UDC 619:602.3:[579.864.1+579.873.13]:615.24.012 DOI 10.36016/JVMBBS-2024-10-2-4

INVESTIGATING THE STABILITY OF A SYMBIOTIC BIOLOGICALLY ACTIVE SUPPLEMENT FOR ANIMALS DURING STORAGE

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Summary. The aim of this work was to determine the stability of a symbiotic biologically active supplement for animals during storage and to establish its shelf life. The stability of the symbiotic was studied under two temperature conditions. The results obtained indicate that the symbiotic biologically active supplement for animals retained its biochemical activity on the day of manufacture and after 3, 6, 9, 12, 15, 18, 21, and 24 months and was harmless to white mice throughout the study period when stored at a temperature of 4 ± 0.5 °C. The drug remained suitable for use for 3 months after manufacture when stored at room temperature (20 ± 2 °C). Taking into account the results of the experimental data, it is recommended to store the symbiotic in the refrigerator at a temperature of 4 ± 0.5 °C for 24 months and at a temperature of 20 ± 2 °C for 3 months

Keywords: *Lactobacillus plantarum* No. 7-317, *Bifidobacterium adolescentis* No. 17-316, quality indicators

Introduction. Preventing and treating gastrointestinal diseases in animals remains a significant challenge in modern veterinary medicine (Vovk et al., 2021; Kolechko et al., 2023; Cherevan et al., 2018). Recently, complex biological drugs based on probiotics and prebiotics, called symbiotics, have been employed in animal husbandry (Gujvinska and Paliy, 2018; Iegorov, Kananykhina and Turpurova, 2021; Mizernytskyi, 2021). The International Scientific Association for Probiotics and Prebiotics (ISAPP) has defined a symbiotic as a mixture containing living microorganisms and substrates selectively used by host microorganisms that are beneficial to the health of the host (Swanson et al., 2020). It should be noted that many probiotics, prebiotics, and symbiotics have been produced recently (AEProbio, 2021; Gibson et al., 2017; Hardy, 2023; Roberfroid, 2000; Taboada et al., 2022; Tavaria, 2017; Gujvinska, 2015; Solovyova and Kaliuzhnaia, 2021).

However, the existing literature contains a lack of information on studies of symbiotic stability. Consequently, the manufacturer's responsibilities include the study of product stability, the results of which determine the shelf life and storage conditions (MHU, 2004). The shelf life and storage conditions of active substances and medicinal products can only be determined based on the results of their stability studies. It should be noted that drug stability studies should be carried out at the stage of development of active substances and medicinal products, as well as during storage.

The study aimed to determine the stability of a symbiotic biologically active supplement for animals during storage.

Materials and methods. The stability of the symbiotic biologically active supplement for animals during storage was studied in the Laboratory of Veterinary Sanitation, Parasitology and Bee Diseases Study of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' (Kharkiv, Ukraine). Six pilot batches of the symbiotic biologically active supplement were manufactured and its stability during storage was studied.

In order to study the stability of the symbiotic biologically active supplement during storage, we conducted several scientific experiments. The shelf life of the symbiotic biologically active supplement was determined by the quality of the symbiotic, as well as by the indicators of microbiological purity (bacterioscopic control and absence of extraneous microflora), harmlessness, specific activity (the number of live bacteria in one dose of the drug).

The control parameters were determined at different storage periods of the biological product, namely on the day of its manufacture and during storage for 3, 6, 9, 12, 15, 18, 21, and 24 months. For this purpose, an archive of 18 drug samples was formed. The symbiotic biologically active supplement for animals was stored at room temperature (20 ± 2 °C) and in a refrigerator at a temperature of 4 ± 0.5 °C. To evaluate the quality of the archived samples of the symbiotic biologically active supplement for animals, three samples of the drug were taken after a certain shelf life.

The symbiotic was tested according to the following criteria: determination of appearance, microbiological purity (bacterioscopic control and absence of foreign microflora), harmlessness, and specific activity (number of live bacteria in one dose of the drug).

Microbiological purity was determined according to DSTU 4483:2005 (DSSU, 2005).

The number of live microbial cells in one dose of the symbiotic product was determined by the method of serial dilutions in saline followed by inoculation of 0.1 cm^3 of bacteria from dilutions of 10^6 on De Man–Rogosa–Sharpe agar (MRS agar) and Blaurock medium.

Cultivation of lactobacilli and bifidobacteria was carried out on MRS agar, Blaurock media, and skim milk for 24–48 h at a temperature of 37 °C.

The determination of biochemical activity was carried out by a generally accepted method.

The symbiotic product should be harmless to white mice weighing 20 ± 1 g when administered orally in an amount corresponding to one dose of the product. The symbiotic was dissolved with 0.9% sodium chloride solution at the rate of 0.5 cm^3 per dose. The resulting solution was orally administered to 15 mice weighing 20 ± 1 g in the stomach (using a special nozzle for a 1 cm^3 syringe) — 0.5 cm^3 each. The mice were observed for 21 days.

All experiments were performed in triplicate. The results were processed by methods of variation statistics using Microsoft Excel for Windows 2010. To compare mean values Student's *t*-test was used (Van Emden, 2019).

Results. For the production of a symbiotic biologically active supplement for animals, a formulation and technological regulations for its manufacture were developed. The formulations of the symbiotic are presented in Table 1.

Table 1 — The studied formulations of symbiotic biologically active supplement for animals

Components	Formulation number					
	1	2	3	4	5	6
Lactulose, %	1.0 ± 0.02	1.2 ± 0.02	1.5 ± 0.03	2.0 ± 0.03	2.5 ± 0.05	3.0 ± 0.06
Inulin, %	1.0 ± 0.02	1.0 ± 0.02	1.5 ± 0.03	2.0 ± 0.03	2.5 ± 0.05	3.0 ± 0.06
Fructose, %	1.0 ± 0.02	1.0 ± 0.02	1.5 ± 0.03	2.0 ± 0.03	2.5 ± 0.05	3.0 ± 0.06
Starch, %	57.0 ± 3.06	52.0 ± 3.01	45.0 ± 2.17	40.0 ± 3.06	36.5 ± 2.04	31.0 ± 2.01
Species and dosage of lactic acid bacteria	A mixture of dried cultures of *Lactobacillus plantarum* No. 7-317 and *Bifidobacterium adolescentis* No. 17-316, not less than 1×10^7 CFU/cm^3					

The ratio of symbiotic components was chosen based on our previous research.

The critical parameter of a symbiotic with a probiotic component is the viability of lactic acid bacteria cells, so the first stage of our research was to determine the number of viable cells in each sample on the day of its manufacture. Our studies were carried out by direct inoculation of the corresponding sample of the product onto the surface of the dense MRS agar. As a control, we used the inoculation of lactobacilli and bifidobacteria dissolved in saline at the same concentration as in the samples with 10^6 CFU/cm^3. The results of the experiments are shown in Table 2.

Table 2 — The number of viable cells of lactic acid bacteria in the experimental samples

The studied formulations of symbiotic biologically active supplement	The number of viable cells, CFU/cm^3
Formulation No. 1	$3.5 \pm 0.11 \times 10^7$
Formulation No. 2	$4.2 \pm 0.13 \times 10^8$
Formulation No. 3	$3.8 \pm 0.14 \times 10^9$
Formulation No. 4	$4.4 \pm 0.15 \times 10^7$
Formulation No. 5	$4.9 \pm 0.13 \times 10^8$
Formulation No. 6	$3.2 \pm 0.18 \times 10^6$
Control *Lactobacillus plantarum* No. 7-317	$3.7 \pm 0.12 \times 10^8$
Control *Bifidobacterium adolescentis* No. 17-316	$4.1 \pm 0.17 \times 10^9$

The results of the experiments showed that the lowest number of viable cells was in sample No. 6 and amounted to $3.2 \pm 0.18 \times 10^6$ CFU/cm^3, which is less than the minimum permissible value of the number of viable cells — 1×10^7 CFU/cm^3. Therefore, this sample was excluded from further studies. Sample No. 3 had the best results, the number of viable cells was $3.8 \pm 0.14 \times 10^9$ CFU/cm^3.

During the long-term storage of the drug under normal conditions at room temperature, control studies were conducted every three months for 24 months of storage. It should be noted that the proposed shelf life of the developed symbiotic is 24 months, which is generally accepted for medicines containing live bacteria.

The microbiological purity of the samples during 24 months of storage at room temperature (20 ± 2 °C) is shown in Table 3.

The study of microbiological purity (Table 3) of samples of symbiotic biologically active supplement for animals showed that the number of contaminating aerobic microorganisms after 6 months of storage was no more than 100 CFU/cm^3, and the number of contaminating yeast and mold fungi was no more than 10 CFU/cm^3. It was experimentally confirmed that microorganisms of the *Staphylococcus aureus* and *Pseudomonas aeruginosa* families were absent.

The analysis of the microbiological purity of the developed product showed that in terms of microbial contamination with foreign microflora, the product meets the requirements of the Technical Specifications of Ukraine only when stored for 3 months.

Table 3 — Symbiotic test results for microbiological purity during storage at room temperature (20 ± 2 °C).

Shelf life	Total amount, CFU/cm^3			
	Aerobic microorganisms	Yeast and mold fungi	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
On the day of manufacture	Absent			
3 months				
6 months	< 100	< 10	Absent	Absent
9 months				
12 months				
15 months				
18 months				
21 months				
24 months				

The quality indicators of the developed symbiotic product, permissible norms, and control methods are summarized in Table 4.

To study the stability of the drug, and determine the storage conditions and shelf life, several experimental samples of the symbiotic were made and stored in the laboratory. Compliance tests were conducted every three months. The results of the symbiotic biologically active supplement analysis are shown in Tables 5 and 6.

Determination of the quality indicators of symbiotic samples for 24 months at storage temperatures of 20 ± 2 °C and 4 ± 0.5 °C showed a non-compliance with one or more indicators. Thus, during the 24 months of the study, when stored at room temperature, the symbiotic samples did not meet the quality control methods for 'Microbiological purity of a drug', 'Quantification of bifidobacteria and lactobacilli', and 'Biochemical activity of a symbiotic biologically active supplement'.

Table 4 — Norms and methods of control of symbiotic biologically active supplement for animals

Indicators	Results obtained	Control methods
Description of a symbiotic biologically active supplement	Homogeneous powder of white or cream color	Visually
Microbiological purity of a drug	The presence of bacterial and fungal microflora is not allowed. The preparation should contain only bifidobacteria and lactic acid bacteria. Gram-stained smears should contain gram-positive bacilli characteristic of bifidobacteria and lactobacilli.	According to DSTU 4483:2005 (DSSU, 2005)
Quantification of bifidobacteria and lactobacilli	Total number of bifidobacteria cells at the end of the expiration date — < 1×10^8 CFU/ cm^3. Total number of lactobacilli cells at the end of the expiration date — < 1×10^8 CFU/ cm^3.	According to the Technical Specifications of Ukraine
Biochemical activity of a symbiotic biologically active supplement	Diluted symbiotic biologically active supplement coagulates skim milk in 18–24 h when incubated in a thermostat at a temperature of 37 ± 0.5 °C	
Harmlessness of a symbiotic biologically active supplement	A symbiotic biologically active supplement should be harmless when tested on white mice	

Table 5 — Results of the analysis of a symbiotic biologically active supplement during long-term storage at room temperature (20 ± 2 °C)

Indicators	On the day of production	3 months	6 months	9 months	12 months	15 months	18 months	21 months	24 months
Description of a symbiotic biologically active supplement	Homogeneous white powder								
Microbiological purity of a drug	Complies with the requirements		Does not meet the requirements						
Quantification of bifidobacteria and lactobacilli	3.8 ± 0.14 × 10^9	3.1 ± 0.11 × 10^8	3.8 ± 0.14 × 10^6	2.7 ± 0.12 × 10^6	1.8 ± 0.15 × 10^6	1.7± 0.12 × 10^6	1.5 ± 0.11 × 10^6	1.1 ± 0.13 × 10^6	1.8 ± 0.20 × 10^5
Biochemical activity of a symbiotic biologically active supplement	Complies with the requirements					Does not meet the requirements			
Harmlessness of a symbiotic biologically active supplement	Complies with the requirements								

Table 6 — Results of the analysis of a symbiotic biologically active supplement during long-term storage at a temperature of 4 ± 0.5 °C

Indicators	On the day of production	3 months	6 months	9 months	12 months	15 months	18 months	21 months	24 months
Description of a symbiotic biologically active supplement	Homogeneous white powder								
Microbiological purity of a drug	Complies with the requirements								
Quantification of bifidobacteria and lactobacilli	$3.8 \pm 0.14 \times 10^9$	$3.7 \pm 0.12 \times 10^9$	$3.5 \pm 0.10 \times 10^9$	$3.1 \pm 0.17 \times 10^9$	$3.9 \pm 0.14 \times 10^8$	$3.2 \pm 0.11 \times 10^8$	$2.9 \pm 0.12 \times 10^8$	$2.6 \pm 0.15 \times 10^8$	$1.7 \pm 0.13 \times 10^8$
Biochemical activity of a symbiotic biologically active supplement	Complies with the requirements								
Harmlessness of a symbiotic biologically active supplement	Complies with the requirements								

It should be noted that when stored in a refrigerator at a temperature of 4 ± 0.5 °C, the samples did not meet the test for 'Quantification of lactobacilli and bifidobacteria', but met the standard for 24 months. Thus, the symbiotic biologically active supplement for animals remains stable for 24 months at a storage temperature of 4 ± 0.5 °C and for 3 months at room temperature (20 ± 2 °C).

Thus, the recommended shelf life of the symbiotic biologically active supplement for animals is 24 months at a temperature of 4 ± 0.5 °C. The quality indicators of the product during 24 months of observation did not change compared to the results at the time of manufacture, which indicates a rationally based formulation of the product and its production technology. It should also be noted that the symbiotic can be stored at room temperature (20 ± 2 °C) for 3 months.

Conclusions. The results show that the symbiotic biologically active supplement for animals on the day of manufacture and after 3, 6, 9, 12, 15, 18, 21, and 24 months retained its biochemical activity and was harmless to white mice throughout the study period when stored at a temperature of 4 ± 0.5 °C. In addition, the symbiotic remains usable for 3 months after manufacture when stored at room temperature (20 ± 2 °C).

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