Food Science and Applied Biotechnology, 2023, **6**(1), 117-126

UFT Academic Publishing House, Plovdiv

Food Science and Applied Biotechnology
e-ISSN: 2603-3380

Journal home page: www.ijfsab.com
https://doi.org/10.30721/fsab2023.v6.i1

OPEN ACCESS

Research Article

Influence of particle size and storage period on the moisture of granular synbiotic products

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Abstract

A synbiotic is a product in which a probiotic and a prebiotic are combined at the same time. The benefits of synbiotics for human health are indisputable and this increases interest in them. This paper investigates the effect of particle size and storage time on the moisture content of granulated lyophilized synbiotic products - "LIO - Strawberry Milk" and "LIO - Aronia Milk". For each of these two products, four variants of mixtures are prepared - Variant 1 - a lyophilized product with added 30% sucrose; Option 2 - a lyophilized product with added 10% sucrose and 2% fructooligosaccharides (FOS); Option 3 - a lyophilized product with added 10% sucrose and 2% locust bean gum and Option 4 - a lyophilized product with added 10% sucrose, 1% locust bean gum and 1% FOS. A two-factor analysis of variance was used for the study. The obtained results show that the two factors - particle size and storage time have no mutual influence on the humidity of the studied products. However, they have a significant impact individually. It was also established that the size of the particles is directly dependent on the type and concentration of the added substances.

Keywords

synbiotic product, particle size distribution, granulometry, moisture, ANOVA

Abbreviations

ANOVA – analysis of variance; HDS – honest significant difference; FOS – fructooligosaccharides

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Article history:
Received 30 January 2023
Reviewed 11 February 2023
Accepted 23 February 2023
Available on-line 17 March 2023

https://doi.org/10.30721/fsab2023.v6.i1.255

Introduction

The term "functional food" was first used in 1984 by the Japanese government (Henry 2010), which defined it as "a food containing an ingredient with health functions and officially approved to claim their physiological effects on the human body". A few years later, first the USA and then the European Union also developed their own legislation, but without giving a clear definition of the concept of functional food. Generally speaking, most foods are functional. One food group may provide protein for muscle repair, another for carbohydrates for energy, and a third for vitamins and minerals for cellular function. This document adopts the following definition - a functional food is a food product or supplement containing a wide range of valuable nutrients that have a direct positive effect on health and help reduce the risk of disease.

After a relatively shaky start, today functional foods are generating serious interest. There is a continuous increase in the number of users who are interested in such products. This is leading to an increase in the global functional foods market. To meet the increased demand, the food industry, supported by the scientific community, is constantly developing new functional food products.

Fermented foods are the most popular functional foods, with yogurt perhaps being among the most commonly consumed products by every person. The health benefits of fermented functional foods are expressed either directly through the interaction of ingested live microorganisms, bacteria, or yeast with the host (probiotic effect) or indirectly as a result of ingestion of microbial metabolites produced during the fermentation process (biogenic effect) (Stanton et al. 2005). Recently, kefir has become more and more popular, claiming that it is even more useful than yogurt. Kefir has been reported to have many health benefits regarding cardiovascular disease, diabetes mellitus, obesity, cancer, immune system disorders, and osteoporosis (Kesenkas et al. 2017). Kefir is full of healthy nutrients like calcium and protein. Also, the microbiological and chemical composition of kefir shows that it provides a complex probiotic effect due to the inherent lactic acid bacteria and yeast (Gao and Li 2016).

A significant drawback of kefir is that it must be refrigerated and usually lasts no more than two weeks before it goes bad. In order to preserve viable probiotic strains and the biologically active components contained in them, it is subjected to freeze drying. This process, also known as lyophilization, has been used for the preservation and storage of microorganisms, starter cultures, and probiotic products and has wide practical applications (Nowak and Jakubczyk 2020). Such processing allows kefir to have fully preserved biological, nutritional and organoleptic properties, to be stored for a long time (over 20 years), to avoid difficulties in its transportation. All this is the reason why more and more manufacturing companies producing functional foods use lyophilization as the main processing method to obtain high-quality products with a long shelf life (Bhatta et al. 2020).

In this paper, two new lyophilized kefir products - "LIO - Milk strawberry" and "LIO - Milk chokeberry" - are studied. Each of them is realized in four variants, using different types and concentrations of added substances (sucrose, fructooligosaccharides, and locust bean gum). The research found that the size of the particles is directly dependent on the type and concentration of the added substances. The effect of particle size and storage time on the moisture content of granulated lyophilized synbiotic products - "LIO - Milk strawberry" and "LIO - Milk chokeberry" was also studied.

Materials and Methods

Kefir culture. The experiments were carried out with goat's milk, previously pasteurized at 85°C for 10 min. The natural sourdough from kefir grains is activated in sterile skimmed milk at 25°C for 24 h, filtered to remove coagulated milk and washed with sterile water. Activated kefir grains are inoculated into pasteurized goat's milk and incubated statically at 25°C for 24 h until a fermented product (kefir) is obtained. The fermentation process takes place at a set temperature of 22-24°C for 24 h. Based on the kefir grains obtained in this way, two synbiotic products - "LIO - Milk strawberry" and "LIO - Milk chokeberry" - were developed, which are the subject of research in the paper.

The production process of granulated synbiotic products. The production process of granulated synbiotic products takes place in the following stages:

1. Lyophilization. The lyophilization process was carried out under conditions presented in Table 1.

Table 1. Lyophilization conditions

Kind of Products Parameters	Milk, Dairy Products, Fermented milk drinks
Thickness of layer, mm	8 -10
Freezing temperature, °C	-32 - -40
Eutectic temperature, °C	-35 - -42
Sublimation temperature, °C	-35 - -40
Desublimation temperature, °C	-60
Total pressure, mm/Hg	0.20 – 0.30
Terminal temperature, °C	28 - 30
Duration of the cycle, h	10 – 14.5
Residual humidity, %	1.5 – 3.5

2. Grinding of the obtained lyophilized products;

3. Addition of the corresponding granulating solutions and homogenization;

4. Granulation of the obtained products in an oscillating granulator "ERWEKA" with a sieve size of 1 mm. (Fig. 1a).

a) **b)**

Figure 1. Wet Granulator with oscillating rotor - a) and ANALYSETTE 22 NanoTec plus - b)

The resulting granulate is dried for 24 h at 30-40°C, after which 1-3% talc is added to it for better possibility of the granules through the particle distribution apparatus.

Each of these two products was developed in 4 different variants using different types and concentrations of granulating components (sucrose, fructooligosaccharides and carob gum).

Variant 1 – lyophilized product with added 30% sucrose.

Variant 2 – lyophilized product with added 10% sucrose and 2% fructooligosaccharides (FOS).

Variant 3 – lyophilized product with added 10% sucrose and 2% locust bean gum.

Variant 4 – lyophilized product with added 10% sucrose, 1% locust bean gum and 1% FOS.

Putting these additional ingredients into the kefir product is done for several reasons. On the one hand, these substances are known as cryoprotectants (Nacheva et al. 2017; Trifonov et al. 2013; Doneva et al. 2022), i.e. help the survival of lactic acid bacteria in the freeze-drying process. On the other hand, they help to achieve an optimal composition of the granulating mixtures, in which minimal dusting can be observed (Doneva et al. 2022). The technology by which the optimal compositions of the new products were obtained is presented in detail in (Solakov et al. 2020).

Granule size measurement is done by the ANALYSETTE 22 NanoTec plus (Fig. 1b), which is suitable for efficient and reliable particle size distribution in production and quality control, as well as in research and development. ANALYSETTE 22 NanoTec plus measures nanoparticles in an extremely wide measurement range (0.01 - 2000 pm). The extremely large measuring range of the ANALYSETTE 22 NanoTec plus with a lower measuring limit of approximately 0.01 pm is due to the Triple-laser technology for forward and backward scattering. ANALYSETTE 22 NanoTec plus always uses all 57 measuring channels of the detector. By combining the different measuring positions, it is possible to perform measurements with up to 165 effective channels, which guarantee a particularly high resolution and sensitivity.

Statistical analysis. All experiments and analyses were done in triplicate and the mean values are presented. The experiments were designed and analysed statistically by ANOVA. Tukey's Honest Significant Difference (HDS) test ($p < 0.05$) was used to determine significant differences among the results. All statistical computations were made using the SPSS v26.0 software package (SPSS Inc., Chicago-Illinois-USA).

Results and Discussion

The study of the dependence of storage moisture on particle size was done using statistical methods. However, several authors (Gherezgihier et al. 2017; Noman et al. 2016; Eftimov et al. 2017) claim that there are some difficulties and problems in the statistical analysis of food data. One such misunderstanding, and a very common one at that, is that when applying a statistical method, the conditions (assumptions) about the data that must be met for the method to apply are not checked. Typically, researchers simply apply a statistical method, in most cases taken from similar published work. As a result, their conclusions may be incorrect. To avoid this, the results obtained in the present study are explained in maximum detail below.

To investigate the influence of two factors - storage time (designated as factor A) and composition and concentration of the inserted granulating components (designated as factor B) - a two-way ANOVA was applied. Both the influence of the two factors A and B separately (the so-called main effect) and their combination (the so-called interaction) are studied. Therefore, two-way ANOVA boils down to testing the following three sets of hypotheses:

1. Influence of factor A. In this case, the main hypothesis states that all mean populations associated with factor A are equal:

$$\left| \begin{array}{l} H_0: \mu_{A1} = \mu_{A2} = \cdots = \mu_{An} \\ H_1: not(\mu_{A1} = \mu_{A2} = \cdots = \mu_{An}) \end{array} \right. \quad (1)$$

2. Influence of factor B. In this case, the main hypothesis states that all mean populations associated with factor B are equal:

$$\left| \begin{array}{l} H_0: \mu_{B1} = \mu_{B2} = \cdots = \mu_{Bn} \\ H_1: not(\mu_{B1} = \mu_{B2} = \ldots = \mu_{Bn}) \end{array} \right. \quad (2)$$

3. Mutual influence of the two factors. In this case, the main hypothesis states that there is no interaction between the two factors A and B.

$$\left| \begin{array}{l} H_0: \mu_{11} = \mu_{12} = \cdots = \mu_{21} = \cdots = \mu_{AB} \\ H_1: not(\mu_{11} = \mu_{12} = \ldots = \mu_{21} = \cdots = \mu_{AB}) \end{array} \right. \quad (3)$$

The acceptance of the main hypothesis H0 in any of the three groups of hypotheses means that the relevant factor does not influence the studied quantity.

The main assumptions that must be respected when applying the two-way ANOVA in order for the conclusions to be sufficiently reliable are as follows:

1. All samples must be drawn from normally distributed populations. To establish this, a normality check was performed. The Shapiro-Wilk test is a more appropriate method for small sample sizes (<50 samples), as is the case here. In this test, the null hypothesis states that the data were taken from a normally distributed population. Thus, when the p-value > 0.05, the null hypothesis is accepted and the data are assumed to be normally distributed. The results obtained for each data set are summarized in Table 2a) and Table 2b) for "LIO - Milk strawberry" and "LIO - Milk chokeberry" respectively. The Sig. value listed in the last column in the tables is the p-value. If the chosen alpha level is 0.05 (which is our case) and the p-value is less than 0.05, then the null hypothesis that the data are normally distributed is rejected. If the p-value is greater than 0.05, then the null hypothesis is not rejected;

Table 2. Tests of normality for a)"LIO - Milk strawberry" and b) "LIO - Milk chokeberry"

Tests of Normality				
	Shapiro-Wilk			
	Time	Statistic	df	Sig.
moisture_lio_milk_strawberry	month 1	0.903	12	0.174
	year	0.944	12	0.550
	variants			
	variant 1	0.903	36	0.389
	variant 2	0.927	36	0.559
	variant 3	0.910	36	0.434
	variant 4	0.854	36	0.169

a)

Tests of Normality				
	Shapiro-Wilk			
	Time	Statistic	df	Sig.
moisture_lio_milk_chokeberry	month 1	0.846	12	0.033
	year	0.921	12	0.290
	variants			
	variant 1	0.934	6	0.609
	variant 2	0.957	6	0.795
	variant 3	0.937	6	0.635
	variant 4	0.930	6	0.577

b)

2. The samples must have a common variance. To verify this, a homogeneity test (Test of Homogeneity of Variance) was made, i.e., hypotheses of equality of population variances were tested using Levene's Test of Equality of Error Variances. For the condition of homogeneity of variances to be met, Levin's test should not be statistically significant, i.e., p-value > 0.05. The homogeneity test results obtained are presented in Table 3a) and Table 3b) for "LIO - Milk strawberry" and "LIO - Milk chokeberry" respectively.

Table 3. Levene`s test of equality of error variances for a) "LIO - Milk strawberry" and b) "LIO - Milk chokeberry"

Levene's Test of Equality of Error Variances[a,b]

		Levene Statistic	df1	df2	Sig.
moisture_lio_milk_chokeberry	Based on Mean	1.01	7	136	0.46
	Based on Median	0.54	7	136	0.79
	Based on Median and with adjusted df	0.54	7	91.176	0.79
	Based on trimmed mean	0.98	7	136	0.48

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a. Dependent variable: moisture_lio_milk_chokeberry
b. Design: Intercept + variants + time + variants * time

a)

Levene's Test of Equality of Error Variances[a,b]

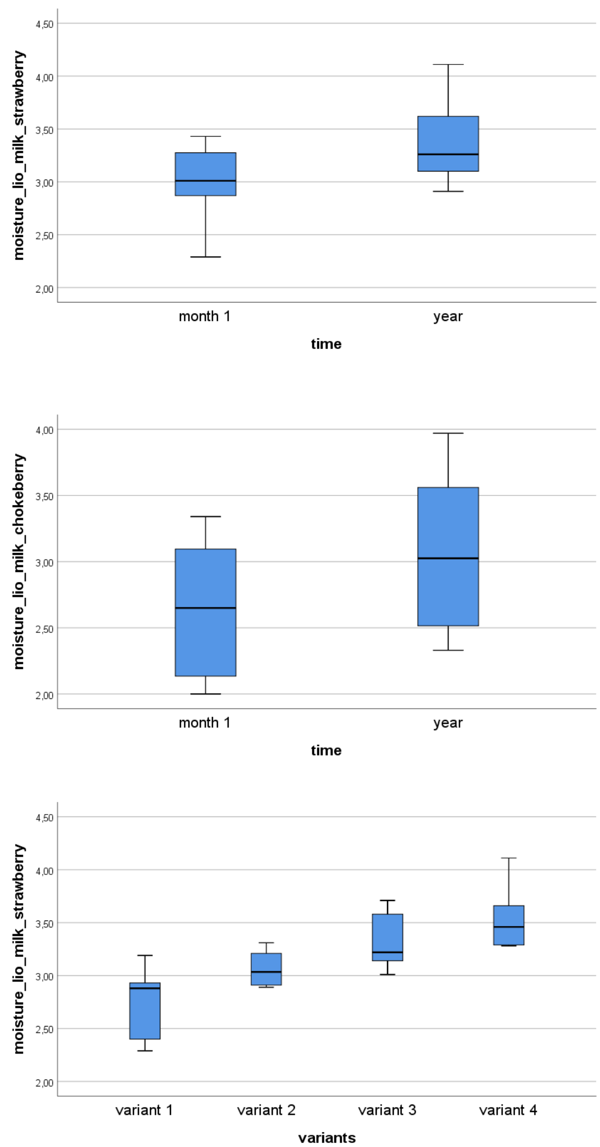
		Levene Statistic	df1	df2	Sig.
moisture_lio_milk_strawberry	Based on Mean	2.621	7	136	0.15
	Based on Median	0.536	7	136	0.8
	Based on Median and with adjusted df	0.536	7	90.02	0.79
	Based on trimmed mean	2.37	7	136	0.07

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.
a. Dependent variable: moisture_lio_milk_strawberry
b. Design: Intercept + variants + time + variants * time

b)

As can be seen from these tables, there are several variants of Levene's test - based on mean, based on the median, based on the median, and with the adjusted degree of freedom, based on the trimmed mean. The choice of an option determines the robustness and power of Levin's test. In principle, one works with the original variant proposed by Levin, which is based on the mean value. In case, however, for all variants, the requirement that the p-value is >0.05 is met.;

3. To have no extreme values. Checking for the presence or absence of extreme values was done using boxplot plots for each group of data. From Fig. 2 it can be seen that there are no extreme values.



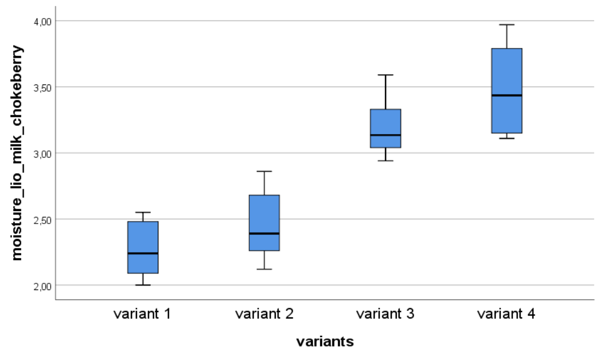
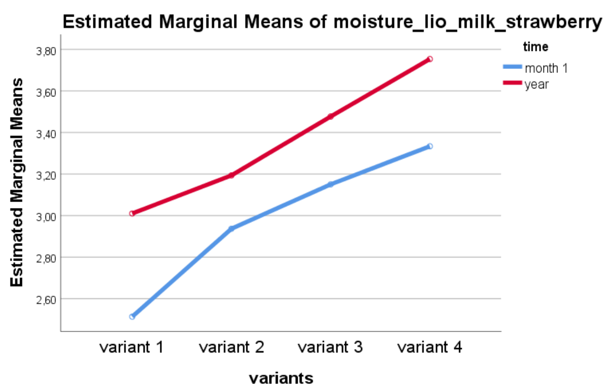


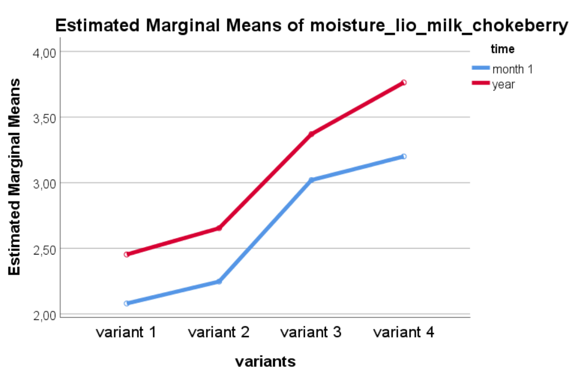
Figure 2. Box plot diagrams

Since all conditions are fulfilled, it can be concluded that the results obtained in the two-way ANOVA will be sufficiently reliable.

The influence of the main factors, as well as the mutual influence, can be judged from Fig. 3.



a)



b)

Figure 3. Estimated marginal means of moisture for **a)** "LIO - Milk strawberry" and **b)** "LIO - Milk chokeberry"

The interaction effect occurs when these two main factors simultaneously affect humidity. Since the two lines are not parallel to the x-axis, therefore the two main factors affect humidity. However, these same two lines are almost parallel to each other, which means that the interaction effect does not significantly affect the humidity. In support of this statement, when testing the hypothesis of an interaction effect, the p-value was 0.768 and 0.638 for "LIO - Milk strawberry" and "LIO - Milk chokeberry" respectively.

It follows that a one-factor analysis of variance can be applied separately to the two factors. A one-way ANOVA was subsequently performed to examine the influence of factor A on the moisture content of a granulated lyophilized synbiotic products "LIO - Milk strawberry" and "LIO - Milk chokeberry". In the one-way analysis of variance, the same assumptions apply as described above. The obtained results are presented in the form of several tables. The Test of Homogeneity of Variance table (Table 4) shows the results of testing hypotheses of equality of population variances by Levin's test, i.e. the second condition described above is verified. Since the p-values are greater than 0.05, it can be considered that the condition of homogeneity of variances is fulfilled. The ANOVA table (Table 5) is the main and most important one. It gives the values of the F-statistic and the significance (Sig., i.e., p-value), which is the probability that the observed differences are due to chance. Since the p-values are very small (almost zero), it can be concluded that there is a statistically significant difference between the groups that are entirely due to the studied factor A. However, a statistically significant result does not provide information about the strength of the result or its size. Therefore, in addition to statistical significance, it is important to know the magnitude of the effect of the independent variable on the dependent variable. It is defined as the strength of the relationship between the independent and dependent variables or the importance of the difference between the levels of the independent relative to the dependent variable.

The magnitude of the effect is calculated using the formula:

$$\eta = \sqrt{\frac{SS_b}{SS_t}} \quad (4)$$

where SSb is the between-group sum of squares and SSt is the total sum of squares. Using the expression (4) for the magnitude of the effect for "LIO - Milk strawberry" 0.75 is obtained, and for "LIO - Milk chokeberry" - 0.896. According to Cohen's scale (Cohen 1988) the effect size for both synbiotic products was interpreted as much larger than typical.

The Multiple Comparisons table (Table 6) contains information on the results of the Tukey HDS post hoc test for each of the two one-way ANOVAs. This test shows exactly where the differences between the groups are (these are the cells colored in beige). It can be concluded that the most significant differences are between the first (Variant 1) and the third and fourth groups (Variant 3 and Variant 4). In other words, the presence of locust bean gum helps prevent the wetting of the granulated synbiotic product during its storage. The benefits of putting locust bean gum into the granule can be sought in other directions as well - it affects the size of the granules, prevents dusting, and improves the taste of the product.

Analogously, a one-way analysis of variance was implemented to verify the influence of factor B (composition and concentration of the inserted granulating components) on the moisture content of the granulated lyophilized synbiotic product "LIO - Milk strawberry" and "LIO - Milk chokeberry". The obtained results are summarized in Table 7 and Table 8. As can be seen from Table 7, the condition of equality of population variances according to Levin's test (Test of Homogeneity of Variance) is again fulfilled. Again, there is a statistically significant difference between the groups, which is entirely due to the studied factor B (Table 8). The strength of the relationship between the independent and dependent variables for "LIO - Milk strawberry" is 0.48 (or much greater than typical), and at "LIO - Milk chokeberry" - 0.37 (larger than typical). In this case, multiple comparisons were not made because there were only two groups.

Table 4. Test of Homogeneity of Variance of factor A

Test of Homogeneity of Variances					
		Levene Statistic	df1	df2	Sig.
moisture_lio_milk_strawberry	Based on Mean	1.078	3	140	0.381
moisture_lio_milk_chokeberry	Based on Mean	0.755	3	140	0.532

Table 5. Analysis of Variance of factor A

ANOVA						
		Sum of Squares	df	Mean Square	F	Sig.
moisture_lio_milk_strawberry	Between Groups	2.026	3	0.675	8.492	0.001
	Within Groups	1.591	140	0.080		
	Total	3.617	143			
moisture_lio_milk_chokeberry	Between Groups	6.110	3	2.037	27.130	0.000
	Within Groups	1.501	140	0.075		
	Total	7.611	143			

Table 6. Multiple Comparisons of factor A

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) variants	(J) variants	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
moisture_lio_milk_strawberry	variant 1	variant 2	-0.303	0.16281	0.275	-0.759	0.1524
		variant 3	-0.552*	0.16281	0.014	-1.007	-0.096
		variant 4	-0.782*	0.16281	0.001	-1.237	-0.326
	variant 2	variant 1	0.303	0.16281	0.275	-0.152	0.759
		variant 3	-0.248	0.16281	0.442	-0.704	0.207
		variant 4	-0.478*	0.16281	0.038	-0.934	-0.023
	variant 3	variant 1	0.552*	0.16281	0.014	0.096	1.007
		variant 2	0.248	0.16281	0.442	-0.207	0.704
		variant 4	-0.230	0.16281	0.506	-0.686	0.226
	variant 4	variant 1	0.782*	0.16281	0.001	0.326	1.237
		variant 2	0.478*	0.16281	0.038	0.023	0.934
		variant 3	0.230	0.16281	0.506	-0.226	0.686
moisture_lio_milk_chokeberry	variant 1	variant 2	-0.183	0.15819	0.659	-0.626	0.259
		variant 3	-0.928*	0.15819	0.000	-1.371	-0.486
		variant 4	-1.215*	0.15819	0.000	-1.658	-0.772
	variant 2	variant 1	0.183	0.15819	0.659	-0.26	0.626
		variant 3	-0.745*	0.15819	0.001	-1.188	-0.302
		variant 4	-1.032*	0.15819	0.000	-1.474	-0.589
	variant 3	variant 1	0.928*	0.15819	0.000	0.486	1.371
		variant 2	0.745*	0.15819	0.001	0.302	1.188
		variant 4	-0.287	0.15819	0.297	-0.729	0.156
	variant 4	variant 1	1.215*	0.15819	0.000	0.772	1.658
		variant 2	1.032*	0.15819	0.000	0.589	1.474
		variant 3	0.287	0.15819	0.297	-0.156	0.729

*. The mean difference is significant at the 0.05 level.

Table 7. Test of Homogeneity of Variance of factor B

Test of Homogeneity of Variances

		Levene Statistic	df1	df2	Sig.
moisture_lio_milk_strawberry	Based on Mean	0.132	1	142	0.719
moisture_lio_milk_chokeberry	Based on Mean	0.191	1	142	0.666

Table 8. Analysis of Variance of factor B

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
moisture_lio_milk_strawberry	Between Groups	0.844	1	0.844	6.694	0.017
	Within Groups	2.773	142	0.126		
	Total	3.617	143			
moisture_lio_milk_chokeberry	Between Groups	1.075	1	1.075	3.619	0.007
	Within Groups	6.536	142	0.297		
	Total	7.611	143			

Conclusions

In this paper, the influence of two factors - storage time and composition of the granulation mixture - on the moisture content of a granulated synbiotic product was studied. These two factors were found to have a significant influence individually, but there was no mutual influence on the degree of humidity. Therefore, two univariate analyses of variance were performed. Since the granulation mixture composition factor had four levels (options 1 to 4), a post hoc Tukey test was additionally conducted to determine which type of mixture this influence was most significant. It was found that there was a statistically significant difference between the first group (Variant 1) and the third and fourth groups (Variant 3 and Variant 4). The main difference in the composition of these mixtures is the presence of locust bean gum in Variant 3 and Variant 4. This ingredient, in addition to improving the taste qualities and being an excellent cryoprotectant, also ensures an optimal size of the granules so that they do not crumble and protects the finished product from moisture during its storage.

Acknowledgement

This study is supported by the Ministry of Education and Science within the National Research Program "Healthy foods for a strong bioeconomy and quality of life", approved by RMS 577/17.08.2018.

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