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PREVALENT AEROBIC BACTERIAL FLORA ISOLATED FROM EMBRYONATED EGGS AND CHICKS FROM EXTENSIVE SYSTEM

FLORA BACTERIANĂ AEROBĂ PREVALENTĂ IZOLATĂ DIN OUĂ EMBRIONATE ȘI PUI NEVIABILI DIN SISTEMUL EXTENSIV

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ABSTRACT

The main sources of infection of eggs and embryos are sick birds and those that have gone through the disease. The paper aimed at the aerobic bacterial flora of the hatching eggs, which can influence the hatching percentage, but also the pathogenicity of the isolated and identified germs. Incubation eggs (547 samples) and non-viable day-old chicks (224 samples) from the Marans, Ameraucana, Kabir, Italiana, Brahma, and Plymouth Rock Barat breeds were examined bacteriologically. The *E coli* strains were tested for antibiotic sensitivity to 10 antimicrobial substances: gentamicin (CN 50), neomycin (N10), colistin (C10), florfenicol (FFC 30), trimethoprim (CTX), amoxicillin (AMC10), tetracycline (T30), enrofloxacin (ENR 5), lincospectin (LCS 10), and doxycycline (DO 30). *Staphylococcus* spp. strains were tested against the following 10 antimicrobials: gentamicin (CN50), kanamycin, florfenicol (FFC 30), trimethoprim (CTX), colistin (C10), amoxicillin (AMC10), tetracycline (T30), enrofloxacin (ENR5), streptomycin (SPT10), and ampicillin (AMP10). Bacterial flora isolated from embryonated eggs and non-viable chicks: *E.coli* 16.96%, *Staphylococcus* spp. 6.7 %, *Proteus* 2.38%, *Bacillus* spp., and *Pseudomonas*. *E. coli* strains were sensitive to: florfenicol (75%), enrofloxacin (66.67%), gentamicin (58.33%), trimethoprim (50%), colistin (41.67%), amoxicillin (33.33%) and were resistant to 100% doxycycline, neomycin, and tetracycline (66.67%).*Staphylococcus* spp. strains were sensitive to amoxicillin (83.33%), kanamycin (75%), gentamicin, and florfenicol (66.67%) and resistant to tetracycline (58.33%), colistin (50%), and enrofloxacin (41.67%).

Keywords: *E. coli, Staphylococcus spp*., antibiotics, embryonated eggs

REZUMAT

Principalele surse de infecție a ouălor și embrionilor sunt păsările bolnave și cele care au trecut prin boală. Lucrarea a vizat flora bacteriană aerobă a ouălor pentru incubație, care poate influența procentul de ecloziune, dar și patogenitatea germenilor izolați și identificați. Au fost examinate bacteriologic ouă de incubație (547 probe) și pui neviabili de o zi (224 probe) din rasele Marans, Ameraucana, Kabir, Italiana, Brahma și Plymouth Rock Barat. Tulpinile de *E coli* au fost testate pentru sensibilitatea la antibiotice la 10 substanțe antimicrobiene: gentamicină (CN50), neomicină (N10), colistină (C10), florfenicol (FFC 30), trimetoprim (CTX), amoxicilină (AMC10), tetraciclină (T30), enrofloxacină (ENR5), licospectină (LCS10) și doxiciclină (DO30). Tulpinile de *Staphylococcus* spp. au fost testate împotriva următoarelor 10 substanțe antimicrobiene: gentamicină (CN50), kanamicină, florfenicol (FFC30), trimetoprim (CTX), colistin (C10), amoxicilină (AMC10), tetraciclină (T30), enrofloxacin (ENR5), streptomicină (SPT10) și ampicilină (AMP10). Flora bacteriană izolată din ouă embrionate și pui neviabili a fost: *E. coli* 16,96 %, *Staphylococcus spp*. 6,7%, *Proteus* 2,38%, *Bacillus spp*. și *Pseudomonas*. Tulpinile de *E. coli* au fost sensibile la florfenicol (75%), enrofloxacină (66,67%), gentamicină (58,33%), trimetoprim (50%), colistin (41,67 %), amoxicilină (33,33%) și au fost rezistente la doxiciclină 100%, neomicină și tetraciclină (66,67%). Tulpinile de *Staphylococcus* spp. au fost sensibile la amoxicilină (83,33%), kanamicină (75%), gentamicină și florfenicol (66,67%) și rezistente la tetraciclină (58,33 %), colistină (50%) și enrofloxacină (41,67%).

Cuvinte cheie: *E. coli, Staphylococcus spp*., antibiotice, ouă embrionate

Incubation means the process of developing the embryo with latent life in the fertilized egg until a day-old chick is obtained, with normal viability, under the influence of certain physical factors, in a certain period, characteristic of each species or bird breeds (26, 29). Fresh eggs are resistant to microbial contamination, but with one condition, to come from healthy, rationally fed birds, sheltered in hygienic conditions and beneficiaries of a proper microclimate (48). However, a major risk that threatens the life of embryos is the microorganisms responsible for triggering infectious diseases and which are transmitted directly from sick

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birds or birds gone through the disease to the embryos by the germinal disc of eggs (13, 26).

The special resistance to microbial contamination of fresh eggs is mainly due to the presence of membranes, which protect the yolk, the most vulnerable structural component of eggs to microbial attack. Additionally, lysozyme contained in egg whites exerts a strong bactericidal action. Unlike fresh eggs, old and incorrectly preserved ones are unsuitable not only for incubation but also for public consumption, be contaminated by microorganisms existing in the environment (26, 28). The main sources of infection of eggs and implicitly of the embryos that develop in these eggs are sick birds and those that have passed through the disease, which eliminates germs through feces for a long time, causing contamination of setters, nests, water, and food (20, 28, 29).

Chicken growth in the extensive system can be exposed to a diverse bacterial flora, as they are raised with other animal species,from which germs can reach birds and then on the eggshell (4, 10, 23, 24, 27).

The paper aimed at the aerobic bacterial flora of hatching eggs, which can influence the hatching percentage, but also the pathogenicity of the isolated and identified germs.

MATERIALS AND METHODS

The study was conducted on 547 embryonated eggs and 224 samples from non-viable day-old chicks (Table 1) collected from the Marans, Ameraucana, Kabir, Italiana, Brahma, and Plymouth Rock Barat breeds, in different western Romania and Hungary locations, such as:

• Location A where Marans eggs were incubated, these eggs came from Hungarian breeders and Ameraucana eggs, these eggs came from Romanian breeders: 77 embryonated eggs and 15 non-viable Marans and Ameraucana day-old chicks;

• Location B where eggs were hatched from Kabir chickens that came from Romanian breeders: 40 embryonated eggs and 10 non-viable Kabir day-old chicks;

• Location C where eggs from Italian hens were from Hungary and Romanian breeders: 86 embryonated eggs and 75 non-viable Italian day-old chicks;

• Location D where eggs from Brahma and Plymouth Rock Barat hens were incubated from breeders in Romania and Hungary: 344 embryonated eggs and 124 non-viable Brahma and Plymouth Rock Barat day-old chicks.

The incubation was done with different incubators and each presented an electronic system for monitoring temperature and humidity. During the incubation, power outages were reported in location D, which increased the number of samples sent to the laboratory. Before incubation, the incubators were sanitized and disinfected. We mention that after the disinfection of the incubators, no samples were collected to control the disinfection efficiency.

From locations A and B, the number of samples that were sent to the laboratory was lower compared to the other two locations (C and D), being between 4.46% non-viable day-old chicks and 14.07% (6.76+7.31) embryonated eggs.

The highest number of samples was recorded in location D, 180 embryonated eggs of the Brahma breed (32.91%) and 107 non-viable chicks (47.77%). A lower percentage was registered in the Plymouth Roch Barat breed, 29.98% embryonated eggs and 7.59% non-viable day-old chicks.

Embryo egg samples were collected separately from each location. Each sample was properly aseptic-ized, placed in a plastic container, and transported to the laboratory for bacteriological examination.

The embryonated egg was wiped with a sterile buffer, with normal saline (0.85%), and finally, the surface was sterilized through the flame. This procedure was necessary to avoid contamination of the egg contents with the germs found on the eggshell (1, 2).

After disinfection, each egg was broken with a sterile spatula, then its contents were aspirated with a sterile Pasteur pipette and placed in a culture medium.

Table 1

Synoptic of the collected samples from embryonated eggs and non-viable day-old chicks

Location	Breed	Embryonated eggs	%	Non-viable day-old chicks	%
A	Marans	37	6.76	7	3.13
	Ameraucana	40	7.31	8	3.57
B	Kabir	40	7.31	10	4.46
C	Italiana	86	15.72	75	33.48
D	Brahma	180	32.91	107	47.77
	Plymouth Rock Barat	164	29.98	17	7.59
Total		547	100	224	100.00

Fig.1. Embryonated eggs

Fig. 2. Embryonated eggs and non-viable chicks

Fig. 3. Unviable chicks

Fig. 4. Embryonated eggs and non-viable chicks

The chicks that died on the first day after hatching were packed in separate plastic containers, depending on the location, and transported to the laboratory for laboratory examination. From each sample (Fig. 1, 2, 3, 4) bacteriological exam was performed on the usual and special culture media.

The pathogens were isolated and identified by culturing and incubation for 24 hours at 37°C. The bacteriological exam was performed in nutrient broth for bacterial growth and then on nutrient agar, MacConkey agar, agar with blood, deoxycholate xylose-lysine agar (XLD), SS agar, Levine agar, and Chapman agar.

The identification of isolated bacteria was performed based on cultural and morphological characteristics. During the morphological examination of the bacteria identified by cultivation and incubation, the following aspects were followed: macroscopic and microscopic morphology, size, shape, grouping, color, and color change on different culture media.

During the bacterioscopic examination, the bacteria were stained by the Gram staining method and examined under a microscope using a 100X cedar oil immersion objective.

Biochemical tests were performed to confirm the diagnosis, including coagulase, catalase, oxidase, MIU medium, and TSI medium.

The definitive identification of the isolated etiological agents was made with Analytical Profile Index (API) diagnostic kits: API 20 E - for the identification of pathogens from the *Enterobacteriaceae* family and API Staph - for the identification of staphylococci (26, 30, 31). The testing of the behavior of various isolated antimicrobial substances in isolated bacteria was performed on Mueller-Hinton agar.

The determination of the antibiotic susceptibility of the isolated strains was performed by the diffusimetric disc method (Kirby - Bauer method), using the Mueller - Hinton medium and antibiotic-impregnated biodiscs, provided by the manufacturing companies.

To test the behavior of *E. coli* strains against antibiotics, the following 10 antimicrobial substances were used: gentamicin (CN50), neomycin (N10), colistin (C10), florfenicol (FFC 30), trimethoprim (CTX), amoxicillin (AMC10), tetracycline (T30), enrofloxacin (ENR5), lincospectin (LCS10), and doxycycline (DO 30).

Table 2

Frequency of bacteria isolated from embryonated eggs and non-viable day-old chicks

Samples	No. exams	No. isolated	Isolated bacteria									
			E. coli		*Proteus*		*Staphylococcus spp*		*Bacillus spp*		*Pseudomonas spp*	
			No.	%	No.	%	No.	%	No.	%	No.	%
Embryonated eggs	547	62	28	5.12	13	2.38	18	3.29	2	0.37	1	0.18
Non-viable day-old chicks	224	59	38	16.96	4	1.79	15	6.70	1	0.45	1	0.45

Staphylococcus spp. strains were tested against the following 10 antimicrobials: gentamicin (CN50), kanamycin, florfenicol (FFC30), trimethoprim (CTX), colistin (C10), amoxicillin (AMC10), tetracycline (T30), enrofloxacin (ENR5), streptomycin (SPT 10), and ampicillin (AMP 10).

The antibiotic-impregnated biodiscs were placed at a distance of 20-30 millimeters between them and a distance of 15 millimeters from the edge of the plate.

The results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The diameter of the inhibition zone was measured with a ruler and expressed in millimeters. Depending on the diameter read, the results were classified as the following: sensitive, intermediate, and resistant (26, 30, 31).

RESULTS AND DISCUSSIONS

The bacterioscopic examination revealed both Gram-positive and Gram-negative bacteria (Table 2). On the culture media, the isolated strains had a characteristic behavior, disturbed the broth, and developed S or R type colonies. *E. coli* strains were easily identified by bacteriological examination.

The isolated strains of *Staphylococcus spp*. had a characteristic behavior, which allowed the classification in the genus *Staphylococcus*. In the smear, they

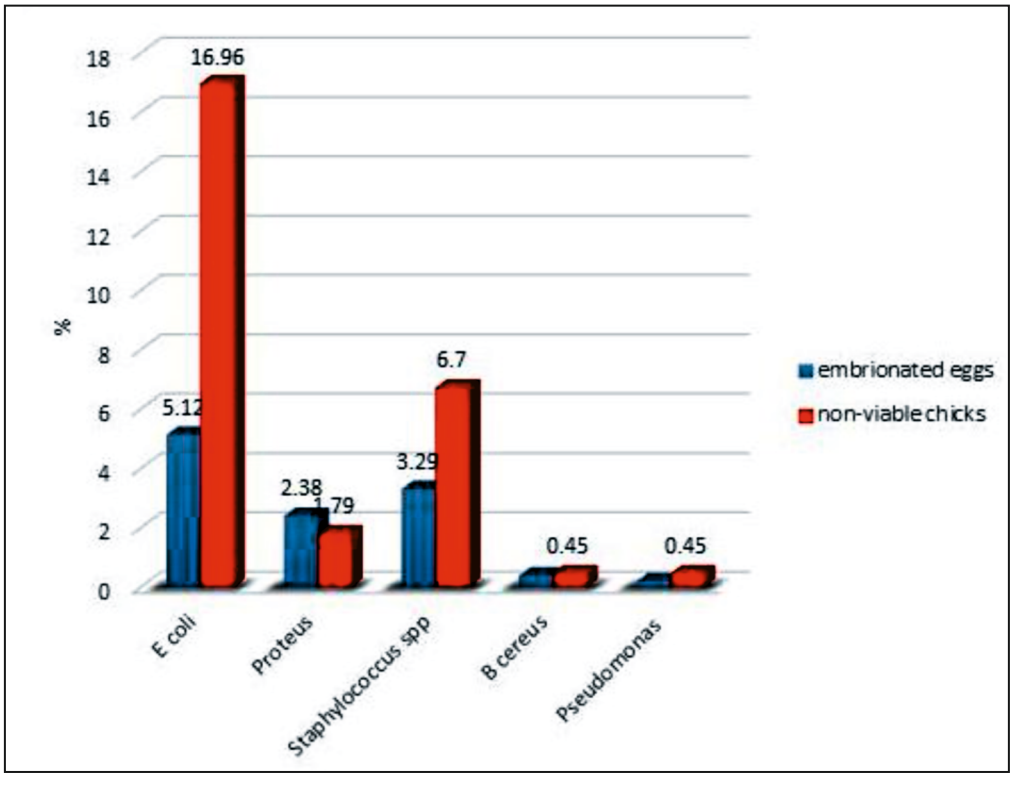

Fig. 5. Distribution of isolated bacteria

Table 3

Synoptic of antibiotic susceptibility of 12 isolated of *E coli*

	Sensibile		Intermediate		Resistant	
Antibiotic	No	%	No.	%	No.	%
Florfenicol	9	75	2	16.67	1	8.33
Neomycin	1	8.33	3	25.00	8	66.67
Gentamicin	7	58.33	4	33.33	1	8.33
Trimetoprim	6	50	4	33.33	2	16.67
Colistin	5	41.67	2	16.67	5	41.67
Amoxacilin	4	33.33	5	41.67	3	25.00
Tetracycline	1	8.33	3	25.00	8	66.67
Enrofloxacin	8	66.67	2	16.67	2	16.67
Lincospectin	3	25	7	58.33	2	16.67
Doxycycline	0	0	0	0	12	100

were grouped in the grape form, and on the bloody agar showed hemolysis. The final identification of *Staphylococcus spp*. was made using the Staph API kit.

From the 547 embryonated egg samples, 28 strains of *E. coli* (5.12%), 18 strains of *Staphylococcus* spp. (3.29%), 13 strains of *Proteus* (2.38%), 2 strains of *Bacillus* spp (0.37%) and one strain of *Pseudomonas* (0.18%) were isolated.

Out of 224 non-viable day-old chicks, 38 *E. coli* strains (16.96%), 15 *Staphylococcus* spp. strains (6.70%), 4 *Proteus* strains (1.79%), one *Bacillus* spp strain and one *Pseudomonas* strain (0.45%) were isolated. Bacterial flora isolated from embryonated eggs showed that the predominant species were: *E. coli* 16, 96%, *Staphylococcus* spp. 6.7%, and from non-viable day-old chicks *E. coli* 5.12%, Staphylococcus spp. 3.29%. Following the bacteriological examination, 66 strains of *E. coli*, 33 strains of *Staphylococcus spp*., 17 strains of *Proteus*, 3 strains of *Bacillus* spp. and 2 strains of *Pseudomonas* were isolated from the 771 samples. The antibiotic susceptibility of 12 *E. coli* isolates was tested against 10 antimicrobial substances: gentamicin, neomycin, florfenicol, trimethoprim, colistin, amoxicillin, tetracycline, enrofloxacin, lincospectin, and doxycycline (Table 3).

The most active antimicrobials were florfenicol (75%), followed in descending order by enrofloxacin (66.67%),gentamicin (58.33%),trimethoprim (50%), colistin (41.67%), amoxicillin (33.33%), lincospectin, tetracycline and neomycin were less active.

The highest resistance was recorded to doxycycline-100% and 66.67% for neomycin and tetracycline.

The 12 *Staphylococcus spp*. isolates were tested against 10 antimicrobials: gentamicin, kanamycin, florfenicol, trimethoprim, colistin, amoxicillin, tetracycline, enrofloxacin, streptomycin, ampicillin (Table 4).

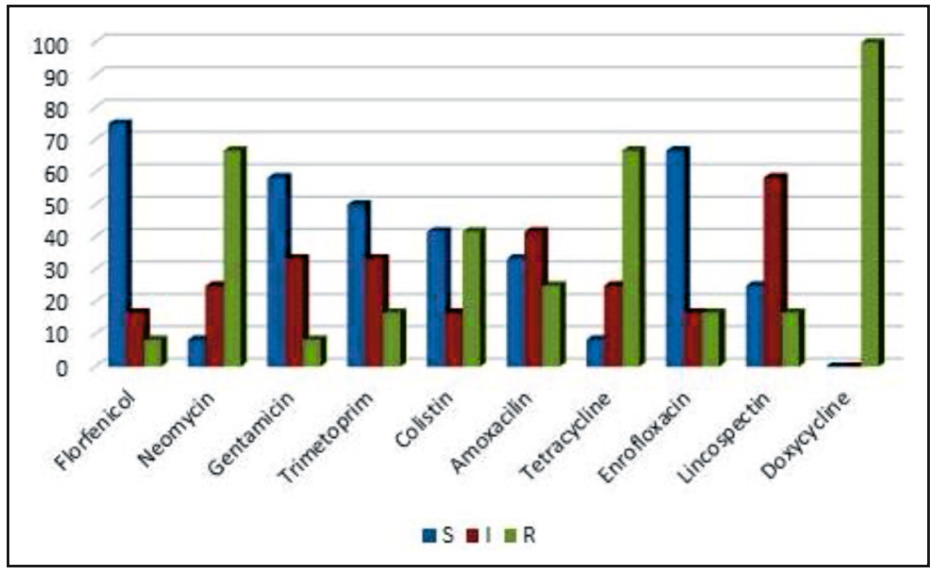

Fig. 6. Dynamics of antibiotic susceptibility of 12 *E. coli* isolates

Table 4

Synoptic of antibiotic susceptibility of 12 *Staphylococcus spp* isolates

	Sensitive		Intermediate		Resistant	
Antibiotic	No.	%	No.	%	No.	%
Gentamicin	8	66.67	3	25.00	1	8.33
Kanamycin	9	75.00	2	16.67	1	8.33
Florfenicol	8	66.67	3	25.00	1	8.33
Trimetoprim	3	25.00	7	58.33	2	16.67
Colistin	4	33.33	2	16.67	6	50.00
Amoxacilin	10	83.33	1	8.33	1	8.33
Tetracyclin	2	16.67	3	25.00	7	58.33
Enrofloxacin	5	41.67	2	16.67	5	41.67
Streptomycin	2	16.67	6	50.00	4	33.33
Ampicilin	10	83.33	1	8.33	1	8.33

As shown in Table 4. and Fig. 7., in the case of *Staphylococcus spp*. isolated, it can be seen that they showed the following antibiotic behavior:

- Sensitive: amoxacillin and ampicillin (83.33%), kanamycin (75%), gentamicin, florfenicol (66.67%);
- Intermediates: trimethoprim (58.33%), streptomycin (50%), gentamicin, florfenicol, tetracycline (25%);
- Resistant: tetracycline (58.33%), colistin (50%), enrofloxacin (41.67%).

The data obtained by us are in accordance with literature, where it is mentioned that: nine (75%) of the 120-day-old chicken samples tested were contaminated with 144 bacteria (5 species), of which 55.56% of these chicks were contaminated with more than one bacterium (1, 29).

Some research concludes that *E. coli* had the highest incidence (68.05%) followed by *Enterobacter spp*. (16.67%), *Citrobacter spp.* (11.11%), *Klebsiella spp*. (3.47 %), and *Bacillus spp.* (1, 2, 15, 21, 29).

The obtained results showed that the antibacterial activity compared with aerobic bacterial flora differs depending on the substances used. Thus, the most active were gentamicin (89.78%), followed in descending order by amoxicillin (87.61%), kanamycin and furazolidone (87.17%), ampicillin (79.38%), chloramphenicol (78.31%), erythromycin (34.51%), tetracycline (28.87%), colistin (28.87%), and streptomycin (8.84%). Streptomycin, colistin, tetracycline and erythromycin are less active, with most isolated strains (42.47 - 77.45%) being resistant (3, 22, 26)

Also, the antibacterial activity of the researched substances also differs depending on the bacterial species examined (2, 5, 12). Thus, the *Proteus vulgaris* strains were sensitive to the action of kanamycin (78.78%), amoxicillin (78.78%), gentamicin (72.28%), furazoli-

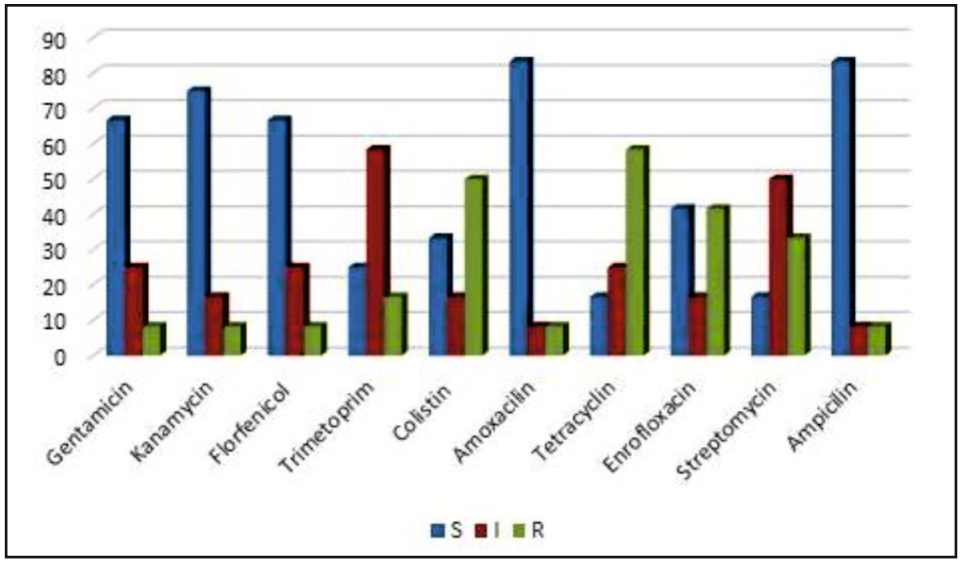

Fig. 7. Dynamics of antibiotic susceptibility of 12 *Staphylococcus spp.* isolates

done (56.07%), and ampicillin (68,78%); intermediate sensitive to streptomycin (39.39%) and erythromycin (37.39%) and resistant to the activity of tetracycline (53.02%) and chloramphenicol (48.50%) (26, 29).

Escherichia coli was very sensitive to the action of gentamicin (71.96%), kanamycin (66.10%), colistin (63.67%), and furazolidone (52.43%); intermediate sensitive to the activity of chloramphenicol (37.81%) and amoxicillin (29.39%); resistant to the activity of tetracycline (63.67%), erythromycin (62.21%), streptomycin (57.33%), and ampicillin (52.43%) (25, 26, 29).

Staphylococcus aureus was sensitive to gentamicin (97.62%), amoxicillin (95.24%), ampicillin (83.34%), and kanamycin (83.24%); furazolidone-sensitive intermediate (53.62%) and colistin (30.94%); resistant to streptomycin (64.48%) and chloramphenicol (57.16%).

Bacillus cereus was sensitive to amoxicillin (93.56 %), kanamycin (87.12%), gentamicin (83.90%), and ampicillin (54.00%); intermediate sensitive to the action of tetracycline (58.14%) and colistin (41.86%) and resistant 100% to the action of erythromycin (41.86%) and chloramphenicol. *Salmonella* spp. sensitive to the activity of amoxicillin (80.00%), gentamicin (80.00%), ampicillin (60.00%), kanamycin (60.00%), and furazolidone (60.00%); tetracycline-sensitive intermediate (80.00%) and chloramphenicol (60.00%); resistant to the activity of 100% amoxicillin, tetracycline and erythromycin.

The susceptibility of the isolated aerobic bacterial flora is evident in chemotherapeutics used to a small extent or not at all for prophylactic-curative purposes or as a biostimulator in poultry feed by applying and rigorously adhering to strategic measures for surveillance and prevention of bacterial infections. Thus, both the percentage of unfertilized eggs (from 1.85% to 0.9%) and the number of dead embryos (from 8.41% to 7.8%) were obtained. Instead, the percentage of viable chicks increased from 63.11% to 77.90% (7, 26, 29). To reduce the rates of bacterial contamination in eggs, risk reduction strategies must be used throughout the production chain (19). These strategies include farm practices that reduce the transport of bacteria, increase hygiene in the incubator, continue to implement HACCP systems, and increase farmers' education efforts (8, 11, 25, 26, 29). Contamination of hatching eggs can reduce the hatching rate and be responsible for the transmission of pathogens from poultry to embryos (26).

CONCLUSIONS

Prevalent aerobic bacterial flora isolated from embryonated eggs and non-viable chicks from growing extensively was: *E. coli* 16.96%, *Staphylococcus spp*. 6.7%, *Proteus* 2.38%, *Bacillus spp*. and *Pseudomonas spp*. *E. coli* strains were sensitive to: florfenicol (75%), enrofloxacin (66.67%), gentamicin (58.33%), trimethoprim (50%), colistin (41.67%), amoxicillin (33.33%) and were resistant to 100% doxycycline, neomycin, and tetracycline (66.67%). *Staphylococcus spp*. strains were sensitive to amoxicillin (83.33%), kanamycin (75%), gentamicin, florfenicol (66.67%) and resistant to tetracycline (58.33%), colistin (50%), and enrofloxacin (41.67%).

At the bacteriological examination of the samples from the four locations, no strain of *Salmonella* spp. was isolated. It is recommended to control the efficiency of disinfection in incubators and disinfection of eggs before incubation.

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