study

Table 6 Number of microorganisms (Lg CFU/gram of feces) in four groups of rats fed with azodicarbonamide (x ±SE, n = 6, duration of the experiment was 20 days, BD – basic diet)

Intestinal microflora	Reference range	BD without azodicarbonamide	BD + 0.25% azodicarbonamide	BD+1.00% azodicarbonamide	BD+4.00% azodicarbonamide
Bifidobacterium spp.	7–9	8.17 ±0.40^{a}	7.83 ±0.31^{a}	8.67 ±0.21^{a}	8.33 ±0.21^{a}
Lactobacillus spp.	5–8	7.66 ±0.40^{a}	7.57 ±0.25^{a}	7.58 ±0.19^{a}	5.99 ±0.37^{b}
Escherichia coli (normal enzymatic properties strains)	7–8	7.47 ±0.54^{a}	5.83 ±0.23^{b}	3.78 ±0.22^{c}	6.07 ±0.51^{ab}
E. coli (weakly fermenting strains)	<25%	4.20 ±0.08^{a}	4.06 ±0.22^{a}	3.78 ±0.35^{a}	3.91 ±0.14^{a}
E. coli (lactose-negative strains)	<5% 2	0.55 ±0.55^{a}	1.18 ±0.55^{a}	1.09 ±0.50^{a}	2.34 ±0.76^{a}
E. coli (hemolytic)	0	0.00 ±0.00^{a}	0.00 ±0.00^{a}	0.00 ±0.00^{a}	0.00 ±0.00^{a}
Enterobacter spp.	2–4	1.92 ±0.62^{a}	2.55 ±0.51^{a}	2.84 ±0.63^{a}	2.67 ±0.87^{a}
Citrobacter spp.	2–4	0.00 ±0.00^{a}	0.00 ±0.00^{a}	0.00 ±0.00^{a}	0.00 ±0.00^{a}
Klebsiella spp.	2–4	3.36 ±0.74^{a}	4.17 ±0.20^{a}	3.26 ±0.87^{a}	4.00 ±0.37^{a}
Proteus spp.	2–4	0.85 ±0.53^{a}	1.33 ±0.62^{a}	1.10 ±0.77^{a}	1.93 ±1.31^{a}
Enterococcus spp.	5–7	4.27 ±0.52^{a}	4.79 ±0.26^{a}	5.28 ±0.33^{a}	5.28 ±0.28^{a}
Clostridium spp.	2	1.43 ±0.33^{a}	1.50 ±0.50^{a}	1.83 ±0.48^{a}	2.33 ±0.21^{a}
Pseudomonas spp.	2–4	2.12 ±0.67^{a}	3.23 ±0.08^{a}	3.49 ±0.23^{a}	2.85 ±0.61^{a}
Staphylococcus aureus	2–5	4.18 ±0.28^{a}	4.31 ±0.14^{a}	4.50 ±0.37^{a}	4.12 ±0.12^{a}
Staphylococcus epidermidis	2–4	3.73 ±0.30^{a}	4.24 ±0.09^{a}	3.95 ±0.52^{a}	3.29 ±0.33^{a}
Candida albicans	2	1.85 ±0.06^{a}	1.92 ±0.09^{a}	2.20 ±0.12^{a}	1.77 ±0.36^{a}
Candida spp.	2–5	4.24 ±0.55^{a}	4.40 ±0.25^{a}	5.25 ±0.17^{a}	5.00 ±0.24^{a}

by Olofinnade et al. (2021) showed that the addition of 1%, 2% and 4% of the substance to a complete diet significantly reduced the weight gain of animals, compared with the control. As conducted by Oser et al. (1965) earlier studies showed no significant effect of 5% and 10% azodicarbonamide supplementation on body weight in dogs compared to controls in a 14-month experiment. In male rats, the high dose (10% azodicarbonamide) at the end of the study showed only mild growth depression with a corresponding decrease in food intake. Similarly, the absence of a significant effect of 2-week repeated and 13-week subchronic inhalation exposure to azodicarbonamide on the final weight of rats is indicated in the results of Medinsky et al. (1990), and in the highest exposure group, final body weight was slightly reduced. In the present study, the addition of different doses of azodicarbonamide to the diet with excessive fat content did not lead to a significant change in the body weight of the animals. Interestingly, when only a high-fat diet was consumed and the minimum dose (0.25%) of azodicarbonamide was added to it, the average daily weight gain was the same (692 µg/day), and when 1% and 4% azodicarbonamide were added, it was insignificantly lower by 19% and 37% respectively.

It is known that the parameters of body weight and the amount of food and water consumed are directly dependent. A study by Olofinnade et al. (2021) showed a significant reduction in the amount of food consumed in the group of animals treated with azodicarbonamide compared to the control, which the authors associated with an increase in the feeling of satiety. In our experiment, the addition of azodicarbonamide to the diet did not affect the amount of food eaten and water drunk.

The change in relative organ mass under the influence of azodicarbonamide was described in a study by Medinsky et al. (1990). Thus, 2-week inhalation exposure to azodicarbonamide at a dose of 200 mg/m^3 caused a decrease in the mass of the liver of male rats, but no macro- or microscopic lesions of the organ were detected. A 13-week subchronic exposure in rats showed an increase in the mass of the lungs and their regional lymph nodes. Histopathological lesions, in this case, were associated with perivascular accumulations of lymphocytes in the lung tissue, which the authors attribute to a possible immune response to an antigen in the lungs. The studies of Olofinnade et al. (2021) showed the toxic effects of azodicarbonamide on the liver and kidney morphology in laboratory animals. So, in rats treated with 1%, 2% and 4% of the substance, dose-dependent damage to the liver (loss of normal structure, swollen hepatocytes with pale colored nuclei, loss of intermediate sinusoids, the appearance of inflammatory cells) and kidneys (loss of normal morphology, shrinkage of the glomeruli, the presence of pale-colored nuclei of tubular epithelial cells, expansion of Bowman's space, expansion of proximal and distal tubules) was observed. Not only azodicarbonamide itself but also its derivatives formed during its breakdown can have toxic effects. For example, studies by Maranghi et al. (2009) showed that oral administration of different doses of semicarbazide (40, 75, 140 mg/kg body weight per day) in rats during the juvenile period for 28 days caused histopathological effects in all studied tissues and organs (ovaries, testes, uterus, spleen, thymus, thyroid gland, adrenal glands). When studying the effect of a diet containing azodicarbonamide on the neurobehavior of rats, morphological changes in brain tissue were not revealed (Olofinnade et al., 2020). In our study, neither low nor high dietary concentrations of azodicarbonamide caused macroscopic pathological abnormalities and changes in relative organ mass.

The site of metabolism for many substances getting into the body with food, including drugs and food supplements, is the liver. It plays a central role in the conversion and purification of chemicals, including xenobiotics, and therefore is susceptible to damage (Lieshchova et al., 2018). The indicators by which the functional ability of the liver is assessed are very important in determining the toxicity and the effect of various substances on the human and animal body. Although many enzymes are present in the liver, Aspartate aminotransferase, Alanine aminotransferase and Alkaline phosphatase are the most informative in determining its functionality, since they are considered specific indicators of liver damage. Studies conducted on rats to determine the toxicity of azodicarbonamide indicate a pronounced hepatotoxic effect. It was shown that serum levels of AST, ALT and ALP were significantly increased in groups of rats fed a diet supplemented with 2% and 4% azodicarbonamide (Olofinnade et al., 2021). At the same time, in a study on the effect of semicarbamide (a by-product of the azodicarbonamide breakdown), even at a dose of 100 or 200 mg/kg of body weight, it showed no effect on liver enzymes in mice (Nestmann et al., 2005). In the present study, the enzymatic activity of rats' blood showed a sharp increase in ALT activity when azodicarbonamide was added to a high-fat diet, while the activity of this enzyme also increased with increasing azodicarbonamide dose. It is interesting that AST activity did not go beyond the physiological normal values, and when azodicarbonamide was added to the diet, it even slightly decreased. As a result, the De Ritis ratio was significantly and prominently reduced in the azodicarbonamide groups. In studies of the azodicarbonamide metabolism in the body, it is indicated that during heat treatment this substance is converted into biourea, which, even at a dose of 100–1000 times higher than the norm, does not cause side effects

on the morphofunctional state of the liver (Ferris et al., 1977). Therefore, we assume that disturbances in the functioning of the liver enzyme system are more associated with excess fat entering rats' bodies than with the action of azodicarbonamide. This is confirmed by other indicators evaluating liver function (lipid and carbohydrate metabolism, protein synthesis function, bilirubin metabolism), which did not differ significantly in the control and experimental animal groups. Even if we assume a negative effect of azodicarbonamide on liver function, the damage is not strong enough and does not go beyond the compensatory capabilities of hepatocytes, even against the background of excessive intake of fat in the body.

One of the most important blood indicators that describe the functional state of the kidneys is the level of creatinine. It is easily measured and is a by-product of protein metabolism, excreted unchanged in the urine. An increase in the level of creatinine in the blood is a marker of kidney insufficient filtration function. The adverse effect of azodicarbonamide at doses of 1%, 2% and 4% on renal function, especially glomerular filtration, was determined in a study in rats. The same study indicated that urea levels also increased significantly after azodicarbonamide ingestion at appropriate doses (Olofinnade et al., 2021). Our studies also showed the effect of azodicarbonamide on blood creatinine levels in rats fed a high-fat diet. So in rats consuming a high-fat diet, the level of creatinine was low (within the reference values of the norm), and with the addition of azodicarbonamide, it increased in proportion to the dose of the substance, reaching maximum values exceeding the physiological norm with the addition of 1% and 4%. The level of urea in the animals' blood of all groups was below physiological values, but no significant difference was found with the addition of azodicarbonamide to the diet.

When assessing the state of the blood system in rats treated with azodicarbonamide on the background of a high-fat diet, no significant differences were found in the indicators of the number of formed elements, haemoglobin, haematocrit and leukocyte formula. But there were study results that showed that the diet supplemented with azodicarbonamide significantly reduces the number of red blood cells at a dose of 1%, and increases at a dose of 2% and 4% while increasing haemoglobin levels in all studied groups of animals. The possible inflammatory, immune and allergic body reactions to azodicarbonamide were also indicated by an increase in leukocyte indices, as well as an increased number of eosinophils and basophils in the blood of the studied rats (Olofinnade et al., 2021).

From the results obtained by us, the most significant is the effect of azodicarbonamide on the functional state of the nervous system. Thus, when azodicarbonamide was consumed against the background of a high-fat diet, significant changes were noted in physical activity, orientational activity, and emotional status. At the same time, the consumption of 4% of the substance after 20 days of the experiment caused a significant decrease in all types of activity and emotional status, and 1% – in physical activity. These results are consistent with studies done in rats in the open-field test of locomotor and rearing activities with azodicarbonamide. Azodicarbonamide has generally been shown to have a concentration-related central inhibitory response, especially when fed with 4% azodicarbonamide, and 2% azodicarbonamide has been shown to increase grooming behaviour (Olofinnade et al., 2020). Semi-carbazide (a breakdown product of azodicarbonamide) is also known to be associated with changes in exploratory behaviour (Maranghi et al., 2009). Therefore, the effect of azodicarbonamide on the functional state of the nervous system and behavioural reactions, in particular, is interesting and requires further research.

The microbiome of the digestive tract of warm-blooded animals, having been formed during centuries of evolution, is quite stable and performs various numerous functions to maintain normal homeostasis of the body and ensure resistance to diseases. However, the quantitative and qualitative composition of the obligate (*Bifidobacterium*, *Propionibacterium*, *Bacteroides*) and facultative (*Escherichia*, *Enterococcus*, *Fusobacterium*, *Peptostreptococcus*, *Clostridium*, *Eubacterium*, etc.) intestinal flora can be changed by diet and environmental influences (Afrc, 1989; Salminen et al., 1995; Molozhavaya et al., 2016; Brygadyrenko et al., 2019). Licht & Bahl (2019) argue that orally administered chemicals can influence the composition of the gut microbiota of individual experimental animals, and thus potentially host health, by selectively suppressing or enhancing certain bacterial species in a complex community. In turn, gut microbes affect the absorption and metabolism of chemicals in different ways. Antagonistic properties and antibacterial action of *Bifidobacterium*, *Lactobacillus*, *Escherichia coli* against pathogenic and opportunistic microorganisms are due to the synthesis of bacteriocins, various enzymes, organic acids and other metabolites. In turn, *E. coli*, by absorbing oxygen from the intestinal lumen, promotes the reproduction of *Bifidobacterium* and *Lactobacillus* (Salminen et al., 1995; Molozhavaya et al., 2016). In this experiment, we observed a change in the qualitative and quantitative composition of representatives of the genus *Escherichia*. Against the background of short-term use of azodicarbonamide, as part of a high-fat diet, in

experimental samples of animal faeces, a pronounced decrease in the number of normal enzymatic properties strains of *E. coli* below the physiological norm was found even at the minimum and average concentration of azodicarbonamide (0.25% and 1%). At the same time, in the group of animals with the maximum concentration of the test substance (4% of the feed weight), the number of bacteria of the genus *Lactobacillus* significantly decreased from 7.66 ±0.40 in the control group to 5.99 ±0.37. Also, in the experimental groups, a slight increase in the amount of facultative (associated) microflora was found: lactose-negative strains *E. coli*, *Enterobacter* spp., *Klebsiella* spp., *Proteus* spp., *Enterococcus* spp., *Clostridium* spp., *Candida* spp. It is representatives of the facultative flora and transient microflora (bacteria that are opportunistic pathogens) that can cause diseases in the event of a decrease in organism resistance. Such and subsequent changes in the qualitative and quantitative composition of the intestinal microflora can contribute to the disruption of the homeostasis of the organism, lead to a decrease in the body's defence against pathogens of infectious diseases, a decrease in intestinal motility, the development of inflammatory processes in the gastrointestinal tract, etc.

4 Conclusions

In conclusion, our results from this study indicate that the addition of various doses of azodicarbonamide to a high-fat diet in model animals does not significantly affect body weight gain, food and water intake, organ mass indices, and most blood parameters. Minor functional disorders of the liver (decrease in De Ritis ratio due to increased activity of Alanine aminotransferase) and kidneys (high creatinine level) were revealed. At a concentration of 1%, azodicarbonamide causes a decrease in physical activity, and a 4% – decrease in physical activity, orientational activity and emotional status. Short-term use of high concentrations (4% of the feed mass) of azodicarbonamide, as part of a high-fat diet, led to a decrease in the number of bacteria of the genus *Lactobacillus*, as well as to a decrease in the number of normal enzymatic properties strains of *Escherichia coli* below the physiological norm, even at the minimum and average concentration of azodicarbonamide (0.25% and 1%).

Further studies will be aimed at analysing the effect of different azodicarbonamide doses on the laboratory animals' body biosystems in different age groups during the long-term experiment.

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