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Penicillin and tetracycline residues in selected fresh and UHT milk with different fat contents

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Abstract

The aim of the present work was to determine the residues of penicillin and tetracycline in selected fresh and UHT milk with different fat contents (< 2, 2, and > 2%). A total of 84 different milk samples (36 fresh and 48 UHT) purchased from retail chains in Silesia Region (Poland) were analysed. Penicillin and tetracycline residues were determined by the ELISA immunoenzymatic method. The obtained results were compared with MRL values adopted in Commission Regulation (EU) No. 37/2010. The results were additionally analysed statistically. Penicillin and tetracycline were found to be common in the analysed milk samples. Both antibiotics were present in low concentrations, and not exceeding their MRL. The median of penicillin residues was 0.298 µg/L, and 0.970 µg/L for tetracycline. For some samples, the determined levels of antibiotic residues did not exceed the limit of detection (LOD): three samples for penicillin (1.15%), and 24 samples for tetracycline (28.57%). A weak negative correlation between the level of penicillin and tetracycline residues was found (p = -0.218), where the decrease in penicillin concentration weakly correlated to the increase in tetracycline concentration. The level of residues of both antibiotics was significantly higher in fresh milk than in UHT milk (by 11.75% for penicillin, and 17.73% for tetracycline); for penicillin, $Z_{corrected}$ = 2.445, and p = 0.0145, and for tetracycline, $Z_{corrected}$ = 3.914, and p = 0.000091.

Introduction

The introduction of antibiotics into clinical practice should be considered as the greatest therapeutic breakthrough in the field of medicine in the 20th century. The discovery of penicillin in 1929 by Sir Alexander Fleming significantly reduced the mortality rates due to bacterial diseases, and limited their spread, which was awarded the Nobel Prize in 1945. Currently, antibiotics are used for therapeutic and prophylactic purposes not only in human medicine but also in veterinary medicine (Ronquillo and Hernandez, 2017; Del Fiol *et al.,* 2018).

The beginning of the use of antibiotics in animals is defined in 1944 - 1945 when penicillin in lyophilised form was used by veterinarians to treat mastitis in cows. It was discovered that the application of these substances caused faster weight gain, and reduced animal mortality (Ronquillo and Hernandez, 2017). In the 1940s, Moore *et al.* (1946) published a study stating that sulfadiazines and aminoglycosides increased the weight of chickens after their inclusion in the standard poultry diet. The inclusion of antibiotics into feed has enabled faster growth of animals, higher body weight gain (even up to 28.0%), lower food consumption (by about 0.8 - 7.6%), lower ammonia and methane emissions, and a significant reduction in economic losses by reducing animal deaths (Del Fiol *et al.,* 2018). The described benefits became the beginning of the mass use of antibiotics in animal breeding, which were called antibiotic growth promoters (AGP).

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The widespread use of antibiotics on an industrial scale in animal production and their increased use in human and veterinary medicine have contributed to the occurrence of bacterial resistance (Ronquillo and Hernandez, 2017). This problem is particularly noticeable among people who are increasingly infected with antibiotic-resistant bacterial strains that do not react to traditional methods of treatment (Przeniosło-Siwczyńska *et al.,* 2015; Ronquillo and Hernandez, 2017). The phenomenon of drug-resistant bacteria associated with the mass use of antibiotics in animal husbandry was first described in Swann's report from 1969. This report led to the division of antibiotics for use in medicine (available only by veterinary prescription) and the so-called feed antibiotics, which could be used on farms under less supervision. Increased awareness on the risk of antibiotic resistance has led to the withdrawal of certain groups of antibiotics from use. As early as 1972 - 1974, penicillin, tetracycline, and streptomycin were banned in some European countries as AGP (Ronquillo and Hernandez, 2017). In the following years, legislators created stricter laws, leading to a total ban on the use of AGP in Europe. In 2003, Regulation (EC) No. 1831/2003 of the European Parliament and of the Council on Additives for Use in Animal Nutrition was adopted on 22nd August 2003 by the European Union (EU, 2003). This had significantly restricted the use of most antibiotics as animal weight enhancers. Finally, under this Regulation, a total ban on AGP (Regulation (EC) No. 1831/2003) (EU, 2003) has been enforced in the European Union since 1st January 2006. Unfortunately, despite numerous evidence in a link between the misuse of antibiotics and the increase in antibiotic resistance, many countries have not withdrawn AGP from use. To date, AGP can still be used in the United States, China, Australia, and Russia (Przeniosło-Siwczyńska *et al.,* 2015; Ronquillo and Hernandez, 2017). According to American sources, about 75% of the antibiotics produced there are used in the treatment and prevention of animal diseases (Del Fiol *et al.,* 2018).

In 2015, the European Centre for Disease Prevention and Control (ECDC), European Medicines Agency (EMA), and European Food Safety Authority (EFSA) have published a joint report which discussed the relationship between the use of antibiotics and the incidence of antibiotic resistance in humans and animals (ECDC/EFSA/EMA, 2015; Osek and Wieczorek, 2015). The report showed that in 2012, in 26 countries of the European Union as well as Norway and Iceland, about 3,400 tons of antibiotics in the form of active substance for human treatment were sold. The same report notes that the use of antibiotics in animal husbandry was 134.8% higher, and amounted to 7,982 tons (Osek and Wieczorek, 2015). The highest use of antibiotics, which significantly exceeded the European average (144.0 mg/kg), was in Cyprus (396.5 mg/kg), Italy (341.0 mg/kg), and Hungary (245.5 mg/kg). The lowest use of antibiotics in animal husbandry was recorded in Norway (3.8 mg/kg), Iceland (5.9 mg/kg), and Sweden (13.5 mg/kg). The same report provided data relating to the use of specific groups of antibiotics. They showed that in 2012, two groups of antibiotics dominated the veterinary medicine, namely tetracycline (2942.6 tons) and penicillin (1779.8 tons). This is disturbing due to the fact that as many as eight of the countries surveyed (including Poland) have seen greater use of antibiotics in veterinary medicine than in human medicine. Finally, the report confirmed that an increase in the use of antibiotics in animal production correlated with the occurrence of resistance in bacteria isolated from animals and foods of animal origin (ECDC/EFSA/EMA, 2015).

Similar data have been published by the Food and Drug Administration (FDA). The FDA reported that 13,500 tonnes of antibiotics have been used in animal production, mainly for cattle, poultry, and pig farming (FDA, 2018). This poses a significant risk of human exposure to antibiotic residues in foods of animal origin. This threat may also occur in the environment, where antibiotics eliminated in faeces and urine are eventually released. It is suspected that every year, only with animal excretion (excluding human waste), approximately 10 million kg of antibiotics are released into the environment. Contamination of the environment also occurs through the use of plant protection products, hospital sewage, and out-of-date drugs, which ultimately contaminate groundwater, and carry the risk of unwittingly taking small doses of antibiotics chronically (Chee-Sanford *et al.,* 2009; Kulkarni *et al.,* 2017; Szekeres *et al.,* 2017). On a global scale, these figures are even more alarming. The data obtained by The State of the World's Antibiotics 2015 showed that for every 100,000 tonnes of antibiotics produced worldwide, 65,000 tonnes are used in animal production, representing 65% of the total production (Singer *et al.,* 2016).

To ensure the health of consumers as well as the quality of zoonotic products, systematic control of antibiotic residues in these products was carried out within the European Union (Gajda *et al.,* 2012). The Maximum Residue Limits (MRLs), which are considered safe for humans, have also been established. The rules for setting the limits and their values are laid down in two regulations. The first one is the Regulation (EC) No. 470/2009 of the European Parliament and the Council adopted on 6th May 2009 (EU, 2009), which specified community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin. The second one is the Commission Regulation (EU) No. 37/2010 adopted on 22nd December 2009, on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin (Commission Regulation (EU) No. 37/2010 and Regulation (EC) No. 470/2009) (EU, 2009). Excessive and irrational administration of antibiotics to farm animals contribute to the occurrence of their residues in animal products (Gajda *et al.,* 2012; Ronquillo and Hernandez, 2017). A withdrawal period has been introduced to protect the population from the harmful effects of antibiotic residues. This is the time when the treated animal or herd cannot be killed or delivered for sale (Giedrojc-Brzana *et al.,* 2017; Patyra *et al.,* 2018). It should be remembered, however, that the withdrawal period is usually determined on the basis of tests carried out on healthy animals, therefore, even if it is observed, there is a certain risk of antibiotic residues in meat and zoonotic products associated with the individual metabolism of the drug by the sick animal (Gajda *et al.,* 2012).

The main aim of the present work was to determine the residues of penicillin and tetracycline in selected fresh and UHT milk samples with different fat contents. Additionally, an attempt was made to verify the relationship between the level of antibiotic residues and the type of milk and fat content.

Materials and methods

Experimental material

Fresh milk (n = 36) and UHT milk (n = 48) samples were purchased from January to April 2019 in 10 retail chains within the Silesia Region (Poland). The fat contents of the milk samples were: < 2% (n = 18), 2% (n = 33), and > 2% (n = 33). Fresh milk with fat content of < 2% was not included in the present work due to its inavailability in selected retail chains (Table 1).

Table 1. The fat contents of milk samples.

Type of milk	**Fat content**		
	< 2%	**2%**	**> 2%**
Fresh	0	18	18
UHT	18	15	15
Total	18	33	33

The purchased milk samples came exclusively from Polish producers, and the analyses were conducted before the expiry date of their shelf-life. The milk samples were stored following the producer's recommendations (fresh milk at 1 - 6°C; UHT milk at 2 - 25°C) until the analyses. Samples of milk with fat content of > 1.5% were skimmed by centrifugation (10 min, 3,000 *g*, 10°C). Prior to analyses, preliminary tests were performed to check the level of selected antibiotics in the samples. The dilutions were selected based on the results of these tests and the information provided in the instructions supplied with the analytical kits. Samples were diluted immediately before the assay.

Analytical methods

The determination of residues of selected antibiotics was performed at the Department of Toxicology and Health Protection, Faculty of Health Sciences in Bytom, Medical University of Silesia, Poland.

The residues of penicillin and tetracycline were determined by a competitive ELISA method, using appropriate commercial kits; Penicillin ELISA (Euro Proxima, the Netherlands) and Ridascreen Tetracyclin (R-Biopharm, Germany). The reagents were prepared with water of first purity degree (PN/EN 3696) (demineraliser Hydrolab HLP series, Smart UV model). The tests were performed following the manufacturer's recommendations. Previously, diluted milk samples (each in two repetitions) were tested. Absorbance was measured with the use of Mindray MW-12A microplate reader at a wavelength of 450 nm. The concentration of antibiotics in milk samples was determined on the basis of a standard curve prepared using a series of standards (RIDA SOFT Win.NET, version 1.99 for tetracycline; Curve Expert 1.4 for penicillin). The values ([absorbance standard / absorbance standard zero] × 100 = B/B0%) calculated for the standards were plotted on the y-axis (y-axis in normal scale for tetracycline; y-axis in logarithmic for penicillin) *versus* concentration (μg/L) on an x-axis (Figures 1 and 2).

The limits of detection (LOD) were 0.08 μg/L for Penicillin ELISA and 0.9 μg/L for Ridascreen Tetracyclin. For calculation purposes, results below the LOD were taken into account as half of the LOD specified by the manufacturers (0.04 μg/L for penicillin, and 0.45 μg/L for tetracycline).

The specificity of the Penicillin ELISA test was 100% for ampicillin and benzylpenicillin

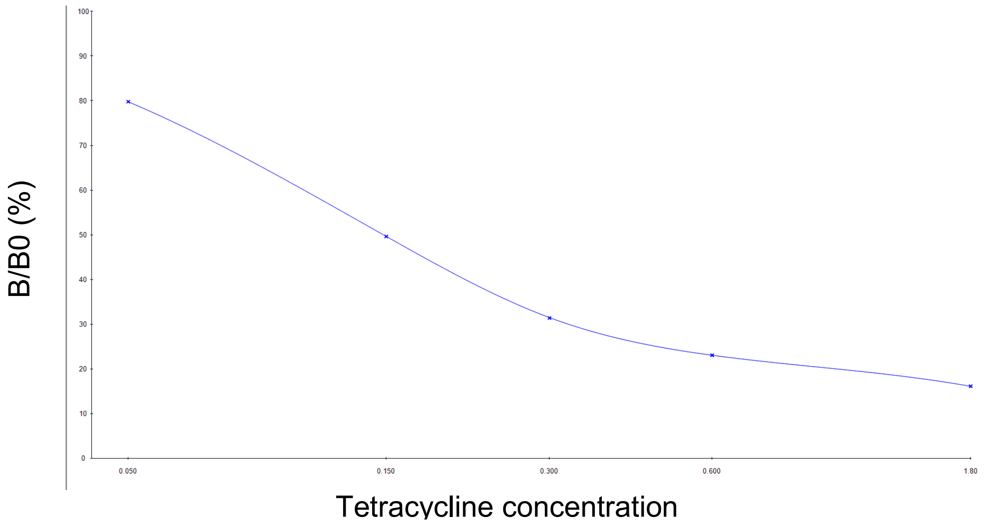

Figure 1. Calibration curve of tetracycline as estimated by ELISA (RIDA SOFT Win.NET, version 1.99).

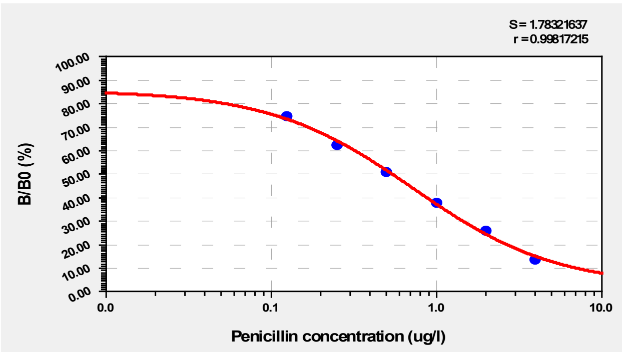

Figure 2. Calibration curve of penicillin as estimated by ELISA (Curve Expert 1.4).

(penicillin G), 99% for azlocillin, and 3 - 88% for other penicillin analogues (in descending order: piperacillin, amoxicillin, penicillin V, oxacillin, cloxacillin, dicloxacillin, and nafcillin). The specificity of the Ridascreen Tetracycline test was 100% for tetracycline (standard substance) and 2 - 70% for tetracycline analogues (in descending order: chlortetracycline, rolitetracycline, demeclocycline, oxytetracycline, minocycline, and doxycycline).

The results obtained were compared with MRLs adopted in Commission Regulation (EU) No. 37/2010. According to the Regulation, the MRL in milk for ampicillin, benzylpenicillin, and amoxicillin is 4 µg/L, and 30 µg/L for oxacillin, cloxacillin, dicloxacillin, and nafcillin. For tetracycline, the MRL in milk is 100 µg/L (tetracycline, chlortetracycline, doxycycline, and oxytetracycline) (Commission Regulation (EU) No. 37/2010). In the present work, all results were given for the sum of penicillin and tetracycline without distinguishing between the active substances.

Statistical analysis

The obtained data were statistically analysed using Statistica 13.3 software (Statsoft; Texas, USA). The data normality was checked using the Shapiro-Wilk test. To evaluate the statistical significance of the differences, Mann-Whitney *U* test (dependence of antibiotic residues on milk type) and Kruskal-Wallis with *post-hoc* test (dependence of antibiotic residues on fat content) were used. The correlation was assessed using Spearman's Rank Correlation Factor (R). The level of significance $\alpha = 0.05$ was assumed.

Results and discussion

The obtained results showed that the levels of penicillin and tetracycline residues in the analysed milk samples were low. The concentrations of penicillin ranged from 0.040 to 0.804 µg/L, while those of tetracycline ranged from 0.450 to 2.520 µg/L. The median (Me) of penicillin residues was 0.298 (Me = 0.298 [0.237:0.359]), and for tetracycline was 0.970 (Me = 0.970 [0.450:1.230]). In the case of some samples, the determined levels of antibiotic residues did not exceed the LOD; three samples for penicillin (1.15%), and 24 samples for tetracycline (28.57%) (Table 2).

None of the milk samples tested were found to exceed the MRLs for both antibiotics (Table 2). For tetracycline, it is very unlikely that the acceptable daily intake (ADI) will be exceeded. The ADI for tetracycline is 0 - 0.03 mg/kg bw/day (WHO, 1999). This means that the average global consumer of 62.0 kg (Walpole *et al.*, 2012) may consume between 0 and 1.86 mg of tetracycline per day for life without any health risk. Assuming that milk would be the only source of tetracycline in the diet, the ADI could only

Table 2. Penicillin and tetracycline residues in milk samples regardless of the type of milk and fat content.

Parameter	Antibiotic residue (µg/L)	
	Penicillin	**Tetracycline**
Median (Me) [Q1:Q3]	0.298 [0.237:0.359]	0.970 [0.450:1.230]
No. of samples found above LOD	3	24
No. of samples detected positive below LOD	81	60
No. of samples found above MRL	0	0

be exceeded if about 738 L of milk with the highest concentration of these antibiotics found in studies (2.520 µg/L ~ 0.00252 mg/L) was consumed. The ADI for penicillin is only for ampicillin (0 - 0.003 mg/kg bw/day) and amoxicillin (0 - 0.002 mg/kg bw/day) (WHO, 2017a; 2017b). In this situation, the daily intake of ampicillin by a global consumer should not exceed 0.186 mg, and in the case of amoxicillin should not exceed 0.124 mg. Assuming that the authors' highest concentration of penicillin (0.804 µg/L ~ 0.000804 mg/L) fully corresponds to ampicillin, the ADI could be exceeded only by drinking approximately 231 L of milk per day, while the results for amoxicillin indicate that the consumption of more than 154 L of milk per day would be potentially dangerous. According to data gathered in 2017, the average world consumption of dairy products (in milk equivalent) is 109.1 kg/man/year, *i.e.,* about only 0.3 L/man/day (FAO, 2019). Therefore, the real threat to the consumer may only occur when antibiotics are supplied in large quantities from other sources for example during antibiotic therapy.

Comparing the present results with those available in the literature, it can be stated that exceeding the acceptable level of tetracycline residues in milk is very rare. Most often, the concentration of these antibiotics does not exceed the MRL, but is found below the detection limit. An example of a Chinese study in which only two out of 26 tested samples showed the presence of tetracycline at 37.08 and 47.7 µg/L (Zhang *et al.,* 2014). Significantly lower levels of tetracycline residues in milk were also found in Croatia (mean: 1.5 µg/L, minimum: 0 µg/L, maximum: 4.26 µg/L) where 12 out of 50 tested samples did not exceed the LOD of 3 µg/L (Vragović *et al.,* 2011). In Czech Republic, although none of the 170 analysed raw milk samples were free from tetracyclines, they did not exceed the MRL (median: 3.73 µg/L, minimum: 0.24 µg/L, maximum: 24.47 µg/L), of which 69.4% of samples contained tetracycline at < 5 µg/L (Navratilova *et al.,* 2009). Gaurav et al. (2014), however, reported that the MRL for tetracycline was exceeded in three out of 133 samples tested. Nevertheless, they also regarded that as a rare occurrence since 115 samples were below the LOD of < 1.5 µg/L (Gaurav *et al.,* 2014).

The levels of penicillin residues in milk are more varied. Since β-lactam antibiotics, especially penicillin, play a key role in the treatment of infectious diseases in dairy cows, milk is most often the main source of these compounds. Prior to the enforcement of legislation which restricted the use of antibiotics in the veterinary industry in 2003, the concentrations of penicillin in milk were often very high. Strasser *et al.* (2003) reported that only 13 out of 186 milk samples tested contained no penicillin residues, and in 10% of the samples penicillin was present in concentrations of > 100 µg/L, significantly exceeding its MRL. Khaskheli *et al.* (2008) used high-performance liquid chromatography to examine 137 commercially available untreated milk samples, and found that 36.5% were positive for penicillin G, ampicillin, and amoxicillin. The mean concentrations of these antibiotics exceeded their MRL values; penicillin G by about 14.9 times (mean = 59.53 µg/L), amoxicillin by about 9.03 times (mean = 36.10 µg/L), and ampicillin by about 11.2 times (mean = 46.91 µg/L). In the following years, a lower percentage of milk samples contaminated with high penicillin concentrations was observed. In Kosovo, during 2009 - 2010, out of 1,734 milk samples tested, 106 samples (6.1%) were positive for penicillin G, amoxicillin, ampicillin, cloxacillin, and dicloxacillin, of which only 17 contained at least one of the analogues above MRL (Rama *et al.,* 2017).

Rama *et al.* (2017) also noted a weak negative correlation between the level of penicillin and tetracycline residues (p = -0.218) where the decrease in penicillin concentration weakly correlated to the increase in tetracycline concentration. The occurrence of such a relationship could not have been accidental, and results from the antagonistic action of penicillin and tetracycline were in relation to each other.

Table 3. Penicillin and tetracycline residues in milk samples based on milk type and fat content.

Antibiotic residue (µg/L) Median (Me) [Q1:Q3]	Type of milk	
	Fresh milk	**UHT milk**
Penicillin	0.315 [0.277:0.365]	0.278 [0.204:0.324]
Tetracycline	1.100 [0.957:1.380]	0.905 [0.450:1.015]

Antibiotic residue (µg/L) Median (Me) [Q1:Q3]	Fat content		
	< 2%	**2%**	**> 2%**
Penicillin	0.247 [0.158:0.296]	0.310 [0.265:0.360]	0.314 [0.256:0.365]
Tetracycline	0.686 [0.450:1.100]	0.970 [0.450:1.260]	0.980 [0.910:1.240]

Therefore, during proper antibiotic therapy, these compounds are not combined because they may lead to the weakening or total inhibition of their respective action (Pejsak and Truszczyński, 2013). Of course, the interaction between penicillin and tetracycline is related to their simultaneous administration, but there are no contraindications for substitution at different times. For this reason, both penicillin and tetracycline (67.85%, 57 samples) were detected in one milk sample many times. One of the factors responsible for this phenomenon may be the ability of tetracycline to accumulate in bone tissue (due to the binding to calcium ions) from which they are released even several years after the end of exposure (Klaassen, 2018).

In the present work, the determined levels of residues of selected antibiotics in milk samples were additionally analysed in relation to the types of milk namely fresh or UHT (Table 3). Results showed that in fresh milk, the penicillin concentrations ranged from 0.100 µg/L to 0.804 µg/L, and the tetracycline concentrations ranged from 0.450 to 2.240 µg/L. In UHT milk, the penicillin concentrations ranged from 0.040 to 0.522 µg/L, and the tetracycline concentrations ranged from 0.450 to 2.520 µg/L. The levels of residues of both antibiotics were significantly higher in fresh milk than in UHT milk; for penicillin, $Z_{corrected} = 2.445$, $p = 0.0145$; and for tetracycline, $Z_{corrected} = 3.914$, $p = 0.000091$. The levels of penicillin residues in fresh milk was 11.75% higher than in UHT milk, whereas for tetracycline, the difference was 17.73%. The observed differences may be the result of the different temperatures of thermal treatment of milk before it was released to the market. During UHT sterilisation process, the temperature of 135 - 150°C is used, whereas the commercially available fresh milk is routinely pasteurised at < 100°C. The thermostability of tetracycline and penicillin varies; but, most often the total heat-decomposition of these antibiotics occurs at 220 - 240°C. It is also worth noting that their heat-decomposition already begins at 50°C. Depending on the prevailing conditions and the duration of the high temperature treatment, the heat-decomposition of antibiotics may already be significant at < 150°C. In the case of penicillin, 150°C degrades up to 30% of the initial compound mass, while for tetracycline, the loss is between 10 - 50% (Hassani *et al.,* 2008; Svahn and Björklund, 2015). The present results are not direct evidence of higher degradation of antibiotics in milk treated with UHT because the analyses included different samples; but, given the numerous literature reports, such conclusions can be made indirectly. At the same time, it should also be remembered that products in which antibiotics decompose as a result of heat treatment are not at all safer for the consumers. On the contrary, studies conducted for many years have indicated the formation of many compounds of unknown toxicity in the process of antibiotic thermodegradation, whose harmfulness to the organism may be much greater than the residues of the original substance (Tian *et al.,* 2017).

The second factor differentiating milk samples in the statistical analysis was fat contents (< 2, 2, and > 2%) (Table 3). The median concentration of penicillin was higher for milk with fat content of 2% and more. Statistical analysis showed that penicillin residues were significantly more frequent in milk with fat content > 2.0% (R = 47.061) than in milk with fat content < 2.0% (R = 29.361; $p = 0.0398$). No similar relationship was found in the case of tetracycline residues. It is difficult to explain the cause of this phenomenon, as there are no available literature on this issue at the moment. Taking into account the hydrophilic character of the analysed antibiotic groups, their binding to the fat present in milk can be excluded. It seems more likely that the above dependence was obtained accidentally. Nevertheless, in the future, it would be necessary to repeat the test with more samples to further ascertain this.

Conclusions

In the present work, the concentrations of penicillin and tetracycline residues in fresh and UHT milk with different fat contents (< 2, 2, and > 2%) were determined. Residues of penicillin and tetracycline were commonly found in the analysed milk samples; but, in low concentrations and not exceeding the MRLs. A weak negative correlation between the level of penicillin and tetracycline residues was found (the decrease in penicillin concentrations weakly correlated to the increase in tetracyclines concentrations). The levels of antibiotic residues were significantly higher in fresh milk than in UHT milk; by 11.75% for penicillin, and by 17.73% for tetracycline. The lower level of antibiotic residues in UHT milk with very few studies in this area indicate the need for further and more advanced research. The obtained results generally indicate that the levels of milk contamination with penicillin and tetracycline are still within the specified limits. However, their concentrations require constant monitoring to prevent future harmful effects on health.

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