ABSTRACT

The Doctoral Thesis entitled ***THE ASSESSMENT OF ANTIBIOTIC AND ELEMENTARY XENOBIOTIC RESIDUES IN MILK AND MILK PRODUCTS AND THEIR RISK FOR CONSUMERS*** has a character of originality by applying new methods of analysis, toxic element determinations through the milk processing technology and the risk assessment of the determined levels.

The thesis includes a total of 238 pages and is structured in two parts.

The first part of *General Considerations* on the topic covers a number of 64 pages, being organized in three chapters that present data from the literature on the chosen subjects: substrates, antibiotherapy and the risk assessments of the antibiotic residues, and ecotoxicology of elementary xenobiotics *(Pb, Cd, Hg, Ni, Cu, Zn).*

Part Two, the *Personal Contributions* covers a total of 116 pages and contains four chapters that present the purpose and importance of the research, materials and working methods, results and the related discussions, and the general conclusions.

The data are illustrated with 13 tables and 18 figures inserted in the *General Considerations*, 39 tables and 48 figures in the *Personal Contributions* part. Refferences contain 270 titles of original paperworks, books, legislative items regarding food safety, analytical methods and internet sites. The thesis contains personal contributions that were published in scientific journals communicate in the Scientific Symposia with international participation, organized by the Faculty of Veterinary Medicine Iassy and in the Romanian Journal of Veterinary Medicine, respectively.

The importance of the chosen object of the thesis comes from the fact that, small quantities and prolonged antibiotic intake can have undesirable effects on human or animal body. The undesirable effects on consumers health can be direct and are manifested as allergic reactions or even as toxic reactions. The indirect effects are also important as they can trigger the bacterial resistance to antibiotics. Some studies showed that polluted milk with antibiotic residues obtained from 20 cows delayed or stopped the fermentative processes of milk collected

from 25.000 cows. Moreover, the samples containing residues of antibiotics are accompanied by increases of somatic cell population and of total number of germs.

The presence of elementary xenobiotics in milk is also dangerous because many of them have cumulative effects and endanger the health of young animals and humans. It should also be noted the mutagenic and carcinogenic effect that they may cause to consumers.

The main objectives of this work were:

- Monitoring antibiotic and elementary xenobiotic residues in milk and milk derivatives according the latest rules on the work methodology and interpretation of results.
- Evaluation of the antibiotic and elementary xenobiotics contamination seasonality and investigation of its correlation with other risk factors.
- Analysis of variation of antibiotic and xenobiotic content of the milk samples collected from two different groups of sources: *milk collection centers* and *dairy farms.*
- The assessment of the risk to which the consumers are exposed, by making a comparison of the residue concentrations determined in this study with their acceptable daily intakes or with their tolerable weekly limits recommanded by the World Health Organization for each antibiotic (except penicillins) and elementary xenobiotics. In the copper and zinc residue case, the extent to which the determined concentrations can meet the daily needs for children and adults were assessed.

Study materials were *milk samples* collected from several milk collection centers and from two dairy farms, from North Moldavia; there were also collected dairy product samples from the processed milk on different technological stages of the milk processing.

Working methods were applied as standards harmonized with EU legislation in force. The identification of the antibiotic residues in milk samples was made by a microbial quality test using the strain sensitivity of *Bacillus stearothermophilus* variety *calidolactis, ATCC 10149.* To confirm the contaminated samples a semi-quantitative test based on the microbial receptors was used as a first step. It allowed the identification of the antibiotic families in the samples. Quantitative confirmation of the antibiotic residues was performed using a competitive immunoassay tests method. For the analysis of the antibiotic residues in dairy products was initially used the semi-quantitative test to identify positive samples, these being quantitatively confirmed lately by the method of competitive enzyme immunoassay tests.

The determination of the elementary xenobiotics *(Pb, Cd, Hg, Ni, Cu, Zn)* from the milk samples was performed by an AA Spectrophotometry. The AA device uses an autosempler and graphite furnace for detection of lead, cadmium, copper and nickel, flame for zinc and hydride generator for mercury. Wet mineralization sample was used and the results were expressed as *µg/kg.*

In accordance with the proposed objectives correlations of the milk contamination with antibiotic and elementary xenobiotics *(Pb, Cd, Hg, Ni, Cu, Zn)* and other risk factors (the growth of the somatic cell and microbial germ population) were established. Determination of somatic cells was performed using a computing equipment for staining and reading microscope blades. The determination of the total number of germs was based on using a specialized device of colony counting, which quantifies color changes of the specifie test tubes.

For an accurate interpretation of analytical data a number of basic statistical indicators were used. Of these, the most readily available ones were the minimum, maximum and mean values, standard deviation, coefficient of variation, the average confidence interval test (chi-square) to determine consistency, Pearson correlation coefficient, the arithmetic means method for calculating the seasonality, the Student test or T - test for variation analysis of data series.

The qualitative examination of antibiotic residues in 2785 milk samples, during a four year period (2006 to 2009), led to the identification of 124 antibiotic (+) samples *(4.45%)* and 130 antibiotic (±) samples *(4.67%).* After applying the heat treatment the presence of antibiotic residues in 109 antibiotic (+) samples *(87.9%)* and 24 antibiotic (±) samples *(18.46%)* was confirmed, the difference being represented by false-positive samples, due to natural inhibitors thermolabile (lactoferrin, lactoperoxidase, lysozyme).

The main identified antibiotic families were betalactams *(in 27.90% of samples)* in average concentrations of 26.65 µg/kg, gentamicin/neomycin *(in 25% of samples)* in mean concentrations of 198.68 µg gentamicin/kg and 2048.53 µg neomycin/kg, tetracyclines *(in 24.42% of samples)* in average concentrations of 271.43 µg/kg, gentamicin/streptomycin *(in 15.11% of samples)* in average concentrations of 198.68 µg gentamicin/kg and 280.61 µg streptomycin/kg and macrolides *(in 7.56% of samples)* in average concentrations of 97.87 µg tylosin/kg.

The milk samples with antibiotic residues showed low seasonality in January *(csi = 0.51)* and July *(csi = 0.59),* and increased in April *(csi = 1.77)* and May *(csi = 1.43).*

A strong correlation between the presence of antibiotic residues and the increasing of somatic cell count was found. The differences between the observed and the expected incidence being 49.54 for the correlation with the somatic cells class with more than 600.000 somatic cells/ml and 30.31 for the correlation with the somatic cells class of somatic cells with 400.000 to 600.000 cells/ml. The analysis of the chi-square indicators showed a stronger correlation between the presence of antibiotic residues and the increased number of somatic cells to contamination with antibiotics and the increased number of microbial germs of the investigated samples.

The analysis of antibiotic residues in dairy products (pasteurized drinking milk, buttermilk, yogurt, cream, cheese and hard cheese) led to the identification of the gentamicin/

neomycin group in one sample of cream and cheese, neomycin being determined later in concentrations of 27.71 µg/kg in cream and of 32.90 µg/kg in cheese. Macrolides were identified in one sample of cheese and hard cheese, tylosin being determined later in concentrations of 51.31 µg/kg, 52.79 µg/kg respectively. The tetracyclines were identified in two samples of cheese and in each one of cream and hard cheese, being determined in concentrations of 22.97 µg/kg and 25.21 µg/kg in cheese samples, 23.74 µg/kg in cream and 24.35 µg/kg in hard cheese.

The quantitatively investigated milk samples came from the samples considered inconsistent since the application of microbial quality test and were not accepted for the human consumption. The determined concentrations of the majority of antibiotic residues do not exceed the acceptable daily intakes established by the World Health Organization to be taken from food substrates.

In the dairy products, the antibiotic residues were identified in 2006-2007, their presence being due to a incomplete operational HACCP system of risk assesment in the milk processing unit. The determined quantities in this study were at levels which do not affect any development of the lactic cultures or consumers' health.

The elementary xenobiotic residues were determined in a total of 98 of milk samples being analyzed every 28 replicates in the years 2006 to 2008 and 14 replicates in 2009 for each metal. *The lead* was identified 71.43 to 85.71% of the samples in the average concentrations of 2.49 - 3 µg/kg, below the legal maximum level. *The cadmium* and *mercury* residues were not encountered in the analyzed samples or they were in lower amounts than the method detection limits. *The nickel* residue was determined in all investigated samples in average concentrations ranging from 11.24 to 11.90 µg/kg, lower than the tolerable levels recommended by the World Health Organization.

The copper residue was determined in all investigated samples and had average concentrations ranging from 237.10 to 303.43 µg/kg that are considered normal for cow's milk. *The zinc* residue was determined in all investigated samples and had average concentrations ranging from 2051.21 to 2628.64 µg/kg that cover the daily human requirements.

The investigated elementary xenobiotics had an increased seasonal incidence in the fall *(csi* between *1.00* and *1.21)* and winter *(csi* between *1.09* and *1.34),* and a low one in spring *(csi* between *0.58* and *0.92).*

The Pearson index analysis showed absence or a weak correlation between the concentrations of the elementary xenobiotic residues and the changes in somatic cell and microbial germ populations in the investigated samples.

The investigation of the elementary xenobiotic residues in dairy products led to the following conclusions:

- *Lead* was found at average concentrations ranging from 2.45 to 2.83 µg/kg in the pasteurized milk and from 3.87 to 4.28 µg/kg in hard cheese; the maximum concentration of lead found in hard cheese was found within tolerable weekly intake of 25 µg/kg for humans.
- *Nickel* was found at average concentrations ranging from 11.88 to 12.13 µg/kg in the pasteurized milk and from 16.10 to 16.55 µg/kg in hard cheese; the maximum concentration of nickel found in hard cheese does not represent a health risk to consumers, being much lower than the acceptable daily intake of 300 µg for an average body weight individual.
- *Copper* was found at average concentrations ranging from 244.52 to 277.25 µg/kg in the pasteurized milk and from 1246.05 to 1332.71 µg/kg in hard cheese; the concentrations encountered in samples of hard cheese could provide, in combination with other sources, the daily requirements of 1 to 1.6 mg for children and of 1.5 to 2 mg for adults.
- *Zinc* was found at concentrations ranging from 2348.18 to 2434.43 µg/kg in the pasteurized milk and 11772.49 to 12334.12 µg/kg in cream; the most residues of zinc found in the analyzed products may meet the daily requirements of 3 to 10 mg for children and of 10 to 15 mg for adults.

Comparisons of the milk quality were made between two groups os samples: *„milk collect centers"* group and *„farm milk"* group. The Student test (T-test) was used for the variation analysis of the investigation data. The milk samples of the former group has higher levels of antibiotic residues as expected. They also had higher levels of lead and nickel residues and lower levels of copper and zinc than the samples of the later group.

The results of in this research were compared with those published in other specialized national and international studies. These studies also confirmed small levels of contaminants in the raw milk.

The survey associated with the present work allowed us to formulate some suggestions to assure toxicological quality of the raw milk and milk products:

- It is highly recommended for the milk processing units to undertake a more rigorous control of the incoming raw milk. It is mandatory to sample the bulk milk at the collection center level and to analyze it for the antibiotic residues. More attention should be payed to the milk supply comming from the population households as it is more susceptible to antibiotic and elementary xenobiotic contamination.
- It is highly recommended that farmers be aware of the correct use of antibiotics, to avoid self - recommended antibiotic treatment of dairy cows and to follow the rules regarding the required withdrawal time for antibiotic clearing of the animal body.
- It is highly recommended the implementation of a regular toxicological control management program for the milk bulk and feed supply to efficiently identify toxic sources and remove them in due time.