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Comparative analysis of different approaches for determining microbiological criteria in feed samples for animals and poultry

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The article presents research results on microbiological criteria of animal and poultry feed conducted under the requirements of the State Monitoring of Animal and Poultry Feed in accordance with the Order of the State Service of Ukraine on Food Safety and Consumer Protection for routine studies of feed samples and for in-depth studies to detect the entire species composition of microorganisms in feed samples coming from feed production enterprises in Ukraine. Isolation and identification of isolates were carried out according to current documentation. Feed is one of the main components of the food chain within the "One Health" concept to which Ukraine is committed. In-depth microbiological studies isolated from animal and poultry feed samples isolates of *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Listeria monocytogenes*, and *Listeria innocua*, confirming high levels of contamination by pathogens indicating potential risks of their spread and danger due to possible antimicrobial resistance (AMR) and potential transmission of acquired resistance to the normal microbiota of animals and humans through feed consumption. Furthermore, animal and poultry feed are not included in the list of objects of the National Strategy of Ukraine for Containing the Development of Antimicrobial Resistance.

Key words: animal and poultry feed, premixes, mixed fodder, bran, meal, fish meal, animal-origin meal, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Listeria monocytogenes*, *Listeria innocua*

Introduction

The intensification of livestock production involves the introduction of biologically complete regulated feeding, which ensures a high level of growth, development, and productivity of animals and prevents diseases associated with metabolic disorders and poor feed quality. Additionally, the contamination of animal feed with conditionally pathogenic and pathogenic microorganisms, including zoonotic ones, which can cause bacterial diseases in animals and poultry, presents a significant problem [4]. The greater danger lies in the detection of antibiotic-resistant pathogens, especially those with acquired resistance. Animal feed is a component of the food chain, thus directly relating to the "One Health" and "Global Health Security" strategies developed by leading global organizations such as the Food and Agriculture Organization of the United Nations (FAO), World Organization for Animal Health (WOAH, founded as OIE), and World Health Organization (WHO). The main priorities of these strategies are to preserve human and animal health and ensure the production of quality and safe products.

Ukraine is also involved in these strategies and conducts monitoring of raw materials and products throughout the agricultural sector, including the quality and safety of agricultural products. Specifically, within these monitoring

studies, information is collected, analyzed, and systematized regarding the contamination of animal and poultry feed with biotic contaminants — bacterial pathogens, including zoonotic ones [2, 11, 19].

However, the feed monitoring system in Ukraine does not cover all risks from feed production, identify critical points in technological processes, nor develop a prediction system for possible bacterial contamination of certain components of animal and plant origin and raw materials from other industries used for feed production. This situation is dangerous as feeding contaminated feed can pose risks of infectious diseases among animals or poultry and have epidemiological consequences. It is known that in EU countries, the USA, Japan, and other highly developed countries, the number of bacterial diseases associated with the food chain, where animal and poultry feed is a major component, is increasing [17, 18, 21]. Therefore, food safety is a high-priority issue in all countries, including Ukraine [8, 12, 19, 20].

Moreover, the most urgent problem of our time is the antibiotic resistance of pathogenic microorganisms. particularly those isolated during microbiological control of cattle, pig, and poultry feed materials, dry feed, and canned feed, as there remains a risk of transmission of such resistance to other animals, poultry, and humans. Although Ukraine has implemented a National Action Plan to combat antimicrobial resistance in line with the World Health Organization's (WHO) Global Strategy to Contain Antimicrobial Resistance, the plan does not cover Ukrainian feed production facilities.

Therefore, it was of interest to establish the entire species spectrum of microorganisms in animal and poultry feed and further test their sensitivity to antimicrobials and check for acquired resistance enzymes.

Materials and Methods

The research was conducted by scientists from the Research Microbiology Department of the State Scientific Research Institute for Laboratory Diagnostics and Veterinary-Sanitary Examination (Kyiv) and the Institute of Animal Biology NAAS (Lviv).

Microbiological monitoring studies were conducted on samples of various types of animal and poultry feed for non-compliance according to the Order of the State Service of Ukraine on Food Safety and Consumer Protection no. 56 dated 21.01.2023 and Order no. 898 dated 27.12.2023 on the approval of the State Monitoring Plan for Feed for 2023 and 2024, respectively.

The determination of microbiological criteria by routine methods for compliance with QMAFAnM, Coliform bacteria, *Staphylococcus aureus*, *Salmonella pathogens*, *Listeria monocytogenes,* and sulfite-reducing clostridia was carried out according to current regulatory documentation, as well as on the demand of manufacturers according to their regulatory documentation.

Additionally, in-depth own studies were conducted to determine the complete species composition of bacterial microorganisms that are feed contaminants. These in-depth studies involved appropriate cultures from enrichment media, previously inoculated with feed samples and incubated at 37°C for 24 hours. Further inoculations were made on various differential-diagnostic media for the detection of *Escherichia coli*, bacteria of the genus *Staphylococcus*, and *Listeria* [3, 5, 6, 13–15].

For the isolation and identification of *E. coli* isolates from the accumulation medium, the cultures were made on special media: Endo, XLD, Ramback, Simons, and trisugar agar (TSA), media with phenol red and carbohydrates glucose, lactose, sucrose, maltose, arabinose, rhamnose, xylose, dulcite, indole was tested and incubated in a thermostat at 37±1.0°C for 24 hours.

To isolate and identify isolates of *Staphylococcus aureus* and *Staphylococcus epidermidis* from the accumulation medium, the samples were streaked on milk-salt agar, egg yolk salt agar, glucose-blood agar, and special Baird-Parker medium to identify the specific growth pattern, plasma-coagulation reaction with sterile rabbit blood plasma was performed, the presence of enzymes for the fermentation of carbohydrates lactose, glucose, mannitol, sucrose, maltose, xylose, arabinose, mannitol was studied, and tests for the detection of catalase and oxidase were performed.

For the isolation and identification of *Listeria monocitogenes* and *Listeria innocua* isolates from the accumulation medium, half Fraser's broth, full Fraser's broth, L-mono medium, selective PALCAM agar, and the CAMP test were performed.

Results and Discussion

During the study period from 01.07.2023 to 01.04.2024, 382 samples of various types of feed were examined for microbiological indicators, including premixes — 36; compound feed and bran — 47; meal and cake — 127; feed for non-productive animals — 66; fish meal — 9; animal-origin meal — 9; other types of feed — 88.

Analysis of the research results for the detection of *E. coli* isolated 21 isolates and identified them. When inoculated on Endo medium, *E. coli* had a characteristic growth in the form of red colonies with a metallic sheen and reddening of the medium beneath them. On XLD medium, *Escherichia* colonies were yellow with an opalescence zone around the colonies. On Rambak medium, green colonies were grown. On Simons medium, the test culture did not grow, and the medium did not change color, which is typical for *Escherichia*.

The growth characteristics of *E. coli* on the slanted column of triple sugar agar (TSA) were characterized by a color change from red to yellow in the slanted part and in the agar thickness due to the fermentation of sugars to acid by *Escherichia* and the change in medium pH. This indicated the characteristic biochemical properties of the studied *E. coli*.

The results of biochemical studies of *Escherichia* isolates to detect enzymes for fermenting glucose, lactose, sucrose, maltose, arabinose, rhamnose, xylose confirmed their presence. The isolates did not ferment dulcine. Biochemical properties confirmed the typical characteristics of *E. coli*. The production of indole, formed during the complete breakdown of proteins and manifested as reddening of the strip impregnated with Kovac's reagent, was confirmed in all *E. coli* isolates.

Thus, the analysis of microbiological research results established that 21 cultures were identified as *E. coli* because they possessed all the typical properties characteristic of this pathogen.

The analysis of the research results for the detection of staphylococcal infection pathogens isolated 38 isolates and identified them. Inoculations from enrichment media on milk-salt agar after cultivation in a thermostat showed the growth of opaque round colonies with even edges, convex, pigmented in yellowish, yellow, and white colors, of small and medium sizes. On yolk-salt agar, staphylococcal colonies grew light-colored, of medium size, with a zone of cloudiness around the colonies with an iridescent fringe, indicating lecithinase production. On Baird-Parker medium, characteristic growth of staphylococcus in the form of characteristic black colonies with a metallic sheen and a clear opalescence zone around them, typical for *S. aureus*, was observed.

Smears were made from individual characteristic colonies on Bad Parker medium to check the purity of the *S. aureus* isolate. The preparations were fixed and stained using the Gram's method. The microscopy of the preparations showed homogeneous Gram-positive cocci located separately, in pairs, in clusters and in packets, which confirmed the purity of the test culture of *S. aureus*. The results of the plasma coagulation test showed complete coagulation of rabbit blood plasma in 16 experimental isolates of staphylococci isolated from samples of premixes, feed and bran and meal. Plasma coagulation was observed after different time periods from 2 h 30 min to 6 h and confirmed one of the characteristic typical properties of *S. aureus*. The plasma coagulation test revealed 22 cultures that did not coagulate blood plasma. During the study of biochemical properties of the experimental isolates, 16 experimental isolates were found to have sucrolytic enzymes for the fermentation of lactose, glucose, mannitol, maltose, which confirm the enzymatic properties typical of *S. aureus*. In 22 isolates, fermentation of mannose, maltose, sucrose and the absence of mannitol fermentation, which are characteristic of *S. epidermidis*, were detected. According to the results of catalase and oxidase tests, 16 test isolates showed catalase production and the absence of oxidase, which confirmed their belonging to the pathogen *S. aureus*.

Taking into account the results of studies on the haemolytic properties of the experimental isolates, complete haemolysis of sheep erythrocytes (β-haemolysis) with a transparent zone around the colonies was detected, which confirms one of the typical properties of *S. aureus*.

Based on the analysis of the results of tests for the detection of listeriosis pathogens, 8 isolates of *L. monocitogenes* and 4 isolates of *L. innocua* were identified. The growth of *Listeria* in Fraser broth one and two was characterised by darkening of the medium, which confirmed the presence of the pathogen. On selective PALCAM agar, the growth of small, dark colonies with a sunken centre and blackening of the medium beneath them was observed. On L-mono medium, *Listeria* bacteria grew in the form of greenish colonies, but a clear zone of opalescence was formed around the *L. monocitogenes* colonies (fig. 2), while such a zone was absent around the *L. innocua* colonies.

The analysis of the CAMP test results showed the presence of the pathogen *L. monocitogenes* by the presence of a zone of hemolysis and a zone of expansion and clearance of hemolysis around the *S. aureus* streak with a narrow zone of hemolysis near the *Rhodococcus equi* streak (fig. 3).

Based on the results of the CAMP test for the identification of *L. innocua*, the absence of haemolysis was detected in relation to the *S. aureus* and *Rh. equi* streaks.

A comparative analysis of the data obtained after the State Monitoring of Animal and Poultry Feed, as well as the data of microbiological tests conducted by routine methods and the results of in-depth studies of animal and poultry feed, revealed that in the first and second cases, the results of the studies do not provide a true picture of the epizootic situation regarding the level of contamination and species circulation of various types of microorganisms at feed production enterprises in Ukraine. In-depth studies have shown a rather problematic picture, since in addition to conditionally pathogenic microorganisms, zoonotic pathogens *E. coli*, *S. aureus*, and *L. monocitogenes* were identified (table).

Analysing the data obtained, it is evident that certain genera and species of pathogens are detected in samples of animal and poultry feed and their number in in-depth microbiological studies is higher than in other data obtained.

The research results showed that monitoring and routine studies do not detect or take into account a significant number of different bacterial contaminants of animal and poultry feed, which creates real risks of infection of animals with opportunistic and pathogenic microorganisms, including zoonotic ones. Our research results are confirmed by scientific data from other researchers [11, 12, 20].

Within the framework of the "One Health" concept, scientists emphasise the need to create a strong feed base, which includes a system and structure of feed production and is one of the main conditions for the economic modernisation of livestock production and ensuring food security in Ukraine [7, 9, 10].

The health, productivity and reproductive capacity of animals largely depend on the quality and suitability of feed for feeding. To determine these characteristics, laboratory and on-farm feed evaluation is mandatory [4, 16].

Table. Results of research on animal and poultry feed samples according to microbiological criteria using different research approaches (n_1=1; n_2=3; n_3=81; piece)

Microbiological non-compliance (type of pathogen)	Total tested/ isolated isolates of positive samples	Premixes	Grain	Compound feed, bran meals	Meals, cake	Feed for unproductive animals	Canned feed for animals	Fish meal	Meal of animal origin	Other types of feed
Results of monitoring studies										
Sulphite-producing clostridia	1	—	—	—	—	—	—	—	—	1 (cattle feed)
Total:	**1**	—	—	—	—	—	—	—	—	**1**
Results of routine studies										
Salmonella spp.	2	—	—	—	2	—	—	—	—	—
Enterobacter spp.	1	—	—	—	—	1	—	—	—	—
Bacterial contamination	3	—	—	1	—	—	—	1	—	—
Total:	**3**	—	—	**1**	**2**	—	—	**1**	**1**	—
Results of our own in-depth research										
Escherichia coli	21	2	6	7	6	—	—	—	—	—
Staphylococcus aureus	16	2	—	5	7	—	—	—	—	—
Staphylococcus epidermidis	22	2	2	9	8	—	—	—	2	1
Listeria monocitogenes	8	—	—	3	4	—	—	—	—	—
Listeria innocua	4	—	—	2	3	—	—	—	—	—
Total:	**81**	**7**	**8**	**26**	**37**	—	—	—	**2**	**1**

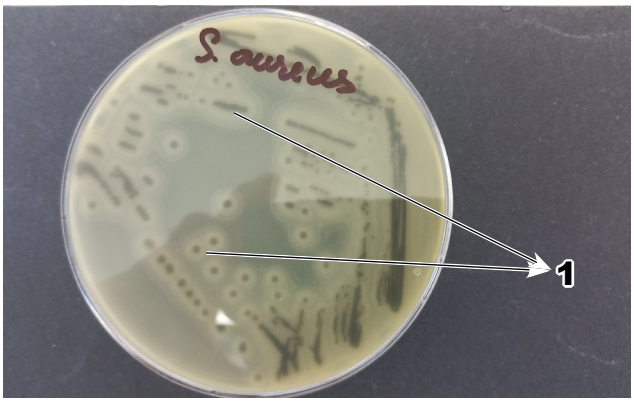

Fig. 1. Growth characteristics of *Staphylococcus aureus* on Baird-Parker medium. 1 — growth of *S. aureus* black colonies with a zone of opalescence

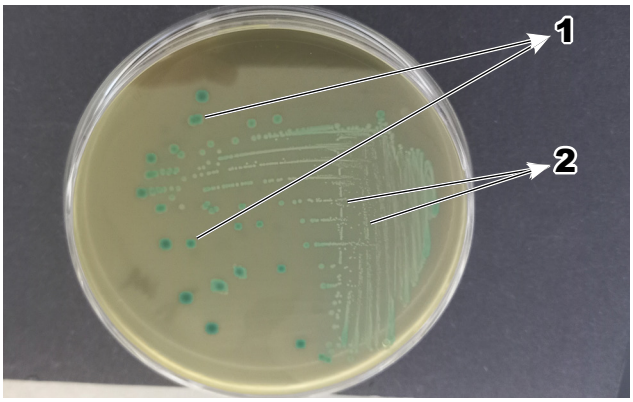

Fig. 2. Typical growth of *Listeria monocitogenes* and *Listeria innocua* colonies on L-mono medium. 1 — growth of greenish colonies with a zone of opalescence for *L. monocitogenes*; 2 — growth of *L. innocua* colonies of light colour and no opalescence zone

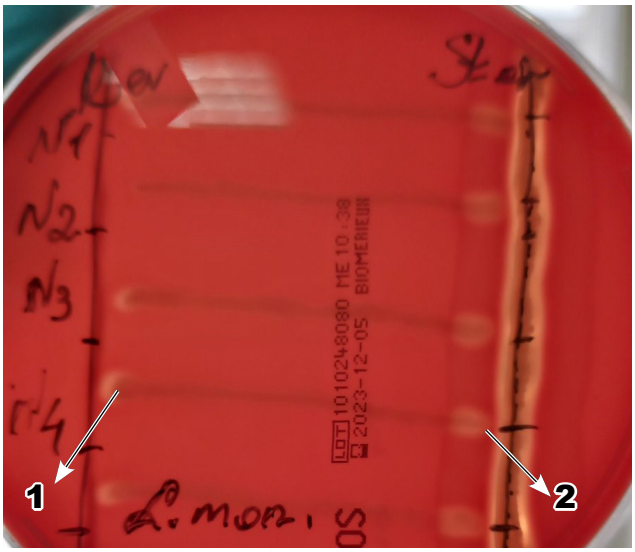

Fig. 3. Features of *Listeria monocitogenes* haemolysis manifestation in the CAMP test. 1 — a zone of expansion and clearance of haemolysis around the *Staphylococcus aureus* streak during the growth of *L. monocitogenes*; 2 — a narrow zone of haemolysis around the *Rhodococcus equi* streak during the growth of *L. monocitogenes*

Even greater risks lie in the fact that microorganisms isolated from animal and poultry feed are not currently monitored for antibiotic-resistant pathogens and acquired resistance to antimicrobials. As both animals and humans are part of the food chain in the "One Health" concept, the problem is exacerbated by the transmission of such acquired resistance to the normal microbiota of the intestinal tract of animals, poultry and humans.

It was found that among the 382 samples of premixes, grain, feed and bran, meal and cake, animal flour and cattle feed examined in depth, 81 samples were contaminated with opportunistic and pathogenic microorganisms that were not identified during monitoring and routine studies.

It was established that in-depth studies of microbiological criteria in animal and poultry feeds revealed a significant proportion of opportunistic pathogens and zoonotic pathogens of bacterial origin, including 21 isolates of *E. coli*, 16 isolates of *S. aureus*, 22 isolates of *S. epidermidis*, 8 isolates of *L. monocitogenes*, 4 isolates of *L. innocua*, which confirms the high level of contamination of animal and poultry feed with pathogens, indicates potential risks of their spread and danger due to the possible presence of antimicrobial resistance and acquired resistance mechanisms.

Prospects for further research are to test the isolated pathogen strains for antibiotic susceptibility, screening of enterobacteria and *Staphylococcus* spp. strains to identify and confirm the production of acquired enzymes, as animals, poultry and humans are integral parts of the food chain.

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Порівняльний аналіз різних підходів з визначення мікробіологічних критеріїв у зразках кормів для тварин і птиці

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В статті представлені результати досліджень мікробіологічних критеріїв кормів для тварин і птиці, виконаних за вимогами Державного моніторингу кормів для тварин і птиці згідно з наказом Державної служби України з питань безпечності та захисту прав споживачів, за проведення рутинних досліджень зразків кормів і за проведення поглиблених досліджень на виявлення всього видового складу мікроорганізмів у зразках кормів, які надходять із кормовиробничих підприємств України. Виділення і ідентифікація ізолятів проведено згідно з чинною документацією. Корми є однією із головних складових частин харчового ланцюга у концепції «Єдине здоров′я», до виконання якої долучена Україна. За поглиблених мікробіологічних досліджень виділені зі зразків кормів для тварин і птиці ізоляти *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Listeria monocitogenes*, *Listeria innocua* підтверджують високий рівень їх контамінації патогенами, вказують на потенціальні ризики щодо їх розповсюдження, на небезпеку через ймовірну наявність у них стійкості до антибактеріальних препаратів (АБП) та ймовірну можливість передачі набутої резистентності нормальній мікробіоті організму тварин і птиці через споживання таких кормів та людині внаслідок споживання сировини і продукції тваринництва. До того ж корми для тварин і птиці не входять до переліку об′єктів моніторингу Державної стратегії України щодо стримування розвитку стійкості до протимікробних препаратів.

Ключові слова: корми для тварин і птиці, премікси, комбікорм, висівки, шрот, рибна мука, мука тваринного походження, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Listeria monocitogenes*, *Listeria innocua*

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