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IDENTIFICATION AND SCREENING OF MICROORGANISMS COMMON IN POULTRY MANURE

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Abstract
The intensification of poultry farming around the world leads to an increase in the volume of waste, particularly poultry manure, which puts a great burden on the environment, polluting the air, soil and water. Composting is the most cost-efficient and environmentally friendly technology among the currently available methods of poultry manure utilization. The resulting product is an organic fertilizer of high quality with a high content of macro and microelements, which not only increases the yield of agricultural crops, but also has a positive effect on the soil structure and its fertility. The process of poultry manure composting can be improved with the addition of effective microorganisms in order not only to reduce the maturation period of compost, but also for their further positive impact on the growth, development and nutrition of plants. In this article, we have isolated and studied microorganisms from fresh chicken manure. The species of microorganisms that have a high cellulose-destroying and growth-stimulating ability has been determined based on a fragment of the 16S rRNA gene. Thus, in the course of this work, the most promising strains of microorganisms have been selected for further development of biologics based on them.

Keywords: poultry manure, waste utilization, composting, effective microorganisms.

INTRODUCTION

The significant growth of the poultry industry around the world is due to the popularity of poultry among the world's population, as well as its relatively low cholesterol content and dietetic meat. The poultry industry is attractive for agribusiness due to its high conversion rate of vegetable protein into animal protein, a short poultry growing cycle, and the possibility of obtaining differentiated products (meat, eggs). Only in the Republic of Kazakhstan, the volume of poultry meat production increased 2.7 times from 2008 to 2016, while the volume of egg production increased by 30% in the period 2012-2016 [1].

Poultry manure contains all the nutrients and trace elements necessary for sufficient plant nutrition, including the most important elements, such as nitrogen (3-5%), phosphorus (1.5-3.5%) and potassium (1,5-3,0%) [2]. The widespread use of poultry manure as organic fertilizers has long been known and is based on their ability to favourably change soil properties, such as plant nutrient availability, soil reaction (pH), increased organic matter content, cation exchange capacity, ability to retain water and maintain soil structure. Thus, the use of poultry manure as fertilizers not only increases crop productivity, but also improves the fertility of the cultivated soil [3].

Composting is a biological process of decomposition of organic substances in a predominantly aerobic environment [4]. In the first stage of the composting process, simple organic carbon compounds are easily mineralized and metabolized by microorganisms, forming CO_2, NH_3, H_2O, organic acids and heat. The accumulation of heat leads to an increase in the temperature in the manure piles, while destroying weed seeds and pathogens. During composting, bacteria, fungi, and other micro-organisms, including microarthropods, break down organic materials into stable, usable organic substances called compost [5]. Another advantage of compost is its ability to suppress crop diseases, mainly due to the presence of the following groups of microorganisms in its composition: *Bacillus* spp., *Enterobacter* spp., *Flavobacterium balustinum* 299, *Pseudomonas* spp., bacteria of the genus *Streptomyces* spp., fungi of the genera *Penicillum* spp., *Trichoderma* spp [6, 7].

Factors that affect the composting process can be divided into two groups: factors that depend on the composition of the composted mixture, such as nutrient balance (mainly C/N ratio), pH, particle size, porosity, and humidity; and controlled factors such as O2 concentration, temperature, and water content. The C/N ratio plays a key role, since microorganisms require a source of energy, in the form of decomposed organic carbon, and nitrogen for their development and life. At the same time, the optimal C/N ratio is considered to be in the range of 25-40, and directly depends on the frequency of mixing of poultry manure [5].

Microorganisms play a key role in composting. They convert organic substances into CO_2 and H_2O, while consuming oxygen. In general, the composting process can be divided into two main phases: the bio-oxidative phase and the maturation phase. Bio-oxidative phase is further divided into three stages: an initial mesophilic phase where sugars, amino acids, proteins, and etc. are decomposed by mesophilic bacteria and fungi, thus quickly increasing the temperature; the thermophilic phase, where thermophilic microorganisms cause the decomposition of fats, cellulose, hemicellulose and some lignin, together with the destruction of pathogens; in the cooling phase, the temperature drops due to the depletion of degradable organic substrates and as a result reduced microbial activity, the composting mass becomes populated by mesophilic microorganisms which are able to degrade the remaining sugars, cellulose and hemicellulose [5].

Various types of poultry manure are known: deep litter manure, broiler manure and cage manure, which have their own characteristics and disadvantages, and their composting requires an individual approach to each type of poultry manure. For example, cage housing of poultry leads to high humidity of the resulting litter due to the lack of ground litter material, as well as to large losses of ammonia, so this type poultry manure should be composted as early as possible. On the other hand, deep litter manure and broiler manure have almost no differences, excepting the fact that broiler manure is changed more often and thus the loss of ammonia is reduced due to organic decomposition [3].

In general, the process of manure composting is quite an expensive procedure and its payback is based only on obtaining high-quality fertilizer. Since microorganisms play a key role in this process, researchers from different countries do not give up trying to develop microbiological biologics for more effective maturation of compost masses, as evidenced by multiple patents and developments [8, 9, 10, 11].

The Russian market is full of products of microbial origin under various brands: Tamir, Baikal EM-1, Sianiye EM products, DROP-T, Vostok EM-1, which, according to its producers, accelerate composting, eliminate offensive odours, destroy pathogenic flora, and improve the physical and chemical indicators of compost [12]. Y.V. Ponamoreva and others have conducted a study on reducing pathogenic microflora in pig manure using Tamir product [13]. Gritsenko showed the effectiveness of using Baikal EM-1 product while composting poultry manure, where high nitrogen content has been found compared to the control and the absence of pathogenic microflora [14].

However, the issues of agriculture environmentalization require immediate measures to introduce existing or develop their own technologies for utilization of poultry manure. In this regard, this article is devoted to finding the effective strains of microorganisms from the chicken manure samples to produce the biological products to be applied in poultry manure composting using manure turner. The isolates have been studied for the presence of cellulose-reducing properties with subsequent identification of each species by sequencing the 16S rRNA sequence, as well as describing the cultural and morphological characteristics of each strain and determining the level of danger for animals and humans.

METHODS

The samples of poultry manure were spread on solid nutrient media of SAA (starch-ammonia agar) and Gause №1 to determine the number and species diversity of microorganisms. The suspensions of samples were prepared from 10 g of sample suspension and 90 ml of sterile water, followed by stirring the suspensions for 20 minutes on a shaker.

Total microbial content was calculated by the number of grown colonies, where the amount of CFU (colony forming unit) in 1 ml was determined by the formula (1):

$M = a \times 10^n/V$, (1)

where a — number of growing colonies;

10^n — dilution;

V — culture dose (0,1 мл).

Some colonies of microorganisms were screened out to obtain pure cultures of microorganisms.

The study of virulence of strains (LD_{50}) was carried out by a conventional method on 8 groups of animals (12 white mice each, 6 females and 6 males weighing 16-18 g) at concentrations from 10^3 to 10^{11} CFU/cm^3 [15].

Cellulose activity of the obtained strains was determined in liquid medium of Hetchenson with the use of filter paper as cellulose substrate. The microorganisms were incubated for 10 days, followed by an assessment of the degree of splitting of the filter paper [16].

The staining was carried out according to Gram procedure [16].

Identification of bacterial cultures was performed by determining the direct nucleotide sequence of the 16S rRNA gene fragment, followed by determining the nucleotide identity with sequences deposited in the international database Gene Bank, as well as formation of phylogenetic trees with the nucleotide sequences of reference strains. Genomic DNA was isolated from pure cultures with the Wizard ® Genomic DNA Purification Kit from Promega (A11125), according to the producer's instructions. The PCR reaction was performed with universal primers 8f/806R [17] (Table 1).

Table 1. – Primers used for amplification of the 16S rRNA gene.

Primer	Sequence-target	Direction
8f	AgAgTTTgATCCTggCTCAg	5’ - 3’
806R	ggACTACCAgggTATCTAAT	3’ – 5’

The nucleotide sequences were analyzed and combined into a common sequence in the SeqMan software (DNA Star). Purification of PCR products from non-bound primers was performed with the enzymatic method using Exonuclease I (Fermentas) and alkaline phosphatase (Shrimp Alkaline Phosphatase, Fermentas) [18]. The reaction was sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applide Biosystems) according to the manufacturer's instructions, followed by fragment separation on an automatic genetic analyzer 3730xl DNA Analyzer (Applide Biosystems). Mega 6 software was used to build phylogenetic trees [19]. The Muscle algorithm was used to align the nucleotide sequences, the tree construction was performed using the Neiighbor-Joining NJ method.

RESULTS AND DISCUSSIONS

As a biological process, composting involves many microorganisms. These microorganisms, their composition and size are important components of the composting process. Changes in different populations of microorganisms such as bacteria, actinomycetes and fungi allow a better understanding of the composting process. Some studies are often aimed at studying microbial changes in different phases of composting, but not the analysis of fresh samples of poultry manure [20].

Selection of microorganisms from poultry manure was performed on two solid nutrient media of SAA (starch-ammonia agar) and Gause and the analysis of the number was presented in the Table 2. According to the results of microbiological analysis, the samples of chicken manure are widely populated by microorganisms of various groups. In fresh chicken manure, the content of microorganisms using mineral forms of nitrogen is quite high, where the amount varied within 3 468,0 – 9 446,0 thousand CFU/ml. The largest number of actinobacteria was detected in variant № 2, and the number was approximately the same in the other variants.

Table 2. – The results of microbiological analysis of broiler manure on solid nutrient media.

№	Samples of broiler manure	Growth on nutrient media (thousand CFU / ml)	
		SAA	Gause
1	№ 1 – the age of the bird is 10 days	3 468,0	230,0

2	№ 2 – the age of the bird is 23 days	6 804,0	1 434,0
3	№ 3 - the age of the bird is 38 days	9 446,0	398,0
4	№ 1 average 3 ages	8 568,0	615,0
5	№ 2 average 3 ages	7 616,0	242,5

Quantitative and qualitative accounting of microorganisms inhabiting fresh broiler manure allowed us to identify pure cultures of microorganisms for further study of their properties and possible use for creating effective biologics based on them. While some of these organisms may be useful for plant growth and soil fertility, others may be pathogenic to humans and animals. All biochemical reactions in the composting process are catalyzed by enzymes. Extracellular enzymes secreted by microorganisms are necessary to catalyze the breakdown of polymer substances such as plant polymers, cellulose, hemicellulose, and lignin, thereby adding nutritional value to compost [20]. It is known that during composting of poultry manure, only a small part of the cellulose decomposes, compared to easily decomposable components such as starch and protein [21].

In this regard, we studied the cellulolytic activity of 29 microorganisms isolated from fresh poultry manure. The results of studies of the cellulolytic activity of strains from fresh broiler manure revealed the presence of 8 strains possessing high cellulolytic activity (table 3). 41% of the strains had average enzymatic activity, of which 3 strains were not used for further research due to their pathogenicity. The remaining 31% of the studied strains had little or no cellulolytic activity.

Table 3. Cellulose-degrading properties of microbial strains isolated from poultry manure

Strain	Cellulolytic activity	Strain	Cellulolytic activity
PM 80 B	+++	PM 92 B	+++
PM 100 B	+--	PM 108 B	---
PM 101 B	++-	PM 109 B	++-
PM 81 B	+++	PM 110 B	+--
PM 82 B	++-	PM 83 B	++-
PM 88 B	+++	PM 111 B	+--
PM 93 B	+++	PM 84 B	++-
PM 104 B	++-	PM 112 B	+--
PM 105 B	---	PM 113 B	+--
PM 106 B	+--	PM 114 B	+--
PM 85 B	++-	PM 89 B	+++
PM 86 B	++-	PM 90 B	+++
PM 87 B	++-	PM 91 B	+++

Only strains with medium and high cellulolytic activity were selected for further research. Based on the results of our research, we selected 5 (PM 88B, PM 93B, PM 90B, PM 80B and PM 86B) most effective strains, the species were determined as a result of sequencing a fragment of the 16S rRNA gene.

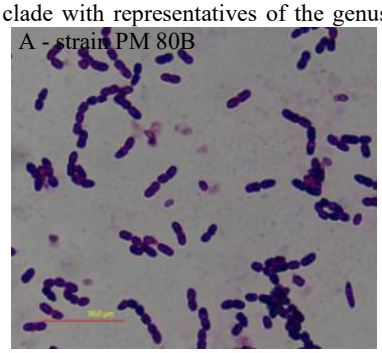
Analysis of 16S rRNA identifies the PM 80B strain as *Bacillus megaterium*. The growth of colonies was observed on the Gause culture medium after 2-3 days at T 30-33°C. The strain was characterized by glossy small colonies of white-grey colour with a diameter of 1-3 mm, convex, velvety, mucosal consistency. This bacterium is gram-positive. Morphological characteristics: rod-shaped bacterium with rounded edges, 3,7-4,3 x 1,5-2 microns in size. It is located singly, in pairs, or in chains (Picture 1-A).

According to the results of the analysis based on a fragment of the 16S rRNA gene, the PM86-B sample is located on the same clade with representatives of the genus *Lentzea*, given that it is most likely that the sample belongs to this genus, and was identified as *Lentzea chajnantorensis* (Picture 2). This species was first isolated and described as a filamentous actinobacterium from the high-altitude soil of the Atacama Desert [22].

Growth of PM 86B culture was observed on the Ashby culture medium for 2-3 days at T 30- 33^{0}C. Culturally, the strain is characterized by small, opaque, elevated, velvety, with a button – like elevation in the centre, white colonies with a diameter of 1-3 mm. Tinctorially the bacterium is gram-positive. Morphological characteristics: branching bacterium, straight branches, hyphae divided by partitions into long bacterial

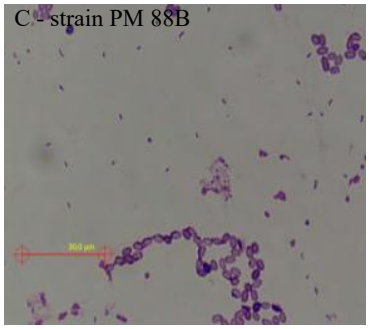
cells, spores are located along the sporangiosus, there is a fragmentation-splitting of hyphae into sticks and single cocci (Picture 1-B).

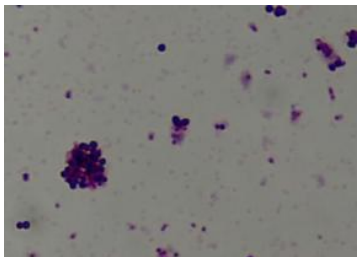
A - strain PM 80B

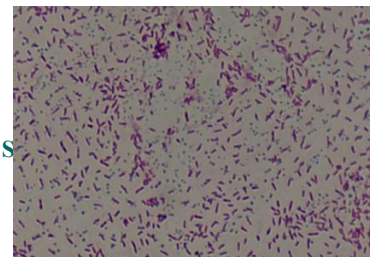


B - strain PM 86B

C - strain PM 88B

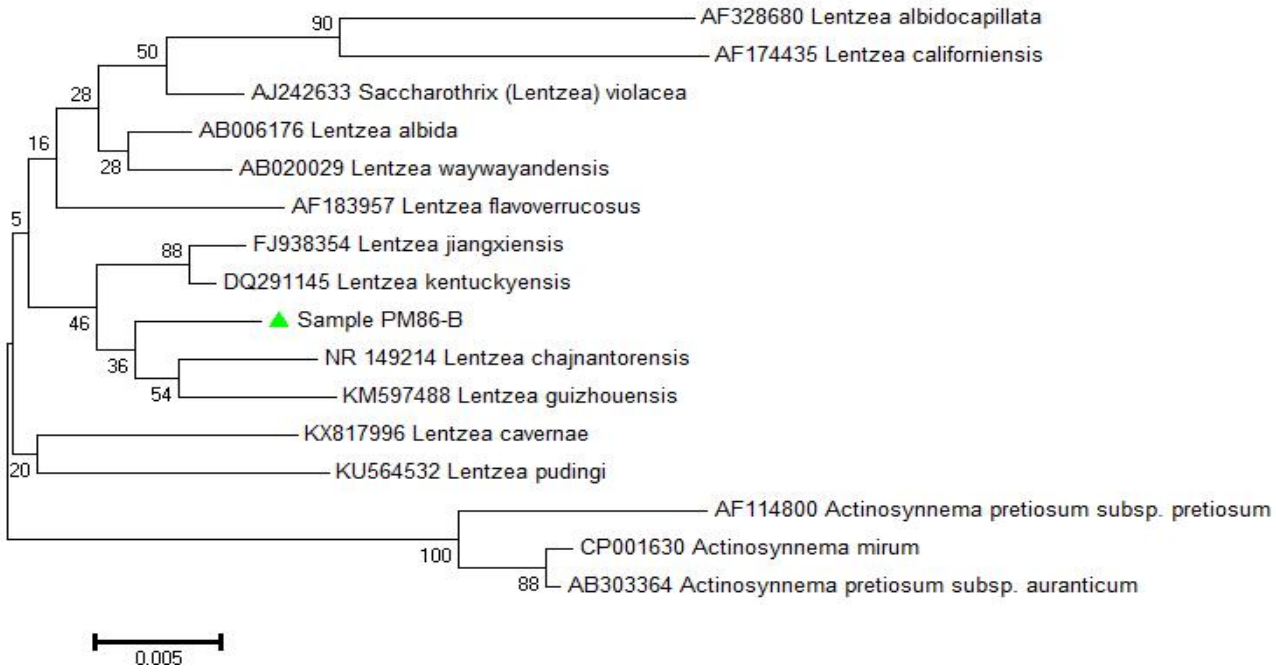






D - strain PM 90B E - strain PM 93B

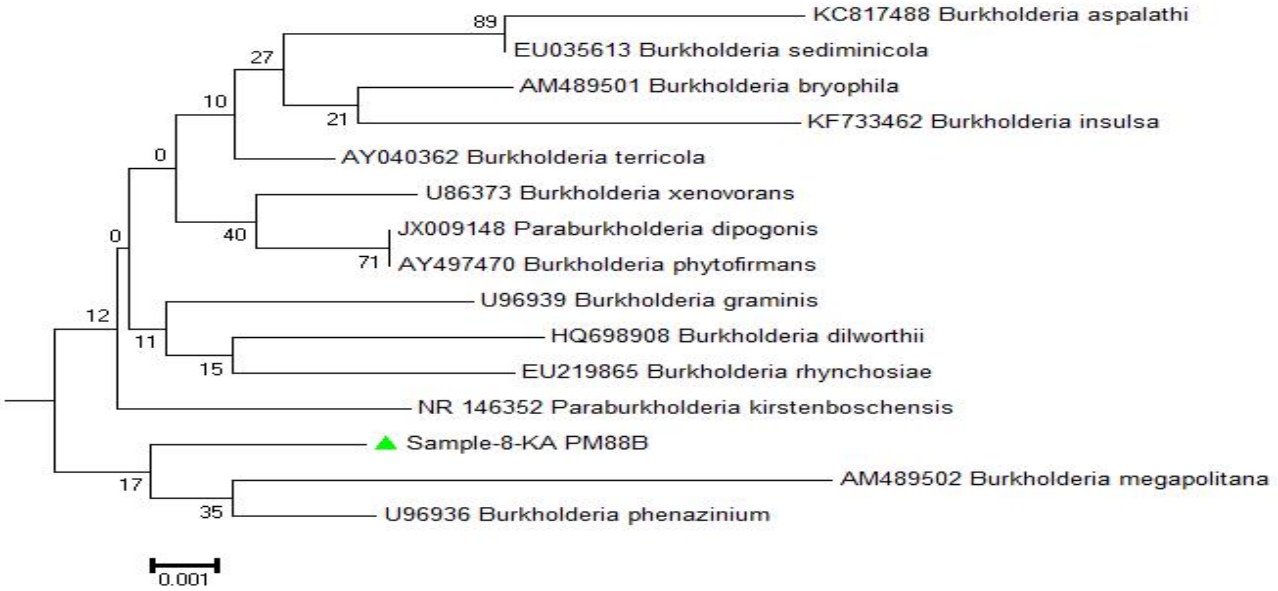
Picture 1. Morphology of isolated strains of microorganisms, magnification×100



Picture 2-a. Phylogenetic tree based on the analysis of the 16S rRNA gene fragment of the RM86-B sample.

Based on the BLAST resource, the PM 88B strain was identified with 100% probability as *Burkholderia xenovorans* (Picture 3). This culture shows the growth on potato agar at 30-33°C, forming mucous colonies, and on the liquid medium – intense turbidity (Picture 1-C). Culture characteristics of the PM 88B strain: bacterial colonies are rounded, convex, with uneven edges, medium-sized 2-4 mm, opaque, lemon-colored with a smooth shiny surface, slimy, superficial. Bacteria of this genus are gram-negative straight sticks, 0,5x1,5-3 mkm in size, locates singly, sometimes in pairs and in short chains.

The genus *Burkholderia*, with more than 60 described species, has a fairly large gene (6-9 MB) with significant protein-coding sequences. This genus consists of both pathogenic and useful representatives, called plants-associated useful and ecological species. Representatives of *Burkholderia xenovorans* were isolated from the rhizosphere of various plants, which is an indicator of its importance for plant growth, in addition, studies show that *B. xenovorans* is a diazotroph [23]. However, the most important and studied function of *Burkholderia xenovorans* is bioremediation of a number of certain xenobiotic compounds, which has led to its widespread use in biotechnology in order to reduce the concentration of chemical pollutants in the soil [24, 25].



Picture 3. Phylogenetic tree based on theanalysis of the 16S rRNA gene fragment of the PM88-B sample.

The PM 90B strain isolated from poultry manure was identified as *Enterobacter hormaechei subsp. xiangfangensis. Enterobacter hormaechei* was first described in 1989 and is characterized as a gram-negative, non-pigmented rod with common characteristics of the *Enterobacteriaceae* family and the *Enterobacter* genus [26]. This species is of great clinical significance, since it is an opportunistic species and most studies are aimed at identifying its sensitivity to antibacterial drugs [27, 28, 29]. The strain is characterized by opaque, rounded, small colonies of 1-3 mm, with a rough, shiny, mucous surface (figure 1-D). The growth of the culture on the solid nutrient medium Gause-1 occurred on 2^{nd}-3^{rd} day at a temperature of 28-30°C, where the colonies have a grainy structure of white color.

According to the analysis of the 16S rRNA fragment of the gene, the PM 93B microorganism was identified as a poorly studied species *Sphingomonas trueperi.* The bacterium secretes a large amount of extracellular polysaccharide, which leads to the formation of characteristic mucous, bright yellow colonies on Petri dishes [30]. Studies have shown that strains phylogenetically closely related to *S. trueperi* are effective nitrogen fixators [30]. The cells of the *S. trueperi* strain were straight, gram-negative rods (0.4–0.75 microns wide and 1.0-3.0 microns long), arranged singly, in pairs, or in a chain. (Figure 1-E). On the Gause culture medium after 2-3 days at T 30-33°C, bacteria forms bright yellow colonies, surrounded by white zones, opaque, convex, round with smooth edges, shiny with a diameter of 3-5 mm, of velvety and mucosal consistency.

Microorganisms that are part of biologics possess significant enzymatic properties and high antagonistic activity against many pathogenic and opportunistic bacteria and toxigenic filamentous fungi. Earlier research has shown that the use of a *Streptomycetes*-based biologics reduces the concentration of pathogenic bacteria in compost [31].

According to the existing classification of strains (Maximum permissible concentrations (MPC) of producing microorganisms, bacterial preparations and their components in the air of the working area Hygiene standards HS 2.2.6.709-98), cultures of *Bacillus megaterium* PM80B and *Lentzea violacea* PM86B isolated from fresh poultry manure belong to the 4th hazard class (Tables 4 and 5).

Table 4. The study results of acute toxicity of Bacillus megaterium PM80B culture with intraperitoneal and oral administration.

№ Variant	Number of testing animals	Administration route	Dose CFU / ml	Sick animals	Dead animals	Survived animals
1	12	Intraperitoneally	103	0	0	12
2	12	Intraperitoneally	105	0	0	12
3	12	Intraperitoneally	107	2	0	12
4	12	Intraperitoneally	109	4	0	12
Control	12	Intraperitoneally	Normal saline	0	0	12
5	12	Oral administration	105	0	0	12
6	12	Oral administration	107	0	0	12
7	12	Oral administration	109	1	0	12
8	12	Oral administration	1011	4	0	12
Control	12	Oral administration	Normal saline	0	0	12

The results of experiments have showed that while applying intraperitoneal administration route of *Bacillus megaterium* PM80B culture at a dose of 10^7 CFU/g, 2 animals got sick, and 4 mice at a dose of 10^9 CFU/g. With oral administration at a dose of 10^8 CFU/g, 1 mouse got sick, and at a dose of 10^{11} CFU/g, 4 mice got sick. In 24 hours after the introduction of the culture, they were noted: lethargy, loss of appetite, thinning of the stool, and woolly hair. On the 3rd day after infection, all the mice recovered. The death of experimental animals was not observed.

Morphological changes of internal organs: the results of animals' autopsies have showed: the liver is dark red, the surface is smooth, slightly hyperemic. The "pattern" of the brain and cortex is clear. Light in structure and volume of the

lobes are normal, the surfaces are smooth, easily separated from each other, there are no adhesions.

Internal organ dissemination: dissemination of internal organs occurs only within the first 72 hours after the introduction of culture.

Allergenic effect on the sensitizing effect: determination of the average allergenic dose was carried out on guinea pigs, which were administered the studied culture at doses of 10^4, 10^5, 10^6 CFU/per animal. The control was a saline solution.

The reaction was accounted for after 10 days by the diameter of the erythema. The average allergenic dose of the studied culture was $6.5x10^4$ CFU per animal. Thus, this strain has almost no allergenic effect.

Local irritant effect: when the studied culture was introduced into the conjunctiva of rabbits ' eyes at a dose of $1x10^9$ CFU/cm^3, a weak positive reaction was observed in the form of injection of sclera and corneal vessels, mucous secretions in the corners of the eyes. On the third day of observation, the above-mentioned phenomena were completely stopped among all animals, and the next 5 days there were no deviations from the physiological norm. Thus, the studied strain of *Bacillus megaterium* PM80B has a weak local irritant effect.

The results of experiments showed that both intraperitoneal and oral administration of all studied doses of *Lentzea violacea* PM86B culture did not cause death of experimental animals. All of them remained active and healthy (Table 5).

Morphological changes of internal organs: the results of autopsies of animals showed: the liver is dark red; the surface is smooth. The "pattern" of the brain and cortex is clear. Light in structure and volume of the lobes are normal, the surfaces are smooth, easily separated from each other, there are no adhesions.

Table 5. Results of the study of acute toxicity of *lentzea violacea* PM86B culture with intraperitoneal and oral administration**.**

№ Variant	Number of testing animals	Administration route	Dose CFU / ml	Sick animals	Dead animals	Survived
1	12	Intraperitoneally	103	0	0	12
2	12	Intraperitoneally	105	0	0	12
3	12	Intraperitoneally	107	0	0	12
4	12	Intraperitoneally	109	0	0	12
Control	12	Intraperitoneally	Saline	0	0	12
5	12	Oral administration	105	0	0	12
6	12	Oral administration	107	0	0	12
7	12	Oral administration	109	0	0	12
8	12	Oral administration	1011	0	0	12
Control	12	Oral administration	Saline	0	0	12

Internal organ dissemination: internal organ dissemination occurs only within the first 24 hours after culture administration.

Allergenic effect on the sensitizing effect: the establishment of an average allergenic dose was carried out on guinea pigs, which were administered the studied culture in doses of 10^4, 10^5, 10^6 CFU/per animal. The control was a saline solution. The reaction was accounted for after 10 days by the diameter of the erythema. The average allergenic dose of the studied culture was $9.5x10^5$ CFU per animal. Thus, the *lentzea violacea* PM86B strain has almost no allergenic effect.

Local irritant effect: when the culture was introduced into the rabbits ' eyes conjunctiva at a dose of $1x10^9$ CFU/cm^3, a weak positive reaction was observed in the form of injection of sclera and corneal vessels, mucous secretions in the corners of the eyes. On the third day of observations, the above-mentioned phenomena were completely stopped in all animals, and the next 5 days there were no deviations from the physiological norm. Thus, the studied strain of *Lentzea violacea* PM86B has a weak local irritant effect.

The purpose of isolating microbial strains from fresh poultry manure was to identify microorganisms with high enzymatic and growth-stimulating activity. Microorganisms with high cellulose-destructive and growth-stimulating activities will be subsequently used to create biologics for accelerated composting of poultry manure in order to obtain high-quality compost.

Thus, it can be concluded that the resulting cultures isolated from samples of fresh broiler manure have high enzymatic activity. These strains have been genetically identified and will be used in the future to create highly effective biologics for composting broiler manure. High enzymatic activity will allow breaking down difficult-to-decompose complex compounds.

According to the conclusion on non-pathogenicity, PM 80B and PM 86B strains are recommended for creating biologics intended for manure utilization and increasing crop yields.

The presence of such strains in ready-made biofertilizers based on broiler manure allows us to conclude that the use of these biofertilizers in the fields will significantly increase the yield of cereals and legumes.

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