ABSTRACT

Nowadays, the use of antibiotics represents a common practice in various domains, such as human medicine, veterinary medicine, livestock industry (intensive animal farming systems). In the veterinary practice, antibiotics are used both for the prevention and treatment of infectious diseases. The use of antimicrobial agents determines a significant selection pressure in appearance of resistant bacterial strains, and the resistance determinants can be transmitted among different microorganisms. Consequently, the necessity of preventive use of antibiotics in livestock is questionable, in conditions of frequent warnings being issued related to the hazards for human health.

The control of antibioresistance emergence implies sustained efforts directed both on detecting resistant bacterial strains and their transmission risk from animals and/or environment to humans, causing affections difficult to treat. This research study is an attempt to assess the transmission of antibioresistance from animals to humans through the consumption of foods of animal origin.

First part - "*Literature review*" is composed of three chapters that synthesize the information from scientific literature, regarding antimicrobial agents, the classification of β-lactams, quinolones (chapter I) and their action mechanisms (chapter II), surveillance programs and susceptibility testing standards (chapter III).

The second part - „*Personal researches*" is composed of five chapters (IV-VIII). Chapter IV presents the aim and objectives of the research, chapter V, VI and VII describe the results of the research, including materials and methods, results and discussions, their interpretation, and conclusions withdrawn from the research work. In chapter VIII are presented the final conclusions, which synthesize the achieved research results, as well as recommendations for the veterinary practitioners. The study includes 64 illustrations, 30 tables and 318 bibliographic references.

The researches in **chapter V,** entitled *„**Susceptibility phenotypical characterization of the studied bacterial markers to antibiotics",*** were conducted by analyzing the strains isolated from 340 samples from animal-foods-human chain. These samples were taken from livestock

(n=144): from intensive farming system were harvested samples from swine (n=60), cattle (n=18), and poultry (n=10); from extensive farming system samples were harvested from cattle (n=32), poultry (n=20) and pigeons (n=4). The samples from foods of animal origin (n=178) are represented by: dairy products (n=100), fresh cheese, telemea type cheese, brie, camembert, gorgonzola type cheese, simple and fruit yogurt and pasteurized milk; and meat products (n=78), harvested from swine and poultry carcasses, salami, pastrami, bacon, squires and sausages. From humans (n=18) there were harvested two oral samples, three fecal samples and ten vaginal samples. The selected bacterial strains have been purified, identified and preserved at -80º C for further testing.

From the total number of samples (n=340), 280 bacterial strains have been isolated: 80 strains of *Enterococcus*, (12.86%) *E. faecalis* and (15.71%) *E. faecium*, 28 *S. aureus* strains (10%), and 172 *E. coli* strains (61.43%). The prevalence of *Enterococcus faecium* strains was superior to *Enterococcus faecalis* strains, for the swine samples. In the case of animal origin products, the prevalence of *Enterococcus faecalis* strains was superior to that of *Enterococcus faecium* strains. The highest occurrence of *Enterococcus faecium* and *Enterococcus faecalis* strains was noticed in fresh varieties of cheese and fresh pork products. Strains of *Staphylococcus aureus* were isolated from samples of fresh varieties of cheese, molded cheese and fresh pork products. Strains of *Escherichia coli* were identified in fresh cheese, bulk salted cheese, and a relatively high percentage was isolated in fresh pork products.

An objective of this chapter was to evaluate the susceptibility of *E. coli* strains to fluoroquinolones (FQ). This was achieved by determination of the MIC for ciprofloxacin, the active metabolite of enrofloxacin. The results were interpreted as recommended by the European EUCAST E. Def. 3.1. standard, being considered as susceptible the strains with MIC ≤ 0.5 mg/L and resistant strains with MIC > 1 mg/L. The *Escherichia coli* ATCC 25922 control strain had values situated within the standard recommended breakpoints. The determinations were made in duplicate, and even in triplicate, in order to confirm each of the obtained values. This method has an extra benefit compared to the disk diffusion method, expressing also the concentration at which an *E. coli* strain is inhibited, thus revealing with precision the susceptibility or resistance degree. Noteworthy, all *E. coli* strains identified as susceptible or resistant by determining the MIC to CIP, coincided with the results obtained with disk diffusion method CLSI M100 S-18.

Determination of the MIC to CIP was performed for all 172 *E coli* strains, the following statistical parameters being also calculated: MIC_{50}, MIC_{90} and GM (geometric mean), as well as the cumulative frequency of MICs.

The MIC_{90} of 2 mg/L for swine and of 64 mg/L for cattle was situated within the resistance value area. The *E. coli* strains isolated from poultry from intensive farming system had

a MIC_{50} value of 16 mg/L and a MIC_{90} value of 128 mg/L, being placed in resistance area, with very high values, all of the *E. coli* tested strains turning out to be resistant. This fact confirms that the *E. coli* strains in poultry become rapidly resistant after the administration of FQ. The *E. coli* strains isolated from humans presented a MIC_{90} value of 1 mg/L, situated within the intermediary-susceptible area, identical to the values obtained for the strain isolated from yogurt and the one isolated from semi-prepared meat, signaling a possible transmission through the consumption of animal origin products. From the presented data of MIC determination to ciprofloxacin for *E. coli* strains, isolated from poultry in the experiment performed in France, the MIC_{50} of the control group was within the susceptibility area and MIC_{50} as well as MIC_{90} of the treated group was situated in the resistance area with a value of 128 mg/L, which demonstrates a rapid induction of resistance to ciprofloxacin as a result of administration in the feed or drinking water. These results may be related to a high resistance degree and thus, therapy failure, as well as the presence of resistant bacterial strains that could contaminate the chicken carcasses on the production line, reaching in human consumption.

The *Enterococcus* (n=80), *S. aureus* (n=28) and *E. coli* (n=172) were tested using disk diffusion method CLSI M100 S-18.

The *E. faecium* strains (n=20) isolated from swine were resistant to linezolid and vancomycin in a percentage of 10%, 15% to ampicillin, 80% to penicillin and enrofloxacin, 85% to erythromycin and 95% to gentamicin. The *E. faecalis* strains (n=6) had resistance values of 16.66% to ampicillin, 33.33% to enrofloxacin, 50% to penicillin and 100% to gentamicin and erythromycin. These strains also proved to be vancomycin-resistant up to 50%, underlining risk of VRE (Vancomycin Resistant Enterococci) strains emergence. The percentage of antibiotic resistant strains of *S. aureus* (n=10) was various, with values of 2% for ciprofloxacin, 80% to penicillin (the strains can produce β-lactamases like penicillinases type), 100% to clindamycin, the strains being susceptible 100% to gentamicin, cefoxitin, oxacillin and vancomycin. The appearance of resistance for clarythromycine up to 80% and 90 % erythromycin, prove the fact that these *S. aureus* strains are resistant to the antibiotics from macrolide class. In the case of *E. coli* strains (n=53), the percentage of antibioresistance had values of 2% to tazobactam+ piperacillin, 4% to cefoxitin, 11% to cefazolin, 13 % to amikacin, 36% to ciprofloxacin, 37.73% to enrofloxacin and chloramphenicol, 49.05% to sulfamethoxazole + trimethoprim, 56.03% to gentamicin, 64.15% to ticarcillin and reached up to 69.81% to ampicillin. The strains were 100% susceptible to amoxicillin + clavulanic acid and to imipenem. The resistance to several β-lactamic antibiotics (ampicillin, ticarcillin, cefazolin) indicates the presence of β-lactamases.

The *E. faecalis* strains (n=6) isolated from cattle presented resistance of 16.66% to enrofloxacin, 17% to penicillin (β-lactamic susceptible), ciprofloxacin and vancomycin, 33% to chloramphenicol, 50% to erythromycin and linezolid and 100% to gentamicin. The *E. faecium* strains (n=6) presented resistance up to 83.33% to gentamicin and in the same percentage of 16.66% to penicillin and ampicillin β-lactams, and enrofloxacin and ciprofloxacin fluoroquinolones. The *E. faecium* strains were susceptible to erythromycin, chloramphenicol, linezolid and vancomycin. The *S. aureus* strains (n=7) isolated from cattle presented an equal percentage of resistance of 57% to penicillin and ampicillin, with a noteworthy susceptibility relation between low-spectrum β-lactams, highlighting the presence of penicillinases. The *S. aureus* strains are susceptible to cefoxitin and to oxacillin, underlining the absence of meticillin-resistance (MRSA negative). Resistance to ciprofloxacin reaches to 29%, similarly to chloramphenicol, clindamycin and sulfamethoxazole. The *E. coli* strains (n=33) isolated from cattle present relatively high percentage of resistance, both for the cattle from intensive and extensive farming systems. It is worth noting that the resistance of the strains isolated from intensive system cattle is obviously higher than of those isolated from extensive system cattle, excepting resistance to ciprofloxacin, which reached to 35 % for first ones and 43.75 % for second, respectively. In the case of *E. coli* strains isolated from intensive farming systems (n=17), the resistance percentages obtained for β-lactamic antibiotics was as follows: 53% to ticarcillin, 41.17% to ampicillin, 41% to cefazolin, 35% to cefoxitin and 29% to tazobactam + piperacillin. These strains presented resistance in percentage of 58.82% to sulfamethoxazole + trimethoprim and gentamicin, 53% to amikacin, 47% to enrofloxacin, 35% to ciprofloxacin, 29% to chloramphenicol and 24% to imipenem. In the case of *E. coli* strains isolated from extensive system cattle (n=16), the resistance to β-lactamic antibiotics reached the following values: 31.25% to ticarcillin and ampicillin, 18.75% to cefazolin and cefoxitin and 12.5 % to amoxicillin + clavulanic acid. These strains were resistant 43.75% to enrofloxacin and ciprofloxacin, 25% to sulfamethoxazole + trimethoprim, amikacin and gentamicin and 18.75% to chloramphenicol. These strains were susceptible to tazobactam + piperacillin and imipenem.

From the strains isolated from poultry, the *E. faecium* strain was enrofloxacin-resistant, and the two *E. faecalis* strains were ciprofloxacin-resistant, these fluoroquinolones being frequently used for curative purposes. The *E. faecium* strain as well as the two *E. faecalis* strains tested, all were resistant to gentamicin and erythromycin. *S. aureus* strains (n=2) isolated from poultry were resistant to penicillin, ampicillin and amoxicillin + clavulanic acid, signaling the fact that β-lactamases had probably been developed due to antibiotic pressure. However, these strains are susceptible to oxacillin and to cefoxitin, fact that indicates the absence of meticillin-resistance (MRSA negative). These strains were susceptible to gentamicin, clindamycin,

vancomycin, ciprofloxacin and sulfamethoxazole + trimethoprim. The *E. coli* strains (n=10) isolated from the samples from intensive farming poultry reached to resistance percentages of 100% to enrofloxacin and ciprofloxacin, values obviously higher compared to the ones of the *E. coli* strains isolated from extensive farming poultry (n=18), which had only 22.22% resistance to both fluoroquinolones. In the case of *E. coli* strains isolated from intensive farming poultry (n=10), the resistance to β-lactams was different, reaching up to 60% to ampicillin and ticarcillin, and being susceptible to amoxicillin + clavulanic acid, cefazolin, cefoxitin, tazobactam + piperacillin and imipenem. In the case of *E. coli* strains from poultry raised in extensive farming system (n=18), the resistance to β-lactams had presented the following values: 83.33% to ampicillin and ticarcillin, and 11% to cefazolin. These strains also presented resistance to other antibiotics: 50% to sulfamethoxazole + trimethoprim and 55.55% to chloramphenicol. The strains were susceptible to amoxicillin + clavulanic acid, tazobactam + piperacillin, cefoxitin, imipenem, gentamicin and amikacin.

In the case of *E. faecium* (n=13) and *E. faecalis* strains (n=13) isolated from dairy products, a high resistance value to gentamicin was noticed, of 84.61% and 100%, respectively. The resistance to penicillin reached to 23.07% for both *E. faecium* and *E. faecalis*. The *E. faecium* strains were resistant 30.76% to erythromycin, 23% to chloramphenicol and 8% to ciprofloxacin, enrofloxacin and vancomycin. The *E. faecalis* strains isolated from dairy products had proved to be sensible to ampicillin, enrofloxacin, ciprofloxacin, chloramphenicol, erythromycin, linezolid and vancomycin. The *S. aureus* strains (n=4) isolated from dairy products were susceptible to all tested antibiotics. From all *E.coli* strains (n=12) isolated from dairy products, one strain isolated from simple yogurt was resistant to ampicillin, ticarcillin, amoxicillin + clavulanic acid and cefazolin β-lactamic antibiotics. Another strain of *E. coli* isolated from fresh cheese, was resistant to ciprofloxacin and enrofloxacin. The two strains of *E. coli* isolated from simple yogurt and fresh cheese were resistant to sulfamethoxazole + trimetoprim. The strains isolated from fresh cheese, simple yogurt and telemea cheese reached up to 25% resistance to chloramphenicol cheese. All strains were susceptible to cefoxitin, tazobactam + piperacillin, imipenem, gentamicin and amikacin.

Two *E. faecium* strains (n=3) isolated from meat products were resistant to vancomycin and gentamicin, and to penicillin, erythromycin, chloramphenicol and ciprofloxacin was resistant only one of them. All *E. faecalis* strains (n=7) isolated from meat products shown resistance to gentamicin and to linezolid only 14%. These strains were susceptible to ampicillin, penicillin, enrofloxacin, ciprofloxacin, erythromycin, chloramphenicol and vancomycin. In the case of *S. aureus* strains (n=3) isolated from meat products, two of them were resistant to penicillin and ampicillin, and to amoxicillin + clavulanic acid was resistant only one of them, signaling the

presence of resistance enzymes which break the β-lactam ring. The *S. aureus* strains are 100% susceptible to oxacillin and cefoxitin, indicators of MRSA (negative). A strain is also resistant to ciprofloxacin. For the *E. coli* strains (n=40) isolated from swine carcasses, meat products, and raw meat, the following resistance values were determined: 42.5 % to amoxicillin + clavulanic acid, 40% to ticarcillin and 22.5 % to sulfamethoxazole + trimethoprim. The resistance values determined for the strains isolated from swine carcasses were similar with the values determined for the strains isolated from live swine originating from the same farm, to which it has been administered β-lactam antibiotics, drawing the conclusion that probably were the same strains which contaminated the carcasses during slaughtering process on the technologic line. From the prepared meat products and raw meat, there were isolated *E. coli* strains, which presented resistance to β-lactams of 5% to cefazolin, 3% to cefotaxim, 3% to cefoxitin, 2.5% to amoxicillin + clavulanic acid, 3% to tazobactam + piperacillin, 3% to both fluoroquinolones, enrofloxacin and ciprofloxacin and to aminoglycosides, 12.5% to gentamicin and 5% to amikacin. All the strains were susceptible to imipenem and to chloramphenicol.

From humans, one *E. faecium* strain was isolated, which was resistant to penicillin, ampicillin, enrofloxacin, ciprofloxacin, erythromycin, gentamicin, and was susceptible to linezolid, chloramphenicol, imipenem and vancomycin. It must be mentioned that this particular strain was isolated from one year old child, that was never treated with antibiotics before and who consumed only dairy products, which apparently are the contamination source. From tested fluoroquinolones, *E. faecalis* strains (n=2) were susceptible to enrofloxacin and one was resistant to ciprofloxacin. One strain was resistant to ampicillin and penicillin, as well as to vancomycin, linezolid and chloramphenicol. In the case of *S. aureus* strains (n=2) isolated from humans, one strains was resistant to penicillin and ampicillin β-lactams, and to clarythromycine, clindamycin, and ciprofloxacin. The *S. aureus* strains were susceptible to chloramphenicol, amoxicillin + clavulanic acid, oxacillin, and cefoxitin (MRSA negative). The *E. coli* strains (n=3) isolated from humans were resistant to ampicillin, ticarcillin, sulfamethoxazole + trimethoprim and to gentamicin only one was resistant. The strains were susceptible to amoxicillin + clavulanic acid, tazobactam + piperacillin, cefazolin, cefoxitin, imipenem, amikacin, enrofloxacin, ciprofloxacin and chloramphenicol.

For the *E.coli* strains, by using the CLSI M100 S-18 disk diffusion method, there were identified multidrug resistant (MDR) strains, 30 strains in swine, 5 in cattle from the extensive farming system and 9 in cattle from intensive farming system, 6 in poultry from the intensive farming system and 10 in poultry from the extensive farming system, 2 in dairy products, 5 in meat products and 2 in humans. It should be underlined the fact that the resistance of the *S. aureus, E. coli, E. faecium* and *E. faecalis* strains is generally encountered to the antibiotics

frequently used in therapy. The bacterial strains isolated in pigeons, as negative control-sample, had proved to be susceptible to all of the tested antibiotics, thus confirming the theory of selective pressure of antibiotic treatments on the occurrence of resistance determinants.

The double discs diffusion method, described by the CLSI M100 S-18 standard, the presence of ESBLs in some of the *E. coli* strains was identified. From the *E. coli* strains (n=53) isolated in swine, only 2 (3.77%), were resistant to aztreonam, cefpodoxim, cefotaxim, ceftazidim and all the strains being susceptible to amoxicillin + clavulanic acid, thus confirming the presence of ESBLs. Within the *E. coli* strains (n=28) isolated from poultry, was identified a strain belonging to poultry from extensive system, resistant to aztreonam, cefpodoxim and to ceftazidim, while being susceptible to amoxicillin + clavulanic acid, imipenem, cefotaxim and cefoxitin, indicating the presence of ESBLs.

Four strains (23.52%) of *E. coli* (n=17) isolated in cattle from the intensive farming system were resistant to aztreonam, cefpodoxim, cefotaxim, ceftazidim, cefoxitin and imipenem, while being susceptible to amoxicillin + clavulanic acid, indicating the presence of carbapenemases from A class. Two *E. coli* strains isolated in cattle from intensive farming system were resistant to all of the tested cephalosporins, including cefoxitin, but proved susceptible to imipenem and β-lactamase inhibitor, amoxicillin + clavulanic acid, emphasizing the presence of ESBLs, CTX-M type. Two strains (12.52%) of *E. coli* (n=16) isolated in cattle from extensive farming system were resistant to aztreonam, cefpodoxim, cefotaxim, ceftazidim, cefoxitin, amoxicillin + clavulanic acid and susceptible to imipenem, thus confirming the presence of ESBLs, *AmpC* type. One *E. coli* strain (6.25%) isolated in cattle from the extensive farming systems was resistant to all cephalosporins, including cefoxitin, but proved susceptible to imipenem and to β-lactamase inhibitor amoxicillin + clavulanic acid, indicating the presence of CTX-M type ESBLs. From the dairy products, a single *E. coli* strain (n=12) was isolated from simple yogurt that presented resistance to cefpodoxim and amoxicillin + clavulanic acid and proved susceptible to aztreonam, cefotaxim, ceftazidim, cefoxitin and imipenem, indicating the presence of *AmpC* type ESBLs. Out of the total *E. coli* strains (40) from meat products, a single strain (2.5%) isolated in sausages was resistant to cefpodoxim, cefoxitin and amoxicilin + clavulanic acid, being susceptible to aztreonam, cefotaxim, ceftazidim and imipenem, indicating the presence of *AmpC* type ESBLs.

Chapter VI, entitled *„**Molecular aspects related to resistance of the evaluated E. coli strains”***, describes an attempt made to identify the resistant determinants for quinolones of the *E.coli* strains. To that effect, molecular biology methods (classic PCR) were used to amplify PMQR genes, *qnrA* and *qnrB*, as well as chromosomal genes, *gyrA*, *gyrB* and parC. All *E. coli* tested strains (n=172), originating from farm animals, products of animal origin and from

humans, were negative for plasmidic resistance genes, *qnrA* and *qnrB*. A number of 172 *E. coli* strains were tested using molecular methods to identify the mutations in *gyrA*, *gyrB* and *parC* and the results were studied after the interpretation of sequencing which marks out if mutations took place and whether these are silent, unexpressed or influence FQ susceptibility.

Chapter VII, entitled ***„Changes in concentration of fluoroquinolones and of ciprofloxacin-resistant Enterobacteriaceae in chicken feces and manure stored in a heap”,*** describes an experiment conducted in France, at the IRSTEA (former Cemagref) Research Unity, Rennes. The aim of this study was to estimate the impact of storing chicken manure on the persistence of FQ resistant *Enterobacteriaceae.* The specific objectives were (i) to measure the concentrations of enrofloxacin and ciprofloxacin and ciprofloxacin-susceptible or resistant *Enterobacteriaceae* in fresh feces and during the storage of poultry manure in a static pile, (ii) to compare the structure of the *Enterobacteriaceae* populations obtained from fresh droppings and in manure using ERIC-PCR and (iii) to check for a relationship between genetic profiles and resistance of the isolates to FQs. The administration of enrofloxacin to chickens led to the emergence of strongly resistant *Enterobacteriaceae* in fresh feces, mainly represented by *E. coli*. However, the two-month period of storage of chicken manure significantly reduce susceptible and resistant *E. coli* in the manure pile. The degradation of enrofloxacin and its metabolite, ciprofloxacin, was not complete during the storage period and a residue of 25 % had persisted. It thus appears that storage of chicken manure in a heap may be an effective treatment to limit the entrance of FQ-resistant *E. coli* into the environment but may still contribute to the dissemination of FQs after land spreading.

Chapter VIII,”***Final conclusions and Recommendations”***, develops all the conclusions and addresses some recommendations in order to avoid the risk of antibiotic resistance.

All the gathered data emphasize the potential of these bacteria (*E. coli, S. aureus, E. faecium* and *E. faecalis*) to affect human population through the food chain (via consumption of foods of animal origins) or the environment.

The results obtained in both CLSI disk diffusion method and MIC determination method to ciprofloxacin of *E. coli* strains, indicate the fact that antibiotic resistance represents an emergence linked to the clinical use of antibiotics, against which the resistance is directed. The presence of bacterial strains resistant to certain antibiotics, isolated in foods of animal origin, represents an extremely critical public health hazard.