UDC 615.33:636.086

DETECTION OF ANTIBIOTICS, ACTIVE AGAINST *BACILLUS SUBTILIS*, IN GRAIN AND FEED

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Received on January 14, 2015

Aim. Detection of antibiotic substances in samples of grain, extraction cake, and oilcake. **Methods.** The bioautography method using strains of *Bacillus subtilis* as test-microorganisms was used to study 102 samples of feed substrates (corn, maize gluten, barley, wheat, sorghum, chaff, dust middling, sunflower oilcake and extraction cake, soybean meal, feed yeast and grains). **Results.** From one to four antibiotic substances, inhibiting the growth of *B. subtilis* and characterized by a wide range of values of chromatographic mobility index, were detected in 95 % of samples of feed substrates. Average areas of the zones of absent growth of a test-microorganism, corresponding to 2.5 g of the sample, were in the range of 52–217 mm^2. **Conclusions.** It was established that feeder grain and other feed substrates are highly contaminated with antibiotics which indicates the necessity of their identification, search for contamination sources, study of prevalence and estimation of the possible impact on the indices of health, performance and reproduction of farm animals and poultry.

Key words: grain, oilcake, extraction cake, antibiotics, bioautography.

INTRODUCTION

The list of maximally acceptable levels of undesired substances in animal feed and fodder in Ukraine includes inorganic pollutants, nitrogen compounds, mycotoxins, toxins of plant origin, chlororganic compounds, dioxins and microelements. The content of coccidiostatics in the composition of pharmaceutical products for veterinary use is specified in case when they are not meant for animals [1]. At the same time the European Union and a number of countries regulate the content of antibiotics in feeds which are prohibited for use as growth promoting agents [2]. Ukraine has not got any restrictions on the content of antibiotics in feeds therefore they are usually not indicated in the list of feed ingredients.

In recent years there have been some communications informing that intentional introduction of veterinary antibiotics into fodder combination is not the only way of contaminating the latter with the substances of antibiotic activity. It was determined that antibiotics may penetrate the agricultural soils with the excrements of animals, fed with the fodder, produced with the addition of antibiotic as growth promoting agents or veterinary antibiotics, prescribed by the veterinary physician [3, 4]. There are published data proving that antibiotics, penetrating the soil from organic fertilizers, may be consumed by the root system of agricultural crops and accumulated in different tissues and organs, including grain [5]. It was determined that microbiocidal agents are capable of being accumulated by plants, cultivated on the soil using solid organic wastes, obtained in the process of purifying wastewater, as fertilizers [6, 7]. Besides, a potential source of antibiotic substances in feeds of plant origin may be metabolites of symbiotic soil [8, 9] and phytopathogenic bacteria [10].

This study was aimed at detecting antibiotics, active against sensitive strains of *Bacillus subtilis*, in the samples of grain and vegetative feed for farm animals and poultry.

MATERIALS AND METHODS

During 2010–2014 102 samples of feed substrates (corn, maize gluten, barley, wheat, sorghum, chaff, dust middling, sunflower oilcake and extraction cake, soybean meal, feed yeast and grains) from the combined feed-processing plants, livestock enterprises and poultry farms of different forms of ownership from eight regions of Ukraine were tested for the presence of antibiotic agents.

The sensitive strain of bacteria was selected out of the strains of *B. subtilis* from the Museum of the Laboratory of poultry feeding and mycotoxicology of the State Poultry Research Station NAAS of Ukraine, remarkable for even growth in the cultivation medium and absence of sensitivity to mycotoxins, namely – aflatoxin B_1, T-2 toxin, HT-2 toxin, deoxynivalenol, fumonisin, zearalenone and aurofusarin. For this purpose the sensitivity of strains of *B. subtilis* ap-2, sn-1, sn-2, sn-3, sn-4 and sn-5 to antibiotics was tested using the disk diffusion method. Such antibiotics as polymyxin B, streptomycin, tetracycline, amikacin, carbenicillin, cefepime, cefuroxime, cefalexin, cefalotin, cefotaxime, chloramphemide, ciprofloxacin, netiline, norfloxacin, ofloxacin or perfloxacin were applied on paper discs in the amount of 5 to 300 μg. The cultures of strains were cultivated in Petri dishes in lawns using meat-and-peptone agar (MPA). The diameters of the delayed growth zones around the disks with antibiotics were measured and their values were ranged. The selection of the most sensitive strain involved calculation of the rank sum, the arithmetic mean value for the diameters of delayed growth zones and the sensitivity to specific antibiotics.

To detect the antibiotic substances, 25 g of ground grain or combined fodder was introduced into the conical 500 ml flask with the addition of 20 ml of the aqueous NaCl solution (10 %, weight/volume) and mixed. Then 120 ml methanol was added; the mixture was shaken for 1 h and filtered through a paper filter. To precipitate protein admixtures, 100 ml of the filtrate was added to 20 ml of the aqueous solution of lead acetate (15 %, weight/volume) and 30 ml of distilled water, mixed and kept for 10 min. The mixture was filtered; 120 ml of the filtrate was transfered to the separation funnel. To isolate the triglycerides, the filtrate was added to 40 ml of hexane and shaken; the lower water-methanol layer was isolated after the phase distribution. The latter was added to 20 ml of hexane twice, shaken each time, and the layer of hexane was isolated after the phase distribution. Then the water-methanol extract in the separation funnel was added 40 ml of chloroform twice, shaken each time. After the phase distribution the lower layer (chloroform) was isolated for further analysis. The chloroform extracts were combined, 5 g of anhydrous sodium sulfate was added for dehydration, shaken and kept for 10 min. The solution was filtered, the filtrate was evaporated. The dry residue was dissolved in 1–2 ml of benzene, evaporated, and the residue was dissolved in 100 μl of benzene.

The Sorbfil slides for thin-layer chromatography (TLC) (Imid Ltd, Russian Federation) were applied 10 μl of the extract of the investigated samples, chromatographed in the system of such solvents as ethyl acetate:toluene (3:1, volume/volume) and dried.

To detect the antibiotic agents, the surface of horizontally placed chromatographic slides was applied the melted MPA, which was inoculated with the suspension of a sensitive strain of *B. subtilis*. The slides were kept in a wet chamber at 32°C for 16–18 h. No growth of a test-microorganism on the slide testified to the presence of an antibiotic agent in the sample. The linear sizes of zones and the indices of their chromatographic motility (R_f) were taken into consideration.

RESULTS AND DISCUSSION

The investigated strains demonstrated different sensitivity to antibiotics (Table 1). *B. subtilis* strains sn-1, sn-3 and sn-4 were insensitive to cefepime, and sn-1 – to cefuroxime. *B. subtilis* strains ap-2, sn-2 and sn-5 demonstrated their sensitivity to each of the investigated antibiotics. *B. subtilis* strain sn-2 was characterized with the the highest sensitivity to antibiotics both in the average value of the diameter of zones of growth inhibition, and in the rank sum, thus it was selected as a test-microorganism for further studies.

The antibiotic agents, capable of inhibiting the growth of *B. subtilis* sn-2, were found in 95 % of the investigated samples of feed substrates (Table 2). Therefore, the bioautographic method of applying the sensitive strain of *B. subtilis*, sn-2, allows revealing the antibiotic substances with different chromatographic characteristics, separating the mixture of several antibiotics and estimating the concentration in relative units of activity. The bioautographic method of applying *Escherichia coli* was previously used for the purpose of quantitative determination of tabtoxin, mangotoxin and phaseolotoxin – *Pseudomonas syringae* metabolites, characterized with antibiotic activity [11]. It should be noted that a considerable amount of *P. syringae* strains are phytopathogenic endophytic microorganisms, causing bacterioses of gramineous plants, due to which they may

be the source of grain contamination with antibacterial activity [12].

Most frequently, namely, in 46 % of cases, the samples of grain and vegetable raw materials were simultaneously contaminated with two substances, inhibiting the growth of *B. subtilis* sn-2 (Fig. 1). Approximately 1.5 times less frequently (i.e. in 29 % cases) there were samples, contaminated with only one antibiotic substance; almost 2.5 times less frequently – samples, contaminated with three antibiotics at the same time. Only the extract of one investigated sample of barley caused four zones of delayed growth of *B. subtilis* sn-2 on the slide for TLC. It is noteworthy that the distribution of frequencies of simultaneous detection of several antibiotic substances in one sample corresponds to prevailing schemes of applying the antibiotic as growth promoting agent individually and in synergistic combinations [13, 14].

The investigated samples of grain and feed were remarkable for a wide variety of antibiotic substances both by the index of chromatographic mobility and

Table 1. The sensitivity of six strains of *B. subtilis* to antibiotics

Antibiotic, μg/slide	Diameter of zones of growth inhibition for *B. subtilis* strains, mm					
	ap-2	sn-1	sn-2	sn-3	sn-4	sn-5
Polymyxin B, 300	12	17	17	13	12	15
Streptomycin, 10	30	26	26	28	29	21
Tetracycline, 10	32	30	30	33	32	30
Amikacin, 10	31	35	35	31	30	27
Carbenicillin, 100	14	28	28	13	13	42
Cefepime, 30	0	16	16	0	0	27
Cefuroxime, 30	0	17	17	10	12	30
Cefalexin, 30	15	37	37	25	13	30
Cefalotin, 30	15	43	43	14	15	34
Cefotaxime, 10	20	24	24	21	20	34
Chloramphemide, 30	33	33	33	35	33	32
Ciprofloxacin, 30	33	40	40	35	35	32
Netiline, 30	31	34	34	33	32	33
Norfloxacin, 10	29	30	30	30	30	25
Ofloxacin, 5	30	37	37	30	30	27
Perfloxacin, 5	28	34	34	28	30	25
Arithmetic mean value	29.8	22.1	30.1	23.7	22.9	29
Rank sum	45	59	31	49	55	63

Table 2. Number of feed substrate samples with antibiotic substances in the extract

Feed substrate	Total number of samples	Samples with different amounts of detected antibiotic agents				
		0	1	2	3	4
Corn	43	1	14	24	4	0
Maize gluten	2	0	0	1	1	0
Barley	11	0	2	6	2	1
Wheat	11	1	3	4	3	0
Sorghum	2	0	2	0	0	0
Chaff	2	0	0	1	1	0
Dust middlings	3	1	1	1	0	0
Sunflower cattle cake, extraction cake	15	0	3	5	7	0
Soybean meal	9	2	4	2	1	0
Feed yeast, grains	4	0	1	3	0	0
Total	102	5	30	47	19	1
%	100	4.9	29.4	46.1	18.6	0.98

the area of delayed growth zones (Fig. 2). Depending on the average area of zones of growth inhibition with different R_f they can be divided for clarity into four groups. It should be noted that the antibiotic substances with the rounded value of chromatographic mobility index of 0.5 are remarkable for the highest average value of the area of growth inhibition zones of the test-microorganism − 217 mm^2. Somewhat lower areas (from 152 to 173 mm^2) were registered for the zones of antibiotic substances with rounded R_f values of 0.3, 0.4 and 0.6. The third place in terms of sizes of growth inhibition zones is taken by antibiotic substances with R_f 0.1 and 0.2, the fourth – with 0.7 and 0.8, and the substances, remaining on the start of the TLC slide.

The results of the studies testify that the antibiotic substances with the chromatographic mobility index of 0.5 and the substances, remaining on the start line in the applied system of eluents are registered the most frequently (Fig. 3). The occurrence frequency for these antibiotic substances is 24 and 23 % respectively. The antibiotics with R_f value of 0.1, 0.2, 0.3 and 0.4 were registered with the frequency from 9 to 12 %, with R_f 0.6 and 0.7 – 6 %, and the least frequently − with R_f 0.8 (frequency of 0.5 %).

The detected antibiotic substances do not decrease the wheat grain quality of rye, barley, triticale, corn, oats, millet, not damaged by insects, fungi and bacteria. They are different in their chromatographic mobility, their capability of inhibiting the growth of test-microorganisms and sizes of zones of absent growth of test-microorganisms (Fig. 4).

One of the ways of antibiotic substances penetrating into grain and secondary products of oil-seed crops processing (oilcake and extraction cake) is through the soil. Recent articles of different authors contain the data on soil contamination by antibiotics for veterinary purposes which penetrate the soil as a result of fertilizing the latter with the excrements of animals, who were kept using antibiotic growth promoting agents or using the schemes of treating the bacterial diseases, presupposing treatment with veterinary antibiotics. For instance, according to the data of Leal *et al*. [15], 30 % of samples of chicken excrements and 27 % of soil samples from Brazil, Austria, China and Turkey were contaminated with enrofloxacin in average concentrations of 6.68 mg/kg and 22.93 μg/kg, respectively. The authors believe that the results obtained indicate veterinary antibiotics to be a potential source of environment pollution, which has been ignored [15]. Recently considerable efforts were taken to elaborate the means for purification of soils and surface water from contamination with sulfonamide antibiotics to prevent the occurrence of resistant strains [16].

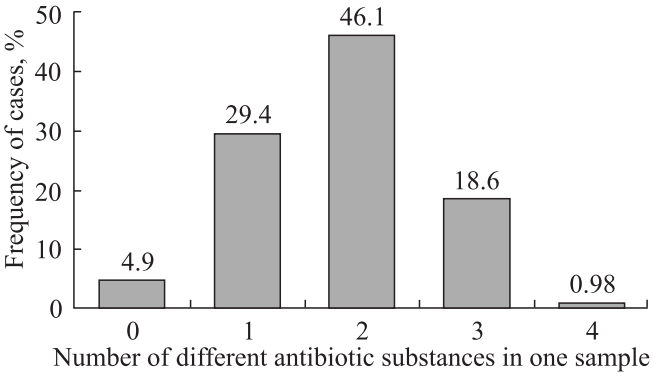


Fig. 1. The frequency of simultaneous detection of several antibiotics in different amounts

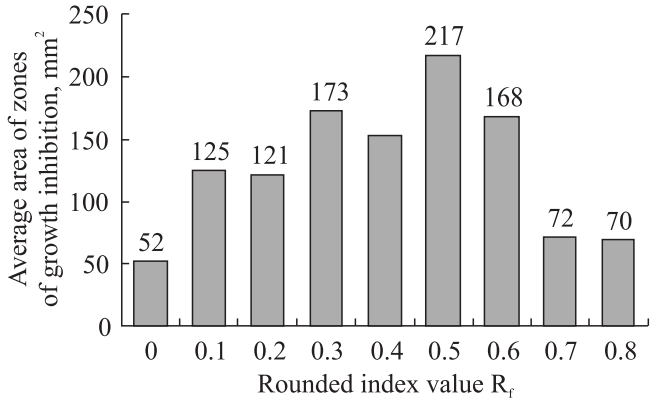


Fig. 2. The average area of zones of growth inhibition for *B. subtilis* sn-2 with different values of R_f

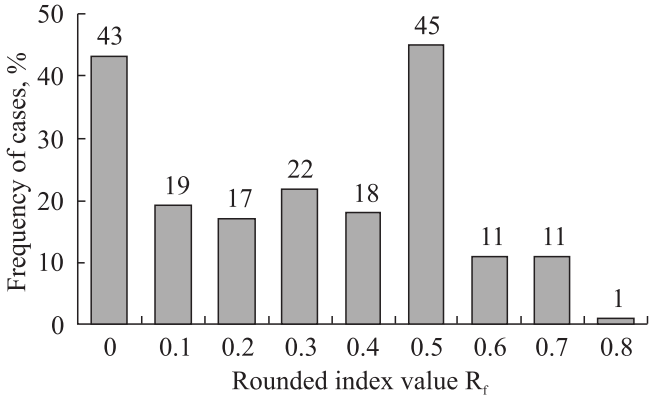
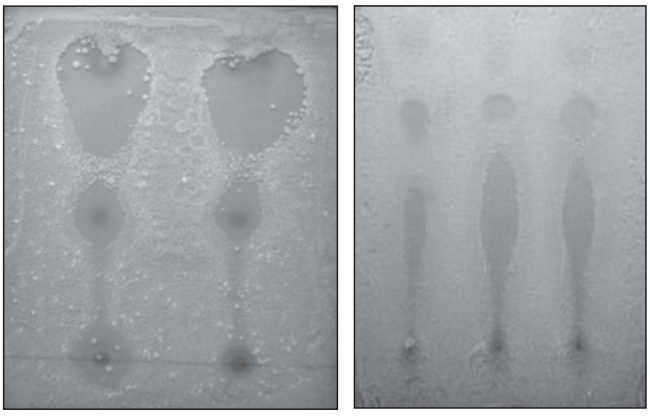


Fig. 3. The frequencies of detecting antibiotic substances with different values of chromatographic mobility

After veterinary antibiotics penetrate the environment, their further destiny is determined by three processes: adsorption by soil particles, biotransformation



A B

Fig. 4. The zones of absent growth of a test-microorganism *B. subtilis* sn-2 in case of biography of extracts of barley (A) and wheat (B)

under the effect of enzymes of microorganisms and bioaccumulation in plant tissues [17]. It was determined that the sorption of sulfadiazine, a veterinary antibiotic, is described with non-linear equations and depends on the level of acidity and type of soil. The study of biotransformation parameters of this antibiotic testified that its half lifetime is in the range from one to six months [18]. According to the experiment, which lasted for 120 days, the periods of half lifetime of erythromycin, oleandomycin, tylosin, tiamulin and salinomycin in soil are 20, 27, 8, 16, and 5 days, respectively. The concentration of roxithromycin did not change during the whole 120-day-period [4]. The results of these studies testify that veterinary antibiotics are stable substances which may remain in soil for a long time.

It should be noted that the experiments, aimed at detecting antibiotics in soil, were conducted in the 50–60s of the previous century to search for new antibiotic substances and their producents. Novel studies, aimed at detecting antibiotics in the soil, emphasize the detection of only veterinary antibiotics which penetrate soils due to human economic activity [19]. The problem of soil contamination with veterinary antibiotics raises the question on the possibility of bioaccumulation of these substances in plant tissues, including agricultural crops. For instance, it was determined that such antibiotics as gentamicin and streptomycin are accumulated in the tissues of carrots (*Daucus carota*), lettuce (*Lactuca sativa*) and radish (*Rhaphanus sativus*), in addition, gentamicin, the molecular mass of which is lower, has more expressed propensity for accumulation [20]. Soybeans may accumulate the substances with antibacterial activity – triclosan, triclocarban and carbamazepine, which are a part of hygiene products and household cleaning products and contaminate soils due to the introduction of products of wastewater cleaning as fertilizers [21].

Then there is a question whether veterinary antibiotic preparations are the only potential source of contamination of plant cultivation products with antibiotics. The substances with antibacterial and antifungal properties are known to be produced by symbiotic bacteria, present in rhizoplane and rhizosphere of cultivated plants and in the root zone of soil [9]. It is quite possible that such antibiotic substances may be absorbed by the root system of plants and get accumulated in different tissues and organs, including fruit and seeds.

One more possible source of antibiotic substances in grain may be phytopathogenic and opportunistic pathogenic endophytic bacteria. Recently there have been some communications on the aggravation of the problem of bacterioses of cultivated crops due to wide-scale application of pesticides with fungicidal activity to protect cultivated plants from fungal diseases [22]. It was determined that fungicides inhibit the development of both fungi as pathogens and representatives of fungal saprotrophic soil microflora. Due to the emptying of ecological niches, previously taken by saprotrophic fungi, namely, crop debris, there is uncontrolled development of bacteria, a considerable part of which belongs to phytopathogenic species.

Phytopathogenic bacteria are capable of penetrating the transport vessels of plants and getting accumulated in grain, producing a wide spectrum of antibiotic substances and compounds, toxic to eukaryotic organisms, including mammals. For instance, *Pseudomonas syringae atrofaciens* is an agent, causing bacteriosis of barley endosperm, one of the consequences of which is the deterioration in quality of grain and flour [23]. An interseasonal factor of the transmission of most agents of bacterial diseases of gramineous plants is seeds, i.e. bacterial cells get accumulated in grain during its development and infect the sprouts during the germination. It is quite probable that the products of bacterial synthesis, including antibiotics, also get accumulated in grain. It is known that *P. syringae* is the producent of antibiotic substances, such as tabtoxin, phaseolotoxin and mangotoxin, harmful both for bacteria [12] and for mammals [24, 25].

CONCLUSIONS

The data obtained testify to high frequency and considerable levels of grain contamination of gramineous plants and secondary products of seed processing of oil-seed crops with antibiotic substances, active against *B. subtilis* strain, rather sensitive to a wide spectrum of antibiotics. The differences in the indices of chromatographic mobility testify to a high variety of physical properties, and therefore, chemical structures of the antibiotic substances revealed. The contamination of grain and vegetative raw material with antibiotics is of potential risk to the environment, health of farm animals and humans. Further studies are planned for the identification of the detected antibiotic substances, the investigation of their impact on the environment and living organisms as well as determination of sources of their penetration into grain and other tissues and organs of cultivated plants.

Виявлення в зерні і кормах антибіотиків, активних відносно *Bacillus subtilis*

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Мета. Виявлення антибіотичних субстанцій у зразках зерна, шрота і макухи. **Методи.** Методом біоавтографії з використанням штамів *Bacillus subtilis* як тест-мікроорганізмів досліджено 102 зразки кормових субстратів (кукурудзи, глютену кукурудзяного, ячменю, пшениці, сорго, висівок, мучки кормової, соняшникової макухи і шроту, соєвого шроту, дріжджів кормових і дробини пивної). **Результати.** У 95 % зразків кормових субстратів виявлено від 1 до 4 антибіотичних субстанцій, які пригнічують ріст *B. subtilis* і характеризуються широким діапазоном значень показника хроматографічної рухливості. Середні площі зон відсутності росту тест-мікроорганізму, що відповідають 2,5 г зразка, були в межах 52−217 мм^2. **Висновки**. Визначено високий ступінь забрудненості фуражного зерна та інших кормових субстратів антибіотиками, що вказує на необхідність їхньої ідентифікації, знаходження джерел забруднення, вивчення розповсюдженості і оцінки можливого впливу на показники здоров'я, продуктивності та репродукції сільськогосподарських тварин та птиці.

Ключові слова: зерно, макуха, шрот, антибіотики, біоавтографія.

Обнаружение в зерне и кормах антибиотиков, активных в отношении *Bacillus subtilis*

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Цель. Выявление антибиотических субстанций в образцах зерна, шрота и жмыха. **Методы**. Методом биоавтографии с использованием штаммов *Bacillus subtilis* в качестве тест-микроорганизмов исследованы 102 образца кормовых субстратов (кукурузы, глютена кукурузного, ячменя, пшеницы, сорго, отрубей, мучки кормовой, подсолнечного жмыха и шрота, соевого шрота, дрожжей кормовых и барды пивной). **Результаты**. В 95 % образцов кормовых субстратов обнаружены от одной до четырех антибиотических субстанций, угнетающих рост *B. subtilis* и характеризующихся широким диапазоном значений показателя хроматографической подвижности. Средние площади зон отсутствия роста тест-микроорганизма, соответствующие 2,5 г образца, были в пределах 52−217 мм^2. **Выводы.** Обнаружен высокий уровень загрязненности фуражного зерна и других кормовых субстратов антибиотиками, что указывает на необходимость их идентификации, нахождения источников загрязнения, изучения распространенности и оценки возможного влияния на показатели здоровья, продуктивности и репродукции сельскохозяйственных животных и птицы.

Ключевые слова: зерно, жмых, шрот, антибиотики, биоавтография.

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