ASSESSMENT OF GUT MICROBIOTA AND SERUM BIOCHEMISTRY PARAMETERS IN RATS FED COMMERCIAL PELLET DIET CONTAINING DIFFERENT PROTEIN CONTENT

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Abstract. The aim of this study was to evaluate the effect of different content of protein in diets on gut microbiota and some blood parameters in male *Wistar* rats. The research was carried out on 20 male *Wistar* rats. Ten rats were allocated to the group I and were fed standard commercial pellet diet with 19.91 % crude protein, ten rats were allocated to the group II and were fed with 21.50 % crude protein from one to ten months old. The animals were individually housed in laboratory cages under standardized environmental conditions. Rats were given *ad libitum* access to the diet and drinking water. The samples of faeces and blood were collected at the end of the experiment. There were no statistically significant changes in the composition of gut microbiota of rats, fed diet with different protein content. There were found elevated statistically significant values of total protein, globulin and total bilirubin in blood of rats, fed with 21.50 % of crude protein in diet. However the higher activities of AST and ALT as well as higher value of Crea were found in blood of rats, fed with 19.91 % of crude protein. Our results showed that feeding of rats with diet containing higher content of crude protein from weaning to the ten months age might have strong effect on some biochemical parameters of blood. The difference between crude protein content in diet has no reliable effect on the gut microbiota of adult rats. It is important fact for further experiments when rats used as animal biomodel for veterinary medicine and biomedical research.

Keywords: rat, diet, protein, gut microbiota, blood biochemistry

Introduction. Rats are an important experimental model in biological research in the context of fundamental science, research, development and determining the quality of products and devices used in human and veterinary medicine, dentistry, as well as in assessing the harmfulness of certain chemicals used in households, industry, agriculture and other safety evaluations (Katica, Gradascevic, 2017; Kurien et al., 2004). The laboratory rat is particularly suitable for biomedical research related to cardiovascular diseases, metabolic and neurological disorders, neurobehavioral studies, organ transplants, autoimmune and renal diseases, to study the susceptibility to cancer, as well as the impact of different doses of radioactivity to tissues and haematological parameters (Hedrich, 2000; Saračević et al., 2004; Abojassim et al., 2015).

Modern housing technologies, various diets compositions, feeding, physical environmental factors, research experiments, human interaction and intervention are parts of the stimuli presented to the laboratory animals every day, influencing their physiology and contributing to their welfare. As the laboratory rat is one of the major species bred and kept for scientific research (Tomas et al., 2012), their health has critical importance on investigation results. Various studies have reported differences in laboratory animal blood biochemistry parameters related to species, strain, sex, age and environment (Dontas et al., 2011; Jeklova et al., 2009). However Tomas et al. (2012) noticed that determining of the non-pathogenic microbial community might be relevant in quality control of laboratory animals. The mammalian intestine harbors a large and diverse community of micro-organisms, known as the intestinal microbiota (Tomas et al., 2012). Flemer et al. (2017) stated that a core of 46 bacterial species is present in all rats but its members' relative abundance progressively decreased with age. Gut bacteria play a critical role in multiple physiological changes of the host related to metabolic disorders, immunity, brain development and many other aspects (O'Hara and Shanahan, 2006). Nicholson et al. (2005) observed that gut bacteria have a close connection with the host through metabolite input to maintain energy homeostasis in the intestinal mucosa.

Carabotti et al. (2015) revealed that between gut microbiota and central brain exit significant relationship, but El Aidy et al. (2017) demonstrated that the neurotransmitter serotonin (5-HT) plays a vital regulatory role in both the brain and gut. Besides its role in the brain, 5-HT has diverse roles in the gastrointestinal tract, ranging from modulation of electrolyte absorption, maintenance of fluid homeostasis, alterations in gastrointestinal motility, and regulation of gut permeability (Coates et al., 2006; Haub et al., 2010; Gill et al., 2013). Moreover, bacteria populating the gut microbiota can release significant amounts of amyloids and lipopolysaccharides, which might play a role in the modulation of signaling pathways and the production of pro inflammatory cytokines related to the pathogenesis of Alzheimer disease (Pistollato et al., 2016).

Although the composition of gut bacteria is relatively stable, but it can be affected by many factors, especially the diet (Carabotti et al., 2015). As one of the major constituents of a diet the proteins can be drawn from a wide range of raw materials according to cost and availability, but its quantity and quality are important considerations in formulating a diet. Protein modifies the profile of fermentation products, but changes in

microbiota composition remain unclear (Kiilerich et al., 2016).

It has been noticed that protein requirements in a diet for rats, used as biomodels in various experiments, must be balanced according to the age. There are controversial opinions on how much protein should be in a rat's diet. Many experts have noticed that the need for protein decreases with age. Food for rats should contain 20 % protein only in young age (Sirois, 2005; Muminović et al., 2006). The reference ranges recommended throughout a rat's lifespan are from 23-24 % for babies and young (before the period of reproductivity) rats down to as low as 5 % for geriatric rats. Protein requirements of rats decline at the age of 50 days and 17 percent of protein in diet is sufficient (Nutrient Requirements, 1995). Moreover, consideration of the protein content needs to be made depending on the sex of the rats. Male rats require less protein than female.

Nowadays there is a wide variety of commercial feeds for laboratory rodents. In spite the fact, that the choice of diet and its composition may influence some health markers of an animal, the most rodents throughout the test period, irrespective of their age and sex, are fed the feed of the same composition, which is usually obtained by organizing public procurement procedures with only one type of feed for the whole year. However in our pilot studies, we found that when rats, regardless of age, were fed with higher dietary protein content, some blood parameters have altered and the tumors of various organs have developed in older animals.

The aim of this study was to evaluate the effect of different content of protein in diets on gut microbiota and some blood biochemical parameters in *Wistar* male rats.

Materials and methods

The experimental study was performed with randomly selected twenty *Wistar* male rats and lasted for nine months. Ten rats from weaning (one month old) were allocated to the group I and were fed diet with 19.91 % crude protein to the age of ten months. Next ten rats were allocated to the group II and were fed diet with 21.50 % crude protein the same period of time. Moreover, during the experiment the composition of the diet had changed neither in group I nor in group II of the rats with respect to their age.

All experimental animals were individually housed in laboratory cages under standardized environmental conditions (temperature 22±2°C, relative humidity 50±10 %, 12:12 h light-dark cycle). Rats were given *ad libitum* access to the commercial standard pellet diet and drinking water.

Experiment with animals was in compliance with the rules of the national Animal Ethics Committee and the protocols were designed according to European Parliament Directive 2010/63 EU.

At the end of the experiment, the samples of faeces and blood of adult rats of ten months age were collected before feeding on the morning. For enumeration of gut microbiota, animals were placed in individual empty cages with no bedding and after normal defecation the four faecal pellets per rat were collected. The faecal samples were delivered to the laboratory immediately after collection.

1 g of faeces sample were homogenized in 9 ml 0.9 % NaCl solution by vortex mixing (IKA mini shaker, MS2, USA). The serial decimal dilutions were made and as parallel duplicate cultures were inoculated into Plate count agar (Liofilchem, Italy) – for enumeration of aerobic and facultative anaerobic bacteria, plates were incubated at 30°C for 24 hours (LST EN ISO 4833:2003); MRS (de Man, Rogosa and Sharpe) agar (Biolife, Italy) - for enumeration of *Lactobacillus* spp., plates were incubated at 30°C for 72 hours under microaerophilic condition (Thermo Scientific™ Oxoid AnaeroGen 2.5L Sachets are anaerobic gas generating sachets for use with Thermo Scientific™ Oxoid 2.5L jar), (LST ISO 15214:2009); Slanetz and Bartley agar +TTC (Liofilchem, Italy) – for enumeration of fecal streptococci, plates were incubated at 35°C for 48 hours (ISO 7899-2:2000); Violet red bile glucose agar (Liofilchem, Italy) – for enumeration of Enterobacteriaceae, plates were incubated at 37°C for 24 hours (ISO 21528-2:2004). Bacteria enumeration was performed by standard method (EN ISO 7218:2007). The mean value of the colony counts (colony forming units (CFU)/g) in the two duplicates was calculated. The numbers of colony forming units are expressed as $\log_{10}$ CFU per gram faeces.

Blood samples were collected in microtubes without anticoagulant by puncturing of the lateral tail vein. Serum was separated by centrifugation (4000 x g, 10 min.) in a EBA-200 centrifuge (Germany) and stored at -20ºC until analysis.

Biochemical parameters as total protein (T-Pro) albumin (Alb), total bilirubin (T-Bil), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine (Crea) were analyzed by automated biochemical analyzer SPOTCHEM EZ SP-4430 (Arkray Inc., Japan). The albumin/globulin (Alb/Glo) ratio was calculated from measured albumin and calculated globulin (Glo) by formula: Glo = T-Pro - Alb (Jolles et al., 2014).

The statistical analysis of the results was carried out using the SPSS (license No. 9900457; version 15, SPSS Inc., Chicago, IL). The data are presented as mean±SEM (standard error of the mean). Differences in gut microbiological and serum biochemical parameters among groups were determined by using *Student's* paired *t* test. The level of statistical significance was set at $P<0.05$.

Results

The data of rats' gut microbiological parameters is presented in Table 1. The total count of aerobic and facultative anaerobic bacteria and *Lactobacillus* spp. were higher by 0.11 $\log_{10}$CFU/g and 0.12 $\log_{10}$CFU/g, respectively, in gut of *Wistar* rats received diet with 19.91 % crude protein (group I) compare with rats, which diet contained 21.50 % crude protein (group II). While the counts of Enterobacteriaceae and faecal streptococci were higher by 0.02 $\log_{10}$CFU/g and 0.04 $\log_{10}$CFU/g, respectively, in group II compare with group I. But the

differences between the groups were not statistically significant (P>0.05).

The data presented in Table 2 demonstrate the elevated values of T-Pro, Glo and T-Bil by 1.35 (P<0.0001), 1.9 (P<0.0001) and 1.33 (P<0.01) times, respectively, without significant change of Alb value (P>0.05) in blood of rats fed with higher content of crude protein (group II) compare with group I. However the higher activities of AST and ALT by 18.76 % (P<0.05) and 59.48 % (P<0.0001), respectively, as well as higher value of Crea by 1.45 times (P<0.0001) were found in blood of rats fed with lower content of crude protein (group I) compare with group II.

Table **1. Gut microbiota of rats, fed with commercial pelleted diet containing different crude protein content**

Parameter	Group I		Group II		P-value
	Mean ± SEM	95% CI	Mean ± SEM	95% CI	
Total count aerobic and facultative anaerobic bacteria, log_{10} CFU/g	6.08±2.15	5.52-6.63	5.97±0.17	5.51-6.43	0.7015
Lactobacillus spp., log_{10} CFU/g	4.81±0.22	4.26-5.37	4.69±0.17	4.25-5.14	0.6706
Enterobacteriaceae, log_{10} CFU/g	4.21±0.17	3.77-4.65	4.23±0.21	3.67-4.78	0.8697
Fecal streptococci, log_{10} CFU/g	4.63±0.19	4.14-5.11	4.67±0.13	4.35-4.99	0.9527
SEM – standard error of the mean; CFU – colony forming units; CI – confidence interval					

Table 2. **Biochemical blood parameters of rats, fed with commercial pelleted diet containing different crude protein content**

Parameter	Group I		Group II		P-value
	Mean ± SEM	95% CI	Mean ± SEM	95% CI	
T-Pro, g/L	55.90±1.29	52.97-58.83	75.20±1.55	71.70-78.70	<0.0001
Alb, g/L	36.00±0.73	34.35-37.65	37.30±1.37	34.21-40.39	0.4125
Glo, g/L	19.90±1.10	17.41-22.39	37.90±2.55	32.14-43.66	<0.0001
Alb/Glo	1.85±0.10	1.63-2.08	1.04±0.10	0.81-1.27	<0.0001
T-Bil, µmol/L	5.70±0.26	5.11-6.29	7.60±0.58	6.29-8.92	0.0080
AST, IU/L	187.60±11.31	162.01-213.19	152.40±8.81	132.46-172.34	0.0245
ALT, IU/L	69.10±3.39	61.4-76.80	28.00±3.25	20.65-35.35	<0.0001
Crea, µmol/L	137.40±4.83	126.46-148.34	94.80±4.85	83.83-105.77	<0.0001
SEM – standard error of the mean; CI – confidence interval; T-Pro – total protein; Alb – albumin; Glo – globulin; Alb/Glo – albumin/globulin ratio; T-Bil – total bilirubin; AST– aspartate aminotransferase; ALT – alanine aminotransferase; Crea – creatinine					

Discussion

Humans and other mammals are colonized by trillions of microorganisms, most of which reside in the gastrointestinal tract, that provide key metabolic capabilities (Gootenberg, Turnbaugh, 2011). The gut microbiota is strongly associated with the well-being of the host (Buhnik-Rosenblau et al., 2011). The vast majority of microbial species (commensals) give rise to symbiotic host-bacterial interactions that are fundamental for human health (Goulet, 2015). Age affects both the composition and function of the gut microbiome *(Lactobacillaceae, Desulfovibrionaceae, Prevotellaceae*, *Enterobacteriaceae)* in mice (Langille et al., 2014).

A balanced diet plays significant role in normal growth and development of juvenile rats, also is essential for the good health of adults (Zemunik et al., 2000). Rats are coprophagic and ingest large number of bacteria along the whole gastrointestinal tract. In the large intestine contains a complex bacteria flora, with the principal groups being the Enterobacteria, enterococci, lactobacilli, bacteroides species, bifidobacteria. A major factor driving the composition and metabolism of the colonic microbiota is diet. The amount, type and balance of the main dietary macronutrients have a great impact on the large intestinal microbiota (Sckott et al., 2013). In our experiment neither 19.91 % of crude protein content in diet of rats, nor 21.50 % of protein did not demonstrate significant changes in the composition of gut microbiota. Our results correspond to the findings of Kiilerich et al. (2016), which revealed significant changes in the composition of the gut microbiota in relation to dietary fat content, but not the protein/sucrose ratio.

Blood protein fractions can change with factors such as age, nutritional status, stress and disease state (Zaias et al., 2009). In our experiment, when rats consumed diet with higher content of protein (21.50 %), it was determined increased concentrations of T-Prot by 1.5 times, Alb by 55.70 % in blood to compare with the literature data. Ujah and others researchers (2013) noted that T-Prot was 49.78±0.21 g/l, Alb – 25.68±1.17 g/l and T-Bil – 21.73±0.76 g/l in blood of rats. Moreover, Aparicio et al. (2013) revealed, that high-protein diet appeared no effect on the concentrations of T-Pro and Alb.

It was noticed, that very high protein intake can lead hepatomegaly and nephromegaly (Jean et al., 2001). Liver and kidneys play a central role in protein metabolism (Ambuhl, 2011; Charlton, 1996). Scientists suggest that

rats fed a moderately high protein diet successfully might adapt to the dietary protein concentration. The specific adaptive processes are involved in the response to variations in the protein content of the diet (Jean et al., 2001). Adaptation to a high protein diet correlated with an increase in the activity of enzymes involved in protein digestion as well as the higher activity of ALT in the liver of rats fed high-protein diet, whereas AST activity was not affected by diet (Erickson et al., 1995; Johnson et al., 2001). Our results clearly indicated statistically significant increase in the activities of enzymes involved in amino acid metabolism, including AST and ALT by 1,2 and 2,47 times, respectively, when rats received diet with 19.91 % crude protein. In contrary, Oarada et al. (2012) found, that refeeding with a 50 % casein diet after 48 h of fasting led to a rapid and abnormal elevation in serum ALT and AST activities in mice. In our study an activity of AST in blood of rats was higher three times compare to Alabi et al. (2017), but our results are in line with Patel and others investigators (2014). ALT results of rats fed diet with 21.50 % crude protein was similar to Alabi et al. (2017), but results of rats received diet with 19.91 % crude protein was similar to that found Patel and others researchers (2014).

Processes regulated by the kidneys are directly affected by dietary protein intake (Ambuhl, 2011). We noticed that concentration of creatinine, which is indicator of kidney damage, was higher by 31 % in rats fed diet with 19.91 % crude protein. However, some authors affirm that the link between protein-induced renal hypertrophy or hyperfiltration and the initiation of renal disease in healthy individuals has not been clearly demonstrated (Calvez et al., 2012).

Conclusion. Our results showed that feeding of rats with diet containing higher content of crude protein from weaning to the ten months age might have strong effect on some biochemical parameters of blood. But it seems, the difference between crude protein content in diet has no reliably effect on the gut microbiota of adult rats. For a good scientific research with rodents is essential that rats would be high genetic quality, have good nutrition status and health, otherwise any work with animals or their organs could give unreliable results. This paper summarizes the currently very few studies addressing to the effects of commercial different diet on gut microbiota, and blood biochemical changes of rats. These results might be significant for further experiments when rats are used as animal biomodel for veterinary medicine and biomedical research.

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