SUMMARY

The use of medicinal substances, for prophylactic and curative purposes, but also to increase the productivity yield, leads to improved animal health, reduced values of impact indicators associated with bacterial infectious diseases, but promotes the development and spread of antibiotic resistance.

One of the consequences of the uncontrolled administration of antibiotics is the modification of the bacterial population, through the continuous selection of resistant bacterial clones, regardless of whether they are pathogenic or commensal.

Increased resistance of zoonotic bacteria to antibiotics has become a global, major public health issue. The risk of ineffective treatment drives the subject of antimicrobial resistant zoonotic bacteria to be a topic of real interest, in both human and veterinary medicine. The holistic approach of the "One Health" concept includes the evaluation of the possible links between animals, humans and the environment. The transmission of resistant bacteria to people living on or near farms, as well as to consumers of products of animal origin, is scientifically proved by identification of resistance genes in strains isolated from both humans and animals.

Among the bacterial processes of continuous adaptation is the increasing resistance to antimicrobials, due to the administration, in some cases excessively, of these drugs. The appearance of ESBL-producing *Enterobacteriaceae* in the faecal microflora of farm animals represents an obvious risk of food contamination, which can become major vectors for the transport of ESBL in the human population.

The continuous detection and characterization of ESBL-producing *E. coli* strains is necessary in terms of the impact on the food chain of healthy pigs, as a possible reservoir of *Enterobacteriaceae*. Currently, in *Enterobacteriaceae*, one of the most important resistance mechanisms, reducing even the efficacy of modern broad-spectrum cephalosporins, is based on the production of enzymes, mediated by plasmids, which inactivate these compounds by hydrolyzing their beta-lactam ring.

The present study demonstrates an increased frequency of ESBL synthesizing *E. coli* strains in pig farms with high consumption of third or fourth generation cephalosporins and indicates plasmid-mediated transfer between pigs and farmers.

Confirmation of interspecific transfer can be made based on the identification of the CTX-M-1 gene in animal and human isolates.

The doctoral thesis entitled "Investigations on antibiotic resistance and zoonotic risk associated with *Escherichia coli* strains isolated from pigs" is structured, according to the standards, in two parts and includes a number of 142 pages.

The first part, which contains the "Current State of Knowledge", comprises 22 pages, being structured in three chapters. It comprises a synthesis of bibliographic data,

necessary to understand the proposed topic. Relevant information on the epidemiology and characteristics of *E. coli* species, the phenomenon of antibiotic resistance through the mechanism of extended-spectrum beta-lactamases (ESBL) enzymes in *E. coli* and the phenotypic identification and molecular characterization of ESBL synthesizing *E. coli* strains are described.

The second part of the thesis covers 92 pages, including 42 images / 21 tables, 249 bibliographic titles and is structured in five chapters.

Chapter 4 briefly describes the organizational and institutional framework in which the research was conducted.

Chapter 5 contains "*The purpose and objectives of the thesis*", which aims to investigate and characterize ESBL synthesizing *E. coli* strains isolated from pigs and farm caretakers, with or without a previous recorded usege of cephalosporins.

The present study assesses the carriage, transmission and zoonotic potential of ESBL-synthesizing *E. coli* strains, analyzes the model of non-beta-lactam antibiotic resistance for some ESBL-synthesizing *E. coli* strains isolates, and establishes the genetic relatedness of the ESBL-producing enterobacteria strains isolated from slaughtered pigs with those isolated from staff working on farms where the slaughtered pigs came from.

The proposed objectives in order to achieve the overall goal were:

- collecting caecal content samples from slaughtered pigs and farm staff;
- microbiological processing of samples by: non-selective preenrichment, selective isolation of presumptive ESBL / AmpC (ESBL / AmpC screening) *E. coli* strains, taxonomic classification of strains isolated from ESBL screening and phenotypic confirmation as producing ESBL / AmpC of identified *E. coli* strains;
- characterization of the resistance to non-beta-lactam antibiotics for presumptive *E. coli* isolates producing ESBL / AmpC;
- molecular characterization of *E. coli* strains, isolated following ESBL detection, in order to identify the main genes encoding extended-spectrum beta-lacatamase enzymes;
- classification in phylogenetic groups of the *E. coli* strains;
- amplification and sequencing of genes involved in the synthesis of these enzymes;
- detection of the types of plasmids on which the genes responsible for the coding of ESBL enzymes are found.

Chapter 6, entitled "*Detection of ESBL producing E.* coli *strains isolated from pigs and humans*", describes the isolation and identification steps of ESBL / AmpC producing strains and their phenotypic confirmation.

During three consecutive years: 2016, 2017 and 2018, 200 samples of caecal content collected from slaughtered pigs and 32 coprological samples collected from medical staff and pig caregivers were examined.

Following ESBL detection, 123 (53.02%) of presumptive ESBL producing *E. coli* strains were isolated from a total of 232 samples, of which 118/200 were of animal origin and 5/32 of human origin. All bacterial isolates (of human and animal origin) developed

on specific cepotaxime-supplemented MacConkey agar medium (MC + CTX) were phenotypically confirmed as ESBL / AmpC.

After analysing the results obtained on samples of animal origin, depending on the county of origin of the samples, the percentage of *E. coli* strains presumably producing ESBL is in the range 51-70, as follows: for Botoşani - 51.72% (45 / 87), for Suceava - 55.56% (25/45) and for Iaşi - 70.59% (48/68). For the samples of human origin, 2 isolates of ESBL / AmpC producing *E. coli* originated from Iaşi County and 3 from Botoşani.

The broth microdilution method was used for phenotypic confirmation of ESBL synthesizing *E. coli* strains. Following the synergy testing, the following phenotypes were obtained between clavulanic acid and ceftazidime and / or clavulanic acid and cefotaxime: ESBL 77/118 (65.25%), ESBL + AmpC 5/118 (4.23%) and AmpC 36 / 118 (30.50%). For the 5 isolates of human origin obtained, the major identified phenotype was ESBL 3/5 (60%), followed by the ESBL + AmpC phenotype 1/5 (20%) and AmpC 1/5 (20%).

Following broth microdilution testing, for the ESBL / AmpC producing strains, phenotypically confirmed, the degree of resistance / sensitivity to other classes of antibiotics was also established, as follows: beta-lactams (100% ampicillin resistance), sulfonamides resistance to sulfamethoxazole 96/118 (81.36%) and 76/118 (64.41%) to trimethoprim. The 5 strains of human origin were resistant to sulfamethoxazole - 3/5 (60%) and trimethoprim - 2/5 (40 %).

Regarding the category of fluoroquinolones, the report shows that the level of resistance to ciprofloxacin and nalidixic acid was over 50%, thus, of the total number of strains of animal origin tested, 66/118 (55.93%) were resistant to ciprofloxacin and 74/118 (62.71%) to nalidixic acid (NAL). Of the isolates of human origin, both for ciprofloxacin and for nalidixic acid, 3/5 (60%) resistant strains were recorded.

Chapter 7, entitled "*Characterization of the molecular substrate of the positive ESBL-producing E. coli strains isolated from slaughtered pigs and farm staff*", includes the molecular analysis performed regarding the main genes encoding the ESBL / AmpC enzymes.

In this chapter, ESBL / AmpC -synthesizing *E. coli* strains were molecularly characterized by identifying the main genes encoding ESBL / AmpC enzymes, identifying genetic markers associated with plasmid-mediated resistance to fluroquinolones or colistin and classifying *E. coli* isolates in the main phylogenetic groups.

Of the 5 main groups of ESBL enzymes of the CTX-M-U type, it was aimed at identifying the most common: CTX-M-1, TX-M-9, TEM, SHV, OXA. Of the 118 strains molecularly tested, in 72/118 (61%) was identified the presence of the $bla_{CTX\text{-}M\text{-}U}$ gene. Of these, 44/72 (61.11%) belonged to the CTX-M-1 group, 18/72 (25%) to the CTX-M-9 group, and 10/72 (13.89%) strains of animal origin, with a positive signal for the gene $bla_{CTX\text{-}MU}$, did not belong to either of the two groups mentioned above.

The presence of bla_{SHV} genes was identified in 44/118 (37.28%) strains of animal origin, while bla_{TEM} genes only in 2/118 strains (1.69%) of the isolates and bla_{OXA} in a single strain of animal origin 1/118 (1.40%).

The prevalence of bla_{CTX-M} genes for **human isolates** is 80% (4/5). For the CTX-M-1 enzyme group, 3/4 (75%) strains were positive, and for the CTX-M-9 group no genes were identified. Also, as in the case of animal isolates, one strain (25%) did not belong to any of the 2 groups of enzymes analysed.

Identification of bla_{TEM}, bla_{SHV} and bla_{OXA} genes in the analysis of the strains of human origin led to the identification of the bla_{SHV} gene, in proportion of 20% (1/5), and the bla_{TEM} and bla_{OXA} genes were not identified in any of the analysed strain.

Due to the fact that the presence of the AmpC phenotype most often masks the ESBL phenotype, the prevalence of positive AmpC strains was calculated following molecular testing. Following molecular investigations targeting the bla_{CIT-M} gene, positive results were obtained for 35/118 (29.66%) strains of animal origin and 1/5 (20%) of human origin.

All strains were tested for the bla_{qnrA}, bla_{qnrB} and bla_{qnrS} genes, which encode fluoroquinolone resistance and *mcr*-1 and *mcr*-2, which encode plasmid-mediated resistance to colistin.

The total number of fluoroquinolone-resistant strains, of animal origin, was 22/118, of which 1/118 (0.85%) positive for the bla_{qnrA} gene, 3/118 (2.54%) positive for the bla_{qnrB} gene and 18/118 (15.25 %) positive for the bla_{qnrS} gene. Dissimilarly to the strains of animal origin analysed, no positive strain was detected for the bla_{qnrA}, bla_{qnrB}, bla_{qnrS} genes in the human isolates.

With reference to colistin resistance, it was observed that 3/118 (2.54%) *E. coli* strains of animal origin are resistant to colistin, based on the identification of the *mcr*-1 gene. No *mcr*-2 gene was reported in any strain. Furthermore, in the case of human isolates, neither gene was identified.

Moreover, the *mcr*-1 gene positive strains were also molecularly analyzed for the presence of genes encoding the CTX-M-1 group, SHV and qnrS genes, which means that these isolates are resistant not only to colistin but also to beta- lactamines, including third-generation cephalosporins and fluoroquinolones.

Our batch of *E. coli* ESBL strains (118 from pigs and 5 from farm staff) were phylogenetically analysed. Phylogenetic analysis showed that all *E. coli* strains fall into the 4 main groups: A, B1, B2 and D, according to the combination of three genetic markers.

The ESBL-synthesizing *E. coli* strains of animal origin were mainly attributed to phylogenetic group A, 37/118 (31.24%), phylogroup B1, 25/118 (21.18%), followed by phylogroup D - 23/118 (19.49%) and phylogroup B2 -20/118 (16.94%). Some strains, 13/118 (11.15%), could not be assigned to any of the previously mentioned groups.

Analyzing the pigs isolates, the proportion of 52.42% of commensal strains (A, B1) is higher than the proportion of extraintestinal strains, 36.43% (B2, D).

In conclusion, the two types of strains evolve simultaneously in the farms from where the slaughtered pigs included in this study originated.

Of the 5 strains of human origin analysed, the detected phylogroups were A and B2, in equal percentages of 40%, phylogroups B1 and D were not identified and one (20%) did not belong to any phylogroup.

Chapter 8 details "*Phylogenetic analysis of ESBL - encoding genes identified in E. coli strains*". To achieve this goal, we selected 21 strains of *E. coli* molecularly characterized for the presence of genes encoding CTX-M enzymes, 17 of animal origin and 4 of human origin. The sequencing was performed using the Sanger technique, outsourced to Macrogen Europe.

Following the analysis of the obtained sequences, it was observed that 11/17 (64.7%) of the strains are carriers of the $bla_{CTX\text{-}M\text{-}1}$ gene, and the rest of the strains presented $bla_{CTX\text{-}M\text{-}15}$ (1/17), $bla_{CTX\text{-}M\text{-}3}$(1/17), $bla_{CTX\text{-}M\text{-}8}$ (1/17) and $bla_{CTX\text{-}M\text{-}9}$ (1/17). For 2 strains we did not obtain results after sequencing. The $bla_{CTX\text{-}M\text{-}1}$ gene was identified in all 4 sequenced strains of human origin.

Moreover, the $bla_{CTX\text{-}M\text{-}1}$ gene was the most predominant in determining the plasmid-mediated resistance to 3rd generation cephalosporins in *E. coli* isolates from slaughtered pigs and in *E. coli* isolates of human origin.

The $bla_{CTX\text{-}M\text{-}8}$ gene, which generally is rarely reported, was identified in our batch of strains from slaughtered pigs, this being the first report in the North-East region of Romania.

Chapter 9 comprises the final 18 conclusions.