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TIMIŞOARA**

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LUCRĂRI ŞTIINŢIFICE

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TIMIŞOARA
VOLUMUL LII (3)**

**SCIENTIFICAL PAPERS
VETERINARY MEDICINE**

To be cited: LUCRARI STIINTIFICE: MEDICINA VETERINARA TIMISOARA (SCIENTIFICAL PAPERS: VETERINARY MEDICINE TIMISOARA)**,** vol. LII (3), 2019

Available online at: **http://www.usab-tm.ro/USAMVBT_Revista_ro_1086.html**
Indexed and/or abstracted in**:** CABI Full Text, CAB Abstracts, Ulrich's Periodicals Directory
Editor: AGROPRINT TIMISOARA ISSN: 1221-5295
Printed by: IMPRIMERIA MIRTON TIMISOARA

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2019**

THE USE OF PARASITICIDE TREATMENTS IN CARP LARVICULTURE

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Summary

The present study aimes to document fish pathology on carp larvae and it was conducted over a period of 10 days. The biological material was produced in Brates Experimental Farm, by the use of controlled semi-natural pond breeding technology, during april-may 2018. Wet mount microscopic analysis revealed parasitic diseases. The parasites were counted and reported as predominant species of protozoan ciliate - *Trichodina sp*. Therefore, the treatment applied on carp larvae was consisted by the administration of bleaching powder - Ca $(ClO)_2$ - in a dose of 10 kilograms per hectare. The treatment had 2 purposes as it follows: prophylactic purpose (0-10 days after hatching) in BR 11 and curative purpose (0-13 days after hatching and 0-16 days after hatching) in BR 12, respectively in BR 11. In order to establish the effectiveness of applied treatments, the degree of fish parasitic infestation was determined. At the end of the experiment period, the highest survival percentage of harvested fish was recorded in the prophylactic treatment option.

Keywords: carp larvae, Trichodiniosis, bleaching powder

Larviculture is known to be of great global significance and has attained an elevated interest in Romania as well, due to the nutritional optimization within the rearing technology and the use of parasiticide treatments, which play a key role in reducing juvenile fish mortality.

Ectoparasitic protozoa manifest variable effects on their hosts. Pathological effects are dependent on parasite population density and the fish physiological condition. Stressful conditions compromise the fish capacity to counteract infection. Heavy infections by ectoparasitic protozoars are mainly found in fish specimen aged less than one year old, reared in overcrowded habitats with poor water quality and under stressful conditions. In these circumstances, opportunistic and ubiquitous parasites species are found. However, infections in adult fish speimen are rare and host-specific species predominate. Heavy infections with ubiquitous trichodinids occur in carp larvae and fry in hatcheries and nursery ponds, and likewise as fish grow, they are replaced by more specialised species.

After the controlled semi-natural pond breeding process of carp (*Cyprinus carpio* Linnaeus, 1758), complications may occur. Decreased functionality of the imunitary system of carp fry may happen after the exogenous feeding shift, due to environmental conditions such as water eutrophication. This fact can cause frequent parasitic disease. Available treatment substances, according to the

present European regulation, are limited. Therefore, scientific researches and studies on the identification of new substances to combat fish parasitism are imperative. For this purpose, an experiment was initiated by using lime chloride as the main substance for the treatment and disinfection of water. The substance used in the indicated dose does not present toxicity for fish, neighter remanence in the water or mud. As well, the substance has not been observed to negatively influence the growth rate of the biological material.

In Brates Experimental Farm, when the water temperature rises, explosive development of *Cyzicus spp*., *Lepidurus apus* and *Streptocephalus spp*. occurs. Also, invasion with the protozoan parasite *Trichodina spp.* is manifested. The necesity of this experiment is based on the immediate need of fish farmers to identify new methods of treatment. It is imperative to determine which substances are most suitable considering the cost-benefit concept and the development stage of the biological material (the larval stages).

Materials and methods

The biological material used consisted of carp larvae, obtained by semi-semi-natural pond breeding technology, in the Brates Experimental Farm of I.C.D.E.A.P.A. Galati. The carp larvae hatched at the end of April 2018. The experiment was carried out for a period of 10 days. The experimental ponds were treated for the prevalent parasite *Trichodina spp*., with 10 kg of lime chloride ($CaOCl_2$) per hectare (6). Experimental variants:

- BR 11 - control pond (absent of parasitic disease) - in which the prophylactic treatment was administered to 10 days old carp larvae, on 08.05.2018;
- BR 12 - (accidental presence of the parasite *Trichodina spp*. on the tegument of the biological material) - in which the curative treatment was administred, of the first sporadic symptoms of parasitic disease, to the 13 days old carp larvae, on 05.11.2018;
- BR 13 – (degree of massive infestation of the biological material) - in which curative treatment was applied–to the 16 days old carp larvae, on 05.14.2018. The treatment was repeated after two days, on 05.16.2018, by using an identical treatment to the previous one. At the end of the experiment, the survival rate of the fish stock was evaluated.

The fish pathology analyses were carried out by collecting biological samples from carp larvae, under the Olimpus SZ 61 dissecting microscope, using a scalpel and dissecting needles (7). The ectoparasitic forms have been detected by direct microscopic examination of skin and gill scrapings from live (or freshly killed) fish larvae (2). Scratched mucus from skin and gills, were spread on a dry clean glass slide with a drop of water, mounted with a coverslip, and examined under (40X) lens of the Oxion Euromex microscope with a Ccmex 3.0 MP camera. The high density trichodinids per microscopic field indicated the trichodinosis disease.

Throughout the experimental period, the basic parameters, as parasitosis prevalence and the parasite incidence in the analyzed fish were studied (5). The prevalence of parasites was analyzed as follows: Prevalence = Number of host species infested with a particular parasite species ×100/ Total number of hosts examined (1).

Water chemistry analysis was determined by standard analysis methods (8) and compared to the maximum admissible values, according to the Order of the Ministry of Environment and Water Management no. 161/2006, concerning the classification of surface water quality, in order to determine the ecological status of water bodies (10). The samples for water chemistry and planktonic analyses (3) were colected from the three experimental ponds, on May 8, 14, 16, 18, before the treatments.

Branchiopoda crustaceans (from Anostraca order-*Streptocephalus spp*.), Filopods (from Spinicaudata class-*Cyzicus spp*., Notostraca class-*Lepidurus apus*) adult forms, and frog tadpoles were counted in the concentrate of 10 liter of filtred water, after administration of Lugol solution, at the stereomicroscope. Naupliar stages in zooplankton samples were counted on the Kolkwitz chamber under microscope (3). In chart 7, the evolution of the Branchiopods and of the batracians competing for the fish feed on the ponds was presented, depending on the treatment periods.

Results and discussions

The prophylactic treatment for Trichodiniosis, was administered by adding 10 kg/ha of lime chloride in BR 11 (in the absence of parasites, to prevent the invasion of *Trichodina spp*.). From the experience of previous years, it is well known that the ponds water during spring-summmer time, recorded a higher rate of eutrophication, compared tot the rest of the year. The treatment was applied with curative purposes in BR 12 (11.05.2018) and BR13 (14.05., 16.05.2018).

The fish pathology analysis from 08.05.2018 did not reveal the existence of ecto-parasites in any of the three experimental ponds.

Fish larvae were sampled for examination in the data of:

- May 11, 2018 (3 days after the prophylactic treatment in BR 11, in BR 12 and BR 13, when parasites were observed in ponds, prior to treatment);
- 05.14.2018 (6 days after treatment in BR 11, 3 days after curative treatment in BR 12 pond and BR 13 before treatment);
- May 16, 2018 (at 8 days after the treatment in BR 11, 5 days after the treatment administration in BR 12 and after 2 days of curative treatment in BR 13);
- 05.18.2018 (from BR 11, at 10 days after treatment, in BR 12 at 7 days after treatment and in BR 13 at 4 days after the first treatment and 2 days after the second treatment).

The parasitic communities consisted from species of Protozoa, which were the most abundant and dominant parasites, Monogenoidea and Crustacea, which are considered like pests for fish.

Taxonomy of predominant parasite (5):

Kingdom-Chromista
Subkingdom-Harosa
Infrakingdom-Alveolata
Phylum-Ciliophora
Subphylum-Intramacronucleata
Class-Oligohymenophorea
Subclass-Peritrichia
Order-Mobilida
Family-Trichodinidae
Genus-Trichodina
Species- *Trichodina domerguei* (Wallengren, 1897)(4).

Trichodinids are peritrichous ciliates (Fig.1), easily recognised by their dome shape, rows of cilia, conspicuous circle of hooklets, and horseshoe-shaped macronucleus (9).

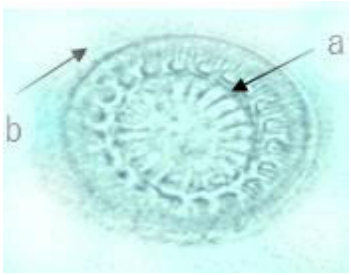

Fig. 1. Description: The protozoan parasite *Trichodina spp*. showing the central oral disc with a crown of denticles (a) and outer ring of cilia (b), under phase contrast microscopy

The parasitological examination was expressed according to the predominant parasite *Trichodina spp.* (Fig. 2.a, b and Fig. 3.a, b) by the parasitation intensity, prevalence and parasitic incidence on the skin and gills.

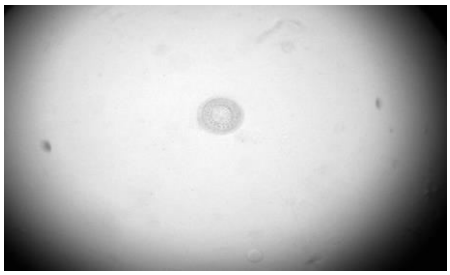

Fig. 2a. *Trichodina spp.* in skin scrape

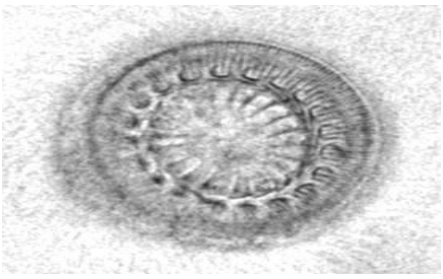

Fig. 2*b. Trichodina spp.* in skin scrape (detailed view)

The results reported for Trichodinosis are centered in tables no. 3, 4, 5, 6, and other parasites are reported in the comments.

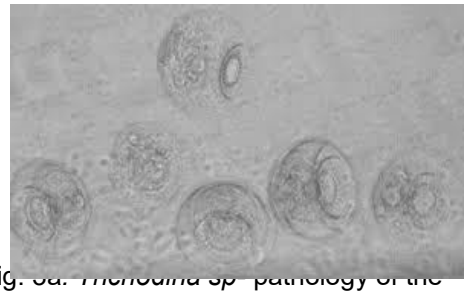

Fig. 3a. *Trichodina sp* pathology of the mucous skin

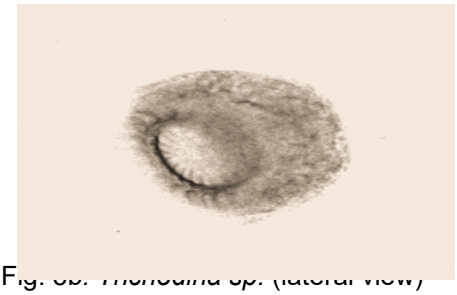

Fig. 3*b. Trichodina sp.* (lateral view)

The systematic classification of the other parasites encountered in the analyzed samples can be found in table 1.

Table 1

Taxonomic classification of fish parasites, exepting the main parasitosis

Phylum subphylum	***Class subclass***	***Order***	***Genus***	***Species***
Ciliophora	Oligohymenophorea (Hymenostomatia)	Peritrichida	Ambiphyra	*Ambiphyra sp.*
Plathelminthes	Monogenea (Monopisthocotylea)	Dactylogyrus	Dactylogyrus	*Dactylogyrus sp.*
Ciliophora	Oligohymenophorea (Hymenostomatia)	Hymenostomatida	Ichthyophthirius	*Ichthyophthirius multifiliis*

The table below shows the mean weights (reported as average±standard deviation) of fish larvae from BR 11, BR 12 and BR 13, weighed on 14.05.2018.

Table 2

Average weight of fish on ponds on 14.05.2018 (g/pcs.)

Pond	B11	B12	B13
Average weight	0.08±0.001	1±0.02	0.9±0.01

Analysis of occurrence and evolution of parasitic diseases have been studied in correlation with the evolution of phyto- and zooplankton, which are dependent on the chemistry of the technological water. Due to the large amount of data, the evolution of planktonic organisms according to the quality of the technological water was presented in six charts (Fig. 1-6). The effects of the administrated treatments on water quality (Fig. 4, 6, 8) and planktonic composition (Fig. 5, 7, 9) are presented as final conclusions. The evolution of abiotic and biotic factors of each pond, is presented vertically in the figures below.

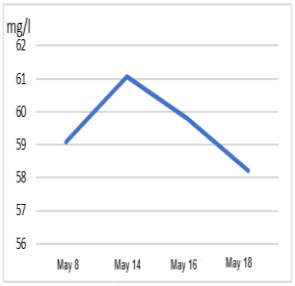

Fig. 4. The evolution of the organic substance mg/l $KMnO_4$ in BR11

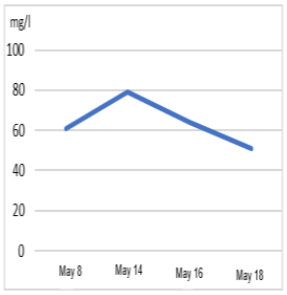

Fig. 6. The evolution of the organic substance mg/l $KMnO_4$ in R12

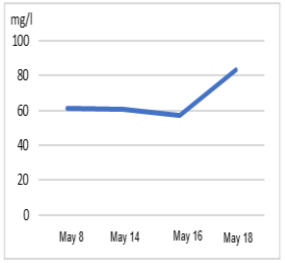

Fig. 8. –The evolution of the organic substance mg/l KMnO4 in BR13

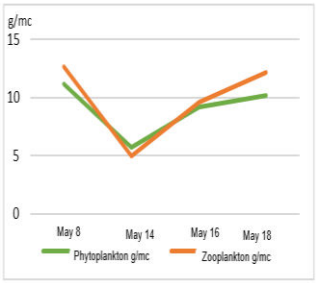

Fig. 5. The evolution of planktonic biomass g/mc in BR11

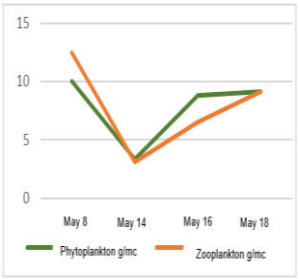

Fig. 7. The evolution of planktonic biomass g/mc in BR12

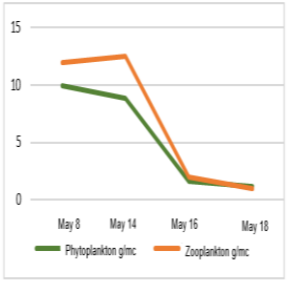

Fig. 9. The evolution of planktonic biomass g/mc in BR13

Assessment of lime chloride treatment effectiveness on 08.05.2018 is directly corelated to the destruction of the *Trichodina spp.*, but also to the disappearance of harmful crustaceans (Fig. 10).

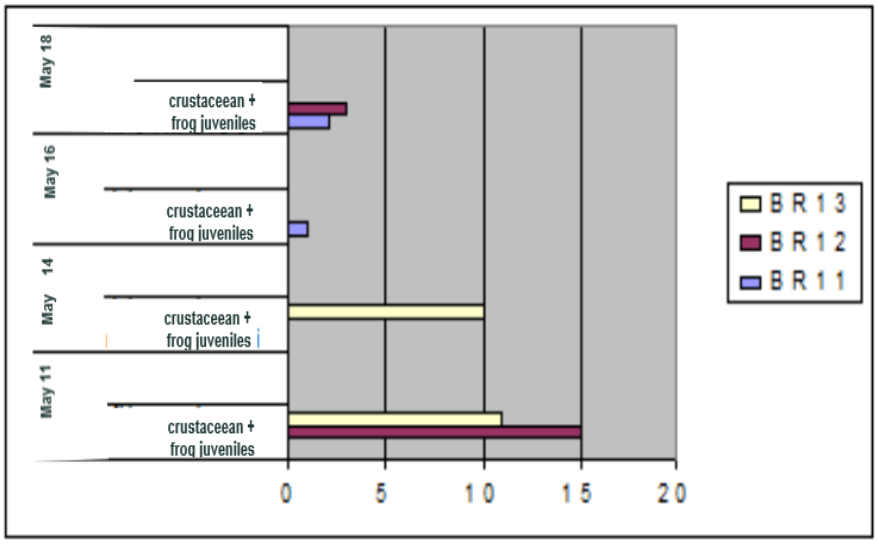

Fig. 10. Evolution of harmful Branchiopods and frogtadpoles after administration of the treatments

Parasite *Trichodina spp*. is present starting with the samples analyzed on May 11, according to table 3.

Table 3

Fish pathology analysis on May 11, 2018

Date of sample collection 05.11.2018						
No	**Pond name**	**Parasitation intensity for *Trichodina spp.***	**Prevalence (%)**	**Tegumentary incidence (%)**	**Branhial incidence (%)**	**Other parasites**
1	**BR11**	**-**	**-**	**-**	**-**	**-**
2	**BR12**	**weak-medium**	**42.85**	**28.57**	**71.43**	**-**
3	**BR13**	**medium**	**71.42**	**92.3**	**7.70**	**-**

Legend: week<5 specimens of parasites in the microscopic field, medium between 5-10 parasites in the microscopic field, >10 specimens of parasites in the microscopic field

Table 4

Fish pathology analysis on May 14, 2018

Date of sample collection 05.14.2018						
No	**Pond name**	**Parasitation intensity for *Trichodina spp.***	**Prevalence (%)**	**Tegumentary incidence (%)**	**Branhial Incidence (%)**	**Other parasites**
1	**BR11**	**-**	**-**	**-**	**-**	**-**
2	**BR12**	**weak**	**33.33**	**75**	**25**	**-**
3	**BR13**	**massive**	**71.42**	**10.34**	**89.66**	**1 organism of *Ichthyophthirius multifiliis* on skin 10 organisms of *Ambiphyra sp*.on gills**

The details regarding the parasites encountered at the pathological examination of fish larvae, except for *Trichodina spp*. are presented in Fig. 11.a and b, Fig.12.a and b, and Fig.3.a and b.

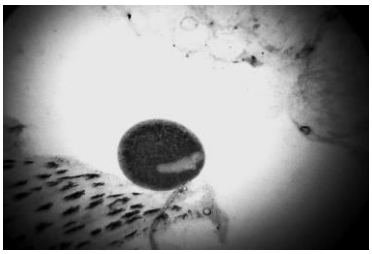

Fig. 11a. *Ichthyophthirius multifiliis*- Fouchet, 1876 on the fins of the analyzed fish

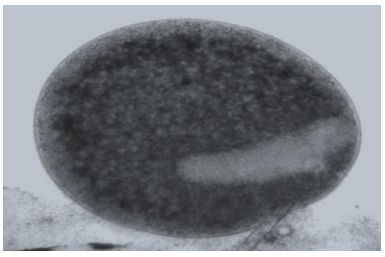

Fig. 11b. *Ichthyophthirius multifiliis* (detailed view)

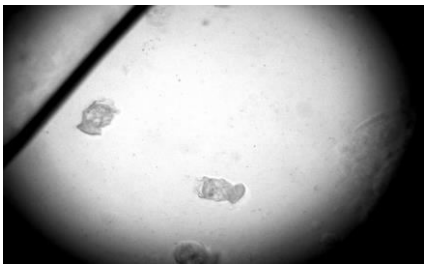

Fig. 12a. *Ambiphyra spp.* in scraped from tegument

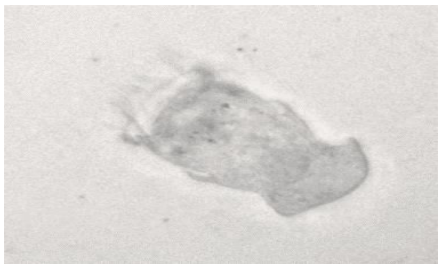

Fig. 12b. *Ambiphyra spp.* (detailed view)

Table 5

Fish pathology analysis on May 16, 2018

Date of sample collection 05.16.2018						
No	**Pond name**	**Parasitation intensity for *Trichodina spp.***	**Prevalence (%)**	**Tegumentary incidence (%)**	**Branhial incidence (%)**	**Other parasites**
1	**BR11**	**accidentally**	**42.85**	**94.11**	**5.89**	**-**
2	**BR12**	**weak-medium**	**57.14**	**69.23**	**30.77**	**2 organisms of *Dactylogyrus sp.* on gills**
3	**BR13**	**medium**	**57.14**	**57.14**	**42.86**	**-**

It is observed in BR 12 the appearance of the monogenean gill flukes *Dactylogyrus spp*., which is not encountered in the BR 11 pond. This fact leads to the conclusion that early administration of the disinfectant treatment had the capacity to destroy the parasitic cycle of the juvenile forms of parasites after hatching, with maximum efficiency, highlighting the importance of the preventive treatment.

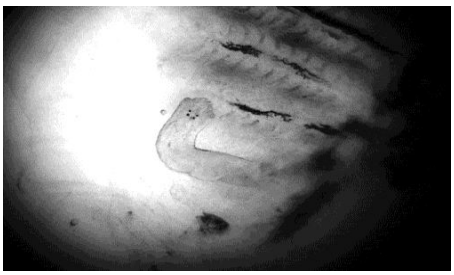

Fig. 13a. *Dactylogyrus vastator*-Nybelin,1924 on the gills of analized fish

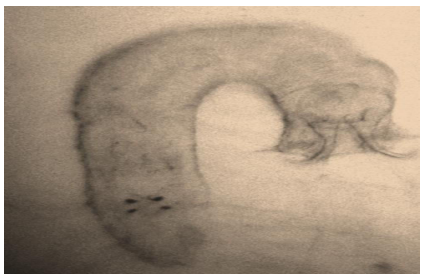

Fig. 13b. *Dactylogyrus vastator* (detailed view)

Table 6

Fish pathology analysis on May 18, 2018

Date of sample collection 05.18.2018						
No	**Pond name**	**Parasitation intensity for *Trichodina spp.***	**Prevalence (%)**	**Tegumentary incidence (%)**	**Branhial incidence (%)**	**Other parasites**
1	**BR11**	**weak**	**45.5**	**58.49**	**41.51**	**-**
2	**BR12**	**weak-medium**	**57.14**	**27.58**	**72.42**	**1 organism of *Ichthyophthirius multifiliis* on gills**
3	**BR13**	**weak**	**10**	**88.88**	**11.12**	**-**

Ten days after the treatment in BR 11, *Trichodina spp*. is present in a weak infestation degree, with a small prevalence of less than 50% and gills incidence also less than 50% (the skin incidence is considered less dangerous compared to the gills incidence, which can cause the fish asphyxiation), fact that confirms the disinfestation efficiency of carp larvae during the experiment.

In BR 12 days, after 7 days of treatment, the parasitation intensity of the biological material was low-medium, with a prevalence of over 50% and an increased incidence on gills (72.42%). This fact leads to the conclusion that treatment effectiveness in BR 12 on May 11th, 2018 (when the infestation was low-medium and prevalence was increasing) was lower compared to treatment effectiveness of BR 11.

In BR 13, 2 days after the treatment, on May 14th, massive infestation of the biological material occured, with the prevalence of 71.42% *Trichodina spp*. Therefore, it was necessary to repeate the treatment within two days after the first one (05.18.2018). The second treatment lead to a decreasing trend of infestation from massive to the medium. Disease prevalence reached 10% on May 18th, however, drastic destruction of planktonic biomass after treatment was observed and minimum values of 1.16 g/mc fitoplankton, respectivelly 0.97 g/mc of zooplankton were recorded.

The treatment of the technological water by the use of lime chloride had a negative impact on the early developed forms of the naupliar stages of Branchiopods and tadpole (see graph 7), but also reached the maximum efficiency of the parasiticide treatment used (see tables 5, 6).

The survival rate of fish stock reared in the experimental ponds (Table 7), following the administration of lime chloride, revealed that the best survival rate was obtained in BR11 (95%), followed by BR 12 (78%) and BR 13 (49%).

Table 7

Larvae survival in the experimental ponds, at the end of the experiment

No	Larvae survival (%)		
	BR11	BR12	BR13
1.	95	78	49

Conclusions

The prophylactic administration of lime chloride in the technological water of ponds, producing 10 days old larvae of carp, prevents massive infestations with *Trichodina spp.*, does not affect the growth rate of fish, has no negative impact on fish larvae (due to the use of one single preventive treatment) and also allows a rapid recovery of zoolpankton organisms, which are necessarry to fish development in the early life stages.

Fish larvae are not negatively affected by the chloride of lime treatment, fact confirmed by the high survival rate recorded in BR 11.

After the water desinfection treatment, the early naupliar stages of crustaceans, the unicellular ectoparasites and a number of freshly hatched organisms (example tadepole or crustacean naupliar forms) which are considered competitors in fish feed, are destroyed.

There is a necessary time for the zoolpanctonic populations to recover, which allows the fish larvae develop, until their mouth size is comparable with the dimension of the live food ingested.

According to the survival rate of the fish produced in the three experimental ponds, it can be concluded that the maximum efficiency of the treatment was recorded in BR 11, with the preventive administration on the 10 days old carp larvae of a disinfectant treatment with 10 kg $CaOCl_2$/ha, while the natural food of the ponds and its development is minimally affected.

The present study demonstrates, through the survival rates of the biological material, recorded in the three experimental ponds, the opportunity of preventive administration of the treatments for desinfection of aquaculture technological water (with eutrophication tendency), during the larval period in carp.

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ISOLATION AND CHARACTERIZATION OF SOME METHICILLIN RESISTANT *STAPHYLOCOCCUS SPP.* STRAINS ISOLATED FROM MASTITIC BOVINES

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Summary

In recent years, a special attention is paid to methicillin-resistant staphylococci strains, which have a pronounced risk and a complex epidemiological circuit, being common in humans, as well. The research was made on 20 samples of mastitic milk taken from primiparous bovines from a cattle breeding farm in Timiș county. From the taken samples, 64 strains belonging to *Staphylococcus* genus were identified and included in 19 species of staphylococci. These species isolated on selective media and definitively identified with the Vitek 2 Compact equipment were tested for resistance to methicillin. Thus, three antibiotics, namely methicillin, oxacillin and cefoxitin, were used, of which the highest resistance frequency wasto methicillin.

Keywords: methicillin, resistant, staphylococci, strains

Starting from the numerous studies conducted at national and international level to demonstrate the extent of multiple resistance to antimicrobial substances phenomenon present at various bacterial species, we consider that such research is necessary because it will generate a valuable set of data from epidemiologically point of view (2, 5, 10, 12).

Methicillin-resistant staphylococci are considered to be zoonotic risk bacteria because methicillin resistance is associated with multiple antibiotic resistance. Methicillin-resistant strains are monitored both in human and veterinary medicine and the study of the animal-human-animal circuit of these strains is a major public health concern (1, 2, 3, 7).

In cattle, staphylococcal infections are common and are represented by clinical and subclinical mastitis, laminitis or other localized infections. Mastitis are produced by both positive and negative coagulase staphylococci, with a correlation between the progressive clinical forms and the pathogenicity of the strains (5).

The research was performed in order to identify the resistance phenotypes of staphylococci strains isolated from primiparous bovines with mastitis.

Materials and methods

Pathological samples, represented by mastitic milk, were taken from primiparous cows that presented mastitis. Primary sowings were performed on

agar with defibrinated blood of 5% sheepand isolated strains were screened based on cultural, morphological and tinctorial characters. To obtain pure staphylococci cultures, the solid Chapman medium was used, while the final identification was made with the Vitek 2 Compact equipment, using identification cards for enterococci, streptococci, staphylococci, and a select group of Gram-positive organisms. Thus, of the 20 collected samples, several strains belonging to the *Staphylococcus* genus and included in different species of staphylococci were identified.

The isolated strains were tested for methicillin resistance by Kirby-Bauer disc-diffusion method using broth, Mueller-Hinton agar and three antibiotics, namely methicillin (5 µg), oxacillin (1 µg) and cefoxitin (30 µg) (Table 1), the results being interpreted according to the standards (6).

Results and discussions

The bacteriological examination, carried out according to thedescribed methodology, followed by definitive identification with the Vitek 2 Compact equipment, allowed the isolation of 64 strains, which were included in 19 species of staphylococci.

The results obtained by testing the behavior against the three β-lactams of the staphylococcal isolated strains are shown in Table 1.

Table 1

Resistance phenotypes to the three antibiotics of staphylococci strains isolated from mastitic cows

Crt. no.	Antibiotic	Antibiogram results						Total strains
		Susceptible		Intermediar		Resistant		
		No.	%	No.	%	No.	%	
1.	Cefoxitin	55	85.93	4	6.25	**5**	**7.81**	64
2.	Oxacillin	20	31.25	8	12.5	**36**	**56.25**	64
3.	Methicilin	18	28.12	9	14.06	**37**	**57.81**	64

The results show that the antibiotic resistance of the tested strains had a variable frequency ranging from 7.81% to 57.81%, the antibiotic susceptibility was between 28.12% and 85.93%, and the strains with intermediate behavior had had a frequency between 6.25% and 14.06%.

These three antibiotics used, namely methicillin, oxacillin and cefoxitin, are part of the β-lactam group and have been selected because they are antibiotics that show methicillin resistance. Oxacillin is resistant to β-lactamases, being preferred for the stability and reproducibility of the results, and since 2004, cefoxitinis also recommended, especially for the identification of *S. aureus* methicillin resistant strains.

Isolated staphylococci strains had a different behavior to **methicillin**, a commonly used antibiotic for the detection of methicillin-resistance. Thus, 57.81% of the tested strains were resistant to this antibiotic, 28.12% of the tested strains were susceptible and 14.06% had an intermediate resistance.

To determine the cross-resistance of staphylococci to the penicillins resistant to penicillinase, oxacillin is also recommended for both its stability and the reproducibility of the results. The results obtained using the oxacillin were as follows: 56.25% were resistant strains, 31.25% were susceptible strains and 12.5% had an intermediate behavior.

The increased frequency of methicillin-resistant staphylococci strains and, in particular, strains of *S. aureus subsp. aureus*, named MRSA strains (Methicillin Resistant *S. aureus*), also determined testing the resistance to cefoxitin, a much more reliable antibiotic than methicillin and oxacillin. The results obtained by testing the isolated staphylococci strains to cefoxitin were as follows: 7.81% of the strains were resistant, 85.93% of the strains were susceptible and 6.25% of the strains had an intermediate behavior (Fig. 1).

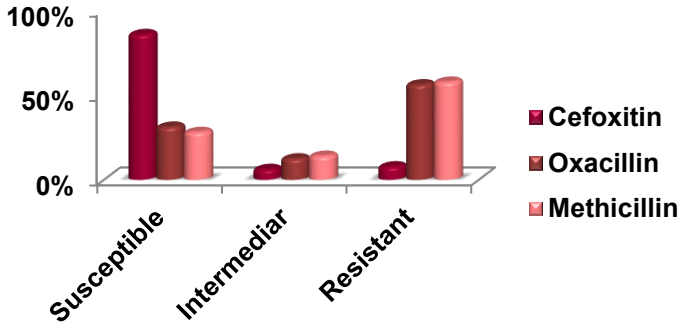

Fig.1. Frequency of resistance phenotypes of the isolated staphylococci strains

The antibiotic resistance to these three β-lactams was different, observing a correlation only between oxacillinand methicillin, whereas only 7.81% of the tested strains were resistant to cefoxitin.

In the tested staphylococci strains, the resistance to methicillin was 57.81%, demonstrating a high proportion of the strains carrying the *mec* gene encoding the resistance to this antibiotic.

Methicillin-resistant staphylococci strains are considered strains with high zoonotic risk and with a complex epidemiological circuit. The *mec* gene encoding the methicillin resistance may be transmitted by the R plasmid to methicillin-susceptible staphylococci strains (intraspecific transmission) strains, but also to

other strains of other bacterial species (interspecific transmission).The results obtained show the correlation between oxacillin and methicillin resistance, but do not prove the correlation between the resistance of these two β-lactams and cefoxitin, of which the antibiotic resistance was very low, phenomenon also reported in the literature (1, 2, 4).

The resistance phenotype to methicillin is commonly found in strains of *S. aureus subsp. aureus*, isolated from humans and animals, strains called MRSA. In recent years, however, this phenotype has been reported more commonly in other staphylococcal species pathogenic to humans and animals (4, 13).

In Brazil, in 2012, Aquino et al. (3) followed prevalence of methicillin resistant staphylococci strains to several species of animals, by taking samples of pathological material from cow milk (36), cattle meat (26), sheep (19), horse (21), pig (23), goats (23) and care staff (13). The authors isolated 161 staphylococcis trains, at which they study the antibiotic resistance against 17 antibiotics, and the *mec* gene was detected with the polymerase chain reaction. Coagulase-negative staphylococci, resistantto methicillin, had a frequency of 31%. The authors did not isolate strains of *S. aureus* but identified a complex epidemiological circuit between animals and humans of methicillin resistant coagulase-negative strains (3).

Mendonça et al. (9) in 2012, studied the resistance patterns of *Staphylococcus spp.* strains isolated from cattle by phenotypic tests (disc diffusion method) and molecular biology. The authors recommend oxacillin to determine the frequency of *Staphylococcus spp.* methicillin resistant strains, considering this method as a standard method, as well as the PCR test for the detection of the *mec*A, *mec*I and *mec*RI genes and the *bla*Z gene.Based on the results obtained, the cited authors found a close correlation between the disk diffusion method with oxacillin and the presence of the mentioned genes (9).

In 2012, in Germany, Fessler et al. (7) have studied the prevalence of MRSA strains isolated from animals and humans. Following the bacteriological examination, 125 MRSA strains were isolated as follows: 46 strains from milk samples, 24 strains from dairy cows, 7 strains from calf, 16 strains from pigs, two strains from dogs and 28 strains from horses, sheep and humans. All strains were included in the CC398 gene line, and the SCC*mec* 4 cassette was detected by the polymerase chain reaction for 48 strains and the SCC*mec* type 5 cassette for 67 strains. Based on the results, the authors believe that there is a complex epidemiological circuit of the MRSA strains among the investigated animal species and humans that can generate pronounced zoonotic risk infections (7).

Bardiau et al. (4), in 2013, in Belgium, studied the frequency of methicillin-resistant strains at a number of 430 staphylococci strains isolated of from cows with clinical and subclinical mastitis. Phenotypic and genotypic tests performed allowed the identification of 19 strains of *S. aureus subsp. aureus* methicillin-resistant. Also, the ST398LA-MRSA clone, considered emerging and with zoonotic risk, was identified by the PCR technique and the pulse field macro-restriction technique (4).

Schlotter et al. (11), in 2013, bacteriologically examined 10421 cattle from 34 farms, being able to isolate 1902 strains of *S. aureus subsp. aureus*. These strains were tested for antibiotic resistance by disc-diffusion method and were tested by PCR for the detection of the genes responsible for coding this phenomenon. A number of 135 strains did not have resistance genes, and at the other strains the *mec*A, *erm*A, *erm*B, *erm*C and *msr*A genes had a variable frequency. The authors believe that *mec*A gene detection is necessary to identify the methicillin-resistant *S. aureus subsp. aureus* strains (11).

In 2017, Mello et al. (8) studied the frequency of resistance to oxacillin and vancomycin in a number of 181 strains of *Staphylococcus spp.*, isolated from subclinical mastitis, as well as the identification of heteroresistance to vancomycin by a screeching method. The authors also identified the *mec*A and *mec*C genes. The results were as follows: 18.2% of the strains were oxacillin-resistant, all isolated strains were vancomycin-susceptible, while the heteroresistance was observed in 13 strains and the *mec*A gene was identified in 8 strains, all included in *S. epidermidis* species. Based on the results obtained, the authors state that it is necessary to evaluate the strains isolated from cows with mastitis from bacteriologically and molecularly point of view, since the presence of *mec*A gene in *S. epidermidis* demonstrates that the cow's milk can be a carrier of resistant strains of human origin, highlighting the epidemiological circuit of these strains (8).

The results obtained confirm the data in the literature on the frequency of resistance patterns of the three resistant beta-lactams and, in particular, the frequency and zoonotic risk of MRSA strains.

Conclusions

The results obtained on resistance phenotypes to three beta-lactams, resistant to penicillinase, have revealed a different frequency of the strains resistant to these three antibiotics.

Testing for resistance to oxacillin detected a higher frequency compared to methicillin resistance testing.

Testing for cefoxitin by the disc-diffusion method revealed a frequency of 7.81% of resistant strains, strains that can be considered MRSA strains.

At the isolated strains, the resistance to methicillin and oxacillin was similar, revealing a high frequency of strains carrying the *mec* gene.

Acknowledgement

The research was carried out in the frame of the *project Bioeconomic approach to antimicrobial agents - use and resistance,* financed by UEFISCDI by contract no. 7PCCDI / 2018, cod PN-III-P1-1.2-PCCDI-2017-0361.

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BIOLOGICAL ACTIVITY OF ARISTOLOCHIA LONGA L. AGAINST SOME PATHOGENIC BACTERIA AND PHYTOCHEMICAL SCREENING

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Summary

This work aims the phytochemical study and biological activity of methanolic, ethanolic and aqueous extracts of *Aristolochia longa* L stems and leaves. Polyphenols were estimated by spectrophotometer and antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl while activity against *Micrococcus luteus* ATCC 14452, *Bacillus subtilus* ATCC 6633, *Staphylococcus aureus* ATCC 43300 and *Escherichia coli* ATCC 25922 was realized by disc impregnation.

The rates of polyphenols (39.01mg.ml^{-1}, 38.25 mg.ml $^{-1}$and 33.78mg.ml^{-1}) and flavonoides (21.56 mg.ml^{-1}; 27.86 mg.ml^{-1} and 28.22 mg.ml^{-1}) for methanolic, ethanolic and aqueous extracts respectively. The antioxidant activity of methanolic, ethanolic and aqueous extracts was 443 µg.ml^{-1}, 860 µg.ml^{-1} and 998 µg.ml^{-1} respectively.

The most significant antibacterial activity in the form of inhibition diameter was observed with methanolic extracts on *B. subtilis* (11.05 mm) and *M. luteus* (9.08 mm). Ethanolic extracts was 12.91 mm on *B. subtilus* and 10.75 mm against *M. luteus* and finally 10.93 mm on *S. aureus*.

Keywords: *Aristolochia longa* L., antioxidant, polyphenols, flavonoids, antibacterial

Conventional antibiotic medications are becoming unable to inhibit pathogenic bacteria that have developed resistance to these antibiotics, threatening the survival of all humanity. To palliate this growing threat, research is focusing on medicinal plants as the main resources of therapeutic agents. In addition to their use in health care by about 80% of the world's population (19), these plants are used in the pharmaceutical, cosmetic, health and nutritional industries (23). Indeed, they are sources of a variety of biologically active compounds; phenolic compounds with antioxidant biological properties and antimicrobial potential (13).

Aristolochia longa L. is a perennial plant with heart-shaped leaves and yellow-labeled flowers, native to central and southern Europe and widespread throughout southwest Asia. This plant is used in traditional pharmacopoeia in the Western Algerian region to treat several diseases. Its roots can inhibit breast cancer in postmenopausal women (3), and pathogenic bacteria due to their richness in bioactive compounds (4, 15).

This work involved the phytochemical study of *Aristolochia longa* L. and its biological effect including the determination of polyphenols, flavonoids and an in vitro study of antioxidant and antibacterial activity against *Staphylococccusaureus*, *Escherichia coli*, *Bacillus subtilus* and *Micrococcus luteus* by the disc impregnation method.

Materials and Methods

Plant material and extracts preparation

The aerial parts (leaves and stems) of *Aristolochia longa*were collected in April 2017 in the region of Tiaret (Algeria) and the identification of the species was performed by the botany laboratory of higher national school of agronomy (Alger). They were then washed and dried in the fresh air, crushed with an electric shredder and stored at room temperature, away from humidity and light. The extracts were prepared by maceration (11), with a ratio of 5 g of powder mixed with 50 ml of the solvent (methanol, ethanol and aqueous) under continuous agitation for 24 hours at room temperature. Then the mixture was filtered on filter paper and the filtrate was evaporated and dried at 40°C.

Phytochemical screening

- **Alkaloids**: Bouchardat test: 2 ml of an extract solution with 2 drops of Bouchardat reagent added. A positive result is revealed by a reddish brown precipitation (26).

Wagner test: The crude extract was mixed with 2 ml of the Wagner reagent. A reddish brown precipitate indicates the presence of alkaloids (1).

- **Tannins**: highlighted by adding 1 to 2 drops of 0.1% diluted ferric chloride solution ($FeCl_3$) to 2 ml of each extract. A dark green color indicates the presence of catechic tannins while blue-green indicates the presence of gallic tannins (12).
- **Steroids**: The presence of steroids was detected by adding 5 ml of the extract to 5 ml of acetic anhydride to which 0.5 ml of concentrated H_2SO_4 is added. The appearance of a purple coloration that turns blue and then green indicates a positive reaction (12).
- **Saponins**: introduce 10 ml of each of the extracts into a test tube. The tube is stirred for 15 S and then left to stand for 15 min. A persistent foam height greater than 1cm indicates the presence of saponosides (positive reaction) (6).
- **Terpenoid** (salkowski test): 0.2 g of dry extract is mixed with 2 ml of chloroform and then 3 ml of concentrated sulphuric acid are carefully added to form a layer. The presence of terpenoid gives a rusty coloration of the interface (16).
- **Cardiac glycosides**: 2 ml of each extract was dissolved with 2 ml of chloroform, and then 3 ml of concentrated sulfuric acid was carefully added to form a dark red to brownish layer, at the interface of the ring indicates the presence of cardiac glycosides (26).

● **Anthocyanins**: 1 ml of each extract was added to 3 ml H_2SO_4 at (10%) and 1 ml NH_4OH (10%). If the coloration increases by acidification and then turns blue in a basic medium, the result was considered positive.

● **Flavonoids:** A few drops of lead acetate solution were added to 3 ml of extract. The formation of yellow precipitate indicates the presence of flavonoids (29).

● **Mucilages**: 1ml of extract was added to 5 ml of absolute ethanol. The appearance of a flaky precipitate indicates the presence of mucilage (18).

Determination of polyphenols and flavonoids

Polyphenols are determined by the Ciocalteufolin method based on electron transfer (24). 0.2 ml of different dilutions (1mg.ml^{-1} to 0.125mg.ml^{-1}) of the extracts was mixed with 1 ml of freshly prepared Ciocalteu foliar reagent (10%) and 0.8 ml of 7.5% sodium carbonate (Na_2 CO_3). The mixture was incubated for 30 min at room temperature and then read against a blank at a wavelength of 765 nm (25).

For flavonoids, 0.5 ml of each extract was added to 1.5 ml of methanol (95%), 100 µl of aluminium chloride ($AlCl_3$) at 10% (m/v) plus 100 µl of sodium acetate (1 M) and 2.8 ml of distilled water. The mixture was agitated and incubated in the dark and at ambient temperature for 30 minutes. The blank was achieved by replacing the extract with 95% methanol and the absorbance was performed at 415 nm using a spectrophotometer. The results were expressed in mg quercetin equivalent/g dry plant matter using the quercetin calibration curve (8).

Antioxidant activity

Antioxidant activity of Aristolochia longa extracts has been achieved by the DPPH (2,2-diphenyl-1-picrylhydrazyl) method described by Braca et al. (7) with some modifications: an equal volume of the different dilutions (from 0.009mg.ml^{-1} to 5 mg.ml^{-1}) of the methanolic, ethanolic and aqueous extracts of *Aristolochia longa* was mixed with a volume of methanolic solution of 0.004% (m/v) DPPH. Then incubation for 30 min at room temperature before reading with at ʎ =517 nm. Percentage of inhibition is calculated by the following equation: $\% \text{ Inhibition} = \frac{\text{Ac} - \text{Ae}}{\text{Ac}} x100.$

Where Ac: absorbance of the control solution which contains an equal volume of methanolic solution of DPPH and methanol, Ae: extract absorbance.

Antibacterial activity

The antibacterial power of *Aristolochia longa* plant extracts was evaluated against four referenced bacteria: *Micrococcus luteus* ATCC 14452, *Bacillus subtilus* ATCC 6633 and *Staphylococcus aureus* ATCC 43300 and Gram negative *Escherichia coli* ATCC 25922 by the disc impregnation method (2) at a concentration of 20mg.ml^{-1}. The Protocol followed is the one described by (14).

Results and discussions

Phytochemical screening

The obtained phytochemical results revealed the presence of tannins, flavonoids, terpenoids, mucilages, alkaloids, cardiac glycosides and the absence of steroids, anthocyanins and saponins (Table 1).

Table 1

Phytochemical screening results for methanolic, ethanolic and aqueous extracts of *Aristolochia longa*

Test	Methanolic extract	Ethanolic extract	Aqueous extract
Alkaloids W	P	P	N
B	P	P	N
Tanins	P	P	N
Steroids	N	P	N
Saponins	N	N	N
Terpenoid	N	P	P
Cardiac glycosides	P	P	P
Anthocyanins	N	N	N
flavonoids	P	P	N
Mucilage	N	P	P

W: Wagnertest, **B**: Bouchardat test, **P**: Positive result, **N**: Negative result.

These results are consistent with several studies; they state that phytochemical screening of aqueous extract of *Aristolochia longa* revealed the presence of polyphenols, flavonoids and saponins and the absence of alkaloids (3).

Other studies have shown the presence of alkaloids, glycosides, phenolic compounds, flavonoids, terpenoids and saponins in *Aristolochia longa* extracts (9).

In addition, others have published that phytochemical screening of *Aristolochia bracteata* reveals the presence of alkaloids, flavonoids, cardiac glycosides and tannins and other compounds such as carbohydrates, proteins and steroids and sterols (5, 10, 28).

Polyphenols and flavonoids

The dosage of polyphenols in methanolic, ethanolic and aqueous extracts of *Aristolochia longa* is 33.01 ±0.19; 38.25 ±0.33 and 33.78 ± 0.38 E AG.g^{-1} and for flavonoids 21.56± 0.075 ; 27.86± 0.19 and 28.22 ±0.15 mg EQ.g^{-1} respectively).

These results are corroborated by several studies that have demonstrated the richness of *Aristolochia longa* extracts in phenolic compounds, as is the case with the studies published by Benarba et al. (3), reveal that the aqueous extract of

Aristolochia longa L. roots contains polyphenols and flavonoids. In addition, other work shows that the aerial part of aqueous extract rich in polyphenols carries methanolic extract and flavonoids (15).

Secondary metabolite products that are found in many foods and have a beneficial effect on human health by protecting against various diseases such as diabetes, infections and cancer due to their antioxidant, anti-inflammatory and anticancerous effects (20, 21).

Antioxidant activity

Antioxidants are molecules involved in the body's protection mechanism against pathologies associated with free radical attack (22).

Screening for antioxidant activity results shows the ability of the different extracts of *Aristolochia longa* to trap free radicals in which IC 50 of 0.86 mg.ml^{-1}, 0.44 ± 0.005mg. ml^{-1}and 0.99 ± 0.05 mg. ml $^{-1}$of ethanolic, methanolic and aqueous extracts respectively.

Solvents used for antioxidant extraction play an important role in determining the sweeping capacity of DPPH for extracts (30).

Antibacterial activity

The antibacterial activity of methanolic extract on *B. subtilis, M. luteus, S. aureus* (11.05 mm, 9.08 mm, 3.11 mm inhibition diameter respectively). The activity of their ethanol extracts was 12.91 mm on *B. subtilus* and 10.75 mm against *M. luteus* and finally 10.93 mm on *S. aureus* (Fig. 1).

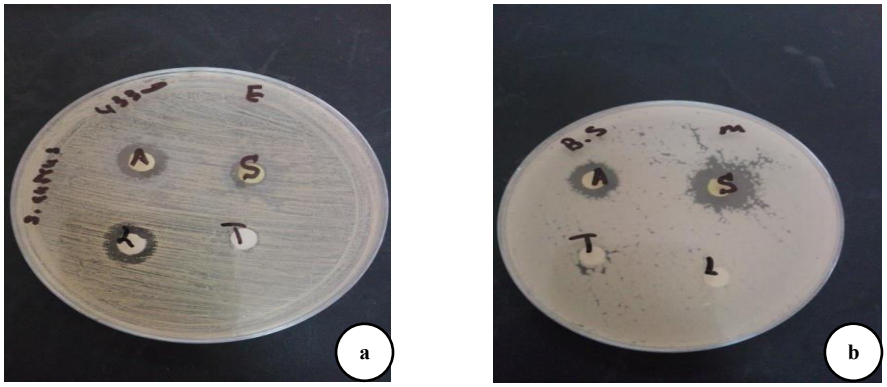

Fig. 1. (a) Antibacterial activity of *Aristolochia longa* ethanolic extract against *Staphylococcus aureus* ATCC 43300, (b) Antibacterial activity of *Aristolochia longa* methanolic extract against *Bacillus subtilus* ATCC 6633

The ethanolic extract of *Aristolochia longa* had a high inhibition power against the four germs tested. This is in line with the results reported by Surendra Kumar et al. (27) on the ethanolic extract of *Aristolochia Indica*. Indeed, it has been demonstrated that the different extracts of *A. Indica* have antibacterial activity

against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* (17).

This antibacterial activity can be explained by the richness of *Aristolochia longa* in bioactive compounds. Plants containing tannins, alkaloids, flavonoids and glycosides have a broad spectrum of antimicrobial activity (4).

In this context, studies prove the biological activity of *Aristolochia longa* extracts and their richness in bioactive compounds, demonstrating that they have good antibacterial activity against Gram positive and Gram-negative bacteria (15).

Conclusion

The results show the richness of the various extracts of the aerial part of *Aristolochia longa* in bioactive compounds including tannins, terpenoids, mucilages, anthocyanins, alkaloids, cardiac glycosides, polyphenols and flavonoids. The latter play also a major role in antioxidant activity with the inhibition of pathogenic bacteria, which gives the possibility to use this plant as natural medication for the treatment of various diseases related to oxidative stress and indeed as effective natural antibiotics against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilus* and *Micrococcus luteus*.

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VALIDATION STUDIES FOR DETERMINATION OF NITROGENPROTEIN FROM BIOLOGICAL PRODUCTS BY KJELDAHL METHOD

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Summary

Nitrogen / protein from tuberculin was determined by the Kjeldahl method following the steps: mineralization of proteins, release of ammonia by decomposition of ammonium sulphate, distillation of ammonia, dosage of nitrogen / protein. Validation studies go through analytical requirements such as repeatability or intra-test accuracy, reproducibility or inter-test accuracy, the accuracy of the method by reference material, the method uncertainty and the value range of the method. The coefficient of variability of the performed determinations was 4.15%, reproducibility limit r was 1.34 and the range of values was: 1.60-1.94 = 0.34 <1.34%. Accuracy was 107.1% and Bias was 7.1%. The sensitivity limit of the method was 0.14 mg N / ml and the protein concentration of the tuberculin tested was determined with an uncertainty of ± 25%. The nitrogen / protein dosing method has been validated according to the demonstration parameters and is appropriate for the intended purpose.

Keywords: nitrogen, validation, uncertainty

Nitrogen is one of the five major elements found in organic materials such as protein. This fact was recognized by a Danish chemist, Johan Kjeldahl, who used it as a method of determining the amount of protein in samples taken from a wide variety of organisms. In 1883 Kjeldahl presented to the Danish Chemical Society a method (much revised since his day) for determining the amount of nitrogen in mixtures of substances containing ammonium salts, nitrate, or organic nitrogen compounds (1, 2, 5).

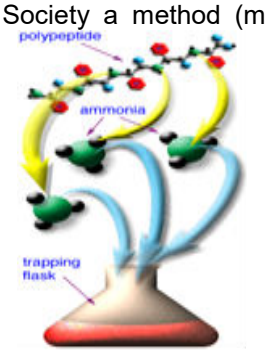


Fig. 1.The Kjeldahl method

The central basis used in this procedure is the oxidation of the organic compound using strong sulfuric acid. As the organic material is oxidized the carbon it contains is converted to carbon dioxide and the hydrogen is converted into water.

The nitrogen, from the amine groups found in the peptide bonds of the polypeptide chains, is converted to ammonium ion, which dissolves in the oxidizing solution, and can later be converted to ammonia gas (5).

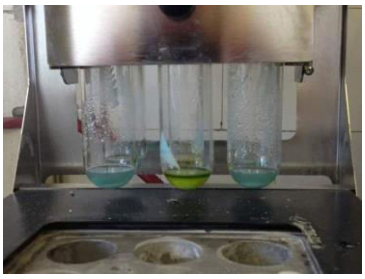
Materials and methods

The Kjeldahl method consists of four steps:

1. Precipitation of proteins in the biological product

Pipette 2 ml of sample and 2.5 ml of 40% trichloroacetic acid into centrifuge cups. Stir well and leave to cool for 4h for complete precipitation.
Centrifuge for 30 minutes at 3000-4000 rpm.
Carefully decant the supernatant and dissolve the precipitate with 0.5 ml of 5N NaOH solution.
After the complete dissolution of the precipitate is transfused into a 200 ml mineralization vial. The cup is washed seven times with a minimum amount of distilled water.

2. Mineralization of proteins



In the sample mineralization flask add 5 ml of concentrated H2SO4 and about 3 g of the catalyst mixture (dipotassium sulfate and copper sulphate pentahydrate in the ratio of 5: 1). Place the vial on mineralization in the Velp digester for 23 minutes at 420ºC (Fig. 2).

Fig. 2. Mineralization of proteins in the Velp digester

Fig. 3. Distillation of ammonia in the Velp distiller

3. Release of ammonia by ammonium sulfate decomposition and ammonia distillation

The release of ammonia by decomposing ammonium sulfate and distilling ammonia is done in the VELP Distiller. Place the distilled water to a volume of 30 ml in the mineralized product vial, then distill it with 60 ml 33% NaOH.
The distillate is taken up in 20 ml H_2SO_4 N / 50 and 0.3 ml Tashiro indicator (0.2 g of methyl red and 0.1 g of methylene blue were mixed and brought to a 100 ml volumetric flask with 96% ethyl alcohol p.a.).

4. Dose of ammonia

The unreacted excess of H_2SO_4 N / 50 with ammonia is titrated with NaOH solution N / 50 until blue coloration (Fig. 4).

Fig. 4. Titration

The difference between the number of ml H_2SO_4 N / 50 initially set and those found by titration with NaOH solution N / 50 is the number of ml of sulfuric acid reacted with ammonia.

Calculation of results

1 ml of H_2SO_4 N / 50 corresponds to 0.28 mg of N_2.

Knowing the volume of H_2SO_4 N / 50 that reacted with ammonia by multiplying by 0.28, we find out the amount of nitrogen in the biological product, expressed in mg / ml.

Protein nitrogen is thought to be on average 16%.

mg protein/ml sample = (V1F1 - V2F2) x 0.28 x 6.25/2

V1 = the volume of H2SO4 N / 50 taken in work, ml

F1 = solution factor H2SO4 N / 50

V2 = volume of NaOH N / 50 used for titration, ml

F2 = factor of NaOH solution N / 50 (3).

Results and discussions

Performance parameters were determined:

- repeatability or intra-test accuracy,
- reproducibility or inter-test accuracy,
- the accuracy by analyzing the reference material (tryptophan),
- the uncertainty of the method,
- the value range of the method (4).

Following total protein testing, repeatedly from 6 samples of Tuberculin A. The coefficient of variability of the determinations carried out was 4.15%, which is below the reference level, of 10%. The repeatability limit, r = 0.3%+0.008 x 1.83= 0.314.

Highest difference between results = 0.31%. Range: 1.75-1.92 = 0.17 <0.31%.

Following total protein testing, repeating 6 samples of the same Tuberculin A sample by two different analysts at one day interval. The coefficient of variability of the determinations performed was 0.10%, values that are below the reference level, of 10%. The repeatability limit r = 1.3+ 0.027 x 1.82= 1.34. Highest difference between results = 0.34%. Range: 1.60-1.94 = 0.34 <1.34%.

A test of 99% tryptophan, lot 10182432, with a certified nitrogen content of 13.7 g% was tested 6 times. The results were obtained: Accuracy%= (2.142/2)*100

= 107.1, Bias%=[(2.142-2) /2] * 100= 7.1. It is found that the bias value is in the range of ± 10% and the accuracy within the range of 80-120%.
According to the Ishikawa diagram, the formula for calculating uncertainty is:

$$U=\sqrt{U^2_{reproducibility} + U_{BIAS}{}^2 + U^2_{volumemasurement} + U^2_{weighing}}$$

Utemp=10*(± 4°C)*2.1*10-4=0.0084. The resulting uncertainty about rectangular temperature distribution: 0.0084/$\sqrt{6}$ =0.00343

U_1 volume measurement = 0.029 ml

Relativized value: U_1 volume measurement /V = 0.029/10=**0.0029**

Upipette = 0.03/$\sqrt{3}$ = 0.0173 ml

Utemp=10*(±4°C)*2.1*10-4=0.0084. The resulting uncertainty about the rectangular temperature distribution: 0.0084/$\sqrt{6}$ = 0.00343

$U_{2\ volumemeasurement}$ = **0.018 ml**

Relativized value: $U_{2\ volumemeasurement}$ /V= 0.018/5 = **0.0035**

U burette =0.03/$\sqrt{6}$ = 0.0122 ml.

U_{temp}=18*(± 4°C)*2.1*10^{-4}= 0.00151.
The resulting uncertainty about rectangular temperature distribution: 0.00151/$\sqrt{6}$ =0.0061

$U_{3\ volumemeasurement}$ = 0.0133 ml

Relativized value: $U_{3\ volumemeasurement}$ /V= 0.0133/18= ***0.00073***

$U_{weighing}$ =0.10/$\sqrt{3}$ = 0.0578 mg.

This value is taken into account twice (bringing to 0 and weighing).

$U_{weighing}$ = 0.0817

Relativized value: $U_{weighing}$/V = 0.0817/3000 = ***0.00002***
The compound uncertainty, U_c:
U_c = 0.125 ***= 12.5 %***

And the expanded uncertainty is: $U_{expanded}$ = k x U_C, where for k = 2 there is a confidence level of 95%.

$$U_{expanded} = 2 \times 12.5\ \% = 25\ \%$$

Thus, the value of the protein concentration of the tested tuberculin sample with the associated expanded uncertainty is: 1.82 ± 0.45 or the uncertainty is ± 25%.

Method Value Range

The protein of Tuberculin A, with a content of 1.82 mg/ml protein and 0.29 mg N/ml, was analyzed. The Tuberculin sample was diluted 2 times, the nitrogen content being 0.14 mg N/ml and a tryptophan solution (reference material) at a concentration of 0.14 mg N/ml (Table 1, Table 2).

The sensitivity limit of the method is 0.14 mg N/ml.

Method sensitivity limit

Table 1

Nitrogen dosing mg/ml, MRC: Tryptophan, 99%, batch 10182432/expire 09.2016

ml NaOH 0.02N	nitrogen concentration, mg/l
18.20	0.50
18.50	0.40
18.90	0.30
19.30	0.20
19.50	0.14
19.50	0.14

Table 2

Nitrogen dosing mg/ml, Tuberculin A lot 321, dilution ½

ml NaOH 0.02N	nitrogen concentration, mg/l
19.00	0.14
18.90	0.15
19.00	0.14
18.90	0.15
19.00	0.14
19.00	0.14

Method sensitivity limit: 0.14 mg N/ml

Calibration results for tuberculin A with L-Tryptophan, 99% are shown in table 3.

Table 3

Calibration with MRC (L-Tryptophan, 99%)

sample number	L-Tryptophan 0.0148 g/ml (ml)	nitrogen (mg/ml)	ml NaOH 0.02 N (ml)	nitrogen (mg/ml)
1	1	2	12.30	2.156
2	1	2	12.40	2.128
3	1	2	12.20	2.184
4	1	2	12.50	2.100
5	1	2	12.30	2.156

Conclusions

The method for nitrogen / crude protein dosing is validated according to the demonstration parameters and is appropriate for the intended purpose.

Today, various scientific associations approve the Kjeldahl method, including the AOAC International (Association of Official Analytical Chemists), AACC (Association of American Cereal Chemists), AOCS (American Oil Chemists Society), EPA (Environmental Protection Agency), ISO (International Standards Organization), and many others. All VELP Scientifica equipment for Kjeldahl nitrogen determination work in accordance with the above-mentioned associations (6).

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RESISTANCE PROFILE SCREENING IN PIG FARMS IN WESTERN ROMANIA

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Summary

Antibiotic resistance is no longer a medical novelty, being heavily studied worldwide by numerous agencies in charge of monitoring, evaluating and implementingprocedures aimed to diminish the effects of this phenomenon in order topreserve the public health. The intense use of antimicrobial agents, as means of preventing or treating colibacili determine more and more frequently the emergence of the antibiotic-resistant strains in swine species. In this respect, the present study intends to follow the evolution of resistance intwo counties from the Western part of Romania, in ten great swine units on piglets, at the weaning age category. After the diagnosis confirmation, by morphopathologic means, the identification and isolation of the etiological agents were followed by the classical methods known in microbiology. Of a total of samples (no. 167), we found pathogenically positive 75.44%,being also identified hemolytic strains (10.77%), and 13.79% negative samples. The (non-hemolytic) positive samples were tested by the Kirby-Bauer disk-diffusion test, where 11 antibiotics were used, and the obtained data were compared with the CLSI / 2009 standard. Results revealed comparatively a diverse evolution of the resistance to the tested antibiotics in both counties visited, probably due to the extensive antibiotherapy applied in these units. The resistance value of the antimicrobial structuresevaluated was relatively similar: in Arad County increased values of resistance to lincospectin and doxycycline were reportedand in Timis County to neomycin and respectively amoxicillin / clavulanicacid, confirming the insidious evolution of this phenomenon in the Romanian swine farms.

Keywords: antibiotic resistance, evolution, swine units

Digestive disorders caused by a series of commensal or pathogenic bacteria can cause symptoms, which have a major impact on growth, meat's quality and decreasing economic profit.

The genus Escherichia is the one that plays an important role in the development of enteric syndromes. Worldwide, these bacteria produce major losses on farms, the percentage of the death being influenced by current legislation, which prohibits the usage of growth promoters in food.

In the current context, particular attention must be paid to youth after weaning, due to the factors which can appear on the individuals by overloading the body with new feed conditions, changing intestinal flora and decreasing immune status.

Thus, functional imbalances could appear by the emergence of pathological processes, an example could be *E. coli* strains in the small intestine at

the piglets (weaning diarrhea). The magnitude of casuistry with diarrheal manifestations in farms has attracted the attention of many researchers, who claim that these diseases are caused by pathogenic bacteria with multiple antibiotic resistance.

The purpose of the present research was to highlight some specific aspects and etiopathogenesis of enteritis around the weaning age of the pig in the breeding units located in the western part of Romania.

Of all pathogens, *E. coli* is an important factor in the appearance of the enteric syndrome, producing significant material damage by decreased productivity, associated with the increasing cost of production and low profitability index.

In order to achieve the proposed goal, the following objectives were addressed: the isolation and identification of *E. coli* which cause enteritis at pigs after weaning until slaughter age and as well as describing the susceptibility and resistance profile of antimicrobial substances of isolated strains as it follows:

• isolation of *E. coli* strains and gender-based biochemical properties;

• assessing the susceptibility and resistance profile of the various antimicrobial agents of isolated *E. coli* strains;

• statistical interpretation of the results with the Anova program (t-test).

Materials and methods

The experiment took part in the most important ten pig breeding and fattening farms in the western part of Romania. The units are constituted as an intensive growth system and structured by age and weight categories. This area of Romania has experienced a strong expansion in the agro-food industry over the past decade, due to its geo-strategic position towards Western Europe, with the benefit of intra-community exchange.

After anatomopathological examination, hemorrhagic injuries were found in focal areas located in the small intestine, predominantly in the anterior third, along the entire duodenum tract and a small part of the jejunum (Fig. 1). In addition to hemorrhagic chromatic changes, can be observed the intestinal distension by gas accumulation.

Identification of the etiologic agent

From the biological material, with the help of a Pasteur pipette, samples have been collected, which were later introduced in bullion, and from there with the help of the Drigalsky loop the samples were moved to nutritive agar cast in Petri dishes. For the best growth of microorganisms, the samples needed controlled temperature for 24 hours at 37 Celsius degrees (Fig. 2, 3).

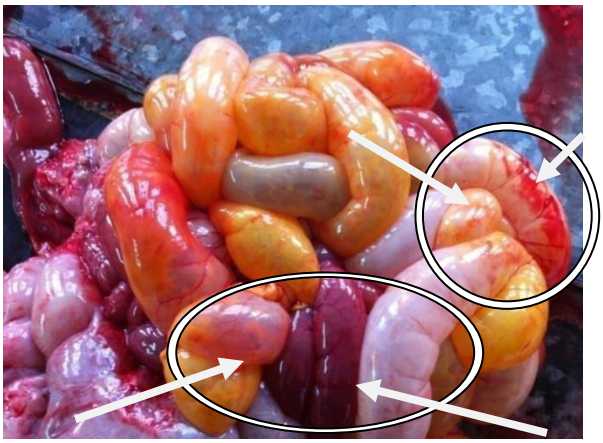

Fig. 1. Haemorrhagic duodenal-jejunitis inn outbreaks with intestinal distension with gas accumulation(Original by Doma)

Fig. 2. Incubation of the samples (Original by Doma)

Fig. 3. *E.coli* cultures identification on selective Levin medium (Original by Doma)

For our study, we have chosen 10 animal raising units from the counties Timis and Arad. In the time period September 2011- November 2014 we examined females with cocobacilarenteritis. The concrete diagnosis was put based on certain symptoms gathered from morphopatological examinations and laboratory exams in which we identified the pathogenic agent.

From the 167 biological samples (sections from the small intestine, liver, kidneys, and lungs) examined, 75.44% turned out positive, from them a really small number of hemolytic routs, 10.77%. The rest of 13.79% turned out negative which makes us confirm that those cases have passed from a different nature than bacteria.

In this study we have taken into consideration only the positive non-hemolytic samples tested with the disk-difusimetric Kirby-Bauer method, which has as the main function principle the diffusion of a substance in a solid medium. Around the tablet, with the active solution it is created a well which is measured with the help of a caliper or a ruler, and the result is compared with the interpretative chart CLSI/2009 (Clinical Laboratory Standard Institute USA/2009).

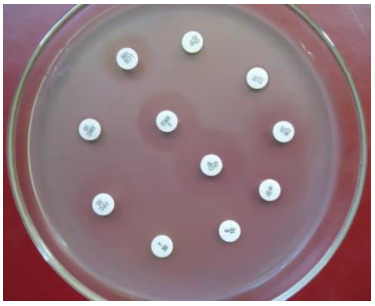

Fig. 4. Disk-Difusimetric Kirby-Bauer method (Original by Doma)

The results are placed in three separated categories:

- sensitive (susceptible-S),
- mild sensitive (intermediate -I),
- resistant(R),
- non-susceptible.

To determine the efficiency/resistance of a bacteriostatic and bactericidal substance we chose 11 antibiotics from Table 1:

Table 1

Commercial disks for antibiogram are from the Oxoid (UK) firm

DO	Doxycycline **- 30µg**
LCS	Lincospectin –109 µg
CT	Colistin – 25 µg
CN	Gentamicin – 10 µg
N	Neomycin –10 µg
CIP	Ciprofloxacin – 30 µg
ENR	Enrofloxacin –10 µg
AMC	Amoxicillin/ Clavulanic Acid – 30 µg
FFC	Florfenicol –30 µg
T	Tetracyclin – 30 µg
E	Eritromicin – 15 µg

In Table 2 is structured the technique used in the isolation and identification of the *E.coli*colonies.

Table 2

Work technique for *E.coli* strains identifications

Nr. Wcrt	Stage	Procedure
1	Bacterial isolation	- confirming the existence of *E.coli* strains; - isolating the colonies(picture 99 & picture 100).
2	Preparing the inoculum.	- the colonies are dissolving in 5 ml of bullion; - incubation at 37 Celsius degrees for 24 hours; - mixing; - adjusting turbidity with standard solution 0.5 McFarland (1,5 x 108 CFU/ml).
3	Insemination of research stems	- on Muller-Hinton agar, using Petri plates - releasing with the help of a sterile cotton-wool pad, successive in 3 directions for a better and a more uniform diffusion - for a better absorption, the plates will be exposed under a source of heat for 10-15 minutes.
4	Application of microcomprimates	- using a plier - the distance between comprimates is approximately 30 mm - the distance between comprimates and the edge of the plate is approximately 15 mm
5	Incubation	- aerobic environment - the temperature of exactly 37C for 24 hours
6	Reading and formulating the results	- using a ruler or a slide ruler - the measuring of the inhibition diameter is made from different directions for 2 up to 3 times - reporting the measurements to CLSI/2009

Fig. 5. Growing bacteria in test tubes with nutrient agar

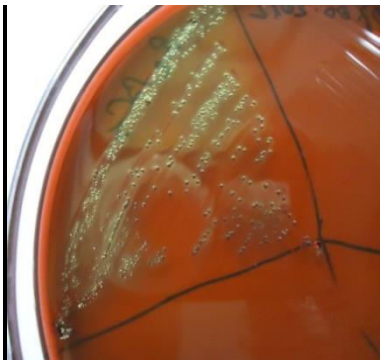

Fig. 6. The microbiological aspect of *E.coli* culture on the selective environment

Results and discussions

Today, the phenomenon of antibiotic resistance does not represent anything new, compared with the last 50 years of the past century, when it was signaled for the first time. There are national and international agencies which is the main scope is to monitor the use of antimicrobial, evaluate the rates of resistance for mating animals and implement measures to mitigate this phenomenon, to maintain public health (1).

The gusty use of substances with antimicrobial basis as a way of preventing colibacilar enteritis has determined the appearance of medicine-resistant stems. This phenomenon continues to expand both locally and globally, so the researcher's attention is turned to implementing some alternative measures to control the disease (3, 6).

The evolution of the resistance phenomenon from the two counties (Timis-84 positive samples taken from 7 farms and Arad- 42 positive samples taken from three farms) of *E.coli* stems isolated from piglets, with ages around ablactation period, compared to the 11 antibiotics used, can be observed in the following figures (Fig.7, 8).

For many researchers, the resistance phenomenon of*E.coli*stems is still an interesting subject, as it is enigmatic.

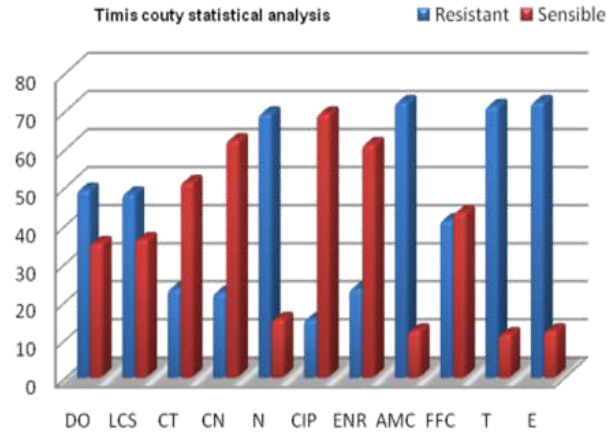

Fig. 7. Statistical interpretation of the results from Timis county through ANOVA program (t-test - P <0.001)

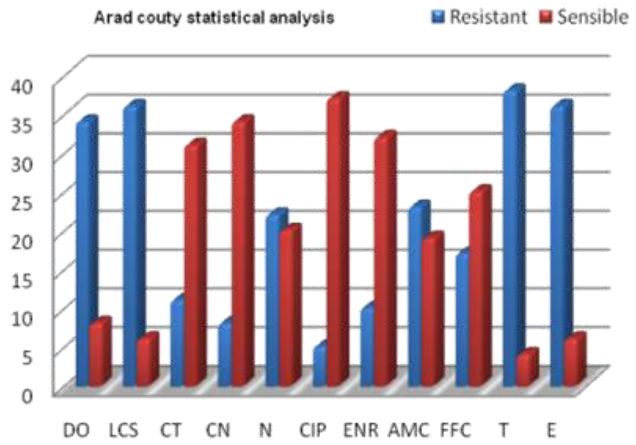

Fig. 8. Statistical interpretation of the results from Arad county through ANOVA program(t-test - P <0.001)

Also, in Croatia, following an experiment, which took part in 8 farms (5), the rising evolution of oxytetracycline, streptomycin and ampicillin resistance was demonstrated. In Romania, following a similar experiment (8) the researchers, through lab exams, claim that they highlighted the resistance of the *E.coli*stem for enrofloxacin, streptomycin, tetracycline, and amoxicillin/clavulanic acid.

Other researchers have highlighted that the feeding of the livestock and elaborating rations based on age category, by lowering the pH with organic acids and adding zinc oxide or peptides, likecolicinE1, can decrease the incidence of ablactation diarrhea (2, 4, 9).

Some literature authors claim that the evolution of antibiotic resistance depends on how often the antibiotics are used (3, 7).

Conclusions

Comparative results of antibiogram have highlighted a different evolution of the resistance phenomenon in Arad and Timis counties, therefore:

In Arad county, the high values of tetracyclin and lincospectin resistance has been highlighted.

In Timiscounty, the values were raised compared to tetracycline, and amoxicillin/clavulanic acid treatment.

The evolution of antibiotic resistance depends on the location of the farms and therapeutic management.

Acknowledgements

Activities under this work were carried out in the frame of project: A bio-economical approach of the antimicrobial agents - use and resistance, code: PN-III P1-1.2-FPRD-2017 **(BioAMR**).

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RESEARCHES REGARDING THE FEEDING OF THE BLACK GOAT *(RUPICAPRA RUPICAPRA)*

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Summary

Some areas of Retezat National Park in which wild goat populations live were considered. Medium samples of natural fodder entering in the regular menu of these animals (grass, leaves, moss) and fecal samples were collected for each region separately. The analysis of the raw chemical composition of the feed showed that there can be considerable differences between the same feed assortments, in the range of 11.34% and 60.38%, depending on the area of origin. It was found that there is a direct correlation between the content in raw protein of grass and feces for each area. Because the black goat is a species protected by law in Romania, because their number is falling and because all aspects of their biology are not sufficiently well known, the researches are recommended to continue.

Keywords: black goat, feed, feces, composition

The black goat is the mammal with the longest history in the fauna of Romania (9). The life of this animal is 15 - 23 years. The black goats are considered rare animals and that's why they are species protected by law (3, 8, 10).

The encounter with such a specimen is quite unlikely both from the point of view of the small number of animals and especially due to the natural environment in which they live. Environment is represented by rocky holes in the ridges of the mountains, places quite difficult to access to man.

The environment in which he lives is not very rich in vegetation due to the altitude. The black goat feeds in winter especially with lichens, blueberries and in summer with various grasses or shrubs. It can withstand two weeks without feed when the snow is too high to find feed. Their feed consists of alpine meadows, buds and shoots of deciduous or coniferous, leaves of different plants, in winter the goats consume mosses or lichens.

Materials and methods

The research was carried out in Retezat National Park, in the Hateg Forestry area, which includes several areas where black goat populations are found. These areas are (according to the local names): Gorganu, Secari, Bradetel and Negru.

From these areas were collected separately samples of fodder: grass, leaves and moss, individually or in mixture trying to imitate the natural way of feeding. Also faecal samples were collected for each region.

The samples were collected from several points in each area. They were mixed and an average sample was retained and sent for analysis to the laboratory.

In the laboratory some parameters of raw chemical composition were determined: raw protein - RP, etheric extract - EE and raw fiber - RF.

Extraction of crude fat from feed was done using ISO 6492 method, and determination of crude protein by EN ISO 5983-2 and AOAC 2001.11. The determination of the percentage of crude cellulose was made according to the method accredited by the international statements no. 92/89 / EEC and ISO 6865.

Results and discussions

The results of the analyzes of the forage samples collected from different areas (the raw chemical composition of the feeds: raw protein - RP, etheric extract - EE and raw fiber - RF) are shown in table 1and were compared with those from other studies (2).

Table 1.

Raw chemical composition of feed

No.	NAME	RP%	EE%	CF%
1	GORGANU grass	12.85	1.52	30.92
2	GORGANU leaves	10.63	4.33	23.96
3	GORGANU – leaves 51% + grass 49%	11.18	3.78	27.44
Average value area GORGANU		11.55	3.21	27.44
4	SECĂRI – mosses	8.91	1.30	35.14
5	SECĂRI – grass	7.89	2.18	34.77
6	SECĂRI – mosses 51% + grass 49%	8.81	1.97	33.08
Average value area SECARI		8.53	1.81	34.33
7	NEGRU – grass	5.75	2.26	35.09
Average value area NEGRU		5.75	2.26	35.09

The forages harvested from several areas were compared with each other. It seems that the most valuable fodder is found in the Gorganu area where the protein is the biggest (RP 11.55%) and the fibre is the least (27.44%).

The average values are recorded in the Secari area while the lowest values are in the Negru area. In the Negru area, the protein and fat are about half of those registered in the Gorganu area, while the fibers are 7-8% higher.

All this analyzes show that the Gorganu area offers the best quality feed.

Due to the very different raw chemical composition, it is recommended to supplement the feed especially during the times when it is harder to find (1, 4, 5, 6, 7).

Elements regarding the gross chemical composition of black goat feces (raw protein - RP, etheric extract - EE and raw fiber - RF) are shown in table 2.

Table 2.

Crude chemical composition of black goat feces

No.	The name of the area	RP%	EE%	RF%
1	GORGANU	11.9	3.22	26.48
2	BRĂDĂȚEL	14.89	3.02	24.75
3	SECĂRI	9.87	3.32	24.22
4	NEGRU – sample 1	13.1	2.07	25.97
5	NEGRU – sample 2	9.51	2.51	22.76

The analysis of the data presented in table 2 shows that the gross chemical composition of the black goat feces has different values depending on the area from which they come.

The most different absolute values are recorded in terms of the percentage of crude protein - that is in the range 9.51% - 14.89%. Although the numbers seem to be the largest in reality, the difference between the fecal content in the protein has a lower variability (56.57%) compared to the fat content. The amount of raw fat has the largest differences between samples (60.38%).

Among the faecal samples, the smallest differences are recorded in terms of fiber content, which varies between 22.76% and 26.48%, thus a difference of 11.34%.

Although black goats are free animals to choose the feed they consume. it is also difficult to appreciate the proportion in which it consumes various fodder supplies.

Table 3 shows the content of raw protein of grass and feces from different areas.

Table 3.

Crude protein content of grass and feces from different areas

No.	The name of the area	RP% from grass	RP% from faeces
1	GORGANU	12.85	11.90
2	SECĂRI	7.89	9.87
3	NEGRU	5.75	9.51

Table 3 attempts to compare the protein content of faeces with that of grass in different areas. The grass is the only forage for which samples were collected from each area.

It is found that there is a direct correlation between the content in raw protein of grass and faeces for each area. The area with the best results remains the Gorganu area, which has the highest protein content.

Conclusions

Black goats are free animals to choose the feed they consume and therefore it is difficult to determine what they eat.

Although it is possible to determine the raw chemical composition of the feed, it is difficult to appreciate the proportion in which it consumes various feed assortments.

The type of feed consumed depends on the quality of the soil in the respective area, the species of plants, the relief, the availability of feed and the safety that the area confers.

The raw chemical composition of the feed can vary greatly depending on the area of origin between 11.34% and 60.38%.

It is found that there is a direct correlation between the content in raw protein of grass and faeces for each area.

The researches can be continued in order to determine biochemical and immunity analyzes from blood samples to better protect these rare animals.

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CLINICAL OBSERVATIONS REGARDING THE USE OF SKIN STAPLER IN OVARYOHISTERECTOMY IN CATS

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Summary

The objective of this study was to determine the efficiency of using skin staples compared to simple interrupted suture and intradermic suture in closing up the surgical wound after ovariohysterectomy. Ovariohysterectomy was used both for spaying the cats and to treat some inflammatory diseases like pyometra. It was also used to remove ovarian cysts. We had a total of 59 cats of ages ranging from 6 months to 11 years. Xylazine and ketamine were used as an anesthetic for this study. We had a post-surgical complication because an intradermic suture failed, and we decided to use the skin stapler to fix the problem.

Keywords: skin stapler, ovariohysterectomy, cats

Skin stapling can be a replacement for classic suture and has a shorter execution time (1, 4, 5). It has been used for a very long time both in human (2) and veterinary surgery (8). By their design, the staples are a way of reducing surgical time and do not react with the tissue (3, 6). The ovariohysterectomy was done by ventral laparotomy on the *linea alba*. The size of the incision was adapted to the size of the uterus determined by an ultrasound exam in such a manner that it would ensure safe removal of the uterus and ovaries. The purpose of this study is to determine the efficiency and the dangers of using skin staples in cats.

Materials and methods

The study took place in a private practice. The cats used for this study were brought to clinic for spaying or because they had different health problems that involved uterus and ovaries. We used a total of 59 cats with ages ranging from 6 months to 11 months. First, the cats were clinically examined using inspection, palpation and thermometry. Moreover, we also did an ultrasound exam to determine the aspect and size of the uterus and ovaries. The cats that were presented for spaying and the cats that had ovariohysterectomy as a therapeutic solution were used for this study.

The cats were fasted 12 hours before the procedure and the water was taken away 8 hours prior to surgery. Anesthesia was obtained using xylazine and ketamine. Cats were put in dorsal recumbency. The abdomen was clipped free of hair and disinfected using diluted Betadine. A sterile field was applied on the abdomen.

The abdominal access was made by making an incision on the *linea alba* by lifting it and stabbing it. Then we used blunt scissors to widen the incision. Where it was possible we used a spaying hook to identify the uterus and where it was not possible we used fingers to identify it. After identifying a uterine horn, we traced it cranially to the ovary. The ovary was exteriorized by gently pulling the horn upward. We made a fenestration in the broad ligament where there was no vascularization. The ovarian suspensory ligament was clamped using hemostatic forceps and it was sutured by a transfixion suture. Another hemostatic forceps was applied closer to the ovary and the incision was made between the two forceps. The suspensory ligament was released back into abdominal cavity checking for possible bleeding. The other uterine horn was exteriorized by using the first one. By gently pulling the second horn, we identified the second ovary and used the same technique of suture.

The uterine body was found by pulling the uterine horns caudally. On the uterine body we placed a clamp and a transfixion suture.

The musculoperitoneal wall was than sutured using polyglycolic acid with a continuous suture.

The skin was sutured in three different ways: simple interrupted using Nylon wire, intradermic (Fig. 2) using polyglycolic acid and with a skin stapler (Fig. 1). The skin staples we used are made of stainless steel and measure 6.9X4.2 mm. After we finished the sutures we sprayed the incision with antibiotic.

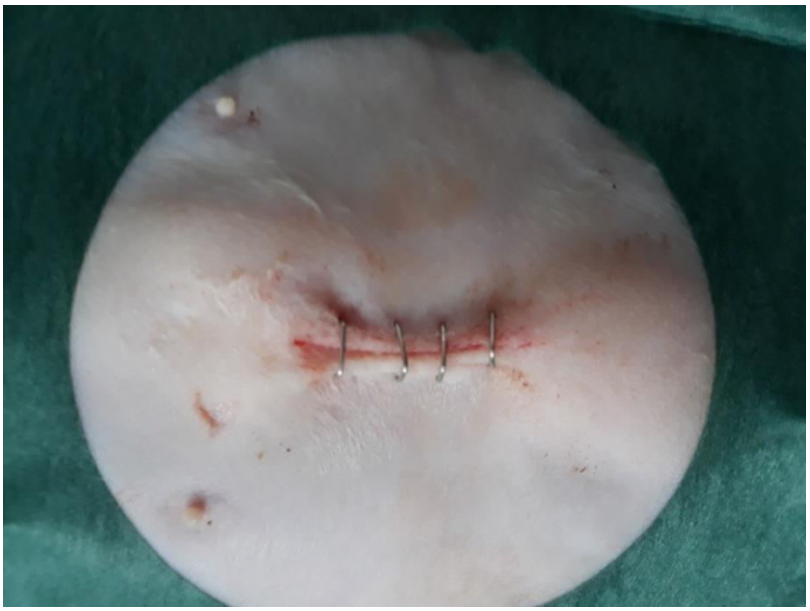

Fig. 1. Skin stapled incision

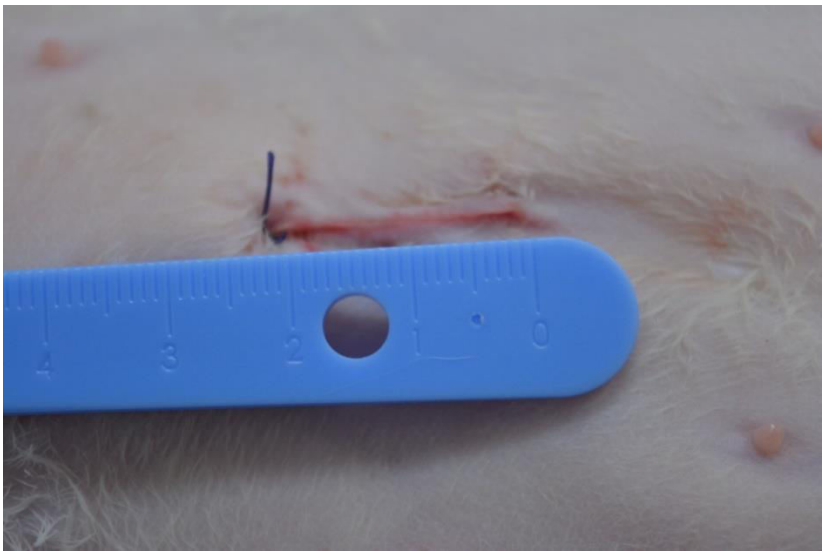

Fig. 2. Intradermic suture

We timed each suture technique to see which one was fastest. The skin suture was inspected on the first and second day post-surgery as well as on the eleventh day when we removed the nylon thread or the staples. We evaluated each animal for discomfort. 60% of the cats from the staples group showed signs of discomfort when we removed the staples. From the cats that were sutured with Nylon only 40 % showed signs of discomfort when the threads were removed.

Results and discussions

All animals included in this study made a full recovery after the surgery. One cat in the intradermic suture group managed to open the surgical wound. We cleaned the wound and refreshed it, and ultimately decided to staple it.

The nylon threads were removed 10 to 12 days after the surgery. The polyglycolic acid threads were not removed. The staples were removed 10 to 12 days after the surgery, but for one cat we removed them after 5 days.

The average incision was three centimeters, ranging from two to five cm. For the average three cm incision, we needed an average of 98 seconds to make the Nylon suture, 115 second to make the intradermic suture and 25 seconds to suture using staples. From the above data we can see that the staples were the fastest way to suture in comparison to the other two. This is a great advantage because it reduces the surgical time. By reducing the time to make the suture we reduce the time the incision would stay open ultimately reducing the contamination.

Another advantage is that staples are better tolerated by the organism than Nylon or polyglycolic acid resulting in a smaller inflammation. Eleven cats sutured with polyglycolic acid showed signs of inflammation, five cats showed less inflammation than those sutured with polyglycolic acid, and the cats sutured with staples showed little to no signs of inflammation. One of the cats that was stapled developed a seroma because we removed some of the abdominal fat and did not suture it back properly. It formed a cavity that got filled with fluid. In time, the seroma got resorbed and the cat showed no sign of discomfort.

The advantage of polyglycolic acid is that it does not need to be removed. Unlike the nylon, polyglycolic acid is embedded in the skin, reducing the chance of contamination. However, the disadvantage is that it needs a longer time of execution and it can lead to a greater local inflammatory response.

Conclusions

The results of this study were that skin stapling requires a shorter execution time (7), followed by the simple interrupted suture and lastly, the intradermic suture, which required the longest time. The intradermic suture had the advantage that it did not require an additional visit to remove the suture threads.

Skin stapling is great because is fast (1, 4, 5), and if the suture is done correctly it requires a shorter time to heal. It also produces a smaller inflammatory response due to the structure of the staple that is better tolerated by the animal.

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RESEARCH REGARDING THE EQUINE INFECTIOUS ANEMIA EVOLUTION IN ARAD COUNTY BETWEEN 2011-2017

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Summary
In the paper are present the serological results obtained between 2011 and 2017 regarding the equine infectious anemia (EIA) in Arad County. The results obtained show that equine anemia prevalence decreases from 2.07% to 0.26% during the analyses period. In each year, a number of positive horses remain in the equine population. We consider that this fact helps the virus maintain in the Arad County equine population reason for the appearance of positive cases in the near future.
Keywords: EIA, prevalence, serological exam

Infectious equine anemia is an infectious disease produced by a specific virus and is one of the most important diseases of the equines. The importance of the disease lies in the losses caused by the costs of prophylaxis and control and, last but not least, by the high costs of diagnosis methods (1, 2, 3).

All equines, irrespective of their race (horses, donkeys, and mules), sex or age are receptive to the EIA virus. Normally, the foals are less expose to insect attack (13) and thus less exposed to the infection. It is possible to persist maternal antibodies that protect animals from infected mothers (3, 5, 6).

Transmission is mainly via blood-feeding insects (*Tabanus spp., Stomoxys spp.*, *Chrysops spp*., possibly *Culicoides spp*.), thus giving the disease a seasonal character with a higher number of new cases occurring during the summer time. Insect vectors have maximum activity in warm and wet seasons, on not very extensive grasslands, close to the forest and not provided with animal shelters (4, 7, 8).

Materials and methods

The present research was made on the existing equine population in the Arad County households.

To obtain primary data about the epidemiological situation of equine infectious anemia, for the period 2011-2017, we received the primary records of the Arad Veterinary Sanitary and Food Safety Directorate (VSFSD). Along with the primary data were analyze: the analysis bulletins issued after the laboratory examinations, the results of the epidemiological investigations, the recovery plans and the records of the epidemiology service from VSFSD Arad.

The routine diagnosis of the disease is represents by agar gel immuno-diffusion (AGID, Coggins test), and in special situations by immunoenzymatic test (ELISA), whose fidelity does not recommend it for mass diagnosis.

Results and discussions

The researches had been extend over seven years (2011-2017) and refer to the equine population in Arad County.

Primary data (taken from the monthly reports of concessionary veterinary surgeons) were processed and interpreted and the significant results were present and summarize in tables and graphics.

We have made it clear from the outset that we have had major difficulties in processing the primary data.

There are numerous inconsistencies between the data in the primary documents, which becomes more obvious when trying to process and interpret these data in terms of epidemiological indicators. VSFSD Arad does not perform epidemiological processing of data collected from concessionary doctors, or from the laboratory. Apart from the fact that the primary data are aggregated (centralized at county level) and reported as such, no further processing of the information gathered from the field is carried out at the central veterinary forums.

In the period 2011-2017 in Arad County, the population of horses ranged from 9083 to 4212 (Table 1).

Table 1

Number of Horses in Arad County between 2011 and 2017

SPECIFICATION	2011	2012	2013	2014	2015	2016	2017
Total number of horses	9083	8563	7427	6457	6457	6522	4212
No of positive horses (old cases)	41	40	27	5	6	5	2
No of positive horses (new cases)	147	129	115	116	26	8	9
Total positive	188	169	142	121	32	13	11
Horses tested positive for infectious equine anemia:							
-sell							
-slaughtered	179	152	128	111	29	10	1
-killed	-	-	-	-	-		-
-dead	-	4	8	2	1	-	-
-remain in the horse population	9	13	6	11	2	3	10

During the study period (2011-2017), the total number of horses in Arad County had fluctuations presented in dynamics in Fig. 1, as follows: in 2011 there were 9083 horses, this year registering the highest number of horses. In the next

two years, there have been decreases of the horses' number, as it follows: in 2012 were 8563, and in 2013 were 7427 horses.

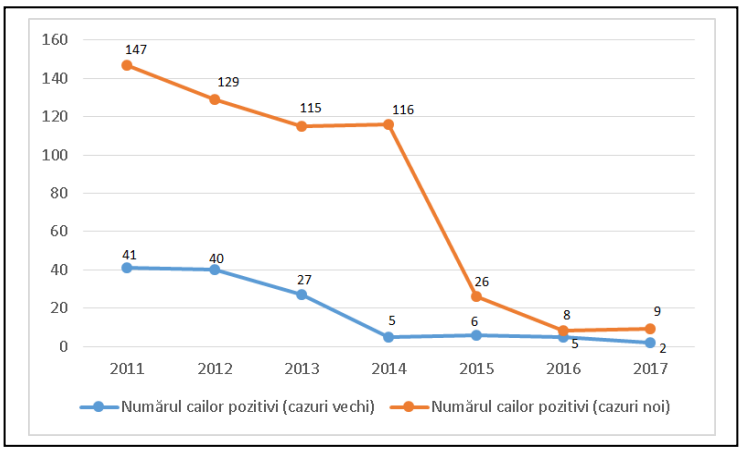

Fig. 1. Dynamic of the serological positive horses in Arad County between 2011 and 2017

According to the data presented in this graphic at the beginning of the study (2011) were 41 old cases of infectious anemia, the number of old cases of disease in the following 3 years was constantly decreasing, so in 2014 there were 5 serological positive, and then their number remained relatively constant.

The number of newly diagnosed cases had a steady downward trend, so that from 147, registered at the beginning of the period of study, the number of newly detected positive horses decreased to 9 in 2017. During the study period 2011-2017 the highest number of new cases was in 2011, with 147 new cases, after that the number of new cases decreased annually, and the lowest numbers respectively 8 new cases, being registered in 2016 (Fig. 1).

Figure 2 presents the evolution of the incidence of equine infectious anemia in Arad County in the period 2011-2017. Incidence as an epidemiological indicator was calculate as a ratio between the number of new cases and the number of animals at risk of contamination. Animals at risk of contamination were calculate by subtracting from the total number of horses, old cases of disease. In fact, Fig. 2 shows the dynamics of the horse population exposed to the risk and the evolution of the incidence.

The incidence as a whole was on an upward trend between 2011 and 2014 from 1.62% to 1.79%, except for 2012 when it fell to 1.51%. After 2014, the incidence fell below 0.40%.

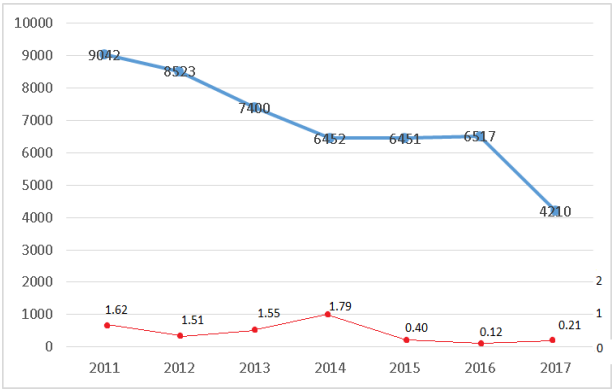

Fig. 2. Dynamic of the incidence of infectious equine anemia in the Arad County between 2011 and 2017

Figure 3 shows the prevalence of equine infectious anemia in Arad County, in the period 2011-2017. Prevalence as an epidemiological indicator was calculate as a ration between the number of new and old cases of disease and the number of animals at risk of contamination. Animals at risk of contamination were calculate by subtracting from the total number of horses, old cases of disease. In fact, Fig. 3 shows the dynamics of the population exposed to the risk and the evolution of the prevalence.

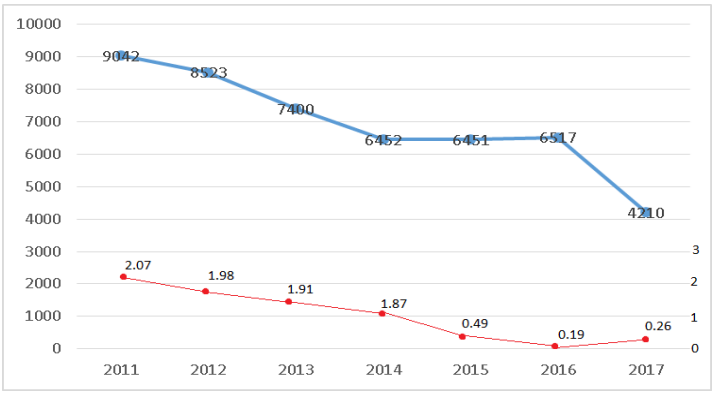

Fig. 3. Dynamic of prevalence of infecious animal equine in the Arad Couny between 2011 and 2017

Prevalence overall had a steady downward trend over the period 2011-2017, with values ranging from 2.07% to 0.26%, with few exceptions, respectively the year 2016, when it dropped to 0.19%.

It can be observe the similarity between the prevalence curve and the curve representing the population exposed to the risk, except for 2016, when a difference between the two curves is observe. This can be mathematically explain based on the observation that the population exposed to the risk has decreased, so the number of old cases has high values, the prevalence was calculate according to the population exposed to the risk, so it is normal to have a curve that justifies their easy approach in 2014.

Figure 4 represents the evolution of the number of remaining positive horses which are not eliminated from the flock in the period 2011-2017.

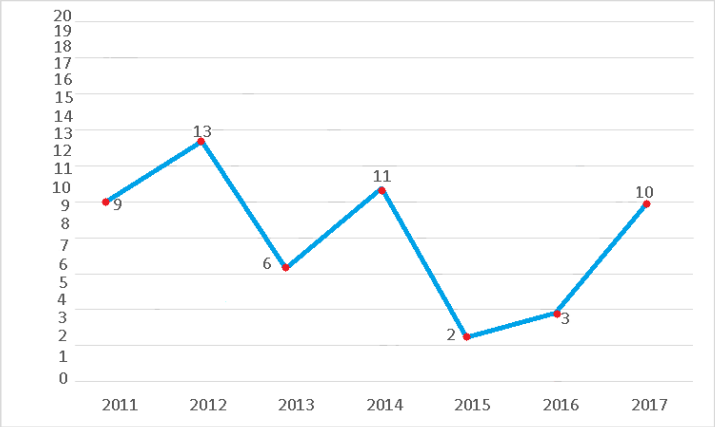

Fig. 4. Dynamic of positive horses for equine anemia infection, which are remain in flocks from Arad County between 2011 and 2017

As it results from the analysis of figure 4, infected horses remained in equine population, constantly, each year.

We consider that their presence in the equine population, in addition to not complying with the current legislation on the control of equine infectious anemia, is a risk factor for cohabiting equine species, given the presence of the virus in the body of these equidae.

Conclusions

In the period 2011-2017 in the equine population of Arad County, serological controls on equine infectious anemia were performed, in each year positive horses being identified.

During the investigated period, the herd of horses in Arad County decreased dramatically, from a number of 9083 horses to 4212 horses.

In the Arad County, the number of positive cases at the beginning of the analyzed period was 147 and at the end of the period the number of cases dropped to 9.

The incidence of infectious equine anemia was at the beginning of the period of 1.62% and oscillated over time, with the highest value - 1.79% in 2014, and at the end of the period, the incidence decreased at 0.21%.

The prevalence of infection equine anemia dropped from 2.07% at the beginning of the period to 0.26% at the end of the analyzed period.

A number of positive horses tested for equine infection anemia remain early in herds maintaining the source of infection among the horse population from Arad County.

Taking into consideration the relatively uniform distribution of equine infectious anemia in Arad County and the fact that annually remain positive horse in equine population, we consider that in the future in Arad County will be new cases of infectious equine anemia, at least in the population of horses around the area where positive horses were diagnosis.

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SOME SERUM PARAMETERS CHANGES IN RATS EXPOSED TO ALUMINUM AND RESVERATROL

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Summary

The aim of the study was to emphasize the serum parameters changes as consequences of aluminum exposure and the possible benefic effect of resveratrol upon these. In this aim 24 Wistar adult rats (280±20 g) were randomly distributed in four groups as follows: C – control that received distillated water, E1 – received aluminum 0.5 g/L as aluminum sulphate (AS) in drinking water, E2 - received aluminum 0.5 g/L and 20 mg/Kg resveratrol in drinking water and CR as blank control - 20 mg/Kg resveratrol in drinking water, the water and substances diluted in water being *ad libitum* for a four weeks period. There were determined albumin (ALB), total proteins (TP), creatinine (CRE) and urea (BUN) using a semiautomatic chemistry analyzer and specific kits. We observed significant ($p<0.05$) decrease of ALB and TP and significant increase of CRE and BUN in rats serum from AS exposed group comparative to control and significant ($p<0.05$) increase of ALB, not significant ($p>0.05$) increase of TP and not significant ($p>0.05$) decrease of CRE and BUN in group that received resveratrol and AS comparative to AS alone exposed group. No significant fluctuations were recorded between the two control groups. We can conclude that resveratrol can be helpful in case of serum parameters changes produced by aluminum exposure, but needs a longer administration period.

Keywords: resveratrol, aluminum, serum parameters

Aluminum represents the third most spread element in the Earth crust after oxygen and silica (2, 8). Aluminum could be found as a constituent of cooking utensils and also in different medical products such as antacids, phosphate binders, buffered aspirins, vaccines, antiperspirants, allergen injection, antiperspirants and deodorants (1, 7).

It has capacity to induce oxidative stress, to produce liver and kidney disorders (14, 15, 16), neurodegenerative disorders such as Alzheimer disease and dementia (7, 12, 17), amyotrophic lateral sclerosis (9).

Resveratrol (3,5,4-trihydroxys-tilbene), is a molecule obtained mainly from grapes and was identified as a biologically active compound in red wine (18). There are numerousin vivo and *in vitro* studies that pointed out the ability of resveratrol to prevent multiple pathophysiological processes (3, 4, 11, 19, 20).

The aim of the study was to emphasize the serum parameters changes as consequences of aluminum exposure and the possible benefic effect of resveratrol upon these.

Materials and methods

Twenty four healthy Wistar albino rats aging six months and 260±20 g, obtained from the authorized animal house of University of Medicine and Pharmacy "Victor Babes" Timisoara, Romania were kept in standard polycarbonate cages and fed *ad libitum* with standard diet. The environmental conditions were maintained at 23 ± 2°C and 12 h light/dark cycle. Before the start of the experiment, animals were kept in the same cages one week for acclimatization and were handled in accordance with Directive 2010/63/EU on the handling of animals used for scientific purposes (6). The experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine from Banat's University of Agricultural Science and Veterinary Medicine from Timișoara (No.120/2018).

The rats were randomly distributed in four experimental groups (n=6) as follows:

- C – control that received distillated water,
- E1 – received aluminum 0.5 g/L as aluminum sulphate (AS) in drinking water,
- E2 - received aluminum 0.5 g/L and 20 mg/Kg resveratrol in drinking water,
- CR as blank control - 20 mg/Kg resveratrol in drinking water.

The resveratrol (Resveratrol 99% purity, 228.24 g mol^{-1}, Sigma-Aldrich, Germany) was dissolved in a carrier-solution containing 5% ethanol and 95% physiological saline. At the end of the experiment all rats were euthanized by overdosing anesthetic agents ketamine (Ketamine 10%, CP Pharma, Burgdorf, Germany) and xylazine (Narcoxyl, Intervet International, Boxmeer, the Netherlands) and blood samples were collected.

The measured parameters were the concentration of total proteins (TP), albumin (ALB), creatinine (CRE) and urea (BUN) in serum by spectrophotometry with a semi-automated analyzer Sinnowa BS3000, according to appropriate standardized procedures, using commercially available kits from ChemaDiagnostica, Italy.

Obtained values were expressed as mean ± SEM (mean's middle error) and for the estimation of the difference between groups one-way ANOVA with the Bonferroni multiple comparison test was used considering the differences are statistically provided when $p < 0.05$ or lower. The software used was GraphPad Prism 6.0 for Windows (GraphPad Software, San Diego, USA).

Results and discussions

Total proteins (TP), albumin (ALB), creatinine (CRE) and urea (BUN) represents important markers of liver and kidney function. There are numerous xenobiotic substances that can affect the liver and kidney function and among them is included also the aluminum. The modification of some serum parameters as TP and ALB are further presented (Table 1, Fig.1, 2).

Table 1

TP and ALB serum levels in rats exposed to aluminum and resveratrol

Group	C		E1		E2		CR	
Parameter	X±Sx	SD	X±Sx	SD	X±Sx	SD	X±Sx	SD
TP	5.57±0.22	0.56	3.96±0,16***	0.39	4.63±0.23* #	0.57	5.56±0.21 ns	0.51
ALB	3.37±0.18	0.44	2.57±0.19*	0.47	3.02±0.05 ns	0.13	3.41±0.21 ns	0.51

Comparative to C: * p<0.05, *** p<0.001, ns-not significant
Comparative to E1: # p<0.05

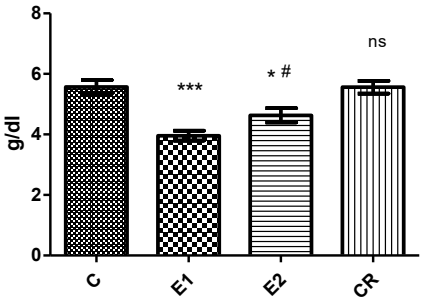

Fig.1. TP level in rats exposed to aluminum and resveratrol
Comparative to C: * p<0.05, *** p<0.001, ns-not significant,
Comparative to E1: # p<0.05

We observed a highly significant (p<0.001) decrease of TP in rats that received AS comparative to control group (-28.90%) and significant increase (p<0.05) in groups that received resveratrol together with AS (+16.91%), but still remaining significantly (p<0.05) lower than control (-16.87%). There were not significant (p>0.05) differences between the control groups.

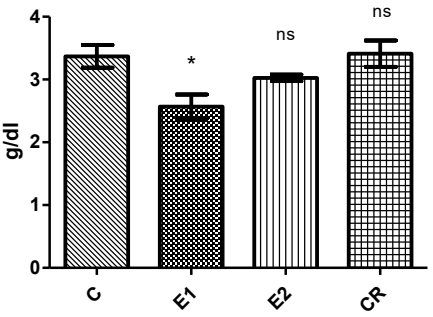

Fig.2. ALB level in rats exposed to aluminum and resveratrol
Comparative to C: * p<0.05, ns-not significant

The ALB level decreased significantly ($p<0.05$) in rats that received AS comparative to control group (-23.73%) but not significant increase ($p>0.05$) in groups that received resveratrol together with AS (+17.51%), remaining not significantly ($p>0.05$) lower than control (-10.38%). There were not significant ($p>0.05$) differences between the control groups.

The transportation, synthesis, and disintegration of protein are related to the function of liver (14, 15, 16, 21), and is known that aluminum impaired the hepatic structure, and by this, caused the reduction of TP and ALB in the serum as was observed in present study and pointed out by some authors in rats and chickens (14, 21).

Katyal et al. (10) noted that aluminum has been implicated in the pathogenesis of some clinical disorders, including the renal dysfunction. The significant decrease in the concentrations of total proteins in rats exposed to aluminum, especially the decrease of the albumin, could be attributed to the reduction of protein synthesis in the liver (5).

The results regarding serum level of creatinine and urea are presented in Table 2 and Fig. 3 and 4.

Table 2

CRE and BUN levels in rats exposed to aluminum and resveratrol

Group	C		E1		E2		CR	
Parameter	X±Sx	SD	X±Sx	SD	X±Sx	SD	X±Sx	SD
CRE	0.31±0.02	0.06	0.93±0,12***	0.29	0.64±0.09*	0.22	0,29±0.02	0.05
BUN	29.84±0.92	2.25	43.21±1.74***	4.28	37.21±1.32**	3.23	28.45±0.84	2.06

Comparative to C: * $p<0.05$, *** $p<0.001$,

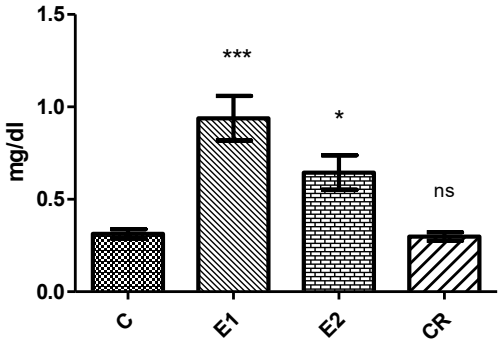

Fig. 3. CRE level in rats exposed to aluminum and resveratrol
Comparative to C: * $p<0.05$, *** $p<0.001$, ns-not significant

In the present study we observed the highly significant (p<0.001) increase of CRE in rats that received AS comparative to control group (+200%) and significant decrease (p<0.05) in groups that received resveratrol together with AS (-31.18%), but still remaining significantly (p<0.05) higher than control (+106.45%). There were not significant (p>0.05) differences between the control groups.

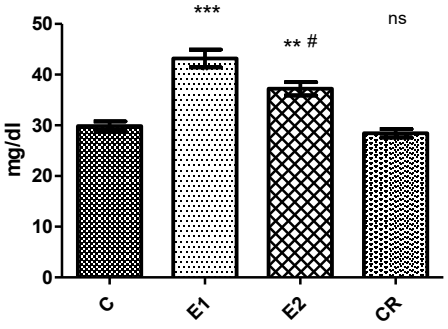

Fig. 4. BUN level in rats exposed to aluminum and resveratrol
Comparative to C: ** p<0.01, *** p<0.001, ns-not significant,
Comparative to E1: # p<0.05

Highly significant (p<0.001) increase of BUN was also observed in rats that received AS comparative to control group (+44.80%) and significant decrease (p<0.05) in groups that received resveratrol together with AS (-13.88%), but still remaining significantly (p<0.01) higher than control (+24.69%). There were not significant (p>0.05) differences between the control groups.

The elevation in serum urea level in aluminum treated rats is considered as a significant marker of renal dysfunction, and this result supported also by the finding of Mahieu et al. (13), who reported that alterations in serum urea may be related to metabolic disturbances (e.g. renal function, cation–anion balance).

The increase in urea concentrations in serum of animals treated with aluminum, as was observed in our study, may be due to its effect on liver function, since urea is the end-product of protein catabolism and this is confirmed by the decrease in serum proteins (14).

Our previous data (15, 16) showed that aluminum could be accumulated in liver and kidney and by this can impair the function of these organs, as also was observed by other authors (13, 14, 21), but different substances with antioxidant properties can protect against this effect of aluminum as it was observed also in this study.

Conclusions

Taking in account the results of the present study and the results obtained by other authors that sustain our study, we can conclude that the resveratrol can regulate the serum parameters of rats exposed to aluminum and offer protection against aluminum organs dysfunction.

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HABITAT INFLUENCES THE BACTERIOME ISOLATED FROM HEALTHY EUROPEAN RED SQUIRRELS (*SCIURUS VULGARIS*)

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Summary

Even though a brief inventory of bacterial, fungal and viral agents would enhance the conduct in managing the main fatality causes, few data is known about the microbiome of the European red squirrel (*Sciurus vulgaris L.).*The present study aims at comparing the bacteria isolated from four red squirrels (n=4), three males and one female, raised in two different habitats. Two of the individuals included in the study were recovered from the wild shortly after being killed in traffic accidents, in different areas of Transylvania. The other two squirrels belonged to different owners and households, having been raised in captivity from one month of age. None of the squirrels showed any signs of an infectious disease at the time the study was conducted. Classical microbiological methods were used for the isolation of the microorganisms sampled from various sites of the body. Samples were obtained from the oral cavity, ears, pads, hair, skin, feces, anal, penile or vulvar region. Identification of the strains was performed by use of Vitek®2 system. Tributary to the 'One Health' concept, which states the interconnection of humans, animals and the environment and the importance of controlling the spread of antibiotic resistance, the antimicrobial susceptibility of all isolates was tested against multiple classes of antibiotics by disc diffusion method. The results revealed a significantly higher antimicrobial resistance in strains isolated from the squirrels that were raised in captivity when compared to those isolated from free-living squirrels.

Keywords: red squirrel, microbiome, antibiotic resistance, wild, captive

Limited information is available on the great variety of bacteria, either pathogenic or opportunistic, in the European red squirrel. High isolation rates are seen in *Staphylococcus aureus* ST49, which causes a fatal exudative and pustular dermatitis in *Sciurus vulgaris* (4, 21). *Staphylococcus aureus* ST49 was isolated in cases of septicaemia (23, 24). The same strain was identified in a red squirrel diagnosed with pneumonia and in the intestine of a case with no relevant findings (12). *Staphylococcus aureus* ST4286 was recovered from a red squirrel with viral enteritis and *Staphylococcus aureus* ST4310 from lesions of purulent dermatitis (12). Growth of non-haemolitic *Staphylococcus spp.* was noted after sampling of squirrels with miscellaneous lesions (21). The red squirrel was unexpectedly identified as an animal reservoir of *Mycobacterium leprae* in the British Isles (2). Infection with *Mycobacterium avium* subs. *avium* was recorded in *Sciurus vulgaris coreae* (17). Pasteurellosis due to *Pasteurella multocida* was diagnosed in a red squirrel with respiratory lesions (15). *Bordetella bronchiseptica* was isolated from 2

cases of mortality due to bronchopneumonia. Isolates of *Pseudomonas mosselii*, *Streptococcus* spp., and *E.coli* from various anatomical sites were documented in squirrels found dead in the UK (21). Microorganisms like *Campylobacter jejuni* (11), multidrug-resistant *Aeromonas spp*. (10) were also recorded in isolated cases in the red squirrel. Fungal infection (candidiasis, adiaspiromycosis, pulmonary phaeohyphomycosis) and even dermatophitosis involving *Trichopyton* spp. seem to be rare in this species (22).

Materials and methods

Four red squirrels (one female and three males), two (male and female) belonging to distinct owners and raised in apartment conditions with no caging, respectively two roadkill found in different areas of Transylvania were sampled for the identification of microbiota. Oral, ear, interdigital, genital and perianal swabs taken from all four cases were inoculated directly onto liquid nutrient broth and incubated in aerobic atmosphere at 37°C for 24h, when cultures were transferred onto Nutrient Agar (Tulip Diagnostics, Verna, India). Following the same regime of incubation, isolated colonies were passed on to selective media: MacConkey Agar (Tulip Diagnostics, Verna, India), Brilliance E. coli/Coliform selective medium (Oxoid, Hampshire, England), Chromogenic UTI medium (Oxoid, Hampshire, England) and Chapman Stone Agar (Himedia, Mumbar, India).

Microscopic examination of bacteria was done subsequently to Gram staining. Microorganisms were evaluated based on their tinctorial properties, morphology and arrangement, and possible means of identification were quested. Isolated colonies were obtained and transferred onto blood agar (Blood Agar Base -Himedia, Mumbar, India) with addition of 5% sterile defibrinated blood. Staphylococci and *Enterococcus faecalis* were confirmed by VITEK® 2 system using GP ID Cards (Gram-positive cocci and non-spore forming bacilli). GN ID Cards (Gram-negative fermenting and non-fermenting bacilli) were implied for Gram negative bacilli. Gram positive spore-forming bacilli were subjected to evaluation by colony morphology (color, shape, margin and surface), haemolitic capacity, presence of catalase, anaerobic growth, growth in 7.0% NaCl and cell morphology (shape, gram reaction and arrangement). The antibiotic susceptibility patterns based on by Kirby-Bauer disc diffusion method results were established according to the CLSI standards. The sensitivity of isolates was tested against chloramphenicol, enrofloxacin, trimethoprim/sulfametoxazole, amikacin, ampicillin, amoxicillin/clavulanic acid, gentamicin, oxytetracyclin and colistin test discs (Bioanalyse, Turkey).

Results and discussions

Out of the 13 bacterial isolates, six strains were recovered from captive and seven were cultured from free-living squirrels (Tables 1, 2, 3 and 4), all with growing interest concerning their role as opportunistic agents in nosocomial infections.

Identified as a skin resident of rainbow trout (18), *Staphylococcus warneri*, was isolated in various clinical cases, such as endocarditis (29), orthopedic infections (7) and osteomyelitis (29).

Previously regarded as an inoffensive commensal microorganism on the human skin, *Staphylococcus epidermidis* is now seen as an important opportunistic pathogen and ranked among the first causative agents of nosocomial infections. Although *S. epidermidis* infections only rarely develop into life-threatening diseases, their frequency, and the fact that they are extremely difficult to treat, represents a serious burden for the public health system (19, 24).

Staphylococcus vitulinus was recovered from human clinical cases, half of the samples comprising urine of the patients (25).

Staphylococcus sciuri has been isolated from the coats of rodents, ungulates, carnivores and marsupials and, occasionally, from other mammals, soil, beach sand, and natural waters. Nevertheless, *S. sciuri* has been associated with serious infections in humans (9, 25), such as endocarditis (13), peritonitis (28), septic shock (14), urinary tract infections (27) and wound infections (26).

Outside its notoriety in association with food poisoning and severe eye infections, *Bacillus cereus* has been incriminated in a multitude of other clinical conditions such as anthrax-like progressive pneumonia, fulminant sepsis, and devastating central nervous system infections (6).

B. licheniformis seems to be uniquely suited as a contaminant and a pathogen. It has been demonstrated to easily colonize catheter implants and then cause progression to infection and bacteremia in humans (5).

Over recent decades, the *Enterobacter* spp. have taken on clinical significance and have emerged as nosocomial pathogens from intensive care patients. *Enterobacter cloacae complex* demonstrates a remarkable ability to acquire resistance determinants, counting for some of the most distressing microorganisms of the current antibiotic era (16, 20).

Pantoea agglomerans was cultured from the bloodstream, abscesses, from joints/bones, urinary tract, peritoneum and from the thorax of human patients. *P. agglomerans* was most associated with penetrating trauma by vegetative material and catheter-related bacteremia (1, 8).

Two strains of *Staphylococcus warneri* (Fig. 2), isolated from each of the captive squirrels, together with *Bacillus cereus* strain (Fig. 1) isolated from one of the free-living squirrel showed the highest antimicrobial resistance (MAR=0.44). *Stahyloccocus sciuri* and *Staphylococcus xylosus* proved susceptible to all antimicrobials included in the test and *Pantoea agglomerans* exhibited a MAR

index <0.2. Discounting the above mentioned three strains in which antibioresistance did not prove worrying, all microorganisms showed resistance to at least two antibiotics (MAR>0.2). 69% of all isolates demonstrated resistance to amoxicillin with clavulanic acid, 53% were resistant to colistin and 30.7% of all bacteria showed no susceptibility to oxytetracyclin and trimethoprim with sulfamethoxazole.

Table 1

Microorganisms isolated and antimicrobial susceptibility results (case 1 – captive male squirrel)

Bacterium / **Antibiotic**	***Staphylococcus warneri*** (interdigital)	***Enterobacter cloacae complex*** (interdigital)	***Enterococcus faecalis*** (perigenital)
Gentamicin	**S**	**I**	**S**
Chloramphenicol	**I**	**S**	**S**
Enrofloxacin	**S**	**S**	**S**
Trimethoprim/sulfametoxazole	**R**	**S**	**R**
Amikacin	**S**	**I**	**S**
Ampicillin	**I**	**S**	**R**
Amoxicillin/clavulanic acid	**R**	**R**	**R**
Colistin	**R**	**R**	**I**
Oxytetracyclin	**R**	**S**	**I**

Table 2

Microorganisms isolated and antimicrobial susceptibility results (case 2 – captive female squirrel)

Bacterium / **Antibiotic**	***Staphylococcus warneri*** (interdigital)	***Enterobacter cloacae complex*** (perianal)	***Staphylococcus epidermis*** (ear and interdigital)
Gentamicin	**I**	**I**	**I**
Chloramphenicol	**I**	**S**	**S**
Enrofloxacin	**S**	**S**	**S**
Trimethoprim/sulfametoxazole	**R**	**S**	**S**
Amikacin	**S**	**I**	**S**
Ampicillin	**I**	**I**	**I**
Amoxicillin/clavulanic acid	**R**	**R**	**R**
Colistin	**R**	**R**	**R**
Oxytetracyclin	**R**	**I**	**R**

Table 3

Microorganisms isolated and antimicrobial susceptibility results (case 3 – free-living male squirrel)

Bacterium / Antibiotic	***Bacillus cereus*** (interdigital)	***Bacillus licheniformis*** (ear)	***Pantoea agglomerans*** (interdigital)	***S. sciuri*** (perigenital)
Gentamicin	**S**	**S**	**S**	**S**
Chloramphenicol	**S**	**I**	**S**	**S**
Enrofloxacin	**S**	**S**	**S**	**S**
Trimethoprim/sulfametoxazole	**R**	**S**	**S**	**S**
Amikacin	**S**	**S**	**S**	**S**
Ampicillin	**R**	**R**	**S**	**S**
Amoxicillin/clavulanic acid	**R**	**I**	**R**	**S**
Colistin	**R**	**R**	**S**	**I**
Oxytetracyclin	**S**	**I**	**S**	**S**

Table 4

Microorganisms isolated and antimicrobial susceptibility results (case 4 – free-living male squirrel)

Bacterium / Antibiotic	***Staphylococcus sciuri*** (perigenital)	***Staphylococcus xylosus*** (perianal)	***Staphylococcus vitulinus*** (perianal)
Gentamicin	**S**	**S**	**S**
Chloramphenicol	**S**	**S**	**S**
Enrofloxacin	**I**	**S**	**S**
Trimethoprim/sulfametoxazole	**S**	**S**	**S**
Amikacin	**S**	**I**	**S**
Ampicillin	**S**	**S**	**I**
Amoxicillin/clavulanic acid	**I**	**I**	**R**
Colistin	**S**	**S**	**S**
Oxytetracyclin	**S**	**S**	**R**

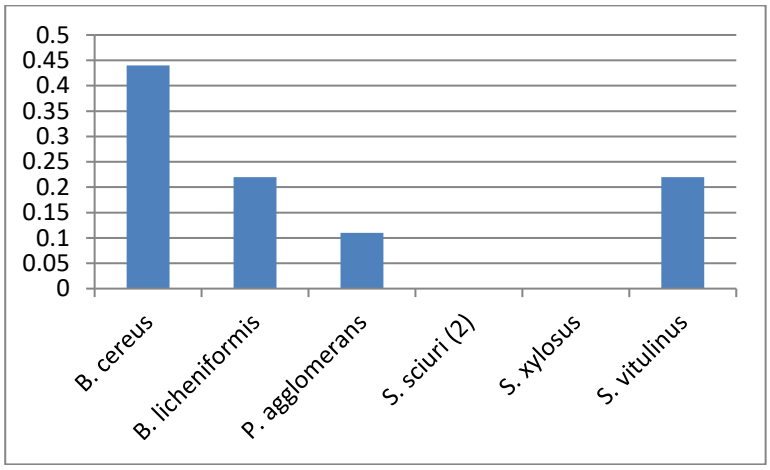

Fig. 1. MAR index of bacteria isolated from free-living squirrels

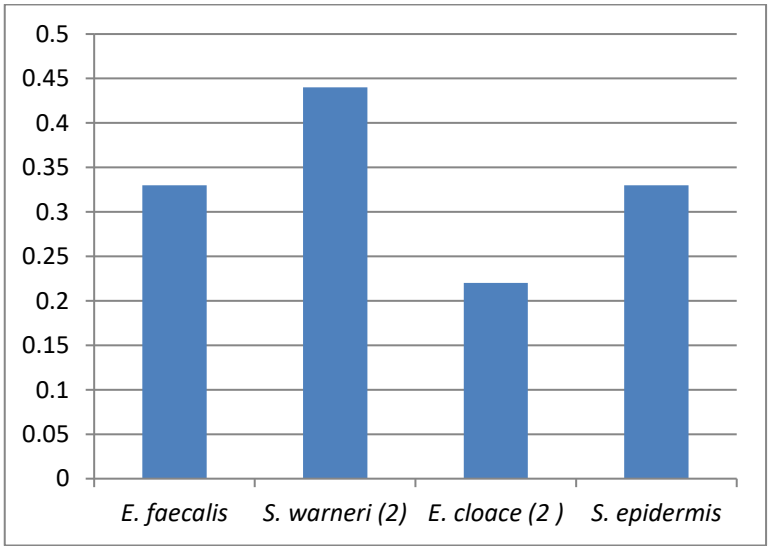

Fig. 2. MAR index of bacteria isolated from captivity raised squirrels

Conclusions

Commensal microorganisms provide metabolic, developmental and immunological functions to the host but homeostasis is breached, invasive symbionts or pathobionts prompt abnormal inflammatory responses potentially causing disease (3).

All of the bacterial isolates recovered the four cases investigated are documented as potential pathogens, not only in animal species, but also in humans. Bacteria isolated from the captive squirrels showed significantly higher overall antibioresistance, suggesting that they were raised in an environment where antibiotic therapy was often implied. Moreover, the majority of the strains show multiple resistance to antibiotics, adding to the worrisome setting of wild animals regarded as reservoirs of multiresistant bacteria. Given the small number of subjects included in this study, further investigation is recommended for the significance increase.

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LUCRARI STIINTIFICE MEDICINA VETERINARA VOL. LII(3), 2019 TIMISOARA

MICROBIOLOGICAL QUALITY OF RAW MINCED BEEF RETAILED IN TIARET CITY (WESTERN ALGERIA)

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Summary

In order to assess the microbiological quality of ground beef retailed in Tiaret, eighty samples were randomly take from butcheries, fort in cold season and forty in warm season. The microbiological studies included Total Aerobic plate counts (APC), Total and Fecal Coliforms (TC and FC), Sulfite-reducing anaerobes (SRC), Staphylococcus aureus (SA), yeasts and salmonella. Expressed in log cfu/g, the overall results were 5.07, 4.98, 3.79, 1.59, 3.40, 3.67 for APC, TC, FC, SRC, SA, yeasts and Salmonella respectively. The samples contamination is higher in warm season for APC, TC, FC and yeasts where. Regarding fecal coliforms, all samples were found to be substandard. Improved hygiene throughout the chain is required to obtain a safe food.

Keywords: minced meat, beef, quality, microbiology, season

Meat, a major source of animal protein highly consumed worldwide is a suitable medium for microorganism growth with a water activity of around 0.99 (30) and required nutrients (25).

Microbial development is greatly affected by meat physical and chemical characters (32) but also by conservation temperature, humidity, and oxygen availability (11).

In traditional abattoir, carcasses are contaminated during the slaughtering process of animals, evisceration, equipment, the water used, and sometimes when gastric reservoirs ruptured. Bacteria already present on meat surface are subsequently scattered throughout the food while preparing ground beef (39).

Thus, minced meat is considered as a medium for transmitting bacteria like *E. coli* and especially Shigatoxin-producing *E. coli* (STEC) (9) and *Salmonella. spp* (34).

Therefore, this study was undertaken to assess the level of microbial contamination of ground beef during at warm and cool season in Tiaret city.

Materials and methods

The study was realized on 80 samples of minced fresh beef during two seasons; 40 samples in warm season (May-July 2018) and 40 samples during the cool one (December-February 2019). The meat is generally minced at time of purchase. The random samples (100 g) were aseptically collected from butcher's shops of the city of Tiaret (Algeria) and sent under cool conditions to the

laboratory. For their preparation, decimal dilutions were carried out according to ISO 6887-2 (17).

Aerobic plate counts (APC), incubation during 72 hours at 30°C in PCA agar (Pasteur Institute, Algiers), microbial colonies between 15 and 300 are counted, and the results expressed in colony forming units per gram of meat (cfu/g) (27).

Total and fecal Coliforms: culture was performed on violet red bile lactose (VRBL) agar medium. Incubation at 37°C for 24-48 hours for the total coliforms (TC) and at 44°C for the fecal (FC) (18).

Sulphite-reducing anaerobes spores (SRC): after heating tubes containing decimal dilution at 75°C for 15 mn, the culture was performed at 37°C for 24 hours on Meat Liver agar (ref). Tubes with blackening from the reduction of sulfites and precipitate of ferric sulfide are considered as clostridia sulphitoreducers (21).

Staphylococcus aureus: culture was performed on Baird Parker agar incubated at 37° C for 48 hours. Black colonies surrounded by clear aureole with thermonuclease and coagulase positive tests indicating Staphylococcus aureus were enumerated (19).

Yeats and molds: deep seeding in 18% Dichloran Glycerol agar (Himedia, India). The counting is performed after 5 to 7 days at 25°C (19).

Salmonella: Isolation was made on Hektoen agar (Pasteur Institute, Algiers) incubated at 37C° for 24 hours. The characteristic colonies underwent morphological and biochemical identification (15).

The confidence interval 95% of prevalence rates of microbes in the minced meat was estimated using an exact binomial distribution. The average logarithms of germs content in the minced beef (warm and cool season) were compared by means of unequal variance Welch test (29).

Results and discussions

Table 1 summarizes the global microbiological meat quality in Tiaret, according to season (warm or cool). Arithmetic mean was used to calculate the averages.

1- Aerobic plate counts

The APC count indicates the degree of global bacterial contamination of meats (31), and is used as a method to monitor the hygienic quality of carcasses (4). It is a criterion for classifying slaughterhouses according to their hygienic quality, but also as a general indicator of bad practices (cold chain not respected, poor cooling, prolonged storage, etc.) and not only as an indicator of alteration in the strict sense (24).

The majority of our of the samples (95.83%) have an acceptable microbiological quality according to the Algerian regulations.

These overall load results (5.07 log cfu/g) are in line with those obtained by Aslam et al. (1) in Pakistan (4.49 to 5.50 log cfu/g) and Siriken (35) in Turkey

where 79% of the samples exceed 5 log cfu/g. Similarly, Heredia et al. (14) and Emswiler et al. (8): 5 log cfu/g and 6 log cfu/g respectively in most samples. However, our previous study (3) conducted in warm season revealed an overall load of 4.88 log cfu/g while in the present one it is of 5.14 log cfu/g. This can be due to sampling differences.

Table 1

Global Microbiological quality of minced meat (log cfu/g)

seaso n	Germs	Global average	Standard (22)	Average	S-deviation	P value (unequal variance Welch's test)
Warm	APC	5.07	5.69	5.24	0.44	0.001*
Cool				4.90	0.45	
Warm	TC	4.98	n.d	5.21	0.50	0.0006*
Cool				4.76	0.63	
Warm	FC	3.79	2	4.01	0.46	0.001*
Cool				3.55	0.73	
Warm	CSR	1.59	1.47	1.74	0.84	0.15
Cool				1.45	0.97	
Warm	Stap	3.40	2	3.57	1.39	0.28
Cool				3.24	1.36	
Warm	Yeast	3.67	4.51	4.07	0.73	0.001*
Cool				3.28	1.35	
Warm	Salmo	6.25	0/25g	7.5		
Cool				2		

APC: Aerobic plate counts, TC: total coliforms, FC: fecal coliform, SRC: sulfite-reducing clostridia, Stap: Staphyloccus aureus, Ye: Yeast and molds, Salmo: Salmonella; n.d.: not determined; * below 5 % significance level

Several works report higher microbial load:

- Oumokhtar et al. (28): 6 log cfu /g in minced meat.
- Skrokki (36): 6.14 log c.f.u/g and 6.62 log u.f.c/g on minced beef and minced beef-pork samples respectively,
- Roberts et al. (31) in the U. K. and Salihu et al. (33) in Nigeria: 6.11 log cfu/g and 9.65 log cfu/g respectively,
- Daabouzi et al. (6): 8.6 log cfu/g in minced beef meat and 7.7 log cfu/g in minced dromedary,
- Salihu et al. (33) In Nigeria reported an average of 9.65 log cfu/g APC in minced beef meat.

Similarly to studies highlighting effect of season on the APC (5, 23) a significant difference (p=0,001) was observed between the two seasons in contrast no season effect was found by Roberts et al. (31) in the UK.

Differences in APC load can be due to variation in hygiene respect at different stages of meat production and preparation. In addition, in our study, most butcheries are not equipped with running water and chilled choppers, in addition, wearing gloves is rarely observed. Such causes would also explain bacterial loads recorded for other studied germs.

This difference may be due to the fact of the high temperature during the summer which favors the multiplication of these bacteria or to the state of slaughterhouses and butcheries during this season (warm) which suffer from poor hygiene and salubrity or an insufficiency of cooling meat.

Thus, an elevated APC level in minced meat indicates high initial bacterial contamination in meat. Indeed, Hamad et al. (13) recorded 2.76 log cfu/g in bovine carcasses at the slaughterhouse of El Oued (southern Algeria) and Bousmaha (2) in Tiaret slaughterhouse (3.15 log cfu/cm²). Another study by Dennai et al. (7) reports a load of 5.15 log cfu/cm^3 on carcasses of freshly slaughtered cattle in Morocco.

Fortunately, not only the regulation prohibits sale of ready minced meat but also consumers hesitate to buy ready minced meat. Nerveless, the break of the cold chain favors and accentuates the contamination of minced meat.

2- Coliforms are group bacteria of fecal origin (e.g. *Escherichia*) or another origin (e.g. *Klebsiella, Enterobacter*). Several studies have indicated that coliforms and *E. coli* are present in ground beef (14, 40). These bacteria reflect the microbiological quality of the meat related to the shelf life of the product or its microbial safety (37) while *E. coli* detection can indicate other pathogens possible presence.

The overall average charge in Total Coliform is of 4.98 log cfu/g, lower those recorded in Morocco in dromedary minced meat and in ground beef with 5.1 log cfu/g and 6 log cfu /g respectively (6). Other studies reported fewer load: 3.2 to 4.6 log cfu/g (23), 4.61 log cfu/g (29) and 4.32 log cfu/g (3).

A significant difference in contamination levels (P=0,001) was observed between the two seasons (5.21 log cfu/g in warm season and 4.76 at the cool one), in contrast to results reported by Dennai et al. (7) where lower results are observed in cool season (2.68 in winter versus 2.56 log cfu/g in summer) where Bousmaha (2): no difference in contamination between the two seasons.

This high rate of Total Coliform in ground beef may be due to inadequate cleaning and disinfection, contaminating material, poor storage conditions, untreated water source, lack of disinfection treatment.

Fecal Coliforms (FC) live in humans and animals intestines; therefore, they are indicators of recent human or animal fecal contamination. As they cannot live outside intestine for a long period, their finding would indicate bad conditions during the slaughter process (Fathy, 1988). All samples were found with microbial load higher than the standard (2 log cfu/g).The average FC load was 3.79 log cfu/g lower than those reported by Roberts (31) in U. K. (4.31 log cfu/g) and by Fathy (1988) in Egypt (4 log cfu/g). Likewise, in Morocco Oumokhtar et al. (28) and Daabouzi et al. (6) recorded 4.75 log cfu/g and 5.5 log cfu/g respectively.

Moreover, less contamination with fecal coliform in ground beef has been reported in Nigeria, 3-5 log cfu/g (33), in Morocco, 3.3 log cfu/g (5) and in Algeria (3): 3.41 log fcu/g.

These different levels of contaminations are likely to be related to the mishandling during slaughtering operation, insufficient material disinfection, or unconformity with decontamination procedure.

The results show the presence of fecal coliforms in all samples, and only 2 taken during the cool season (2.5%) are of acceptable quality. Their rate is significantly higher in the warm season (P = 0.001).

Even on bovine carcasses, fecal coliforms can be found with variable load: In Algeria, 1.75 log cfu/g and 1.66 log cfu/g by Bousmaha (2) and Hamad (13) respectively and 3.89 log cfu/g in Morocco (7).

The presence of fecal coliforms in meat may originate in slaughterhouses. In fact, contamination of carcasses seems inevitable during slaughtering operations, especially during evisceration, by workers' hands and equipment, the water used or the eventual rupture of gastric reservoirs. A lack of care at this level may lead to direct or cross-contamination in addition that occurring during carcass transport, in the butcheries, break of the cool chain or during the mincing.

3- Clostridia and Sulfite-reducing anaerobes (CSRA) were detected in 82.5% (64/80) of the samples, however in total 37.5% have acceptable quality and no significant difference has been observed between the two seasons (P=0.15) similarly to Dennai et al. (7).

Overall load in CSR was of 1.59 log cfu/g greater than that (0.63 log cfu/g) reported by Roberts et al. (31). Likewise, Cohen et al. (5) and Oumokhtar et al. (28) recorded 1.3 log cfu/g and 1.54 log cfu/g respectively. In contrast, Daabouzi et al. (6), 2.7 log cfu/g in ground beef meat and 2.2 log cfu/g for ground dromedary.

In contrast to other results showing a great level of contamination in warm season versus the cool one (5, 7), no effect of the season was observed here (P=015).

Spores of anaerobic sulfite-reducing microorganisms (clostridia) are widely distributed in the environment. Because of their sporulation and massive presence in the soil, these germs can easily contaminate the meat.

The contamination of the meat can occur following the reflux of the digestive contents, during the evisceration and by the contact with the soil and the leathers since the evisceration is done on the ground.

Improve hygiene measures in the abattoir and for the food chain are a condition sine qua no to reduce infection rate.

4- Staphylococci are usually present on the skin and upper respiratory tract of humans and animals and can easily contaminate meats (12)

Staphylococcus had a similar level of contamination in the warm and cool seasons (P=0.28). Similarly, for Dennai et al. (7), the recorded results do not allow to conclude to a significant effect of the season. In contrast, in the United Kingdom, Roberts et al. (31) found that the number of *Staphylococcus aureus* is high in the cool season than warm.

Minor and Marth (26) found that high levels of *S. aureus* (5-6 log cfu/g) are necessary to produce a pathogenic dose of enterotoxin.

The average Staphylococcal load in minced meat is 3.4 log cfu/g higher than the value recorded by ourselves previously: 4.61 log cfu/g (2). Oumokhtar et al. (28) and Roberts et al. (31) with 2.27 log cfu/g and 0.5 log cfu/g on bovine minced meat samples. Our results are higher than the Algerian standards (2 log cfu/g).

On bovine carcasses, Bousmaha (2) and Dennai et al. *(*7) reported an average of 2.13 log cfu/cm² and 2.57 log10 cfu.

Bovine minced meat contamination with Staphylococci may be due to poor hygiene of staff, *Staphylococcus aureus* carriers (abscesses on the skin of handlers), or inadequate handwashing equipment (soap, warm water).

5- Salmonella: These Enterobacteriaceae are pathogenic for humans and animals. Their research is important because minced meat that reaches the consumer must not contain it.

Salmonella was isolated in 6.25 % of the total samples whereas no salmonella was isolated in our last study (3) as also in Bucharest (38).

Variable contamination were reported elsewhere: 2.8% (5), 1.81% (15), 10% (35) and 17.5% (28).

The differences could be due to seasonal factors, geographical region, and isolation method. Many researchers have indicated that season is a factor in the contamination of ground beef with Salmonella spp.

The contamination of ground beef with Salmonella is still considered a major problem in food hygiene. Dennai et al. (7) and Bousmaha (2) recorded the presence of Salmonella on cattle carcasses with rates of 2.17% and 6.58% respectively, probably due to slaughter unfold in poor conditions or poor hygiene when mincing meat.

6- Yeasts and Molds: Some are part of the normal flora of various food products. They are found on improperly cleaned equipment or as a contaminant in the air. When they reach excessive levels, yeasts and molds can cause product deterioration (taste, texture, appearance) and result in significant economic losses.

The obtained average (3.67 %) is in line with the result (3.84 to 4.50 log cfu) recorded by (1) whereas Daabouzi et al. (6) reported 6.6 log cfu /g and 6.4 log cfu/g in minced beef meat and minced dromedary meat respectively.

Yeasts and molds are present in 91.25% (73/80) of the samples but all meet the standard.

A significant difference between yeasts during the warm and cool season ($p = 0.001$) can be explained by the temperatures favorable to their development.

The contamination of minced meat by yeasts and molds is probably due to long-term preservation, reduction of freshness or initial contamination of the meat from slaughter.

Conclusions

Contamination and pathogen bacteria were present in minced meat with different rates in relation with gestures such as smoking and scraping are very common among butchers, the wearing of gloves during handling is exceptional.

However, appropriate hygienic measures can minimize or eliminate this problem. Samples showing high contamination levels indicate poor hygiene and mishandling. Of course, the consumption of such is a risk for human safety.

Adequate measures and good hygiene practices are needed to guarantee good quality meat.

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SWINE GRANULOSA CELLS: TOOLS FOR REGENERATIVE MEDICINE?

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Summary

Our current study was designed to isolate and assess the swine granulosa cells as potential tools for regenerative therapy. Ovaries from sows were collected in authorized slaughterhouse and transported to the laboratory in 0.9% normal sterile saline supplemented with antibiotic and antimycotic (Sigma-Aldrich) at 10–15°C. The granulosa cells were collected from large antral follicles. Isolated cells were treated with Dispase (Sigma-Aldrich) at 37°C for 30 min. The cells suspension were cultured in DMEM/F12 supplemented with 20%FCS, 1%antibiotic-antimycotic at 37°C in a 5 % CO2 atmosphere. After 5 passages the cells were analyzed by FACS (FACS Canto II, Becton Dickinson) and the proliferative ability and differentiation potential were also evaluated. Our results indicate the potential of isolated cells and thus can be further tested for multiple therapeutic applications.

Keywords: swine, oocyte, granulosa cells, regenerative medicine

The functional component of the ovary is the ovarian follicle where the oocyte resides surrounded by granulosa cells (GCs) (1). The GCs are endocrine cells which are primarily involved in follicle growth and development (1, 5). Granulosa cells isolated from different species are the most intensively studied cells due to their commitment in the control of follicular growth, ovulation and corpus luteum function (2, 11). GCs have multiple functions, including secretion of estradiol during follicular growth in response to follicle stimulating hormone (FSH) and progesterone after ovulation (12, 13), and also capacity to form intercellular connections with cells surrounding an oocyte (13). Earlier studies revealed that cellular contents of follicular aspirate consist of granulosa cells (single and in aggregates), leukocytes and large epithelial cells (3, 10). In the assisted reproduction programme GCs are considered useless which are currently discarded. Recent studies have shown that the subpopulation of GCs holds exceptionally plasticity and showing stem cell characteristics (5, 8). Our current study was designed to isolate and assess the biological characteristics of swine GCs as potential tools for regenerative therapy. Also the transdifferentiation capacity was evaluated.

Materials and methods

Collection of ovaries

Ovaries from sows were collected in authorized slaughterhouse and transported to the laboratory in 0.9% normal sterile saline supplemented with antibiotic and antimycotic (Sigma-Aldrich) at 10–15°C. The GCs were isolated using the method described previously by Kossowska-Tomaszczuk et al., 2009 (9) adapted for the sows ovaries.

GCs isolation and culture

The ovarian cortical tissues were excised and oocyte-and-granulosa cell complexes were collected from large antral follicles using a dissection microscope. The oocyte and granulosa cells complexes were added in sterile saline supplemented with 10% fetal calf serum and were centrifuged for 5 minutes at 1500 rpm. GCs were separated from other cells by density gradient centrifugation on 5 ml of Histopaque 1077 (Sigma-Aldrich) for 20 minutes at 3000rpm. GCs were collected from the interphase layer, washed in PBS (Sigma-Aldrich) supplemented with 10% FCS (fetal calf serum) and centrifuged again at 1500 rpm for 5 minutes. Isolated cells were treated with Dispase (Sigma-Aldrich) at 37°C for 30 min and then filtered through a 40-µm nylon mesh and centrifuged at 1200 rpm for 5 min at room temperature. The cells concentration and viability were assessed using a hemocytometer and trypan blue solution. The purified cells ($2x10^5$) were placed in propagation medium DMEM/F12 (Sigma-Aldrich) supplemented with 20% FCS (Sigma-Aldrich), fibroblast growth factor (4ng/ml) (Sigma-Aldrich) 1% antibiotic-antimycotic (Sigma-Aldrich) at 37°C in a 5 % CO2 atmosphere. After 72 hours, the nonadherent cells were removed and the medium was changed.

Chatacterization of isolated GCs

The cells morphology and the attachment capacity were observed microscopically. Cell proliferation was assayed with Cell Counting Kit-8 (Sigma-Aldrich). 10 µl CCK-8 solutions were added into each well of a 96-well culture plate containing cells in 200 µl of culture medium. The plates were incubated for 4 h at 37°C with 5% CO_2. At the end of the incubation period, the absorbance was recorded at 450 nm using ELISA plate reader. The absorbance was directly proportional to the number of living cells in culture. Each treatment for the assay was performed in triplicate.

After 5 passages the cells were analyzed by FACS (FACS Canto II, Becton Dickinson) and the proliferative ability and differentiation potential were also evaluated.

Colony-forming assay (CFU)

The colony forming capacity of the isolated cells were assessed after the

first and fifth passages. Cells were plated at a concentration of $1x10^2$ in normal propagation medium. The medium were changed every 2 days and after 14 days the cells were fixed with 4% paraformaldehyde for 10 min and stained with 0.5% Crystal violet (Sigma–Aldrich) in methanol for 5–10 min at room temperature. The study was performed in triplicate. CFU-F efficiency was estimated according to the formula: CFU-F efficiency = (counted CFU-F/cells originally seeded) × 100.

Flow cytometry

Surface marker analysis was performed using FACS Canto II (BD, Biosciences). Cells were labeled with antibodies for: Oct3/4, CD44, CD105 and CD49f. The device calibration was done automatically using the BD Cytometer Setup & Tracking Beads and the CS & T application included in the FACS Diva program. Compensation was performed for PerCyP, APC, FITC, PE fluorochromes. Data acquisition and analysis were performed using the FACS Diva software (BD Biosciences) and the results were presented as histograms.

***In vitro* differentiation assay**

For osteogenic induction GCs were grown in DMEM supplemented with 10% FBS, 10 mM b-glycerol phosphate (Sigma-Aldrich), 0.1 mM dexamethasone (Sigma-Aldrich), and 200 mM ascorbic acid (Sigma-Aldrich) for 21 days. Cultures were fixed with 70% ethanol for 30 minutes and the mineralization was confirmed using Alizarin Red staining (Sigma-Aldrich). The Alizarin Red was solubilized in cetylpyridinium chloride (Sigma-Aldrich, St. Louis, USA) for 1 h and the absorbance was measured at 550 nm using a spectrophotometer (Bio-Rad, Hercules, CA, USA). GCs cells cultured in normal expansion medium were used as control.

Adipogenic differentiation was performed in monolayer. The isolated GCs were grown in DMEM containing 10% FBS, 1 mM dexamethasone (Sigma-Aldrich), 10 mg/mL insulin (Sigma-Aldrich), 5 mM isobutylxanthine (Sigma-Aldrich), and 200 mM indomethacin (Sigma-Aldrich) for 25 days. The culture were fixed with 10% formalin for 20 minutes and washed with 60% isopropanol and then stained with Oil Red O (Sigma-Aldrich) for 10 min. The Oil Red was solubilized with 100% isopropanol, and the absorbance was measured at 450 nm using a spectrophotometer (Bio-Rad, Hercules, CA, USA). GCs cells cultured in normal expansion medium were used as control.

Chondrogenic differentiation was performed in three-dimensional (3D) cell cultures. GCs at a concentration of $5x10^4$/ml were aggregated using hanging drops method. After 48h, the aggregates (spheroids) were washed and incubated in chondrogenic induction medium: DMEM/F-12 (Sigma-Aldrich) supplemented with 1% ITS (Sigma-Aldrich), 50 nM L ascorbic acid 2-phosphate (Sigma-Aldrich), 100 nM dexamethasone, 10 ng/ml of transforming growth factor (TGF-b; Sigma-Aldrich) and 1% antibiotic/antimycotic (Gibco Life Technologies). After 21 days the production of sulphate glycosaminoglycans was measured using Alcian blue

binding assay and absorbance was read at 630 nm. GCs cells cultured in normal expansion medium were used as control.

Statistical analysis

Statistical analysis (t-test) was performed in order to observe differences in the behavior of isolated cells using GraphPad Prism 6 software (Manual Graph Pad Prism 5.0, GraphPad Software). A value of $p < 0.05$ was considered statistically significant. All data were expressed as the mean ± SD.

Results and discussions

In order to demonstrate the proliferative ability of GCs, we performed daily microscopic evaluation. After 12 and 24h the cell started to attach on the surface and showed a marked heterogeneity of which the fibroblastic, elongated, triangular and round cells were predominant.

After 72 h of culture, the cells begin to form small clusters and the cell acquired a fibroblastic-like morphology (Fig 1.).

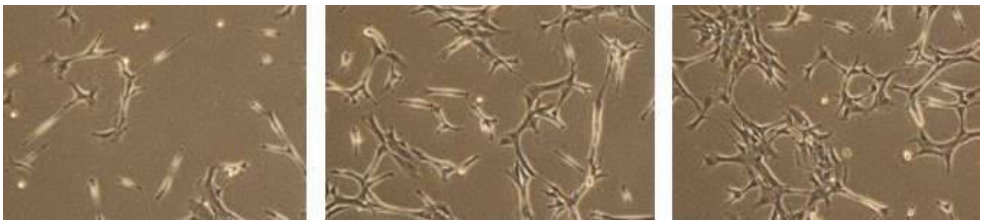

Fig.1. GCs isolated from large antral follicle and cultured in normal propagation medium (DMEM/F12 supplemented with FCS, fibroblast growth factor and antibiotic-antimycotic) at 37°C in a 5 % CO_2 atmosphere

The number and size of cell clusters increased exponentially with the cultivation period, after 7 days reach 50-60% confluence (Fig.2). The highest rate of proliferation was highlighted for fibroblast-like cells.

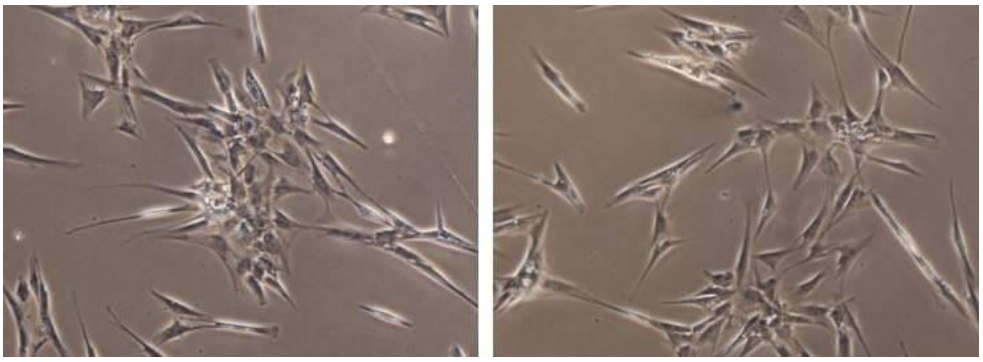

Fig. 2. Morphological evaluation of GCs using phase-contrast microscopy

The colonies reached confluency (90-100%) after 10 days after isolation (Fig 3.). The cell morphology during several passages underwent successive transformations, consequently the degree of cell proliferation indicated different changes.

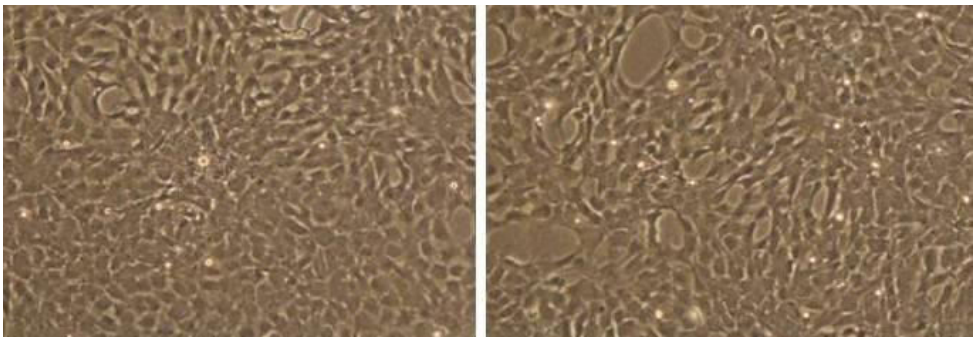

Fig 3. Confluent culture after 1st passages; GCs isolated from large antral follicles cultured in normal propagation medium

The average of doubling time was 2.33 ± 0.28 days. The number of round cells showed a marked decrease and at the end of the 3th passages a homogeneous culture was obtained. The frequency of colony-forming capacity of large antral follicles derivate GCs after 1st passage was 41.67±1.8. After 5th passages the CFU were higher 64.33±4.09 in similar plating condition. The differences are not statistically significant (Fig.4).

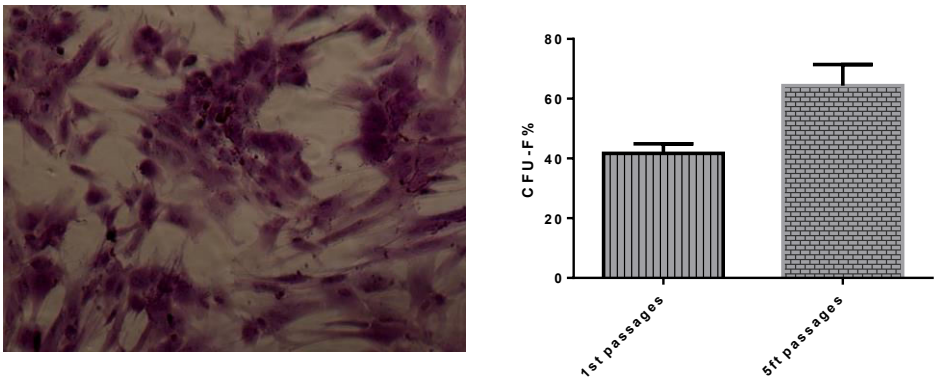

Fig. 4. Colony-forming efficiency after 1st and after 5th passages of large antral follicles derived GCs

In order to assess cell viability and adhesion capacity GCs were cultured in normal propagation medium. After 3 and 7 days the culture medium was changed and CCK8 assays were performed. In both evaluation periods the cells showed higher proliferation capacity. The results of proliferation assay are shown in Fig. 5.

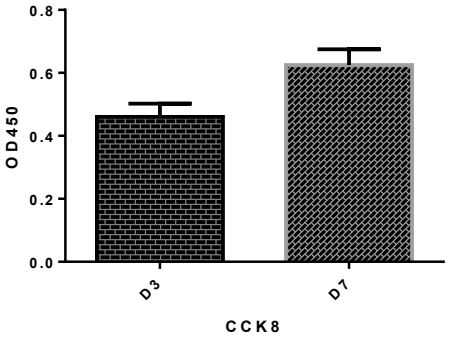

Fig.5. Proliferation capacity of GCs isolated from large antral follicles

The phenotypic evaluations of isolated cells were assessed using FACS Canto II. GCs cells isolated from large antral follicles were assessed for several surface antigens proposed as standard phenotype by the International Society for Cellular Therapy for adult stem cells (4). GCs were positive for Oct3/4, CD105, CD44, and CD49f (Fig.6).

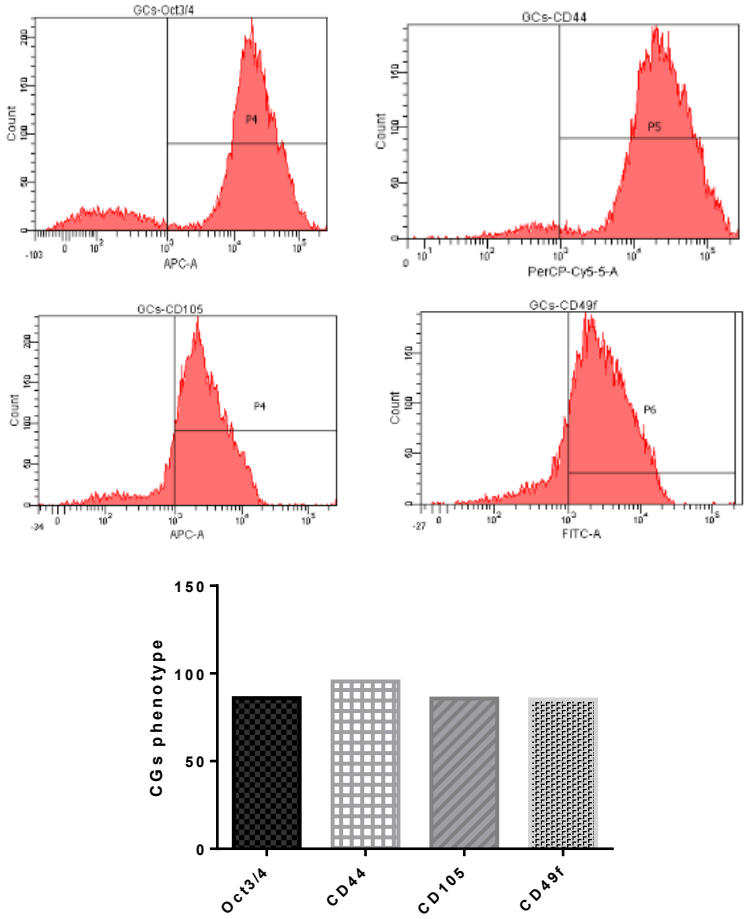

Fig.6. GCs phenotype was detected by FACS analysis after 5th passages

Our results showed a high OCT3/4 (85.9%) positivity after several passages. Preliminary studies demonstrate the OCT3/4 marker expression in the freshly collected luteinizing GCs and remained expressed in the luteinizing GCs throughout the culturing (5). GCs were positive for various markers of mesenchymal stem cells: CD29, CD44, CD90, CD105, CD117 and CD166, but not for CD73 (5, 8). Similar results were obtained for expression of CD90, CD105 and for polymorphic and polyfunctional transmembrane glycoprotein CD44 (9).

The differentiation capacity of GCs was assessed. The cells were differentiated *in vitro* to osteoblastic, adipogenic and chondrogenic lineages,

respectively. After 14 days of incubation in osteoinductive medium, cells changed their morphology, compact cell clusters was observed. The calcium deposits of cultured GCs were assessed by alizarin red staining. Alizarin Red was solubilized with cetylpyridinium chloride and the absorbance was measured. The average of absorbance was 0.178 ± 0.02 for negative control and 1.68±0.060 for treated wells (Fig.7).

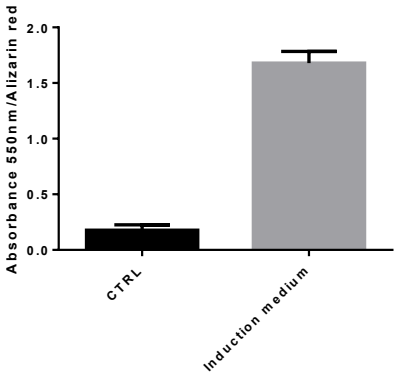

Fig.7. GCs cultured in osteoinductive medium, the mineralization was assessed by Alizarin red staining

In adipogenic medium after 21 days the presence of intracellular lipid droplets was highlighted using Oil Red staining. For measuring, the cells were solubilized with 100% isopropanol and the absorbance was measured. Cultures treated with normal culture medium were negative for Oil Red staining. The average of absorbance was 0.178±0.20 for negative control, 2.494±0.27 for treated cells ($p<0.05$). The difference was statistically significant (Fig.8).

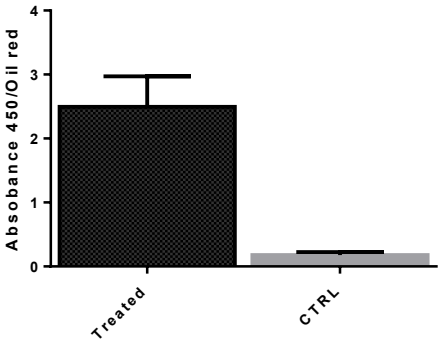

Fig.8. Adipogenic differentiation of GCs stained with oil red

To determine chondrogenic induction the cells were aggregated in hanging drops in order to form the pellets. After 21 days Alcian Blue staining indicated the presence of glycosaminoglycans contained in the chondrocytes' extracellular matrix was measured in an Alcian blue binding assay. The average of absorbance was 0.229±0.13 for negative control, 3.011±0.15 for treated cells ($p<0.05$). The difference was statistically significant (Fig.9).

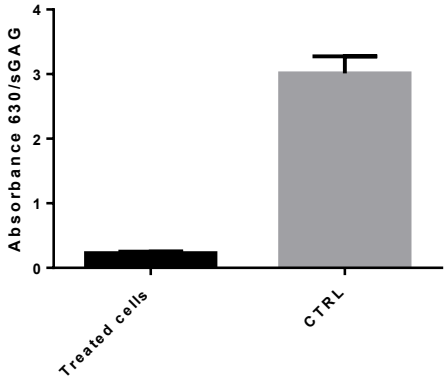

Fig.9. Chondrogenic differentiation, Alcian Blue staining indicated the presence of glycosaminoglycans

Our results indicate the successful isolation of GCs from large antral follicle, cells that possess multiple functions and transdifferentiation capacity. GCs are somatic cells that are involved in oocyte development and form the multiple layers surrounding the ovocyte (1). The importance of GCs consists in their ability to secrete growth factors and hormones and play a crucial role in oocyte development and function. The most important source for obtaining GCs is follicular fluid and can be easily harvested while performing various procedures during *in vitro* fertilization (10, 11). After ovulation, GCs are luteinized and produce progesterone to maintain viability of the endometrium during gestation (6, 8). Do to their function GCs are frequently used to evaluate the multiple aspects of ovarian function and reproduction biology (7). Isolation and purification method are essential for the correct interpretation of data obtained from different experiments. We used density gradient centrifugation followed by enzymatic treatment and incubation in normal propagation medium supplemented with fibroblast growth factor. Isolation of pure populations of GCs are essential to facilitate functional and transdifferentiation studies. Because these anatomical structures are vascularized, therefore cells derived from the follicular fluid are contaminated with vascular components (7), resulting in a heterogeneous cell population. Several strategies have been recently developed for GCs isolation. Combined use of specific surface

markers and fluorescence-activated cell sorting (FACS) represent the gold standard method (6). IVF techniques require follicular aspirate, which can be consider as a potential source of different cell populations. Previous studies have shown that follicular aspirates consist of single or aggregated granulosa cells, leukocytes and large epithelial cells (3). Recently, it has been shown that primitive granulosa and germ cells can differentiate from the cytokeratin-positive mesenchymal progenitor cells exist in the ovarian tunica albuginea (16) and may contribute to the generation of epithelial cells similar to granulosa cells. Luteinizing GCs was considered to be terminally differentiated, undergo cell death by apoptosis after a few days after ovulation (17).

Studies conducted by Kossowska-Tomaszczuk and Zhang et al. (9), demonstrated that ovarian granulosa cells, contain a subpopulation of follicular cells, who have the ability to remain viable *in vitro* over prolonged periods when cultured in the presence of leukaemia-inhibiting factor (LIF) (8, 13).

Our results demonstrated the proliferative and self-renewal capacity of GCs. Additionally isolated cells were differentiated into osteoblasts, adipocytes and chondrocytes using standard *in vitro* culture-differentiation conditions. Differentiation into osteoblasts, chondrocytes and adipocytes was confirmed by spectrophotometric analysis after staining of mineralized matrix, the intracellular lipid droplets and the presence of glycosaminoglycans contained in the chondrocytes' extracellular matrix with Alizarin Red, Oil Red O and Alcian blue, respectively. Better understanding of GCs proliferation could also play a critical role in the developing of cell therapies and tissue (5).

Conclusions

Because of their characteristics, these cells can be used in various *in vitro* studies, including cytotoxicity and cloning assays. Also, these cells can be used as a source of adult cells for the production of induced pluripotent stem cells.

We suggest that GCs cells provide a useful model for studying the mechanisms of dedifferentiation and acquired multipotency of stem cells.Our results indicate the potential of isolated cells and thus can be further tested for multiple therapeutic applications.

Acknowledgments

This work was additionaly supported by the Domus Hungarica Scientiarum and Artium research fellowships.

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MICROBIOLOGICAL AIR CONTAMINATION IN RELATION WITH BIRD AGE IN A MEAT CHICKEN FARM

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Summary

The aim of this study was to assess the level of microbiologic air contamination in relation with bird age in broiler houses. The investigations were carried out in two houses from a meat chicken farm. The determination of the bacteria and fungi numbers was performed in the first, third and the sixth week of the production cycle by specific methods. Air temperature and relative humidity were simultaneously measured. The data were statistically processed using the SPSS software, version 17. The concentration of studied parameters in indoor air were as follows: total mesophilic bacteria ranged from 6.88 x 10^5 to 4.97 x 10^6 CFU/m^3, the Staphylococci concentration was between 3.35 x 10^5 - 2.71 x 10^6 CFU/m^3, Streptococci between 7.49 x 10^4 - 2.52 x 10^5 CFU/m^3, gram-negatives from 9.88 x 10^3 to 1.53 x 10^4 CFU/m^3 and fungi from 1.33 x 10^4 to 3.05 x 10^4 CFU/m^3. Staphylococci were 48.69 - 82.49%, streptococci 6.14 - 10.89%, and gram-negatives 0.39 - 2.59% within the total mesophilic bacteria. In both houses significant differences ($P<0.05$) were recorded between the three periods of the determinations for all microbiological parameters. The results show that microbial air contamination increased with poultry age.

Keywords: airborne bacteria, airborne fungi, broiler age

Simultaneously with the development of the poultry industry for meat production, researchers' concerns about bird health and welfare have become increasingly apparent in many countries around the world. It is generally accepted that most welfare issues are caused by genetic factors, environmental factors and interactions between them. Among the environmental factors, microbial air quality is a key factor for bird health, as well as for health workers and the environment.

The air in broiler houses is usually heavily contaminated by large quantities of microorganisms (bacteria and fungi), dust and toxic gases (8, 10, 16, 17, 20). High levels of microbial contamination may contribute to lower immunity and susceptibility to infections (13). Numerous studies have shown that poultry workers are usually exposed to high concentrations of airborne microorganisms (5, 12), as can cause respiratory diseases (7, 18). Furthermore, the dust may transfer different microorganisms from one livestock building to another (5, 12). In addition, the contaminants emitted by poultry farms have negative environmental consequences (8).

The concentration of microorganisms in the indoor air of broiler houses depends on different factors such as the type of the housing, the number of birds, the litter quality, the type of ventilation and the microclimatic conditions (temperature, humidity, airflow velocity, and dust concentration) (22). Improper

management and hygienic conditions can represent causes of considerable microbial air pollution in broiler houses (22).

In recent years there has been a significant increase in the world-wide scientific database on air pollution in poultry houses and environmental farming (1, 2, 5, 16, 17, 20). However, few studies have investigated the level of air pollution on poultry farms with regard to bird's age and productivity (15, 16, 20).

The aim of this study was to assess the level of microbiologic air contamination in relation with bird age in broiler houses.

Materials and methods

The investigations were carried out in two houses (H1, H2) from a meat chicken farm from Transylvania, Romania. Each house has an area of 1.680m^2 and accommodates 32.000 ROSS 308 broiler chicks (39 kg/m^2). Both houses are equipped with appropriate automatic facilities (for feeding, watering, heating, and ventilation) for the rearing technology of broilers on chopped straw bedding. Broiler's slaughtering is done at 44 days.

In each house six determinations were performed (in three areas of the house, in two different days in the first, third and the sixth week of production cycle).

Bacterial counts in air samples were determined with a Merck MAS-100 device (Merck, Germany). Bacteria and fungi were collected and grown in Petri dishes on different standard culture mediums: Columbia agar for mesophilic bacteria, Chapmann agar for staphylococci, Endo agar for gram negative bacteria, blood agar for hemolytic bacteria and Sabouraud agar for fungi. Air was sampled in a volume of 1 L. Plates with the usual bacterial nutrient Columbia agar and with selective culture mediums were then incubated for 24 h in an incubator at 37°C. The material sampled on Sabouraud agar was incubated for 5 days at 22°C. The grown colonies were calculated using a mechanical optic colony counter, and results were corrected using the conversion formula devised by Feller (6). The average number of bacteria and fungi was calculated as colony-forming units in one cubic metre (CFU/m^3).

Air temperature and relative humidity were measured using a Testo 400 device (GmbH & Co device) on the same days as the air samples were collected.

All data were analyzed by SPSS version 17 software, including descriptive statistics (mean, standard deviation, median, minimum, and maximum) and Kruskal-Wallis test. It was calculated also the percentage of staphylococci, streptococci, and gram-negatives within the overall number of mesophilic bacteria.

Results and discussions

The results obtained for bacteria and fungi in two broiler houses in week 1, 3 and 6 of the production cycle are shown in Table 1. Data analysis shows that

both the number of bacteria and fungi varied in the two shelters, with significant differences ($P<0.05$) between the studied periods. In both houses, the number of bacteria and fungi increases with age, even if the differences were not significant in all cases.

Table 1

Descriptive statistical indicators for bacteria and fungi in two broiler houses in relation with bird age

	Parameter	Age (week)	Mean	Standard deviation	Median	Minimum	Maximum
H 1	Mesophilic bacteria (CFU/m^3)	1	6.88×10^5	1.95×10^4	7.19×10^{5a}	4.11×10^5	9.02×10^5
		3	8.78×10^5	5.67×10^4	8.82×10^{5b}	7.89×10^5	9.42×10^5
		6	4.97×10^6	4.68×10^5	5.99×10^{6c}	2.88×10^6	5.00×10^6
	Staphylococci (CFU/m^3)	1	3.35×10^5	6.63×10^4	3.62×10^{5a}	2.04×10^5	3.80×10^5
		3	6.90×10^5	1.84×10^4	6.92×10^{5b}	6.62×10^5	7.12×10^5
		6	2.45×10^6	1.83×10^5	2.47×10^{6c}	2.14×10^6	2.65×10^6
	Streptococci (CFU/m^3)	1	7.49×10^4	2.77×10^3	7.57×10^{4a}	6.95×10^4	7.69×10^4
		3	8.92×10^4	3.06×10^3	8.90×10^{4b}	8.50×10^4	9.30×10^4
		6	2.52×10^5	9.86×10^3	2.55×10^{5c}	2.35×10^5	2.61×10^5
	Gram-negatives (CFU/m^3)	1	1.18×10^4	2.56×10^4	7.73×10^{3a}	6.24×10^3	7.01×10^4
		3	1.22×10^4	2.71×10^3	1.20×10^{4b}	8.00×10^3	1.60×10^4
		6	1.23×10^4	2.66×10^3	1.15×10^{4b}	9.00×10^3	1.60×10^4
	Fungi (CFU/m^3)	1	1.65×10^4	7.21×10^3	1.95×10^{4a}	1.89×10^3	2.04×10^4
		3	2.90×10^4	3.63×10^3	2.85×10^{4b}	2.50×10^4	3.50×10^4
		6	3.05×10^4	3.62×10^3	3.00×10^{4b}	2.60×10^4	3.60×10^4
H 2	Mesophilic bacteria (CFU/m^3)	1	1.08×10^6	1.35×10^5	1.05×10^{6a}	9.40×10^5	1.27×10^6
		3	2.17×10^6	4.13×10^5	2.13×10^{6b}	1.59×10^6	2.63×10^6
		6	3.94×10^6	5.60×10^5	3.97×10^{6c}	3.85×10^6	3.99×10^6
	Staphylococci (CFU/m^3)	1	6.88×10^5	2.40×10^4	6.86×10^{5a}	6.54×10^5	7.24×10^5
		3	1.27×10^6	7.8×10^4	1.26×10^{6b}	1.15×10^6	1.39×10^6
		6	2.71×10^6	6.48×10^4	2.71×10^{6c}	2.60×10^6	2.80×10^6
	Streptococci (CFU/m^3)	1	7.76×10^4	9.04×10^3	7.97×10^{4a}	6.38×10^4	8.72×10^4
		3	1.59×10^5	1.39×10^4	1.55×10^{5b}	1.46×10^5	1.78×10^5
		6	2.42×10^5	4.04×10^3	2.41×10^{5c}	2.37×10^5	2.48×10^5
	Gram-negatives (CFU/m^3)	1	9.88×10^3	7.84×10^2	1.00×10^{4a}	8.45×10^2	1.06×10^4
		3	1.20×10^4	2.62×10^3	1.13×10^{4a}	8.00×10^3	1.53×10^4
		6	1.53×10^4	3.72×10^3	1.55×10^{4b}	1.00×10^4	2.10×10^4
	Fungi (CFU/m^3)	1	1.33×10^4	4.86×10^2	1.32×10^{4a}	1.28×10^4	1.39×10^4
		3	1.67×10^4	5.01×10^3	1.65×10^{4a}	1.10×10^4	2.30×10^4
		6	2.23×10^4	5.20×10^3	2.25×10^{4b}	1.50×10^4	2.90×10^4

H1, H2 - house 1, house 2; ^{abc} Values in a some group of bacteria and fungi with no common superscript are significantly different ($P<0.05$)

In Table 2 are presented the results obtained for temperature and relative humidity in all investigated periods. Increased values were recorded for both parameters, especially at week 6.

The concentration of microorganisms in the air of the houses presented in the literature varies considerably, probably due to the different methods of

determination used. The results obtained in this study are in agreement with those found by other researchers who reported concentrations from 10^4 to 10^7 CFU/m^3 for mesophilic bacteria, from 10^2 to 10^4 CFU/m^3 for gram negative bacteria and from 10^2 to 10^2 CFU/m^3 for fungi (16, 17, 19, 20).

Table 2

Descriptive statistical indicators for microclimate parameters (temperature and relative humidity) in two broiler houses in relation with bird age

	Parameter	Age (week)	Mean	Standard deviation	Median	Minimum	Maximum
House 1	Temperature (°C)	1	31.82	0.31	31.90[a]	31.40	32.20
		3	29.92	0.58	30.00[a]	29.00	30.50
		6	24.75	0.58	24.85[b]	24.00	25.60
	Relative humidity (%)	1	64.37	1.74	64.60[a]	62.00	67.00
		3	68.57	1.02	68.70[a]	67.00	70.00
		6	74.35	3.28	74.80[b]	69.00	78.00
House 2	Temperature (°C)	1	31.97	0.51	32.00[a]	31.20	32.60
		3	29.75	0.42	30.00[a]	29.00	30.00
		6	25.20	0.52	25.10[b]	24.60	26.00
	Relative humidity (%)	1	60.67	1.97	61.00[a]	58.00	63.00
		3	69.00	2.00	70.00[b]	65.00	70.00
		6	80.82	2.13	80.75[c]	78.00	84.00

[abc] Values of some parameter with no common superscript are significantly different (P<0.05)

The microbial air load expressed by the total number of mesophilic bacteria is influenced by a lot of factors such as: number of housed animals, breeding technology, floor and litter type, microclimate quality, dust concentration, and ventilation level (1, 11, 22). Many epidemiological evidence has suggested that the increase of bacteria and fungi concentration in the air may affect the health of chickens and workers (19). In addition, large quantities of pollutants produced on the farm are emitted to the environment, where they can affect the health of the population in the area or lead to air pollution (17). For these reasons, their concentration should be kept as low as possible with effective ventilation and good management practice.

The large variability in the number of mesophilic bacteria in the air is the reason why there is no internationally accepted bacterial standard for the number of bacteria so far. However, the recommendation of most authors, valid also in our country, is that the total number of mesophilic bacteria does not exceed 1 x 10^5/m^3 (4) in the inside air for farm animals. The results of this study indicate values of mesophilic bacteria 40 to 50 times higher than those admitted at week 6. High density of animals and improper ventilation are the main causes of such a microbial air load.

In our study, microbial air contamination increases simultaneously with the fattening period. This finding was also reported by other researchers (9, 16, 20).

Bacterial and fungal counts varied between weeks of the rearing period, most likely due to changes in dust levels and ventilation efficiency (21).

Temperature and relative humidity influence the thermal comfort of birds; therefore they must be kept at the recommended level (temperature 26-32 °C in the first period, then 16-20 °C, relative humidity 70%). At older ages, increased humidity determines litter moisture and related issues. Increased humidity associated with high temperature, as found in this study (Table 2), causes discomfort to the offspring due to the combined effect of the two physical factors of the microclimate (14, 16).

The temperature and relative humidity of the air, as well as other microclimate factors, are influenced by the flock density and the ventilation type. In one study, it was found that the percentage of birds that died throughout the growing period was positively correlated with humidity and temperature in weeks 3-5 (3). The conclusion of this study was that the housing conditions (litter quality, air temperature and humidity) were more important for the welfare of the chickens than the stocking density.

The proportion of staphylococci, streptococci and gram-negative bacteria within the total mesophilic bacteria in each house is shown in Fig. 1.

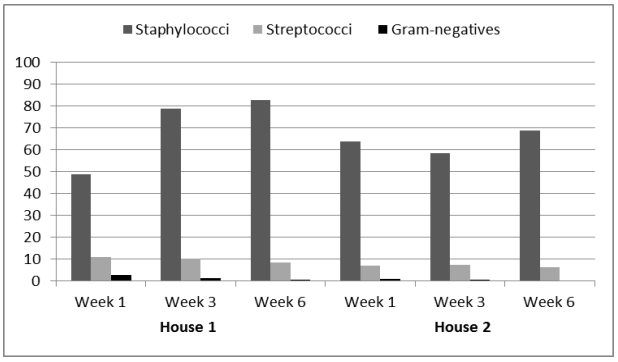

Fig. 1. Percentage of staphylococci, streptococci and gram-negative bacteria within the total mesophilic bacteria in each house

Hartung (8) shows that gram positive bacteria such as staphylococci and streptococci are in larger amounts in the air. Our results confirm this finding, in the studied houses gram positive bacteria were found up to 83%. It is also stated that the gram negative bacteria isolated from the air represent a minor proportion of the total germ, between 0.02 and 5.2% (23). This was also found in our study (0.39-2.59%).

Conclusions

The results of this study show that microbial air contamination increased with poultry age. The poor microbiologic quality of the air in the investigated broiler houses is mainly caused by improper ventilation.

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HISTOLOGICAL ASPECTS OF LINGUAL GLANDS IN OSTRICH

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Summary

Compared to other avian species, the ostrich tongue reveals structural, anatomic and histologic differences. The tongue is provided with mucosa, support cartilaginous lining and striated muscle layer attached to it. It is coated with squamous, stratified, non-keratinized epithelium, both on the dorsal and ventral sides. The ostrich tongue is equipped with several salivary glands, arranged in large lobules on the ventral side and small lobules on the dorsal side. From the histological structure point of view, the mucus-secreting glands are simple, branched, tubulo-alveolar, interconnected through abundant connective tissue septa. The glandular epithelial cells cytoplasm is bubbly and abundant, with spherical or flat nuclei, located at the bottom. The salivary glands of the dorsal side of the tongue are located in the shallow side, within lamina propria, while the ones on the ventral side are located deeper.
Keywords: ostrich, tongue, salivary glands

Several publications have communicated peculiarities regarding the histological structure of the digestive system of the ostrich, as increasing economic interest has been characterizing breeding this species. Compared to other avian species, the ostrich tongue reveals structural, anatomic and histologic differences (1, 2, 3).

Materials and methods

Five organs from ostrich of different ages were analyzed. The dissection took place at the discipline of Anatomy of the Faculty of Veterinary Medicine in Bucharest, and within the discipline of Histology and Embryology the samples were prefixed in special containers in a neutral soil form. 10%, after which they were adjusted and refitted in the same type of fixator for 24 to 48 hours. The samples thus fixed were included in the paraffin, then they were cut using the microtome and finally the samples were stained using several coloring methods: PAS, AA, HEA, HE, Gomori. After staining, the histological preparations thus obtained were examined using the MoticPanthera microscope and photographed.

Results and discussions

The tongue of the ostrich, compared to other bird species, presents structural differences, both anatomical and histological.

The results we obtained from applying the technique of processing for the lingual tissue samples harvested from the studied ostriches to which the histological and / or histochemical staining methods were applied. The results of examination indicate the existence of many branched tubulo-alveolar salivary glands, of various sizes, all of which have mucus secretion, revealing both the alicyanophilic and positive PAS characteristics of the gland cells, as well as their trajectories and their discovery at the surface of the epithelium (Fig. 1, 2, 3, 4, 5, 6, 7). The taste buds are absent on the surface of the tongue.

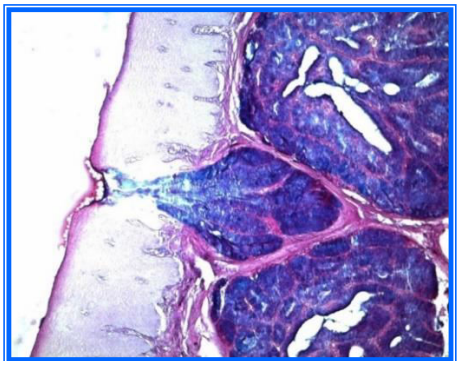

Fig. 1. Tongue. Ostrich. Tract of elimination secretion of the mucous tubular glands PAS stain, x 100 (original)

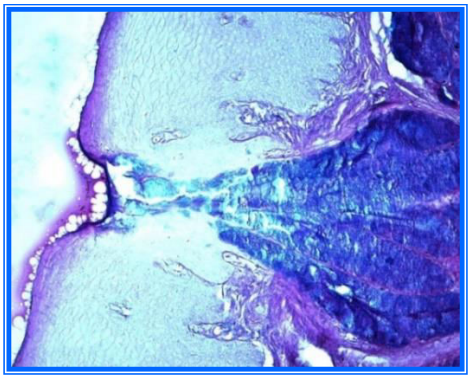

Fig. 2. Tongue. Ostrich. Albastru alcianofilic reaction at the level of the lingual structure AA stain, x 200 (original)

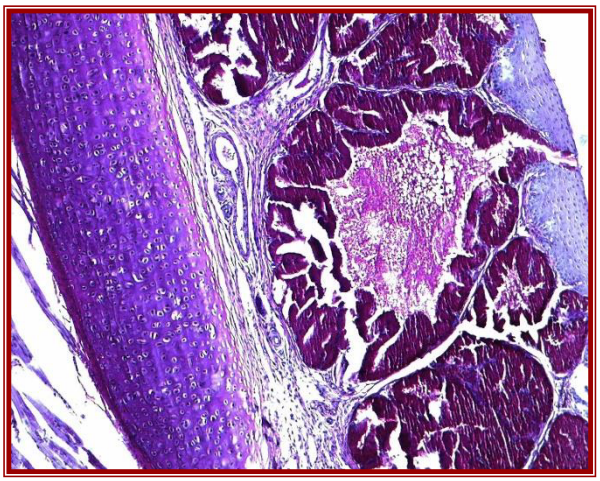

Fig. 3. Tongue. Ostrich. Histochemical reaction PAS + and AA + by demonstrating neutral and acid mucopolysaccharides AA-PAS stain, x 200 (original)

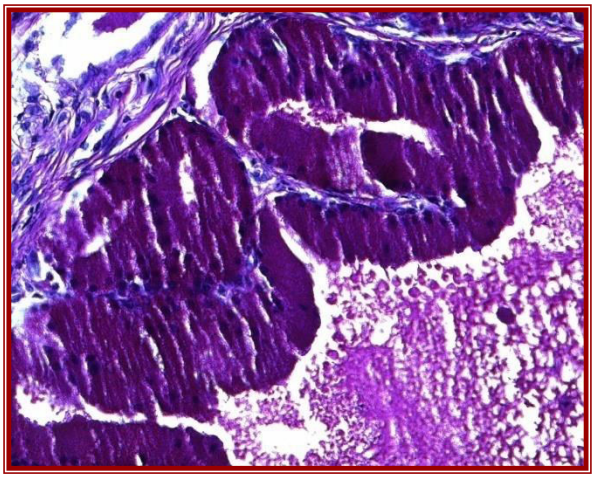

Fig. 4. Tongue. Ostrich. Detail of the glands situated on the ventral face of tongue AA-PAS stain, x 630 (original)

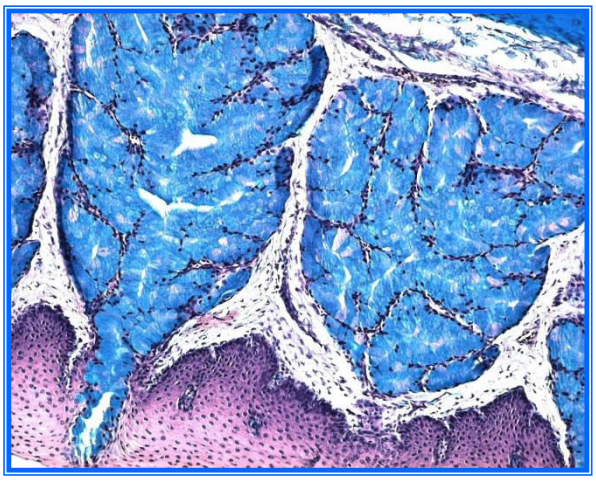

Fig. 5. Tongue. Ostrich. Ventral face epithelium, with large glandular lobe
AA stain, x 100 (original)

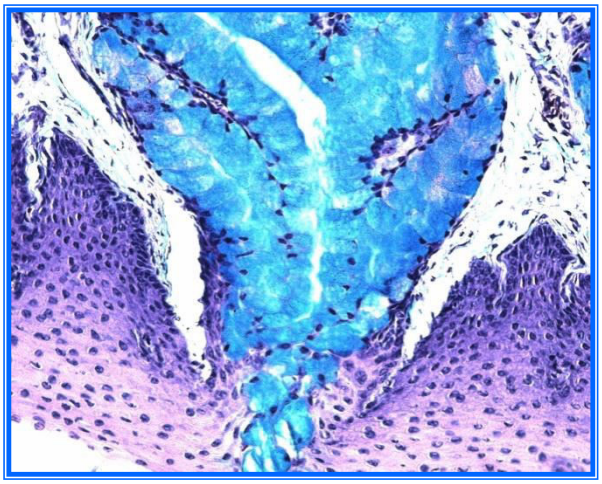

Fig. 6. Tongue. Ostrich. Detail of mucus elimination by excretory duct of theventral
mucosa level AA stain, x 200 (original)

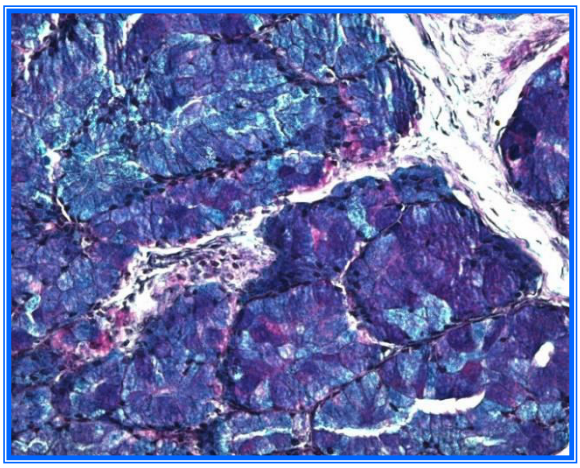

Fig. 7. Tongue. Ostrich. Aspects of mucus secreting cells positive AA and PAS positive AA-PAS stain, x 200 (original)

The histostructure of the tongue includes the mucosa, the cartilaginous support piece and the striated muscles attached to it (Fig. 8). On both the dorsal and ventral side, the tongue is covered with a squamous, stratified, non-keratinized epithelium (Fig. 8, 9), with the indication that the dorsal part of the epithelium is thickened.

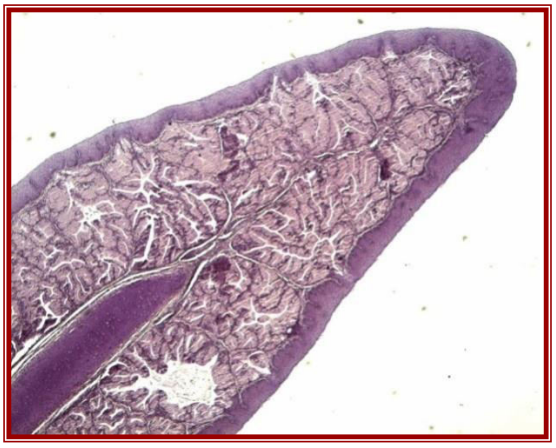

Fig. 8. Tongue. Ostrich. Cross section of the tongue, ventral surface of the tongue base at the junction with the caudal process. Stem cartilaginous support of soft tissue. HE stain, x 50 (original)

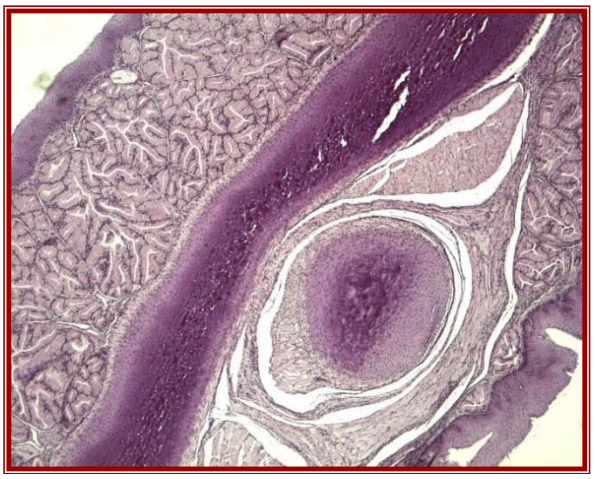

Fig. 9. Tongue Ostrich. General view of the ostrich tongue, with evidence of the layered squamous non-keratinized epithelium and hyaline cartilage in the top of the tongue ring, HE stain, x 50 (original)

In its structure we encounter more salivary glands, both on the dorsal and ventral side. The glands show large lobules on the ventral face and on the dorsal they are grouped as small lobules (Fig. 10, 11).

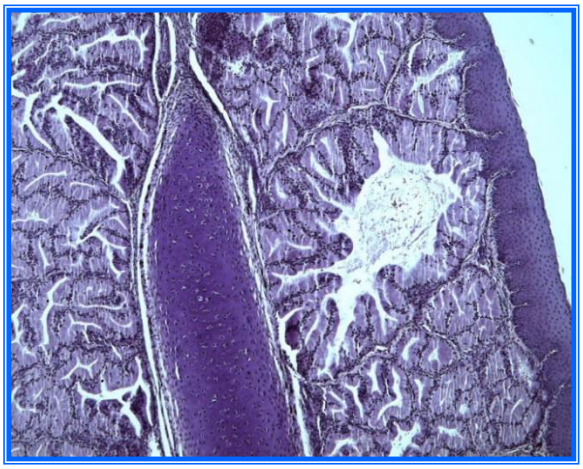

Fig. 10. Tongue. Ostrich. Section carried out in the middle portion of the tongue, with detail of mucous glands and highlighting glandular lumen, AT stain, x 100 (original)

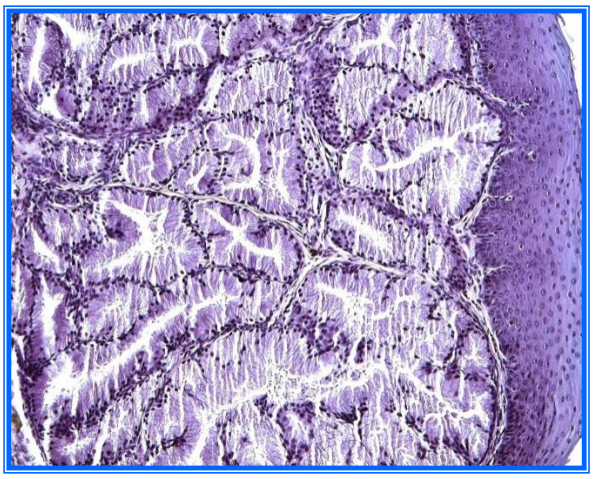

Fig. 11. Tongue. Ostrich. Glandular lobules on the underside of the tongue. H.E.A. stain, x 200 (original)

The mucus-secreting glands are simple, branched, tubular-alveolar, linked to each other by connective tissue sepsis in which the presence of collagen fibers, fibroblasts and blood vessels in common colors (Pph and HEA) is observed (Fig. 12, 13) and in special colors (Gomori) the presence of elastic connective fibers is obvious, they surround the glandular lobules (Fig. 14).

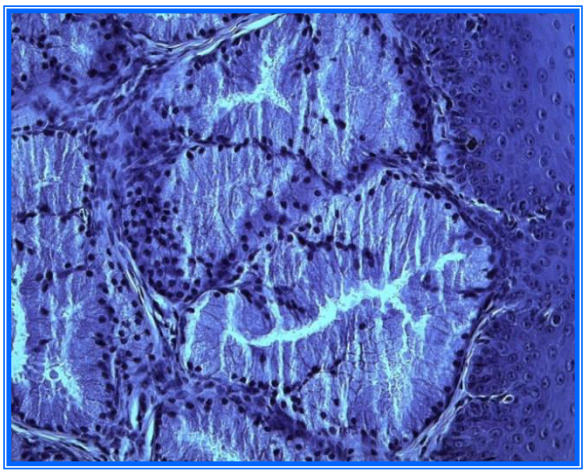

Fig. 12. Tongue Ostrich. Tip of the tongue, detail of simple tubulo-alveolar glands, Pph stain, x400 (original)

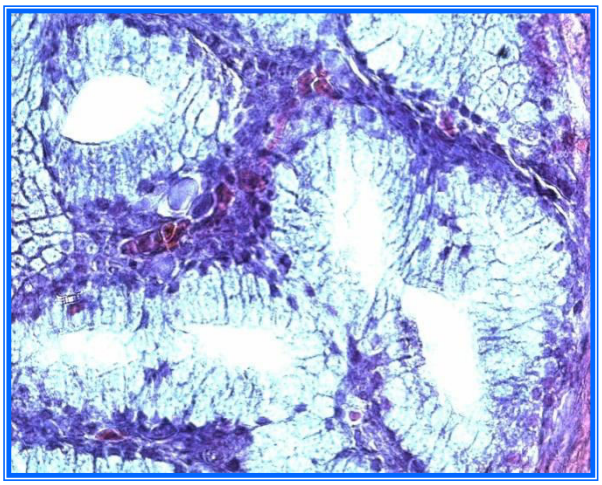

Fig. 13. Tongue. Ostrich. Histological structure aspect of the salivary tubulo-alveolar mucosal glands. Lamina propria with irregular conjunctive tissue containing collagen fibers, blood vessels and fibroblase, HEA stain x 630 (original)

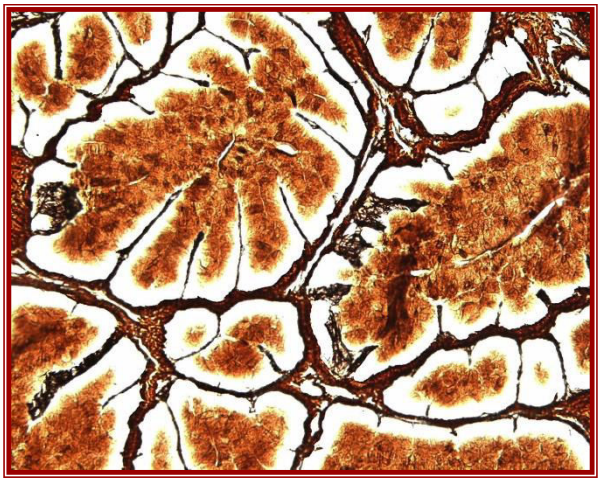

Fig.,14. Tongue. Ostrich. Detail of silver impregnation response of the structure of the language, Gomori stain, x 200 (original)

The branched tubular-alveolar glands are lined with a simple columnar epithelium (Fig. 15, 16) - the cytoplasm of glandular epithelial cells is sparse, abundant, and spherical or flattened nuclei appear basal (Fig. 17, 18).

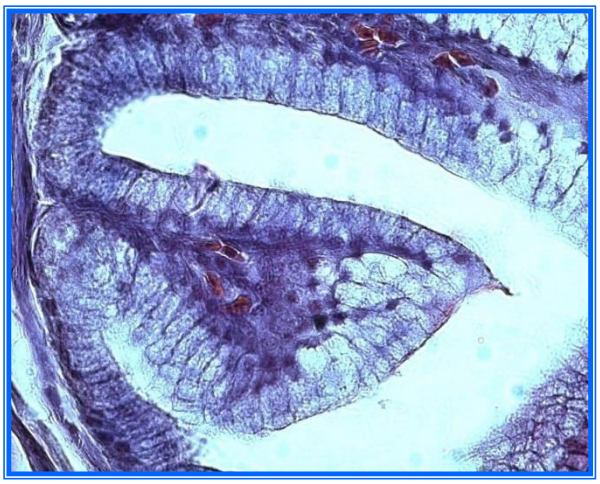

Fig. 15. Tongue. Ostrich. The aspect of the glandular simple columnar epithelium. Blood vessels in the lamina propria, gland lumen HEA stain, x 630 (original)

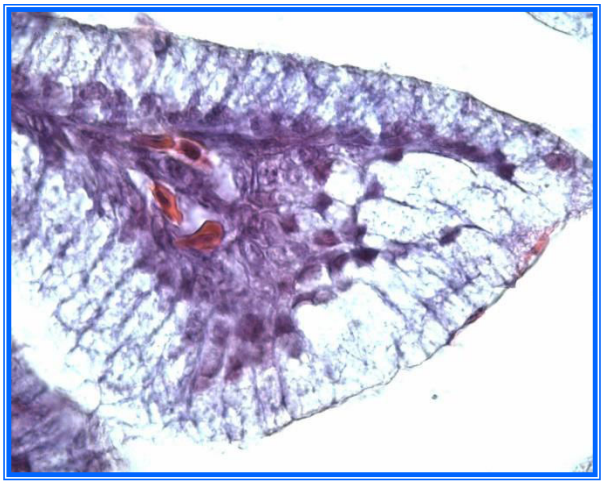

Fig. 16. Tongue. Ostrich. Aspect of the top apical of the salivary gland HEA stain, x 630 (original)

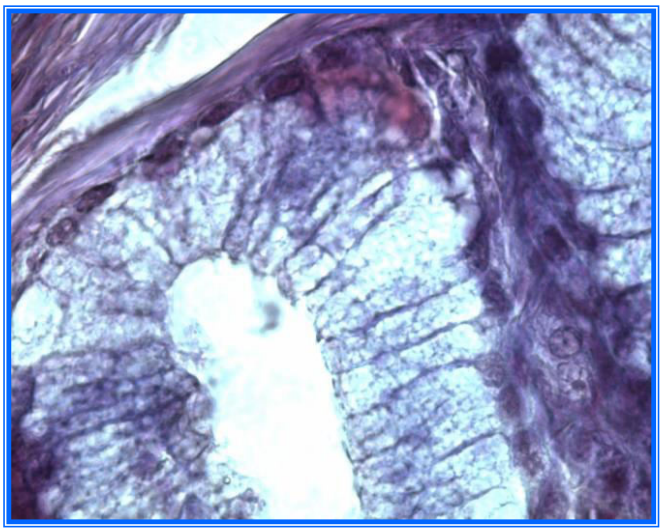

Fig. 17. Tongue. Ostrich. Detail of the salivary glands. There is foamy cytoplasm of epithelial cells HEA stain, x 1500 (original)

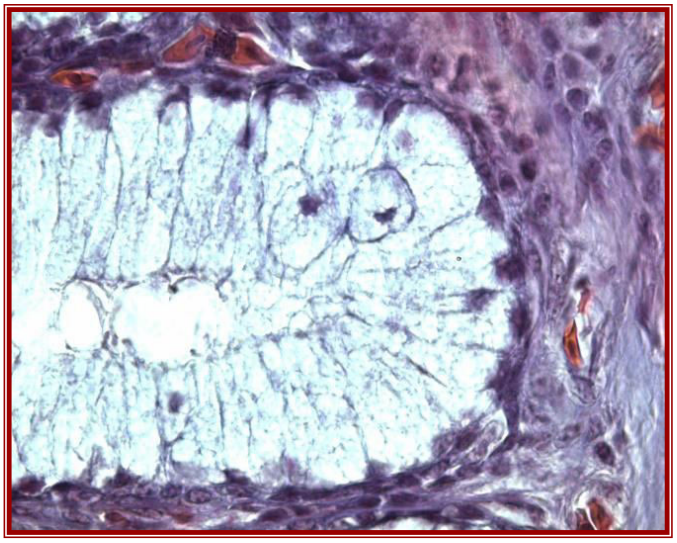

Fig. 18. Tongue. Ostrich. Detail of glandular epithelium, with highlighting round nuclei, willing basal. HEA stain, x 1000 (original)

Conclusions

At the level of the tongue, both the dorsal face and the ventral face, we encounter numerous alveolar salivary glands.

The glandular epithelium is simply prismatic, the cells exhibit the vacuolar cytoplasm with oval shaped nuclei disposed at the basal pole.

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APPLICATION OF HACCP IN A FISH UNIT - SMOKED FISH

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Summary

The purpose of these paper is to identify and monitor the critical points on the technological flow in a fish processing unit and to identify the potential microbial agents that may affect the final product, implicitly the health of the consumer (*E. coli, L. monocytogenes, Salmonella, Staphylococci, coagulase-positive*). The implementation of a HACCP plan in fish product establishments has been beneficial both for the economic operator and the end consumer. By applying this food safety system, the consumer confidence increases for products in this category, given that fish is a perishable product with a risk of contamination during the technological flow, regardless of the form under which it is sold (smoked, marinated fish, fish pastrami, various types of salads, etc.)

Keywords: HACCP, smoked fish, hazard

Fishery products, which are of great importance for human nutrition worldwide and provide clear health benefits, can also act as a source of food-borne pathogens (2). Food poisoning organisms in fish are often divided into two groups: those that are naturally present in the freshwater environment, referred to as indigenous bacteria, and those associated with pollution of the aquatic environment. A third group includes bacteria introduced into fish and fish products during post-harvest handling and processing (3).

For centuries, smoking has been a popular way to preserve fish. The applications of salt, smoke and, in some products, nitrate imparts a characteristic texture and flavor that is enjoyed throughout the world. With the advent of refrigeration, these products now contain less salt and smoke and have higher concentrations of moisture. In addition, packaging systems such as vacuum packaging with high barrier films have extended shelf life.

To ensure that smoked products do not contain *L. monocytogenes* or *Salmonella spp*., processors must develop and implement HACCP plans along with comprehensive cleaning and sanitation programs.

The objective of our study was to detail the flow diagram, to assess physical, chemical and biological hazards and to identify critical control points for *Cyprinus carpio* on processing line.

Materials and methods

Description of product

Smoked carp, processed in the form of fillets that can weigh between 100-400 grams; the smoking process is warm and hardwood sawdust is used. The packaging of the final product can be done in polypropylene bags by means of vacuuming. Fish smoking is performed in automatic smoking units, the smoke results from burning of beechwood sawdust, taking into account the steps of drying, smoking and cooling an also the temperature, time and the humidity parameters. Smoke phases are monitored in the "HACCP Daily Record", keeping the product at 63ºC for at least 30 minutes. Cooling is considered to be finished when the product has a temperature of less than 35°C. With regard to organoleptic characteristics, the meat has a compact appearance, free of grease and odors or unpleasant tastes. From the chemical point of view, the meat has a total protein content - minimum 18%, max. 5.5%. Following the analysis of the carp fillet, it has been found that the percentage of fat can vary between 4% - 8%, depending on the geographical area it originates and depending on the type of food consumed and the season.

Organoleptic characteristics

Appearance: Smoked carp fillets are cut transversely cut, packed in a polypropylene bag by means of vacuuming. The fillets have a smooth surface, with no spots and notearing of the skin; in section, the color is yellowish-white, without traces of blood or viscera, compact muscle structure. The weight of the packaged product may vary between 150 and 400 grams.

Consistency: Muscle texture is compact without fat clumps.

The color of the skin after smoking is golden, uniform and the muscles are brownish-reddish.

The taste and flavour of the product - by smoking the product gets a pleasant taste and smell.

Chemical composition

Sodium chloride: maximum 5-7%;
Fat: 5.5%;
Water: 70 - 75%;
Protein: min 18%;
Mineral substances: 1.1%

Technology Flow Diagram

Quantitative and qualitative fish receptionis done in vivo, the fish beingtransported from fish farms into hydrocontainers and fish must be ready for processing within 3 hours as of entering the unit. One checks the water temperature, the documentation of each batch of fish, it visually appreciates the freshness, the existence of external parasites. The result of the reception is recorded in the register "Reception of raw materials" and "Aquaculture reception form", the quantity and the organoleptic characteristics are recorded by entering

the corresponding/inappropriate rating and the batch number is assigned. In fish farms prior to live fish delivery to processing units, they arenot fed for at least 24 hours by keeping them in cages specially designed for this purpose, the effect being the elimination of gastrointestinal content, thereby diminishing the intensity of proteolytic activity of fish after fishing.

Fish conditioning is carried out in stainless steel pools with water cooled with ice flakes, the temperature in the basin's thermal center must be up to 2ºC.

Fish storageis an intermediate stage and it aims at keeping the product's quality characteristics during storage at a temperature of -1 to +2°C. The deposit is made in PVC valves in alternative ice layers.

The primary processing of fish consists of manually executed operations: removal of scales, head, tail, evisceration, milt and egg collection and filleting.

Scale removal is done manually and the temperature in the room where these operations take place must be at maximum 10ºC. Technological waste is collected in crates lined with household bags, which are periodically discharged through the waste disposal facility provided with a chopper and a transport pump into the landfill at a temperature of +2 ... + 8°C;

The removal of the head is done by performing a V-incision, besides the opercula, the resulting head is collected in PVC shutters lined with household bags placed under the work table, on a shelf specially arranged for this stage and the fillets are collected in platic crates, to the evisceration table;

Evisceration is done by cutting the abdomen along the fish, from the head to the anal opening, and the collected waste is evacuated in the same way as the scales;

The milt and eggs are collected during evisceration by separating them from the rest of the gastrointestinal mass in vessels destined for this purpose. During the evisceration, a visual inspection of the parasites is performed continuously and at the same time eliminating them with the abdominal content;

Rinse the fish - after evisceration, the resulting fish trunk is rinsed with a mixture of water and ice flakes and collected in plastic crates and transported into the filletingroom;

The fish trunk filleting is executed manually by practicing an incision on the dorsal side of the fish along the spine, in addition to the dorsal fin and entering with the blade of the knife under the fillet, above the bones that delineate the abdominal cavity, until the total detachment of the fillet. The resulting files are collected in plastic crates and each layer is covered with ice flakes.

Salting of fish fillets in the hot smoking process is done in order to preserve and improve the gustative qualities. The quantity of salt is 5 - 7% of the weight of the fish, to which 10% of the sodium benzoate is added to the salt. Salting is done in stainless steel tanks, as follows: sprinkle a thin layer of salting mixture at the bottom of the basin, place a thin layer of skin with the skin down, place the next layer of fillets,filling the smaller spaces, the salt is evenly distributed until the fillets are exhausted, the last layer being salt. The salting method used is

the traditional, dry salting. Salting time is minimum 16-24 hours. Salting time depends on the size of the fish, the fishing period, the ambient temperature and the salting method. When ambient temperature is low, dry salting is completed with wet salting.

Smoked fillets are placed on trolleys provided with stainless steel sieves, the space between the fillets must be 3-5 cm to ensure an uniform smokingof the fillets. Smoked fish fillets are made in the automated smoking unit, the entire process of thermal processing being controlled by the electronic microprocessor, which has the possibility to fulfill all the requirements of the smoker operation and of the set parameters (time, temperature, humidity).

Smokingkeeps the tradition of hot smoking at a temperature of 60-75°C inside the unit during the actual smoking and baking operations when the temperature of 65°C is reached in the product's thermal center, maintaining it for at least 30 minutes. Drying at 45°C provides the necessary conditions for depositing smoke compounds on the surface of the fish, removing free water, coagulating proteins in the superficial layer of the meat, limiting water evacuation. The timing of drying and humidity is programmed to ensure uniform smoke and the specific color. The composition of the smoke is influenced by the type and humidity of the wood used (generally sawdust from beech wood).

Smoked fish maturation aims to interrupt the action of high temperature on fish meat and lead to weight loss reduction. This phase begins when the smoked fillet is cooled below the 35ºC in the smoking unit. The smoked fish is matured by storing in the storage room for 16-24 hours, up to a temperature of 0 ... + 4°C for uniformity of taste and flavor and then after packing up to 0 ... + 4°C.

Packaging of the finished product is performed by forming a 1-sided cross-cut package with golden outer skin, inserted into 100/90/10 mm bags closed by vacuum to maintain the shelf life, this operation being performed in the hall packaging the product.

Table 1.

HACCP principles

No.	Principles
1	Establish the potential hazards and conduct a hazard analysis
2	Determine the Critical Control Points (CCP)
3	Establish critical limits for each CCP
4	Establish a monitoring system to control each CCP
5	Establish the corrective action to be taken when monitoring indicates that a particular CCP is not under control
6	Establish verification procedures to confirm that the HACCP system is working effectively
7	Establish documentation concerning all procedures and records

Weighing and labeling are carried out to determine the weight of the finished product. Labeling is done for the purpose of identifying the finished product and executed manually in the same room as the packaging.

The storage of the product is carried out in cooled rooms at a temperature between 0 to + 4ºC to ensure its validity for 45 days. The codex protocol for the application of the HACCP system includes seven principles (Table 1).

Results and discussions

Smoked fish is a product that is generally obtained from fresh fish, frozen or salted by hot or cold smoking. In general, for the production of a quality product, it is recommended for smoking to use wood sawdust, which has the property of preventing the spoilage of the fish, also offering special organoleptic properties**.** Hot smoking is the process used in the majority of smoked fish products. Hot-smoked products are fully cooked and can reach temperatures as high as 82°C. Because of the high temperatures, hot smoking takes only a short time, depending on the internal temperature of the product. Different species of fish tolerate heat differently, so the hot smoke process is not the same for all products. The process is tailored to the species, the processing equipment used, market demand, distribution considerations and regulatory requirements. Hot-smoked fish are most and juicy when properly finished. Because of this, they have a relatively short shelf life and must be refrigerated.

Current guidance from health regulatory agencies recommends a minimum internal temperature of 63°C for 30 minutes. Following smoking, the products should be stored at or below 3°C. Hot-smoked products should also contain a minimum water phase salt concentration of 2.5% if aerobically packaged and 3.5% salt if anaerobically packaged.

Hotsmoked fish receives a cook, for example, 62.8°C (145°F) for 30 min that should inactivate vegetative pathogens. The issue of *Listeria monocytogenes* in hot-smoked fish is the need to prevent recontamination after the cook through plant sanitation and other methods.

The flow in the processing plant must adequately separate the finished product from all raw product in order to prevent cross contamination with pathogens, especially *Listeria monocytogenes*. This is particularly important after the heating step where there are no competing spoilage bacteria on the product.
Other factors that may affect the survival of *L. monocytogenes* include the formation of a “pellicle,” where the surface dries before the application of smoke which decreases the inhibitory effect of smoke. The use of liquid smoke may also provide an inhibitory effect.

Salmonella spp. are not recognised as part of the normal flora of the temperate aquatic environment and their presence in aquaculture products is related to rearing practices, as well as to faulty hygiene practices during post-harvest handling and processing (2).

Contamination with E.coli, is also an important health indicator, can be crossed, during evisceration or after post processing of the product, contamination can occur in contact with the other products or with the persons who are carrier and germ-eliminating. must be taken into account that the germs of *E.coli* are destroyed at temperatures above 60 degrees as presented in Tabel 2, below.

Table 2

HACCP Plan

Stage	Important hazards	Control measures	PCC /PC	Critical limits	Monitoring procedures			Corectie/Actiuni corective		Documents/ Records
					Method	Frequency	Responsible	The action	Responsible	
Reception over fresh fish / frozen fish	*Listeria monocytogenes* *Salmonella* *E.coli*	Checking vendor analysis bulletins Supplier selection Annual analysis by a neutral laboratory	**PC1**	Salmonella 25/g absent E coli- abs	Verification of vendor quality certificates	At each batch Once a year	Reception Commission Veterinarian.	Reject batch Supplier selection	Chief Purchasing Officer	Registers of raw materials, auxiliaries and packaging
Storage	*Listeria monocytogenes* *Salmonella* *E.coli*	Temperature monitoring	**PCC1**	-18 ºC +/- 3ºC Max 1 an	Freezer temperature monitoring	continuously	Veterinarian.	Partial thawing - direct routing to machining Switch to own electrogen group Adjustment of freezing equipment maintenance Personal trening	Department head F Refrigeration Technician	Computer memory
Defrost	*Listeria monocytogenes* *Salmonella* *E.coli*	Temperature monitoring	**PC2**	Max + 15 ºC	Temperature monitoring	2 times / batch	Defrosting Operator	Temperature adjustment maintenance Personal training Immediate processing over	Department head Refrigeration Technician	Temperature chart thawing

Salting	*Listeria monocytogenes* *Salmonella* *E.coli*	Correct dosage Temperature monitoring and duration Laboratory tests	**PC4**	Min. 8 % Max 15ºC Max 96 h	Weighing Visual control	For each batch	Soil operator	Salt concentration correction Verification metrological scales Personal training	Head of department	Data / register
Smoking	*Listeria monocytogenes* *Salmonella* *E.coli*	Monitoring product temperature and duration smoking process	**PCC2**	Min 68ºC for min 1 min Smoke cycle duration 4 hours	Temperature and duration monitoring	For each bounce	Smoking operator	Resume process Personal training maintenance Smoke cell adjustment	Mechanic Department head	Vacuum documentation
Vacuum	*Listeria monocytogenes* *Salmonella* *E.coli*	Monitorizare presiune Programare corecta	**PCC3**	Presiune min. 570 mb Timp 2 sec	Visual inspection	For each packing unit	Vacuum operator	Repackagin, -vacuuming Adjusting the vacuuming device Training the maintenance personnel	Department head Mechanic	Vacuum documentation
Storage of finished product	*Listeria monocytogenes* *Salmonella* *E.coli*	Monitoring temperature	**PCC4 ?**	0ºC - +4ºC	monitoring temperature	Continuously	Veterinarian. Administrator	Immediate intervention by regulating refrigeration installations Own electrogen group	Refrigeration Technician	Computer memory Temperature chart

Conclusions

Implementation of the HACCP system in smoked fish line proves to be a necessary tool for improving the safety and quality characteristics of fish products.

Following hazard analysis, four CCP (critical control points) were established for smoked fish: fish reception, fish storage, smoking of fish and cold storage of the fish products .

The application of the HACCP system is not a stand-alone system, but it should be seen as an element of food safety management. It complements basic good hygienic practices in food safety assurance by targeting product-specific hazards and devising control measures necessary for managing risks relevant to the product and conditions of operations

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BACTERIA OVERGROWTH ASSOCIATED WITH PARVOVIROSIS IN DOGS

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Summary

Parvovirosis in young dogs is one of the most serious and frequent viral diseases encountered in young dogs. Various bacteria can be involved, leading to over-infection of the gastrointestinal tract, increasing the risk of potentially fatal outcome. Our study aimed to isolate, characterize and identify bacteria associated with CPV-2b infection in dogs that can aggravate the health of patients suffering from parvoviral gastroenteritis. The research included 9 dogs admitted to our clinic, with the clinical and epidemiological suspicion of gastroenteritis. The fecal samples collected were used for the snap test band confirmation of the disease and microbiological examination. Qualitative and quantitative evaluation of the faces confirmed secondary bacterial overgrowth for 88,88% of the dogs included in the study (8/9). Compared to the literature, coliforms were found in association with other species of bacteria, both Gram positive and Gram negative. These results underline the importance of target antimicrobial therapy in order to reduce the septicemic and toxemic complication, thus increasing the survival chances. Further studies are intended to monitor the emergence of antimicrobial resistance in case of bacteria isolated from dogs with parvovirosis.

Keywords: *parvovirosis, bacteria, dogs, gastroenteritis*

Canine parvovirosis is regarded worldwide as one of the most important infectious diseases encountered in dogs due to the severity of clinical signs and the lethal course (9). The virus is small non-enveloped, containing a single stranded DNA of circa 5.2 kb in length (10).

Research has proven that only dogs with ages between six and twelve week old are prone to infection (3). A study conducted in 2004 by Jennifer Prittie has shown that dogs with ages between 6 weeks and 6 months are at ages when they are the most unprotected to the action of the virus. It is believed that older dogs are immune to the virus, either by natural infection or immunization (8).

Some breeds are more at risk than the others in contracting the disease. Rottweilers, Doberman Pinschers, American Pit Bull terriers, Labrador retrievers, and German Shepherds are breeds most frequently cited as sensible to parvoviral gastroenteritis (7). Studies have proven that there is a seasonality of the occurrence of parvovirosis, which is between July and September. Also it appears that sexually intact males are more susceptible to the disease than the unspayed females (2, 5).

The infection can occur through two ways, a direct one and an indirect one. The direct one takes place through fecal-oral route and the other one is via exposure to infected objects, clothes, and people. The virus first replicates in the local lymphoid tissue and in the oropharynx (7).

After three or five days the virus spreads in the target tissues that contain rapid cell turnover (15). These aforementioned cells are found in the intestinal epithelium, the bone marrow and the lymphoid tissues. Sure, if the infection occurs in neonates then the heart is as well affected (3).

The most characteristic microscopic intestinal lesions consist in the atrophy of the intestinal villi, the disruption of the lamina propria and the necrosis of the intestinal crypts (4). Parvovirosis may be more severe when bacterial overgrowth occurs. Normally the canine gastrointestinal tract is the “home” of a large population of bacteria, viruses and fungi (21). This microbiota is beneficial to the host, being like a defense barrier, protecting the immune system (10). Disbalances to the intestinal microbiota can aggravate the course of the disease (18).

In 1990 Turk and collaborators isolated *E.coli* from the lungs and liver of almost all the dogs that succumbed to CPV. It is believed that bacterial endotoxin and Gram negative bacteria are potent triggers to the inflammatory processes that lead to SIRS, sepsis and death (17, 22). Literature findings show that the most frequently encountered secondary invaders are *β-hemolytic E.coli, Cl. Perfringens or Difficile* and *Salmonella spp.* In addition to this parvovirosis may be more rapid or severe in dogs with an already compromised gastrointestinal barrier, as seen with concurrent endoparasites or canine coronaviral infection (11).

Diagnosis of this pathology is usually based on clinical signs, although literature cites the use of commercial snap tests (1). This test is a rapid enzyme immunoassay that detects the canine parvovirus antigen in the canine feces. The company that manufactures the tests have a sensitivity and a specificity of 95 % (Idexx laboratories).

The treatment of this disease, it being a virosis, it is strictly symptomatic. Fluid therapy is extremely important in order to combat dehydration and electrolyte imbalance. Fluid therapy usually consists in a mix of a sterile, balanced electrolyte solution, with of a right amount B-complex vitamins, crystalloid or colloid fluids. In addition to this,antiemetics and broad-spectrum antibiotics are also used (12).

The prevention and control of parvovirosis can be performed with good results by vaccination and rigorous disinfection (6, 16).

Materials and methods

The study included 9 dogs that were diagnosed with canine parvovirosis, using commercial snap tests at the same time relying on the clinical signs. Fecal samples were collected on the first day of treatment, using sterile swabs. Using standard microbiological techniques, the samples were processed in the Infectious Diseases Laboratory from FMV Cluj-Napoca. First of all they were inoculated onto

sterile broth, then on MacConkey medium and simple agar medium. Incubation takes place at 37°C for 24 hours.In samples that were cultured on the sterile broth, we observe the degree of turbidity, the presence or absence of the deposit or ring on its surface. On the simple agar we count for the color of the colonies, their size and appearance. Moreover, on MacConkey we can differentiate the bacteria based on lactose fermentation.

It is also necessary for us to make smears, using Gram stain, in order to check the morphology and tinctorial proprieties of isolated bacteria from our patients.

Results and discussions

Out of the dogs included in our study 88,88% of the dogs were positive to secondary bacterial overgrowth. In sterile broth we could observe the moderate turbidity in all of the samples, with the presence of a white deposit (Fig.1). On simple agar the colonies appear white, smooth of variable sizes, meaning that there is more than one type of bacteria involved (Fig. 2). On MacConkey agar, the colonies are red, smooth, lactose-negative (Fig. 3), but also lactose-positive. On the Gram stain smears different types of bacteria (Fig. 4) described in Table 1.

Table 1.

Description of bacteria of the Gram stain smears

Sample	Bacteria
1	**Coccobacilli +**
2	**Bacilli G -**
3	**Cocci G-**
4	**Negative**
5	**Bacilli bipolar**
6	**Coccobacilli – and +**
7	**Bacilli g+**
8	**Bacilli g – and cocci +**
9	**Coccobacilli -**

Fig. 1. Aspect on broth

Fig. 2. Colonies aspect on simple agar

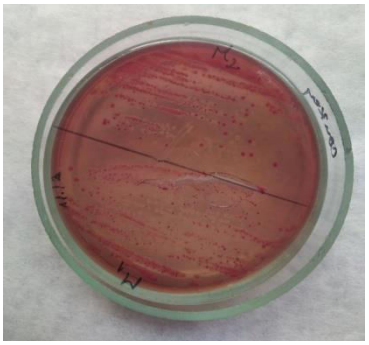

Fig. 3. Aspect on MacConkey agar

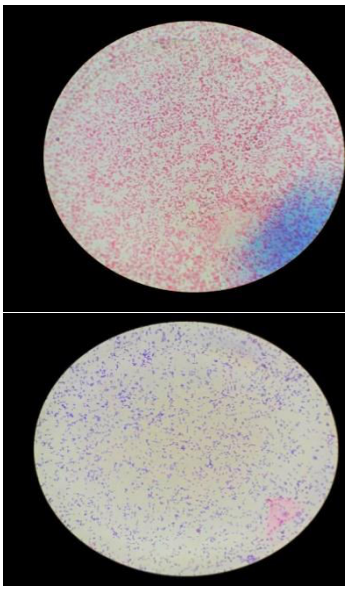

Fig. 4. Gram – (up), Gram + (down)

Conclusions

These results underline the importance of target antimicrobial therapy in order to reduce the septicemic and toxemic complication, thus increasing the survival chances. Further studies are intended to monitor the emergence of antimicrobial resistance in case of bacteria isolated from dogs with parvovirosis.

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