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Effects of standard diets from different sources on growth and some organ parameters of rats

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ABSTRACT. This study aims to determine the effects of open and closed formulated standard diets supplied from different sources on growth performance and internal organ development of laboratory rats. Five-week-old 32 Wistar rats were used. A special control group diet was produced in accordance with the criteria determined by the National Research Council (NRC) (1995). Three different most preferred commercial open and closed-formula diets produced by international and local companies were used as trial groups' diets. The experiment was carried out for 12 weeks. Weekly feed consumption, body weight change, internal organ weight, intestinal organ weights and lengths, intestinal villi height and crypt depth were measured in groups. The body weight values of the control group and the first group fed with open-formula diet were found at the highest level ($P < 0.05$). The control group diet had a positive effect on small intestine villi height and crypt depth ($P < 0.05$). The nutrient contents and energy values of the diets of experimental groups were determined as different from the commercial firm notifications. As a result of the research, it is concluded that the diets prepared with open-formula give more reliable results in the growth performance and development of internal organs of Wistar rats.

Keywords: Diet, growth performance, internal organ, open formula, wistar.

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INTRODUCTION

The number of studies using live animal is increasing day by day in order to get the most realistic results in many areas, especially in health and science (Genc, 2017). Alive animal model is the best model that can best reveal the cell, tissue, organ and system integrity. However, the fact that alive animal model is accepted as an appropriate model depends on the health status of the animals. The selection of healthy animals and the monitoring of their health status during the research are also dependent on many external factors and one of them is nutrition (Faith and Hessler, 2006). Inappropriate changes in feeding dynamics of laboratory animals cause physiological and behaviorally rapid and evident reactions, and may also prevent the healthy conduct of research. Thus, the result of the experiments may also be affected by this situation. That is why, alive animals used in research are subject to internationally accepted feeding procedures and fed (Barnard et al., 2009) with the well known and appropriate diets.

Commercial diets are collected under three main headings based on the principle of nutrition of laboratory animals, production methodology and reporting of nutrient content: open, closed and fixed-formulas. All nutrients and energy values contained in diets with open-formula are presented in numerical form, while other diets do not provide this information (Barnard et al., 2009). Open-formula diet manufacturers can prepare dietary formula to meet different requirements of the researchers' needs. On the other hand, because their content are not indicated barely, other commercial formulations, impose a limitation on the use of diets of these formulations in order not to adversely affect animal health and research outcome. Due to the heat sterilization process that is still widespread in the production of laboratory animal diet, protein levels are kept high during production because of high levels of nutrient loss (Barszcz et al., 2014; Bielohuby et al., 2010; Schaafsma, 2005). Ten to fifteen percent of the energy taken from a normal diet comes from proteins. However, in the case of high-protein diet, this rate increases to 20-30% (Plantenga et al., 2009). High protein with carbohydrates also increases the activity of glucagon-like peptide-1 (GLP-1) which provides insulin release (Lejeune et al., 2006; Plantenga et al., 2009) thus, the feeling of satiety increases. The same effect is explained (Veldhorst et al., 2008; Plantenga et al., 2009) primarily by the increase in oxygen consumption and body temperature, formation of lack of oxygen, and consequently in the feeling of saturation. As

the type and amount of nutrient contents of diets to be used in the study may affect the metabolism, developmental levels, hormonal patterns and behaviors of animals; it is important that, these parameters are known by the researchers in advance and can be changed.

In some countries, pellet feed production mostly address the ruminant and poultry sector. Due to commercial and economic production practices, production of at least 1-2 tons per factory in each batch can not meet the need for different types of research and dietary requirements of 20-25 kg in laboratory animal trials (Genc, 2017). In such a case, the researchers are making improper manipulations in diets with "standard" characteristics to form different dietary groups. The variety of feed materials used in the production of laboratory animal diets may create differences in nutrient composition. Even if the nutrient values of the diets are provided at an appropriate level, the differences in macro and micro nutrient levels may affect the animal health and performance (Andreoli et al., 2016; Nagano et al., 2016; Ronis et al., 2016; Genç, 2017). The diets produced with closed-formula -those are not reporting of micro-nutrients and raw materials or not conforming to the standards- appear to be a greater risk to influence the result of the research. Yet, there are studies reporting that micro-nutrients may have an effect on the fetus during DNA methylation stage, especially with inadequate or unbalanced intake in the fetal period (Mc Kay et al., 2012; Vanhees et al., 2014).

The aim of this study was to determine the nutritional profile and nutritional effects of standard mice and rat diets produced with open and closed formula obtained from three different sources in an *in vivo* trial.

MATERIALS AND METHODS

All procedures that were used on animals in this research had been approved by Ondokuz Mayıs University Animal Experimentation Ethics Committee with Ethics committee acceptance number: 2017-40.

Data on the number of animals used to form the research groups is given in Table 1. In this study, 5-week-old 32 Wistar rats consuming group feeds with their mothers since the birth to the end of weaning period (0-5 weeks) were used.

After the weaning (5th week), 4 male and 4 female rats were selected randomly from each mother rat and feeding was continued with 1 control and 3 experimental groups with 8 animals per group. Mothers and other offspring were excluded from the study after 5th

week. The study was carried out until the rats reached to the 12 weeks old by taking into consideration the age and body weight of adult rats. Animals of both sexes were used to eliminate the gender factor. After the formation of the groups, male and female animals were fed their own group diets in different cages.

Before the study, each mother was fed with group feed for 10 days in advance. Number of mother (n), litter (n) and litter size (n), female and male rat ratio (n/n) of the groups between 0-5th and 6th-12th weeks of study and G-power analysis values prior to forming groups were given in Table 1. The number of animals used was determined with the G-Power test by reference to a previously conducted similar study (Idoko et al., 2015).

Diets in all groups were preferred to identify diets produced as standard mice and rat feeds. The control group has an open-formula and was prepared by a commercial company in accordance with the characteristics reported in the NRC (1995). The control group diet ration contains soybean meal, bonkalite (wheat), corn, rice bran, calcium carbonate, molasses, soybean oil, dicalcium phosphate, salt (NaCl), vitamin-mineral premix, probiotic (*Saccharomyces cerevisiae*), and mycotoxin binder. The diet of the first experimental group was formed by a globally preferred open-formula commercial diet consist of wheat, dehulled extracted toasted soya, wheatfeed, barley, dehulled cooked soya, soya oil, calcium carbonate, dicalcium phosphate, salt (NaCl), and vitamin-mineral premix supplement. The diet of the 2nd group was formed with a closed-formula based on cereals, oilseed residues, bonkalit, calcium carbonate, saccharose, alfa alfa meal, pellet binder, choline, salt, antioxidant and vitamin-mineral premix and sold by an international company. The 3rd group's diet is a closed-formula product of a company that sells in the local market and based on soybean meal, wheat, corn and vitamin-mineral premix. The feedstuffs of the experimental groups were given as declared by the companies. The nutrient contents of all diets are given in Table 2.

The animals were born in Ondokuz Mayıs University Experimental Animal Research and Application Center and were housed in standard plexiglass cages at 20 - 23 °C heat, 50-60% humidity and 12 hours light and 12 hours dark conditions. Wood chips were used as bedding material in all groups during the study. Throughout the research, water and diet were provided as *ad-libitum*.

Nutrient analysis

The nutrient analysis of the control and experimental groups' diets used in the study was carried out at the Ondokuz Mayıs University Faculty of Veterinary Medicine, Department of Animal Nutrition and Nutritional Diseases Laboratories. All groups consumed dry matter (DM), nutrient levels (crude ash (CA), crude protein (CP), crude cellulose CC)) determined according to Weende (AOAC 2012), while the ether extract (EE) level was determined by Soxhlet extraction device according to the method reported by Keskin (1975). Metabolic energy (ME) value was determined according to the formula reported by Yalçın (2011).

$$ME, MJ/kg DM = (0,01465 \times \text{crude protein}) + (0,03558 \times \text{ether extract}) + (0,01465 \times \text{Nitrogen-free substance})$$

The nutrient analysis values were compared with the companies' own statements (Table 2).

Evaluation of performance and internal organ parameters

Weekly measures of individual body weights of animals were performed with A&D Weighing GF 3000 Balance (0.01g readability). The daily intakes of the group's diet were carried out by group weighing weekly. Body weight gain and feed consumption were calculated from the difference of weight value from the previous week. In the postmortem period, the weights of the internal organs (heart, liver, spleen, stomach and intestines) and the length of the digestive tract sections were evaluated with A&D Weighing GF 3000 Balance (0.01g readability) and tape measure respectively.

Histopathological examination

At the end of the study, all rats were euthanized with appropriate anesthetic (Ketamine 150 mg/kg + Xylazine 30 mg/kg body weight) and intestinal organs were taken in accordance with proper asepsis and antiseptic rules. The intestines taken under appropriate conditions were fixed for 12-24 hours in the 10% formaldehyde solution and were passed through the routine histological tissue follow-up procedures and embedded in paraffin. Section in 5µ thickness taken from organs which were dried in drying oven and Crossmon's (1937) tripple staining method was applied in order to see the normal histological structures of the intestines. Height of intestinal villi and depth of crypts were measured. Preparations were photographed with The Nikon digital-sight imaging system and the Nikon 50I research microscope.

Table 1. Number of mother (n), litter (n) and litter size (n), female and male rat ratio (n/n) of the groups between 0-5th and 6th-12th weeks of study and g-power analysis values prior to forming groups.

| 0-5 weeks | | | | 6-12 weeks |
|----------------|--------|-------------|-------------|-------------|
| Mother (Group) | Litter | Litter size | Female/Male | Female/Male |
| Control | 1 | 9 | 5/4 | 4/4 |
| 1 | 1 | 11 | 6/5 | 4/4 |
| 2 | 1 | 12 | 4/8 | 4/4 |
| 3 | 1 | 9 | 4/5 | 4/4 |

g-power: $\alpha=0.05$; $1-\beta= \%80$; $n^0=8$ (Estimation of approximately unit number in study).

Table 2. Dry matter (DM) (%), crude ash (CA) (%), crude protein (CP) (%), ether extract (EE) (%), crude cellulose (CC) (%), and metabolic energy (kcal/kg ME) values of diets.

| Diet | DM | | CA | | CP | | EE | | CC | | ME | |
|---------|------|------|-----|-----|------|------|-----|-----|-----|------|------|------|
| | A | B | A | B | A | B | A | B | A | B | A | B |
| Control | 92.0 | 92.0 | 8.0 | 8.0 | 24.0 | 24.0 | 3.0 | 3.0 | 4.0 | 4.0 | 2920 | 2920 |
| 1 | 92.0 | 93.0 | 7.0 | 6.9 | 20.0 | 19.1 | 2.0 | 4.7 | 4.0 | 3.8 | 2906 | 2921 |
| 2 | 92.0 | 92.5 | 6.0 | 6.1 | 20.0 | 19.2 | 4.0 | 4.1 | 7.0 | 6.1 | 2937 | 3226 |
| 3 | 92.0 | 93.0 | 7.0 | 7.2 | 19.0 | 24.0 | 2.0 | 4.7 | 6.0 | 13.0 | 2830 | 2600 |

A: Nutrient values reported by the diet companies. B: Nutrient values found in the feed analysis.

Statistical Analysis

In this study, the conformity of the data to normal distribution was determined by Kolmogorov Smirnov test. After the weaning of the control and experimental groups, the body weight values from 6 to 12 weeks old age, feed consumption, weight and length of some organs and the histological values of organs were analyzed by using variance analysis. TUKEY HSD test was used for the importance control of the difference between the groups.

RESULTS

Nutrient analysis and Metabolic Energy values

The nutrient contents of the diets and the results of the analysis of energy values (A) are given in Table 2 and compared with the declaration of the companies (B). The percentage values of nutrients and ME values reported by the companies in the diets of the research groups were found different from the results

of the analyzes. The difference in CP value was acceptable in the 1st and 2nd trial groups, but was high in the 3rd trial group. Ether extract percentages were found higher than the values reported in all experimental groups. In the 1st and 2nd trial groups, it was found that the values of CC were close to the reported values but in the 3rd trial group, the values reported were more than doubled. Metabolic energy values were found close to those reported in the first group diet with an open formula, higher than those reported in the second group diet and much lower than those reported in the third group.

Performance Parameters

The weekly amount of feed consumption after weaning (6th-12th weeks) is given in Table 3. The highest feed consumption was observed in the animals fed with the 3rd group diet and the lowest feed consumption was observed in the animals fed with the 2nd group diet.

Table 3. Weekly feed consumption (FC) (g) values of groups (n = 8) from 6th to 12th week.

| Week | Groups | | | |
|------|---------|---------|---------|---------|
| | Control | Group 1 | Group 2 | Group 3 |
| 6 | 1195 | 1115 | 980 | 865 |
| 7 | 1256 | 1195 | 1007 | 901 |
| 8 | 1300 | 1213 | 1098 | 1265 |
| 9 | 1348 | 1299 | 1176 | 1365 |
| 10 | 1400 | 1365 | 1256 | 1456 |
| 11 | 1489 | 1402 | 1398 | 1501 |
| 12 | 1545 | 1490 | 1450 | 1605 |

Table 4. The weekly body weight (BW) values of the control (n=8) and experimental groups (n=8) from 6th to 12th week (Mean \pm SE).

| Week | Groups | | | | P value |
|------|--------------------|--------------------|-------------------|--------------------|---------|
| | Control | Group 1 | Group 2 | Group 3 | |
| 6 | 120.87 \pm 3.22 | 95.12 \pm 9.61 | 97.87 \pm 2.83 | 92.25 \pm 4.19 | 0.005 |
| 7 | 150.87 \pm 8.37 | 114.12 \pm 11.07 | 120.12 \pm 3.07 | 107.75 \pm 4.67 | 0.002 |
| 8 | 179.12 \pm 12.39 | 133.50 \pm 16.06 | 133.00 \pm 3.04 | 117.37 \pm 5.80 | 0.002 |
| 9 | 195.87 \pm 14.94 | 159.62 \pm 16.00 | 136.25 \pm 3.73 | 129.25 \pm 8.73 | 0.002 |
| 10 | 207.00 \pm 16.13 | 180.50 \pm 20.94 | 142.62 \pm 5.29 | 145.75 \pm 9.50 | 0.10 |
| 11 | 217.37 \pm 17.89 | 204.75 \pm 23.69 | 150.00 \pm 9.77 | 165.12 \pm 10.49 | 0.022 |
| 12 | 230.12 \pm 18.88 | 228.75 \pm 26.49 | 168.75 \pm 9.16 | 183.00 \pm 14.43 | 0.50 |

Table 5. Internal organ weight (g) and length (cm) values (Mean \pm SE) in groups (n=8)

| Internal organs | Groups' Weight (g) | | | | P value |
|-----------------|--------------------|-------------------|-------------------|-------------------|---------|
| | Control | Group 1 | Group 2 | Group 3 | |
| Heart | 0.78 \pm 0.05 | 0.84 \pm 0.04 | 0.66 \pm 0.03 | 0.69 \pm 0.02 | 0.016 |
| Liver | 10.38 \pm 1.01 | 9.97 \pm 0.82 | 7.74 \pm 0.46 | 7.50 \pm 0.44 | 0.014 |
| Lien | 0.58 \pm 0.03 | 0.59 \pm 0.04 | 0.43 \pm 0.02 | 0.44 \pm 0.02 | 0.002 |
| Stomach | 1.30 \pm 0.04 | 1.47 \pm 0.08 | 1.21 \pm 0.05 | 1.41 \pm 0.05 | 0.026 |
| Small intestine | 8.72 \pm 0.33 | 7.63 \pm 0.13 | 7.56 \pm 0.14 | 7.06 \pm 0.32 | 0.001 |
| Large intestine | 3.03 \pm 0.14 | 2.66 \pm 0.12 | 2.92 \pm 0.10 | 2.96 \pm 0.13 | 0.230 |
| | Length (cm) | | | | P value |
| | Control | Group 1 | Group 2 | Group 3 | |
| Small intestine | 105.93 \pm 1.83 | 107.00 \pm 2.91 | 100.27 \pm 1.97 | 100.75 \pm 1.99 | 0.085 |
| Large intestine | 20.31 \pm 0.74 | 20.62 \pm 0.95 | 20.62 \pm 0.99 | 21.37 \pm 0.30 | 0.812 |

Table 6. Small intestine crypts (SC) depth (μ m), small intestine villi (SV) height (μ m) and large intestine crypt (LC) depth (μ m) values (Mean \pm SE) of the groups (n = 8).

| Crypt & villus | Groups | | | | P value |
|----------------|--------------------|--------------------|--------------------|--------------------|---------|
| | Control | Group 1 | Group 2 | Group 3 | |
| SC | 84.11 \pm 2.79 | 69.70 \pm 4.46 | 78.66 \pm 1.56 | 81.85 \pm 4.13 | 0.032 |
| SV | 634.29 \pm 48.80 | 434.08 \pm 31.73 | 350.95 \pm 26.60 | 478.36 \pm 12.81 | 0.000 |
| LC | 154.42 \pm 12.32 | 138.85 \pm 5.95 | 164.31 \pm 13.74 | 170.55 \pm 6.39 | 0.164 |

The weekly body weight changes of the groups after the weaning are given in Table 4. At the end of the experiment, it was observed that the body weight values were significant ($P < 0.05$) at the 6th, 7th, 8th, 9th and 11th weeks, and the difference between the control group and the 2nd group at the 12th week was statistically significant ($P < 0.05$). In groups, hierarchy fights and related stress symptoms were observed at the 8th and the 10th weeks. However, no serious injuries and deaths were observed in the groups.

Internal organ weight and length values are given in Table 5. Heart, liver, spleen, stomach and small intestine weights were significantly different between groups ($P < 0.05$), while large intestine weight and length of both intestines did not make a significant difference between groups ($P > 0.05$).

Histopathological Findings

At the end of the experiment, the small intestine crypt (SC), large intestine crypt (LC) depths and small intestine villi (SV) height of the groups were measured and given in Table 6. Images of intestinal villi and crypts of control and experimental groups given in Figure 1. Small intestine villi height and crypt depth were found significant ($P < 0.05$) and among the groups, the highest villus height and crypt depth were found in the control group. Crypts lengths of the large intestine did not make a significant difference between the groups ($P > 0.05$). No pathological finding was observed in the internal organs and intestines in any group.

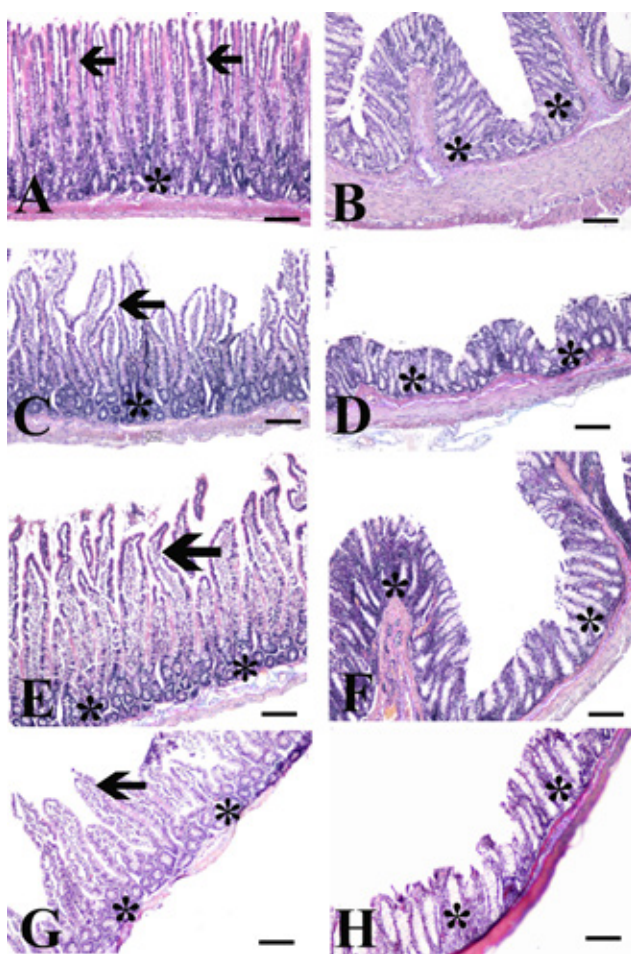


Figure 1. Images of intestinal villi and crypts of control and experimental groups.

A: Control group small intestine; B: Control group large intestine; C: 1. group small intestine; D: 1. group large intestine; E: 2. group small intestine; F: 2. group large intestine; G: 3. group small intestine; H: 3. group large intestine, Crossman's Trichrome Stain. *: crypt, arrow: Villus intestinalis, Bar: 100µm.

DISCUSSION

The standard mouse-rat diets, obtained from three commercial firms, have different levels of nutrient content (Table 2). The use of different raw materials in the diets as a source of energy and protein naturally give rise to such a result. In addition, the given percentages of nutrient contents and metabolic energy values of the research diets by the companies were found different from our research analyzes. This finding is in agreement with the results obtained from Barnard et al. (2009)'s research, in which different standard diets were formed using different raw materials. If the rations where natural raw materials are not purified, the plants in the same harvest period have significant potential ($P<0.05$) in nutrient values in different batches and have the potential to affect the physiological parameters of animals (Thigpen et al.,

2004; Thigpen et al., 2007). Although the crude protein content in research diets is within the legal limits according to NRC (1995), the fact that the diets characteristics stability should be considered in the studies to ensure the repeatability of the study.

During the period between 6th and 12th weeks when individual weighing was made, feed loss was higher due to the natural feeding behavior observed in rodents. Due to the continuous extension of the incissive teeth of rodents, their diets are made of hard feeds in the form of pellets and are important in terms of dental health. However, in the conventional feeding method, feed residues are produced which are not consumed during the gnawing of pellet feed and these residues are taken together with the litter and feed consumption among the groups does not show homogeneity. For these reasons, feed consumption value is not a sufficient parameter in the evaluation of nutritional performance of rodent laboratory animals. Thus, feed consumption rate values were not evaluated in this study.

The body weight gain was at the highest level in the control group for each week, but only the 10th week was statistically insignificant ($P>0.05$). The difference on body weight gain between the control and the 2nd group at the 12th week was statistically significant ($P<0.05$). The animals in the 1st group fed with the open-formula diet showed that the body weight increase was mathematically closer to the control group than the other groups. Differences in performance values may be caused by unspecified raw material sources in the diets of the manufacturing company with closed-formula or lack of macro-micro nutrients. In a study, Pichon et al. (2008) investigated the effects of different protein sources on dietary consumption and body weight gain. It was shown that body weight gain and dietary consumption were significantly ($P<0.05$) lower in rats fed with a diet containing beta lactoglobulin than those fed with dietary alpha lactalbumin. Faipoux et al. (2008) showed that protein-related saturation sensation is provided with vagal feedback. Because of the similarity (Plantenga et al., 2009) of saturation between the groups in case of dietary nutrition with a high level protein or amino acid source, the effect of other parameters in the diet may be covered. Bowen et al. (2006a; 2006b) in their research on the effects of high-carbohydrate diets showed that there was no difference in dietary consumption between the groups fed with high casein and wheat protein. Yürük (2014) pointed out that, the metabolism of active substances used in research should be well known and reported that, feed consumption of animals fed with

diets using fructose as a source of energy is similar to rats fed with other standard diets.

It is reported (Garlick et al., 1991; Tome, 2004) that, the rats can distinguish between the taste of some essential amino acids and their dietary consumption varies depending on the presence of these amino acids. The presence of such a detection mechanism also emphasizes the physiological importance of providing a sufficient amount of protein synthesis (Plantenga et al., 2009) and its effect on feed consumption.

Diets should be fed to animals after being analyzed and reported for antinutritional substance contents. Most of the diets with closed-formula have no antinutritional substance report. The phytoestrogen levels, which are one of these substances, may differ between the raw material of the plant during the same harvest period. Phytoestrogens can be effective on feed and water consumption, anxiety behaviors, stress, insulin, leptin and thyroid hormone levels. (Lephart et al., 2004; Torre et al., 2008).

The effects of protein and fat imbalance on growth performance can also be affected by gender factor. It has been reported in a study (Bellinger et al., 2005) that low protein intake causes less fat consumption in female offspring but no such effect has been observed in males. Krishnakumari et al. (1979) reported that the rats consuming the diets prepared by using different raw materials consume more delicious ingredients. It is reported (Kasaoka et al., 2004) that high levels of histidine in diets may reduce dietary consumption.

Studies on the effects of standard diets on internal organ weights, lengths and development of intestinal villi and crypt are very limited. In our study, it was observed that the liver and small intestine weights were highest in the control group ($P<0.05$), and the heart, spleen and stomach weights were highest in the 1st group ($P<0.05$). According to Bailey et al. (2004) liver weight value is the most proportional among the internal organs to the body weight value. Findings in our study are in compliance with this information that the highest liver weight is in the control group with the highest body weight value. Small intestines in digestive system are the highest ($P<0.05$) in control group. These results may be explained by the fact that they consume the control diet, which is prepared under control due to the right and balanced requirement of nutrients. Belobrajdic et al. (2014) reported that there was no significant effect on internal organ weights ($P>0.05$) in rats consuming open-formula diets containing different protein sources. The different results and notifications between experimental studies can be

caused by the fact that animals are of different strains and genders. Physiological and anatomical individual differences in outbred strains such as Wistar are quite common. Therefore, it can be said that inbred strains are more suitable for performance studies.

In our study, the small intestine crypt depth and villus height were found the highest ($P<0.05$) in the group fed with the open-formula control group diet contains 24% CP content. This data is in agreement with the declaration of Pelizzon (2016) on the effects of open-formula diets on the morphology of the intestinal system in rats and mice. In a study (Syme, 1982) performed with rats consuming isoenergetic rations, especially after 4th week, higher protein level effected the intestinal structure, villus height and crypt depth improvement better. Same results reported by King et al. (1983) in a study performed by rats consumed diet contains different levels of aminopeptidase and isomaltase. The effects of dietary crude protein levels on villus height and crypt depth values are explained by the increase in enterocyte activity due to the density of amino acids. (Syme, 1982; King et al. 1983).

Although intestine villi height and crypt depth values showed statistically significant ($P<0.05$) differences, there was no significant difference ($P>0.05$) between intestinal lengths. These parameters can be evaluated with a longer research period.

CONCLUSION

As a result, it was concluded that open formula diets had a positive effect on body weight gain, small intestine villi height, crypt depth and internal organ weight in rats during the developmental period compared to the diets produced with closed formula. Based on the data obtained, it may be suggested that the laboratory animal diets to be used in the research should be selected from the open formula products whose nutrient content is specified. Although open formula diets are more costly, it can be said that, they are more advantageous than closed formula diets because they give more positive results on animal development. In addition, it may be recommended to conduct a longer term research using other inbred species.

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CONFLICT OF INTEREST

None declared.

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