

LIFE FACTORY ENGINEERS – PROBIOTIC MICROORGANISMS IN JOY DAY – FOREST FRUITS DRINK

Summary

Probiotic microorganisms are an important element of the diet not only of humans but also of animals. Their presence in the body is associated with its proper functioning. Numerous, although not fully recognized relationships between pro-health microorganisms and body systems, organs and cells are increasingly complex. Probiotic microorganisms play the role of specific engineers, performing many important functions (directly or indirectly) in the factory of life, which is a properly functioning organism. The aim of this study was to characterize the JOY DAY - forest fruits liquid probiotic drink of Living Food Sp. z o.o. The product is one of many from the large range of biofunctional products offered by the manufacturer and is a fluid obtained through the fermentation process of plant raw materials by carefully selected strains of lactic acid bacteria. First, the morphology of bacterial colonies included in the product and cell phenotype was assessed. The cell phenotype was typical and corresponded to the species composition of microorganisms declared by the manufacturer. The number of probiotic microorganisms and the stability of this indicator proving the quality of the product during the storage for 12 months were also determined. Another important feature of the product that proves its health-promoting properties is the antimicrobial activity against potentially pathogenic microorganisms, what was demonstrated against bacteria from the species *Listeria monocytogenes*, *Escherichia coli* and *Salmonella typhimurium*.

Keywords: probiotics, postbiotics, vegetable raw materials, fermentation

INŻYNIEROWIE „FABRYKI ŻYCIA” – DROBNOUSTROJE PROBIOTYCZNE W NAPOJU JOY DAY – OWOCE LASU

Streszczenie

Drobnoustroje probiotyczne to ważny element diety nie tylko człowieka, ale także zwierząt. Z ich obecnością w organizmie wiąże się jego prawidłowe funkcjonowanie. Liczne, choć nie do końca poznane, zależności między drobnoustrojami prozdrowotnymi a układami, narządami, komórkami organizmu są coraz bardziej złożone. Drobnoustroje probiotyczne pełnią rolę swoistych inżynierów, pełniących wiele ważnych funkcji (w sposób pośredni bądź bezpośredni) w „fabryce życia” jaką jest prawidłowo funkcjonujący organizm. Badany napój probiotyczny JOY DAY – owoce lasu firmy Living Food Sp. z o.o. jest jednym z wielu preparatów biofunkcyjnych oferowanych przez producenta. Jest to produkt płynny uzyskany na drodze procesu fermentacji surowców roślinnych przy udziale skrupulatnie wyselekcjonowanych szczepów bakterii fermentacji mlekowej. W pierwszej kolejności oceniono morfologię kolonii bakterii wchodzących w skład produktu i fenotyp komórek, który był typowy i odpowiadał zadeklarowanemu przez producenta składowi gatunkowemu drobnoustrojów. Następnie określono liczebność drobnoustrojów probiotycznych oraz stabilność tego wskaźnika świadczącego o jakości produktu w czasie przechowywania przez 12 miesięcy. Ważną cechą produktu, świadczącą o jego prozdrowotnym charakterze jest aktywność przeciwdrobnoustrojowa wobec mikroorganizmów potencjalnie patogennych, co też wykazano dla bakterii z gatunku *Listeria monocytogenes*, *Escherichia coli* i *Salmonella typhimurium*.

Słowa kluczowe: probiotyki, postbiotyki, surowce roślinne, fermentacja

1. Introduction

Microorganisms are an integral and inseparable element of human life, they inhabit many environments from the human body, through plants, animals to the soil and water. They determine the proper functioning of all living creatures, being an integral part of them, as well as provide appropriate living conditions for them. Additionally, they are specific indicators, markers of natural environment quality. Disturbance of their biodiversity often results in unfavorable changes of a very wide range.

The human body is naturally inhabited by desired, undesirable and opportunistic microorganisms (gastrointestinal tract, sexual system, skin). The state of microbiological

balance ensures and even, as some claim, determines the protection and proper functioning of the organism. Qualitative and quantitative disturbances of microorganisms, and above all, a significant decrease in the number of the desired ones (dysbiosis), has an adverse and multidimensional impact on the functioning of many systems and is a cause of many civilization diseases. The condition of dysbiosis is a consequence of inadequate diet, the effect of abuse of pharmacological agents (mainly antibiotics and paracetamol) and increasingly often dietary supplements, intake of stimulants, limited physical activity and permanent stress. Probiotic microorganisms are one of the possibilities to effectively support the functioning of the organism and eliminate the effects of adverse environmental impact on the

human organism. A probiotic is defined as a single or mixed culture of living microorganisms, which, when taken in an appropriate dose, have a positive effect on the host organism, thanks to ensuring the proper balance of the microbiota that inhabits the digestive tract. Probiotics include some strains of *Bifidobacterium*, *Lactobacillus* and yeasts [1-3]. They are found in fermented food products, in products having the status of drugs and in food supplements. There is a lot of scientific evidence confirming that the condition of intestinal microbiota affects the protection of the organism against gastrointestinal diseases and ailments from other systems [1, 2]. Thanks to the use of probiotic bacteria cultures, the growth of desired microorganisms is stimulated, pathogenic microorganisms are degraded and natural defense systems of the body are strengthened [1-3].

The aim of the study was the initial characterization of the liquid preparation JOY DAY - forest fruits (Living Food Sp. z o.o.) containing fermented plant materials and microorganisms with probiotic potential.

2. Materials and Methods

2.1. Materials. Composition of the probiotic drink JOY DAY - forest fruits

The probiotic drink JOY DAY - forest fruits is a product which is a result of fermentation of plant raw materials such as the leaves of blackberry, black currant, wild strawberry, horsetail herb, black currant and blackberry fruit, enriched with apple juice (Living Food Sp. z o.o., Trzciel, Poland). The fermentation process was conducted by a unique consortium of probiotic bacteria, which are characterized in Table 1.

Table 1. Characteristics of probiotic bacteria strains in the JOY DAY – forest fruits drink composition
Tab. 1. Charakterystyka szczepów bakterii probiotycznych wchodzących w skład napoju JOY DAY – owoce lasu

Probiotic strain	Colony phenotype and cell morphology	Distinctive features of the strain
<i>Lactobacillus rhamnosus</i> GG	Gram positive bacteria, chains milky white, tear-like, embedded in the substrate	modulation of the immune response
<i>Lactobacillus rhamnosus</i> LR 04		modulation of the immune response
<i>Lactobacillus rhamnosus</i> LR 05		modulation of the immune response
<i>Lactobacillus acidophilus</i> LA 1		contributes to the alleviation of ulcerative colitis
<i>Lactobacillus plantarum</i> LP 01		alleviation of symptoms of irritable bowel syndrome (IBS), increased frequency of bowel movements in children with functional constipation; strong antimicrobial properties, including against <i>E. coli</i> strains
<i>Lactobacillus plantarum</i> LP 02		alleviation of IBS symptoms
<i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i>		positive effect on intestinal peristalsis
<i>Lactobacillus fermentum</i> LF 2		ability to synthesize high concentrations of exopolysaccharide (EPS), thus contributing to lowering blood cholesterol levels; anticancer, anti-ulcer and immunomodulatory properties
<i>Lactobacillus casei</i> 101/37		positive effect on intestinal peristalsis
<i>Bifidobacterium breve</i> BL 10	Gram positive bacteria, chains, tiny brown colonies with a white coating	alleviation of IBS symptoms
<i>Bifidobacterium breve</i> Bbr 8		alleviation of IBS symptoms
<i>Bifidobacterium longum</i> BL 03		positive effect on intestinal peristalsis
<i>Bifidobacterium animalis</i> ssp. <i>lactis</i> Bi MDX		improving intestinal peristalsis alleviation of IBS symptoms
<i>Streptococcus thermophilus</i> 9Y	Cocci, gram positive, beads, fine, white	modulation of the immune response, maintaining remission in patients with ulcerative colitis and alleviation of IBS symptoms
<i>Streptococcus thermophilus</i> Z 57		alleviation of IBS symptoms

Source: own study / Źródło: opracowanie własne

– Evaluation of antimicrobial activity

In order to determine the antagonism of products containing probiotic microorganisms to indicator microorganisms, tests were carried out, which included preparation of indicator microorganisms and testing of the activity of isolates by the well method.

The indicator microorganisms listed in Table 2 were used in the experiment. In the first place the indicator strains were transferred to the broth medium with 2% glucose to multiply the biomass. The cultures were carried out at the temperature of 30-37°C for 24 hours (depending on the species). Then, in order to obtain a clear bacteria layer, the liquefied agar medium was inoculated with 10% (v/v) 24-hour indicator culture and poured on Petri dishes. 20 µL of the tested probiotic drink JOY DAY - forest fruits was applied on the surface of solid medium inoculated with indicator microorganisms. The incubation was carried out at 30-37°C for 24 hours under anaerobic or relatively anaerobic conditions (depending on the species). Next, the diameters of the inhibition zone or growth limitation of indicator bacteria were measured. The inhibition of the growth of indicator microorganisms, manifested by complete brightness around the site of culture liquid application, was indicative of the bactericidal activity of the tested strain. The bacteriostatic properties were determined on the basis of reduced bacterial growth density (indicator strain growth limitation).

B. Analytical methods

– Qualitative and quantitative determination of postbiotic compounds

The quantitative and qualitative composition of probiotic drink was determined by high-performance liquid chromatography (HPLC) using the Agilent Technologies 1200 series chromatograph. The chromatograph system included: automatic sample feeder G1329B, double pump G1312B with refractometer detector G1362A. The separation was made on Rezex ROA column. 10 µL samples were applied to the column, the mobile phase was 0.005 N H₂SO₄. The flow rate was 0.6 mL/min at 40°C. Identification of the determined compounds was carried out using the external standard method, measuring the surface area under the peaks (measurement and computer integration with ChemStation for LC 3D systems, Agilent).

– Storage tests

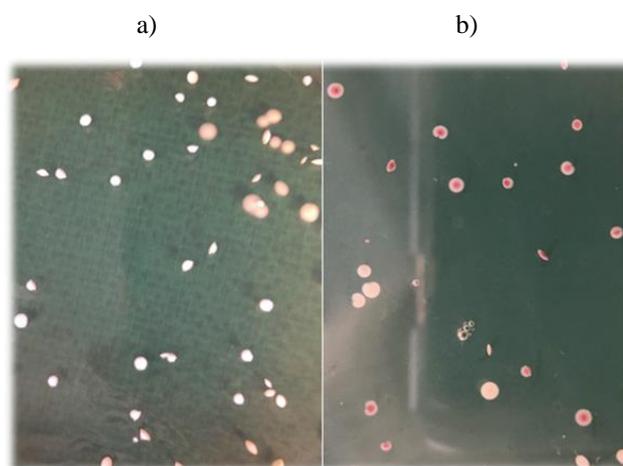
Probiotic drink JOY DAY - forest fruits was stored at room temperature and refrigerated for 12 months. Microbiological analysis was performed at time intervals - on the second day after the confection (t₀), 1 month after production (t₁), 3 months after production (t₃), 6 months after production (t₆) and 12 months after production (t₁₂). Microbiological analyses of the products were performed in the direction: the total number of psychophilic and mesophilic microorganisms, the total number of psychophilic and mesophilic moulds and yeasts, the number of lactic acid fermentation bacteria, the number of bacteria of *Bifidobacterium* genus, the presence and number of spore-forming bacteria (including *Bacillus* genus), the presence and number of anaerobic bacteria (including *Clostridium* genus), the presence of *Listeria monocytogenes*, the presence of *Salmonella* bacteria and the presence of coliforms.

3. Results

3.1. Probiotic products quality evaluation

One of the key factors that determines the commercial success of a product is its quality. In the case of liquid probiotic

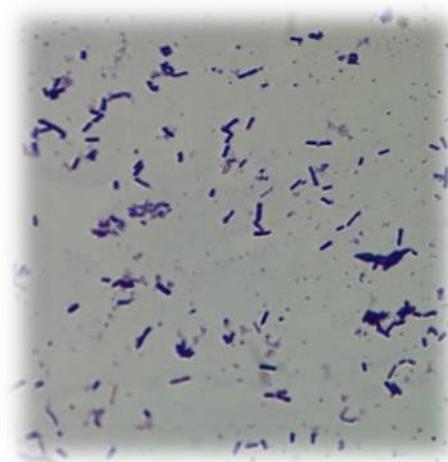
products, the quality is determined by the number of microorganisms over the entire shelf life of the product, their vitality, as well as the preserved sensory characteristics of the product. The appropriate number of probiotic microorganisms in the product determines its biofunctionality and also determines its market position. The tested product was analyzed for the number of lactic acid fermentation bacteria, *Bifidobacterium* and also for undesirable microorganisms such as yeasts, moulds, bacteria of the genus *Salmonella sp.*, *Listeria sp.* or coliforms. The analysis shows that the tested product meets the requirements for probiotic products. The number of microorganisms from *Lactobacillus* genus was 2.6 x 10⁷ cfu/ml, while *Bifidobacterium* 4.8 x 10⁶ cfu/ml. Moreover, no other microbial groups were found. The bacterial colony phenotype was also analyzed, and it was typical and at the same time indicative of good physiological condition of the cells (Table 1). It is worth pointing out that well visible colonies with an intense color and typical smell indicate good condition of the microorganisms (Fig. 1 and Fig. 2).



Source: own study / Źródło: opracowanie własne

Fig. 1. Growth of bacterial colonies on solid media: a) *Lactobacillus*, b) *Bifidobacterium*

Rys. 1. Wzrost kolonii bakterii na podłożu stałym: a) *Lactobacillus*, b) *Bifidobacterium*



Source: own study / Źródło: opracowanie własne

Fig. 2. Microscopic image of the probiotic drink JOY DAY – forest fruits (magnification 1000 x)

Rys. 2. Obraz mikroskopowy napoju probiotycznego JOY DAY – owoce lasu (powiększenie 1000 x)

Small, tiny, poorly visible colonies may indicate that the constituent microorganisms were exposed to environmental stress either at the culture stage or during the analysis. The physiological state of the cells of probiotic microorganisms is not negligible. Even when the number of probiotic cells in the product is high, poor vitality may consequently prevent the desired function in the body from being performed.

3.2. Characteristics of the post fermentation fluid

Each group of microorganisms is characterized by a specific profile of metabolites that results from their metabolism. The microorganisms take nutrients from the substrate to maintain their life processes, including reproduction. By metabolizing the components of the substrate, they synthesize metabolites such as organic acids, alcohols or glycerol. Metabolites secreted outside the cell are used by microorganisms capable of producing them, as part of a system that protects the cell or ensures homeostasis. The presence of probiotic microbial metabolites in the finished products is important in terms of their biofunctionality and guarantees their stability and persistence. The most recent literature indicates biofunctionality not only of the microbial metabolites themselves, but also attributes these properties to cell wall components or proteins present in lysates of probiotic strains, calling all these elements postbiotics [4]. Short chain fatty acids such as butyric acid and lactic acid are considered the basic postbiotics. Both compounds have very beneficial effects on the digestive tract. They restore the structural and functional integrity of intestines and proper gastrointestinal motility and accelerate the regeneration of damaged intestinal epithelial cells. Additionally, they increase the absorption of sodium and water, thanks to which they are important in mitigating the effects of diarrhea of different origin. Moreover, research has shown that butyric acid has anti-inflammatory properties as well as protects against the translocation of bacteria from the intestines into the bloodstream [5].

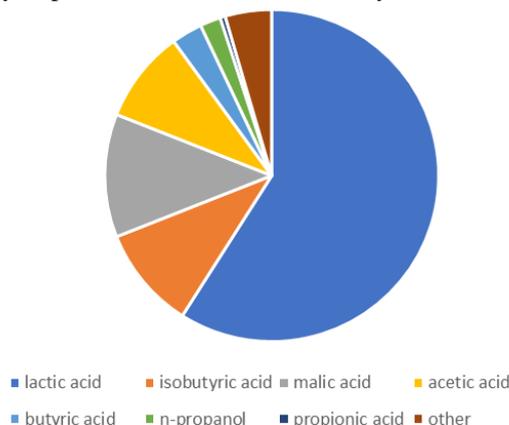
It should be pointed out that the probiotic drink JOY DAY - forest fruits, in addition to living and vital probiotic microorganisms, is a mixture of about 40 chemical compounds, of which 11 (valeric acid, heptanoic acid, acetic acid, benzoic acid, ethyl ester, 2-methyl-butyl ester, 2-ethyl-hexyl, methyl ester, benzoic aldehyde, 1,2-propanediol and dodecanol) has proven health-promoting properties including antiseptic ones. The compound present in the highest concentration is lactic acid, which is closely related to the metabolism of lactic acid fermentation bacteria (Fig. 3).

Another interesting, and recently functioning term is paraprobiotic, i.e., inactivated probiotic bacteria cells. Paraprobiotics come from microorganisms that completely lost their vitality (DNA degradation, cell membrane rupture, mechanical damage to the cell envelope, inactivation of key enzymes and deactivation of cell membrane selectivity). Paraprobiotics modulate intestinal microbiota, inhibit the growth of pathogens, support the therapy of colitis or inhibit the growth of gastrointestinal cancers. Additionally, paraprobiotics have a better immunomodulation effect than probiotics. Due to the low survival rate of some probiotics in the gastrointestinal tract, paraprobiotics seem to be an interesting topic for further research [6].

A. Antimicrobiological activity

The antagonistic activity of probiotic bacteria is closely related to the synthesis of specific products inhibiting the growth of undesirable microorganisms (e.g. organic acids),

which act synergistically. It should be noted that the antimicrobial activity of bacteria is a strain-dependent characteristic resulting from specific interaction between the bacterial and indicator strains. The mechanism of antagonistic activity of probiotic bacteria is not exactly known.



Source: own study / Źródło: opracowanie własne

Fig. 3. Profile of chemical compounds of postbiotic nature included in the probiotic drink JOY DAY – forest fruits

Rys. 3. Profil związków chemicznych o charakterze postbiotyków wchodzących w skład napoju probiotycznego JOY DAY – owoce lasu

It is assumed that it consists in changing environmental conditions under the influence of the production of organic acids and other metabolites unfavorable to the growth of undesirable microorganisms. Such environmental factors include: temperature, pH, composition of the medium in which lactic acid bacteria produce products with antifungal and antibacterial activity (Table 2, Fig. 4).

The probiotic drink JOY DAY – forest fruits shows the highest antibacterial activity against *Listeria monocytogenes*, *Escherichia coli* and *Salmonella typhimurium*, 43, 39 and 38 mm, respectively. The highest antifungal activity was found against *Candida albicans* (39 mm). Importantly, the highest antimicrobial activity was shown by the fluid containing the biomass of probiotic bacteria together with their metabolites (postbiotics).

B. Quality stability of probiotic products during the storage

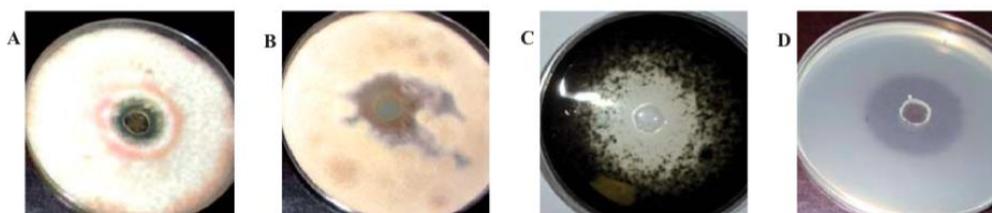
Quality and unique taste and smell characteristics are the main aspects that make consumers decide to buy a product. In order to maintain the quality advantage, it is essential to maintain microbiological purity, use high-quality organic raw materials, use unique sets of probiotic bacteria in the production process and then, after confectioning, maintain the assumed quality indicators until the end of shelf life. In the case of production of liquid probiotic products, it is crucial to maintain hygiene and sanitary regimes at all stages of production, as well as to precisely control the conditions of the production process according to a unique recipe. Each product intended for consumption has a specific expiry date. Therefore, it is important to know how the product changes and how durable it is during the storage. In the case of products containing probiotic microorganisms, the key indicator is the maintenance of an adequate number of microorganisms from the desired groups (Figs. 5, 6). The probiotic product tested was characterized by good microbiological stability during the storage, both in refrigerated conditions and at room temperature.

Table 2. Antimicrobiological activity of the probiotic drink JOY DAY – forest fruits
 Tab. 2. Aktywność antymikrobiologiczna napoju probiotycznego JOY DAY – owoce lasu

No	Indicator microorganisms	Probiotic drink *	Probiotic drink **
		Growth inhibition zone (mm)	
1.	<i>Clostridium difficile</i> ATCC 9689	24	17
2.	<i>Clostridium butyricum</i> ATCC 860	30	25
3.	<i>Listeria monocytogenes</i> ATCC 7644	43	11
4.	<i>Bacillus subtilis</i> ATCC 23857	24	15
5.	<i>Enterococcus faecalis</i> ATCC 29212	18	6
6.	<i>Staphylococcus aureus</i> ATCC 25923	27	32
7.	<i>Staphylococcus pyrogenes</i> ATCC 19615	31	18
9.	<i>Escherichia coli</i> ATCC 25922	38	21
10.	<i>Klebsiella pneumoniae</i> ATCC 31488	13	28
11.	<i>Proteus mirabilis</i> ATCC 12453	31	17
12.	<i>Salmonella typhimurium</i> ATCC 14028	39	18
13.	<i>Pseudomonas aeruginosa</i> ATCC 27853	0	0
14.	<i>Enterobacter aerogenes</i> ATCC 13048	12	8
17.	<i>Candida krusei</i> ATCC 14243	25	12
18.	<i>Candida albicans</i> ATCC 10231	39	16
19.	<i>Fusarium</i> sp.	21	11
20.	<i>Alternaria</i> sp.	17	9

* probiotic product containing biomass of probiotic bacteria and postbiotics, ** post-fermentation fluid free of probiotic bacteria biomass
 * produkt probiotyczny zawierający biomasę bakterii probiotycznych i postbiotyki, ** płyn pofermentacyjny pozbawiony biomasy bakterii probiotycznych

Source: own study / Źródło: opracowanie własne



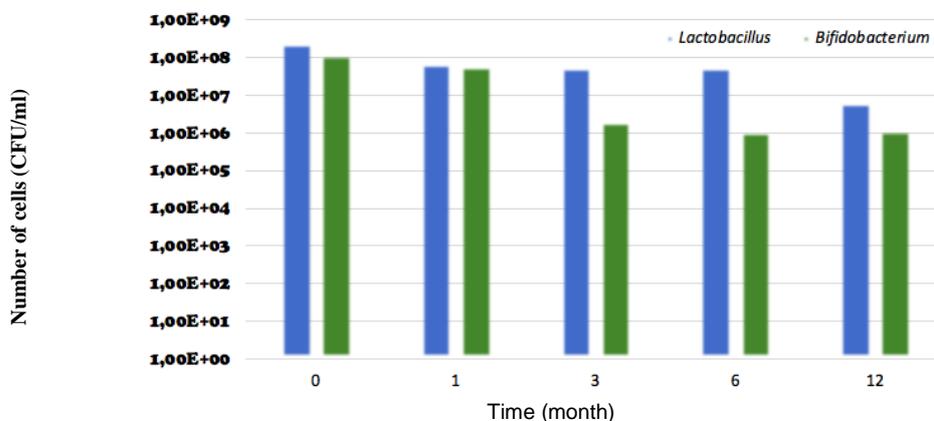
Source: own study / Źródło: opracowanie własne

Fig. 4. Antimicrobial effect of the probiotic product on moulds of the genus: A) *Fusarium* sp., B) *Alternaria* sp., C) bacteria *Clostridium butyricum*, D) *E. coli*

Rys. 4. Przeciwdrobnoustrojowy wpływ produktu probiotycznego na pleśnie z rodzaju: A) *Fusarium* sp., B) *Alternaria* sp., C) bakterie *Clostridium butyricum*, D) *E. coli*

Differences in microbial counts during the storage are visible in the 12 months after production and the results obtained suggest that the product should be stored refrigerated. *Lactobacillus* counts were 8.8×10^6 cfu/mL and *Bifidobacterium* 1.2×10^6 cfu/mL, one year after the production process. For com-

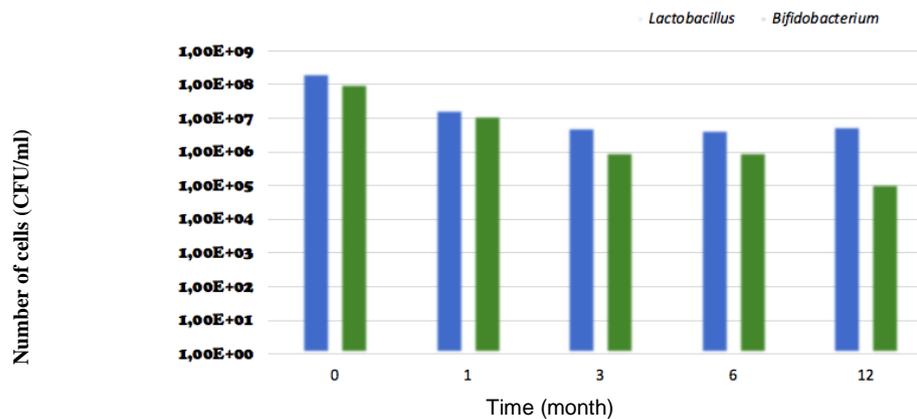
parison, the counts at room temperature were equal to 4.8×10^6 cfu/mL for *Lactobacillus* and 3.8×10^5 cfu/mL for *Bifidobacterium*. During the storage, just as immediately after the production process, no undesirable microorganisms of the genera *Salmonella*, *Listeria* and *Bacillus* or moulds were found.



Source: own study / Źródło: opracowanie własne

Fig. 5. Kinetics of changes in the number of lactic acid fermentation bacteria in the probiotic drink JOY DAY – forest fruits during cold storage

Rys. 5. Kinetyka zmian liczby bakterii fermentacji mlekowej wchodzących w skład napoju probiotycznego JOY DAY – owoce lasu w czasie przechowywania w warunkach chłodniczych



Source: own study / Źródło: opracowanie własne

Fig. 6. Kinetics of changes in the number of lactic acid fermentation bacteria in the probiotic drink JOY DAY – forest fruits during the storage at room temperature

Rys. 6. Kinetyka zmian liczby bakterii fermentacji mlekowej wchodzących w skład napoju probiotycznego JOY DAY – owoce lasu w czasie przechowywania w temperaturze pokojowej

To sum up, the preliminary characteristics of the probiotic product JOY DAY - forest fruits indicate that it may have a significant pro-health value. The probiotic microorganisms used in the fermentation process of raw materials were reliably selected from a pool of commercially available microorganisms on the basis of literature data and statements of producers. The raw materials subjected to the fermentation process are certified organic and their botanical selection is related to the purpose of the finished product. Additional value of the product is increased by the post-fermentation fluid containing postbiotics. The quality is ensured by a stable and relatively high number of microorganisms during the storage of the finished product.

Moreover, it should be pointed out that probiotic microorganisms are only one of many useful groups of microorganisms, and the human gastrointestinal tract is one of many niches in which biodiversity is increasingly disturbed. The fact of microbiological degradation concerns many other ecological niches, which are indirectly connected with human life. Inevitable progress of civilization, overexploitation of the environment associated with limited human awareness and knowledge in a degrading way affects the natural ecosystem. This fact requires the implementation of

corrective actions, including well thought-out and effective interference with the use of natural and organic methods.

4. References

- [1] Saarela M., Mogensen G., Rangne F., Mättö J., Mattila-Sandholm T.: Probiotic bacteria: safety, functional and technological properties. *J. Biotechnol.*, 2000, Vol. 84 (3): 197-215.
- [2] Markowiak P., Śliżewska K.: Effects of probiotics, prebiotics, and synbiotics on human health. *Nutrients*, 2017, Vol. 9 (9): 1021.
- [3] Manigandan T., Mangaiyarkarasi S.P., Hemalatha R., Hemalatha V.T., Murali N.P.: Probiotics, prebiotics and synbiotics - a review. *Biomedical and Pharmacology Journal*, 2012, Vol. 5 (2): 295-304.
- [4] Ruszkowski J., Szewczyk A., Witkowski J.: Przegląd doustnych prebiotyków, probiotyków, synbiotyków i postbiotyków dostępnych na polskim rynku aptecznym. *Farm. Pol.*, 2018, Vol. 74 (2): 114-122.
- [5] Banasiewicz T., Borycka-Kiciak K., Dobrowolska-Zachwieja A., Friediger J., Kiciak A., Krokowicz P., Małecka-Panas E.: Clinical aspects of sodium butyrate application in dietary treatment of bowel diseases. *Gastro Rev.*, 2010, Vol. 5 (6): 329-334.
- [6] Almada C.N., Almada C., Martinez R.C.R., Sant'Ana A.S.: Paraprobiotics: evidences on their ability to modify biological responses, inactivation methods and perspectives on their application in foods. *Trends Food Sci. Technol.*, 2016, Vol. 58: 96-114.