Research article

Use of chromium nanoparticles as a protector of digestive enzymes and biochemical parameters for various sources of fat in the diet of calves

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Abstract: The gastrointestinal tract acts as a digestive conveyor and carries out a specific stage of food processing throughout its length. A non-traditional approach to feeding cattle unprotected vegetable fats/oils may involve minerals (Ca and/or Mg). In our study, we studied the biological effects associated with the use of chromium nanoparticles at a dose of 200 μg/kg feed, together with soy and sunflower oils, in the diet of 10-month-old Kazakh white-headed breed calves. The calves were surgically fitted with duodenal fistula and pancreatic duct. Results demonstrated that inclusion of chromium nanoparticles in the diet increased the digestibility of crude protein, NDF, ADF and crude fat ($p \leq 0.05$). At the same time, inclusion of chromium nanoparticles in the diet led to changes in the blood serum: a 2-fold increase in aspartate aminotransferase ($p < 0.05$), a 42.2% increase in lactate dehydrogenase and alanine levels similar to control values. We noted that the addition of lipids in the feed led to the mobilisation of triglycerides and cholesterol. At the same time, the inclusion of chromium nanoparticles contributed to a decrease in the level of total lipids in serum and inhibited peripheral metabolic pathways (a 2-fold increase in the de Ritis coefficient). These data may represent additional evidence of the activation of anabolic processes when chromium nanoparticles are introduced into the diet. The increase in the enzyme index was associated with an escalation of lipid peroxidation processes and a decrease in antioxidant defence activity. We showed that vegetable oils enhanced lipolytic and proteolytic activity of pancreatic juice enzymes in the gastrointestinal tract. Adding chromium nanoparticles to the diet reduced lipase activity, especially when used with soybean oil. These data indicate the inhibitory effect of chromium on the activity of pancreatic lipolytic enzymes.

Abbreviations: ADF: acid detergent fiber; ALaT: alanine aminotransferase; ASaT: aspartate aminotransferase; CAT: catalase; CP: crude protein; DC: digestibility coefficient; DM: dry matter; GGT: gamma-glutamyltransferase: KK: creatinine clearance; LDH: lactate dehydrogenase; NDF: neutral detergent fiber; NPCr: chromium nanoparticles; SOD: superoxide dismutase

1. Introduction

An important reserve for increasing the productivity of farm animals is their assimilation of nutrients from the utilised feed. This process depends on many factors: the technology of harvesting feed and preparing it for feeding; the structure of diets and the level and ratio of minerals in them; the level of productivity; physiological conditions; and individual characteristics of animals [1]. Dietary nutrient digestibility is an important indicator of animal metabolism: high digestibility contributes to active growth and increased productivity of animals. The researchers note that increasing the digestibility of feed in the digestive tract in cattle is achieved as a result of the selection of the optimal structure of the diet, the ratio of all ingredients in it, as well as a certain amount of each nutrient, including minerals. The nutrient digestibility depends on on a number of factors: the type of animal, age, quantity and composition of the feed, preparation of feed and the presence of minerals [2].

The use of fat is of great importance for feeding farm animals. A lack of fat in the diet leads to stunted growth, impaired reproductive function, decreased productivity and reduced product quality. Lipids are an unconventional component in the diets of ruminant; thus, their excess inclusion leads to disturbances in the digestive system. In addition, free fatty acids can form insoluble or soluble compounds that reduce the absorption of fatty acids and trace elements [3–5].

Mineral components of the feed affect protein, carbohydrate and fat metabolism; and also support the protective functions of the body and participate in the regulation of the digestive system [6–7]. Such a specificity in relation to the action of minerals is necessary in the body to transfer the energy of fats to increase the growth rate, excluding their deposition. Chromium is an important microelement involved in lipid metabolism [8–9]; its positive effects on human and animal health are well understood [10].

The introduction of chromium positively correlates with a high growth rate and an increase in the activity of the antioxidant defense system in the body. Chromium also reduces lipid peroxidation, reduces cholesterol and fat in the abdominal cavity [11]. As you know, chromium is the main component of the glucose tolerance factor (GTF). The ability of GTF to regulate glucose levels by activating insulin secretion ensures proper metabolic conversion of carbohydrates, proteins and lipids [12]. Insulin metabolism is known to affect lipid peroxidation [13] and therefore, Cr as a cofactor of insulin can exhibit antioxidant activity. Chromium participates in the metabolism of nucleic acids; increases muscle area; and leads to the accumulation of chromium in tissues [14].

At a dose of 50–200 µg/day [15–16], chromium stimulates the metabolism of proteins, fats, carbohydrates and enzymes [17–19]. Although only 1.91%–8.56% of the adopted chromium is digested [20], its use in animal husbandry to grow calves contributes to more intensive growth and development. Chromium has positive effects on the processes of cicatricial digestion; the use of nitrogen, calcium and phosphorus in feed; milk production; and milk quality indicators and its chemical composition [21]. In combination with proteins and nucleic acids, chromium forms strong

bonds with oxygen- and sulfur-containing ligands and strengthens the immune system. In particular, a low protein content [22], as well as a high fat content in the diet [23], cause chromium deficiency.

Based on the literature, low molecular weight organic chromium complexes, such as picolinic acid and nicotinate salt forms, provide higher bioavailability than inorganic forms that are most often used as a food additive [24]. The prospect of replacing traditional sources of trace elements with ultrafine forms of metals is determined by the presence of a high specific surface area, greater reactivity and bioavailability. Given the small size and high penetrating power of nanoparticles, it is necessary to understand that each section of the gastrointestinal tract has a unique environment, which includes its own set of enzymes and pH level. Nanoparticles must be able to overcome these obstacles in order to exert their biological activity in the intestine [25]. Thus, it becomes necessary to regulate digestion by introducing mineral components into the diet. It is assumed that the addition of nanoparticles to the diet of cattle will help control the synthesis and amount of digestive enzymes.

This study determined the feasibility of using chromium nanoparticles (NPCr) as modulators of metabolic activity when using vegetable fat in the diet for ruminants.

2.   Materials and methods

2.1. Animals and diets

The studies were carried out on 10-month-old calves of the Kazakh white-headed breed, with an average weight of 210–220 kg, on the farm of the Federal Scientific Center for Biological Systems and Agricultural Technologies of the Russian Academy of Sciences. Animals were kept in separate metabolic cages (1.0 m × 2.2 m) in a room with optimal temperature and humidity parameters for this species with free access to water. Animal services and experimental studies were performed in accordance with instructions and recommendations: Russian Regulations, 1987 (Order of the Ministry of Health of the USSR No. 755 of 12.08.1977) and *The Guide for Care and Use of Laboratory Animals* (National Research Council [NRC], 1996).

Five groups of three heads were formed in each group: the calves of the control group during the experiment received a normal diet (T1) (Table 1), formed in accordance with the recommendations of the NRC (2000).

According to published data, at 200–300 μg/kg feed, chromium has the most pronounced biological effect on the physiological parameters of animals [14,26]. The inclusion of NPCr at a dose of 200 mg/kg of feed in the diet was carried out by initially mixing NPCr with water in a ratio of 1: 10, followed by inclusion in the cereal part of the diet (with thorough mixing). The introduction of chromium with water is necessary for a better uniform distribution of the trace element over the mass of the mixture and to ensure an animal takes in the correct amount. The average moisture content of the feed mixture was 56%.

NPCr were obtained by plasma chemical synthesis (Platina LLC, Moscow; d = 91 nm, specific surface area 9 m^2/g, Z-potential 93 ± 0.52 mV). Before incorporation into the feed mixture, NPCr was dispersed in physiological saline in ultrasonic to prevent aggregation of NP (35 kHz, 300 W, 10 μA, 30 min).

Table 1. The structure of the recipe and indicators of the quality of the diet.

Indicators	Group		
	Standard diet	Sunflower oil diet	Soybean oil diet
Mixed grass, kg	7.0	7.0	7.0
Concentrates, kg	2.0	2.0	2.0
Sunflower oil, kg	-	0.3	-
Soybean oil, kg	-	-	0.3
Feed molasses, kg	0.6	0.6	0.6
Premix PK-60, kg *	0.06	0.06	0.06
Salt, kg	0.02	0.02	0.2
Nutritional value of the diet (in kg)			
Dry matter	8.42	8.42	8.42
NDF	1.997	1.997	1.997
ADF	0.563	0.563	0.563
Crude fat	0.244	0.355	0.355
Crude protein	0.72	0.66	0.66
Nitrogen-free extractives	5.4	5.0	5.0
Calcium	0.99	2.14	2.19
Phosphorus	0.69	1.13	1.26
Metabolic energy, MJ	63.0	71.1	71.1

Note: *: vitamin and mineral premix (0.06 kg. the content of trace elements per 1 kg of concentrates: Mn-48 mg; Zn-36 mg; Fe-60 mg; Cu-10 mg; Se-0.24 mg; Co-0.12 mg; vitamin content per kg of concentrate: vitamin A (VA)-2640 M; vitamin D (VD)-302 IU; vitamin E (VE)-17 mg).

In addition to the normal diet, sunflower oil was added to the diet of group T2; sunflower oil + NPCr (200 mg/kg of feed) were added to group T3; soybean oil to group T4; and soybean oil + NPCr (200 mg/kg of feed) to group T5. Oil was introduced at a rate of 3% of the DM of the diet by replacing the concentrate part of the diet with the evaluated oils. The animals were fed twice a day, in equal proportions, in the morning and in the evening. The studies were carried out in three replicates.

2.2 Determination of the activity of digestive enzymes

To study pancreatic secretion, a surgical operation was performed according to [27]. An operation to implant fistulas was performed in compliance with the principles of a humane attitude towards animals using sedatives and painkillers. After inhalation (intubation) anaesthesia, a 4–5 cm long segment of the site of the confluence of the pancreatic duct into the duodenum was resected and a plastic fistula fixed into it. To collect the chyme, another fistula was implanted, which was connected with a rubber tube ('bridge') to the first fistula and formed an external anastomosis to return pancreatic juice to the duodenum. During the experiments, the 'bridge' is disconnected and pancreatic juice is collected in a test tube from the fistula tube of an isolated segment of the pancreas. In the period when experiments are not carried out, the ends of both tubes are connected and pancreatic juice flows from an isolated area of the duodenum into the small intestine.

Before the start of the experiment, the animal was placed in a special machine for fixing during the experiment and kept there for 8 hours without food, but with access to water. Then they received

feed. Machine is a frame structure with side walls. The front locking gates are removable, double-leaf, made of lattice sections. They serve for cervical fixation, with a fixation on the upper base. Remote opening of the front gate leaves in the upper part of the machine frame from the entrance side is carried out by means of a lever, which is pivotally connected to the rods of the gate leaves. The frame of the machine and the gate leaves are made of rectangular steel pipes. On the side walls of the machine from the side of the entrance and at the height of the hock of the animal there is a mechanism for supporting the hind legs. A detachable chain-fence is fixed on the side posts in the middle of the entrance opening. Animal bedding was made from wheat straw.

The animal was fed for 3 days with a control diet (without vegetable oils and NPCr), to ensure each animal had the same background indicators. Subsequently, the animals of the experimental groups were transferred to diets (T2, T3, T4 or T5). Biosubstrates were sampled after 15 days (pancreatic juice, chyme, blood and faeces). The daily dynamics of pancreatic secretion were determined in the following order: the pancreas juice was collected for the first 60 minutes before receiving the feed, and then every 60 minutes for 480 minutes. Pancreatic juice and chyme were collected in a bottle immersed in dry ice. Studies were carried out «in situ» in three replicates.

Laboratory studies were carried out in the laboratory 'Agroecology of technogenic nanomaterials' and the Test Center (FSC, Biological Systems and Agricultural Technologies of the Russian Academy of Sciences', accreditation certificate RA.RU.21PF59 from 02.12.15).

2.3. Digestibility determination

Digestibility was assessed according to Hashemi et al. [28] during 10–15 days of the experiment by carrying out balance experiments based on the food consumed by the animals, uneaten residues and the amount of excreted faeces. DC was calculated (in %) as the ratio of digested nutrients to the received nutrients. Faeces were collected and stored at −20 °C until further analysis. After freezing, drying and homogenising, the faeces and feed were analysed for the nutrient content–DM, CP, NDF, ADF, fat and ash–in accordance with the recommendations of AOAC [29].

2.4. Hematologic studies

Blood was collected from animals in the morning, on an empty stomach, on days 15 of the experiment from the jugular vein into vacuum tubes with a coagulation activator (thrombin). The studies were carried out on a CS-T240 automated analyser (DIRUI Industrial Co., Ltd, China) using commercial veterinary kits from DiaVetTest (Russia) and Randox Laboratories Limited (United Kingdom). The enzyme index (IF) is determined by the formula:

$$IF = [(ASaT/ALaT) + (ASaT/LDH) + (KK/LDH)] \qquad (1)$$

2.5. Measurement of pancreatic enzyme activity

Determination of amylase–according to Smith Roy [30] in the modification for determining high enzyme activity, proteases–by hydrolysis of purified casein according to [31] with calorimetric control (wavelength 450 nm), lipase on a CS-T240 automatic biochemical analyser (DIRUI Industrial Co., Ltd, China) using commercial biochemical kits for veterinary medicine from DiaVetTest (Russia).

2.6. 16S sequencing of the intestinal microflora calves

The samples content of intestine was placed into the sterile Eppendorf microtubes with the snap-on lid (Nuova Aptaca S.R.L., Italy); Total genomic DNA was extracted from the samples by phenol–chloroform method. DNA purity (according to OD260/OD280) was assessed with a NanoDrop spectrophotometer (Thermo Scientific, USA), for measuring concentration (ng/µL) a Qubit 2.0 fluorimeter (Invitrogen/Life Technologies, USA) was used. DNA concentration was measured 3 times: after extraction, after the first polymerase chain reaction with the specific 16S prokaryotic primers, and after the second PCR with the adapters and indices of Nextera XT protocols. The analysis of microflora was made by metagenomic sequencing (Illumina MiSeq, Illumina, USA) with the MiSeq® Reagent Kit v3 (600 cycles). The bioinformatic processing of the results was performed with PEAR software (Pair-End AssembeR, PEAR v0.9.8).

2.7. Statistical analysis

The studies were carried out in three replicates. Statistical analysis was performed using the ANOVA technique (Statistica 10.0 software package, StatSoftInc and Microsoft Excel, USA). Statistical processing included calculation of the mean (M) and standard errors of the mean (±m). The significance of differences between the compared indicators was determined by the t-student criterion. The level of significant difference was set at $p \leq 0.05$.

3. Results

3.1. Digestibility of feed

The differences in the fatty acid composition of the vegetable oils used in the study are presented in Table 2.

Table 2. Fatty acid composition of oils, %.

Fatty acid	Sunflower oil	Soybean oil
C16: 0 palmitic	6.3	10.6
C18: 0 stearic	4.0	4.6
C18: 1 oleic	18.8	24.3
C18: 2 linoleic	70.8	52.5
C18: 3 linolenic	0.1	7.3

The digestibility of nutrients was better in groups where NPCr was used with vegetable oils: the digestibility of CP increased by 10.5%, crude fibre by 20%–30% ($p \leq 0.05$) and crude fat by 40% ($p \leq 0.05$). At the same time, in the groups receiving vegetable oils, the digestibility of crude fat decreased by 27.9% (T2) and 7.9% (T4) and fiber by 2%, as the oils had a specific effect on the components of feed particles (which caused reduced availability for rumen bacteria [32]) (Table 3).

Table 3. Digestibility of dietary nutrients, %.

Index	Groups				
	T1	T2	T3	T4	T5
Crude protein	76.3 ± 3.6	81.37 ± 2.3*	86.8 ± 1.2	72.8 ± 1.02	88.1 ± 1.3
Crude fat	72.7 ± 1.23	44.87 ± 1.88	72.6 ± 1.31[(*)]	64.8 ± 0.75	73.1 ± 0.9[(*)]
NDF	31.3 ± 0.12	30.25 ± 0.11	49.2 ± 0.12*	28.9 ± 0.15	51.6 ± 0.24*
ADF	16.1 ± 0.1	15.22 ± 0.09	25.4 ± 0.08*	14.8 ± 0.1	26.3 ± 0.12*

Note: *: $p \leq 0.05$, when comparing the control with the experimental group. (*): when comparing T2/T3 and T4/T5.

3.2. Hematological indicators

The addition of vegetable oil produced a hyperglycemic effect, denoted by a 5% increase in glucose. The biological feature of chromium in the body of calves was manifested by a 1.5–2-fold increase in protein synthesis ($p \leq 0.05$) and a metabolic decrease in glucose to control values, which is consistent with the results of studies by [33] (Table 4).

Adding vegetable oils stimulated the production of ALaT by 25.2% and 17.4% ($p \leq 0.05$) in groups T2 and T4, respectively, with a tendency to increase the level of ASaT. The addition of NPCr led to an almost 2-fold increase in ASaT ($p \leq 0.05$), while ALaT activity was close to the control value. There were significant 21.0%–42.2% increases in LDH levels in the experimental groups ($p \leq 0.05$) with the simultaneous inclusion of NPCr. GGT indices increased independently of the acting factor.

Table 4. Biochemical parameters of the blood of calves when fats are included in the diet (n = 5, M ± m).

Index	Groups				
	T1	T2	T3	T4	T5
Total protein, g/L	72.05 ± 3.98	86.85 ± 4.51*	147.5 ± 3.41*	99.43 ± 6.98	124.22 ± 5.45
Albumin, g/L	29 ± 6.1	36 ± 5.8*	36 ± 4.2*	42 ± 5.3*	41.6 ± 3.9*
Glucose, mmoL/L	3.41 ± 0.87	3.52 ± 0.63	3.12 ± 0.02	4.21 ± 0.74*	3.27 ± 0.23*
Triglycerides, mmoL/L	0.29 ± 0.07	0.37 ± 0.03	0.14 ± 0.02	0.45 ± 0.09*	0.21 ± 0.02*
Cholesterol, mmoL/L	2.67 ± 0.19	3.63 ± 0.31*	1.45 ± 0.28*	4.99 ± 0.81*	1.68 ± 0.21*
ALaT, U/L	23.8 ± 4.3	31.8 ± 5.1*	27.9 ± 3.2	28.8 ± 2.1*	26.1 ± 2.8
ASaT, U/L	44.2 ± 5.9	52.3 ± 6.3	113.5 ± 7.8	54.9 ± 5.8	98.2 ± 4.6
Total bilirubin, μmoL/L	2.43 ± 0.07	3.25 ± 0.08	1.17 ± 0.01	3.67 ± 0.09	1.75 ± 0.06
Bilirubin Direct, μmoL/L	1.11±0.13	1.72 ± 0.18	3.26 ± 0.12	1.59 ± 0.16	2.96 ± 0.2
LDH, U/L	3049 ± 56.5	5272 ± 64.3*	4648 ± 55.2*	4098 ± 63.7*	3856 ± 42.2*
a-Amylase, U/L	415 ± 23.1	471 ± 63.1*	573 ± 42.6*	423 ± 21.9	497 ± 18.1
Lipase, U/L	17.3 ± 3.4	16.8 ± 1.2	6.2 ± 0.8	28.4 ± 3.9	16.2 ± 2.9
Urea, mmol/L	3.2 ± 0.7	5.1 ± 0.9*	5.4 ± 0.6*	4.6 ± 0.9	5.1 ± 0.5

Index	Groups				
	T1	T2	T3	T4	T5
Creatinine, µmoL/L	74.5 ± 6.3	88.7 ± 7.2*	100.7 ± 8.6*	89.6 ± 7.2	96.5 ± 8.6
Alkaline phosphatase, U/L	100 ± 19.6	114 ± 21.1	161.2 ± 56	158 ± 20.6*	198.6 ± 43.2*
GGT, U/L	18.3 ± 2.6	32.4 ± 4.1	26 ± 3.4	23.6 ± 3.1	21.6 ± 2.8
Uric acid, µmoL/L	15.5 ± 3.2	18.9 ± 4.3	7.2 ± 1.4	16.1 ± 3.9	6.9 ± 1.6
Iron, µmoL/L	19.2 ± 3.8	33.4 ± 5.1*	57.5 ± 6.3*	34.6 ± 4.3*	41.5 ± 3.7*
Magnesium, mmoL/L	1.22 ± 0.2	1.73 ± 0.3	0.84 ± 0.08	1.68 ± 0.9	0.88 ± 0.7
Calcium, mmoL/L	2.45 ± 1.1	3.01 ± 1.2	2.6 ± 1.4	2.71 ± 0.8	2.06 ± 0.02
Phosphorus, mmoL/L	1.54 ± 0.04	2.03 ± 0.06	2.53 ± 0.2	1.68 ± 0.6	1.92 ± 0.4

Note: *: $p \leq 0.05$, when comparing the control with the experimental group.

The addition of oils to the feed composition led to an increase in triglyceride and cholesterol mobilization by 44.4% and 54.4%, respectively. The inclusion of NpCr in the diet helps to reduce the level of total lipids in the blood serum: triglycerides and cholesterol in the blood, in the T3 group by 62.2% and 60%, in the T5 group by 55.6% and 66.3%, respectively.

The de Ritis coefficient (ASaT: ALaT ratio) (Figure 1), as an indicator of the type of metabolism in experimental calves, did not change significantly compared to the control group of calves (1.64) when sunflower (1.85) and soybean (1.81) oil was introduced into the diet. The maximum coefficients were in the T3 (4.06) and T5 (3.76) groups.
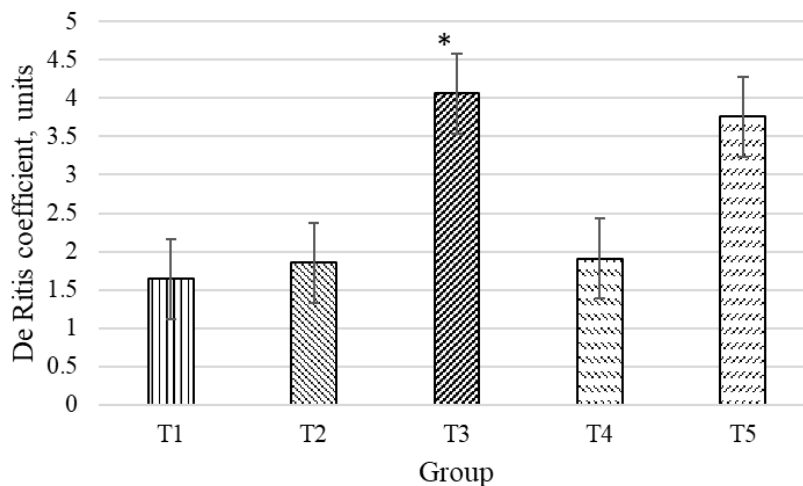

Figure 1. De Ritis coefficient in experimental calves. Units.
Note: *: $p \leq 0.05$, when comparing the control with the experimental group.

To assess the general condition and direction of the course of metabolic processes, the enzyme index was determined (Figure 2).

An increase in the enzyme index indicates an aerobic shift in cellular metabolism due to a decrease in the activity of anaerobic mechanisms. This phenomenon occurred in groups T3 (56.3% increase

compared with control; p ≤ 0.05) and T5 (55% increase compared with control). The enzyme index when adding sunflower (4.63%) and soybean oil (18.91%) to the diet did not change significantly compared with the control group.
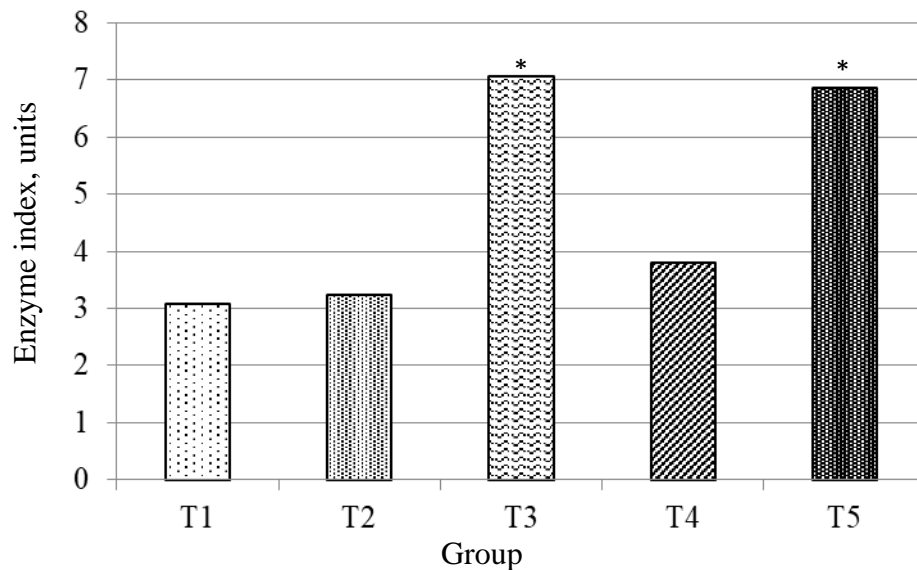

Figure 2. Changes in the enzyme index in calves with the introduction of fats and NpCr into the diet. Note: *: p ≤ 0.05, when comparing the control with the experimental group.

3.3. The antioxidant activity of the blood

With regard to antioxidant enzymes (Table 5), there was a decrease in the activity of CAT; however, SOD activity increased by 14.6% (p ≥ 0.05) with the inclusion of sunflower oil and by 13.3% (p ≥ 0.05) with the inclusion of soybean oil.

Table 5. Antioxidant activity in serum of blood when fats are included in the diet (n = 5, M ± m).

Group	SOD, % inhibition	Catalase activity, μM	Malondialdehyde, μM/L
T1	64.1 ± 5.8	26.5 ± 3.7	0.09 ± 0.08
T2	75 ± 6.2*	20.1 ± 6.7**	0.12 ± 0.06
T3	66.96 ± 4.2	19.8 ± 5.4	0.19 ± 0.02**
T4	73.9 ± 6.7*	13.3 ± 2.4*	0.05 ± 0.003
T5	73.91 ± 3.8*	13.42 ± 2.4*	0.24 ± 0.04*

Note: when *: p ≤ 0.05, when comparing the control with the experimental group.

The use of NPCr increased the level of malondialdehyde (MDA), an indicator of lipid peroxidation, 2.2–2.6 times compared with control (p ≤ 0.05). These data indicate the presence of oxidative stress associated with a high degree of metal oxidation in the presence of fats and the active degradation of polyunsaturated fatty acids (PUFA) by reactive oxygen species. Thus, the intake of 200 μg Cr/kg feed along with vegetable oils contributes to an increase in lipid peroxidation processes and a decrease in antioxidant defence activity.

We used the fistulation method to study the secretion of pancreatic juice and to analyse the content of digestive enzymes in real time–in response to various components of the diet. When soybean oil was added to the diet, the level of trypsin in the pancreatic juice increased by 14.5% compared with the diet with sunflower oil. The inclusion of NPCr in the diet increased trypsin content in groups T3 and T5 relative to the control group by 46.5% ($p \leq 0.05$) and 43% ($p \leq 0.05$), respectively (Figure 3).
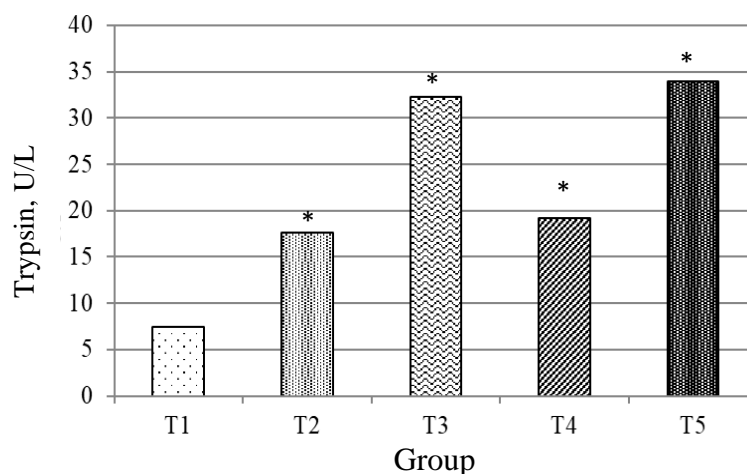

Figure 3. Trypsin content in calf pancreas juice. U/L.

Note: *: $p \leq 0.05$, when comparing the control with the experimental group.

The metabolic processes in the gastrointestinal tract are significantly affected by nitric oxide (NO), which mediates a cascade of physiological processes, including regulation of vascular tone, plasma and platelet units of hemostasis, neurotransmission and the formation of an immune response, inhibition of smooth muscle cell proliferation. When NPCr was introduced with vegetable oil, there was an increase in the level of nitric oxide metabolites in all animals. There was a 29.4% increase in the NO level in the T3 diet (sunflower oil + NPCr) relative to the control group. Differences in blood metabolites suggest that they may be associated with animal nutrition and, as a consequence, unequal levels of albumin, urea and globulin in the blood of calves (Figure 4).
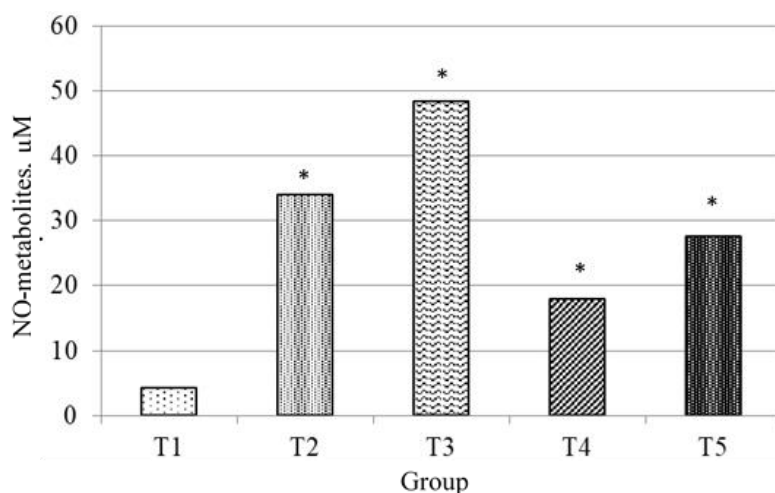

Figure 4. The content of NO-metabolites in the blood serum of calves. μM.

Note: *: $p \leq 0.05$, when comparing the control with the experimental group.
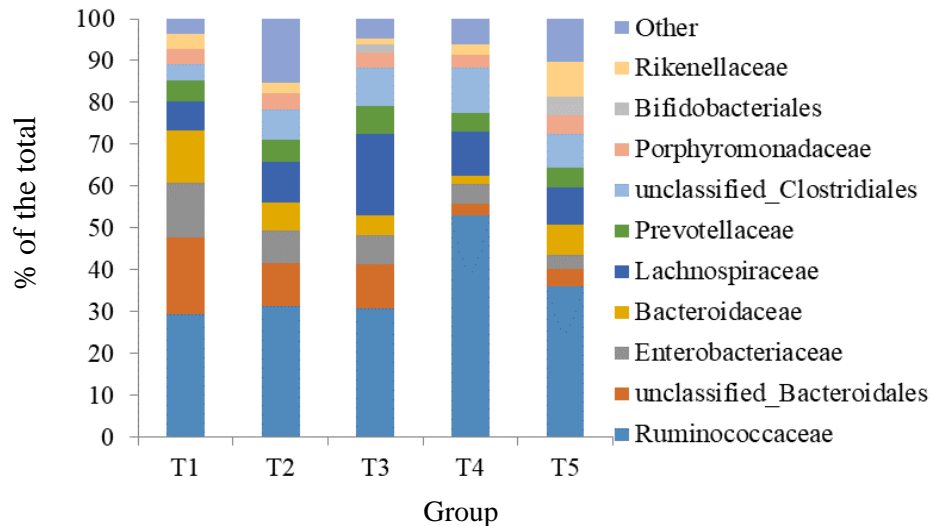
Figure 5. Genetic diversity of calf intestinal microflora.

3.4. Enzymatic activity of the pancreas and chyme

A large number of digestive enzymes are secreted by the pancreas; due to their unique function, their quantity and ratio can be regulated [18]. The participation of chromium in metabolic processes cannot be ruled out considering the complex digestion mechanisms in cattle (Tables 6 and 7) [34]. The inclusion of sunflower oil in the diet increased lipase activity 8.5 fold in the pancreatic secretion ($p \leq 0.05$) and 5.6 fold in the chyme; this phenomenon is a response to the high content of free fatty acids. The addition of soybean oil to the diet was accompanied by a pronounced increase in proteolytic activity: 33.5% in pancreatic juice and 25% in chyme, compared with control values. In addition, there was a 7.3-fold increase in lipase activity in juice ($p \leq 0.05$) and a 1.1-fold increase in chyme relative to control values. The use of NPCr in the diet reduced the enzymatic lipolytic activity 10 and 53.6% compared with groups T2 and T4 by ($p \leq 0.05$), respectively. There was a 2–3-fold decrease in amylolytic activity in all comparison groups.

Table 6. Activity of enzymes in pancreatic juice of calves (n = 5, M ± m).

Index	Ration				
	T1	T2	T3	T4	T5
Amount of pancreatic juice, mL	59.5 ± 4.8	45.9 ± 6.9	65.7 ± 4.61	52.1 ± 5.65*	84.2 ± 7.84
Lipase, U/L	90.9 ± 18.2	773 ± 14.8*	696 ± 30.18*	667 ± 37.0*	310 ± 30.68*
Amylase, mg/mL/min	5137 ± 450	2537 ± 400	3668 ± 416.97	1931 ± 69	2704 ± 658.34
Proteases, mg/mL/min	133 ± 24.3	249 ± 21.1*	255 ± 38.75*	200 ± 12.6	173 ± 51.08
Total protein, g/L	0.46 ± 0.12	0.18 ± 0.007	0.52 ± 0.03	0.33 ± 0.01	0.71 ± 0.15
Phosphorus, mmoL/L	0.14 ± 0.02	0.03 ± 0.004	0.45 ± 0.02	0.08 ± 0.01	0.45 ± 0.04*
Calcium, mmoL/L	2.33 ± 0.12	2.43 ± 0.22*	2.07 ± 0.04	2.39 ± 0.1*	2.01 ± 0.08
a-Amylase, U/L	416 ± 4.8	578 ± 11.5	4141 ± 607.45	767 ± 13.8	3218 ± 296.36

Note: when *: $p \leq 0.05$, when comparing the control with the experimental group.

When assessing the calcium content in the pancreatic secretion and chyme, the introduction of vegetable oils increased the indicator by 5–6%. By contrast, the inclusion of NPCr in the diet reduced the calcium level by 10.8 and 32.0% ($p \leq 0.05$) compared with groups T2 and T4, respectively.

Table 7. Activity of enzymes of duodenal chyme in when fats are included in the diet (n = 5, M ± m).

Index	Ration				
	T1	T2	T3	T4	T5
Lipase, U/L	23.2 ± 0.42	129 ± 22.5	28.43 ± 10.34*	26.2 ± 10.4*	24.11 ± 3.17**
Amylase, mg/mL/min	4780 ± 110	4309 ± 375*	2837.5 ± 654	1746 ± 443	2709 ± 514*
Proteases, mg/mL/min	96.8 ± 43.5	95.1 ± 17.2*	94.80 ± 25.0*	129 ± 49.4	181.35 ± 53.5
Total protein, g /L	0.4 ± 0.06	2.7 ± 0.07	0.51 ± 0.09	0.71 ± 0.19	0.73 ± 0.14
Phosphorus, mmoL/L	0.12 ± 0.02	0.05 ± 0.005	0.17 ± 0.05*	0.23 ± 0.05	0.26 ± 0.02
Calcium, mmoL/L	2.46 ± 0.05	2.61 ± 0.02	2.33 ± 0.07*	2.73 ± 0.61	2.39 ± 0.04*
a-Amylase, U/L	277 ± 2.55	2318 ± 28.7	2957.67 ± 216.65	2998 ± 297	3954.15 ± 29.38

Note: when *: $p \leq 0.05$, when comparing the control with the experimental group.

3.5. Metagenomic studies

When studying the genetic diversity of the intestinal microflora of calves, representatives of the *Ruminococcaceae* family (29.3%–53.12%) were the most common. The T4 group showed the highest number of these bacteria (81.2% higher than the control). The number of representatives of the *unclassified_Bacteroidales* taxon was the largest in the control, but in the experimental samples, their number decreased by 45.2 and 42.7% in the calves fed T2 and T3, respectively. The largest decrease in this taxon was noted in the variant with the addition of soybean oil and soybean oil with chromium (by 85.6% and 77.7%, compared with the control). The number of representatives of *Enterobacteriaceae* decreased in all experimental variants (by 39.2, 46.4, 63.7 and 73.5% for T2, T3, T4 and T5, respectively) compared with the control group. The number of representatives of the taxon *Bacteroidaceae* decreased in the experimental groups by 61.6%–84.1%. In turn, the number of the *Lachnospiraceae* family increased in experimental variants by 26.9%–173.9%. The number of representatives of the taxon *unclassified_Clostridiales* increased in all experimental groups by 1.83–2.76 fold compared with the control. The abundance of *Porphyromonadaceae* did not significantly differ from the control values.

4.   Discussion

Until recently, it was assumed that the diet of cattle provides enough chromium to meet the needs. Recent research suggests that, in some situations, practical diets may not contain enough chromium to maximize biological processes in ruminants. It is known that additional chromium affects lipid metabolism. Several forms of supplemental chromium have been investigated in cattle diets, including chromium-nicotinic acid complex, chromium tripicolinate, high chromium yeast, amino acid chelated chromium and chromium chloride [35]. One problem is that little is known about the potential use of chromium nanoparticles in feed commonly used in cattle diets. Further research is needed to determine if chromium supplementation will affect the digestibility of ruminants.In our experiment, we recorded a trend towards an increase in the de Ritis coefficient when NPCr was added to the diet. Notably, the

high de Ritis coefficient value occurred due to a decrease in ALaT in the studied groups. This finding may indicate inhibition of peripheral metabolic pathways (glycolysis, measles cycle, etc.) in the cells with unaltered central metabolic pathways. This fact may be additional evidence of the activation of the processes of anabolism with the introduction of NPCr in a dosage of 200 µg/kg.

There is an opinion [36] about the negative effects of chromium on the body. The main doubt has emanated from the results of a study published in [37]. The researchers found that dietary chromium content did not significantly affect body weight, feed intake, glucose or insulin levels. In addition, it must be taken into account that the reaction to the presence of a trace element can be variable, that is, the introduction of 200 µg/kg of chromium into the diet can be accompanied by its multidirectional effect on biochemical processes. This usually directly depends on the composition of the diet. An increase in the ASaT/ALaT ratio may indicate a pronounced biological effect of the central metabolic link that regulates the flow of substrates into the Krebs cycle. This cycle provides aerobic oxidation of substrates, a detoxifying function in relation to ammonia, and also provides the restoration of the content of aspartate in tissues, which decreases with an imbalance of amino acids [38–40].

The most studied aspect of food adaptation in ruminants includes the ratio of the diet and the level of pancreatic α-amylase. The activity of this enzyme may vary depending on the composition of the feed additive [41]. The results from this study demonstrated that the inclusion of vegetable oil and NPCr resulted to 1.21- and 1.17-fold increase in the level of α-amylase for the T3 and T5 groups, respectively.

The inhibitory effect of chemicals is manifested by their ability to interact with free radicals of an oxidising substance with the formation of less active radicals, or with hydroperoxides and their radicals with the formation of stable products. In addition, the inhibitory effect is caused by the binding of prooxidants–metal ions of variable valency [33]. In our experiment, the inclusion of 200 µg Cr/kg of feed for cattle in the diet together with vegetable oils enhances the processes of lipid peroxidation and decreases the activity of the antioxidant defense. The decrease in SOD activity in the chromium-containing diet is probably associated with a decrease in the amount of lipid peroxidation intermediates (POL) accumulated in the blood [42].

Known, for a soy-based diet, a critical amount of protein secretion (e.g. trypsin) is required for appropriate digestion; however, this phenomenon is not necessary with a diet that includes sunflower oil. The present results are the first associated with the resistance of soy protein to enzymatic degradation. Second, after digestion in the stomach, the flow rate and kinetics of postprandial secretion increase in response to a diet with a different composition [43].

The activity of enzymes changes significantly with an alteration in food composition, and specifically in response to the introduction of appropriate fatty components into the diet against the background of a mineral supplement [44]. The inclusion of vegetable oils in the diet enhanced the lipolytic and proteolytic activity of pancreatic juice enzymes. The inclusion of NPCr in the diet reduced lipase activity, especially when provided with soybean oil. Our experiment indicates the inhibitory effect of chromium on pancreatic lipolysis.

The introduction of vegetable oils increased the calcium content in the pancreas secretion and chyme by 5–6%. On the contrary, the inclusion of NPCr in the diet decreased its level by 10.8 and 32.0% ($p \leq 0.05$) compared with groups T2 and T4, respectively. The manifestation of this effect is possibly associated with a few factors. First, there may be a high need for calcium due to an increase in protein metabolism – which, according to Weiser [45], stimulates urinary calcium excretion. Second, there may be increased calcium absorption in the rumen [46] due to the ability of free fatty acids to form

complexes with minerals, followed by the formation of insoluble or soluble soaps that reduce the availability of fatty acids and Ca for absorption [3–4]. We draw attention to the fact that the pancreas, which secretes the two main phospholipases, also requires the presence of calcium ion as a cofactor [47]. We confirmed this phenomenon with our results, where the level of calcium increased in chyme and blood, with a simultaneous decrease in pancreatic secretion. These data confirm the participation of calcium in lipid metabolism.

It is believed that the effectiveness of additional Cr in the regulation of impaired glucose and lipid metabolism depends on various factors, such as the chemical form [48] and dose of chromium (and its bioavailability), the degree of insulin resistance or hyperglycemia, as well as individual sensitivity and genetics, factors that have not been completely determined. The use of vegetable oils in cattle diets is not traditional due to the competition of bacteria for lipids and food nutrients. The addition of feed lipids led to the mobilisation of triglycerides and cholesterol due to PUFA that inhibit hepatic lipogenesis, by which acetyl-CoA is converted to triglycerides [49–50]. Furthermore, NPCr supplementation to a calf's diet might improve body lipid metabolism [10] due to high-density lipoprotein (HDL), which is primarily synthesised in the liver and small intestine. HDL plays an important role in eliminating serum cholesterol [14]. A decrease in serum lipids may be due to a decrease in the synthesis and/or increased hydrolysis of triglycerides [51], which is associated with an improvement in blood lipid metabolism. The content and relative concentrations of these fatty acids are mandatory for nutrition. Notably, the content of oleic acid, a monounsaturated fatty acid, is 5.5% higher in soybean compared with sunflower oil. This difference exerts a positive effect, since the oil's heat resistance increases, which makes it more digestible in the gastrointestinal tract. The biological role of PUFA is determined by their participation as structural components in the phospholipids of cell biomembranes, different positions and the number of double bonds cause different physiological effects of fatty acids.

In a metagenomic study of the intestinal microflora of calves, there was a decrease in the occurrence of *Enterobacteriaceae* representatives in experimental samples. Given that the members of pathogenic genera are also represented in this family, one can discuss the positive effect of diets with fat additives with NPCr on the intestinal microflora, which can serve as a basis for revising approaches to antibiotic therapy of intestinal diseases. In addition, it is assumed that oils as fat additives can be used as modulators of microbial community compositions in young cattle. Given that the composition of the intestinal microbial communities in newborns and young animals tends to fluctuate until it is stably established at a later age, it is more likely to respond to manipulations at these early stages of growth [52]. Thus, the goal of modulating the early composition of the intestinal microbiome would be to provide long-term benefits for the productivity and health of adult animals [53]. In this case, further studies are necessary, because the current accumulated data on the effect of fatty acids on the growth and development of bacterial populations is contradictory. For example, bacteria, especially *Lachnospira* spp. and *Prevotella* spp., react differently to the application of oils in different concentrations [54].

5.   Conclusion

In our studies, the inclusion of various fatty components of food in the diet affected the activity of digestive enzymes, namely, elevated lipolytic activity and intestinal protease activity against the background of a decrease in the amylolytic activity of pancreatic juice and chyme. However, with the

introduction of NPCr, lipase activity decreased with an increase in the activity of amylase when both sunflower and soybean oils were included in the diet. This phenomenon reduced the load on the pancreas and contributed to the normalisation of digestion. The ratio of diets with different fat composition with the introduction of ultrafine particles of chromium with the activity of digestive enzymes is an example of adaptation of the digestive system. The data obtained in the experiments can be used to correct diets in the industrial production of high-quality beef.

Ethics approval of research

All experiments on live animal in our experience were performed in accordance with relevant guidelines and regulations (Russian regulations (Order of the Ministry of Health of the USSR1 755 of 12.08.1977) and "The Guide for Care and Use of Laboratory Animals (National Academy Press, Washington, D.C., 1996)". Every effort was made to minimize the suffering of the animals and reduce the number of used samples.

The experiments and animal care protocol were approved by the animal welfare committee of Institutional Committee for Animal Care of Federal Research Centre of Biological Systems and Agrotechnologies of the RAS.

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Conflict of interest

All authors declare no conflicts of interest in this paper.

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