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ADAPTATION OF BIOSTATISTICAL METHODS FOR CROSS-SECTIONAL OR LONGITUDINAL EXPERIMENTAL PROTOCOLS

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Abstract

In recent years, biostatistics has emerged as an indispensable tool in biomedical research, playing a very important role in the design, analysis, and interpretation of data from biological and medical studies. Biostatistics encompasses a diverse range of statistical methodologies adapted to address the unique challenges presented by complex biological systems.

This paper aims to explore and contribute to the further development of biostatistics by deepening into the difference between statistical analysis tests.

The general purpose of this paper is to compare different tests for the analysis of the differences between the means of the control group and the experimental groups, in order to identify the optimal biostatistical test, correlated with the study and typology of the collected data.

To perform these statistical tests and subsequently compare them, data were collected from a total of 60 BALB/c mice divided into 3 groups with equal numbers of individuals (one control group and two experimental groups) in whose food was introduced monosodium glutamate at different concentrations.

Key words: biostatistics, longitudinal and cross-section protocol, monosodium glutamate

INTRODUCTION

In recent decades, biostatistics has emerged as an important interdisciplinary field within biomedical research, providing essential tools for the design, analysis, and interpretation of data from biological and medical studies.

Statistics, by its object and method, is part of the sciences that study the quantitative aspects of phenomena and processes within nature, technology and society (Spinei Larisa, 2009).

Biostatistics can be defined as consisting of probabilistic methods used for the collection, description, analysis, interpretation and presentation of medical or biological data in general (Boiculese G., 2007).

This field holds a broad array of statistical methodologies tailored to address the specific challenges posed by complex biological systems.

Within scientific research, there are several classifications of the studies carried out, according to certain criteria: temporal, the characteristics of the methods used, the purpose of the research, the reactivity of the criteria and the place and role played in the experiment.

Within the temporary criterion, we have:

a) transversal research, which establishes the links and variables of a phenomenon at a certain moment in time;

b) horizontal research: the same experiment is performed, but on different samples, at different temporal moments (example: the same experiment in successive years);

c) longitudinal studies: allow observation and data collection in an experiment that takes place over a longer period of time, and the same subjects are used in the experiment.

In conducting any experiment, regardless of the safety measures taken to control the environment and other factors, the results obtained will have some degree of variability.

The measured values may vary depending on one or a multitude of parameters for the phenomenon under study.

All experimental data derived from measurements or methods of gathering such data are generally subject to errors.

According to the cause that produces them, they can be divided into three categories (Oancea Sevilia, 2007):

1. Errors given by measuring devices;
2. Errors due to the experimenter;

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3. Errors due to the method or theoretical model used.

This study seeks to advance biostatistical methodology by examining distinctions among statistical tests used for analyzing data. Specifically, this paper aims to compare different tests for evaluating differences between the means of control and experimental groups, with the goal of identifying the most suitable biostatistical test based on study design and data typology.

The Student's test (also called the T-test) is used to compare the means of a quantitative, measurable characteristic between two groups, and there is no need for multiple comparisons because P is unique, the Student's test highlighting the significance of the difference (Prabhaker Mishra et al., 2019).

The ANOVA test (analysis of variance) was introduced by the statistician R.A. Fisher and is a generalization of Student's t test. Given the fact that some processes can be influenced by several factors that can act at the same time, in order to highlight the extent to which they act on a studied characteristic, one resorts to the use of dispersion analysis (variance analysis) (Daniel Wayne W., 1995).

To perform these statistical tests and subsequently compare them, data were collected from a total of 60 BALB/c mice divided into 3 groups with equal numbers of individuals (one control group and two experimental groups).

MATERIAL AND METHOD

The experimental research was carried out within the bio facility of the Regional Advanced Research Center for Emerging Diseases, Zoonoses and Food Safety – ROVETEMERG from ”Ion Ionescu de la Brad” Iași University of Life Sciences (*figure 1*).

Figure 1 **ROVETEMERG Research Center - IULS**

The housing, breeding and use of the mice were done in accordance with the European legal norms transposed into the Romanian legislation (Directive 2010/63/EU, Law no. 205/2004, Law no. 43/2014) regarding the assurance of living conditions and well-being as well as the protection of animals used for scientifically purposes.

In this experiment, the animals used came from the Muridae family, genus Mus musculus (mouse), namely 60 BALB/c strain mice, certified animals that were purchased from the "Cantacuzino" National Institute of Medical and Military Research and Development in Bucharest.

The cages in which the animals were kept are made of polycarbonate, type 1284L EUROSTANDARD TYPE II L, with dimensions of 365 x 207 x 140 mm, having a useful surface of 530 cm2, and an occupancy rate of 4 animals per cage.

The animals were kept at constant temperature 22 + 2°C, relative humidity (55 + 5%) and day-night cycles of 12 hours light (07:30 to 19:30) and 12 hours dark (UK Law on Code of practice for the housing and care of animals bred, supplied or used for scientific purposes, 2014 ; Pritchett-Corning R. et al., 2015).

The bedding used in the cages was purchased from authorized suppliers and meets the specific requirements of use for animals used for scientific purposes. Shredded paper and tannin-free softwood sticks were used to enrich the rodents' living environment (RSPCA, 2011).

The bedding and enrichment media were autoclaved at 121 °C for 20 min and changed weekly.

Treated pellets were administered ad libitum in the cage support, and water was at the discretion of the rodents.

All cages used were machine washed using only approved detergents and rinse solutions.

To ensure the quality of living conditions, food and water, all operations performed in the animal room were monitored, recorded and kept in accordance with legal requirements and laboratory standards.

To ensure animal welfare, two daily checks (morning and afternoon) were carried out during normal working hours and a single check at weekends. Thus, the daily checks included: ensuring the ambient temperature and humidity, compliance with the lighting schedule, the level of food and water, but especially the health status of the animals. Temperature and humidity values were collected and stored in the register of each animal room.

Also, each intervention or operation carried out (by the caretaker, veterinarian or researcher) was recorded in the same register by the person concerned, and the final checks were carried out by the bio facility supervisor (Directive 2010/63/EU).

The animals were divided into 3 groups and given monosodium glutamate (E 621) aqueous solution sprayed on the pellets. The 50% lethal dose (LD50) for this compound is 15 grams per kilogram of body weight (Buzescu Anca et al., 2013) or 0.45 grams per animal with an average weight of 30 grams, as follows: the first experimental group Exp1 - in a concentration of

1/20 of the LD50, the second experimental group Exp2 - a concentration of 1/10 of the LD50, and the control group benefited only from normal food.

The rodent diet consisted of compound feed pellets purchased from the same institution as the research animals in the study.

Mono sodium glutamate in crystal form was purchased commercially.

To determine the gravimetric values, the rodents and the administered food were weighed, using a scale equipped with the tare function, with a precision of two decimal places, and a graduated cylinder was used to determine the water consumption.

Each animal in the experiment was weighed at the beginning of the experimental period, then weekly at: 7, 14, 21 and 28 days.

The same weighing and measuring schedule was used for the feed and water administered to the animals.

An electronic caliper was used to determine body measurements and these measurements were taken at 7, 14 and 28 days.

The value of interest was the length measured from the tip of the animal's nose to the base of the tail (Rogers P., et al., 1979; Stephens D.N., 1980) and was used along with weight to determine the LEE obesity index (Chizuko Hioki et al., 2010):

$$\text{Lee index} = \sqrt[3]{\text{weight}}/(\text{body length}) \times 100$$

For the statistical interpretation of the data, the Student test (t-test) and the ANOVA test (variance analysis test) were used, using two programs: Microsoft Excell ® and Graph Pad Prism ® - version 10.2.2.

RESULTS AND DISCUSSIONS

The nutrition of the rodents during the experiment was qualitatively different (the presence of monosodium glutamate), following the evolution of their body weight over a period of 4 weeks, emphasizing the highlighting of some differences in the evolution of the rodents' weight depending on the concentration of mono sodium glutamate applied over the pellets of compound feed administered.

The evolution of body weight is an indicator of the quality of food, the degree of assimilation of nutrients, as well as the state of health. Rodent weight was monitored starting on the first day of the experiment, then weekly for a period of four weeks.

Results regarding the evolution of the cross-sectional study

Weight gain followed a normal upward curve for the control group but also for the other two groups (although with a noticeably higher growth rate, taking into account that the animals used in the present research are in the growth phase (*figure 2*).

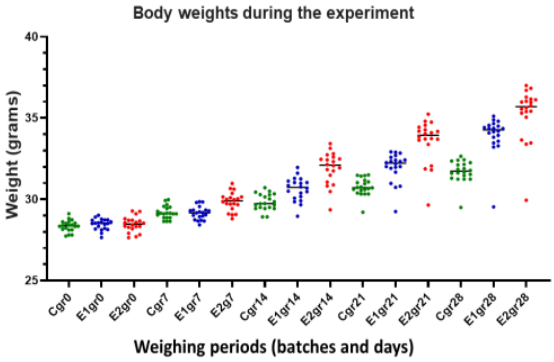

Figure 2 **Body weights throughout the experiment**

The water consumption and the food consumption are presented in water and feed consumption charts (*figure 3* and figure *4)*, for the 3 groups (C, E1 and E2), so it can be observed the increase in consumption with the aging of the mice, but also the increased consumption of the experimental groups compared to that of the control group.

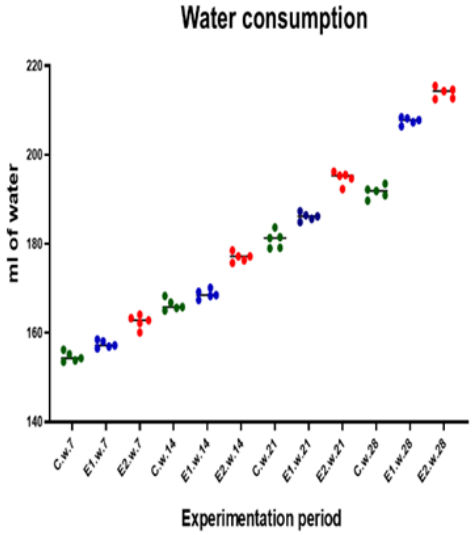

Figure 3 **Water consumption in the experiment**

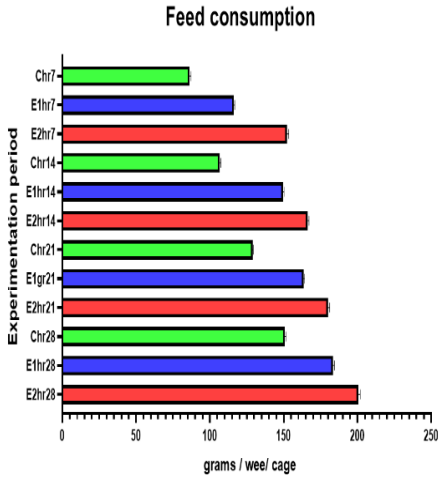

Figure 4 **Feed consumption for the whole period**

Table 1

Experimental values at 7 days

First weighing (7 days)		Control	Exp. E1	Exp. E2
Water consumption (ml)	$\bar{X}$	154.6	154,7	162,5
	$\pm s_{\bar{x}}$	0,4130	0,3736	0,6760
	sd	1,102	0,8355	1,512
	cv%	0,713	0,5307	0,9302
Feed consumption (grams)	$\bar{X}$	86.30	116.2	152.2
	$\pm s_{\bar{x}}$	0.4196	0.2748	0.5600
	sd	0.9383	0.6144	1.252
	cv%	1.087	0.5286	0.8228
body weight (grams)	$\bar{X}$	29.19	29.16	29.86
	$\pm s_{\bar{x}}$	0.08878	0.09104	0.1286
	sd	0.3970	0.4071	0.5752
	cv%	1.360	1.396	1.926
Lenght (cm)	$\bar{X}$	9.861	9.883	9.895
	$\pm s_{\bar{x}}$	0.06195	0.04811	0.05089
	sd	0.2771	0.2151	0.2276
	cv%	2.810	2.177	2.300
Lee index	$\bar{X}$	306.1	305.7	305.3
	$\pm s_{\bar{x}}$	1.932	1.607	1.738
	sd	8.640	7.189	7.773
	cv%	2.823	2.352	2.546

Note: Exp. E1 = Experimental group 1
Exp. E2 = Experimental group 2

As can be seen in table above (*table 1*), at the time of the first weighing (7 days from the beginning of the experiment) the average water consumption was 154.6 ml/week in the control group, 154.7 ml/week in the E1 group and of 162.5 ml / week for batch E2.

By applying the Student test in 1 to 1 combination, very significant differences resulted for all possible combinations ($p<0.001$).

The second method of analysis (ANOVA) revealed significant differences for $p<0.001$ in the overall comparison (C x E1 x E2).

In the case of food consumption, it is observed that the control group had an average amount of food consumed of 86.3 grams while the experimental group E2 had an average consumption of 152.2 grams/week. Both methods of statistical interpretation revealed very significant differences between the groups, with a value of $p<0.001$.

As for body weight, at the end of the first week the experimental group E2 had an average weight of 29.86 grams/animal, the experimental group E1 a weight of 29.16 grams/animal and the control group 29.19 grams/animal, revealing a highly significant statistical difference between groups C x E2 and E1 x E2, but a statistically insignificant difference between groups C x E1 (difference insignificant observed in both Anova and Student's tests).

In the case of body length (naso-caudal), the average between the groups being approximately 9.87 cm, no statistical differences could be established between the groups.

Due to the relatively close appearance of body lengths and weights at the first week, the Lee body development index was statistically insignificant in all combinations in both statistical analysis tests.

Table 2

Experimental values at 28 days

Final weighing (28 days)		Control	Exp. E1	Exp. E2
Water consumption (ml)	$\bar{X}$	191.6	207.6	213.9
	$\pm s\bar{x}$	0.6384	0.3583	0.5748
	sd	1.428	0.8012	1.285
	cv%	0.7449	0.3859	0.6008
Feed consumption (grams)	$\bar{X}$	150.6	183.5	200.4
	$\pm s\bar{x}$	0.4503	0.3885	0.6518
	sd	1.007	0.8687	1.457
	cv%	0.6685	0.4735	0.7272
body weight (grams)	$\bar{X}$	31.72	33.97	35.29
	$\pm s\bar{x}$	0.1528	0.2606	0.3624
	sd	0.6833	1.165	1.621
	cv%	2.154	3.431	4.593
Lenght (cm)	$\bar{X}$	10.67	11.51	12.25
	$\pm s\bar{x}$	0.04649	0.02900	0.03898
	sd	0.2079	0.1297	0.1743
	cv%	1.948	1.127	1.423
Lee index	$\bar{X}$	293.2	278.0	264.0
	$\pm s\bar{x}$	1.478	1.176	1.144
	sd	6.611	5.260	5.116
	cv%	2.255	1.892	1.938

Note: Exp. E1 = Experimental group 1
Exp. E2 = Experimental group 2

In second table (*table 2)*, at the last weighing (28 days) the average water consumption was 191.6 ml/week in group C, compared to 213.9 ml/week for group E2. By applying the T-test and ANOVA all combinations (C x E1, C x E2, E1 x E2 and C x E1 x X2) came out highly significant, including the overall value for the ANOVA test.

In the case of food consumption, the average difference between the control group and the experimental group E2 was approximately 50

grams / week per cage, (from 150.6 grams to 200.4 grams) which indicates a very significant statistical difference for this aspect.

The average body weight at 28 days for the control group was 31.72 grams/animal, in the experimental group E1 33.97 grams/animal and the experimental group E2 35.29 grams/animal.

Following the application of statistical analysis tests, a distinctly significant difference emerged between the experimental groups E1 and E2, with a value of p=0.0053 for the Student test and a value of p = 0.0032 following the ANOVA test, and for the group control and the other experimental groups resulted in highly significant differences with a value of p<0.001.

Due to the aspect of length (10.67 cm/animal in the control group and 12.25 cm/animal in the experimental group E2) and body weights, the Lee's index had a value of 293.2 for the control group, 278 for the experimental group E1 and a value of 264 for the experimental group E2, which reveals a more pronounced body development in the two experimental groups.

Results regarding the evolution of the longitudinal study

In this experiment, in addition to the cross-sectional studies conducted at 7, 14, 21 and 28 days, a longitudinal study from the beginning of the period to 28 days was also considered.

In the longitudinal study, body weight and Lee body development index were considered for all three groups separately: control group C, experimental group E1 and experimental group E2.

Thus, for the control group C the values shown in *Table 3* reveal an increase in the average body weight from 29.19 grams/animal in the first week to 31.72 grams/animal at the end of the fourth week.

Table 3

Longitudinal values for Control group

		7 days	14 days	28 days	Student test		ANOVA	
Weight (grams)	$\bar{X}$	29.19	29.83	31.72	C-7 vs. C-14	<0.01 **	Global	<0.001 ***
	$\pm s_{\bar{x}}$	0.3970	0.1130	0.1528	C-14 vs. C-28	<0.01 ***	C-7 vs. C-14	0.012 **
	sd	0.3970	0.5052	0.6833	C-7 vs. C-28	<0.001 ***	C-14 vs. C-28	<0.001 ***
	cv%	1.360	1.694	2.154			C-7 vs. C-28	<0.001 ***
Lee index	$\bar{X}$	306.1	286.7	293.2	C-7 vs. C-14	<0.001 ***	Global	<0.001 ***
	$\pm s_{\bar{x}}$	1.932	0.5114	1.478	C-14 vs. C-28	0.002 **	C-7 vs. C-14	<0.001 ***
	sd	8.640	2.287	6.611	C-7 vs. C-28	<0.001 ***	C-14 vs. C-28	0.0059 **
	cv%	2,823	0,7978	2.255%			C-7 vs. C-28	<0.001 ***

After performing the Student statistical interpretation tests and ANOVA, for the combination pair C at 7 days x C 14 days a distinctly significant difference emerged, with a more accurate value of the ANOVA test of p=0.012, compared to the T test with the value of p=0.01.

In the case of the value of the Lee index, very significant differences emerged for both tests, for all combinations (C 7 days x C 14 days x C 28 days) but for the combination C at 14 days x C at 28 days in the Student test the value of p = 0.002 and in the ANOVA test, the value of p =0.0059.

In the *Table 4* it can be seen that for body weight, following the statistical analysis for all combinations (E1 7 days x E1 14 days x E1 28 days) for the T-test and ANOVA the results were very significant.

After analyzing the values of the Lee body development index only for the pair of values between 14 and 28 days, the result was statistically insignificant, the values of the Lee index being 279.8 at 14 days and 278 at 28 days, at Student's test p = 0.2835 (p>0.05) and for ANOVA p = 0.6215 ((p>0.05).

Table 4

Longitudinal values for E1 group

		E1 7 days	E1 14 days	E1 28 days	Student test		ANOVA	
Weight (grams)	$\bar{X}$	29.16	30.63	33.97	E1 -7 vs. E1 - 14	<0.001 ***	Global	<0.001 ***
	$\pm s_{\bar{x}}$	0.09104	0.1607	0.2606	E1 – 14 vs. E1 - 28	<0.001 ***	E1 -7 vs. E1 - 14	<0.001 ***
	sd	0.4071	0.7188	1.165	E1 – 7 vs. E1 -28	<0.001 ***	E1 – 14 vs. E1 - 28	<0.001 ***
	cv%	1.396	2.347	3.431			E1 – 7 vs. E1 -28	<0.001 ***
Lee index	$\bar{X}$	305.7	279.8	278.0	E1 -7 vs. E1 - 14	<0.001 ***	Global	<0.001 ***
	$\pm s_{\bar{x}}$	1.607	1.052	1.176	E1 – 14 vs. E1 - 28	0.2835 n.s.	E1 -7 vs. E1 - 14	<0.001 ***
	sd	7.189	4.706	5.260	E1 – 7 vs. E1 -28	<0.001 ***	E1 – 14 vs. E1 - 28	0.6215 n.s.
	cv%	2.352	1.682	1.892			E1 – 7 vs. E1 -28	<0.001 ***

For values at 7 days versus 14 and 28 days, respectively, the resulting values showed a highly significant statistical difference.

Table 5 shows the values of weight and Lee's index for experimental group E2 from 7 days to the end of the experiment, i.e. 28 days.

The average weight per animal / week increased from 29.86 grams to 35.29 grams and the Lee index decreased from 305 to 264.

Following statistical analysis, both the Student's t test and the ANOVA test show a highly significant statistical difference with a value of p < 0.001 for all combinations.

Table 5

Longitudinal values for E2 group

		E2 7 days	E2 14 days	E2 28 days	Test Student		ANOVA	
Weigh (grams)	$\bar{X}$	29.86	31.91	35.29	E2 -7 vs. E2 – 14	<0.001 ***	Global	<0.001 ***
	$\pm s_{\bar{x}}$	0.1286	0.2216	0.3624	E2 – 14 vs. E2 – 28	<0.001 ***	E2 -7 vs. E2 - 14	<0.001 ***
	sd	0.5752	0.9911	1.621	E2 – 7 vs. E2 -28	<0.001 ***	E2 – 14 vs. E1 - 28	<0.001 ***
	cv%	1.926	3.106	4.593%			E2 – 7 vs. E1 -28	<0.001 ***
Lee index	$\bar{X}$	305.3	272.1	264.0	E2 -7 vs. E2 – 14	<0.001 ***	Global	<0.001 ***
	$\pm s_{\bar{x}}$	1.738	1.331	1.144	E2 – 14 vs. E2 – 28	<0.001 ***	E2 -7 vs. E1 - 14	<0.001 ***
	sd	7.773	5.951	5.116	E2 – 7 vs. E2 -28	<0.001 ***	E2 – 14 vs. E2 - 28	0.005 **
	cv%	2.546	2.187	1.938			E2 – 7 vs. E2 -28	<0.001 ***

However, following the ANOVA test, the predictability value for the pair E2 at 14 days and E2 at 28 days was p = 0.005, denoting a distinctly significant statistical difference compared to the value of p<0.001, highly significant, following the Student's test.

Also, as a continuation of the present research, in addition to the use of body mass development indices, serum biochemistry tests and analyzes can be used regarding some metabolic values, interpreted with the standard values of the basal metabolism in the studied species or with a control group conducted without the presence of the experimental factor.

CONCLUSIONS

The evolution of body weight is a very valuable indicator of assessing the quality of food, the degree of assimilation of nutrients and the state of health of the respective animal.

The average body weight/animal in all groups at the beginning of the experiment was 28.39 grams, reaching the following weights at the end of the experiment: the control group 31.72 grams/animal, the experimental group E1 33.97 grams/animal and the experimental group E2 35.29 grams/animal (p < 0.001 in most comparisons).

The use of mono sodium glutamate in the preparation of foods potentiated their taste and aroma, leading to increased consumption of food, beyond the caloric needs of the consumers in the experiment.

Water consumption increased from an average of 158.16 ml/cage in the first 7 days to a batch average of 204.37 ml/cage at 28 days, with consumption approximately 22 ml higher at 28 days in the experimental group E2 (213.9 ml) compared to the control group (191.6 ml) and with a highly significant statistical difference (p<0.001).

Food consumption followed the same upward curve, from a mean value of 86.3 grams in the first week in the control group to a mean value of 200.4 grams in the fourth week in the experimental group E2. Thus, statistical differences were highly significant (p < 0.001) in all combinations and for both tests used: Student and ANOVA.

In the case of body lengths measured on days 7, 14 and 28 of the experiment, a dynamic similar to body weight was observed; at the end of the experiment, the mice in the experimental group E2 showed an average body length of 12.25 cm compared to 10.67 cm in the control group (p<0.001).

Due to the increase in body weight and implicitly the length measured from the tip of the nose to the base of the tail, the Lee index for body development had a decrease from initial values of about 305, finally reaching values of 264 in experimental group E2, with highly significant statistical differences with a significance probability value of differences between means greater than 99.9% (p<0.001).

The Student's test can interpret the statistical significance only for two strings of values, while the single factor ANOVA test (analysis of variance) can interpret more than two groups of values, both between them grouped two by two but also globally, between all groups).

The ANOVA statistical analysis test, due to the different way of calculating the predictability, will always give more sensitive, more accurate results of the probability, therefore closer to the three values of the confidence threshold (95%, p<0.05 ; 99%, p< 0.01 and 99.9%, p<0.001), compared to the results obtained with the Student's test.

Following the experiment undertaken, in addition to the conclusions shown, some recommendations can also be made, such as:

1. The use of statistical interpretation tests should be done in accordance with the type of study performed (cross-sectional or longitudinal), the number of experimental groups used and the accuracy of the desired interpretation data;

2. For cross-sectional studies (time section, without chronological variations in dynamics) it is recommended to use the Student's test for 1-to-1 comparisons between experimental groups;

3. For longitudinal type studies (expansion over time, with chronological variations in dynamics) it is recommended to use the ANOVA test, followed by Tukey's post hoc discriminatory test or another test (Benferoni, Fortnyte) for comparisons of several experimental moments (T0, T1 ...Tn);

4. For longitudinal studies, the experimental data can be entered into a biostatistical linear regression model whose algorithm could predict a

probable dynamic of the studied character, with a certain probability threshold of repeatability between 95-99.99%, on based on the data recorded in the previous readings;

5. To facilitate the processing of collected experimental data, it is recommended to use special statistical interpretation programs (eg GraphPad Prism®, IBM-SPSS®, Microsoft Excel® with Data Analysis modules;

6. In order to appreciate the effect of the studied food additive on the chosen biological material, it is indicated that the Lee index of body development should not be used on its own, but should be associated with other values for determining the body mass index.

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