Destruction of Penicillin residues in waste milk

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Abstract

To destroy the antibiotic residuals in waste milk on dairy farms, the aim of this study was to develop an implementable approach for the degradation of Penicillin G in waste milk on the farm, working efficiently and without posing a risk to the user or the environment. Commercially available bovine pasteurized and homogenized milk with 3.8 % of milk fat was spiked with Penicillin G sodium-salt to an initial concentration of Penicillin G in the milk of 4 µg/kg to 500 mg/kg. Degradation of Penicillin G was performed by two physico-chemical treatments, namely 1) exposure to heat and 2) exposure to heat after prior acidification and by 3) enzymatic degradation by β-lactamases from *Escherichia* (*E.*) *coli* strains tested positive for extended spectrum β-lactamases (ESBL) production. Heating to 95 °C for 120 minutes led to a degradation of 4 µg/kg Penicillin G below the detection limit of 2 µg/kg. A higher Penicillin G concentration of 8 µg/kg could not be sufficiently degraded anymore. Heating the samples to 65 °C at pH 4.0, a maximum of 64 µg/kg were degraded after 120 min, while at pH 4.5 a maximum of 8 µg/kg was degraded. When heating to 75 °C at pH value 4.0, a maximum of 500 µg/kg Penicillin G was degraded after heating for 120 min and 100 µg/kg at pH value 4.5, respectively. Heat exposure to 80 °C at pH 4.0 for 120 min caused the degradation of 80 mg/kg Penicillin G, at pH 4.5 1 mg/kg, and at pH 5.0 32 µg/kg. When heating to 90 °C, at pH 4.0 for 60 min, a degradation of 80 mg/kg Penicillin G was detected, while it was 100 mg/kg after 90 min and 500 mg/kg after 120 minutes of heating. Heat treatment for 120 minutes at pH 5.0 resulted in a degradation of 500 µg/kg, whereas it was 64 µg/kg pH 5.5. 6 *E. coli* strains were capable of degrading 25 mg/kg Penicillin G within 8 hours. In conclusion, it is technically possible to degrade Penicillin G in waste milk. Sole heat treatment did not lead to the degradation of sufficiently high amounts of Penicillin G. A combination of acidification of the milk and subsequent heating lead to a degradation of very high antibiotic concentrations but assumes a high level of technical equipment, time, and energy supply. The enzymatic degradation process of the antibiotic by β-lactamases promises better results than heating and acidification and requires lower temperatures than the methods without enzymatic degradation. A possible transmission of plasmids from the enzyme solution must be considered and needs to be solved.

Keywords: waste milk; antibiotic residues; β-lactam antibiotics; β-lactamases; enzymatic degradation

Introduction

Clinical mastitis (CM) is one of the most prevalent health problems in dairy cattle and is also a major reason for culling [1]. The annual incidence of CM amounts to approximately 50 % [2] and therefore mastitis treatment reclaims about 68 % of all the antibiotics used in the dairy sector [3]. Usually, in order to cure CM, antibiotic udder injectors are applied to the affected udder quarter, whereby β-lactam antibiotics such as penicillins are widely used due to their high effectiveness and their currently unproblematic antimicrobial resistance situation [4]. The advantage of a local antibiotic therapy is the high drug concentration in the udder parenchyma without systemic absorption and, therefore, minimized side effects [5].

Independently of the advantages of the local mastitis therapy, it is known that during and after treatment, almost 50 % of the applied antibiotic doses are excreted non-metabolized via the milk. The antibiotic amount can reach 25 mg Penicillin G per kilogram milk after treatment of all quarters depending on the milking frequency [6]. Milk, obtained from cows treated for mastitis, is waste milk and must not enter the food chain within the withdrawal period. For this reason, maximum residue levels (MRL) for pharmacologically active drugs in animal tissues and their products (VO (EG) No. 470/2009) were determined by the European Union.

Waste milk is either disposed on agricultural areas with the liquid manure or it is fed to calves [7]. Both procedures lead to severe ecological consequences. In case manure containing waste milk is used as fertilizer, the antibiotic residues or their metabolites enter the soil where they can persist or reach the groundwater [8,9]. Subsequently, they might reach the human food chain by plants cultivated on these areas. It has been reported that the use of manure containing waste milk affects the bacterial microbiome of the soil [7,8] and that the transfer of resistance genes is favored by the presence of antibiotics over a long period and at subtherapeutic concentrations [10,9]. The practice of feeding waste milk to calves is considered a potential risk factor for the selection of antimicrobial drug resistant bacteria [11]. An increased proportion of resistant *E. coli* in feces from pre-weaned calves was found when they had been fed with pasteurized waste milk compared to the feces of calves that had received pasteurized bulk milk [12].

In order to prevent these undesirable side effects, the antibiotic residuals in waste milk should be destroyed. In general, β-lactam antibiotics like penicillins can be degraded easily by opening the β-lactam ring through hydrolysis because of their relatively instable molecular structure [6]. Since this ring is the active part of the drug and therefore responsible for the antibiotic activity, changes in its structure cause the loss of all antibiotic activity. Hydrolysis can occur due to physico-chemical processes such as heat or changes of the pH value, but also due to enzymes called β-lactamases. These are the most important resistance mechanism against β-lactam antibiotics [13]. They are produced by many Gram-positive bacteria such as staphylococci and gram-negative bacteria such as coliform bacteria as well and can be encoded in the bacterial genome or on plasmids. After its random finding by Fleming in 1928 [14], Penicillin G was already used for the treatment of bovine

mastitis by the veterinarians in the late 1940s. In 1940 it was detected that *E. coli* produces an enzyme capable to inhibit the Penicillin G and was called *Penicillinase* [15] and in the 1950th, first experiments with the enzyme *Penicillinase* in bovine milk were made. Application of β-lactamases to degrade Penicillin G has also been performed successfully, but only with low antibiotic concentrations [16,13].

This study aims to develop an implementable approach for the degradation of Penicillin G – the prototype of the penicillins – in waste milk on dairy farms which works efficiently without posing a risk to the user or the environment. The application should achieve an antibiotic concentration below the MRL after a short time.

Materials and Methods

The first part of the study deals with the degradation of Penicillin G in bovine milk by two physico-chemical treatments, namely 1) exposure to heat and 2) exposure to heat after prior acidification, while the second part is concerned with the 3) enzymatic degradation by β-lactamases.

Preparation of spiked milk samples with Penicillin G

For all experiments commercially available pasteurized and homogenized bovine milk with 3.8 % of milk fat was used. Previous to the trials, each new milk charge was analyzed for the absence of inhibitors by the Delvotest BR Brilliant (Milku Tierhygiene, Bovenden, Germany). Spiked milk samples were prepared freshly every test day. The respective amount of Penicillin G sodium-salt (P3032-10MU, Sigma-Aldrich, Taufkirchen, Germany) was dissolved in 100 g phosphate buffered saline (VWR International GmbH, Hanover, Germany). Then the amount of dissolved Penicillin G needed for the target concentration was transferred in a beaker which was filled up to 100 g with milk. Finally, 10 g of spiked milk were transferred in test tubes. The initial concentration of Penicillin G before degradation in the milk was 4 µg/kg.

Detection of Penicillin G residues in treated milk samples

In order to detect Penicillin G residues in the treated milk samples, Delvotest BR Brilliant was chosen as test system since it is the official German detection method (L 01.01-5) for antibacterial substances in milk according to the German Food and Feed Code (§ 64). Test ampoules contain solid agar medium with *Geobacillus stearothermophilus* varies *calidolactis* and brilliant black. The growth of the test bacterium, indicated by a color change from purple to yellow or transparent, shows a negative result – the tested milk is free of inhibiting substances. Test ampoules remaining purple indicate that the growth of the test bacterium has been inhibited by anti-infective substances and thus result as positive. The detection sensitivity for Penicillin G is 2-3 µg/kg. In the following, the term degradation of Penicillin G means the reduction of the antibiotic concentration below the detection level of the applied test. In our research, Delvotest BR Brilliant was set after the two different treatments and the test ampoules were placed in a dry incubator at 62 °C for 3 hours together with a negative control. Finally, the optic evaluation was conducted and documented.

Degradation of Penicillin G in milk by exposure to heat

The heating process of the milk samples was performed in a thermostatic water bath (VWB 18, VWR International GmbH, Hanover, Germany) at 5 different temperature levels, namely 75 °C, 85 °C, 90 °C, 92 °C, and 95 °C. The milk samples were heated in the water bath for 10, 30, 60, 90, and 120 minutes. Once removed from the water bath, the test tubes were transferred in iced water immediately to avoid a possible subsequent antibiotic degradation process. Each tested combination of temperature and time interval was performed in a threefold approach.

Degradation of Penicillin G in milk by exposure to heat after prior acidification

Previous acidification added to the heating process was performed to point out an eventual synergism between the parameters heating temperature and pH value concerning the degradation of Penicillin G in milk. The temperature levels chosen were 65 °C, 75 °C, 80 °C, and 90 °C and the heating time was 30, 60, 90, and 120 minutes. Before heating, the milk samples were acidified at pH level 4.0, 4.5, 5.0, and 5.5 by the dropwise addition of 30 % lactic acid (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) using a pH meter (Mettler Toledo FiveEasy, Met- tler Toledo, Gießen, Germany). The initial concentration of Penicillin G in the samples was 4 µg/kg. The concentration of Penicillin G was increased stepwise for combinations of heating temperature, pH value and heating time when it had resulted in a negative Delvotest. The highest tested antibiotic concentration was 500 mg/kg.

Subsequent to the heating, samples were cooled in iced water. Following that, the pH value was re-adjusted to a value about 6.5 by the dropwise addition of 1 mol/l sodium hydroxide (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), since this corresponds approximately to the pH value of bovine milk and since the test bacterium contained in the Delvotest can grow only under neutral pH conditions.

Enzymatic degradation of Penicillin G in milk by β-lactamases

Coliform bacteria isolated from milk samples which had been send to the microbiological lab of the University of Applied Sciences and Arts Hannover for bacteriological examination were selected with the aim to find an appropriate β-lactamase with the following conditions: The enzymatic activity must be high enough to degrade at least 25 mg/kg Penicillin G in milk as this is a realistic antibiotic concentration in waste milk after local mastitis treatment [6]. Furthermore, the sub- strate spectrum should be as wide as possible because udder injectors contain also other β-lactam antibiotics as ampicillin or cloxacillin for example. Since ESBL can be found often in gram-negative bacteria and since they are characterized by resistance to cephalosporines of the third generation [17], the database was scanned for bacterial strains showing resistance to the β-lactam antibiotics Penicillin G, Cloxacillin, Cefalexin, Cefquinome and the third generation cephalosporine Cefoperazone. Eleven bacterial strains were selected for the trials.

Screening of coliform bacterial strains for ESBL-production and isolation of the β-lactamases

Screening for ESBL-production was performed using the double disk approximation test by Jarlier et al. [18], which is an agar diffusion test. Bacterial strains showing ESBL-production were identified as strains of *E. coli* by Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (MALDI Biotyper MTB Smart, Bruker, Bremen, Germany) and used for the trials.

E. coli strains showing ESBL-production were cultivated in brain heart infusion (Merck Chemicals GmbH, Darmstadt, Germany) and the permeate was added to the milk samples after sterile filtration with 25 mg/kg Penicillin G to a final concentration of 2 %, 1 % and 0.5 %.

Characteristics of the enzyme solutions of *E. coli* strains tested positive for ESBL production

The enzyme solutions (sterile filtrates produced from the *E. coli* strains containing β-lactamase of *E. coli* strains tested positive for ESBL production were characterized concerning their pH optimum, their ther-

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mostability and their shelf life under different storage conditions. The degradation capacity of 25 mg/kg Penicillin G in milk at 37 °C within 2 hours was chosen as reference for optimal conditions.

Table 1: Results of the Delvotest of the Penicillin G degradation in milk spiked with 4 µg/kg Penicillin G at different pH values, at different temperature levels and at different incubation times.

Temperature	pH value	Delvotest after 30 min	Delvotest after 60 min	Delvotest after 90 min	Delvotest after 120 min
65 °C	4.0	+	-	-	-
	4.5	+	-	-	-
	5.0	+	+	+	+
	5.5	+	+	+	+
75 °C	4.0	-	-	-	-
	4.5	+	-	-	-
	5.0	+	+	-	-
	5.5	+	+	+	+
80 °C	4.0	-	-	-	-
	4.5	-	-	-	-
	5.0	+	-	-	-
	5.5	+	+	+	+
90 °C	4.0	-	-	-	-
	4.5	-	-	-	-
	5.0	-	-	-	-
	5.5	+	-	-	-

-: negative result, Penicillin G is degraded+: positive result, Penicillin G is not degraded

Results

Degradation of Penicillin G in milk by exposure to heat

Only heating to 95 °C for 120 minutes led to a degradation of 4 µg/kg Penicillin G. A higher Penicillin G concentration of 8 µg/kg could not be efficiently degraded anymore.

Degradation of Penicillin G in milk by exposure to heat after prior acidification

The results of the Penicillin G degradation in milk by exposure to heat after prior acidification are shown in table 1. Table 2 shows the maximal degraded Penicillin G concentrations in milk with pH value 4.0 after different heating time intervals and Table 3 shows the maximal degraded Penicillin G concentrations with pH 4.5 after different heating time intervals.

Table 2: Maximal degraded Penicillin G concentrations in milk samples with pH value 4.0 after different heating time intervals.

Temperature	Maximal degradation after 60 min	Maximal degradation after 90 min	Maximal degradation after 120 min
65 °C	4 µg/kg	8 µg/kg	64 µg/kg
75 °C	64 µg/kg	100 µg/kg	500 µg/kg
80 °C	8.000 µg/kg	64.000 µg/kg	80.000 µg/kg
90 °C	80.000 µg/kg	100.000 µg/kg	500.000 µg/kg

Table 3: Maximal degraded Penicillin G concentrations in milk samples with pH value 4.5 after different heating time intervals.

Temperature	Maximal degradation after 60 min	Maximal degradation after 90 min	Maximal degradation after 120 min
65 °C	4 µg/kg	4 µg/kg	8 µg/kg
75 °C	8 µg/kg	32 µg/kg	100 µg/kg
80 °C	100 µg/kg	500 µg/kg	1.000 µg/kg
90 °C	8.000 µg/kg	64.000 µg/kg	350.000 µg/kg

Maximal concentrations for 30 minutes of heating were not analyzed, because no satisfactory outcome was expectable after the previous studies. When heating the samples to 65 °C, the maximum degraded antibiotic concentration was 64 µg/kg in milk samples with pH 4.0 after 120 minutes. With the pH value 4.5 after 120 minutes it was 8 µg/kg. Due to these findings, increasing the antibiotic concentration in milk adjusted at pH value 5.0 and 5.5 was not performed. When heating to 75 °C, in milk samples with pH value 4.0 a maximum concentration of 500 µg/kg Penicillin G was degraded after heating for 120 minutes. Under the same conditions at pH value 4.5 the maximum concentration degraded was 100 µg/kg.

Heat exposure of 80 °C for 120 minutes caused the degradation of 80 mg/kg Penicillin G in milk samples with pH value 4.0, of 1 mg in samples with pH value 4.5 and of 32 µg/kg in samples with pH value 5.0. When heating to 90 °C, in milk samples with pH value 4.0 a degradation of 80 mg/kg Penicillin G was detected after 60 minutes, while it was 100 mg/kg after 90 minutes and 500 mg/kg after 120 minutes of heating.

Heat treatment for 120 minutes in milk samples with pH value 5.0 resulted in a degradation of 500 µg/kg, whereas it was 64 µg/kg in samples adjusted to pH value 5.5.

Enzymatic degradation of Penicillin G in milk by β-lactamases

8 of the 11 *E. coli* strains screened were tested positive for ESBL production and were therefore used for the trials (strains 5368, 6634, 6635, 7124, 13111, 13373, 13512, and 15072).

After adding 2 % of enzyme solution to the milk samples, the enzyme solutions of 6 *E. coli* strains were capable of degrading 25 mg/kg Penicillin G within 8 hours at 37 °C. Hence, the enzyme solutions from *E. coli* strain 6635, 7124, 13111, 13373 and 13512 were used for further experiments. The enzyme from the *E. coli* strain 7124 was the only one capable of denaturing 25 mg/kg Penicillin within 2 h at 37 °C.

Table 4: Characteristics of the 3 enzyme solutions.

Analyzed characteristics	Enzyme solution from *E. coli* strain 7124	Enzyme solution from *E. coli* strain 13111	Enzyme solution from *E. coli* strain 15072
Optimum temperature	25 °C–37°C	25 °C–37 °C	25 °C–37 °C
Denaturation at	60 °C	50 °C	50 °C
pH optimum	5.5 to approx. 6.8	5.5 to approx. 6.8	5.5 to approx. 6.8
Minumum value of shelf life at			
- 18 °C (freezer)	4 weeks	4 weeks	4 weeks
+ 8 °C (fridge)	3 weeks	3 weeks	weeks

Characteristics of the enzyme solutions of *E. coli* strain 7124, 13111 and 15072

The results of the characterization of the enzyme solutions concerning their thermostability, pH optimum, and minimum value of shelf life under different storage conditions are shown in table 4.

Discussion

The objective of this study was to find a degradation method for Penicillin G residues in milk, practicable on dairy farms. Background of this question is the need of a method directly applicable on dairy farms after local antibiotic treatment of mastitis. The approach should not require expensive technical equipment, should work fast and reliably without posing a risk neither to the user nor to the environment. For this purpose, 2 physico-chemical degradation methods and an enzymatic degradation process of the antibiotic were tested under laboratory conditions.

Based on a study of Knappstein et al. [6] who examined the excretion of several antibiotics via the milk after local treatment with udder injectors depending on the milking frequency, 25 mg/kg Penicillin G were considered as a realistic antibiotic concentration for a necessary degradation capacity. This antibiotic concentration was the average daily concentration found in the milk of a small group of 5 cows with healthy udders during the treatment and the withdrawal period.

Cows were treated for 3 days twice a day after milking. Though it is rare that cows suffer from mastitis in all quarters simultaneously and therefore antibiotic residues in milk probably are much lower, this concentration was chosen as base for this study. Furthermore, it must be noted that a constant excretion of the antibiotic is assumed, but it is probable that the excretion of the antibiotic decreases significantly within the withdrawal period.

Initially, sole heat treatment of milk samples spiked with the very low concentration of 4 µg/kg Penicillin G was analyzed. It became obvious that even heating at 95 °C for 2 hours could not lead to the degradation of higher Penicillin G amounts than 4 µg/kg. Therefore, sole heat treatment is no option for this purpose. These results correspond to the findings of other researchers dealing with heat inactivation of Penicillin G. A study of Roca et al. [19] for instance, occupies with the application of different heating processes commonly used in the dairy industry for milk spiked with several β-lactam antibiotics. It was reported that only sterilization causes a significant degradation of Penicillin G, while the other treatments such as pasteurization evoke only minor losses of the antibiotic concentration [19].

The high impact of the pH value in milk samples on the degradation of Penicillin G becomes obvious comparing the antibiotic concentration degraded at 75 °C within 2 hours: After sole heat treatment it was only 4 µg/kg, but in milk adjusted to pH value 4.0 it was 500 µg/kg which is the 125-fold. Nevertheless, heating temperatures of 65 °C and 75 °C even combined with the pH values 4.0 and 4.5 in milk were not sufficient to reach an antibiotic degradation in the mg-range. Furthermore, there was no degradation in the mg-range in milk samples with pH value 5.0 and 5.5 even at a temperature of 90 °C, probably because these pH values are too close to the pH value of 6.0 when Penicillin G is most stable [20]. The observation that a pH value of 4.5 in combination with high heating temperatures leads to the degradation of high antibiotic concentrations, is based on the poor stability of Penicillin G at pH values below 4.0. By the acidification of the milk samples at pH values 4.0 and 4.5 and subsequent heating at 80 °C and 90 °C, very high antibiotic concentrations can be degraded.

Considering these results, a combined procedure of acidification and heat treatment states an effective approach to degrade realistic amounts of Penicillin G in milk. However, the approach must be suited for the application on dairy farms. A serious disadvantage of this treatment is the technical equipment required for the heating process performing a temperature up to 90 °C. On the farms, heating is possible by means of pasteurizer for calf milk, but only a few of them can reach this temperature. For example, the pasteurizer sold by Holm & Laue (Westerrönfeld, Germany) is one of the most common products on German dairy farms. It features a pasteurization treatment of about 35 min at 63 °C and the possibility for a heat treatment at 60 °C for about 60 or 70 min. The high heating temperature and the long heating time periods will need a high energy supply which might be expensive. Therefore, it is highly unlikely that this procedure will meet with the approval of dairy farmers.

In order to avoid the high temperatures required for the thermic degradation, it might be an alternative to acidify the waste milk and then store it at ambient temperature until the antibiotic is degraded. This was not analyzed in our research, since this would take much time and so large amounts of waste milk would accumulate on the farms. Nevertheless, acidification of the waste milk can be performed easily by the farmers using lactic acid or other organic acids such as formic acid, which is often used to produce sour milk for calves to prevent neonatal diarrhea. Organic acid is a cheap product and the required amount for a precise pH value can be calculated through dosage schedules. The acidified milk could be used as sour milk for the calves but usually the pH value should not fall short pH value 5.5 to avoid problems of taste acceptance by the calves.

The enzymatic degradation process of the antibiotic promises better results than heating and acidification. The required amount of enzymatic solution could be added to the waste milk and heated at 37 °C in a milk taxi with integrated stirring unit for 2 hours. In the second step, the milk could be pasteurized at 75 °C to denature the β-lactamases. Compared to the process of acidification and heating of milk with Penicillin G, a much lower temperature is needed, which can be reached easily with a milk taxi. Furthermore, a lower temperature means a lower energy supply and less costs. Even though the enzymatic degradation with attached pasteurization works effectively, the treated milk must not reach the human food chain, so a reliable management on the farms is essential. A possible measure might be the use of food coloring to mark the treated milk. With regard to the practical applicability on dairy farms, the use of enzymatic solutions containing β-lactamases produced by *E. coli* is a much more appropriate approach to degrade Penicillin G residues in waste milk than the heat treatment of acidified milk.

An important aspect to take into consideration is the possibility that the enzyme solutions might contain plasmids, since ESBL from gram-negative bacteria are mostly located on plasmids and the filter used for sterile filtration withholds only particles bigger than 0.2 µm, whereas the size of plasmids is in the nm-range [21]. The presence of plasmids in the enzyme solutions is disadvantageous because through its application in waste milk with Penicillin G and the subsequent exposure on agricultural fields with the liquid manure or the use as milk for calves, plasmids can be incorporated by other bacteria. This process is called transformation and can enlarge the resistance spectrum of bacteria. Unfortunately, plasmids cannot be destroyed by pasteurization. One possibility to destroy them is the addition of a desoxyribonuclease or the application of a physical separation process as density gradient centrifugation to separate plasmids and enzyme solution.

The fact should not be overlooked that the tested methods only de-

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grade Penicillin G. Preine et al. [22] started a survey in 2022 to gather information about the most commonly used antibiotic udder injectors on farms in Germany. The results show, that preparations with a combination of Cephalexin/Kanamycin or Amoxicillin/clavulanic acid/prednisolone are most common. Kanamycin is not part of the β-lactam antibiotics and therefore not susceptible for the enzymes. Clavulanic acid has no antimicrobial effect but structural similarity to β-lactam antibiotics with a β-lactam ring. As a result, clavulanic acid acts as competitive inhibitor of bacterial β-lactamases. This inhibition restores the antimicrobial effect of β-lactam antibiotics.

Conclusions

It is technically possible to degrade β-lactam antibiotics in waste milk. Sole heat treatment seems to be no option for this purpose because even heating to 95 °C for 2 hours could not lead to the degradation of sufficiently high amounts of Penicillin G. A combination of acidification of the milk samples at pH values 4.0 and 4.5 and subsequent heating at 80 °C and 90 °C, very high antibiotic concentrations can be degraded. Nevertheless, this approach assumes a high level of technical equipment, time, and energy supply. The enzymatic degradation process of the antibiotic by β-lactamases promises better results than heating and acidification and needs lower temperatures than the methods without enzymatic degradation. However, an attached pasteurization is needed to denature the β-lactamases. Also, the possibility that the enzyme solutions might contain plasmids has to be considered, because after the exposure of the treated milk on agricultural fields or the use as milk for calves, plasmids can be incorporated by other bacteria. This process can enlarge the resistance spectrum of bacteria. Further research is needed to develop practicable methods to destroy them, maybe by the addition of a desoxyribonuclease or the application of a physical separation process as density gradient centrifugation to separate plasmids and enzyme solution.

Disclosure of conflicts of interest

The authors declare no potential conflicts of interest.

Compliance with Ethical Standards

This study has been conducted in compliance with ethical standards.

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