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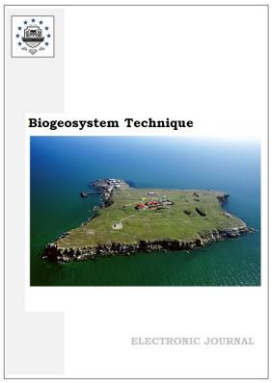

Searching for Effective Antagonists Against Toxin-Forming Microscopic Fungi and Bacteria

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Abstract

The paper presents the studies in the field of toxin-forming phytopathogens of plant raw materials prevention. The studied isolates can be used as potential agents of biological control of fungal infections (*Fusarium, Verticillum, Alternaria, Chaetomium,* etc.) in agriculture. In the experiment, when silage was contaminated with the phytopathogen *Chaetomium*, sanitary indicators deteriorated, namely: the crude protein content when exposed to the phytopathogen *Chaetomium* became 26 % below control; the concentration of acid-detergent fiber when exposed to phytopathogen became lower than control by 28 %; the moisture content in the second group increased by 11 %; the concentration of dry matter when exposed to phytopathogen became lower than control by 36 %; the silage acidity index when exposed to phytopathogen became higher than control by 22 %; the concentration of lactic acid in the group with the use of the phytopathogen *Chaetomium* decreased by 20 %, the concentration of acetic acid decreased by 17 % compared to the control group. The use of isolates in corn silage contributed to the improvement of chemical parameters and increased nutritional value. Also, strains of Bacillus spp., Lactobacillus spp. Lactococcus spp. and 5-hydroxy-6-methyluracil can be used in the development of new effective drugs to combat various groups of pathogenic microorganisms.

Keywords: plant feed, corn, silage, dry protein, antagonism, opportunistic microflora, isolates.

1. Introduction

The principal methods of combating phytopathogenic microorganisms are the use of chemical preparations or biological agents, including probiotics and antibiotics (Sharma et al., 2009; Valiullin et al., 2020; Mukhammadiev et al., 2021). Phytopathogens in food raw materials can form secondary metabolites or mycotoxins that are toxic to humans and animals and highly stable in the environment. Mycotoxins of fungi of the genus *Fusarium, Verticillum, Alternaria, Chaetomium*, etc. are common in temperate latitudes because climatic conditions are suitable for development and reproduction in plant raw materials. Agricultural products and feeds affected by

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microscopic fungi change their nutritional value and become toxic to human and animal health. Such products and feeds can cause disorders of the nervous system, liver, kidneys, immune system, and disrupt reproductive ability. Chemicals are widely used in agriculture to protect food raw materials of plant origin, but they can accumulate in soil and food products, which negatively affects human health. Biological agents are safer, but their use is not always effective due to limited use and low stability of action (Valiullin et al., 2020; Tao et al., 2023; Bikmullin et al., 2023). Recently, studies have been conducted that have shown the possibility of using nanoparticles to combat phytopathogenic microorganisms (Li et al., 2019). Nanoparticles are able to penetrate into fungal cells and destroy them, thereby reducing damage from plant diseases (Miedaner et al., 2017; Zgadzay et al., 2021; Afordoanyi et al., 2022). However, the use of nanoparticles can also have a negative impact on the environment and human health, so it is necessary to conduct additional studies to assess their safety.

In general, the development of new methods for improving the safety of feed and combating phytopathogenic microorganisms is an urgent problem of agriculture, which requires an integrated approach and the introduction of innovative technologies (Valiullin et al., 2020; Miftakhov et al., 2022; Kosolapov et al., 2023).

In this regard, the purpose of this study was to search and develop a microbial community, assess its potential as an inhibitor of the development of phytopathogens, as well as improve the quality indicators of plant feeds.

2. Methodology

An isolate antagonistic activity was determined using the agar blocks method with assessment and delayed antagonism. The isolates were sown in Petri dishes on a meat-peptone agar (MRS, HMS media) surface at a temperature 37°C until a "continuous lawn" formation. Then, with a sterile cork drill, the isolate blocks were cut out of the agar surface and transferred to the MPA surface (Chapek growing medium), which was previously seeded with a pathogen test culture in another Petri dish. A pathogen cultivation without an isolate was a control option. The dishes were incubated in a thermostat at a temperature circa 26-–37°C. The data were recorded on the 2nd, 4th and 7th days of experiment. The studies were carried out using the methods of microbiological analysis in accordance with the methodology presented in (Nielsen et al., 1999; Fogle et al., 2007; Cheremisin et al., 2021).

Two experiment in the different options were carried out.

Experiment I: 1 – silage not subjected to isolate addition – control; 2 – silage preserved using an isolate containing *Lactococcus lactis*; 3 – silage preserved using an isolate containing *Lactobacillus plantarum*; 4 – silage preserved using an isolate containing *Bacillus subtilis*; 5 – silage preserved using isolates containing *Lactococcus lactis, Lactobacillus plantarum* and *Bacillus*.

Experiment II: 1 – silage not subjected to phytopathogen *Chaetomium* and isolates – control; 2 – silage contaminated with fungi *Chaetomium*; 3 – silage contaminated with mushroom *Chaetomium* and canned using an isolate containing *Lactobacillus plantarum*; 4 – silage contaminated with fungi *Chaetomium* and canned using an isolate containing *Lactococcus lactis*; 5 – silage is contaminated with *Chaetomium* fungus and preserved using an isolate containing *Bacillus subtilis*; 6 – silage contaminated with *Chaetomium* fungus and preserved using isolates containing *Lactococcus lactis, Lactobacillus plantarum* and *Bacillus subtilis*; 7 – silage contaminated with *Chaetomium* fungus and preserved using isolates containing *Lactobacillus plantarum* and *Bacillus subtilis*; 8 – silage contaminated with *Chaetomium* fungus and preserved using 5-hydroxy-6 compound-methyluracil; 9 – silage contaminated with *Chaetomium* fungus and preserved using isolates containing *Lactobacillus plantarum, Bacillus subtilis* and the 5-hydroxy-6-methyluracil compound.

The organic acid concentration was determined in accordance with the GOST R 55986-2014 https://files.stroyinf.ru/Data2/1/4293772/4293772192.pdf. The silage pH value was measured in extracts using a pH meter (150-MI). The silage dry matter content was determined according to the GOST 31640-2012 https://internet-law.ru/gosts/gost/52337/. The nitrogen and crude protein contents were determined according the GOST 13496.4-2019 https://internet-law.ru/gosts/gost/71526/.

3. Results

A bacterial strains *Bacillus spp., Propionibacterium spp., Lactobacillus spp.* and *Streptomyces* potential as biological means for plant raw materials protection was first described in the early 20th century [15]. The researchers have noticed that the of lactic acid bacteria strains protect harvested plant raw materials from a development of the microscopic fungi producing mycotoxins and pathogenic microorganisms. The data on the antagonistic activity of isolated microorganisms study against the toxin-forming fungi are presented in the Table 1.

The microorganisms №3, 6, 8, 10, 14, 16, 18, 22 and 27 with the most biochemical activity (proteolytic, cellulolytic, etc.) were selected to further research focusing on their ability to suppress the growth and development of toxin-forming microscopic fungi in food raw materials of plant origin.

Table 1. Antagonistic activity of isolated microorganisms to toxin-forming fungi

Test microorganism culture	Isolate								
	3	6	8	10	14	16	18	22	27
Fusarium sp.	+	-	-	-	+	-	-	+	-
Verticillum sp.	-	+	-	-	-	-	-	-	-
Alternaria sp.	-	-	-	+	+	-	-	-	+
Pinicillum sp.	+	-	-	-	+	-	-	-	-
Chaetomium sp.	-	+	-	-	-	+	-	+	-
Aspergillus sp.	+	-	+	-	-	-	-	-	+

Note: "-" – absence; "+" – antagonistic activity

The data of the Table 1 proved an overwhelming resistance to *Fusarium sp.* in the isolates 3, 14 and 22. The isolate №6 showed an antagonism to the phytopathogen *Verticillum sp.* The isolates 10, 14 and 27 developed an antagonism towards *Alternaria sp.* The isolates 3 and 14 showed a suppression of a toxin-forming micromycete *Penicillium sp.* growth. The isolates 6, 16 and 22 showed an antagonism to the phytopathogen *Chaetomium sp.* The isolates 3, 8 and 27 suppressed a toxin-forming micromycete *Aspergillus sp.* growth.

The study of isolated microorganism antagonistic activity to the pathogenic bacteria is presented in the Table 2.

Table 2. Antagonistic activity of isolated microorganisms to pathogenic bacteria

Test microorganism culture	Isolate				
	3	6	8	14	Combination
Clostridium perfringens	+	-	-	-	+
Salmonella	-	+	+	+	+
Escherichia coli	-	+	-	-	+

Note: "-" – absence; "+" – antagonistic activity

From the results of the Table 2, it can be seen that a suppressive ability to *Clostridium perfringens* was shown by the isolate 3 and a combination of isolates. The isolates 6 and 8 and a

combination of isolates showed antagonism to the Salmonella pathogen. An antagonism towards Escherichia coli was shown by the isolate №6 and a combination of isolates.

Further, we conducted the experiments to study a use of isolates in the preparation of silage in laboratory models.

Experiment I

The qualitative indicators of silage when using bacterial isolates are presented in the Table 3.

Table 3. Qualitative indicators of silage when using bacterial isolates

Silage sample	Raw protein,%	KDK, %	Humidity, %	Dry matter, %	pH	Organic acids, %		
						lactic acid	acetic acid	butyric acid
1	63,4±3,9	283,5± 2,9	67,2±1,8	32,74± 2,9	3,92± 0,7	64,1±4,5	22,3±0,2	-
2	63,7±4,1	284,1± 2,9	66,1±1,9	33,09± 2,8	3,80± 0,6	63,7±4,3	23,0±0,1	-
3	63,8±4,0	283,0± 2,9	67,4±1,8	32,46±2,6	3,75± 0,5	64,1±4,8	22,9±0,3	-
4	63,5±3,6	285,0± 2,9	66,7±1,6	33,26±2,7	3,70± 0,4	66,3±3,6	20,1±0,1	-
5	64,9±3,9	285,0± 2,9	67,51±1,9	32,49±2,4	3,83± 0,4	3,29±3,8	1,33±0,1	-

* - $p \leq 0,05$

From the data of the Table 3, it can be concluded that the best quality indicators of silage are obtained when it is preserved using the isolates of experiment I, option 5 – *Lactococcus lactis, Lactobacillus plantarum and Bacillus subtilis*. However, it should be borne in mind that a silage quality may vary depending on the storage and making conditions, as well as on the quality and composition of raw materials. Therefore, it is recommended to conduct a regular silage quality laboratory assessment and monitor the process of its preparation.

Microscopic fungi of the Chaetomium species are ubiquitous in the environment and are among the most common pollutants of plant raw materials due to the high activity of cellulolytic enzymes (Fogle et al., 2007). In addition to the biological destruction of the plant substrate, Chaetomium micromycetes can form mycotoxins of the cytochalazine family, chaetoglobosins A and C, at levels up to 50 mkg/ml, which poses a high risk to public health (Nielsen et al., 1999).

To study the effectiveness of protective action of plant raw materials and isolates, an experiment of artificial fungal contamination has been carried out.

Experiment II

The silage contamination with the phytopathogen *Chaetomium* at a concentration of 1×10^2 CFU/ml: option 1 – silage not subjected to the phytopathogens and isolates (control); option 2 – silage contaminated with fungi *Chaetomium*; option 3 – silage contaminated with mushroom *Chaetomium* and canned using an isolate containing *Lactobacillus plantarum*; option 4 – silage contaminated with fungi *Chaetomium* and canned using an isolate containing *Lactococcus lactis*; option 5 – silage contaminated with *Chaetomium* fungus and preserved using an isolate containing *Bacillus subtilis*; option 6 – silage contaminated with *Chaetomium* fungus and preserved using

isolates containing *Lactococcus lactis, Lactobacillus plantarum and Bacillus subtilis*; option 7 – silage contaminated with *Chaetomium* fungus and preserved using isolates containing *Lactobacillus plantarum and Bacillus subtilis*; option 8 – silage contaminated with *Chaetomium* fungus and preserved using 5-hydroxy-6 compound-methyluracil; option 9 – silage contaminated with *Chaetomium* fungus and preserved using isolates containing *Lactobacillus plantarum, Bacillus subtilis* and the compound 5-hydroxy-6-methyluracil.

The qualitative indicators of silage in a case of contamination with *Chaetomium* phytopathogens against the background of the use of bacterial isolates are presented in the Table 4.

Table 4. Qualitative indicators of silage in case of contamination with *Chaetomium* phytopathogens against the background of the use of bacterial isolates

Silage sample	Raw protein, %	ADF, %	Humidity, %	Dry matter, %	pH	Organic acid, %		
						lactic acid	acetic acid	butyric acid
1.	63,4±5,2	283,5 ±22,7	67,2±5,8	32,7±2,7	4,91± 0,28	64,1 ±4,5	22,3 ±1,9	0,05 ±0,001
2.	47,2±4,1 *	204,3 ±19,3*	74,9±5,7	21,0±2,1 *	5,98± 0,32*	51,3 ±3,2*	16,3 ±1,2*	0,26 ±0,002*
3.	51,8±4,6	221,2 ±16,9	72,5±4,9	27,5±2,6	5,97± 0,34*	53,7 ±3,5	18,1 ±1,3	0,23 ±0,002*
4.	50,3±4,7	216,6 ±16,9*	73,9±4,6	27,12±2, 4	5,91± 0,35*	53,4 ±3,7	17,5 ±1,3	0,25 ±0,002*
5.	50,2±4,9	219,3 ±18,4	73,2±4,5	27,24±2, 9	5,92± 0,34*	54,2 ±3,5	18,0 ±1,3	0,24 ±0,002
6.	53,4±5,1	224,1 ±19,3	73,1±6,1	29,23±2, 5	5,63± 0,29	55,1 ±3,5	18,7 ±1,3	0,17 ±0,002*
7.	55,1±5,4	228,2 ±20,3	72,3±5,7	30,49±2, 8	5,31± 0,26	56,2 ±3,5	19,8 ±1,4	0,14 ±0,002*
8.	54,0±4,9	227,4 ±21,6	73,3±6,2	27,12±2, 4	5,57± 0,37	53,4± 3,5	19,1 ±1,4	0,12 ±0,002*
9.	57,1±4,7	231,5 ±20,7	71,2±5,7	32,17±2, 6	5,22± 0,32	58,1± 4,1	20,3 ±1,5	0,08 ±0,002*

* ADF – acid-detergent fiber; $p \leq 0,05$

4. Discussion

From the data obtained in the Table 4, it can be seen that a crude protein content in the silage when exposed to a phytopathogen in the second group was 26 % lower than that in control. In the third group, the concentration of crude protein in the silage when using *Lactobacillus plantarum* was 17 % lower than that in the control. In the fourth group, the crude protein content in the silage when using *Lactococcus lactis* was 21 % lower than that in the control. In the fifth group, the concentration of crude protein in the silage when using *Bacillus subtilis* was 20 % lower than that in the control. In the sixth group, the content of crude protein in the silage when using *Lactococcus lactis* microorganisms, *Lactobacillus plantarum* and *Bacillus* were 16 % lower than that in the control. The concentration of crude protein in the silage in the seventh group when using *Lactococcus lactis, Lactobacillus plantarum* and *Bacillus* microorganisms was 17 % lower than that in the control. In the eighth group, the crude protein in the silage content when using the drug 5-hydroxy-6-methyluracil was 16 % lower than that in the control. In the ninth group, the crude protein content in the silage with the use of *Lactobacillus plantarum, Bacillus* microorganisms and the preparation 5-hydroxy-6-methyluracil was 10 % lower than that in the control.

The ADF concentration in the silage when exposed to phytopathogen in the second option was lower than that in the control by 28 %. In the third option, an ADF concentration in the silage when using *Lactobacillus plantarum* was lower than that in the control by 22 %. In the fourth option, an ADF content in the silage when using *Lactococcus lactis* was lower than control by 24 %. In the fifth option, an ADF concentration in the silage when using *Bacillus subtilis* was 23 % lower than that in the control.

The ADF content in the silage in the sixth option when using *Lactococcus lactis, Lactobacillus plantarum* and *Bacillus* microorganisms was 21 % lower than that in the control. In the seventh option, an ADF concentration with the use *of Lactococcus lactis, Lactobacillus plantarum* and *Bacillus* was 20 % lower than that in the control. In the eighth option, the ADF content when using the drug 5-hydroxy-6-methyluracil was 20 % lower than that in the control. In the ninth option, the ADF content with the use of *Lactobacillus plantarum, Bacillus* microorganisms and the preparation 5-hydroxy-6-methyluracil was 10 % lower than that in the control.

A moisture content in the silage in the second option increased by 11 % compared to the control. The moisture content in the silage in the third, fourth, fifth, sixth, seventh options with the use of microorganisms was higher circa 7 to 9 %, respectively to control.

A concentration of dry matter in the silage when exposed to phytopathogen in the second option was 36 % lower than that in the control. In the third option, a concentration of dry matter when using *Lactobacillus plantarum* was 16 % lower than that in the control. In the fourth option, a dry matter content when using *Lactococcus lactis* was 18 % lower than that in the control. In the fifth option, a concentration of dry matter in silage when the use of *Bacillus subtilis* was lower than that in the control by 17 %. A dry matter content in the sixth option when using the microorganisms *Lactococcus lactis, Lactobacillus plantarum* and *Bacillus* was lower than that in the control by 11 %. In the seventh option, a concentration of dry matter when using *Lactococcus lactis, Lactobacillus plantarum* and *Bacillus* microorganisms was 7 % lower than that in the control. In the eighth option, a content of dry matter when using the drug 5-hydroxy-6-methyluracil was 18 % lower than that in the control. In the ninth option, a content of ADF with the use of *Lactobacillus plantarum, Bacillus* microorganisms and the preparation 5-hydroxy-6-methyluracil was 5 % lower than that in the control.

A silage acidity index when the silage exposed to phytopathogen in the second option was 22 % higher than that in the control. In the third option, an acidity index of the silage when using *Lactobacillus plantarum* was 21 % higher than that in the control. In the fourth and fifth options, an acidity index of the silage was 20 % higher than that in the control. In the sixth option, an acidity of the silage when using *Lactococcus lactis, Lactobacillus plantarum, Bacillus* was 15 % higher than that in the control. In the seventh option, an acidity index of the silage when using microorganisms *Lactococcus lactis, Lactobacillus plantarum, Bacillus* was 8 % higher than that in the control. In the eighth option, the dry matter content when using the drug 5-hydroxy-6-methyluracil was 13 % lower than that in the control. In the ninth option, a content of ADF with the use *of Lactobacillus plantarum, Bacillus* microorganisms and the preparation 5-hydroxy-6-methyluracil was 6 % lower than that in the control.

The lactic acid concentration in the second option decreased by 20 % compared to the control. A lactic acid concentration in the third, fourth, fifth, sixth, seventh and eighth options with the use of microorganisms was lower by 16, 17, 16, 15, 13 and 17 %, respectively to the control. In the ninth group, the lactic acid content was 10 % lower relative to the control.

The acetic acid concentration in the second option decreased by 17 % compared to the control. A lactic acid concentration in the third, fourth, fifth, sixth, seventh and eighth groups with the use of microorganisms was lower by 19, 22, 20, 17, 12 and 14 %, respectively to the control. In the ninth group, a lactic acid content was 9 % lower relative to the control.

A butyric acid concentration in the third option decreased by 12 % compared to the second option. A butyric acid concentration in the fourth, fifth, sixth, seventh and eighth options when using microorganisms was lower by 19, 22, 20, 17, 12 and 14 % compared to the second option. In the ninth option, a butyric acid content was 9 % lower relative to the second option where the phytopathogen was used.

5. Conclusion

The studies conducted have shown that the most isolated microorganisms with antagonism to pathogens and microscopic fungi belonged to bacteria *Bacillus, Lactobacillus, Lactococcus* genera. The use of *Bacillus, Lactobacillus* and the preparation strains of 5-hydroxy-6-methyluracil for silage conservation provided the most positive result in terms of the crude protein, ADF, dry matter content and organic acids ratio in the finished silage.

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