SUMMARY

of the of doctoral thesis entitled

OPTIMIZATION OF THE FAST ASSESSMENT OF CERTAIN INHIBITORS IN THE COLLECTION MILK

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The doctoral thesis is structured on two distinct main parts (*Bibliographic study* and *Personal researches*), together with the contents, the abstract, introduction and bibliography.

PART I, BIBLIOGRAPHIC STUDY, includes two chapters, respectively:

CHAPTER I - MILK, A BASIC COMPONENT OF HUMAN NUTRITION, describes the essential issues of milk importance in the balanced human diet, as well as the main features of milk, being structured on the following sub-sections*: Nutrition and health, The importance of milk, Organoleptic characteristics of milk* (Aspect and consistency of milk, The color of milk, The taste of milk and The smell of milk), *Physico-chemical characteristics of milk, Chemical composition of milk* (Water, Lipids, Carbohydrates, Proteins, Mineral substances, Vitamins and Enzymes), *Parameters of quality for the acceptance of raw milk in handling and / or processing units, The management of milk quality assurance.*

CHAPTER II - INHIBITORY SUBSTANCES IN MILK have issues related to the main inhibitors that may be present in milk, as well as the adverse issues of the antibacterial substance residues of the milk on human's health and industrial processing of milk, being structured on the following sub-sections: *General considerations on the presence of inhibitory substances in milk, Classification of antibacterial substances, Spectrum of action, Mechanisms of action, Pharmacokinetics, Food safety and chemical xenobiotics, The consequences for consumers of inhibitory substances presence in milk* (The negative effects of antibiotic residues on humans, Technological implications, Maximum permitted limits of antibiotic residues in milk).

PART II, PERSONAL RESEARCHES includes three chapters, respectively: *Materials and methods, Results and discussions, Conclusions and recommendations*.

The three main purposes of this doctoral thesis were:

- presence or absence monitoring of residues of certain antibacterial substances (mainly antibiotics) in the raw milk by fast tests relying on microbiological methods (microbial qualitative tests);
- identification of the antibiotics groups and sulphamide, respectively, whereof residues were identified on the positive tests, using radioimmunoanalysis method (semi-quantitative test Charm III based upon microbial receivers);
- quantification of antibacterial substance residues (by the immunoenzymatic method ELISA) and comparison of the obtained values to the maximum allowed limits by the national and European legislation in force.

CHAPTER III - MATERIALS AND METHODS

For the development of studies, raw milk tests were taken from five collection centers and two bovine farms located around Bucharest City (Ilfov county), which constantly supply raw milk for the consumption of Bucharest people, but also raw material milk to the processing units. The tests intended to the establishment of inhibitor residues of the milk were sampled observing certain sampling, labeling, packaging, transport rules and input time of tests into process.

The researches were issued during 6 years, between 2008 and 2013, on 2214 raw milk tests, sampled from five collection centers and two farms located around Bucharest City, as follows: 421 tests in 2008, 378 tests in 2009, 397 tests in 2010, 227 tests in 2011, 404 tests in 2012 and 387 tests in 2013.

For the purposes of determining the traces of inhibitory substances of the raw milk tests, a succession of working stages was applied, respectively:

1. Microbial qualitative test

It was used for the screening (monitoring) of inhibitory substances present in the raw milk. Being a qualitative test, it does not provide precise information on the relevant inhibitory substance, nor on its quantity in the test, but has major advantages: it detects the positive samples, may remove the negative ones, is quite cheap, does not need an expensive machinery, may be applied both under the land conditions, and laboratory conditions, and the results are supplied within about 3 hours. This test allowed the identification of presence of the most of antibacterial substances used in the veterinary therapy and with a higher milk

contamination risk.

The test used for this study (Delvotest SP NT 100) allowed the identification of presence in the raw milk tests of the following anti-bacterial substances: penicillin G, cloxacillin, dicloxacillin, oxacillin, nalcillin, ampicillin, cefapirin, cefalonium, cefalexin, cefacetril, cefoperazon, ceftiofur, tetracycline, oxytetracyclin, chlortetracyclin, tylosin, eritromicin, lincomycin, spiramycin, gentamicin, neomycin, di-hydro-streptomycin, kanamycin, cloramfenicol, sulfametazine, sulfadimetoxine, sulfathiazole, sulfadiazine, dapson, trimetoprim, being currently considered the most performing test of this kind.

2. Semi-quantitative test Charm II, based on microbial receptors

This test was used in the case of tests that positively reacted to the microbial qualitative test and relies on the irreversible binding of functional groups of antibacterial substances of the test to the receptor sites of tested bacteria. The test also involves antibacterial substances, radioactively marked by ^{14}C or ^{3}H that compete against the test inhibitors.

3. Immunoenzymatic quantitative test

The ELISA test was used as assessment method for the establishment of antibacterial substance residues of milk. The competitive immunoenzymatic method relies on the antigen-antibody reaction, and the obtained results may be quantified for the quantitative assessment of inhibitory substance residues of the analyzed milk tests. The kits for the establishment of anti-bacterial substances supplied by R-Biopharm (Germany) and Bioo Scientific (U.S.A.) were used for the researches.

CHAPTER IV - RESULTS AND DISCUSSIONS

By using the microbial qualitative test Delvotest SP NT 100, during 2008 and 2013 there were analyzed 2214 raw milk tests, sampled from five collection centers and two farms located around Bucharest City (Ilfov county). Out of the 2214 analyzed tests, 2032 tests (91.78%) had negative results, 94 tests (4.25%) had doubtful results, while 88 tests (3.97%) had positive results.

For the detection of the possibly false-positive reactions and for the clarification of the situation of doubtful tests, the tests that had positive and doubtful results upon the initial application of Delvotest SP NT 100 test were re-analyzed after the 10 minutes thermal treatment at 82°C. After the re-testing the samples that had originally shown a positive and doubtful reaction, after the thermal treatment, 84 tests (3.79% of the total) were established, which showed on the first test a false-positive reaction, being finally considered negative

tests.

Therefore, upon the use of microbial qualitative test Delvotest SP NT 100, 2116 tests (95.57% of the total of analyzed tests of the 2214 analyzed raw milk tests, showed a negative result, while 98 tests (4.43% of the total of analyzed tests) were detected as positive, respectively having anti-bacterial substance residues of a higher concentration than the detection limit of the method.

Related to the correlation of positive tests to the season, it resulted the fact that, during the period considered for the study, the most of the positive tests have been registered during the spring months (March - May term). Upon the statistic processing of obtained results, a gradual decrease of the number of positive tests for the antibacterial substance residues was noticed at about the beginning of summer and during it, as well as a new increment at the beginning of autumn, but not at the levels registered during the spring months. Compared to October month, the dynamics of positive tests expressed a descending line during the winter, the anti-bacterial substance residue contamination being placed during this season at the lowest level, per whole, of the entire year.

The semi-quantitative establishment of the antibacterial substance **residues** of the raw milk tests was issued by **the radioimmunoanalysis method Charm II,** for the purpose of identifying the groups of antibacterial substances, whereby the residues present in the tests established as positive, upon the application of microbial qualitative test fall within.

The used method allowed the falling within the present anti-bacterial substances as residues in the raw milk tests, analyzed in 7 distinct groups: aminoglycosides type I, aminoglycosides type II, amphenicols, betalactamines, macrolide/lincosamide, tetracyclin, sulfonamide.

Upon the analyses, 69 tests (70.41%) were contaminated by one class of anti-bacterial substances, of the 98 analyzed tests, and 29 tests (29.59%) have an anti-bacterial substance residues content fallen within two different groups.

On the whole of the term considered for the study, on the positive tests contaminated by one group of antibacterial substances, the beta-lactam antibiotics were identified in 38 tests (55.07%), tetracyclines in 11 tests (15.94%), aminoglycoside type I (neomycin, gentamicin) in 5 tests (7.25%), aminoglycoside type II (streptomycin, dihydrostreptomycin, gentamicin) in 7 tests (10.14%), macrolides/ lincosamides in 5 tests (7.25%), and sulphamides in 3 tests (4.35%). The antibiotics of the group of Amphenicols were not identified by any analyzed test.

Regarding the tests whereby anti-bacterial substance wastes, belonging to two distinct

groups were identified, the very high frequency of association between the beta-lactam and aminoglycoside, being very well known that these two groups of antibiotics complete each other very well the area of activity; this combination was identified by 20 of the 29 analyzed tests, from the total of associations established within the experiments. There have been also identified tests whereby the associations between various aminoglycoside, as well as the associations between aminoglycoside and tetracyclines were established, this being closely related to the use of these antibiotics for the treatment of mastitis at milk cows.

According to the global results, 60 milk tests were identified, including antibiotic residues of the group of beta-lactam (47.24%), 40 milk tests including aminoglycoside residues (31.50%), 15 milk tests including tetracyclin residues (11.81%), 9 milk tests including macrolide / licosamide residues (7.09%) and 3 milk tests including sulphamide residues (2.36%). No milk tests were identified, which include antibiotics residues of the group of amphenicols.

Globally, during 2008-2013, the anti-bacterial substance residues have been quantitatively established in 127 raw milk tests, **by the ELISA technique.** 60 of these 127 tests were positive for the residues of *betalactamine*; the lowest value of residues was 6 μg/kg, and the highest value was 74 μg/kg, averagely 26.13 μg/kg.

Although the ELISA kit used for this study did not allow the establishment of each representative of the betalactamine class, but only of the group, globally, the established levels may be considered high, taking into account the fact that the maximum allowed limits on milk for penicillin G, ampicillin and amoxicillin are 4 μg/kg, provided that these antibiotics are admitted as the most commonly used betalactamines in therapeutics.

With respect to the *aminoglycoside* wastes, 40 positive tests have been analysed during the period considered for the study. It should be noted that the used method allowed the exact identification of the aminoglycoside antibiotic; therefore, the gentamicin, streptomycin/di-hydro-streptomycin and neomycin antibiotics were identified by the analysed tests.

The gentamicin residues were established by 7 milk tests; the lowest value of residue was 179 μg/kg, and the highest value was 287 μg/kg, averagely 221.14 μg/kg. All the 7 tests showed residues over the maximum allowed limit (100 μg/kg).

The neomycin residues were established by 12 milk tests; the lowest value of residue was 309 μg/kg, and the highest value was 2.280 μg/kg, averagely 849.83 μg/kg. It should only note that only two tests showed higher values of neomycin residue than the maximum allowed limit (1.500 μg/kg).

Globally, during 2008-2013, 21 positive tests have been registered for the residues of streptomycin/ dihydrostreptomycin; the lowest value of residue was 302 μg/kg, and the highest value was 673 μg/kg, averagely 409.95 μg/kg. All the 21 tests showed residues over the maximum allowed limit (200 μg/kg). The streptomycin/ dihydrostreptomycin residues could neither be noticed as having a clear descending progress tendency of the concentrations established during the six years of study.

During the 6 years of study, 15 positive tests have been registered for the residues of tetracyclines; the lowest value of residue was 113 μg/kg, and the highest value was 443 μg/kg, averagely 224.27 μg/kg. All the 15 tests showed residues over the maximum allowed limit (100 μg/kg).

Out of the 127 positive tests for antibacterial substance wastes, the *macrolides* were identified and quantified in 9 raw milk tests.

The thylosin residues were established in 6 milk tests; the lowest value of residue was 62 μg/kg, and the highest value was 113 μg/kg, averagely 88.17 μg/kg. All the 6 tests showed residues over the maximum allowed limit (50 μg/kg).

The erythromycin residues were established in 3 milk tests; the lowest value of residues was 59 μg/kg, and the highest value was 97 μg/kg, averagely 78 μg/kg. All the 3 tests showed residues over the maximum allowed limit (40 μg/kg).

The *sulphamides* residues were established by 3 milk tests; the lowest value of residue was 75 μg/kg, and the highest value was 180 μg/kg, averagely 125.33 μg/kg. Out of the three positive tests, only two showed residues over the maximum allowed limit (100 μg/kg).

CHAPTER V. CONCLUSIONS AND RECOMMENDATIONS presents 17 conclusions and recommendations detached from the obtained results.