Effects of cephalexin residues on the starter culture's microbial activity during the fresh cheese making process

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Keywords: cephalexin, cheese, whey, carry-over, LC-MS/MS

1. SUMMARY

The aim of our study was to investigate the carry-over of cephalexin from cow's milk to cheese and whey, as well as, to study the potential impact of its presence on the microbial activity of the starter culture. Before cheese-making, the raw milk was artificially contaminated to different antibiotic levels. Cephalexin concentrations and the pH values were measured all along the process. It was found that cephalexin was transferred less into the cheese curd (1.8-4.3 % of the original amount) than into the whey (29.3-42.8 %). According to the results the concentration of cephalexin did not influence substantially the pH changes during curding nor the activity of the starter culture. However, pH of the fresh cheese showed significant ($p < 0.05$) differences compared to the control suggesting that antibiotic residues even below MRL level may influence the quality of product.

2. Introduction

Dairy products form an important part of the healthy everyday diet, being consumed in high and growing amounts worldwide. Cheese production process involves the formation of lactic acid from lactose as an essential step. Acidification of the cheesemilk is usually initiated by selected starter cultures of lactic acid bacteria. The rate and extent of acidification can significantly influence the quality of cheese, including its texture, by having effect on the microbial biota of the developing product. Whey is the main by-product of cheese making, with utilization options as an ingredient in human foodstuff and animal feed production as well as in agricultural applications **[1]**. Therefore, the presence of antibiotic residues in cheese whey could have negative implications for human or animal healthcare or environmental safety.

Ensuring livestock health is of utmost importance for combating hunger and assuring appropriate food for the population, and antibiotic treatments are still an important part of this effort. Therefore, the fate of veterinary drug residues in foodstuffs remains of increasing concern due to their possible negative implications for consumer health such as allergic reactions or disturbances in the intestinal flora **[2, 3, 4]**. In addition to the direct negative effects on human health, antibiotic residues may contribute to the development of antimicrobial resistance **[5, 6, 7]**.

In spite of the concerns outlined above, maximum residue levels (MRLs) of veterinary drug residues in dairy products, such as cheese or whey, is not regulated by the relevant Commission Regulation (EU) No. 37/2010 **[8]**. This lack of regulation is even more incomprehensible, when the actual data on antibiotic residues found in dairy products are taken into account **[9, 10, 11]**.

Cephalexin is a first-generation cephalosporin antibiotic belonging to the wider group of β-lactams with a broad spectrum of activity against both Gram positive and Gram negative bacteria **[12]**. In the European Union, it is set out in the Commission Regulation (EU) No. 37/2010 **[8]** with a maximum residue limit of 100 µg/kg for bovine milk. Since it can penetrate to soft tissues in significant ratio it has been widely applied in the treatment of dairy cow mastitis either alone or in combination products **[13]**.

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Our study was aimed to investigate the carry-over of cephalexin from cow's milk to cheese and the whey produced from it, as well as, to study the potential impact of its presence on the pH of the internal products and the fresh cheese made from raw and heat-treated cow's milk, respectively. Our further aim was to study the possible effects of cephalexin residues on the microbe count of cheesemilk and thus the initial operation of starter culture for cheese production.

3. Materials and methods

3.1. Milk spiking and cheese making

Raw cow milk was purchased at a local market at the beginning of every trial day. It was quick-tested for possible antibiotic residues by DelvoTest® (DSM Food Specialties B.V. Delft, The Netherlands). It was also tested specifically for residues of 11 veterinary antibiotics by an LC-MS/MS method developed previously **[14]**. From the raw milk no positive sample occurred during the trials.

Fresh cheese was prepared from 10 litres of control raw milk itself (without additives or heat treatment) in every trial, to serve as control samples for analysis. Furthermore, for each trial, 10 litres of milk were artificially contaminated to concentration levels of 50, 100 and 500 ng/mL cephalexin (by Fluka, purchased from Sigma-Aldrich, USA) respectively, resulting in theoretical contamination levels of 0.5·MRL, 1·MRL, 5·MRL values of this antibiotics (Low, Medium and High trials, respectively). After this, the milk was divided into two equal portions (two times 5 litres), from which one portion was subjected to heat treatment (72 °C, 15 sec) industrially applied in case of this cheese type, the other one was processed without it (see ***Figure 1***). Heat treatment was carried out in a temperature controlled cheese vat with continuous slow stirring in order to maintain balanced heat distribution. After heat treatment, fresh cheese was made from the milk with lyophilised starter culture (CHOOZIT™ RA 22 LYO 125 DCU by Danisco DuPont) containing *Lactococcus lactis subsp. lactis*, *Lactococcus lactis subs. cremonis* and *Streptococcus salivarius subsp. thermophilus.* The starter culture was added in 0.313 DCU/5 L (260 mg/5 L) dosage. Every cheese making trial was repeated three times.

3.2 pH measurements

The pH of the milk was monitored during the cheese manufacture with Thermo Orion 2 Star benchtop laboratory pH meter. The pH values of the original milk and all milk batches serving as raw material were recorded. For milk portions used in spiked trials, the pH was recorded following the addition of cephalexin both before and after the heat treatment. The pH values of cheesemilk, whey and the fresh cheese were also recorded. The latter one was recorded immediately after the 30 minute pressing.

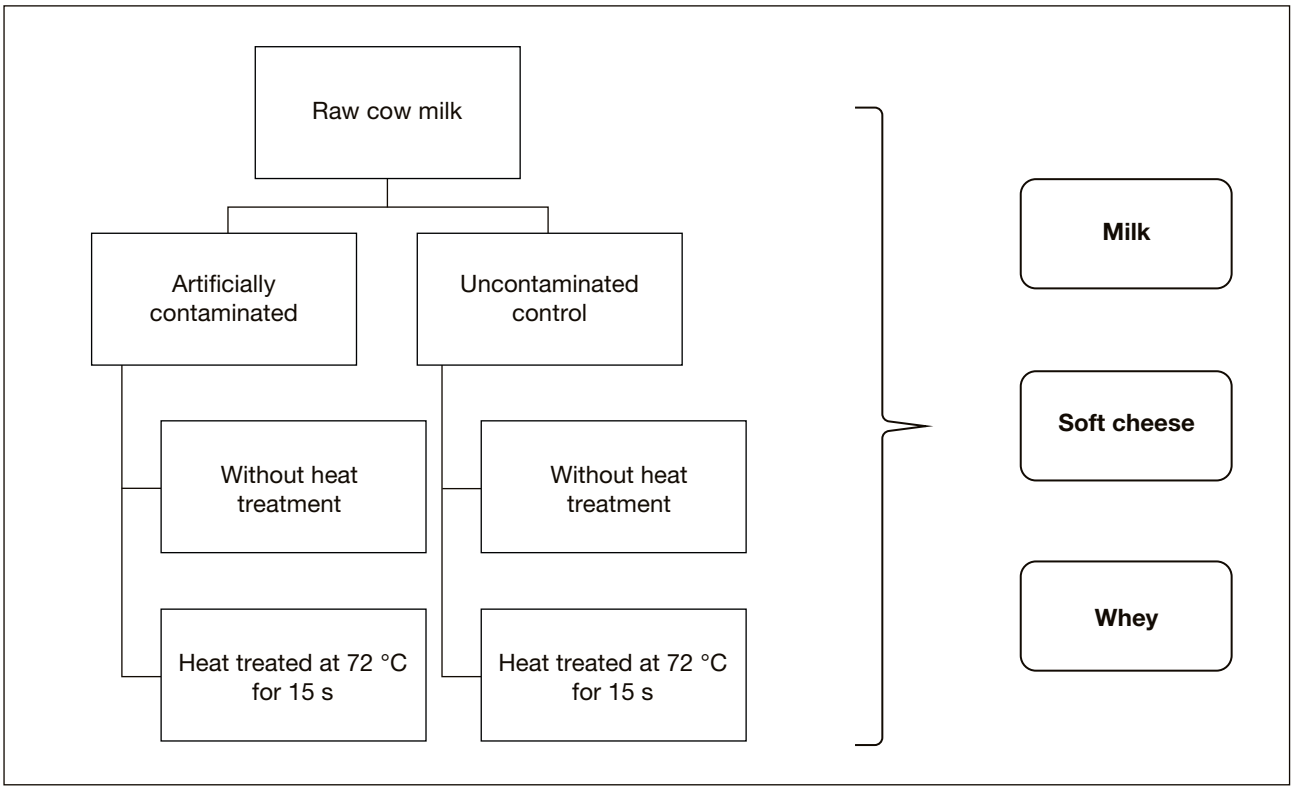


Figure 1. Design of the trials

3.3. Sample preparation and analysis

3.3.1. Milk and whey

The liquid samples (milk and whey) from each trial were kept in refrigerator (6 °C) before analysis and analysed together with the fresh cheese samples. From each individual milk and whey sample, nine sub-samples were taken. Internal standard (penicillin-V; by Fluka, purchased from Sigma-Aldrich, USA) was added to 1,000 μL of sub-samples to obtain 1,500 $ng \cdot mL^{-1}$ final concentration before sample processing. 80 μL 10 % (v/v%) acetic acid was added to 1,000 μL milk sample. It was vortexed for 30 s, and then 15 μL 1 M NaOH solution was added to it. The mixture was vortexed again for 15 s, and then it was centrifuged at 16,100 g and 15 °C for 10 min. The supernatant was filtered through 0.22 μm syringe membrane filter and then analysed by liquid chromatography – tandem mass spectrometry (LC-MS/MS) as described in section 2.4. All chemicals in the sections 2.3.1 and 2.3.2 excluding the analytical standards were from VWR International Ltd, Hungary.

3.3.2. Fresh cheese

Quantification of the cheese's antibiotic level was made via standard addition method. The calculation method for quantification is described in the section 2.4. Nine times 10 grams of fresh cheese were removed from randomly selected sites of the whole fresh cheese and measured into beakers. Three samples were treated in themselves, three of them had been spiked with medium levels of cephalexin (additional 80 μg/kg), and the last three had been spiked with high levels of the target compound (400 μg/kg). The spiking was carried out by a single-use syringe and needle, injecting small portions of the spiking solution (total volume 1.000 μl; cephalexin solved in water) into different parts of the fresh cheese pieces.

The samples were kept in refrigerator (6 °C) for a night and were processed on the next day. 20 ml extracting solvent consisting of 10 % acetonitrile, 89.5 % water and 0.5 % methanol with 5 % ammonia were added to every sample then manual homogenisation followed by stainless steel homogenising tools. Before the homogenization started 200 μL penicillin-V (50.000 ng/mL) solution was added to each sample as internal standard (ISTD). Homogenisation went until the size of fresh cheese pieces decreased below ~1 mm and the extracting solvent and fresh cheese pieces were mixed evenly.

Then the manually homogenised samples were replaced into Erlenmeyer flasks with screw caps. The content of beakers was rinsed with an additional 5 ml of extracting solvent into the Erlenmeyer flasks. The samples were shaken in a thermoset water bath shaker at 40 °C for 2 hours. Then the content of the Erlenmeyer flasks was transferred into centrifuge tubes and been centrifuged at 7,100 g and 15 °C for 10 min. After this 1,000 μL from the supernatant was subjected to the same procedure as described in the section 2.3.1 for milk and whey with except for internal standard addition.

3.4. Analytical chemical method

The cephalexin content of milk, whey and cheese extracts was analysed by a high performance liquid chromatograph coupled with a tandem quadrupole mass spectrometer (HPLC-MS/MS) system, according to a validated method described in details elsewhere **[14]**. A Shimadzu LCMS-8030 system with a Kinetex C18, 100 x 4.6 mm ID (2.6 μm particle size) column and a 4 x 2 mm C18 guard column (both from Phenomenex, Inc., USA) was used for the analysis. Lowest level of quantitation (LOQ) for cephalexin was 1.0 ng/mL in the case of milk and whey, and 2.5 μg/kg for fresh cheese.

The calibration curves of cephalexin measurements were carried out by the LabSolutions software of the Shimadzu 8030 LC-MS/MS apparatus using internal standard quantification method and $1/C^2$ weighing. The quantified data were then transferred and processed in MS Excel software. For the quantification of data for cheese, the standard addition method was used in order to minimise the mistakes that may originate from the heterogeneity of the sample. After the spiking and extraction described in the section 2.3 and analysis described above, the measured concentrations of cephalexin were plotted against the level of spiking made (i.e., zero, 80, 400 μg/kg), and the intercept of the straight gave the concentration level of the antibiotic in the given cheese.

3.5. Effect of cephalexin on the microbe count of the inoculated cheesemilk

The effect of cephalexin on cheese starter culture was investigated through measuring the microbe count (in colony forming units – CFU) of the inoculated cheesemilk with a MicroTester apparatus (Microtest Ltd, Hungary) operating on the principle that during growth in the test cell microorganisms decrease the redox potential of the medium as a consequence of their metabolic activity. Principles of operation and the methods of calculation are described in details elsewhere **[15]**. 10 mL sub-samples of inoculated cheesemilk were removed from the cheese vat just after the inoculation and homogenisation and sent to microbe count testing. Investigation was carried out both with the raw and heat treated milk trials. The total colony number of blank, raw milk was in the 10^3 CFU order, the same value for the heat treated blank milk was zero. The total colony number of the starter culture in the amount given to the milk (see section 2.1) was in the 10^8 CFU order.

3.6. Statistical analysis

Multi-way and one-way ANOVA methods were used to investigate the significance of differences ($p < 0.05$) between the measured parameters in the trials and those of the blank samples. Correlation of specific datasets and significance ($p < 0.05$) of correlation were also calculated. Statistical analysis of the results was performed by Microsoft Excel and R program (version 3.1.3.).

4. Results and discussion

4.1. Measured cephalexin concentrations

The cephalexin concentrations in milk, whey and cheese are presented in ***Table 1***. In every case the background cephalexin level of the untreated milk as well as the whey and cheese prepared from it (blank trial) were also checked. None of the blank trials showed cephalexin levels above the detection limit.

The differences between cephalexin concentrations in heat treated and untreated milk agree well with previous research in the field of heat stability of veterinary antibiotics **[14, 16]**. Based on these previous studies, cephalexin can be considered as a medium heat stable compound and this was supported also by our present results. At every concentration level the measured cephalexin concentrations of the heat treated milk differed significantly from the untreated portions' concentrations ($p = 2.92 \cdot 10^{-2}$, $5.22 \cdot 10^{-4}$ and $6.09 \cdot 10^{-7}$ for the Low, Medium and High concentration level trials, respectively). However, the extent of heat degradation is far from being adequate for completely removing cephalexin from the milk.

The cephalexin concentrations in whey and fresh cheese were in good accordance with the concentration of cephalexin in the milk itself with correlation factors of 0.9997 for whey and 0.9480 for cheese. Both correlations showed high significance ($p = 0.00001$ and 0.00399 for the correlations of milk-whey and milk-cheese concentrations, respectively). This supports that products originating from milk containing higher concentration of cephalexin will contain proportionally higher concentration of the antibiotics, too.

In every trial, whey contained significantly less cephalexin than the original milk ($p = 1.65 \cdot 10^{-7} – 2.64 \cdot 10^{-12}$), and cheese contained also significantly less antibiotics than the whey ($p = 1.49 \cdot 10^{-4} – 6.25 \cdot 10^{-12}$). This finding supports that cephalexin is retained less in the cheese curd and is transferred more into the whey. Giraldo et al. **[17]** found it differently in goat milk, concluding that cephalexin was the only β-lactam being retained in the cheese curd. Cabizza et al. **[18]** found that around 60 % of the original amount of oxytetracycline was found in the 1-day old cheese they studied. In our research, in the case of cephalexin, this ratio ranged between 1.8-4.3% by the original mass of cephalexin added to the milk, indicating a much lower retention of this compound in the cheese curd. On the other hand, whey contained 29.3-42.8 % of the original cephalexin amount. 52.9-67.7 % of the original cephalexin amount was lost during the cheesemaking process. As no significant microbial inhibitory effect was observed (see section 3.4) this loss may be attributed mainly to chemical decomposition occurring in the acidified environment. Investigation of the possible metabolites originating from this decomposition could be an important issue of future research.

The mass balance calculations of cephalexin process were carried out for the cheese making. The volumes of the original milk and the resulting whey , as well as the weight of the fresh cheese were recorded. From the measured cephalexin concentrations and the volume and weight data, amounts of cephalexin in every tested matrix were calculated in µg. Considering the cephalexin amount of the original milk as 100 %, carry-over ratios were also calculated (see ***Figure 2*** and ***Table 2***).

Table 1. Measured cephalexin levels

Trial	Heat treatment	$C_{N, ceph}$ [2]	Measured concentration in		
			milk	whey	soft cheese
		ng/mL	ng/mL	ng/mL	µg/kg
Blank control	untreated	0	< LOQ[1]	< LOQ[1]	< LOQ[1]
	72 °C		< LOQ[1]	< LOQ[1]	< LOQ[1]
Low	untreated	50	51.0±2.8	27.5±2.4	16.5±1.1
	72 °C		46.3±1.9	23.2±1.1	5.5±0.4
Medium	untreated	100	104.1±2.0	41.5±0.8	25.3±0.8
	72 °C		88.8±3.0	39.3±0.7	22.8±0.5
High	untreated	500	493.1±5.5	185.9±11.3	65.9±1.3
	72 °C		438.2±6.2	166.0±2.2	62.0±1.1

([1]LOQ: level of quantitation; [2]$C_{N, ceph}$: nominal cephalexin concentration in the milk)

Mass balance expressed as ratio of average total amounts of cephalexin in the given medium. The width of arrow heads is proportional to the average ratio of cephalexin.

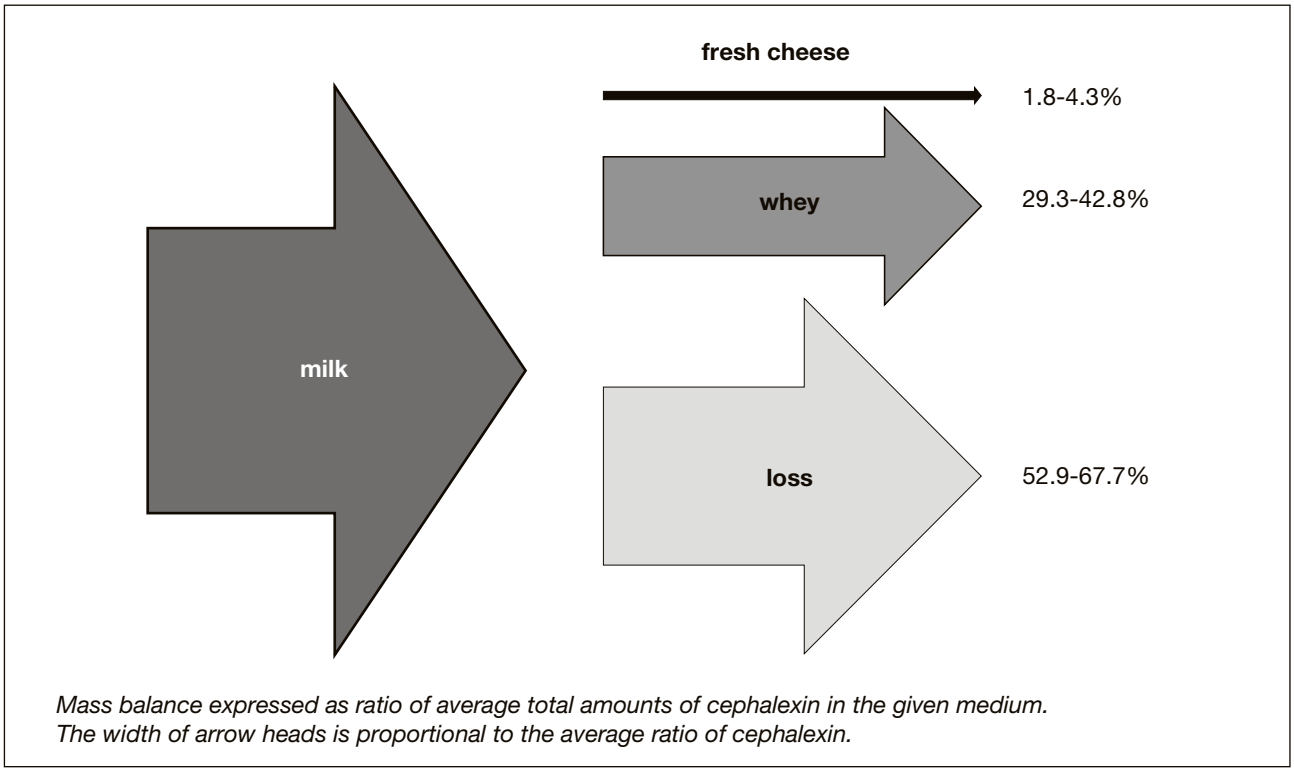
Since the legal background for maximum residue levels of veterinary antibiotics in processed foodstuff is incomplete, it is hard to compare the cephalexin levels measured to any official requirements. Acceptable daily intake (ADI) values as set by of Australian **[19]** and European **[12]** competent organisations mention 0.01 mg/kgbw/day (bw: body weight). However, the documents emphasise that since the limited toxicology data are not sufficient to establish a toxicological ADI, therefore microbiological ADI is given. The cephalexin levels measured in our trials can contribute to the acceptable daily intake amount up to 0.3 % in the case of an adult of 60 kg average body weight and 29.8±0.9 g fresh cheese consumption per day **[20]**; or up to 0.9 % in the case of a child of 20 kg average body weight and 25.5±1.1 g fresh cheese consumption per day **[20]**.

4.2. pH measurements

The optimal pH range for the fresh cheese we prepared is between 5.7-5.8. The pH of fresh cheese prepared in our trials was 5.24±0.03, 5.46±0.02 and 5.78±0.05 for the Low, Medium and High concentration level trials, respectively, in the case

Table 2. Carry-over ratios of cephalexin

Trial	Heat treatment	Volume of original milk	Volume of whey	Weight of cheese	Cephalexin concentration in the milk	Cephalexin concentration in the whey	Cephalexin concentration in the cheese	∑ cephalexin in the milk	∑ cephalexin in the whey	∑ cephalexin in the cheese	∑ cephalexin in the process milk	∑ cephalexin in the process whey	∑ cephalexin in the process cheese	∑ cephalexin in the process loss
		mL	mL	g	ng/mL	ng/mL	mg/kg	µg			%			
Low	untreated	5000 ± 2	3967 ± 53	670 ± 6	51.0 ±2.8	27.5 ±2.4	16.5 ±1.1	255.1 ± 5.5	109.1 ± 3.2	11.1 ± 0.6	100.0	42.8 ± 1.7	4.3 ± 0.4	52.9 ± 2.1
Medium	untreated	5000 ± 2	3679 ± 71	700 ± 31	104.1 ±2.0	41.5 ±0.8	25.3 ±0.8	520.3 ± 4.0	152.5 ± 2.8	17.7 ± 0.9	100.0	29.3 ± 1.1	3.4 ± 0.2	67.3 ± 3.2
High	untreated	5000 ± 2	4022 ± 53	718 ± 20	493.1 ±5.5	185.9 ±11.3	69.0 ±1.3	2465.4 ± 11.0	747.7 ± 6.1	49.6 ± 1.1	100.0	30.3 ± 1.4	2.0 ± 0.1	67.7 ± 2.8
Low	72 °C	4791 ± 6	3644 ± 27	738 ± 16	46.3 ±1.9	23.2 ±1.1	5.6 ±0.4	221.6 ± 8.3	84.6 ± 1.9	4.1 ± 0.1	100.0	38.2 ± 1.0	1.8 ± 0.1	60.0 ± 2.5
Medium	72 °C	4796 ± 9	3627 ± 24	690 ± 13	88.8 ±3.0	39.3 ±0.7	22.8 ±0.5	425.9 ± 15.2	142.4 ± 3.0	15.7 ± 1.7	100.0	33.4 ± 1.9	3.7 ± 0.1	62.9 ± 1.9
High	72 °C	4808 ± 5	3953 ± 18	624 ± 34	438.2 ±6.2	166.0 ±2.2	62.0 ±1.1	2107.0 ± 22.4	656.3 ± 4.3	38.7 ± 2.1	100.0	31.1 ± 1.4	1.8 ± 0.2	67.0 ± 2.1



Mass balance expressed as ratio of average total amounts of cephalexin in the given medium. The width of arrow heads is proportional to the average ratio of cephalexin.

Figure 2. General mass balance pattern of cephalexin in the cheese making process

of raw milk; 5.81±0.12, 5.71±0.05 and 5.91±0.02 for the Low, Medium and High concentration level trials, respectively, in the case of heat treated milk. The pH of the original milk was 5.99±0.13 and 6.15±0.06 for the raw and heat treated milk, respectively. As it can be seen, the optimal pH range was reached in some trials, but in specific trials the pH of product fell outside of this range (see the details in ***Table 3***). In each trial carried out with cephalexin in the milk, the pH of fresh cheese differed significantly from the blank trial's value irrespectively to the concentration of antibiotic ($p=7.76 \cdot 10^{-5}$ and $2.42 \cdot 10^{-4}$ for the raw and heat treated milk trials, respectively). It may suggest that cephalexin residues even well below the MRL level can change the pH of resulting cheese into unfavourable directions. Too low pH of cheese may result in texture problems during the ripening process.

Contrary to the expectations, presence of the antimicrobial agent did not lead to considerable delay or reduction in pH-changes during the cheese making process that would indicate lower level of activity of the starter culture. Our results showed that the concentration levels of cephalexin tested in the trials did not influence substantially the way as pH changed during the cheese making process. On the one hand, it is generally accepted that antibiotic residues in the milk may cause significant technological problems in the dairy industry **[21]**. Our results concerning the pH of fresh cheese seem to support this opinion. In the case of more susceptible yoghurt starter strains **[22]** it was proved among laboratory conditions that the presence of cephalexin in the ewe's milk causes imbalances in pH and in the L(+)/D(–) ratio of lactic acid isomers, resulting in yogurts less assimilable for consumers **[23]**. On the other hand, it was proved that lactic acid bacteria in cheese may be more resistant to certain individual antibiotics **[24]**, and it is also discussed **[12]** that cephalexin causes disturbances in pH development during cheese making only at higher concentrations (range of mg/kg). Our results are in accordance with this finding.

4.3. Effect of cephalexin on the microbe count of the inoculated cheesemilk

Testing the possible effects of cephalexin residues in milk on the cheese starter culture according to the examinations described in the section 2.6 (see ***Figure 3***.) did not result in significant reduction in the starter culture's microbial activity. The microbe count of cheesemilk (N; expressed in CFU – colony forming unit per mL) was not affected by the level of cephalexin in the medium.

Changes of pH values during cheese making are in strong relation with the activity of the starter culture's microbes, therefore this finding about the unchanged microbe count is in accordance with the fact that the pH changes were very similar in the case of control trials and those made with cephalexin containing milk. Moreover, this result is in accordance with previous findings **[12]** and supports also earlier research's conclusions that veterinary antibiotics alone may be less effective in inhibiting dairy industrial starter cultures than added in mixture **[24]**. As it is described in the section 3.2, it is a widely accepted supposition that antibiotic residues are harmful for the dairy product technologies without considering deeper the chemical group of the drug or the microbe strains [**25, 26]**. It is an undisputable fact that certain antibiotic residues may have severe impact on the final quality of dairy products or may even render the whole production process to fail **[23, 24]**. However, it shall be noted that the chemical-toxicological characteristics of the given antibiotics may strongly influence this picture as well as the microbiological sensitivity of the affected microbe strains **[22]**.

Table 3. Changes of pH values during and after the cheese making process

Trial	Heat treatment	$C_{N, ceph}$ [1]	pH of					
			milk	cheesemilk at			whey	cheese
				start of the process	half of the process	before cutting		
		ng/mL						
Blank control	untreated	0	6.81±0.02	6.79±0.02	6.48±0.05	6.37±0.03	6.38±0.08	6.30±0.03
	72 °C		6.74±0.09	6.80±0.04	6.55±0.03	6.48±0.03	6.52±0.06	6.62±0.08
Low	untreated	50	6.84±0.11	6.67±0.02	6.58±0.04	6.45±0.05	6.53±0.05	5.24±0.03
	72 °C		6.79±0.04	6.84±0.11	6.59±0.10	6.55±0.02	6.50±0.06	5.81±0.12
Medium	untreated	100	6.56±0.04	6.69±0.02	6.35±0.07	6.34±0.06	6.34±0.03	5.46±0.02
	72 °C		6.66±0.08	6.66±0.05	6.58±0.09	6.48±0.03	6.48±0.07	5.71±0.05
High	untreated	500	6.62±0.01	6.79±0.14	6.52±0.06	6.50±0.11	6.21±0.09	5.78±0.05
	72 °C		6.77±0.02	6.49±0.07	6.20±0.08	6.21±0.08	6.52±0.08	5.91±0.02

([1] $C_{N, ceph}$: nominal cephalexin concentration in the milk)

5. Conclusions

From the results outlined above it may be concluded that the effect of certain veterinary antibiotic residues in the milk cannot be described by general observations, and statements aiming at all antibiotics may be misleading. The cross-effects between the given antibiotic ingredient – or veterinary treatment – and dairy industrial culture, or process shall be investigated in themselves. Further research on the fate of veterinary antibiotics during dairy manufacturing processes is needed to clarify all the important interconnections that may occur. On the other hand, it is important to emphasise that dairy products made from milk containing antibiotic residues will also contain these residues although in some cases in lower concentrations. This fact is independent of the effect these residues may have on the texture and quality of the product. By-products and dairy industrial wastes may also be contaminated with antibiotics in this way, thus posing further threats to human health and the environment. These consequences draw our attention to the importance of keeping the withdrawal periods and examining the milk for antibiotic residues before letting it into the food chain.

6. Funding

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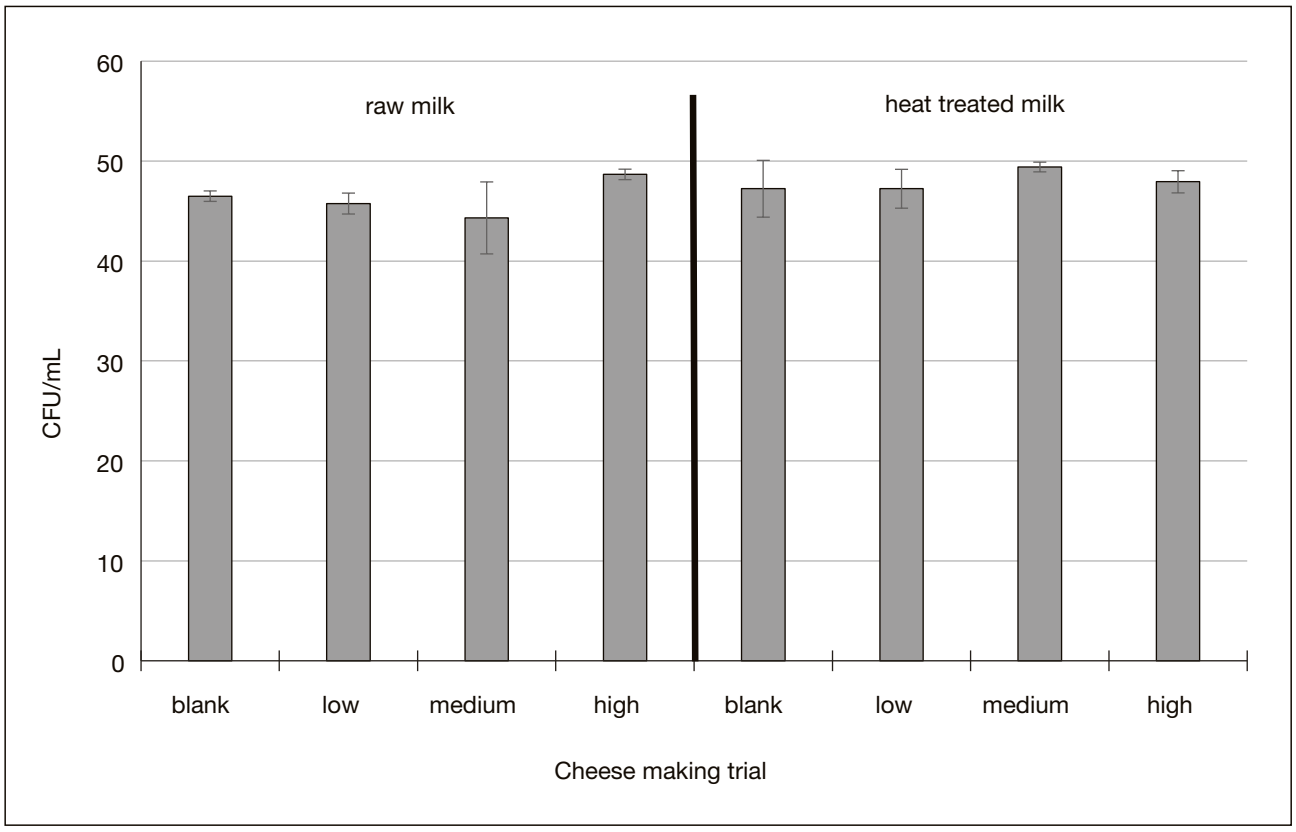

Number of colony forming units per 1 mL (CFU/mL) as a function of cephalexin concentration in the milk just following the inoculation.

Figure 3. Connection between cephalexin level and cheese starter culture microbe count

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