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The inhibitory effect of Ukrainian honey on probiotic bacteria

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ABSTRACT

Honey is used in the food industry as a natural sweetener and has therapeutic effects on the human body. Obtaining quality honey involves using organic preventive and treatment agents in beekeeping. The most common of these agents are probiotic supplements. This research aimed to study honey's interaction with an inhibitory effect on the growth of microorganisms from the probiotic supplement Immunobacterin-D under laboratory and experimental field conditions. At the first stage of the research, we assessed the effects of ten honey varieties (buckwheat, sunflower, meadow and forest plants, linden) on *B. subtilis* and *B. licheniformis* from the dry probiotic supplement. The honey-containing nutrient media had an inhibitory effect on the growth of *B. subtilis* colonies. After 24 hours of cultivation under aerobic conditions, the concentration of *B. subtilis* decreased, on average, from 5×10^{12} colony-forming units in 1 g to 3.2×10^{4} and 2.1×10^{5} CFU/g in samples with monofloral and polyfloral honey, respectively. These results emphasize the need for further research on the symbiotic role of microflora in the stability of the microbiota of the hive and bee colony ecosystem. The next stage of the study investigated the probiotic effect on bee colonies in the field. Observations were made on the sanitary conditions of the hives and the behaviour of bees at the Petrodolyna demo apiary. No differences were found at the macro hive-bee colony ecosystem level between control bee colonies (n = 5) and the experimental ones (n = 5) that had received carbohydrate feeding with added probiotics. This confirms the inhibitory effect of honey on the development of bacteria, which eliminates the risk of uncontrolled growth of *B. subtilis* and *B. licheniformis* strain colonies inside the hive and the bacteria getting into bee products. The probiotic had positive effects, increasing the live weight of worker bees by 9.15% by the end of the apiary season compared to the control. This can improve the viability of the bees during wintering. At the last stage of the research, the honey obtained from the experimental colonies was checked for the spores of *B. subtilis* and *B. licheniformis* using melissopalynology.

Keywords: bee family, microbiota, safety, *Bacillus subtilis, Bacillus licheniformis*

INTRODUCTION

Honey production in Ukraine is export-oriented. More than 70% of the honey produced is exported. Up to 86% of it is delivered to the markets of European countries. Safety, quality, and honey botanical identification research methods are a hot topic in the food industry **[1]**. Ukrainian market operators compete fiercely with other producers with proven safety, naturalness, and product quality. The safety of honey and its value for the buyer are the biggest concerns. Considering the international requirements, the need for alternatives to antibiotics in beekeeping is also increasingly pressing **[2]**. Most of the research is concerned with the use of probiotics. There are positive results for using *B. subtilis* in shrimp and fish aquaculture production systems **[3]**. This bacterium is used in various living organism interaction mechanisms, such as synergistic, antagonistic, competitive exclusion, and immune-stimulating effect systems. Various applications of this bacterium include its use as a probiotic, bioremediating agent, bioflocing agent, and, potentially, live vaccine vehicle in aquaculture **[4]**.

It is known that some strains of *Bacillus*, particularly the *B. subtilis* strain DSM32324 or the *B. subtilis* strain DSM32325, are used as direct-fed microbial (DFM), premix, animal feed additive, and animal feed **[5]**.

Other researchers **[6]** have shown that *Bacillus licheniformis* can reduce the incidence of diarrhoea and modify cecal microbiota composition in weaning piglets. This suggests that *Bacillus licheniformis*-fermented feed additive has good potential as a suitable alternative to antibiotics in the swine industry. Scientists have developed a symbiotic bacterium for bee intestines, *Snodgrassella alvi*, to induce eukaryotic RNA interference (RNAi) immune responses **[7]**. They have shown that engineered *S. alvi* can kill parasitic *Varroa* mites by triggering the

mite RNAi response. While the normal flora composition of the main productive animals has been studied extensively, the microflora of hives is currently insufficiently understood. At the same time, a shift of the microflora toward pathogenic or conditionally pathogenic agents with virulence against macroorganisms leads to a significant increase in the bee incidence rate **[8]**.

In recent years, the use of probiotic supplements has also become widespread in beekeeping. It was found that the use of a *Lactobacilli*-containing hive supplement may reduce enzootic-pathogen-related hive losses **[9]**. Samples of honey stomachs, honey, bee bread, bee pollen, and royal jelly from different species of honey bees (*Apis ceranaindica* Fabricius*, Apis melliefra* Linnaeus*, Apis florea* Fabricius*, Apis dorsata* Fabricius*, Tetragonula iridipennis* Smith) were examined for the presence of probiotic lactic acid bacteria **[10]**. The results confirmed that *Enterococcus*, *Micrococcus*, *Streptococcus*, *Pediococcus*, *Lactobacillus*, *Lactococcus*, and *Leuconostoc* typically live in the bee habitat and beekeeping supplies. However, considering the complexity of the individual bee body's biological functions and the whole bee colony as an integrated biological and technological production unit, the use of probiotics as a preventive measure is still little understood.

There are recorded cases of colonies of *Bacillus* strains growing excessively under certain cultivation conditions **[11]**. Given the great variety of modern probiotics and the microorganisms in them, more research is needed on the impact of different species and strains on the quality of the final product, honey **[12]**. New methods for isolating bacteria from honey are also being investigated. For example, it was found that HiCrome *Bacillus* agar combined with simple microbiological tests was beneficial for rapid and reliable identification of most *Bacillus*, *Brevibacillus*, *Lysinibacillus*, and *Paenibacillus* species commonly found in honey samples, facilitating their isolation from polymicrobial honey **[13]**. In the probiotic supplement formula, *B. subtilis* is mixed with *B. licheniformis*. Due to their ability to form spores, these bacteria are resistant to acids, alkalis, sudden temperature changes, and some antibiotics. Numerous studies have shown that they are harmless to animals, even in high concentrations; they have antagonistic activity against a wide range of pathogenic and conditionally pathogenic microorganisms; they have high enzymatic activity, which can have significant regulatory and stimulating effects on digestion; they can carry out antiallergenic and antitoxic actions; they are technological in production, and stable during storage **[14]**. We were interested in the product called Immunobacterin-D, which contains, per 1 kg, bacteria of the species *B. subtilis* and *B. licheniformis* at not less than 6×10^{12} CFU/kg (6×10^{9} CFU/g); xylanase (300.000 U/kg); protease (5000 U/kg); amylase (1000 U/kg); and a filler. This product is widely studied and used in Ukrainian animal husbandry. It was found that in calves, the components of Immunobacterin-D accelerate the population of microflora and the development of ruminal digestion by a factor of 2.5 compared with calves in the control group **[15]**. The use of Immunobacterin-D in cows increases their milk yield by 0.7 – 2.5 L per day **[16]**.

Our research aimed to study the interaction and inhibitory effect of honey on the growth of microorganisms from the probiotic product Immunobacterin-D under both laboratory and experimental field conditions.

Scientific Hypothesis

The main hypothesis of this research is that the presence of aerobic transient spore-forming probiotic bacteria would improve various stages of honey production from the bees *A. mellifera*. There is a possibility of excessive growth of *B. subtilis* inside hives during the vegetation season. This probiotic product is so far untested for use in beekeeping, and it is possible that unanticipated action of the probiotic bacteria would compromise the quality of the honey or interfere with the functioning of the bee colonies. Also, if the bacteria are found in excess in the honey, its naturalness could be compromised.

MATERIAL AND METHODOLOGY

Samples

Sampling of honey was performed according to DSTU 4497 : 2005 **[17]** and GOST 20264.4-89 **[18]**.

Honey samples for group 1 (n = 10) for the research on inhibitory effects were obtained from beekeepers in Odesa Oblast, Ukraine.

Honey samples for group 2 (n = 10) were taken at the end of the beekeeping season from experimental and control colonies on the Petrodolyna demo apiary (Odesa Oblast), where the field experiments were performed to create the average samples for *B. subtilis* and *B. licheniformis* residue testing.

Immunobacterin-D.

Chemicals

The following chemicals were used: sodium chloride (NaCl), c.p.; nutrient agar (composition: enzymatic peptone, meat extract, sodium chloride, agar), brand HMH-Agar Dry Culture Medium; standard set for melissopalynology.

All chemicals were obtained from Khimtest Ukraine TOV, Ukraine.

Animals and Biological Material

The water-soluble probiotic product Immunobacterin-D (dry) was used for the researche. It contains the bacteria *Bacillus subtilis* AX20 and *Bacillus licheniformis* EA22 at a concentration of 5×10^{12} CFU/g, as well as a water-soluble filler (xylanase, amylase, and protease enzymes and nutrient-rich substances) to stimulate the rapid growth of vegetative forms of the bacteria after metabolism restoration.

Immunobacterin-D was manufactured in accordance with TSU 24.4-32430604-001: 2009 (manufacturer KronosAhro TOV, Ukraine). The bacterial strains of *Bacillus subtilis* AX20 and *Bacillus licheniformis* EA22 were initially deposited in the Depository of Institute of Microbiology and Virology of Ukraine with a conclusive confirmation of non-pathogenicity.

10 bee colonies were used (Petrodolyna educational demo apiary, Odesa Oblast, Ukraine).

Instruments

Petri dishes (diameter 60 mm, sterile) and other laboratory glassware (Standard-Lab TOV, Ukraine).

150-mL container for biological samples (plastic) (Khimlaborreaktyv TOV, Ukraine).

Water thermostat Elmi TW-2.03 (Latvia, supplier MEDTECHNIKA TOV, Ukraine).

Water-soluble filler manufacturer VNP (Ukrzoovetprompostach PRAT, Ukraine).

Sigeta Biogenic Led Trino Infinity microscope (China).

Rotator Multi Bio RS-24 (Latvia, distributor BioLabTech Ltd., Ukraine).

Laboratory centrifuge SM-3M.01 (Torhivelnyi Dim Mikromed TOV, Ukraine).

KERN ABJ 220-4NM (220 g/0.1 mg) analytical balance (Kern & Sohn, Germany).

Qc PASS (100 g/0.01 g) lab balance (Biomed LTD TOV, Ukraine).

Laboratory Methods

Method for determining the quantity of aerobic microorganisms present

The research was conducted in a certified veterinary laboratory (Ukrmetrteststandard SE № PT-446/19 certificate of measurement capabilities). We measured the amount of *Bacillus subtilis* bacteria in 1 g of culture medium with an added honey sample. Honey and probiotic sampling were performed in accordance with the requirements of GOST 20264.4-89 **[18]**. The number of viable cells the quantity of mesophilic aerobic and facultative anaerobic microorganisms (QMAFAnM) was determined by the test method GOST 10444.15-94 **[19]**.

The method consists of diluting 1.0 g of Immunobacterin-D aseptically in 100 cm^3 of sterile 0.85% sodium chloride solution, followed by suspension. Two series of ten-fold dilutions were prepared from this dilution so that the predicted number of microorganisms in 1.0 g of product could be determined.

The culture media were prepared according to the manufacturer's instructions and aseptically poured into Petri dishes. 1 g of each honey test sample was added to a culture medium. The ten-fold dilutions were then applied on the surface of the solidified nutrient agar, and the Petri dishes were incubated in a water thermostat aerobically for 24 h at +37 ±1 °C.

During the growth phase, bacteria formed individual characteristic colonies, counted selectively. Colony-forming units (CFU) were calculated for the Petri dishes, each held 20 to 300 colonies, considering their morphology. 10 Petri dishes with colonies were taken for counting, based on the number of honey samples. The procedure was repeated two times.

Botanical origin and bacterial residue in the honey after probiotic feeding

Botanical origin was determined according to the adapted harmonized methods of melissopalynology **[1]** and **[21]** using a Sigeta Biogenic Led Trino Infinity microscope (China) with 400× and 2000× magnification, based in the laboratories of the Department of Certification and Standardization of Agricultural Products, NULES of Ukraine, following DSTU 4497:2005 **[17]**.

The method for measuring the bacterial residue in the honey after feeding with Immunobacterin-D involved looking for the presence of an excessive amount of endospores that could generate *B. licheniformis* and *B. subtilis* in a high-sugar medium (i. e., in honey). Melissopalynological analysis shows that Ukrainian honey usually contains no more than 0.3% fungal spores, 0.1% yeast, and 0.3% of other spores, microalgae, and other biological inclusions of natural origin **[1]**. During the melisopalynological analysis, we assumed that bacterial spores, depending on their species, can be up to 2 μm long **[22]**.

Description of the Experiment

Sample preparation: Preparation of honey sample was performed according to DSTU 4497 2005 **[17]** and GOST 20264.4-89 **[18]**.

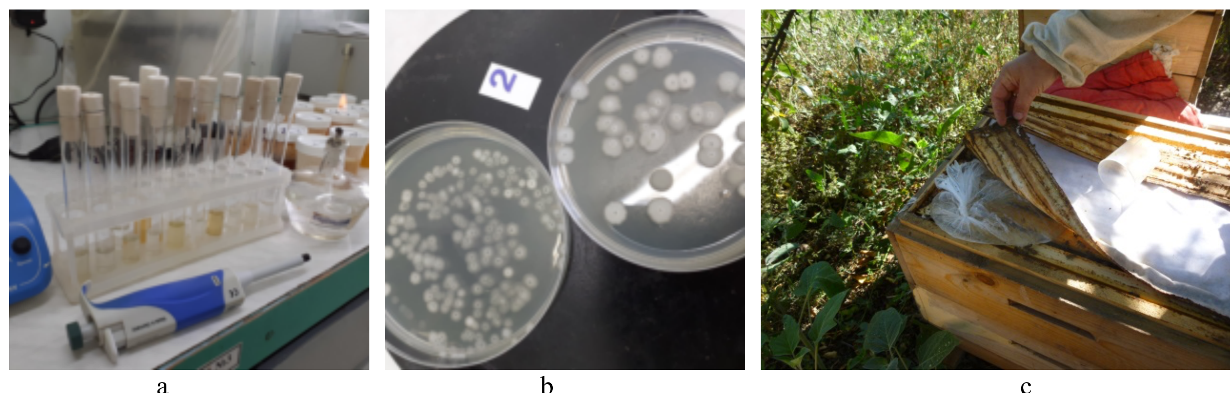
Number of samples analyzed: 20

Number of repeated analyses: 2

Number of experiment replication: 2

Design of the experiment: At the first stage of the research, we assessed the effect of ten different varieties of honey on *B. subtilis* and *B. licheniformis* from the dry probiotic Immunobacterin-D. The second stage of the study

assessed the probiotic effect on bee colonies in the field. Observations were made of the sanitary condition of hives and the behavior of bees at the Petrodolyna demo apiary (Odesa Oblast). At the third stage of the research, the honey obtained from the experimental colonies was checked for the presence of spores of *B. subtilis* and *B. licheniformis* using melissopalynology (Figure 1).



a b c

Figure 1 Selected stages of the research. Note: a – sample preparation; b – growth of *B. subtilis* on the culture medium with honey after 24 hours of cultivation; c – feeding sugar syrup with added probiotic.

Bee feeding arrangement with probiotic

Ten bee colonies were used (5 experimental and 5 control). One feeding was done daily for 5 days, starting on August 22, 2020. Twenty-five doses with Immunobacterin-D were used.

The following feeding procedure was used. Before mixing, the dry Immunobacterin-D was weighed on a KERN ABJ 220-4NM analytical balance. 25 5-g doses were weighed out and aseptically packed. Each dose was diluted in a clean vessel with 5 mL of filtered drinking water (t +20 ±1 °C. The mixture was mixed with syrup (250 mL) and added to a bee feeder. The syrup residue was measured in 12 h (the following day).

Field research procedure

The field (second) research stage was conducted over the summer of 2020 on the Petrodolyna educational demo apiary (Odesa Oblast). The weather was arid, and fodder was low due to poor flowering and insufficient nectar secretion from plants. Five control and five experimental colonies were identified, in which bee colonies were given carbohydrate feed (sugar syrup diluted 1:1 with purified drinking water). 250 mL of feed was given per colony for 5 days. For the experimental colonies, pre-diluted Immunobacterin-D was added to the syrup before feeding, 5 g for each colony. In both the control and experimental colonies, the bees consumed all the feed, as nature had insufficient feed resources within range of a productive summer of bees (r = 2.5 km).

Visual observations

From the beginning of the experiment (August 22, 2020) until October 1, 2020, the experimental and control colonies' condition was observed to determine possible residual effects. The sanitary conditions of the hive were recorded according to the generally accepted rules in terms of the inner surface of the walls and honeycombs, the appearance of the bees, their behavior, flight activity, and aggression/peacefulness during the examination.

Live weight of worker bees

The worker bees were weighed before the first feeding (on August 22, 2020) and again in 21 days, on September 14, 2020. The weighing was carried out at the Petrodolyna demo apiary (Odesa Oblast), in warm, dry weather without wind, between 12:00 noon and 13:30. Two samples of 10 worker bees from each colony were weighed. The sample of worker bees was taken from the middle bee space of the nest. The bees were placed into a container for biological samples, in which ventilation holes had previously been made. The bees did not show aggressive behavior or other atypical reactions during weighing.

Statistical Analysis

Fundamental statistical analysis was carried out with the help of the software package Statistica-6.1 (Sentinel System 7.5.7, V6.1). Student's t-test determined the probability of similarity between the group averages.

RESULTS AND DISCUSSION

After melissopalynological analysis, the honey samples were divided into monofloral (buckwheat and sunflower) and polyfloral (meadow flowers, forest plants, a mixture of linden and clover, and a mixture of linden and maple). During the counting of *B. subtilis* colonies, different numbers of viable cells were found in samples grown in culture media with different varieties of honey (Table 1).

Table 1 The growth of *B. subtilis* colonies on culture media containing honey of different botanical origins.

Monofloral honey (n = 5)		Polyfloral honey (n = 5)	
Botanical origin	**Results**	**Botanical origin**	**Results**
Buckweat[a]	2.7×10^4	Meadow plants[a]	2.0×10^5
Sunflower[a]	6.5×10^4	Meadow plants[b]	6.6×10^4
Buckweat[b]	5.7×10^3	Forest plants	1.5×10^5
Sunflower[b]	2.8×10^4	Linden+melilot	3.1×10^5
Sunflower[c]	3.6×10^4	Linden+maple	3.4×10^5
$\bar{x} \pm s_{\bar{x}}$, CFU.g	$3.234 \pm 0.958 \times 10^4$**	$\bar{x} \pm s_{\bar{x}}$, CFU/g	$2.132 \pm 5.064 \times 10^5$
σ	2.14	σ	11.32
Cv, %	66.3	*Cv*, %	53.1

Note: a, b, c – honey samples belonging to the same botanical variety but having different geographical origins within Odesa Oblast, Ukraine; ** $p < 0.01$ – probable difference from polyfloral honey; $\bar{x}$ – arithmetic mean; $s_{\bar{x}}$ – arithmetic mean error; σ – standard deviation; *Cv* – coefficient of variation.

The average results from our research indicate that monofloral honey has a high inhibitory effect on bacterial growth. The strongest bactericidal properties were observed in buckwheat honey obtained from the Shyriaieve Raion of Odesa Oblast. Other authors **[23]** found that Ziziphus honey had bactericidal effects against *B. cereus* ATCC 10876 and other gram-positive and gram-negative bacteria. In addition, some authors have reported contamination of honey by spore-forming anaerobes, including *B. cereus* and *C. botulinum* **[24]**. However, the research on bactericidal and bacteriostatic properties of honey has focused on pathogenic microorganisms, mainly *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* **[25]**. Ukrainian honey has also been analyzed for the pathogenic microorganisms *Staphylococcus aureus* CCM 4223, *Listeria monocytogenes* ATCC 7644, *Salmonella enterica serovar Typhimurium* CCM 3807, and *Escherichia coli* ATCC 25922 29 **[26]**.

Our results (Table 1) suggest that introducing honey of any type into the culture medium has an inhibitory effect on the development of the bacteria we studied. Immunobacterin-D contained an initial concentration of 5×10^{12} CFU/g *B. subtilis*; after daily contact with media with honey, their average concentration decreased to 3.2×10^4 in monofloral honey and 2.1×10^5 in polyfloral honey. Monofloral honey had significantly higher inhibitory activity compared to polyfloral one ($td = 3.509$; $p < 0.01$). Our results confirm results from the previous studies on the bactericidal properties of honey **[27]**, **[28]**, **[29]**. There are also significant variations in the extent of the inhibitory effect of honey, which correspond to different botanical and regional origins.

The next research stage was to test bees' reaction to consuming the Immunobacterin-D preparation by observing the bee colonies. The results are shown in Table 2. No difference was observed between the behavior and condition of the control and experimental bee colonies. As of February 1, 2021, all colonies were normally wintering (the winter dormancy period for bees in Ukraine lasts from mid-November till the end of February).

During the examination of the hives, their sanitary condition was found satisfactory. There were no microorganism colonies on the hive walls, honeycombs, or brood frames. There were no visible symptoms of disease or parasitism. There were no observable differences in propolis production between bee colonies, except for normal individual fluctuation.

Similar positive results had been obtained by researchers who studied the effect of feeding *B. licheniformis*, *B. subtilis*, and other bacteria on the survival of bees during their infection with *P. larvae* spores (American foulbrood) **[29]**. It has also been shown that after bees infected with *Nosema* spp. were fed the commercial probiotic EM® Probiotic for Bees, the number of spores in the colonies decreased significantly, and the bees' strength increased **[30]**.

Immunobacterin-D normalized the digestive functions of the experimental bee colonies. An analysis of the weight of the worker bees shows that there is no significant difference between the average live weight values of individuals from the experimental and control groups. But there is a positive trend of the average live weight of bees remaining constant in the experimental group, which received Immunobacterin-D, in comparison with the control group (Table 3).

It should be noted that these results were obtained in the summer of 2020 under unfavorable environmental conditions (Odesa Oblast, Ukraine). The conditions included insufficient feed resources for bees; an extremely dry summer (June–August) during the bee-keeping season; and low nectar secretion by honey plants due to a lack of precipitation and low humidity. But even with chronic nutritional stress, the bees retained their body mass, indicating better preparation for winter.

Table 2 Hive sanitary conditions, signs of disease, and behavior of bees after the use of carbohydrate feed with the addition of Immunobacterin-D.

Indicator*	Bee colony groups	
	Experimental (n = 5)	Control (n = 5)
Behavior of bees during honey flow and examinations	no change	no change
Increase in the aggressiveness of bees	n/a	n/a
Increase in dead bees	none	none
Consumption of the daily portion of feed	full	full
Condition of the inner surfaces of the beehive walls	no change	no change
Signs of disordered digestive systems in bee colonies	none	none
Signs of bee brood disease	none	none
Growth of microorganism colonies on the beehive walls, honeycombs	none	none
Change in bee propolis activity	no change	no change
Varroa mite infestation	no change	no change

Note: * – observations of changes in the condition of experimental and control bee colonies were conducted from August 22 till October 1, 2020.

Table 3 The weighing results of worker bees before and after feeding with Immunobacterin-D.

Weighing date	Indicator	Bee colony groups	
		Experimental (n = 5)	Control (n = 5)
August 22, 2020	$\bar{x} \pm s_{\bar{x}}$, mg	114.80 ±4.97	113.80 ±5.46
	σ	11.12	12.21
	Cv, %	9.68	10.73
September 14, 2020	$\bar{x} \pm s_{\bar{x}}$, mg	115.31 ±5.38	103.50 ±3.86
	σ	11.84	8.63
	Cv, %	10.3	8.3

Note: $\bar{x}$ – arithmetic mean; $s_{\bar{x}}$ – arithmetic mean error; σ – standard deviation; *Cv* – coefficient of variation.

Similar results with experimental hives indicate that *B. subtilis subsp. subtilis* Mori2 improved the performance of bees. The micro-organisms stimulated egg-laying by the queen bee, which led to an increase in the number of bees and, consequently, the amount of honey. They also reduced the prevalence of two important bee diseases found worldwide (nosemosis and varroosis) **[31]**.

The changes in the microbiota of the bee digestive system after feeding the probiotic require further research. Similar studies have been performed with the autochthonous strain *Lactobacillus brevis* B50 Biocenol™ (CCM 8618), which had been isolated from the digestive tracts of healthy bees **[32]**. Some strains of *Lactobacillus* have been shown to have a positive effect on bee health **[9]**. Positive results were also obtained using the strain *Bacillus subtilis subsp. subtilis* Mori2 **[33]**. This strain has been shown to cause probiotic effects in bee colonies, including a constant increase in egg laying by the queen bee; high yields of honey; and a decrease in the incidence of nosema and varroosis diseases.

The results may indicate the different modes of action of the strains *Bacillus subtilis* AX20 and *Bacillus licheniformis* EA22, which are part of the probiotic Immunobacterin-D, under different interaction conditions with a functioning bee colony and with honey.

Because of reports of bacterial contamination in honey, including contamination by some species of *Bacillus* spp. (e.g., *B. cereus*) **[24]**, we conducted a microscopic study of the honey, looking for the excessive presence of *Bacillus* spp. spores. The spores could get into commercial honey due to feeding Immunobacterin-D to the bees. It is important to know whether that is the case, given that the supplement would be used in the spring or summer to produce commercial honey.

Honey samples were taken from experimental and control bee colonies and combined to make an average sample, used to compare the microscopic spectrum. The results are shown in Table 4.

The results of the melissopalynological analysis of the honey confirm that Immunobacterin-D is safe to use. No bacterial spores were detected in either sample (control or experimental). The pollen spectrum of the honey was similar in both samples, with a predominance of *Helianthus* spp., but in terms of botanical origin, it was defined as floral polyfloral.

Table 4 The microscopic analysis of honey in percentages.

Parameter	Control	Experimental
Predominant pollen	none	none
Secondary pollen (%) (16 – 49%)	35 (k 40) *Helianthus* spp. (Asteraceae) 16 (k 19) *Onopordum acanthium* (Asteraceae)	23 (k 26) *Helianthus* spp. (Asteraceae)
Minor pollen (%) (≤15%)	6 (k 7) *Bunias orientalis* (Brassicaceae) 4 (k 5) *Erigeron* spp. (Asteraceae) 4 (k 4) *Draba nemorosa* (Brassicaceae) 4 *Artemisia* spp. (Anthemideae) 3 (k 4) *Silene* spp. (Caryophyllaceae) 3 (k 3) *Cirsium arvense* (Asteraceae) 3 (k 3) *Rosa* spp. (Rosaceae) 2 (k 2) *Tripolium pannonicum* (Asteraceae) 2 (k 2) *Helichrysum* spp. (Asteraceae) 2 (k 2) *Inula britannica* (Asteraceae) 2 *Atriplex* spp. (Atripliceae) 2 (k 2) *Marrubium vulgare* (Lamiaceae) 2 *Secale cereale* (Poaceae)	14 (k 15) *Onopordum acanthium* (Asteraceae) 9 (k 10) *Bunias orientalis* (Brassicaceae) 7 (k 7) *Brassica napus* (Brassicaceae) 5 (k 5) *Erigeron* spp. (Asteraceae) 4 (k 4) *Barbarea vulgaris* (Brassicaceae) 4 (k 4) *Helichrysum* spp. (Asteraceae) 3 (k 3) *Cirsium arvense* (Asteraceae) 3 (k 3) *Draba nemorosa* (Brassicaceae) 3 (k 3) Fabaceae 2 *Artemisia* spp. (Anthemideae) 2 (k 2) *Inula britannica* (Asteraceae) 2 *Atriplex* spp. (Atripliceae) 2 *Vinca major* (Apocynaceae) 2 (k 2) *Tripolium pannonicum* (Asteraceae) 2 (k 2) *Urtica* spp. (Urticaceae) 2 (k 2) *Carduus* spp. (Asteraceae)
Trace pollen (≤1%)	*Urtica* spp. (Urticaceae); *Origanum* spp. (Lamiaceae); *Vicia cracca* (Fabaceae); *Senecio vulgaris* (Asteraceae); *Cichorium intybus* (Asteraceae); *Carduus* spp. (Asteraceae); *Centaurea cyanus* (Asteraceae); *Berteroa incana* (Brassicaceae); *Cruciata* spp. (Rubiaceae); *Geranium sylvaticum* (Geraniaceae); *Salvia tesquicola* (Lamiaceae); *Genista tinctoria* (Fabaceae); *Vinca major* (Apocynaceae); *Iris pseudacorus* (Iridaceae)	*Impatiens spp.* (Balsaminaceae); *Silene* spp. (Caryophyllaceae); *Marrubium vulgare* (Lamiaceae); *Centaurea cyanus* (Asteraceae); *Berteroa incana* (Brassicaceae); *Geranium sylvaticum* (Geraniaceae); *Eupatorium cannabinum* (Asteraceae); *Rosa* spp. (Rosaceae); *Senecio vulgaris* (Asteraceae); *Cichorium intybus* (Asteraceae); *Iris pseudacorus* (Iridaceae); *Taraxacum officinale* (Asteraceae); *Centaurea jacea* (Asteraceae); *Echium vulgare* (Boraginaceae)
Damaged pollen, not identified (%)	3	2
HD-Elements, fungal spores	few (0.3)	none
HD-Elements, other spores	none	none
HD-Elements, algae	few (0.3)	none
Yeast content	few (0.1)	none
Starch grains	none	none
Other solid constituents	none	none

Note: k = counts without nectarless plants; HD = honeydew in per 300 pollen; spore, algae, starch graine, yearst content and other solid constituents in per 300 pollen.

Our previous research on the botanical composition of honey **[1], [20]** showed that most Ukrainian honeys contain pollen grains of *Helianthus* spp. in various quantities. Other scientists also mention that sunflower honey is common in Ukraine **[24]**, **[34]**. This is because Ukraine currently ranks first in the world in sunflower production, with a share of 29.3% (40.57 million tons) of total world sunflower production **[35]** and **[36]**.

Furthermore, the proportion of sunflower hybrids is increasing **[37]**. The practicing beekeepers who participated in our research state that sunflowers, especially the drought-resistant varieties, have stopped secreting nectar, and produce only a large amount of pollen, contaminating the honey of other botanical origins.

HD-elements were detected in the control samples of honey from bee colonies that were not fed probiotics; this requires additional research. It may be due to the antagonistic action of spore-forming bacteria. After entering the anterior digestive system of bees (mouth, pharynx, oesophagus, honey sac), the bacteria probably entered a vegetative state. They actively reduced the number of fungal spores on the inner surface of the organs. This is probably the reason for the improved microbiological purity of the honey. However, the research results are not conclusive so the experiment will be continued.

A gamma irradiator is used to sterilise honey in contamination with *Clostridium botulinum* and *Bacillus subtilis* spores **[38]**. Short-wave ultraviolet light (UV-C) has been studied for inactivating vegetative cells of *Escherichia coli* (CECT 405) and spores of *Bacillus subtilis* (CECT 12) and *Clostridium sporogenes* (CECT 553) in honey, inoculated at 104 – 105 CFU/g **[39]**. In addition, it is known that *Bacillus subtilis* and other bacteria normally exist in the digestive systems of honey bees **[40]** and Malaysian stingless bees *(Heterotrigona itama)* **[41]**, **[42]**.

In our opinion, the microbiological purity of honey should be reconsidered, and individual strains of bacteria should be included in the list of permitted bacteria for use in natural honey as a raw material. Some microorganisms are also used for the geographical identification of honey **[43]**.

CONCLUSION

The nature of the microbiota, both in individual bees and in the whole bee colony, remains insufficiently studied. The negative impact of pathogenic or excessive microflora is a threat to the safety and quality of honey, which requires further research.

The objective of the research was to investigate the effects of *Bacillus subtilis* AX20 and *Bacillus licheniformis* EA22 strains, contained in the probiotic supplement Immunobacterin-D, on both the microlevel (honey) and the macrolevel (the sanitary conditions of the hive interior, the bees' behavior, their health, and the products obtained). We confirmed that these strains have different effects on honey and on the bee colony. Cultivation of *B. subtilis* on culture media with added honey showed that honey had an inhibitory effect on colony growth. The initial concentration of 5×10^{12} CFU/g in the probiotic preparation decreased over 24 h, with final values between 2.1×10^5 and 3.2×10^4 CFU/g. Immunobacterin-D had no ill effects on colony health or the sanitary conditions of the hive. On the other hand, the probiotic had significant positive effects on most of the bees despite an extremely unfavourable (dry) apiary season. Furthermore, a comprehensive analysis showed that Immunobacterin-D is safe to use during commercial honey production.

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