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Antifungal And Antagonistic Properties Of The Bioconservant “Imbiocon”

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ABSTRACT

The antifungal and antagonistic properties of the most effective microorganisms of lacto, bifido and spore bacteria, which are part of the bioconservant “IMBIOCON”, have been studied. It was shown that the studied cultures had the highest antagonistic activity against conditional pathogens such as *Pseudomonas aeruginosa*, *Proteus morganii* 399, *Serratia marcescens*, *Listeria monocytogenes* as well as antifungal activity against *Aspergillus flavus*, *Verticillium dahlum* and *Alternaria alternata*.

KEYWORDS

Lactobacteria, Bifidobacteria, Spores, Conventionally Pathogenic Bacteria, Fungi.

INTRODUCTION

Nowadays, one of the priorities of modern agricultural production is to provide the population with sufficient quantities of high-quality livestock products. In the structure of the cost of livestock products, the largest share

is occupied by feed costs. Livestock production continues to grow due to inflated feed import costs, as well as increasing wages.

Silage is one of the most famous ways to preserve feed. Pre-silage feed intended for silage usually contains a small amount of lactic acid bacteria (LAB). Therefore, it is necessary to use starter cultures of selected strains. Traditionally, starter cultures of LAB have been used to reduce pH through the production of lactic acid and to suppress the growth of undesirable epiphytic microorganisms due to competition for nutrients. Epiphytic microflora characteristic of plant raw materials used for silage production and sources of undesirable microflora in the process of ensiling. Among them, the main described trends were the prevention of the growth of mycelial fungi and the detoxification of mycotoxins with the help of LAB inoculants, inhibition of yeast growth by means of LAB present in the preparations and limiting the development of pathogenic bacterial microflora [1]. The authors [2] conducted studies on the analysis of bacterial dynamics associated with whole wheat silage with and without inoculants. Whole wheat was ensiled under laboratory conditions with and without addition of *Lactobacillus* (*L. plantarum*, *L. buchneri*) for 3 months. After 3 months of silage, *Lactobacillus* dominated the bacterial population of silage and reached 59.5, 92.5 and 98.2% in untreated silage treated with *L. plantarum* and *L. buchneri*, respectively. The lactic acid content was higher in silage with *L. plantarum* compared to untreated silage and silage treated with *L. buchneri*. Silage of corn by LAB with a combination of *Lactobacillus buchneri* NCIMB40788 and *Lactobacillus hilgardii* CNCM-I-4785 was carried out [3], which contributed to an increase in aerobic stability. Four treatments were tested; Control, *Lb. buchneri*, *Lb. hilgardii* and a combination of two strains that were fermented for 1, 2, 4, 8, 16, 32 and 64 days. Microbiota analysis using

metasequencing of 16S and ITS amplicon showed that inoculation brought the dominant *Leuconostocaceae* population to day 1 with 48.1%. *Lactobacillaceae* predominated in sequence by day 4 with 21.9%. After 32 days, inoculation with both strains had the lowest level of bacterial alpha diversity: 29.0 observed compared to 61.3 for control. The works of the authors [4] are aimed at studying the adaptation and competition of bacteria *Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Enterococcus faecalis*, in the silage of alfalfa. Before ensiling, alfalfa was inoculated with *L. plantarum* (Lp), *P. pentosaceus* (Pp), *E. faecalis* (Ef) and in combination with (Lp Pp, Lp Ef, LpPp Ef). Samples were taken on days 1, 3, 7, 14 and 60 of ensiling. On the 60th day of fermentation of the silage, the silage inoculated with Lp, Pp- and Lp Pp cultures had a lower pH. High concentrations of lactic acid were observed in silage with the addition of the association of cultures Pp, Lp, Ef and Lp Pp Ef. The quantitative composition of the microflora of silage on the 14th day showed the dominance of *L. plantarum*, *Weissella cibaria* and *L. pentosaceus* after fermentation in all treatment options. Silage was carried out [5] with fresh and withering leaves of *Moringa oleifera* with the addition of LAB *Lactobacillus farciminis* LF and *Lactococcus lactis* LL for 1, 7, 14, 30 and 60 days. As a result, silage with bacteria of the species *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Enterococcus*, *Leuconostoc* and *Enterobacter* improved the fermentation quality of wilted and fresh silage from *M. oleifera* leaves by accelerating the production of lactic acid and lowering pH. A decrease in dry matter loss and suppression of the growth of yeast and coliform bacteria was observed throughout the entire fermentation

process. Researchers [6] constructed a strain of *Lactococcus lactis* with highly effective secretory-expressing cellulase genes from *Trichoderma reesei* and studied the effects of a combination of transgenically engineered *L. lactis* strains HT1/pMG36e-usp45-bgl1, HT1/pMG36e-usp45-cbh2 and HT1/pMG36e-usp45-egl3 (HT2) on fermentation quality, structural degradation of carbohydrates and kinetics of fermentation of nonstructural carbohydrates of alfalfa silage with a high moisture content. In [7], 104 strains of LAB were isolated from rumen fluid and feces of six dairy cows, of which four strains (*Lactobacillus plantarum* F1, *L. plantarum* F50, *Lactobacillus salivarius* L100, and *Lactobacillus fermentum* L120) and one commercial inoculant (GFG) were isolated from feed. The laid alfalfa silage treated with F1 had the lowest ($P < 0.05$) pH value and the highest lactic acid content in all variants. Microbiological analysis showed that the amount of *Lactobacillus* in silage treated with F1 increased to 60.32%, which is higher than with other silage treatments (5.12–47.64%). Silage [8] inoculated with isolated LAB (LpE) provides better fermentation, including low pH, high levels of lactic acid and water-soluble carbohydrates, which have been demonstrated that LpE inoculant can help maintain nutrition and regulate fermentation. In silage inoculated with LpE prevailed *Lactobacillus* (95.71%) and *Weissella* (0.19%). For use as biofuel, a mixture of air-dried corn straw and cabbage waste was ensiled to preserve lignocellulose biomass [9]. At the same time, the use of formic or acetic acid in an amount of 0.3 and 0.6% in silage resulted in higher fractions of water-soluble carbohydrates than in the control for 170 days. The transition of bacteria from *Lactobacillus* and *Enterobacter* in silage biomass to

Lactobacillus and *Paralactobacillus* was also observed. In particular, *Enterobacter* disappeared after 130 days of storage. The effect of four sources of food; sugar, fructose, pectin and starch on the microbiota and metabolome of alfalfa silage (*Medicago sativa*) has been examined in a study [10]. On the 60th day of fermentation, the pH values with fructose and pectin were lower than that of sucrose and starch. A higher concentration of lactic acid was observed in silage with added sucrose and pectin. In silage, *Weissella* (47.44%) and *Lactobacillus* (42.13%) were identified as the dominant species in all four groups of carbohydrates. It was shown that the addition of pectin and fructose improved the quality of alfalfa silage by stimulating *Leuconostoc*, *Pantoea* and *Microbacterium*, as well as inhibiting *Pediococcus*, *Turicibacter*. The researchers [11] studied a plant of the legume genus *Oxytropis glabra* sharkfish, which dominates with a high natural yield on degraded pastures of Inner Mongolia. Fermentation of a silage mixture of *oxytropis* and corn was carried out, which had a positive effect on the quality of fermentation and detoxification of *svainsonin*. The highest amount of *Lactobacillus plantarum* was found in all silage variants in an optimal ratio of 1:1. From the studied cultures strains *L. amylovorus* and *L. plantarum* had a higher rate of removal of the immunomodulator and *svainsonin* inhibiting glycoside hydrolases, in particular, N-linked glycosylation. A test was conducted out [12] using whole grain corn silage in silo bags and fodder. Silage sown with cultures of *Lentilactobacillus buchneri* NCIMB 40788 (*Lactobacillus buchneri*) and *Lentilactobacillus hilgardii* CNCM-I-4785 (*Lactobacillus hilgardii*) on day 159 of fermentation showed a high content of *saccharomyces* and the presence of

lactic acid cultures of *Lactobacillus* throughout the analysis. In the control silage, there was an increase in the amount of yeast assimilating lactate (*Pichia* and *Issatchenkia*, as well as *Acetobacter* and *Paenibacillus*). In [13], investigations have been conducted to study the effect of various additives on the fermentation quality of microflora and aerobic stability of a silage of a mixed version containing moist corn gluten feed and corn straw. Inoculation was performed using LAB with the addition of a fibrolytic enzyme. Silage analysis showed that the addition of LAB improved the aerobic stability of the mixed silage, as evidenced by the high lactic acid content and lower pH. In some other investigations [14] are described the composition and diversity of the bacterial communities of the silage process of the Mexican corn variety Amarillo Zamorano compared with the commercial hybrid Antilope. Grown corn of both types was crushed and packed into laboratory micro-bins. Physicochemical parameters have been studied, and DNA was isolated from juice in microcircuits. Lactobacillales predominated in both variants. In a silage with commercial hybrid Antilope, cultures of Enterobacteriales, Lactobacillales have been identified. The influence of the moisture index on the dynamics of fermentation parameters and microflora during alfalfa silage was studied [15]. Noculant LAB was used as an additive. On the 15th and 30th days of silage readiness in the control, a high pH and a high concentration of ammonia nitrogen ($\text{NH}_3\text{-N}$) were noted. In the experimental version of the silage, significant accumulations of lactic and acetic acids were observed and a decrease in pH on the 60th day of silage, and the number of *Lactobacillus* significantly increased while the number of

bacteria *Garciella* and *Anaerosporebacter* decreased. The authors of [16] conducted studies to assess the effect of two strains of *Lactobacillus* species producing class IIa bacteriocins on silage fermentation, microbial population, chemical composition and aerobic stability. As a result, it was found that lactic acid bacteria producing class IIA bacteriocin improve the quality of fermentation of silage, reduce the amount of mold and yeast aerobic stability, to a greater extent than inoculation with *Lactobacillus plantarum* MTD/1, a proven and widely used inoculant, which does not produce bacteriocin. Experiments were carried out [17] with piglets that were fed with liquid unfermented feed and fully fermented with the addition of 40% unfermented coarse cereals. Studies of the microbiota of the digestive tract of the small and large intestine and faeces showed that Lactobacillaceae prevailed in the digestion of the small intestine of pigs receiving fermented feed (relative abundance was up to 95%). In the contents of the colon, the amount of Lactobacillaceae was significantly higher only in pigs receiving fermented liquid feed with the addition of non-fermented coarse cereals. In this variant, an increase in the number of bacteria belonging to the genus *Lactobacillus* and *Bifidobacterium* was observed. The authors [18] studied the effect of silage on the nutritional composition and fermentation characteristics of two species of brown algae, *Fucus vesiculosus* and *Saccharina latissimi* using *Lactobacillus plantarum* inoculant. The suspended algae were placed in laboratory-scale silage tanks for 90 days. The silage with *Saccharina latissimi* had a high fermentation ($\text{pH} < 4$), with a predominance of lactic acid (50-60 g/kg of dry matter) and a relatively low content of acetic acid compared to the *Fucus vesiculosus* silage.

At the same time, a decrease in crude protein (CP, -32%) and ash (-36%) was observed in the silage with *Saccharina latissima*. In an investigation [19], a method of preparing corn straw for processing *Irpeus lacteus* (*I. lacteus*) was used in order to improve its ability to decompose in the rumen in vitro under non-sterile conditions. The following investigations [20] were carried out to study the antimicrobial activity of 13 strains of LAB and the presence of antibiotics in them. An effective composition was selected containing 1.0% EO of thyme and strains of LAB: *Lactobacillus plantarum* LUHS122, *Enterococcus pseudoavium* LUHS242, *Lactobacillus casei* LUHS210, *Lactobacillus paracasei* LUHS244, *Lactobacillus plantarum* LUHS244, *Lactobacillus plantarum* LUHS135, *Lactobacillus plantarum* LUHS135 which are recommended for poultry farming as feed ingredients or for treating surfaces contaminated with *Salmonella* strains. A review [21] describes the many stresses faced by LAB and presents the experimental context used to study the stress responses of LAB. Systemic strategies have been developed to characterize the "stressome" of LAB and to develop new nutritional and probiotic LAB with improved stress resistance. The use [22] of subtherapeutic doses of antibiotics as a growth stimulant in animals enhances the emergence and spread of antimicrobial resistance in bacteria. An alternative to antibiotics in animal feed are LAB as probiotics. A study was conducted to determine the anti-*Salmonella* activity and the suitability of LAB isolated from bovine faeces in Nigeria as potential probiotics in cattle feed. The use of [23] LAB in raw and minimally processed fruits and vegetables helps to better maintain their quality by increasing their shelf life. Bacteriocins, either alone or in combination

with edible coatings, are considered a very promising approach for microbiological quality and safety in post-harvest raw and minimally processed fruits and vegetables. The addition [24] of sub-therapeutic doses of antibiotics to cattle feed to stimulate growth is a contributing factor to antibiotic resistance, therefore an alternative to antibiotics is needed in animal feed supplements. A study [25] aimed at determining the antifungal activity of *Lactobacillus plantarum* MYS6 against the fumonisin-producing fungus *Fusarium proliferatum* MYS9. The isolate was subjected to standard tests to determine its probiotic properties and antifungal properties *L. plantarum* MYS6 which developed well at pH 3.0 and 6.0 and showed strong resistance up to 3% bile. The bioprotective feature of the isolate was manifested in the inhibition of the development of fungi in corn kernels treated with the cell-free supernatant *L. plantarum* MYS6. Lactic acid bacteria [26] are important probiotics for preventing certain infections. The authors studied the effect of selenium dioxide on the antifungal activity of *Lactobacillus plantarum* and *L. johnsonii* against *Candida albicans*. In this regard, the aim of the investigation was to study the antagonistic and antifungal activity of probiotic strains of microorganisms, preserving feed, promising for the preparation of the biological product "IMBIOCON".

MATERIALS AND METHODS

The research material was lactic acid producing bacteria: *L. rhamnosus*-J.S.2, *L.c.*K7/4, *L. plantarum* AB-1 *L.c.* P6/2, *L. plantarum* ET 2, *L.c.* K7/3, *L. plantarum* K3.3, *L.c.* CO1, *L. acidophilus*, *Bifidobacterium animalis* and *B. subtilis* spore bacteria isolated from vegetables, sauerkraut,

various types of cheese, cottage cheese, and yogurt). Selected samples were crushed, placed in MRS broth and cultured at 37°C for 48 hours for enrichment. Serial dilutions were prepared from the storage broth and made on a continuous lawn on MRS agar, 2 cups for each dilution. One dish of 2 replicates was placed in a thermostat under aerobic conditions of cultivation, the second in an anaerostat, where the air was replaced with gaseous nitrogen (under anaerobic conditions). They were cultivated at 37°C for 48 hours. After cultivation, isolated colonies differing in morphological characteristics were selected from the plates, and then subcultured onto a plate with MRS agar containing 2-3 drops of an alcohol solution of the indicator bromocresolpurpur to establish acid formation. Plates were incubated at 37°C until growth appeared. Isolates that change the color of the medium from violet to yellow were used for further studies.

To determine the antagonistic activity, a drip technique was used. The daily culture of the probiotic was applied to the surface of the nutrient medium with a bacteriological loop 3 mm in diameter on petri dishes with MRS medium. The cups were left at room temperature until the drop was completely absorbed. After that, having retreated 1-2 mm from the edge of the first spot, a drop of the culture of the tested microorganism was applied. The melted drop of the test culture flows onto the culture spot of the probiotic culture strain up to half the diameter. In the part where droplets overlap, competing cultural relationships arise. After the drops dry, the dishes are incubated in an anaerostat under microaerophilic conditions at 37°C. The determination of the test results was carried

out after 24 hours of incubation in a thermostat at a temperature of 37°C according to the size of the zones (mm) of inhibition of the growth of test cultures. The presence and degree of antagonistic activity in the tested probiotic culture was judged by the size of the zone of inhibition of the test strain.

Antifungal activity was determined by the method of agar blocks; an exponential culture of the studied strain was sown on the surface of the agar medium in a Petri dish and incubated on MRS medium; the culture time of the studied cultures was 72 at its optimum temperature (37°C). The antagonistic properties of the isolated lactic acid cultures were studied on the Czapeka Doksa solid nutrient medium: by the method of agar blocks, the experiments were carried out in two replicates. The antagonistic activity of bacteria was judged by the size of the zone of no pathogen growth. A 6-day suspension of conidia of the tested pathogenic fungi was sown with a lawn in Petri dishes with agar Czapek's medium. 15-20 minutes after sowing: 1) blocks of a 3-day culture of lactic acid bacteria were applied to the test culture. The dish was again incubated, but now under conditions (temperature and duration) favorable for the growth of the test culture. The determination of the test results was carried out after 24 hours of incubation in a thermostat at a temperature of 37°C according to the size of the zones (mm) of inhibition of the growth of pathogen cultures. The presence and degree of antagonistic activity in the tested probiotic culture was judged by the size of the zone of inhibition of the test strains on the border with the block. The isolated strains were identified using a MALDI-TOF mass spectrometer [28].

RESEARCH RESULTS

For the study of morphological and cultural characteristics and selection of the most effective microorganisms, strains of lactic acid, bifido and spore bacteria *L. rhamnosus*-J.S.2, *L. casei* isolated in the laboratory of "Microbiology and biotechnology of probiotics" were used. K7/4, *L. plantarum* AB-1, *L. casei*. P6/2, *L. plantarum* ET 2 *L. casei* K7/3, *L. plantarum* K3.3, *L. casei* CO1, *L. asidophilus*, *Bifidobacterium animalis* and spore bacteria *B. subtilis* preparation of high quality silage and haylage from various forage crop [29]. In the isolated cultures, the antagonistic activity was studied by the drop method on the MRS nutrient medium; the cultivation time of the studied cultures was 72 hours. The results were interpreted as follows: zone of inhibition of pathogen growth ≤ 15 mm - low antagonistic activity, 15-30 mm - medium activity, ≥ 30 mm - high activity. As the results show, the cultures we selected in Table 1 selectively suppressed the growth of all opportunistic pathogenic strains we tested; *Proteus morganii* 399, *Serratia marcescens* 367, *E. coli* NC 101, *Pseudomonas aeruginosa*, *Citrobacter freundii*

002801/27, *Klebsiella pneumoniae* B-1823, *Staphylococcus aureus*, *Enterococcus faecalis* OGIFR I, *E. faecium* K50, *Listeria monocytogenes*. The highest was the antagonistic activity of lactobacilli. Thus, *L. rhamnosus* completely suppressed the growth of 10 tested pathogens. This was especially evident in relation to *Proteus morganii* 399 with an inhibition zone of 38mm, *Pseudomonas aeruginosa* 40mm and *L. monocytogenes* 30mm. The same indicators were observed in the *L. casei* strain. K7/4. A comparatively high drop in growth with a size of 40 mm was found in *Ps. aeruginosa* and *Citrobacter freundii* in *L. plantarum* ET 2. Strain *L. casei*. K7/3 showed the highest suppression of all pathogens tested on *Listeria monocytogenes* where the area was 42 mm. The antagonistic activity of the *B. subtilis* spore culture was manifested on *Serratia marcescens* 367 *E. faecium* K50 and *Listeria monocytogenes* with a growth inhibition zone of 36.35 and 34 mm, respectively. The bifidobacterium *Bifidobacterium animalis* was significantly different in relation to *Pseudomonas aeruginosa* 34 mm and *Listeria monocytogenes* 42 mm.

Table 1

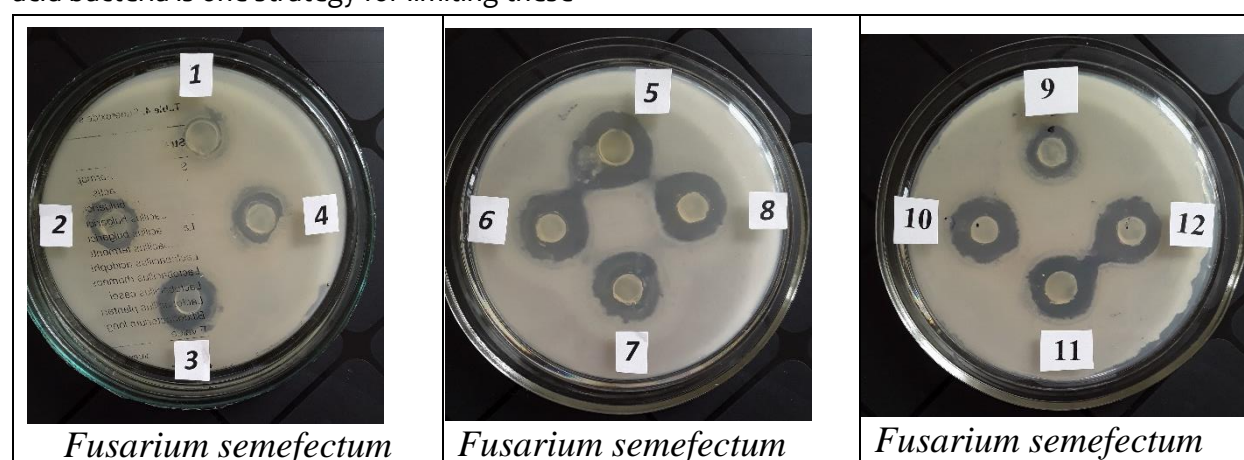
Antagonistic activity of bacteria to opportunistic bacteria (zone of growth retardation of pathogenic cultures, mm)

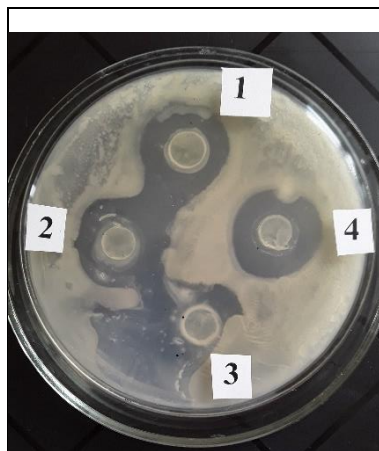
Culture	Diameter of the growth inhibition zone,mm									
	<i>Proteus morganii</i> 399	<i>Serratia marcescens</i> 367	<i>E.coli</i> NC 101	<i>Pseudomonas aeruginosa</i> 003841/114	<i>Citrobacter freundii</i> 002801/27	<i>Klebsiella pneumoniae</i> B-1823	<i>Staphylococcus aureus</i> 003594/wood	<i>Enterococcus faecalis</i> OGIFR I	<i>E.faecium</i> K50	<i>Listeria monocytogenes</i> ATCC 1911

<i>L.rhamnosus</i> -J.S.2	38	25	24	40	25	20	15	40	25	30
<i>L.casei</i> K7/4	40	32	18	30	16	22	20	0	25	30
<i>L.plantarum</i> AB-1	26	38	25	40	0	12	10	30	0	30
<i>L.casei</i> . P6/2	30	36	24	33	0	12	15	0	35	34
<i>L.plantarum</i> ET 2	20	37	30	40	40	26	15	26	15	30
<i>L.casei</i> .K7/3	20	30	20	34	24	26	28	25	20	42
<i>L.plantarum</i> K3.3	25	25	13	0	15	0	21	0	0	0
<i>L.casei</i> CO1	25	30	20	28	30	27	40	32	35	20
<i>B.subtilis</i>	30	36	24	33	0	12	15	0	35	34
<i>L.asidophilus</i>	26	38	25	40	0	12	10	30	0	30
<i>Bifidobacterium animalis</i>	20	30	20	34	24	26	28	25	20	42
<i>L.plantarum</i> AB 18/1	19	24	18	25	20	23	26	23	17	30

Aerobic spoilage of silage after fattening leads to mycotoxin contamination and deterioration of its quality. Inoculation of silage with lactic acid bacteria is one strategy for limiting these

effects. In this regard, studies were carried out to study the antifungal activity in relation to the phytopathogens of the LAB.

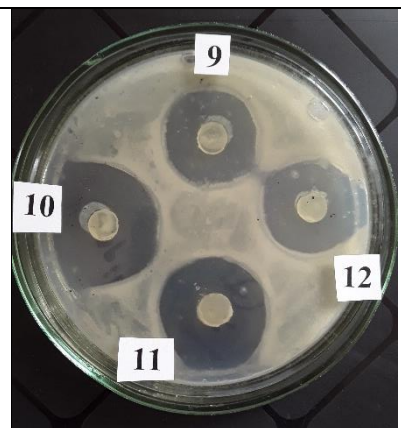




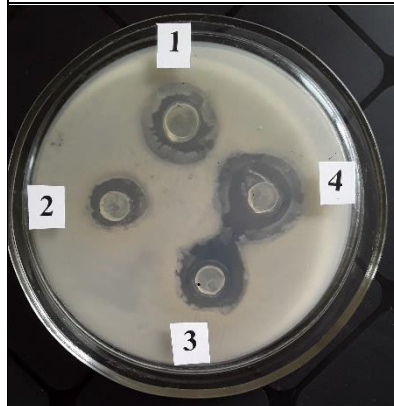
Verticillium dahliae



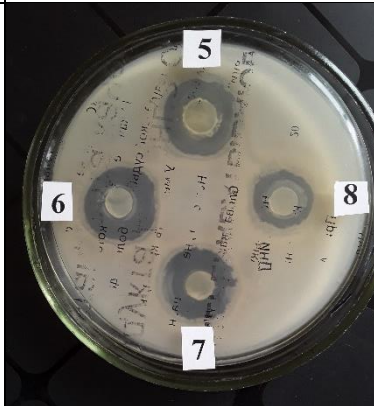
Verticillium dahliae



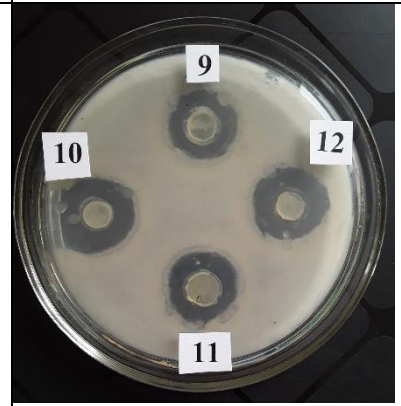
Verticillium dahliae



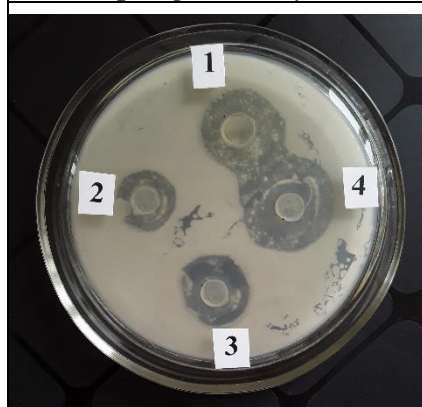
Aspergillus oryzae



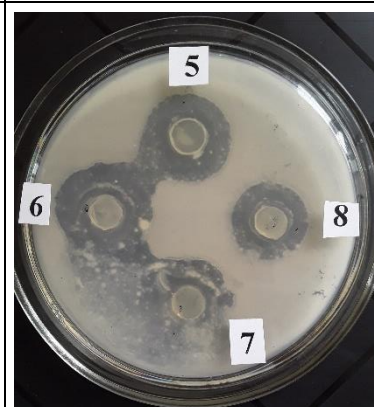
Aspergillus oryzae



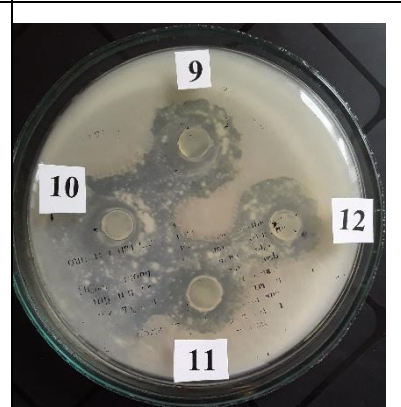
Aspergillus oryzae



Aspergillus sp



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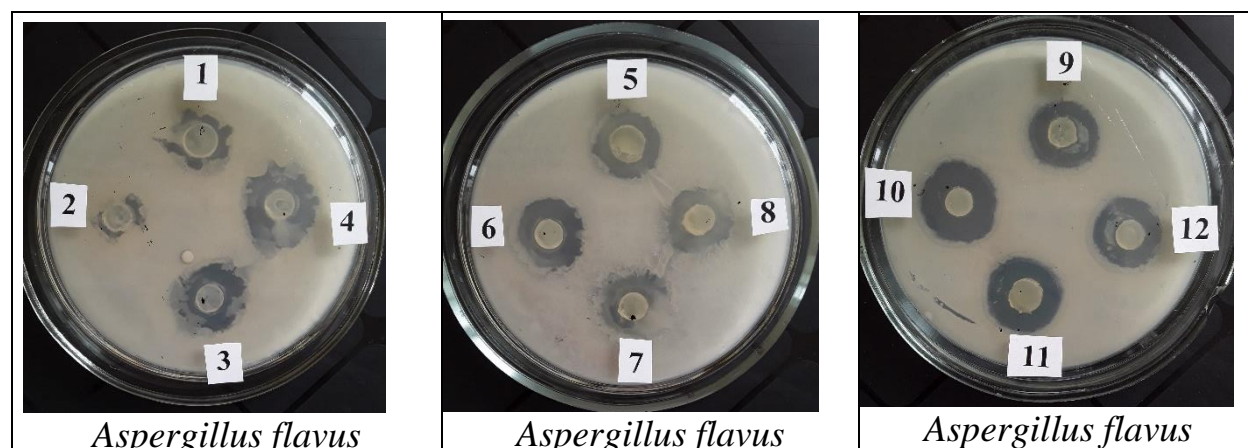


Fig.1 Antifungal activity of local strains of lactobacilli included in the bioconservative IMBIOKON.

The results of the studies showed that the studied lactic acid bacteria exhibit moderately pronounced antagonistic activity in relation to *Alternaria alternata* of the culture *L. plantarum* AB-1, *L. plantarum* ET 2, *L. plantarum* K3.3 where the zone of absence of growth in them was 28, 27, 25 mm.

Relatively low rates, noted for the phytopathogen *Fusarium* species, in this

variant, the *L. casei* P6 / 2 strain was active with an area of action of 25 mm.

A pronounced antagonistic activity was detected on *Verticillium dahliae* in lactic acid cultures *L. rhamnosus*-J.S. 2 - 22 mm, *L. casei* K7 / 4 - 33 mm *L. casei*. P6 / 2 - 27 mm, *L. plantarum* ET 2 - 29 mm. The studied cultures showed similar results in relation to *Aspergillus flavus*.

Table 2.
Antifungal activity of bacteria against phytopathogenic fungi

Culture	Growth inhibition zone, in mm			
	<i>Aspergillus flavus</i>	<i>Verticillium dahliae</i>	<i>Fusarium species</i>	<i>Alternaria alternata</i>
<i>L.rhamnosus</i> - J.S.2	17	25	15	20
<i>L.casei</i> .K7/4	22	33	19	25
<i>L.plantarum</i> AB-1	26	25	15	28
<i>L.casei</i> P6/2	22	27	25	22
<i>L.plantarum</i> ET 2	22	29	12	27
<i>L.casei</i> K7/3	24	24	23	20
<i>L.plantarum</i> K3.3	27	22	22	25
<i>L.casei</i> CO1	23	23	21	23

<i>B.subtilis</i>	20	18	20	22
<i>L.asidophilus</i>	22	20	18	23
<i>Bifidobacterium animalis</i>	19	21	17	19
<i>L.plantarum</i> A5 18/1	24	23	12	20

It is noted that the most pronounced antifungal effect on the studied phytopathogens was shown by strains of lactic acid bacteria cultures, which have different magnitudes and spectrum of action. In relation to phytopathogens *Alternaria alternate*, *Verticillium dahliae*, *Aspergillus flavus*, *Fusarium* species.

Thus, the domestic bioconservative "IMBIOKON", developed by the Institute of Microbiology Academy of Sciences of the Republic of Uzbekistan, has a wide spectrum of antagonistic activity against both opportunistic bacteria and phytopathogenic fungi. The practical use of a bio-preservative helps to improve the composition, quality of silage fodder and increases the productivity of farm animals.

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