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ANTIBIOTIC RESIDUES IN MILK AND ASSESSMENT OF HUMAN HEALTH RISK IN ROMANIA

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Abstract

Antimicrobial resistance is expected to make many more victims in the future than cancer, which requires constant, rigorous and thorough control of all factors that contribute to the growth of this particularly worrying phenomenon. Animal products and in particular milk, especially the one obtained in the households of the population, could be especially contaminated with antibiotic residues. The present paper aims at the qualitative and quantitative screening of 284 samples sold by individual producers from the entire territory of Romania, in a period of 3 months (January-March this year). The qualitative test was a microbiological test in tubes with spores of Geobacillus stearothermophilus. 6.33% (n = 18) of the samples confirmed positively in the qualitative test, the quantitative test performed using chromatography showed the presence of beta-lactams and tetracycline in quantities exceeding the maximum residue limit (MRL), (21, 50 µg / kg milk for penicillin and on average 115 µg / kg milk for oxytetracyclines). Among aminoglycosides, gentamicin was on average 294.5 µg / kg milk far exceeding the MRL.

Key words*: antibiotic residues, health, milk, Romania.*

INTRODUCTION

Even though antibiotics have been banned from being used as growth promoters in animals, certain conditions still require such medications. Excessive and inappropriate use even for such conditions causes a transmission of these drug residues in organs, muscles and other animal products such as eggs and milk. This phenomenon contributes to the spread of antimicrobial resistance, a worrying and burdensome phenomenon of health systems around the world (Bassetti et al., 2020). Antibiotic residues in food have been reported in several recent studies (Tiseo et al., 2020; Treiber et al., 2021). Eggs (Mbodi et al., 2014; Nonga, et al.,2010), fish (Wang et al., 2019, Gaspar,et al., 2019), meat (Al-Ghamdi et al., 2000; Er et al., 2013; Ezenduka, 2019; Monger et al., 2021), seafood and especially milk (Ondieki et al., 2017; Pogurschi et al., 2015; Prado et al., 2015; Vragović et al., 2011) from various parts of the world have been reported as foods in which antibiotic residues were found. Milk, in particular, is a food consumed by vulnerable age groups such as children and the elderly. In addition, milk is also a source of protein and minerals essential for childhood and old age. Moreover, milk has an affordable price, which makes it present in today's diet almost daily or several times a day. All these considerations lead to the conclusion: milk placed on the market must be a safe product, without contaminants and drug residues, especially antibiotic residues. Previous research has shown the presence of different groups of antibiotics in milk in a well-defined legal context. All these considerations, together with the major health risks of antibiotic residues, make the monitoring of the hygienic quality of milk continuous and the monitoring systems to be correlated and upgraded periodically according to the reported determinations. The eating habits of the Romanian population are constantly changing (Iordachescu et al., 2020), and the source of acquisition has become increasingly important. Family producers, suppliers of agri-food products are in the preferences of the modern consumer. However, the lack of monitoring of their products has

negative consequences on health; this is unfortunately known by few consumers. The present study presents the results of the determinations of the presence of antibiotics in milk marketed by Romanian individual producers in the first 3 months of 2022.

MATERIALS AND METHODS

Out of a total of 300 milk samples collected from individual producers, 284 samples were subjected to determinations. The rest of the samples did not present an optimal condition to be analysed, probably due to the conditions during the transport to the laboratory. 250 samples tested negative for qualitative testing for antibiotics. A number of 16 tests had uncertain results, being false positive, which is why they were excluded from the following determinations. Only 18 samples were confirmed positive. The period analysed in this study was January - March 2022.

The qualitative screening

The qualitative screening of antibiotics was based on their inhibitor action against the in vitro growth of the bacterium *G. stearothermophilus* at 64 ± 0.5^0C. In the determination tube containing spores of the bacterium *G. stearothermophilus* preheated to the mentioned temperature 1 ml of milk sample was placed. The sample was left at the room temperature for 20 minutes for the possible present antibiotic to action. The tube with the milk sample to be analysed was reintroduced in incubator at 64 ± 0.5^0C for 3 - 3.5 hours for the second incubation. The colour differences between the milk sample tube and the control tubes represented the confirmations of the presence of antibiotics in the analysed milk samples (Figure 1).

The qualitative screening

Equipment and reagents

The equipment used were: Boeco centrifuge C-28A (Germany), ABI Digital Vortex - Genie 2 vortex mixer (USA), Elmasonic S 50 R ultrasonic bath (Germany), Heidolph Rotary Evaporator Laborota 4000 (Germany), Kern Analytical balance ABJ 220-4NM (Germany), Agilent 1260 Infinity II LC System (Germany), B 30 Water purification system ADRONA (Latvia).

Figure 1. Positive confirmed milk samples

The chromatographic grade reagents were: methanol (Honeywell Burdick & Jackson, USA), acetonitrile (Baker, Mexico), and triethylamine (Vetec, Brazil). The grade reagents were citric acid (Reagen, Brazil), EDTA disodium salt (Labsynth, Brazil), oxalic acid crystal (Dinamica, Brazil), anhydrous dibasic sodium phosphate (Nuclear, Brazil), and trichloroacetic acid (Reagen, Brazil). Ultrapure water for solutions preparation was obtained in the laboratory using B 30 Water purification system ADRONA (Latvia).

The study focused on the main groups of antibiotics that are widely used in both dairy and human medicine – B-lactams (Penicillin), Tetracycline (Oxytetracycline) and Aminoglycosides (Gentamicin). The operating procedures for storage and handling of the standards have been followed according to the manufacturer's instructions. Stock solutions were prepared by dissolving standards in methanol by using class A glassware (Final volume 25 ml) so that effective concentration remained more than 100 µg/mL. Standard solutions of different concentrations were stored in a deep freezer at -18°C. For preparation of standard solutions, the maximum residue limits (MRLs) prescribed by European Union Commission (EU, 2010) and Codex Alimentarius Commission of WHO (Codex, 2015) for all antibiotics were considered. Based on these MRL values, a linearity range (50, 100, 150, 200, 250 µg/kg) was selected to cover the lowest MRLs for all the analyte molecules.

LC instrumentation and condition

Agilent, 1260 Infinity II LC system used to determine milk antibiotics was equipped with: 1260 vialsampler and 1260 Flexible pump connected to C18 column (Poroshell 3.0 mm x 100 mm porosity 2,7 um) housed in 1260 MCT column oven with ELSD 1260 Infinity detector was used throughout the experiment. The system was controlled by Open Lab CDS 2.6 Software. The working technique was adapted to determine the three antibiotics studied - penicillin, oxytetracycline and gentamicin at sub MRL levels. The method was validated for specificity, precision, recovery and linearity. The extracted samples were centrifuged for 20 minutes at 3000 rpm in Eppendorf tube followed by filtration using 0.2 nm MFS filters. The final extracted samples were set to run in the LC system described above.

Risk assessment

To assess the risks associated with the presence of the three antibiotics determined in milk consumed the following equations were used in accordance with Juan et al., 2010 and Rahman et al., 2021.

$$\text{Hazard Quotient} = \frac{\text{EDI}}{\text{ADI}}, \text{ where}$$

EDI- Estimated Daily Intake
ADI- Acceptable Daily Intake

$$\text{EDI} = \frac{\text{(concentration of residue as µg/ kg) x (daily intake of food in kg/person)}}{\text{Adult body weight (60 kg)}}$$

The mean level of antibiotic concentrations in milk sample was calculated, then, the value of the mean concentration and average daily milk consumed based on 60 kg body weight were taken into consideration. The ADI values set by Codex Alimentarius Commission (2015) are: 3µg/kg BW/day for oxytetracycline, 4 µg/kg BW/day for gentamicin and 30µg/person/day for penicillin. According to data provides by National Institute of Public Health the daily milk consumption in Romania, was in 2020 109, 0 ml/man/day and 113, 2 ml/woman/day (https://insp.gov.ro/RAPORT-Sanatate-Mediu-2020.pdf).

If the Hazard Quotient value is higher than 1 then the likelihood of harm is stated but it is not the statistical probabilities of occurrence. A negligible hazard is when the value of the hazard quotient is less or equal to 1.

RESULTS AND DISCUSSIONS

In the present study, three of the most widely used classes of antibiotics used in both animals and humans were considered (Figure 2).

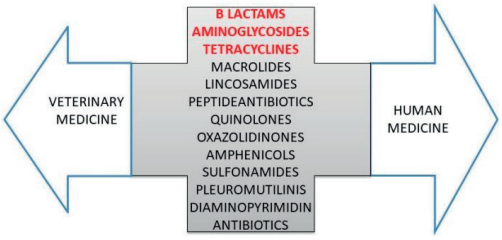

Figure 2. Classes of antibiotics used in human and veterinary medicine in Europe

284 of the milk samples subjected to qualitative screening, 6,34% (n=18) were positive, 88,03% (n=250) were negative and 5,63% (n=16) had false positive results, which is why they were removed from subsequent determinations. Table 1 and Figure 3 show the proportion of each sample category.

Table 1. The percentage distribution of tested milk samples

Specification	n	%
Milk samples	284	100
Positive milk samples	18	6,34
False positive milk samples	16	5,63
Negative milk samples	250	88,03

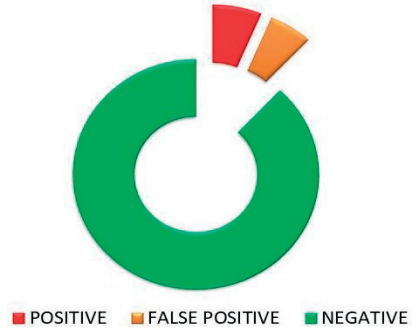

Figure 3. The percentage distribution of tested milk samples

After the successful completion of the qualitative testing, the samples tested positive for the three (Penicillin, Oxytetracycline and

Gentamicin) antibiotics were subjected to quantitative determinations. The overall occurrence of antibiotics residues is presented in Table 2.

Table 2. The overall occurrence of antibiotics residues in positive samples milk

Positive samples milk n=18		
Penicillin	Oxytetracycline	Gentamicin
n=5	n=9	n=4
27,77%	50,00%	22,23%

Oxytetracycline is the antibiotic present in half of the positive samples (n=9), while the milk samples tested positive for penicillin represented a little more than a quarter of the total samples (27,77%).

Gentamicin was tested positive for a number of 4 samples, which represents 22% of the total positive samples (Figure 4).

These results are similar to those obtained in 2015 by Pogurschi et al., in the metropolitan area of the Romanian capital, where 28.37% of the samples were contaminated with beta-lactams, a group to which Penicillin belongs and 71.43% of the samples were reported contaminated with tetracycline, group to which oxytetracycline belongs.

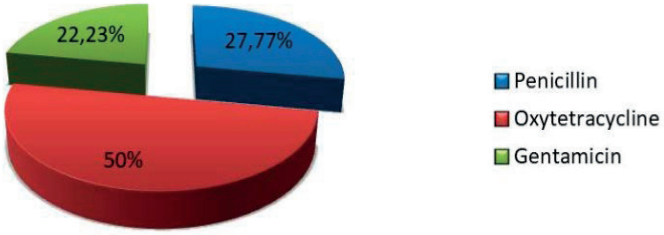

Figure 4. The proportion of samples tested positive for the three antibiotics studied

The total number of positive samples is lower when comparing to others (Rahman et al., 2021) but the difference is not significant, respectively 6.34% compared to 7%. Zanella et al., 2010 reported significantly high presence of tertacyclines in milk, 18,5% (n=48) of the samples being contaminated with tetracyclines.

The results of the quantitative test performed using Agilent 1260 Infinity II LC System are shown in Table 3.

Table 3. Mean concentration of antibiotics residues in milk

Group of antimicrobials	Analytes	Mean Conc.[a] (µg / kg)	MRL value[b] (µg / kg)
B-lactams	Penicillin	21. 50	4
Tetracycline	Oxytetracycline	115.00	100
Aminoglycosides	Gentamicin	294.5	100

[a] The average concentration of positive samples
b MRL value was collected from EU regulation no.37/2010

As can be seen from the data presented in Table 3, the mean Penicillin concentration (21, 50 µg/kg) was more than 5.37 times higher than the MRL level, while Gentamicin (294.5 µg/kg) was more than 2.95 times higher than the MRL level.

The level of oxytretracycline, the antibiotic detected in 50% of the milk samples, exceeded the MRL values, but this excess is a small one, 1,15%. The amount of antibiotics detected in these samples may be due to the fact that the milk from individual producers does not undergo heat treatments. Milk treatments, such as pasteurization, can reduce the final milk content of residues.

Pasteurized milk, therefore subjected to a heat treatment, contains fewer contaminants. This is confirmed by several researchers including Rasooli et al., 2014, in whose study of 432 pasteurized milk samples only 1.62% (n=7) contained tetracycline residues above MRL.

In order to assess the risk through the intake of raw analysed milk in the present study, the (EDIs) for consumers were calculated. Based on the mean value of residues in analysed milk samples and the daily milk consumption in Romania, reported in 2020, the risk of dietary exposure to penicillin, oxytetracycline and gentamicin through the milk consumed has been assessed. The results are presented in Table 4. The lowest EDI value was calculated for penicillin.

Table 4. Evaluation of risk assessment

Antibiotic	EDI (µg /kg/day)		ADI (µg/kg/day)		Hazard Quotient	
	Woman	Man	Woman	Man	Woman	Man
Penicillin	0.041	0.039	200		0.00021	0.00019
Oxytetracycline	0.217	0.208	30		0.0072	0.0069
Gentamicin	0.555	0.535	30		0.0018	0.0178

The highest individual calculated EDI was obtained for gentamicin, followed by oxytetracycline. For the three studied antibiotics, the Hazard Quotient is less than 1, which is why it can be concluded that the detected levels of residues in milk could not be considered as a public health issue with regards to these veterinary antimicrobial substances.

CONCLUSIONS

The methodology used to determine antibiotics residues in milk was in accordance with the protocol recommended by the U.E. legislation, which requires two steps: qualitative microbiological procedure and quantitative procedure for confirmation of the results obtained in the first stage. The most common contaminant was oxytetracycline, an antibiotic whose residues was found in half of the confirmed positive samples. Residues measured for gentamicin were far above the maximum residue limits (MRLs) set by law. The Hazard Quotient calculated for all three antibiotics was less than 1. This indicates that the milk marketed by individual producers in Romania, in average, contains low levels of veterinary drugs, and therefore, it could be considered as safe for human consumption. Regarding the milk consumption, this should be doubled in order to reach 200 ml / person / day, an amount that can be considered acceptable as part of a healthy diet and lifestyle.

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