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# WILD RUMINANTS` INTESTINAL MICROFLORA IDENTIFICATION BY THE MALDI-TOF-MASS SPECTROMETRY PROTEOMIC TECHNIQUE

## IDENTIFICAREA MICROFLOREI INTESTINALE LA RUMEGĂTOARELE SĂLBATICE PRIN TEHNICA PROTEOMICĂ MALDI-TOF-Ms

Luminița COSTINAR[1)], Narcisa MEDERLE[1)], Monica CRÂSTIU[1)], Lucia Elena IONESCU[2)], A.A. MUNTEAN[2)], M. NECȘULESCU[2)], Corina PASCU[1)], V. HERMAN[1)]

**ABSTRACT | REZUMAT**

Wild ruminants, such as Carpathian fallow deer (*Dama dama*) and Carpathian chamois (*Rupicapra rupicapra carpatica*), represent an important part of the natural ecosystem of Romania. These animals had a significant impact on biodiversity and the well-being of the ecosystem through their pasture behaviour and interaction with the surroundings. Because of their ecological importance, wild ruminants are hosts for microorganisms and bacteria. The subject of the study was to research and compare the bacterial profiles of wild ruminants, including Carpathian fallow deer and Carpathian chamois. This study used common identification and isolation methods, as well as modern identification techniques like proteomic technique, and MALDI-TOF Ms technique, for high-accuracy information regarding the bacterial flora of the digestive system. This paper aimed to study and observe newly developed antibiotic resistance profiles in bacterial flora isolated from these wild species and to compare our data with the existing ones in the literature. Our goal was to understand the relationship between these wild animals and their intestinal microbiota, and to know the potential microbial risks associated with their late-developed antibioresistency phenomenon. The low resistance to the tested antimicrobials denotes that the wild animals (Carpathian fallow deer and Carpathian chamois) from which the bacterial strains originated have not yet been subjected to high infectious pressures, especially those obtained from fallow deer (*Dama dama*).

**Keywords**: antibiotic resistance, Carpathian fallow deer, Carpathian chamois, *E. coli*, *Serratia spp.,* MALDI-TOF Ms

Rumegătoarele sălbatice, cum ar fi cerbii lopătari (*Dama dama*) și caprele negre (*Rupicapra rupicapra carpatica*), reprezintă o parte importantă a ecosistemelor naturale din România. Aceste animale au o influență semnificativă asupra biodiversității și funcționării ecosistemelor prin intermediul comportamentului lor de pășunat și prin interacțiunea cu mediul înconjurător. Pe lângă importanța lor ecologică, rumegătoarele sălbatice sunt, de asemenea, gazde pentru diverse microorganisme, inclusiv bacterii. Scopul acestei lucrări a fost de a investiga și compara pe cât posibil, profilurile bacteriene ale rumegătoarelor sălbatice, de la cerbi și capre negre. Am utilizat atât metode de izolare și identificare bacteriană uzuale cât și tehnici moderne de identificare bacteriană și anume, o tehnică proteomică, tehnica MALDI-TOF Ms, pentru a obține informații deosebit de importante despre bacteriile prezente în tractul digestiv al acestor animale. Pe de altă parte ne-am propus prin această lucrare să contribuim la stabilirea unor profiluri de rezistență la antibiotice a florei bacteriene izolate de noi, comparând datele noastre cu cele deja existente în literatura de specialitate. Raționamentul nostru a fost de a înțelege relația dintre aceste animale sălbatice și microbiota lor intestinală, precum și să cunoaștem potențialele riscuri microbiene asociate care pot să apară ca urmare a acestor fenomene de antibiorezistență.

**Cuvinte cheie**: rezistența la antibiotice, capra neagră, cerb lopătar, *E. coli*, *Serratia spp*., MALDI-TOF Ms

The phenomenon of antimicrobial resistance in both humans and domestic animals is growing and has reached alarming levels, making the presence of bacteria carrying antimicrobial resistance genes (AMR) an increasingly serious and complex threat. Today, due to the expansion of urban populations, this event is spreading and contact between humans, domestic animals, and wildlife is very close. It is therefore of utmost importance to study this aspect of antimicrobial resistance from a "one health" perspective. This perspective must be a multidisciplinary, integrated approach, with particular emphasis on issues related to identifying key priorities for combating antimicrobial resistance. Even if wild animals are unlikely to be treated with antibiotics, the overlap between wildlife, domestic animals,

1) University of Life Sciences "King Mihai I", Faculty of Veterinary Medicine, Timişoara, Romania
2) INCDMM Cantacuzino, Bucharest, Romania
*) *Corresponding author: corina_pascu_ro@yahoo.co.uk*

and human habitats inevitably increases the transmission of antibiotic-resistant bacteria. In Romania, there is very little research on aspects of the antibiotic resistance phenomenon in wild animals.

However, even though many studies in the literature from other countries state that wild animals are reservoirs and dispersers of antimicrobial-resistant bacteria, this role is not yet well established. To make this claim, including in our country, further epidemiological research is needed, because the mere fact that they are carriers of AMR bacteria does not mean that they can be a vehicle of contagiousness for humans or other animals. Therefore, we aimed through this work to contribute to the establishment of antibiotic resistance profiles in our isolated bacterial flora, comparing our data with those already existing in the literature. Our rationale was to understand the relationship between these wild animals and their gut microbiota, as well as the potential associated microbial risks that may occur as a result of these antibiotic resistance phenomena.

## MATERIALS AND METHODS

To reach the purposed aim, bacterial strains were isolated from the duodenum provided by 3 Carpathian fallow deer (*Dama dama*) and 3 Carpathian chamois (*Rupicapra rupicapra carpatica*). The primary isolation and biochemical characterization were done on different culture media: Columbia blood agar (Oxoid), Uriselect 4 (Bio-Rad, chromogen agar), Mueller-Hinton agar (Oxoid), Nutritive broth (Oxoid), and selective culture media EMB, MIU, TSI, and XLD (Oxoid). To inhibit the invasion phenomena of *Proteus spp* and to confirm the isolates' purity, the samples were also cultivated on chromogenic media. Proteomic identification with the MALDI-ToF-Ms technique was realized based on the related methodology described further:

• Each dispersion from the initial plate was examined by minimum 2 examinators and one sample from each isolated colony, with individual morphological aspect, resulting after incubation at 37°C, 18-24 h, was taken and applied on the MALDI-ToF-MS plate in duplicate.

• After drying, was applied 1 µL matrix 4-*HCCA (acid α-Cyano-4- hydroxycinnamic, α-Cyano-4-hydroxycinnamic acid*) and waited till dry again. The plate has been put to analysis for identification using MALDI-ToF-MS Bruker mark, model auto Flex Speed.

Sensibility testing on antimicrobial substances was realized on disk-diffusion method (Kirby-Bauer) using primary culture or subculture following standard procedure, adopted by the Clinical and Laboratory Standard Institute (CLSI), USA.

For testing was selected next substances:

1. Amoxicillin (AML), 2. Ticarcillin (TIC), 3. Piperacillin (PRL), 4. Amoxicillin-Clavulanate (AMC), 5. Ticarcillin -Clavulanate (TCC), 6. Piperacilin-Tazobactame (TZP), 7. Cefoxitin (FOX) 8. Moxalactam (MOX) 9. Mecilinam (MEL), 10. Cefotaxime (CTX), 11. Ceftazidime (CAZ), 12. Cefepime (FEP), 13. Temocillin (TEM), 14. Aztreonam (ATM), 15. Ertapenem (ETP), 16. Meropenem (MEM), 17. Imipenem (IMP), 18. Ceftazidime-Avibactam (CZA), 19. Ceftolozan-Tazobactam (C/T), 20. Gentamicin (CN), 21. Tobramycin (TOB), 22. Amikacin (AK), 23. Netilmicin (NET), 24. Ciprofloxacin (CIP), 25. Levofloxacin (LEV), 26. Rifampicin (RD) 27. Chloramphenicol (C), 28. Nitrofurantoin (F), 29. Fosfomycin (FOS), 30. Trimethoprim-sulf (SXT), 31. Colistin (CT).

## RESULTS AND DISCUSSIONS

After the incubation, the cultures were analysed and interpreted based on their cultural aspects. Figures 1 and 2 present cultures obtained from Carpathian fallow deer samples, and Figures 3 - 6 present cultures obtained from Carpathian chamois samples.

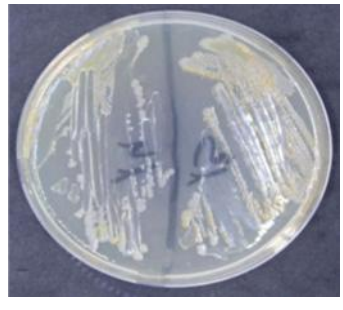

**Fig. 1.** The aspect of primary cultures on nutritive agar

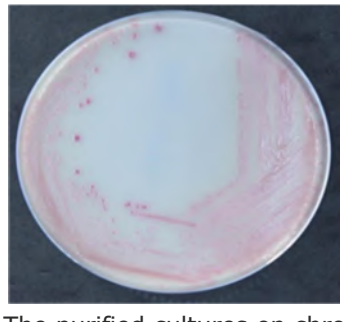

**Fig. 2.** The purified cultures on chromogen culture media Uriselect 4

**Fig. 3.** The aspect of primary cultures on nutritive agar

Were isolated and identified, in total, 6 isolates, *E. coli* – 4 isolates, 3 from Carpathian fallow deer and one from Carpathian chamois, and *Serratia* spp. – 2 isolates, both from Carpathian chamois.

Identification and classification *E. coli* strains were made based on morphological, biochemical, and tinctorial characteristics. Figure 4 presents characteristic aspects of *E. coli* isolated from Carpathian fallow deer.

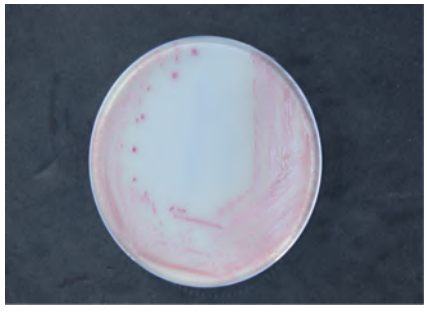

**Fig. 4.** The characteristic aspect of *E. coli* strain isolated from Carpathian fallow deer on chromogenic agar

Isolated *E. coli* strains were mobile, and produced indole, fermented glucose, lactose, and sucrose with gas release. Also, they didn't produce hydrogen sulphide. To easily differentiate isolated strains from both wild ruminants' species, passages were made on Rambach agar. On this medium, it was obtained green colonies, greenish-blue colonies, and pale pink-orange colonies. Since, based on the cultural aspects of the usual and special culture media, it was not possible to precisely identify the bacterial genera, the API 20E test was used for the biochemical differentiation of bacteria from the family *Enterobacteriaceae* isolated from the duodenum of wild ruminants (Fig. 5).

**Fig. 5.** *E. coli* strain aspect obtained on API 20 E from Carpathian fallow deer

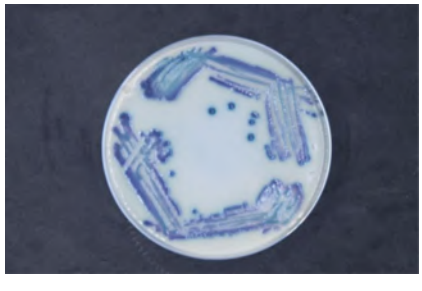

**Fig. 6.** Redispersion of the obtained strain on chromogen agar – *Serratia spp.*

For better differentiation between the two *Serratia* strains, chromogen media and proteomic technique MALDI-TOF Ms were used.

In this study, we isolated two species of *Serratia marcescens* and *Serratia liquefaciens* from *Rupicapra rupicapra carpatica*. Our results are in concordance with other results obtained by other researchers from Slovenia (48). In another study conducted by Bucur et al. (7) five bacterial species where identified: *E. coli, Listeria spp., Enterococcus spp., Salmonella spp*., and *Staphylococcus spp.* Antibiotic resistance is a global problem that is rising continuously. The past period revealed facts like that some bacteria, including commensal enterococci and *Escherichia coli*, could be used as efficient indicators to monitor antibiotic resistance in different bacterial populations and to evaluate the transfer of resistant bacteria in different environments (1, 7, 13, 19, 20).

*E. coli* colonises the gastrointestinal tracts of numerous animal species, including wild ruminants, and serves as a reservoir of antimicrobial resistance genes, revealing an epidemiological role in resistance dissemination (8, 9, 11, 18, 30). The results of the antibiograms are presented in Table 1 and Fig. 7, 8. It could be observed that isolated *E. coli* strains presented low resistance, with only one isolate being resistant to ceftolozan-tazobactam. All the other *E. coli* isolates were sensitive to 9 antibiotics and to ceftolozan-tazobactam.

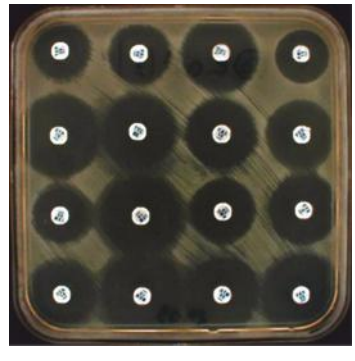

**Fig. 7.** *E. coli* isolated from Carpathian fallow deer (*Dama dama*)

**Fig. 8.** *E. coli* isolated from Carpathian chamois (*Rupicapra rupicapra carpatica*)

**Table 1**

**Resistance patterns of bacterial strains isolated from wild ruminants**

| Antibiotic | *E. coli* | *E. coli* | *E. coli* | *E. coli* | *Serratia marcescens* | *Serratia liquefaciens* |
|---|---|---|---|---|---|---|
| Amoxicillin-clavulanic ac | S | S | I | S | R | S |
| Mecilinam | S | S | S | S | I | I |
| Cefoxitin | S | S | S | S | S | R |
| Temocillin | I | S | I | I | I | I |
| Ceftolozan-Tazobactam | S | I | S | R | S | S |
| Moxalactam | I | I | I | I | I | I |
| Amikacin | S | S | S | S | R | S |
| Tobramycin | S | S | S | S | I | S |
| Netilmicin | S | S | S | S | I | S |
| Nitrofurantoin | S | S | I | S | I | I |
| Tigecycline | S | S | S | S | I | I |
| Fosfomycin | S | S | S | S | I | I |
| Colistin | I | S | I | I | I | I |
| Rifampicin | I | I | S | I | I | I |

This low-level resistance to antibiotics may be the result of low infectious pressure in wild ruminants compared with domestic ruminants. Our data is in concordance with data obtained by other researchers (5, 7, 10, 24), even if other researchers detected multiple antibiotic-resistant *E. coli* strains, which can be a real problem when we refer to the dissemination of these strains from wild animals to domestic animals and humans (27, 29, 31). In this respect, extended-spectrum beta-lactama-ses (ESBLs) deserve special attention, as they confer resistance to many of the beta-lactam antibiotics commonly used in human and veterinary medicine and can be easily transferred between different strains due to the frequent plasmid localization of the corresponding genes (2, 5, 7, 23, 29).

The *Serratia spp.* isolates present resistance to amoxicillin-clavulanic acid, amikacin, and cefoxitin, and most of the isolates were intermediate, this could be an aware signal because this may be an increased tendency of the resistant isolates in wild animals.

## CONCLUSIONS

The present study completes the existing data in the literature from our country regarding the bacterial flora isolated from wild ruminants. The low resistance to the tested antimicrobials denotes that the wild animals (Carpathian fallow deer and Carpathian chamois) have not yet been exposed to high infectious pressures, especially those obtained from Carpathian fallow deer (*Dama dama*). The appearance of antibiotic resistance to amoxicillin-clavulanic acid, cefoxitin, and amikacin in Carpathian chamois (*Rupicapra rupicapra carpatica*) raises an alarm signal regarding the phenomenon of the expansion of antibiotic resistance in the wild animal population in Romania. Bacterial proteomic identification by the MALDI-TOF Ms technique is an efficient and rapid method for the identification of bacteria isolated from wild animals.

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