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ATTEMPT AT APPLYING PARAMETRIC STATISTICAL TESTS FOR THE ASSESSMENT OF THE DECLARATIVE PROBIOTIC BACTERIA NUMBER IN SELECTED DAIRY PRODUCTS

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During this study quality and declared health features of probiotic yogurt milk were assessed. Using the methods of statistical analysis a tool was developed allowing verification of formulated statistical hypotheses. It was established that no statistically significant differences were present in the assessment of the number of microorganisms dependent on the dilutions applied. It was shown that the number of live bacteria in the product during storage decreases and that those changes are statistically significant. It was determined that the average number of live probiotic bacteria in the product in all samples during storage was significantly higher than declared by the producer m value and it indicated appropriate health quality of the tested product.

INTRODUCTION

Increasing awareness of nutrition influence on human health and fitness has resulted in a dynamic development in production of functional food also known as health food [Szepieniec-Puchalska, 2001].

The development of diet science resulted in the situation where food is no longer perceived in the traditional way as a source of energy and nutrients that serve satisfying human needs but it is also seen as food products capable of a positive influence on human health [Kostogrys *et al.*, 2002].

Recently supplies of food products containing health additives, most frequently in the form of synthetic vitamins and mineral components, have increased significantly. They may also contain bioactive components possessing known favorable health properties: food cellulose, oligosaccharides, polyols (polyhydric alcohols) amino acids, peptides, proteins, polyunsaturated fatty acids, vitamins, mineral components, choline and lecithin, lactic fermentation bacteria, phytochemical substances and many others [Ochocki & Stańczyk, 2003]. Such products are referred to as "functional products".

Functional food should be treated as a supplement to a balanced diet and not as a substitute. Consumers should not believe that their diet is healthy and balanced if they consume a certain volume of functional food products [Lutomski, 2003]. Because of the specific composition, functional food can be divided into a number of groups which include, among others, probiotic food [Kostogrys *et al.*, 2002].

In recent years, new generation probiotic products, which, thanks to proven health properties, are recommended by the Institute of Food and Nutrition, have been floated to the food market. They contain live cultures of bacteria: traditional strains of yogurt bacteria – *Lactobacillus bulgaricus* and *Streptococcus thermophilus* as well as *Lactobacillus casei defensis* strain, which, as a consequence of its particular probiotic properties, has been registered with the Pasteur Institute in Paris.

With the large supply of products referred to as "health-promoting" or "functional" food, the consistency of offered products quality declared by the manufacturer and their real quality have become an issue. Achieving the appropriate product quality requires the application of adequate manufacturing processes, means of production and controls during the production process and of the final product. That is linked to increasingly wide application of modern quality control methods, among them statistical methods based on parametric tests [Koronacki, 1999].

This study aimed at developing an application in the spreadsheet environment and verifying its applicability to assessment and controlling the significance of difference of consistency in numbers of live probiotic bacteria *Lactobacillus casei defensis* in the product during its shelf life and the number declared by the manufacturer, by applying the parametric tests method.

MATERIAL AND METHODS

Subject and scope of studies. The data for statistical tests was obtained by determining the number of live *Lactobacillus casei defensis* bacteria in natural flavor yogurt milk. Product for the tests was purchased once, on day 13

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since the production date in the amount of 40 containers (100 mL in capacity each). The purchased product was divided into 5 batches of 8 samples. In the first batch, the number of live *Lactobacillus casei defensis* bacteria was determined on the day of purchase. The other batches were stored at refrigerated conditions (4°C) until the end of shelf life declared by the manufacturer and during that time the number of live *Lactobacillus casei defensis* bacteria was determined after 18, 23, 28 and 33 days of storage.

Microbiological analysis. In the tested yogurt milk the number of live *Lactobacillus casei defensis* cells expressed as cfu/cm^3 was determined using the method of surface inoculation on sterile Petri dishes with solid MRS medium. Each determination was done in two parallel repetitions [PN-A-86034-15:1998].

To this end, consecutive decimal dilutions were prepared until obtaining the final dilutions of 10^6 and 10^7. From the final dilutions surface inoculation to Petri dishes with solidified MRS medium was done. The dishes were incubated in anaerobic conditions in a laboratory CL-135 incubator at 37°C for 72 h. On completion of incubation the number of colonies obtained from the tested product dilutions was counted [PN-EN ISO 8261:2002].

The results obtained were used as data for statistical calculations.

Statistical analysis. The majority of hypotheses verification methods are based on χ^2, Snedecor's-F and Student-t distributions [Sobczyk, 2004]. Significance Student-t and Snedecor's-F tests based on standard formulas were used as the method for verification of microbiological determinations results [Volk, 1973]. Statistical calculations were done using the obtained results of microbiological determinations grouped in samples with n=16.

RESULTS AND DISCUSSION

Description of the application

The application allowing automated verification of the null hypothesis was designed using Microsoft Excel software. In that program the working file was established in which spreadsheet "Dane_0" offers a table for input of the determined number of *Lactobacillus casei defensis* colonies obtained from final product dilutions of 10^6 and 10^7 and for individual storage periods. Next the spreadsheet "Dane" was developed allowing automated conversion of the input data to *Lactobacillus casei defensis* cfu per 1 cm^3 of product and presentation of that number in the form of common logarithm.

The following application development stages involved design of spreadsheets allowing calculation of individual statistical parameters and verification of the formulated null hypotheses in the applied significance tests on the basis of Excel statistical functions and designed mathematical and logical formulas.

All cells containing calculation and logical formulas were protected against changes and as a result the tool is protected against corruption in use.

In the spreadsheets testing the formulated hypotheses the user can access only the cells describing the data, standard value m and the significance level α.

After input of determination results into "Dane_0" spreadsheet, the tool automatically calculates the required statistics allowing fast and objective statistical analysis of results.

Analysis of results

The average number of *Lactobacillus casei defensis* cfu per 1 cm^3 of yogurt milk tested and the value of the logarithm of that number are presented in Table 1.

TABLE 1. Average values of the number of *Lactobacillus casei defensis* cfu per 1 cm^3 and logarithm of that number calculated from 10^6 and 10^7 dilutions during product's storage.

Storage time at 4°C (days)	Number of *Lactobacillus casei defensis* (cfu/1 cm^3 calculated from dilution)		Logarithm of *Lactobacillus casei defensis* (cfu/1 cm^3 calculated from dilution)	
	10^6	10^7	10^6	10^7
13	8.9E+08	10.4E+08	8.9398	9.0035
18	6.8E+08	6.3E+08	8.8296	8.7741
23	4.3E+08	4.9E+08	8.6276	8.6408
25	2.8E+08	3.1E+08	8.4197	8.4480
33	2.2E+08	2.1E+08	8.3395	8.2964

Analysing the data presented it was determined that the average number of *Lactobacillus casei defensis* cfu per 1 cm^3 calculated from final 10^6 and 10^7 dilutions decreased during storage from: 8.9E+08 to 2.2E+08 and from 10.4E+08 to 2.1E+08 respectively, which is reflected in the decrease of logarithmic value of that number.

Aiming at objective determination of differences found between average values of cfu/cm^3 calculated from final 10^6 and 10^7 dilutions, the Student-t test concerning significance of the difference between two averages was applied. Equity in variance of test populations is the necessary condition for applying that test [Brandt, 1999].

Comparison of variances in cfu/cm^3 number calculated from final dilutions was done applying Snedecor's-F test with the null hypothesis that variances of both populations are equal (H_0: $s^2(x_1) = s^2(x_2)$).

Results for verification of that hypothesis are presented in Table 2.

TABLE 2. Results of variances equality test for cfu/cm^3 number and variance of its logarithm for *Lactobacillus casei defensis* calculated from 10^6 and 10^7 dilutions during storage of the yogurt milk samples tested.

Storage time at 4°C (days)	α=0.05 Hypotheses tested	
	cfu/cm^3 H_0: $s^2(x_1)=s^2(x_2)$	Logarithm of cfu/cm^3 H_0: $s^2(x_3)=s^2(x_4)$
13	Accept	Accept
18	Accept	Accept
23	Accept	Accept
25	Accept	Accept
33	Accept	Accept

Sample coding: $s^2(x_1)$ – cfu/cm^3 number variance – 10^6 dilution; $s^2(x_2)$ – cfu/cm^3 number variance – 10^7 dilution; $s^2(x_3)$ – cfu/cm^3 number logarithm variance – 10^6 dilution; $s^2(x_4)$ – cfu/cm^3 number logarithm variance – 10^7 dilution

Analysing the variance equality test results presented in Table 2 it was established that the H_0 hypothesis was accepted for all samples tested meaning that tested variances were equal and the condition for conducting the significance test of the difference of two averages was satisfied.

The significance test of the difference of two averages was carried out formulating the null hypothesis that the tested averages were equal H_0: $x_1=x_2$ (averages from compared samples are equal) against the alternative hypothesis H_1: $x_1<>x_2$ (averages from compared samples are different).

The verification results for those hypotheses are presented in Table 3.

Analysing the results of the significance test of the difference of two averages it was determined that the H_0 was accepted for all tested samples meaning that the values of average cfu/cm^3 number calculated for final 10^6 and 10^7 dilutions as well as logarithms of that number showed no statistically significant differences.

TABLE 3. Results of the test of the difference between averages of the cfu/cm^3 number and values of its logarithm for *Lactobacillus casei defensis* calculated from 10^6 and 10^7 dilutions during storage of the yogurt milk samples tested.

Storage time at 4°C (days)	α=0.05 Hypotheses tested	
	cfu/cm^3 H_0: $x_{śr1}=x_{śr2}$	Logarithm of cfu/cm^3 H_0: $x_{śr3}=x_{śr4}$
13	Accept	Accept
18	Accept	Accept
23	Accept	Accept
25	Accept	Accept
33	Accept	Accept

Sample coding: $x_{śr1}$ – cfu/cm^3 number calculated for 10^6 dilution; $x_{śr2}$ – cfu/cm^3 number calculated for 10^7 dilution; $x_{śr3}$ – logarithm of cfu/cm^3 number – 10^6 dilution; $x_{śr4}$ – logarithm of cfu/cm^3 number – 10^7 dilution

On the basis of the above-presented null hypothesis verification concerning equality of averages, the value of the

TABLE 4. Results of the test of the difference between the declared m value and the average *Lactobacillus casei defensis* cfu/cm^3 number per 1 cm^3 of the tested product during its storage.

H_0: $x_{śr}\leq m$ The average is equal or less than the m value
H_1: $x_{śr}>m$ The average is higher than m value

		α=0.05			
Sample number	1	2	3	4	5
Value m=	1.0E+08	1.0E+08	1.0E+08	1.0E+08	1.0E+08
Storage time	13 days	18 days	23 days	28 days	33 days
$x_{śr}$=	10.4E+08	6.3E+08	4.9E+08	3.1E+08	2.1E+08
$l_{x(1-\alpha)}=\pm$	1.3E+08	1.3E+08	1.4E+08	7.0E+07	3.1E+07
s(x) =	2.5E+08	2.4E+08	2.6E+08	1.3E+08	5.7E+07
$s^2(x)$ =	6.25E+16	5.56E+16	7.00E+16	1.72E+16	3.29E+15
N =	16	16	16	16	16
Compared	1 with m	2 with m	3 with m	4 with m	5 with m
$t_{obl.}$ =	15.0000	9.0100	5.9547	6.4875	7.4077
P(t) =	9.706E-11	9.669E-08	1.320E-05	5.126E-06	1.096E-06
$t_{\alpha;f}$ =	1.7531	1.7531	1.7531	1.7531	1.7531
H_0:	Reject	Reject	Reject	Reject	Reject

TABLE 5. Results of the test of the difference between the average and the declared m value – logarithm of *Lactobacillus casei defensis* cfu/cm^3 number per 1 cm^3 of the tested product during its storage.

H_0: $x_{śr}\leq m$ The average is equal or less than the m value
H_1: $x_{śr}>m$ The average is higher than the m value

		α=0.05			
Sample number	1	2	3	4	5
Value m =	8	8	8	8	8
Storage time	13 days	18 days	23 days	28 days	33 days
$x_{śr.}$ =	9.0035	8.7741	8.6408	8.4480	8.2964
$l_{x(1-a)}$ = ±	0.05821	0.08134	0.11626	0.11978	0.07201
s(x) =	0.10924	0.15264	0.21817	0.22478	0.13514
$s^2(x)$ =	0.01193	0.02330	0.04760	0.05053	0.01826
n =	16	16	16	16	16
Compared	1 with m	2 with m	3 with m	4 with m	5 with m
$t_{obl.}$=	36.74567	20.28474	11.74809	7.97301	8.77399
P(t) =	2.062E-16	1.285E-12	2.887E-09	4.488E-07	1.356E-07
$t_{a;f}$ =	1.75305	1.75305	1.75305	1.75305	1.75305
H_0:	Reject	Reject	Reject	Reject	Reject

average *Lactobacillus casei defensis* cfu number per 1 cm^3 of product and logarithm of that number calculated for 10^7 dilution was assumed for further statistical analyses.

The declared number of live bacteria per 100 cm^3 of product was not less than 10×10^9 cells ($1\times10^8/cm^3$). In the application 1×10^8 cfu/cm^3 was assumed as the m value. Aiming at verification of statistical differences between the average cfu/cm^3 value and the m value the hypothesis H_0: $x_1 \leq x_2$ (the average is less or equal to the m value) was assumed while the alternative hypothesis was H_1: $x_1 > x_2$ (the average is more than the m value).

Results of null hypothesis verification concerning the difference between the average cfu/cm^3 number and the declared m value are presented in Table 4 while the results of the test of difference between the average value – logarithm of cfu/cm^3 number and the m value expressed as the logarithm in table.

Analysing the results presented in Table 4 it was established that the null hypothesis was rejected for all samples. That means that the average cfu/cm^3 number in all tested yogurt milk samples during the storage time was higher than the declared m value and it indicates appropriate health quality of the product.

The obtained H_0 hypothesis verification results are identical with those presented in Table 4.

Analysing average values presented in Tables 4 and 5, a decrease in the cfu/cm^3 number and the logarithm of that number during the storage period from 10.4E+08 to 2.1E+08 and from 9.0035 to 8.2964, respectively, was identified.

Despite the decrease in the cfu/cm^3 number during storage, the average number of live cells was higher than the manufacturer declared value. That means that the tested product, even on the last day of its shelf life, shows adequate quality.

CONCLUSIONS

1. The average number of live probiotic bacteria in yogurt milk determined during tests at 1×10^9 cfu/cm^3 decreased by one order of magnitude during 33 days of storage at 4°C reaching 2×10^8 cfu/cm^3 and on the basis of the difference significance test Student-t was significantly higher than declared, which confirms probiotic properties of the product tested.
2. The prepared application also allowed verification of significance of the difference in average numbers of probiotic bacteria in the product calculated for consecutive dilutions. It was established that there were no statistically significant differences between values calculated for 10^6 and 10^7 dilutions.

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ZASTOSOWANIE PARAMETRYCZNYCH TESTÓW STATYSTYCZNYCH DO SZACOWANIA DEKLAROWANEJ AKTYWNOŚCI PROZDROWOTNEJ WYBRANYCH PRODUKTÓW MLECZARSKICH

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W niniejszej pracy oceniono jakość oraz deklarowane cechy prozdrowotne mleka jogurtowego. Korzystając z metod analizy statystycznej, zbudowano aplikację, umożliwiającą weryfikację postawionych hipotez statystycznych. Stwierdzono, iż nie występują istotne statystycznie różnice przy ocenie liczby drobnoustrojów w zależności od stosowanych rozcieńczeń w metodzie płytkowej. Wykazano, że liczba żywych bakterii w produkcie w okresie przechowywania ulega obniżeniu, a zmiany te są statystycznie istotne. Pomimo obniżenia liczby żywych bakterii podczas przechowywania, stwierdzono, że średnia liczba żywych bakterii probiotycznych zawartych w produkcie we wszystkich próbach w okresie przechowywania jest istotnie wyższa niż wartość deklarowana m (deklarowana przez producenta $10\times10^9/100$ cm^3) i wskazuje na odpowiednią jakość prozdrowotną badanego produktu. Wykorzystane testy statystyczne t-Studenta i F-Snedecora po-zwalają ocenić zmiany liczebności komórek bakterii fermentacji mlekowej w badanym produkcie przy mniejszej ilości analizowanych próbek (rozcieńczenie szóste i siódme zamiast ósmego, dziewiątego czy dziesiątego). Zbudowana na ich bazie aplikacja może być pomocna przy ocenie jako-ści produktów tak na etapie produkcji jak i w okresie przechowywania.