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DEVELOPMENT OF TECHNOLOGY FOR THE PRODUCTION OF SYMBIOTIC BIOLOGICALLY ACTIVE SUPPLEMENT FOR ANIMALS BASED ON *LACTOBACILLUS* AND *BIFIDOBACTERIUM*

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Summary. The study aimed to develop a technological procedure for the production of a symbiotic biologically active supplement for animals based on *Lactobacillus* and *Bifidobacterium*. Three pilot batches of the symbiotic preparation were manufactured. The technology for the production of a symbiotic biologically active supplement for animals has been developed. The scheme of the technological process of manufacturing a symbiotic is proposed: production of nutrient media and working solutions; cultivation of cultures of lacto- and bifidobacteria for the preparation of a symbiotic biological supplement for animals; freeze-drying of cultures of lacto- and bifidobacteria for a symbiotic biological supplement for animals; obtaining mass for a symbiotic biological supplement for animals; control of the finished product before release; packaging, labeling, transportation and storage of a symbiotic biological supplement for animals. The formulation of a symbiotic biologically active supplement is proposed: a mixture of freeze-dried cultures of *Lactobacillus plantarum* No. 7-317 and *Bifidobacterium adolescentis* No. 17-316 (55–65%), inulin (1.0–2.5%), lactose (1.0–2.5%), fructose (1.0–2.5%), starch (42–27.5%). The number of microbial cells per 1 cm^3 of symbiotic is lactobacilli ≥ 10^8, bifidobacteria ≥ 10^8

Keywords: *Lactobacillus plantarum* No. 7-317, *Bifidobacterium adolescentis* No. 17-316, freeze-drying

Introduction. Scientific literature indicates that an imbalance between members of the intestinal microbial community and the host organism leads to dysbiosis (Gujvinska and Paliy, 2018; Floch et al., 2011; Khyzhniak, 2017). It should be noted that the use of antibiotics, various pharmaceuticals, and chemotherapy leads to the development of diseases of the gastrointestinal tract and other organs and systems of the animal body (Gujvinska, 2015, 2019; Starovoitova et al., 2012). As a result of scientific research, veterinary medicine has been enriched with biological bacterial preparations that have proven to be effective in the prevention and treatment of gastrointestinal diseases (Roberfroid, 2000).

According to researchers, until recently, only probiotic drugs were available on the market. Today, the range of probiotic drugs is expanding (Vastano et al., 2013; Ohland and MacNaughton, 2010). Domestic medical science has not ignored probiotics and has contributed its priority materials to the world of medical science. Special merits in this area are Kovalenko N. K., Kigel N. F., Shenderov B. Ya., Pidhorskykh V. S., and others. Research institutes have published many works on probiotic drugs.

Recently, complex biological preparations based on probiotics have become increasingly popular. Many scientists claim that symbiotics are preparations containing a combination of probiotic cultures and prebiotics (Krupytska and Kaprelyants, 2016; Krupytska et al., 2018; Candela et al., 2010; Vitali et al., 2010). From the above, it can be concluded that the effect of symbiotics is the synergistic effect of living bacteria and non-living biologically active factors (Kianifar et al., 2014; Maydeo, 2010; Samuylenko et al., 2011). With the help of symbiotics, probiotic microorganisms colonize the epithelium of the gastrointestinal tract of animals, and their own microbiota is stimulated.

The work aimed to develop a technology for the production of a symbiotic biologically active supplement for animals based on *Lactobacillus* and *Bifidobacterium*.

Materials and methods. The development of technological regulations for the production of a symbiotic biologically active supplement for animals based on *Lactobacillus* and *Bifidobacterium* was carried out in the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine'. Three pilot batches of the symbiotic drug were produced. To create the symbiotic, we considered the following cultures: *Bifidobacterium bifidum* No. 3, *Bifidobacterium longum* No. 5, *Lactobacillus plantarum* No. 7-317, *Bifidobacterium adolectentis* No. 17-316, *Lactobacillus casei* No. 12. The bacteria were cultured on nutrient media in various combinations.

Comparing the level of the main biological parameters (number of microbial cells, acid formation activity, milk coagulation rate, etc.), *Bifidobacterium*

adolectentis No. 17-316 and *Lactobacillus plantarum* No. 7-317 strains were selected for the production of the symbiotic. Additional prebiotic substances are inulin, lactose, fructose, and starch.

Based on the research, a scheme for the production of a symbiotic biologically active supplement for animals was developed. Scheme of the technological process of symbiotic production: production of nutrient media and working solutions; cultivation of lacto- and bifidobacteria cultures for the preparation of a symbiotic biological additive for animals; freeze-drying of lacto- and bifidobacteria cultures for a symbiotic biological additive for animals; obtaining mass for the animal symbiotic; control of the finished product before release; labeling, packaging, transportation, storage of a symbiotic biological supplement for animals.

Lactobacilli and bifidobacteria were cultured on De Man–Rogosa–Sharpe agar (MRS agar), Blaurock medium, and skim milk for 24–48 h at a temperature 37 °C.

Microbiological control of the symbiotic was carried out at all stages of cultivation of probiotic cultures from the stage of bacterial recovery to the stage of control of the finished product. Control was performed by Gram staining of smears. Bifidobacteria and lactobacilli are colored purple.

Freeze-drying of bifidobacteria and lactobacilli was carried out on the LZ-4527 unit. The drying was performed according to the following technological regime: the temperature was increased from –72 °C to +26 °C, and the duration of drying of bacteria was from 26 h to 28 h.

The following composition of the symbiotic is proposed: dry biomass of *Lactobacillus plantarum* No. 7-317 and *Bifidobacterium adolectentis* No. 17-316, inulin, starch, lactulose, fructose. The content of lyophilized bacteria in the symbiotic supplement is not less than 10^8 CFU.

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

Appearance and color were determined visually.

Microbiological purity was determined in accordance with DSTU 4483:2005 'Veterinary Immunobiological Preparations. Methods for Determination of Bacterial and Fungous Contamination' (DSSU, 2005). The symbiotic should not be contaminated with bacterial and fungal microflora. The symbiotic should not contain any microflora other than lacto- and bifidobacteria.

To determine the number of live microbial cells in 1 dose of the symbiotic drug, the contents of the sachet were dissolved in 0.9% sodium chloride solution at a rate of 1.0 ml per 1 dose of the drug. The number was then determined by serial dilutions of the resulting suspension in saline followed by inoculation of 0.1 cm^3 of bacteria from 10^6 dilutions onto MRS agar and Blaurock medium.

The biochemical activity was determined by a conventional method. The symbiotic was inoculated into test tubes with skim milk (inoculation dose — 0.2 cm^3 per 5 cm^3 of milk). The inoculated symbiotic should curdle the milk within 48–72 hours with the formation of a characteristic solid clot without gas puffs. A symbiotic that does not meet the requirements is rejected.

The symbiotic drug should be harmless to white mice weighing 20 ± 1 g when administered orally in an amount corresponding to one dose of freeze-dried drug. The symbiotic was dissolved in 0.9% sodium chloride solution at the rate of 0.5 ml per dose. The resulting solution was orally administered to 12 mice weighing 20 ± 1 g into the stomach (using a special nozzle on a 1 ml syringe) — 0.5 ml each. The mice were observed for 21 days.

Experiments on animals were conducted following the recommendations of the 'European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes' (CE, 1986) and Council Directive 2010/63/EU (CEC, 2010), and in accordance with Art. 26 of the Law of Ukraine No. 3447-IV of 21.02.2006 'About protection of animals from cruel treatment' (VRU, 2006) and basic bioethical principles (Simmonds, 2017). The research program was reviewed and approved by the Bioethics Committee of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' under the current procedure.

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 and discussion. To create a symbiotic dietary supplement for animals, according to the results of preliminary studies, we have selected *Lactobacillus plantarum* No. 7-317 and *Bifidobacterium adolectentis* No. 17-316 as the most promising probiotic cultures. Additionally, the product contains prebiotics — inulin, starch, and lactulose — which accelerate, stabilize, and enhance the vital activity of lactic acid and bifidobacteria in the gastrointestinal tract.

A formulation of a symbiotic dietary supplement was developed: a mixture of freeze-dried cultures of *Lactobacillus plantarum* No. 7-317 and *Bifidobacterium adolescentis* No. 17-316 (55–65%), inulin (1.0–2.5%), lactose (1.0–2.5%), fructose (1.0–2.5%), starch (the rest). The content of lyophilized probiotic bacteria in the finished product is not less than 10^8 CFU. Three pilot batches of the symbiotic dietary supplement for animals were prepared for the experiments (Table 1).

Based on literature data and our research, the most promising sample of symbiotic No. 2 was obtained (Table 2).

Table 1 — Formulation of a symbiotic dietary supplement

Experimental series	Formulation of a symbiotic dietary supplement, %				
	Bacterial biomass	Inulin	Lactulose	Fructose	Starch
1	40.0±2.5	1.0±0.02	1.0±0.02	1.0±0.02	57.0±3.06
2	50.0±3.0	1.5±0.03	1.5±0.03	1.5±0.03	45.0±2.17
3	60.0±3.2	2.5±0.05	2.5±0.05	2.5±0.05	32.5±2.01

Table 2 — Biological characteristics of the bacteria included in the symbiotic preparation

Indicators	Bacteria that are part of a symbiotic dietary supplement	
	Lactobacillus plantarum No. 7-317	*Bifidobacterium adolescentis* No. 17-316
Number of live bacteria, CFU/cm^3	$3.7 \pm 0.12 \times 10^8$	$4.1 \pm 0.17 \times 10^9$
Acid formation activity, °T	250 ± 5	230 ± 7
Milk fermentation rate, h	18–24	18–24

Table 2 shows that in the experimental series, the number of live bacteria ranged from $3.7 \pm 0.12 \times 10^8$ CFU/cm^3 to $4.1 \pm 0.17 \times 10^9$ CFU/cm^3, and the acid formation activity ranged from 230 ± 7 °T to 250 ± 5 °T. It should be noted that the rate of milk fermentation for lactobacilli and bifidobacteria was within 18–24 h.

The next stage of our work was to develop a technology for the production of a symbiotic biologically active additive for animals. The scheme of the manufacturing technology:

Preparation of nutrient media for the cultivation of bacteria *Lactobacillus plantarum* No. 7-317 and *Bifidobacterium adolescentis* No. 17-316

↓

Sterilization of culture media 110 ± 1.0 °C for 20 min

↓

Cooling of culture media up to 30 °C

↓

Introduction of 5% daily cultures *Lactobacillus plantarum* No. 7-317 and *Bifidobacterium adolescentis* No. 17-316 in a ratio of 1:1

↓

Cultivation of bacterial mass at a temperature of 37.0 ± 1.0 °C for 48 h

↓

Freeze-drying of cultures of lacto- and bifidobacteria

↓

Obtaining mass for symbiotic biologically active supplement for animals

↓

Control of the finished product before its release

↓

Labeling, packaging, transportation, storage of symbiotic biologically active supplement for animals

During the production of a symbiotic, one of the most important conditions is the control of the technological process. A symbiotic dietary supplement for oral use is controlled by the following indicators: appearance, microbiological purity (bacterioscopic control and absence of foreign microflora); harmlessness; specific activity (number of live bacteria in one dose of the symbiotic), and acid formation activity of cultures (Table 3).

Table 3 — Standard quality indicators of symbiotic dietary supplements for animals

Indicators	Obtained results	Control methods
Description	Powder of white-cream color	Visually
Microbiological purity of a symbiotic dietary supplement	The product was not contaminated with bacterial and fungal microflora and contained only lacto- and bifidobacteria	In accordance with DSTU 4483:2005 (DSSU, 2005)
Harmlessness	Symbiotic was harmless when tested on white mice	In accordance with the TUU
Specific activity	The number of lactobacilli was ≥ 10^8, the number of bifidobacteria was ≥ 10^8.	
Biochemical activity of the drug	The diluted preparation coagulates skim milk in test tubes within 18–24 h. Incubation at a temperature of 37 ± 0.5 °C	

The test results showed that it was a white-cream-colored powder. The symbiotic dietary supplement was not contaminated with bacterial and fungal microflora, containing only lacto- and bifidobacteria. Based on the experiments, it is clear that twelve white mice weighing 20.0 ± 1.0 g remained alive and healthy for 21 days after oral administration of the diluted symbiotic dietary supplement at a dose of 0.5 cm^3. During the experiments, it was found that the inoculated symbiotic on skim milk curdled it within 18–24 h with the formation of a characteristic solid clot without gas puffs. The number of

microbial cells in 1 cm^3 of the symbiotic was: lactobacilli $\geq 10^8$, bifidobacteria $\geq 10^8$. The symbiotic dietary supplement was packed in plastic sachets, labeled, and then placed in boxes of 10. The symbiotic was stored in a dry place, protected from direct light, at a temperature between +4 °C and +8 °C.

Conclusion. The formulation of a symbiotic dietary supplement was developed: a mixture of freeze-dried cultures of *Lactobacillus plantarum* No. 7-317 and *Bifidobacterium adolescentis* No. 17-316 (55–65%), inulin (1.0–2.5%), lactose (1.0–2.5%), fructose (1.0–2.5%), starch (42–27.5%).

References

Candela, M., Maccaferri, S., Turroni, S., Carnevali, P. and Brigidi, P. (2010) 'Functional intestinal microbiome, new frontiers in prebiotic design', *International Journal of Food Microbiology*, 140(2–3), pp. 93–101. doi: 10.1016/j.ijfoodmicro.2010.04.017.

CE (The Council of Europe). (1986) *European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes*. (European Treaty Series, No. 123). Strasbourg: The Council of Europe. Available at: https://conventions.coe.int/treaty/en/treaties/html/123.htm.

CEC (The Council of the European Communities) (2010) 'Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes', *The Official Journal of the European Communities*, L 276, pp. 33–79. Available at: http://data.europa.eu/eli/dir/2010/63/oj.

DSSU [State Committee for Technical Regulation and Consumer Policy] (2005) DSTU 4483:2005. *Veterinary Immunobiological Preparations. Methods for Determination of Bacterial and Fungous Contamination [Preparaty veterynarni imunobiolohichni. Metody vyznachannia bakterialnoi i hrybkovoi kontaminatsii]*. Kyiv: Derzhspozhyvstandart Ukrainy. [in Ukrainian].

Floch, M. H., Walker, W. A., Madsen, K., Sanders, M. E., Macfarlane, G. T., Flint, H. J., Dieleman, L. A., Ringel, Y., Guandalini, S., Kelly, C. P. and Brandt, L. J. (2011) 'Recommendations for probiotic use—2011 Update', *Journal of Clinical Gastroenterology*, 45, pp. 168–171. doi: 10.1097/MCG.0b013e318230928b.

Gujvinska, S. O. (2015) 'Determination of storage stability of the probiotics' [*Vyznachennia stabilnosti probiotyka u protsesi zberihannia*], *Veterinary Medicine [Veterynarna medytsyna]*, 101, pp. 208–211. Available at: https://jvm.kharkov.ua/sbornik/101/10_61.pdf. [in Ukrainian].

Gujvinska, S. (2019) 'Study of the biological properties of lactobacilli and bifidobacteria', *4th Annual BTRP Ukraine Regional One Health Research Symposium*, Kyiv, Ukraine, 20–24 May 2019: abstr. Kyiv, p. 328.

Gujvinska, S. O. and Paliy, A. P. (2018) 'Determination of antagonistic and adhesive properties of *Lactobacterium* and *Bifidobacterium*' [Vyznachennia antahonistychnykh ta adhezyvnykh vlastyvostei laktobakterii ta bifidobakterii], *Microbiological Journal [Mikrobiolohichnyi Zhurnal]*, 80(1), pp. 36–44. doi: 10.15407/microbiolj80.01.036. [in Ukrainian].

Khyzhniak, O. S. (2017) *Development of the Composition and Biotechnology of the Multipurpose Probiotic Preparation [Rozrobka skladu ta biotekhnolohii otrymannia kompleksnoho probiotychnoho preparatu].* The dissertation thesis for the scientific degree of the candidate of pharmaceutical sciences. Kharkiv: National Pharmaceutical University. Available at: https://nrat.ukrintei.ua/searchdoc/0417U002705. [in Ukrainian].

Kianifar, H., Ahanchian, H., Grover, Z., Jafari, S., Noorbakhsh, Z., Khakshour, A., Sedaghat, M. and Kiani, M. (2014) 'Synbiotic in the management of infantile colic: A randomised controlled trial', *Journal of Paediatrics and Child Health*, 50(10), pp. 801–805. doi: 10.1111/jpc.12640.

Krupytska, L. O. and Kaprelyants, L. V. (2016) 'Fatty acid composition of dietary supplements with the inclusion of propionic acid bacteria' [Zhyrnokyslotnyi sklad biolohichno aktyvnykh dobavok z vkliuchenniam propionovokyslykh bakterii], *Visnyk of Lviv University. Biological Series [Visnyk Lvivskoho universytetu. Seriia biolohichna]*, 73, p. 442. Available at: http://nbuv.gov.ua/UJRN/VLNU_biol_2016_73_123. [in Ukrainian].

Krupytska, L. O., Kaprelyants, L. V., Kylymenchuk, O. O. and Velichko, T. O. (2018) 'Non-waste biotechnology of obtaining a symbiotic and a metabolite on the basis of *Bifidobacterium longum* – Ya 3 and *Propionibacterium shermanii* – 4' [Bezvidkhodna biotekhnolohiia otrymannia symbiotyka i metabiotyka na osnovi *Bifidobacterium longum* – Ya 3 ta *Propionibacterium shermanii* – 4], *Scientific Messenger of Lviv National University of Veterinary Medicine and Biotechnologies named after S. Z. Gzhytskyj. Series: Food Technologies [Naukovyi visnyk Lvivskoho natsionalnoho universytetu veterynarnoi medytsyny ta biotekhnolohii imeni S. Z. Gzhytskoho. Seriia: Kharchovi tekhnolohii]*, 20(85), pp. 148–154. doi: 10.15421/nvlvet8527. [in Ukrainian].

Maydeo, A. (2010) 'Lactitol or lactulose in the treatment of chronic constipation: Result of a systematic', *Journal of the Indian Medical Association*, 108(11), pp. 789–792. PMID: 21510584.

Ohland, C. L. and MacNaughton, W. K. (2010) 'Probiotic bacteria and intestinal epithelial barrier function', *American Journal of Physiology. Gastrointestinal and Liver Physiology*, 298(6), pp. 807–819. doi: 10.1152/ajpgi.00243.2009.

Roberfroid, M. B. (2000) 'Prebiotics and probiotics: Are they functional foods?', *The American Journal of Clinical Nutrition*, 71(6), pp. 1682-1687. doi: 10.1093/ajcn/71.6.1682S.

Samuylenko, A. Ya., Shkolnikov, Ye. E., Pavlenko, I. V., Rayewsky, A. A., Eremets, V. I., Skichko, N. D., Anisimova, L. V. and Andrianova, E. N. (2011) 'Development of symbiotic lysine-synthesizing preparation for growing broiler chickens' [Razrabotka simbioticheskogo preparata na osnove *E. coli* VL-613 i primenenie ego v broylernom ptitsevodstve], *Veterinary Medicine [Veterynarna medytsyna]*, 95, pp. 183–185. Available at: http://nbuv.gov.ua/UJRN/vetmed_2011_95_80. [in Russian].

Simmonds, R. C. (2017) 'Chapter 4. Bioethics and animal use in programs of research, teaching, and testing', in Weichbrod, R. H., Thompson, G. A. and Norton, J. N. (eds.) *Management of Animal Care and Use Programs in Research, Education, and Testing*. 2nd ed. Boca Raton: CRC Press, pp. 35–62. doi: 10.1201/9781315152189-4.

Starovoitova, S. O., Skrotska, O. I., Penchuk, Yu. M. and Pyroh, T. P. (2012) *The Technology of Probiotics [Tekhnolohiia probiotykiv]*. Kyiv: National University of Food Technologies. ISBN 9789666121274. Available at: https://dspace.nuft.edu.ua/handle/123456789/10384. [in Ukrainian].

Van Emden, H. F. (2019) *Statistics for Terrified Biologists*. 2nd ed. Hoboken, NJ: John Wiley & Sons. ISBN 9781119563679.

Vastano, V., Capri, U., Candela, M., Siciliano, R. A., Russo, L., Renda, M. and Sacco, M. (2013) 'Identification of binding sites of Lactobacillus plantarum enolase involved in the interaction with human plasminogen', *Microbiological Research*, 168(2), pp. 65–72. doi: 10.1016/j.micres.2012.10.001.

Vitali, B., Ndagijimana, M., Cruciani, F., Carnevali, P., Candela, M., Guerzoni, M. E. and Brigidi, P. (2010) 'Impact of a synbiotic food on the gut microbial ecology and metabolic profiles', *BMC Microbiology*, 10(1), p. 4. doi: 10.1186/1471-2180-10-4.

VRU (Verkhovna Rada Ukrainy) (2006) 'Law of Ukraine No. 3447-IV of 21.02.2006 'About protection of animals from cruel treatment' [Zakon Ukrainy № 3447-IV vid 21.02.2006 'Pro zakhyst tvaryn vid zhorstokoho povodzhennia'], *News of the Verkhovna Rada of Ukraine [Vidomosti Verkhovnoi Rady Ukrainy]*, 27, art. 230. Available at: https://zakon.rada.gov.ua/laws/3447-15. [in Ukrainian].