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ANTAGONISTIC ACTIVITY OF PROBIOTIC *BACILLUS* STRAINS ON PLANKTONIC FORMS OF BIOFILM-FORMING BACTERIA AND FUNGI

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Summary. The presence of microbial biofilms of pathogenic fungi and bacterial contaminants in animal feed can lead to disruption of the intestinal microflora and the development of infectious diseases. A promising field of study is the examination of the antagonistic effect of bacteria from the genus *Bacillus* on microbial biofilms and planktonic forms of pathogenic microorganisms in feed. The objective of this research is to investigate the antimicrobial and antifungal activity of the probiotic complex of bacteria from the genus *Bacillus* against planktonic forms of biofilm-forming pathogenic fungi and microorganisms isolated from pig feed. The antagonistic activity of five probiotic strains against the test cultures *Pasteurella multocida* type D No. 07, *Neisseria meningitidis* No. 18, *Streptococcus haemolyticus* No. 14, *Escherichia coli* No. 21, *Actinobacillus pleuropneumoniae* No. 12 was studied by the method of delayed inoculation (perpendicular strokes) in three replicates. The antifungal activity against the test fungi *Aspergillus niger* No. 1 and *Aspergillus candidus* No. 2 was evaluated by the injection method. According to the results of the study, it was determined that the strain *B. licheniformis* UNCSM-033 showed a pronounced antagonistic effect on the bacteria *N. meningitidis* No. 18 with an inhibition level of 26.7 ± 1.2 mm. Inhibition of growth and reproduction of *S. haemolyticus* No. 14 at a high level of intensity was determined in four probiotic strains with diameters of growth inhibition from 28.7 ± 1.2 mm to 34.0 ± 1.2 mm. A sufficiently high level of antagonism against the test culture *E. coli* No. 21 was shown by five experimental probiotic strains in the range of 24.7 ± 1.2 mm to 30.7 ± 2.3 mm, respectively. A very high level of antagonistic properties of the probiotic complex of bacteria of the genus *Bacillus* from five experimental strains against five types of pathogenic microorganisms from 38.3 ± 0.9 mm was shown in *A. pleuropneumoniae* No. 12 and up to 47.3 ± 0.9 mm in *P. multocida* type D No. 07. The highest degree of antagonistic activity against five test cultures of biofilm-forming microorganisms and antifungal effect against two test strains of pathogenic fungi, *A. niger* No. 1 and *A. candidus* No. 2, was demonstrated by the probiotic complex of bacteria belonging to the genus *Bacillus* (five strains). The pronounced antimicrobial properties of the five strains of the probiotic complex of bacteria of the genus *Bacillus* allow for the potential development of drugs based on them as an alternative to antibiotics

Keywords: antifungal effect, antibiotic substances, inhibitory activity

Introduction. The organization of animal feeding should provide conditions that facilitate the efficient use of feed and regulate microbiological digestive processes. Incomplete and unbalanced diets, as well as animal feeding with contaminated feed containing microbial biofilms of pathogenic fungi and bacterial contaminants, can disrupt the intestinal microflora and lead to the development of infectious diseases. This, in turn, can result in dysbiosis in pigs, which may reduce their natural resistance and productivity. One potential solution to this problem is the inclusion of probiotics in animal diets. Spore-forming bacteria from the genus *Bacillus*, which exhibit antagonistic activity, are increasingly used as probiotic cultures against planktonic forms and biofilms of pathogenic and opportunistic microorganisms of the genera *Escherichia*, *Salmonella*, *Shigella*, *Serratia*, *Streptococcus*, *Staphylococcus*, *Klebsiella*, *Proteus*, *Pseudomonas*, *Citrobacter*, *Candida* and *Aspergillus* fungi by producing antibiotics, bacteriocins, lysozyme, participate in digestion by synthesizing hydrolytic enzymes (analogues of macroorganism digestive enzymes) and at the same time do not inhibit the growth of lacto- and bifidobacteria (Kolchyk et al., 2022; Petrova and Sauer, 2016). Antagonism is ensured by the production of more than 200 antibiotic substances (polymyxins, bacitracins, gramicidin C, subtilin, microbacillin, biosporicin). Participation in the digestive process occurs due to the synthesis of extracellular hydrolytic enzymes proteases, amylases, pectinases, cellulases, and lipases. Probiotic functions are performed not only by vegetative bacilli cells but also by germinating spores (Cutting, 2011; Kotowicz et al., 2019; Chechet et al., 2022).

The vast majority of *Bacillus* species are harmless to animals. Strains that produce toxins and have pronounced adhesive and invasive properties are not used in the production of probiotics.

A promising area is the study of the antagonistic effect of bacteria from the genus *Bacillus* on microbial biofilms and planktonic forms of pathogenic microflora of feed for the further design of probiotic agents to break the epizootic chain of not only bacterial but also viral infections in pig production.

The study aimed to investigate the antimicrobial and antifungal activity of a probiotic complex of bacteria of the genus *Bacillus* against planktonic forms of biofilm-

forming pathogenic fungi and microorganisms isolated from pig feed.

Materials and methods. Microbiological studies of feeds were carried out in the Laboratory for Pig Diseases Study of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' (Kharkiv, Ukraine) using modern methods. The object of the study was the probiotic complex of bacteria of the genus *Bacillus*, namely five strains in its composition (*B. amyloliquefaciens* ALB 65, *B. pumilus* UNCSM-026, *B. subtilis* UNCSM-020, *B. subtilis* var. *mesentericus* UNCSM-031 and *B. licheniformis* UNCSM-033).

The test cultures of pathogenic and opportunistic bacteria *Pasteurella multocida* type D No. 07, *Neisseria meningitidis* No. 18, *Streptococcus haemolyticus* No. 14, *Escherichia coli* No. 21, *Actinobacillus pleuropneumoniae* No. 12 were used as test cultures for determining the antagonistic activity of the five strains of the probiotic complex of bacteria of the genus *Bacillus in vitro.* To determine the antifungal activity, micromycetes of the genus *Aspergillus*, namely *A. niger* No. 1 and *A. candidus* No. 2, were used. Field isolates of the above microorganisms were isolated from 38 industrial batches of feed (grain, barley, and corn) from four pig farms positive for reproductive and neonatal infections in pigs in two regions of Ukraine.

Cultivation of five strains of the probiotic complex of bacteria of the genus *Bacillus* was carried out on meat-peptone broth (MPB) and meat-peptone agar (MPA) with the addition of 1% glucose, test cultures of microorganisms: *E. coli* No. 21 and *N. meningitidis* No. 18 — MPB, MPA, Endo agar; *P. multocida* type D No. 07 — Hottinger broth and agar; *S. haemolyticus* No. 14 — 5% blood agar; *A. pleuropneumoniae* No. 12 — 5% blood agar with 10% yeast extract; *A. niger* No. 1 and *A. candidus* No. 2 — Czapek medium.

The antagonistic activity of *B. amyloliquefaciens* ALB 65, *B. pumilus* UNCSM-026, *B. subtilis* UNCSM-020, *B. subtilis* var. *mesentericus* UNCSM-031, and *B. licheniformis* UNCSM-033 strains was studied by the method of delayed inoculation (perpendicular strokes) in three replicates (Ivchenko, 2004). To determine the antagonistic activity, the antagonist strains were inoculated on the surface of the agar and a day later, after incubation at a temperature of 37.0 ± 0.5 °C, the test culture of microorganisms was inoculated perpendicularly to it at a distance of no more than 2 mm. The growth of the test cultures was controlled by their parallel inoculation on plates with the same media but without antagonist strains.

The activity of bacilli antagonists to the test fungi *A. niger* No. 1 and *A. candidus* No. 2 was evaluated by the injection method (Sidorova, 2020). A suspension of fungal spores of each species of the test system was washed off with sterile water from 10-day-old colonies (100 μL at a spore density of 10^5 spores/ml) and spread over the surface of the Petri dish medium on Czapek agar. Then, 24-hours-old biomass of antagonist bacteria was inoculated onto the agar surface. The plates were incubated at a temperature of 28.0 ± 0.5 °C for three days.

The antifungal activity of five strains of the probiotic complex of bacteria belonging to the genus *Bacillus* was evaluated by measuring the diameter of the inhibition zone around the colonies of isolates of microorganisms and pathogenic fungi.

Results. The results of the study on antagonistic activity revealed that a high level of growth inhibition of the test culture *P. multocida* type D No. 07 was observed in all five strains of the probiotic complex of bacteria belonging to the genus *Bacillus*. This was evidenced by the diameter of the growth inhibition zones, which ranged from 27.3 ± 0.9 mm to 35.3 ± 2.0 mm (Table 1).

Table 1 — Antagonistic activity of five experimental strains of the probiotic complex of bacteria of the genus *Bacillus* against planktonic forms of biofilm-forming microorganisms *in vitro*

Bacteria culture	Diameter of the zone of inhibition of growth of biofilm-forming microorganisms, mm				
	P. multocida type D No. 07	*N. meningitidis* No. 18	*S. haemolyticus* No. 14	*E. coli* No. 21	*A. pleuropneumoniae* No. 12
B. amyloliquefaciens ALB 65	27.3 ± 0.9	17.7 ± 1.2	28.7 ± 1.2	26.3 ± 1.5	16.3 ± 0.3
B. pumilus UNCSM-026	35.3 ± 2.0	22.0 ± 1.7	21.3 ± 2.3	29.3 ± 0.9	22.3 ± 0.9
B. subtilis UNCSM-020	29.3 ± 1.5	14.3 ± 3.2	32.3 ± 2.6	24.7 ± 1.2	18.7 ± 1.2
B. subtilis var. *mesentericus* UNCSM-031	34.0 ± 2.3	23.3 ± 2.6	28.7 ± 0.7	27.3 ± 2.0	26.0 ± 0.6
B. licheniformis UNCSM-033	31.3 ± 3.8	26.7 ± 1.2	34.0 ± 1.2	30.7 ± 2.3	20.3 ± 1.5
Probiotic complex of bacteria of the genus *Bacillus* (five strains)	47.3 ± 0.9	40.3 ± 2.6	45.3 ± 0.9	39.3 ± 1.5	38.3 ± 0.9

Note. Inhibition level, mm: up to 13 — low; 14–26 — medium; 27–36 — high; 37 and more — very high.

The *B. licheniformis* UNCSM-033 strain demonstrated a notable antagonistic impact on *N. meningitidis* No. 18, with an inhibition level of 26.7 ± 1.2 mm. This was higher than the corresponding value of the *B. subtilis* var. *mesentericus* UNCSM-031 strain by 12.7%, *B. pumilus* UNCSM-026 by 17.6%, *B. amyloliquefaciens* ALB 65 by 33.7% and *B. subtilis* UNCSM-020 by 46.4%, respectively.

Inhibition of growth and reproduction of *S. haemolyticus* No. 14 at a high level of intensity was determined in four probiotic strains *B. licheniformis* UNCSM-033 — 34.0 ± 1.2 mm, *B. subtilis* UNCSM-020 — 32.3 ± 2.6 mm, *B. amyloliquefaciens* ALB 65 — 28.7 ± 1.2 mm and *B. subtilis* var. *mesentericus* UNCSM-031 — 28.7 ± 0.7 mm, while the strain *B. pumilus* UNCSM-026 showed an average inhibition level of 21.3 ± 2.3 mm.

A sufficiently high level of antagonism concerning the test culture *E. coli* No. 21 was shown by five experimental probiotic strains. Thus, the spore-forming strain *B. licheniformis* UNCSM-033 with a diameter of growth inhibition 30.7 ± 2.3 mm did not have a significant difference from the values of *B. pumilus* UNCSM-026 (29.3 ± 0.9 mm), *B. subtilis* var. *mesentericus* UNCSM-031 (27.3 ± 2.0 mm), *B. amyloliquefaciens* ALB 65 (26.3 ± 1.5 mm) and *B. subtilis* UNCSM-020 (24.7 ± 1.2 mm), respectively.

Antimicrobial activity concerning *A. pleuropneumoniae* No. 12 was observed in all experimental probiotic strains with an average level of antagonistic effect: in strain *B. amyloliquefaciens* ALB 65 (16.3 ± 0.3 mm) and differed from this indicator in *B. subtilis* UNCSM-020 by 14.7%, in *B. licheniformis* UNCSM-033 by 24.5%, *B. pumilus* UNCSM-026 by 36.8% and *B. subtilis* var. *mesentericus* UNCSM-031 by 59.5%, respectively. In addition, it is necessary to note a very high level of antagonistic properties of the probiotic complex of bacteria of the genus *Bacillus* from five experimental strains against five types of pathogenic microorganisms from 38.3 ± 0.9 mm in *A. pleuropneumoniae* No. 12 and up to 47.3 ± 0.9 mm inhibition in *P. multocida* type D No. 07.

At the next research stage, the results of the antifungal activity of five probiotic strains of *Bacillus* were obtained. As a result of the studies, it was found that the average level of antifungal activity was observed in two probiotic strains *B. subtilis* var. *mesentericus* UNCSM-031 (15.7 ± 0.7 mm and 19.3 ± 2.6 mm) and *B. licheniformis* UNCSM-033 (17.3 ± 2.0 mm and 24.7 ± 0.7 mm) against pathogenic fungi *A. niger* No. 1 and *A. candidus* No. 2. Strains *B. amyloliquefaciens* ALB 65 (23.3 ± 0.9 mm) and *B. pumilus* UNCSM-026 (26.0 ± 1.7 mm) had an average level of inhibition only against *A. niger* No. 1, respectively (Table 2).

Table 2 — Antagonistic activity of five experimental strains of the probiotic complex of bacteria of the genus *Bacillus* against pathogenic fungi *in vitro*

Bacteria culture	Diameter of the zone of inhibition of pathogenic fungi growth, mm	
	A. niger No. 1	*A. candidus* No. 2
B. amyloliquefaciens ALB 65	23.3 ± 0.9	31.0 ± 1.2
B. pumilus UNCSM-026	26.0 ± 1.7	32.3 ± 0.9
B. subtilis UNCSM-020	28.3 ± 0.9	33.3 ± 2.2
B. subtilis var. *mesentericus* UNCSM-031	15.7 ± 0.7	19.3 ± 2.6
B. licheniformis UNCSM-033	17.3 ± 2.0	24.7 ± 0.7
Probiotic complex of bacteria of the genus *Bacillus* (five strains)	30.7 ± 1.2	37.8 ± 2.0

Note. Inhibition level, mm: up to 13 — low; 14–26 — medium; 27–36 — high; 37 and more — very high.

High fungicidal activity was observed in the probiotic strain *B. subtilis* UNCSM-020 with a zone of growth inhibition of 28.3 ± 0.9 mm against *A. niger* No. 1, and against *A. candidus* No. 2 this value was higher by 17.7%. Two probiotic strains *B. amyloliquefaciens* ALB 65 (31.0 ± 1.2 mm) and *B. pumilus* UNCSM-026 (32.3 ± 0.9 mm) had a high level of mycelial destruction in *A. candidus* No. 2. The highest level of antifungal activity was found in the probiotic complex of bacteria of the genus *Bacillus* (37.8 ± 2.0 mm) against the fungal strain *A. candidus* No. 2 and high against *A. niger* No. 1 (30.7 ± 1.2 mm), respectively.

Thus, according to the results of the studies, five experimental strains of the probiotic complex of bacteria of the genus *Bacillus* (*B. amyloliquefaciens* ALB 65, *B. pumilus* UNCSM-026, *B. subtilis* UNCSM-020, *B. subtilis* var. *mesentericus* UNCSM-031, and *B. licheniformis* UNCSM-033) demonstrated antagonistic activity against opportunistic and pathogenic microorganisms and fungicidal effect against pathogenic fungi. The most sensitive to the five antagonist strains were the test strains of *P. multocida* type D No. 07, *S. haemolyticus* No. 14 and *E. coli* No. 21. The highest degree of antagonistic activity against five test cultures of biofilm-forming microorganisms and antifungal effect against two test strains of pathogenic fungi *A. niger* No. 1 and *A. candidus* No. 2 was shown by the probiotic complex of bacteria of the genus *Bacillus* (five strains).

Discussion. The antimicrobial properties of *Bacillus* strains of the probiotic complex are due to their ability to destroy (lyse) certain bonds in the peptidoglycan structure of cell walls of different microorganism types. The mechanism of antagonistic action of probiotic strains is due to the presence of their properties useful for the macroorganism, in particular, the ability to synthesize various biologically active compounds (Cairns, Hobley and Stanley-Wall, 2014; Sumi et al., 2015; Khardziani et al., 2017). These include antibiotic substances and extracellular enzymes, including proteases, amino acids, and polysaccharides. *B. subtilis* and *B. licheniformis* strains can synthesize cyclic lipopeptide antibiotics. The main components of these antibiotics are surfactin, fengicin, and bacitracin, which are characterized by multiple biological activities (AlGburi et al., 2017; Chen et al., 2024). Lipopeptides from bacilli also exhibit pronounced inhibitory activity against various types of pathogenic fungi.

The antimicrobial activity of bacilli is determined not only by the synthesis of antibiotics, but also by lytic enzymes that cause the lysis of cells of gram-positive and gram-negative bacteria, as well as pathogenic fungi, which together or separately are capable of destroying the cell wall of microorganisms (Adeniji, Aremu and Babalola, 2019; Kadhum and Hasan, 2019; Mardonov et al., 2021)

In addition, bacilli can form biofilms and form associations with several microorganisms (five antagonist strains) and thereby inhibit the rate of biofilm formation in test cultures of *P. multocida* type D No. 07, *N. meningitidis* No. 18, *S. haemolyticus* No. 14, *E. coli* No. 21, *A. pleuropneumoniae* No. 12 (Aguilar et al., 2010; Arnaouteli et al., 2021). Taken together, the results confirm the probiotic potential of *Bacillus* bacteria, emphasizing its metabolic advantage over pathogens.

Conclusions. 1. The strains *B. pumilus* UNCSM-026, *B. subtilis* UNCSM-020, *B. subtilis* var. *mesentericus* UNCSM-031, *B. licheniformis* UNCSM-033 have versatile inhibitory and fungicidal activity against opportunistic and pathogenic test cultures of microorganisms. A very high antagonistic activity (38.3–47.3 mm) was found in the probiotic complex of bacteria of the genus *Bacillus* to planktonic forms of test cultures of *P. multocida* type D No. 07, *N. meningitidis* No. 18, *S. haemolyticus* No. 14, *E. coli* No. 21, *A. pleuropneumoniae* No. 12 and biofilm-forming fungi *A. candidus* No. 2 (37.8 mm).

2. The pronounced antimicrobial properties of five strains of the probiotic complex of bacteria of the *Bacillus* genus present a promising avenue for developing drugs based on them as an alternative to antibiotics. This is particularly crucial in the context of the growing prevalence of antibiotic-resistant pathogens and the declining efficacy of numerous antibiotics.

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