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BIOLOGICAL PROPERTIES OF MICROORGANISMS ISOLATED FROM DRONE MILK OF HONEYBEES

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ABSTRACT

This article presents the studied results of the biological properties and biosafety indicators of probion strains isolated from drone milk of honey bee (Apismellifera). It was established that the isolated cultures of microorganisms (Bifidobacterium asteroides strain DFM5, Fructobacillus fructosus strain DF74, and Lactobacillus kunkeei strain DFM21) reacted differently to aggressive media, with the best tolerance to experimental biological fluids being shown by strain intact. As a result of repeated reseeding, antibiotic resistance in each culture was increased. The isolated cultures of microorganisms have low toxicity for laboratory animals, both in acute and in chronic experience, since prolonged use of microbial compositions does not negatively affect the general condition of mice, their clinical status, morphological and biochemical blood parameters, and there are no negative effects on organs and tissues of experimental animals.

Disciplinary: Microbiology, Biotechnology, Animal Science, MicrobioScience.

1. INTRODUCTION

The normal intestinal microflora provides a wide range of metabolic, trophic, and protective functions of honeybees, which play an important role in protecting the bees against pathogenic microorganisms, affect reproduction and honey activity. Recently, the use of beneficial microbiota in the composition of probiotic preparations, which have a positive effect on increasing the resistance of bees to a pathogenic agent, helps activate their immunological defense, relevant in beekeeping [6].

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The mechanism of action of microbial additives lies in the fact that the probion strains exhibit highly adhesive properties to the intestinal enterocytes of the host, and due to the production of several antibiotic substances, they cause a decrease in the growth and development of opportunistic microorganisms, thereby dominating them and contributing to an improvement in the vital activity of the host [14-15, 17, 18].

Both in Russian and foreign practice of beekeeping, probiotic supplements are widely used to combat a specific disease or to comprehensively affect the bee's body and prevent infectious and invasive diseases, as well as stimulating top dressing, which allows a targeted effect on the dynamics of morphofunctional, ultrastructural indicators organism of honey bees in ontogenesis. These measures contribute to the successful overcoming of critical periods of growth of the bee family and its better preparation for honey collection [9].

In this regard, the development of new highly effective drugs based on living beneficial microorganisms for beekeeping is promising, and the study of the biological properties of microflora is not only scientific, but also practical.

This research work aims to study the biological properties and biosafety assessment of probion strains isolated from drone milk of honeybees.

2. METHOD

The objects of research were normoflora - Lactobacillus kunkeei strain DFM21, Bifidobacterium asteroides strain DFM5, and Fructobacillus fructosus strain DF74, isolated by modern molecular genetic and microbiological methods from samples of drone milk of honey bee (Apismellifera).

To identify the probiotic potential of the studied cultures, their resistance to bile, a solution of phenol, and sodium chloride were studied by adding these compounds to the composition of the microbial nutrient medium [5]. Separately studied the effect on the viability of the studied cultures of gastric juice. To do this, 1.0 ml of culture with an activity of at least 1.0×10^9 CFU/ml was poured into a test tube and 9.0 ml of gastric juice was added (Equin medicine). The tubes were thermostated at 37°C for 2 hours, and then the viability of microorganisms was analyzed [7]. Microflora growth or its absence was recorded according to the turbidity standard.

The resistance of the strains was determined to therapeutic doses of antimicrobial substances by the disk diffusion method in agar using standard disks [2, 3, 7].

The antibacterial properties of the cultures used were individually studied for E. coli (Escherichia coli) and Staphylococcus aureus (Staphylococcus aureus). The result was taken into account by the size of the growth inhibition zone of the test microbe under the influence of the studied microorganisms [7].

A study was made of the quantitative and qualitative composition of organic acids produced by the studied microflora. The studies were carried out by capillary electrophoresis on a Kapel-105 M automated instrument.

The study of the toxicity parameters of the studied microorganisms was carried out in accordance with the requirements and recommendations [11, 13]. The study of acute and chronic toxicity was

performed on clinically healthy laboratory mice that had previously been quarantined for 14 days. Laboratory animals were divided into groups according to the principle of pair-analogues. The number of animals in each group was 10 animals of both sexes; the content of females and males was separate. Laboratory animals of all groups were kept in accordance with sanitary and epidemiological requirements for the design, equipment, and maintenance of experimental biological clinics (vivariums) [12].

The data obtained in the experiments were subjected to biometric processing using Microsoft® software. The reliability criterion was determined according to the student table. The difference was considered significant at $P < 0.05$.

3. RESULT AND DISCUSSION

3.1 RESISTANCE OF PROBION STRAINS TO EXPERIMENTAL BIOLOGICAL FLUIDS

An important indicator of the probiotic properties of beneficial microflora is their ability to colonize the gastrointestinal tract, as well as their resistance to the biological fluids of the host organism [1, 4, 10]. The latter indicator was visualized by the presence or absence of growth of the studied microflora in a liquid nutrient medium containing horse's natural gastric juice (Equin preparation), medical bile of cattle (20%, 30%, 40%), phenol (0.4%)) and NaCl (2%, 4%). The research results are presented in Table 1.

Table 1: Resistance of probion strains to aggressive environments

Strain	Growth in culture medium containing						
	NaCl		Bile			Phenol (0.4 %)	Gastric juice
	2 %	4 %	20 %	30 %	40 %		
Lactobacillus kunkeei strain DFM21	+	+	+	+	±	+	+
Bifidobacterium asteroides strain DFM5	+	+	+	±	–	±	±
Fructobacillus fructosus strain DF74	+	±	+	±	–	±	±

Note: * "+" - good growth; "±" weak growth; "-" lack of growth.

The results of the studies showed that the studied microorganisms reacted differently to aggressive environments. So Bifidobacterium asteroides strain DFM5 showed weak growth in an environment containing 30% bile, phenol, and gastric juice. Fructobacillus fructosus strain DF74 showed weak growth in a medium containing 4% sodium chloride, 30% bile, phenol, and gastric juice, while only one of the strains showed a lack of growth at 40% bile in a nutrient medium. The best experimental results were obtained with the cultivation of Lactobacillus kunkeei strain DFM21, which showed weak growth in only one case, namely, availability in a nutrient medium containing 40% bile. In all other cases, the studied strain showed high tolerance to aggressive components of the nutrient medium.

Thus, the studies showed that the strains isolated from drone milk of the honey bee react differently to the experimental biological fluids of the host organism, while the probiotic strain Lactobacillus kunkeei showed the best results.

3.2 PHENOTYPIC PROFILE OF ANTIBIOTIC RESISTANCE OF MICROORGANISMS

Important technological and probiotic properties of normoflora include its resistance to

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antibiotics since the use of these drugs is every day. In this connection, the sensitivity of the used strains to therapeutic doses of some antibiotics was studied. The results are shown in Table 2.

Table 2: Sensitivity of microorganisms to antibiotics

Antimicrobial substance	Dosage in the disk, mcg	The diameter of the zone of growth inhibition, mm		
		Lactobacillus kunkeei strain DFM21	Bifidobacterium asteroides strain DFM5	Fructobacillus fructosus strain DF74
Penicillin	10	6.0 ± 0.2	8.0 ± 0.2	9.0 ± 0.3
Erythromycin	15	6.0 ± 0.3	8.0 ± 0.1	10.0 ± 0.2
Lincomycin	15	9.0 ± 0.3	9.0 ± 0.3	11.0 ± 0.3
Cephalotin	30	8.0 ± 0.2	11.0 ± 0.2	11.0 ± 0.2
Tetracycline	30	8.0 ± 0.1	10.0 ± 0.2	11.0 ± 0.2
Streptomycin	10	4.0 ± 0.1	6.0 ± 0.1	9.0 ± 0.2
Ampicillin	10	4.0 ± 0.2	7.0 ± 0.3	8.0 ± 0.1

The results of evaluating the antibiotic sensitivity of the selected strains showed that growth inhibitory drugs acted to varying degrees on the studied microorganisms. In this case, I would like to note that according to the criteria for assessing the sensitivity of lactobacilli, taking into account the diameter of the zone of inhibition of their growth, all used microorganisms can be classified as “resistant” to the action of the applied antimicrobial substances [7, 8]. Only under the action of lincomycin, tetracycline and cephalothin on Fructobacillus fructosus strain DF74, as well as cephalothin on Bifidobacterium asteroides strain DFM5, a growth inhibition zone of 11 mm was detected, which is the minimum value to classify the cultures used in relation to these antimicrobial substances "Intermediate resistant."

To increase the antibiotic resistance of the studied probion strains, they were repeatedly cultivated with the addition of previously used antibiotics. Total 54 transfers were carried out. The results of the antibiotic sensitivity of microorganisms after reseeding are presented in Table 3.

Table 3: Antibiotic sensitivity of probion strains after repeated passages

Antimicrobial substance	Dosage in the disk, mcg	The diameter of the zone of growth inhibition, mm		
		Lactobacillus kunkeei strain DFM21	Bifidobacterium asteroides strain DFM5	Fructobacillus fructosus strain DF74
Penicillin	10	–	–	3.0 ± 0.1
Erythromycin	15	4.0 ± 0.1	3.0 ± 0.1	3.0 ± 0.1
Lincomycin	15	3.0 ± 0.1	3.0 ± 0.2	4.0 ± 0.2
Cephalotin	30	2.0 ± 0.1	4.0 ± 0.1	3.0 ± 0.1
Tetracycline	30	–	4.0 ± 0.1	5.0 ± 0.1
Streptomycin	10	–	–	4.0 ± 0.1
Ampicillin	10	–	3.0 ± 0.1	4.0 ± 0.1

Note: * "-" - no growth retardation.

As a result of selection, it was possible to reduce the zones of growth inhibition of the studied strains to the used antibiotic concentrations by at least 2 times, and in some cases, no zones of inhibition were formed. Thus, there were no zones of growth inhibition of selected cultures when exposed to Bifidobacterium asteroides strain DFM5 antibiotics penicillin and streptomycin; Lactobacillus kunkeei strain DFM21 growth retardation zone was absent under the action of penicillin, tetracycline, streptomycin, and ampicillin. The worst results were revealed with the action of antibiotics on Fructobacillus fructosus strain DF74, while growth retardation zones decreased after repeated reseeding, but the inhibitory reaction remained on all antimicrobial substances.

Thus, due to repeated reseeding of selected cultures, it was possible to increase their antibiotic resistance, which will ensure the maximum therapeutic effect of the developed biological product.

3.3 ANTAGONISTIC AND ACID-FORMING PROPERTIES OF CROPS

The manifestation of the antibacterial activity of the culture against pathogenic microorganisms is one of the main parameters providing a therapeutic and prophylactic effect.

It was established that the used microorganism cultures exhibit an antibacterial property with respect to Escherichia coli and Staphylococcus aureus. However, the best results were revealed when cultivating Lactobacillus kunkeei strain DFM21 and Bifidobacterium asteroides strain DFM5. The growth retardation zone of Escherichia coli was 5.4 and 4.1 mm, and Staphylococcus aureus was 5.2 and 3.9 mm, respectively. Fructobacillus fructosus strain DF74 also showed antagonistic properties, but to a lesser extent, the growth inhibition zone of Escherichia coli was 3.2 mm and Staphylococcus aureus was 3.4 mm.

One of the main parameters of the antibacterial activity of microorganisms in relation to pathogenic microflora is their ability to produce antibiotic substances, in particular, organic acids. In this regard, we studied the assortment and content of acids that produced the selected culture. The research results are presented in Table 4.

Table 4: Quantitative and qualitative composition of organic acids, mg / l

Organic acid	Probing Strain		
	Lactobacillus kunkeei strain DFM21	Bifidobacterium asteroides strain DFM5	Fructobacillus fructosus strain DF74
Lactic acid	1856.47	1127.95	895.21
Acetic acid	577.85	354.53	276.49
Propionic acid	–	577.59	313.18
Butyric acid	–	157.67	–
Formic acid	–	134.16	–

The results of the studies showed that each studied probion strain produces one or another organic acid. The main share falls on lactic and acetic acids, which are the main substances in the manifestation of antibacterial properties. It was found that the largest amount of lactic and acetic acids produces Lactobacillus kunkeei strain DFM21 (1856.47 and 577.85 mg/l). To a lesser extent, lactic and acetic acids are synthesized by the Bifidobacterium asteroides strain DFM5 1127.95 and 354.53 mg/l, however, this organism produces additional organic acids propionic, butyric and formic. The strain Fructobacillus fructosus produced the least amount of lactic and acetic acids (895.21 and 276.49 mg/l).

Thus, it can be noted that the isolated cultures of microorganisms exhibit antibacterial activity against a pathogenic test culture, which can provide a high therapeutic and prophylactic effect in diseases caused by an imbalance of the microflora in the gastrointestinal tract, however, the best effect is shown by Lactobacillus kunkeei strain DFM21.

3.4 ACUTE TOXICITY OF PROBION STRAINS

Toxic in honey products and bees have been reported [16]. This study, the acute toxicity of the isolated cultures was determined by intragastric administration of the liquid form of the supplement to nonlinear white mice (with an initial body weight of 18-22 g) after 12-hour fasting

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once using a metal probe. Animals of the control group were intragastrically injected with physiological saline in a similar volume. The observation was carried out for 14 days. The general condition, behavior, and feed intake were taken into account. The results of the acute toxicity of the studied crops are presented in Table 5.

Table 5: Acute toxicity of microorganisms

Group	Number of mice, heads	Fluid volume, ml	The number of microbial cells, CFU/ml	The test result (heads)		
				got sick	died	survived
Control	10	1.0	–	0	0	10
1st experimental (Bifidobacterium asteroides strain DFM5)	10	1.0	7.5×10^{8}	0	0	10
2nd experimental (Fructobacillus fructosus strain DF74)	10	1.0	8.1×10^{8}	0	0	10
3rd experimental (Lactobacillus kunkeei strain DFM21)	10	1.0	8.3×10^{8}	0	0	10

As a result of the studies, it was found that a single injection of microbial components with a different number of microbial cells did not cause a clinical picture of toxicosis in mice, animals were not killed in the experimental groups. The condition of all experimental animals remained satisfactory, the appetite was not disturbed, the mice were mobile, the reaction to external stimuli remained the same.

Thus, in the course of determining the acute toxicity of the isolated microorganisms on white mice, no pronounced toxicosis was detected in experimental animals; therefore, the studied strains can be attributed to the group of low toxic drugs.

3.5 CHRONIC TOXICITY OF PROBION STRAINS

The determination of chronic toxicity was carried out on 14-day non-linear white mice. The liquid form of the microbial composition was administered to laboratory animals orally for 28 days, followed by observation. Since the semi-lethal dose was not established in the determination of acute toxicity, twice as high doses of microorganisms were used as the daily dose (Table 6).

Table 6: Chronic toxicity of microorganisms

Group	Dosage, ml/day	Number of mice, heads	The test result (heads)		
			got sick	died	survived
Control	–	10	0	0	10
1st experimental (Bifidobacterium asteroides strain DFM5)	5.0×10^{9}	10	0	0	10
2nd experimental (Fructobacillus fructosus strain DF74)	6.2×10^{9}	10	0	0	10
3rd experimental (Lactobacillus kunkeei strain DFM21)	6.6×10^{9}	10	0	0	10

Studies have shown that experimental animals tolerated the studied suspensions of microorganism cells. The death of mice was not observed in any of the groups: they were clinically healthy throughout the experiment, there were no disturbances in food and water intake. The experimental animals were mobile and active, the coat was smooth and characterized by a characteristic shine. Observation of the mice throughout the experiment did not reveal any changes in their behavior compared with the control group.

Table 7 presents the studied results of the bodyweight of experimental and control laboratory animals, as well as an increase in live weight, safety.

Table 7: Effect of microorganisms on the growth and development of laboratory animals

Group	Bodyweight g		Growth per experience, g	Safety, %
	at the beginning of the experiment	at the end of the experiment		
control	6.17 ± 0.33	18.21 ± 0.55	12.04	100
1st experience	6.21 ± 0.23	19.89 ± 0.40*	13.68	100
2nd experience	6.09 ± 0.17	19.55 ± 0.51*	13.46	100
3rd experience	6.11 ± 0.14	19.75 ± 0.37*	13.64	100

Note: * - the difference with the control is significant (P <0.05).

According to the data obtained, the weight of the mice at the end of the experiment was statistically significantly (P <0.05) higher in the groups where the isolated cultures were introduced. So, in the 1st experimental group, the weight of mice was higher by 9.2% compared to the analogue from the control, in the 2nd by 7.4% and the 3rd by 8.5%. The safety of animals in the control and all experimental groups was 100%.

Studies of some morphological and biochemical blood parameters of control and experimental mice indicate that in all animals these parameters were within normal limits (Table 8).

Table 8: Effect of microbial components on the blood parameters of mice

Index	Group			
	control	1st experience	2nd experience	3rd experience
Erythrocytes, 10^{12}/l	8.0±0.7	9.2±0.3	9.1±0.3	9.6±0.3
Platelets, 10^{9}/L	267.2±11.0	331.1±14.1	292.1±15.7	324.1±15.1
White blood cells, 10^{9}/l	8.7±0.7	9.1±0.5	9.4±0.4	9.3±0.5
Hemoglobin, g/l	143.4±4.9	160.1±3.9	162.7±4.2	147.4±4.2
Total protein, g/l	50.1±2.1	56.2±2.9	53.7±2.4	56.4±2.3
Urea, mmol/l	22.22±0.90	19.12±0.57	19.62±0.63	19.69±0.79
Cholesterol, mmol/l	3.35±0.11	3.17±0.10	3.16±0.11	3.24±0.13
Calcium, mmol/l	2.13±0.10	2.31±0.16	2.30±0.09	2.25±0.10
Phosphorus, mM/L	1.39±0.11	1.43±0.10	1.51±0.12	1.46±0.11

However, it is worth noting that the content of red blood cells, platelets, and leukocytes in the experimental groups was higher than in the control. The content of cholesterol and alkaline phosphatase in the blood serum of experimental mice was lower, compared with the control group but did not go beyond the normal range. There was a slight increase in the experimental groups in the content of calcium and phosphorus.

When conducting pathomorphological studies in nonlinear white mice, in the experimental groups where the isolated strains were used, no abnormalities were detected. The location of the internal organs was correct, fluid in the pleural and abdominal cavities was not found. The lumen of the trachea and bronchi is free, the tissue of the lungs is pink. The mucous membrane of the intestines and stomach is gray-pink colors without ulceration and hemorrhage. The cortical and medulla of the kidneys in the section are clearly distinguishable, the adrenal glands are unchanged.

Histological studies of the organs of white mice are as follows:

Stomach - integument epithelium preserved; cytomorphology of the main and parietal glands

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allows us to talk about the absence of dystrophic processes in them and the normal acid-forming function.

Small and large intestine - in the villi of the small intestine, in some cases, desquamation of the integumentary epithelium is noted; no edema, plethora; no increase in lymphohistiocytic infiltration; the wall of the large intestine without features, has the usual histological structure.

Liver - the beam pattern is well defined; granular and eosinophilic cytoplasm of hepatocytes; activation and proliferation of cells of the reticuloendothelial system is not observed.

Kidneys - glomerular apparatus without features; the Shumlyansky – Bowman capsule is free; in nephrocytes, dystrophic changes are not observed; in the lumen of the tubules, the amount of protein mass is not increased.

Adrenal glands - no dystrophic changes are observed; thin capsule; subcapsular blastema is not determined; bunching is well expressed; medulla without features.

Heart - myocardium is represented by correctly oriented bundles of fibers; dystrophy, atrophy, fragmentation, deformation of cardiomyocytes was not observed.

Spleen - the plethora of red pulp; the boundaries of the follicles are clear; proliferation centers are expressed; the pulp is rich in cellular elements.

Pancreas - pathological changes in the structure of the endo- and exocrine gland apparatus are not observed.

Thymus - has a lobed structure; cortical and medulla are well differentiated; a decrease in the density of lymphocytes in the cortical substance is not observed.

The thyroid gland is a plethora, follicles are polymorphic; colloid light, foamy; many small follicles devoid of clearance; the epithelium is cubic, in some follicles, it is hyperplastic, multirow.

Lungs - moderate plethora; the gaps of the alveoli and small bronchi are free; in the lumen of the bronchi accumulation of leukocytes; fresh hemorrhages occur as a result of decapitation.

Thus, the determination of the chronic toxicity of microbial components in non-linear white mice, the assessment of some morphological and biochemical blood parameters of experimental animals, as well as the study of pathological changes in the structure of the internal organs of mice, did not reveal the toxic effect of the studied cultures on the organism of experimental animals.

4. CONCLUSION

The results of the experiments showed that the probion strains isolated from drone milk of the honey bee (Bifidobacterium asteroides strain DFM5, Fructobacillus fructosus strain DF74 and Lactobacillus kunkeei strain DFM21) reacted differently to aggressive media with the best biological conditions. The studied microorganism strains showed high resistance to many antibiotic drugs, but as a result of repeated reseeding, antibiotic resistance in each culture was increased. The probion strains showed antagonistic properties against pathogenic test cultures, which were expressed by the growth inhibition zone of Escherichia coli and Staphylococcus aureus when grown with Lactobacillus kunkeei strain DFM21 at 5.4 and 4.1 mm when grown with Bifidobacterium asteroides strain 5 DM, 2 and 3.9 mm, when grown with Fructobacillus fructosus strain DF74 - 3.2 and 3.4 mm. When analyzing the production of organic acids, it was found that of the assortment present, the

highest value was accounted for by lactic and acetic acids, the level of which was 1856.47 and 577.85 mg / l (Lactobacillus kunkeei strain DFM21), 1127.95 and 354.53 mg / l (Bifidobacterium asteroides strain DFM5), 895.21 and 276.49 mg/l (Fructobacillus fructosus strain DF74). The isolated cultures of microorganisms are not toxic to laboratory animals, both in acute and in chronic experience, since long-term use of microbial compositions is not affected negatively on the general condition of mice, their clinical status, morphologist and biochemical parameters of blood, and there are no negative effects on the organs and tissues of the experimental animals. According to the complex of the studies, the probiotic strain − Lactobacillus kunkeei strain DFM21 should be noted, since in several experiments it showed the best results.

5. AVAILABILITY OF DATA AND MATERIAL

Data can be made available by contacting the corresponding authors

6. ACKNOWLEDGEMENT

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