PhD THESIS

Integrated milk quality assessment studies by complex analytical methods

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I. INTRODUCTION

Milk has been used by humans since the beginning of the 7th millennium BC. (EVERSHED, 2008; DUDD, 1998), this being a white, nutritious liquid secreted by the mammary gland of mammals (ZHANG, 2021).

Milk is a complete food, being the only food that, consumed by the newborn organism, provides it with all the necessary nutrients for growth and development. It is one of the basic foods for all age groups and the raw material for an extremely diverse range of products (GUZUN, 2001).

Milk is one of the food components in the daily diet of the population of all ages. The consumption of one liter of milk covers the daily requirement of an adult in lipids, calcium and phosphorus, 53% of the protein requirement, 35% of the vitamin A, C, B requirement and 26% of the energy requirement (GUZUN, 2001). Evidence has shown that milk has a wide range of physiological functionalities, including anti-carcinogenic (PARODI, 1997), anti-inflammatory (DA SILVA, 2015), anti-oxidant (SULTAN, 2018), anti-adipogenic (MILARD, 2019), anti-hypertensive (HE, 2011), anti-hyperglycemic (O'CONNOR, 2019) and anti-osteoporotic (CADOGAN, 1997).

The quality of raw milk is the main factor influencing the compositional characteristics and hygienic quality of dairy products (GUZUN, 2001).

Milk is a food as important and irreplaceable - considered a sanogenesis factor, as delicate and easily exposed to the risks of spoilage and contamination - thus potentially becoming a factor of pathogenesis.

Due to its composition, chemical and biological structure, milk is an extremely favorable culture medium for the development of various types of microorganisms. The presence of microorganisms in milk is of particular importance for quality, sanitation and freshness. These microorganisms can increase or reduce the quality of products or make them inedible, either through their pathogenic action or through degradation and the production of toxic metabolites.

Consuming contaminated milk and milk products could have serious health implications for consumers. Consumer demand for safe and high-quality milk has placed a significant responsibility on dairy producers to produce and market milk and milk products safely (MENNANE, 2007).

Considering this context, this doctoral research topic aimed to fulfill the following objectives:

- Evaluation of the main hygiene parameters (NTG, NCS) in raw milk obtained from large capacity breeding units (farms) through mechanized milking and milking with robots and from private households through manual milking.
- Evaluation of the presence of aflatoxins in the milk chain.
- Evaluation of some endocrine disrupting compounds with potential for bioaccumulation and high toxicity: phthalates and the elaboration, development, validation and implementation of complex, fast, sensitive and ecological analytical methods for determining these compounds present in milk and dairy products.

II. THESIS STRUCTURE

The thesis entitle "Integrated studies of milk quality assessment by complex analytical methods" is structured in two main parts, namely the current state of knowledge and the personal contribution. The current state of knowledge includes 3 chapters (1,2,3), has 33 pages and represents 28% of the thesis. In this part, data from the literature are described regarding the importance of milk and the compositional markers of cow's milk, its physico-chemical characteristics and microbiological characteristics; hygienic quality parameters of milk and chemical contaminants present in drinking milk, as well as their degree of toxicity on human health.

The personal contribution is composed of 7 chapters (4,5,6,7,8,9,10) and bibliography. It contains 82 pages and represents 72% of thesis. The 7 chapters contain data on the materials and methods used in this research, results and discussions, as well as the conclusions of these studies. The last chapter emphasizes the originality and contribution of this research.

III. CURRENT STATE OF KNOWLEDGE

The first part of the thesis is structured in 3 chapters:

Chapter 1 includes data on the general framework regarding the existing information in the literature on the compositional part of milk, from a physico-chemical and microbiological point of view.

Chapter 2 presents information about the sanitary quality parameters of milk and their importance.

Chapter 3 contains information on potential chemical contaminants of milk and their toxicity.

IV. PERSONAL CONTRIBUTION

The second part of the thesis consists of the personal contribution and includes 7 chapters:

Chapter 4 presents the main objectives of the study:

I. Evaluation of the main hygiene parameters in raw milk obtained from different breeding units, which includes other secondary objectives:

- Dynamic evaluation of the total number of germs and the number of somatic cells in a farm with manual milking;
- Dynamic evaluation of the total number of germs and the number of somatic cells in a farm with mechanized milking;
- Dynamic evaluation of the total number of germs and the number of somatic cells in a farm with milking performed by means of robots.

II. Determination of mycotoxins in the milk supply chain in a regional surveillance system; especially the assessment of the prevalence of aflatoxins and the chemical risk represented in the analyzed milk samples.

III. Evaluation of some endocrine disrupting compounds with potential for bioaccumulation and high toxicity – phthalates, as well as

- The development and implementation of fast, sensitive and ecological complex analytical methods for the determination of these compounds present in milk and dairy products.

- Validation of a method for quantifying the presence of phthalates in dairy products;
- Evaluation of the chemical risk represented by the presence of phthalates in milk.

Chapter 5 is represented by the biological material used in the work and the research methodology used for each individual determination.

- **Material and method used to determine hygienic quality parameters (TVC, SCC)**

Raw material milk was monitored in order to establish the total viable count and the number of somatic cells for a period of 6 months. During our study, 180 individual samples were collected from 10 cows, being collected individually, from 3 categories of farms, with different types of milking systems: a farm where milking is done manually; a farm where milking is done mechanized with the intervention of the caretaker; and an animal farm where milking is done mechanized by means of the robot, without human intervention.

For the determination of TVC/ml, the technique for establishing the number of microorganisms in products intended for human consumption is applied by counting the colonies obtained in the solid medium after thermostating in aerobiosis at 30ºC. The preparation of the sample to be analyzed and the initial suspension is carried out according to the provisions of point 3.1. and 3.2. from SR EN ISO 6887-1:2017.

To determine the number of somatic cells in milk, the fluoro-opto-electronic method is applied, which is performed according to the provisions of points 4, 5, 6, 7, 8 and 9 of SR EN ISO 13366-2/2007 and the instructions in the User's Manual Bentley Instruments. The technology used is flow cytometry, using the SOMACOUNT 150 device.

- **Material and method used for the determination of aflatoxins in milk**

A total of 150 raw milk samples were obtained from milk collection units and animal breeding units in the period 2013-2017, from Cluj County. All milk samples were prepared using the method shown in the ELISA kits and according to the official method specified in the Aflatoxin M_1 ELISA system (RIDA Screen aflatoxin M_1). The ELISA method (enzyme-linked immunosorbent assays) is based on the identification of enzyme-conjugated secondary antibodies, which are meant to amplify the immuno-reactivity of the primary antibodies and, therefore, to increase the sensitivity of protein determination through their specific antibodies.

- **Material and method used for the determination of the phthalates in milk and milk products**

For this study, samples of milk and milk products were randomly collected at several stages of the production chain, from the farm, the milk collection center and the retail level.

Milk samples were collected from several specific sources: milk obtained by manual milking, milk obtained by mechanical milking - collected from the cooling tank and commercial milk.

To investigate the phthalates at the retail level, samples purchased from the supermarket were ad represented by cream, yogurt, butter, whipped cream, and 5 different varieties of cheese.

For the analysis of phthalates in milk samples, a liquid-liquid extraction of the samples was first performed with methanol - n-hexane solvent (1:2 v / v). For each dairy product studied, the ultrasound-assisted extraction method was used.

After these procedures, phthalate analysis was performed on a gas chromatograph with a mass spectrometer system (Agilent GC system 6890 series, Agilent MS detector series 5975).

- **Statistical analysis**

Statistical analysis was performed with Origin 8.5 software (OriginLab Corporation, Northampton, MA 01060, USA). Mean differences between dairy products were analyzed using ANOVA analysis of variance. Results were expressed according to standard deviation (SD), with a significance level set at $P < 0.05$. Post hoc test comparison was performed using Bonferoni, Tukey's and Scheffe.

Chapter 6 represents **Study 1 - Analysis of milk compliance parameters according to the milking system** and includes the resulting data on the hygienic-sanitary quality of milk obtained by using three different milking systems - manual milking, mechanical milking and robotic milking.

The main aim of this study was to evaluate raw milk in three cattle farms with different milking systems, by comparative monitoring of total germ count and somatic cell count.

Results

Out of the total of 180 samples collected from the three farms under study, 29 samples were obtained that exceeded the values imposed by the European standards and 151 samples complied with them, regarding the total number of germs. Most of the samples with exceeded TVC values came from the farm with the manual milking system.

During the summer months (June, July, August), a significant increase in the TVC value is observed in the individual milk samples obtained by manual milking, the weight of the samples exceeding the maximum allowed value being 60% of the total samples studied in these months .

From the total of 60 samples analyzed between March and August 2017, from the farm with robotic milking, all the samples fall within the EU standards, which means a geometric mean of TVC below 100,000 cfu/ml.

During the 6 months of the study, a number of 60 individual samples of raw milk were examined. Therefore, the average number of somatic cells obtained in March was 400,897 cells/ml, in April 420,320 cells/ml, and in May the average value was 459,051 cells/ml. In all these 3 months of milk, the average SCC obtained from the individual samples exceeded the sticking value imposed by the legislation. The geometric mean of the 3 months was 426,080 cells/ml, thus resulting in milk not compliant with EU standards, being removed from the market and denatured. In the following months, the geometric mean dropped below the maximum allowed value of 400,000 cells/ml. This improvement was possible due to the efficient monitoring of the mammary gland health of the animals.

In the farm with mechanized milking, the geometric mean in the first 3 months of the SCC had a value of 53,040 cells/ml, a value well below the maximum allowed limit. In the last 3 months, the value obtained was 47,527 cells/ml.

In the farm with robotic milking, all samples collected during the 6 months of the study were compliant with EU standards, obtaining values well below the maximum allowed limit.

Conclusions

In the farm with manual milking, a percentage of 41.67% of the samples exceed the normal value, which signifies the lack of hygiene conditions and milk contamination, compared to mechanical milking where only 6.66% of the samples exceed the value allowed by the legislation.

Following the comparative analysis of the total viable count in the studied holdings, it is observed that in the farm with mechanized milking and in the farm with mechanized milking by means of the robot, most samples are below 100,000 cfu/ml, in accordance with the EU standards (Reg. CE 853/2004).

In farms with mechanized milking and with mechanized milking by means of the robot, 97.50% of the samples had values below 60,000 cells/ml.

In the case of the farm with manual milking, only 60% of the individually collected samples fell below the value of 400,000 cells/ml, this proves that in this farm there is no program of prophylaxis and combating mammary gland diseases.

The robotization of the milking system in a dairy cow farm leads to obtaining a larger amount of milk, with values much lower than the maximum allowed limit in terms of hygiene parameters TVC and SCC, which means a quality milk.

Chapter 7 presents **Study 2 - The incidence of aflatoxin M_1 in milk originated from a regional surveillance system** and reveals information on the prevalence and content of aflatoxins in milk from the monitored area.

The aim of this study was to evaluate the frequency of AFM_1 contamination of milk in a surveillance area established in Romania, due to the precariousness of research on the prevalence and level of aflatoxins in milk, as well as the need, imposed by the increased toxicity, to evaluate the contamination of milk with aflatoxins in order to protect human health.

Results

The positive samples were detected only in 2013. The material used in 2013 was represented by 61 raw milk samples, 22 were obtained from milk collection units and 39 from breeding units. The study shows that 60.65% of the samples analyzed in 2013 were positive.

The samples collected from the milk collection centers were positive in proportion to 68%, two of which showed values that exceeded the maximum limit allowed by the current legislation.

The minimum value detected, respectively the maximum value of the contamination level of milk with AFM_1 was 0.010 μg/kg, respectively 0.089 μg/kg.

Due to the drastic measures taken in the period 2014-2017, both by producers and by the authorities, the level of aflatoxin M_1 decreased to undetectable values.

Conclusions

Following the study conducted, it was possible to observe the presence of aflatoxin M_1 in milk in 60.65% of the samples analyzed in 2013, both at the level of milk collection centers and at the level of farms. Exceeding the maximum allowed limit at the level of milk collection units can be explained by the receipt of aflatoxin-contaminated milk from several sources, which, by combining them, led to the increase of the aflatoxin level above the allowed limits.

The present study reveals that the risk of contamination of milk with aflatoxins may increase if animal feed is stored in improper humidity and temperature conditions. This risk can be significantly reduced by using feed that is not contaminated with aflatoxin. In addition, it is important that forages are frequently tested for aflatoxins before they are fed to animals.

Due to the fact that dairy products are consumed by both children and infants as well as adults, the presence of aflatoxins in milk and dairy products can have serious consequences for human health, generating imbalances in the reproductive, nervous system, as well as growth problems and development.

Chapter 8 represents **Study 3 - Evaluation of the level of contamination with phthalates in milk and dairy products found on the Romanian market** and provides data on the presence of phthalates in milk and dairy products, the mode of transfer and their degree of toxicity.

Given the lack of knowledge about the level of phthalates in dairy products produced in Romania and the importance of this topic, **our aim** was to investigate a specific milk production chain and assess the seriousness of this chemical hazard.

Results

Only four of the six phthalates studied were present in the samples.

In the case of commercial milk, the highest concentration of phthalates (DOP), respectively 0.3152 ± 0.2441 mg/kg, was found in a milk sample with a fat content of 3.5% and the lowest, of 0.0201 ± 0.0349 mg/kg (DBP) was determined in the case of milk samples with 1.5% fat content.

Based on statistical analyses, higher levels of phthalates were observed in raw milk at 4%, followed by pasteurized milk at 3.5%, raw milk at 3.5% and pasteurized milk at 1.5% fat content. Significant differences were observed only when comparing 4% raw milk versus 1.5% pasteurized milk ($p = 0.01$). Thus, there is a direct correlation between the level of total phthalates and the fat content.

In the case of cream, the highest concentration of phthalates (DMP), respectively 0.0831 ± 0.0214 mg/kg, was found in a sample with 20% fat. By comparing the values obtained for the cream samples, significant differences were observed for BBP ($p = 0.035$) and DOP ($p = 0.0089$). In the yogurt samples, the only significant difference was observed when comparing the total amount of phthalates ($p = 0.001$).

For fresh cheese, based on statistical analyses, significant differences were calculated in terms of the level of phthalates by comparing BBP and DEHP ($p = 0.002$), BBP and DOP ($p = 0.00074$), respectively BBP and DBP ($p = 0.006$). Higher total phthalate levels were observed in ripened cheeses (0.5905 ± 0.0175 mg/kg), followed by bellows cheese (kneaded cheese), cream cheese, fresh full-fat cheese and Telemea cheese.

According to the analyses, the lowest number of total phthalates was detected in yogurt with 0.1% fat - 0.042 ± 0.0052 mg/kg, and the highest concentration was recorded in butter with 85% fat - 0.683 ± 0 .0072 mg/kg.

Conclusions

Even if the total phthalate levels in all analyzed samples do not exceed the maximum limit (60 mg/kg), full-fat milk and dairy products could be considered a high-risk food category, being a potential source of human exposure to phthalates. Analyzes need to be carried out on a more extensive level to accurately assess the degree of this risk to human health.

Of the six phthalates examined, DEHP was the most frequently detected compound.

It could also be seen that the highest levels of phthalates were found in the samples where the fat concentration was higher (milk with 3.5% fat, cream cheese, fresh and matured cheese, cream and butter respectively).

Chapter 9 includes the general conclusions resulting from the studies carried out in Chapters 6, 7 and 8.

Chapter 10 reveals the original elements of this study, represented by important evidence about the quality of milk obtained by using modern milking technology with the help of robots, and also the determination of the presence of toxic compounds for human health, which come either by metabolizing them from the feed, reaching in milk-aflatoxins, either due to their lipophilic characteristics, end up in the composition of milk from packaging materials, respectively phthalates.

V. GENERAL CONCLUSIONS

- From the total number of individual milk samples processed during 2017, over a period of 6 months, a total number of 29 samples, respectively 16.11%, represented milk samples that exceeded the maximum value allowed by EU standards.
- The comparative determination of the number of somatic cells reveals the fact that for farms with mechanized milking and with mechanized milking by means of a robot, where 97.50% of the samples had values below 60,000 cells/ml, it denotes the fact that all ailments at the level of the mammary gland, respectively clinical and subclinical mastitis are detected in time, and the sick animals are isolated, treated accordingly and the milk is not used during this period,
- In the case of the farm with manual milking, only 60% of the samples fell below 400,000 cells/ml, which proves that in this farm there is no program for the prevention and combating of mammary gland ailments, the milk being sold regardless of the health of the animal.
- Following the comparative analysis of the total number of germs in the farms studied, it is observed that in the farm with mechanized milking and in the farm with mechanized milking by means of the robot, most samples are below 100,000 cfu/ml. In the case of the farm with manual milking, a percentage of 41.67% of the samples exceed the normal value, which signifies the lack of hygiene conditions and contamination of the milk, compared to mechanical milking where only 6.66% of the samples exceed the value allowed by the legislation.
- From the total samples with values above the limits, regarding the TVC value, 86.12% of the samples that exceeded this value came from farms where manual milking was performed. Regarding the SCC, its value was exceeded in 88.88% of the milk samples from the same origin. In this unit, during the summer, the geometric mean obtained for TVC exceeded the maximum value imposed by legislation, obtaining a milk that does not comply with EU standards, the same being the case of SCC.

- No correlation was observed between the two determinations, which indicates that the increased value of the TVC is due to poor hygiene during milking, and the high number of the SCC appears against the background of unidentified subcynic mastitis.
- Periodic monitoring of the hygienic quality of milk by determining SCC through individual samples facilitates the discovery of cows with chronic mastitis. Milk from cows with chronic mastitis has the greatest influence on the cell population in mixed milk, due to the fact that it does not present sensory changes.
- The robotization of the milking system in a milk cow farm leads to obtaining a larger amount of milk, with values much lower than the maximum allowed limit in terms of TVC and SCC hygiene parameters, thus avoiding the amount of milk removed from marketing and increasing the profit obtained by the farmer by reducing the costs incurred in the case of administering treatments to cows with mastitis.
- As with other milk production technologies, the robotic milking system has been designed to achieve advantages in terms of saving the labor of the farmer, improving the quality of life style and increasing milk production without harming health and well-being. animals.
- Overall, the benefits of automation in dairy farms can be seen through improved profitability, animal health, milk quality and farmers' lifestyles. Therefore, a robotic milking is extremely important and up-to-date. Manual work is partially replaced by management and control, the presence of the operator at regular milking being no longer necessary.
- Aflatoxin M_1 in milk was found in 60.65% of the samples analyzed in 2013, both at the level of milk collection centers and at the level of farms. Exceeding the maximum allowed limit at the level of milk collection units can be explained by the receipt of aflatoxin-contaminated milk from several sources, which, by combining them, led to the increase of the aflatoxin level above the allowed limits.
- The present study reveals the fact that the risk of contamination of milk with aflatoxins can increase if animal feed is stored in inappropriate humidity and temperature conditions. This risk can be significantly reduced by using feed that is not contaminated with aflatoxin. In addition, it is important that forages are frequently tested for aflatoxins before they are fed to animals.

- It is important that at the level of milk collection centers and milk processing and processing units only milk is received that meets the conditions imposed by the legislation in force, by complying with Regulation (EC) no. 165/2010, this is possible by taking precautions and using rapid tests to identify the presence of aflatoxins in raw milk.
- Due to the fact that dairy products are consumed by children and infants as well as adults, the presence of aflatoxins in milk and dairy products can have serious consequences for human health, generating imbalances in terms of the reproductive system, nervous system, as well as problems of growth and development. For these reasons, it is essential to avoid the consumption of milk and contaminated products by applying all measures to prevent their occurrence in milk.
- Even if the total phthalate levels in all analyzed samples do not exceed the maximum limit (60 mg/kg), full-fat milk and dairy products could be considered a high-risk food category, being a potential source of human exposure to phthalates. Analyzes need to be carried out on a more extensive level to accurately assess the degree of this risk to human health.
- Among the six phthalates examined, DEHP was the most frequently detected compound, being blamed for the occurrence of cancer, toxicity on the reproductive, nervous and immune systems, and also having effects of disrupting the endocrine system.
- It could also be observed that the highest levels of phthalates were found in the samples where the fat concentration was higher (milk with 3.5% fat, cream cheese, fresh and matured cheese, cream, respectively butter) which proves the lipophilic character of these compounds.
- In order to highlight the transfer rate of phthalates in milk and dairy products, it is necessary to evaluate the dynamics of these compounds over the entire shelf life of the products, because there are products with a long shelf life, in which case the amount of phthalates found in the products would it could increase quantitatively in direct proportion to the exposure period or there could be the possibility of degradation of these compounds over time.

VI. ELEMENTS OF ORIGINALITY AND CONTRIBUTION

The research is original in its integrity and is based on the following aspects:

- A study was carried out at the primary production level regarding the attestation of milk conformity according to the milking systems, thus providing a necessary database for a chemometric evaluation;
- Development of the knowledge base in the interest of producers, as well as of the official control authorities regarding the quality certification of milk obtained under particular conditions in Romania;
- Quantification of the studied substances in cow's milk and dairy products from different milk processing units;
- Determination of the transfer characteristics of phthalates in milk and their capacity to accumulate in the food chain;
- Assessing the risk to the health of the exposed population, due to the ingestion of milk, by comparing it with the maximum standard values allowed both in the case of aflatoxins and in the case of phthalates;
- Formation of a database on the footprint of phthalates monitored in milk and dairy products.