T2R2 東京科学大学 リサーチリポジトリ
Science Tokyo Research Repository

論文 / 著書情報
Article / Book Information

Title	Effect of milk on antibacterial activity of tetracycline against Escherichia coli and Staphylococcus aureus isolated from bovine mastitis
Authors	KuangYing, JiaHaoyu, Kazuhiko Miyanaga, YASUNORI TANJI
Citation	Appl. Microbiol. Biotechnol., Vol. 84, , pp. 135-142
Pub. date	2009,
Note	This is a post-peer-review, pre-copyedit version of an article published in Appl. Microbiol. Biotechnol.. The final authenticated version is available online at: http://dx.doi.org/http://dx.doi.org/10.1007/s00253-009-2008-6.

Powered by T2R2 (Science Tokyo Research Repository)

Ying Kuang, Haoyu Jia, Yasunori Tanji*

Effect of milk toward antibacterial activity of tetracycline against *Escherichia coli* and *Staphylococcus aureus* isolated from bovine mastitis

Graduate school of Bioscience and Biotechnology, Tokyo Institute of Technology,
4259-B-47 Nagatsuta-cho, Midoriku, Yokohama 226-8501, Japan

* Corresponding author

E-mail address: ytanji@bio.titech.ac.jp

Tel.: +81-45-924-5763, fax: +81-45-924-5818

Abstract

The susceptibility of mastitis-causing *Escherichia coli* and *Staphylococcus aureus* to two commonly used antibiotics, tetracycline and penicillin G was tested in raw milk and in Muller-Hinton medium by introducing a pH indicator, Bromocresol purple, which was shown to be a simple, sensitive and quick method. The minimum inhibitory concentration (MIC) of penicillin G in milk were the same as those in MH, whereas the MIC of tetracycline in milk were 4-32 times those in MH. An irreversible binding between tetracycline and large molecules of milk which might probably due to the hydrophobic interaction was demonstrated by a dialysis test, suggesting the observed impairing effect was due to the action of milk on the tetracycline being tested. Further investigation reveled that much of the reduction of tetracycline's activity in milk appears to be attributed to the milk casein protein while other heat-sensitive components in milk also play some role.

Introduction

Bovine mastitis has generally been considered as the most costly disease in dairy industry, loses caused by mastitis have been estimated at 100 million dollars annually in Hokkaido, one of Japan's main milk production areas (Yamane et al. 2006). The economic impacts, together with diversified bacterial etiologies of disease, have led to development of various therapeutic strategies. Among them, the identification of pathogens along with their antibiotic treatment is the most frequently used therapy (Grave et al. 1999), of which

tetracycline is one of the most extensively used drugs because its relative safety, low cost and broad-spectrum activity against Gram-positive bacteria, Gram-negative bacteria, and atypical organisms such as Mycoplasmas (Al-Nazawi 2006). However, results from in vitro susceptibility tests for mastitis pathogens were not comparing well with therapeutic outcomes. The inefficacy has been considered to be due to the factors such as limited knowledge of its pharmacokinetic properties for lactating dairy cows, decreased antibiotic activity in the milk phase and appearance of resistant bacteria (Constable et al. 2003).

Currently, methods for determining the antibiotic susceptibility are based on dilution of selected substances in artificial broth or agar to determine the minimum inhibition concentration (MIC) of antibiotics. However, it is accepted that the closer the test medium to the real environment of bacterial infections, the more relevant the test results would be to the clinical outcome, but technical issues need to be considered when determining bacterial growth inhibition in milk because milk is a turbid fluid. Previously, direct plate counting was frequently used to determine the bacterial growth, but it is time-intensive and labor-consuming. In view of this, many assays such as reduction of triphenyltetrazolium chloride (Ali-vehams et al. 1997) and β–glucuronidase assays (Fang et al. 1995a) have been developed. However, these methods can only apply to a specific group of pathogens. Traditionally, pH indicator method is frequently employed to monitor the contamination of phage in the dairy fermentation industry due to its simplicity and easiness to handle (Kutter

and Sulakvelidze 2005). Based on this concept, a commercially available MASTik test was also developed for antibiotic susceptibility test for mastitis which demonstrates a wide-range application for most mastitis pathogens (George et al 1993).

Up to now, many researchers have reported reduced activity of tetracycline in milk in vitro compared with activity in artificial broth media (Owens et al. 1986, Fang et al.1995b, 1996)., but the reason why tetracycline behave differently in milk is till unknown. Concerning this issue, hypotheses have been proposed as following: the decreased activity of tetracycline could be ascribed to its binding to casein micelles and cream or its chelating by bivalent calcium in milk. However, no reliable study has been reported to verify the hypotheses (Fang et al. 1995b, 1996).

In this study, we investigated the antibacterial activity of tetracycline against bovine mastitis isolates in raw milk by using a pH indicator, bromocresol purple. As a comparison, the activity of another widely used antibiotic, penicillin G, was also examined in parallel. Finally, we attempted to clarify the mechanism of the effect of raw milk toward these antibiotics` activity.

Materials and methods

Bacterial isolates

A total of 6 *S. aureus* isolates and 6 *E.coli* isolates from bovine mastitis were studied. Mastitic milk samples were aseptically taken from affected quarter of cows by Rakuno

Gakuen University in Hokkaido. 100μl of properly diluted samples were plated onto selective agar including brain-heart agar and Chromocult® Coliform(CC). Isolates were presumptively identified as *S. auresus* and *E. coli* by phenotypic methods like morphology, catalase, oxidase and coagulase assays. *E. coli K12* and *S. aureus* JCM 2151 (ATCC6538) were used as reference strains.

Milk and milk fraction preparation

Raw bovine milk was aseptically collected from clinically healthy cows with milk somatic cell counts 70,000 cells/ml by Rakuno Gakuen University in Hokkaido.

For preparation of skim milk, milk samples were centrifuged at 3,000×g at 4˚C for 10 min, the fat layer removed with a sterile spatula and the skim milk was transferred into another sterile tube, the procedure was repeated until all the fats have been removed.

For preparation of whey, skim obtained above was subsequently subjected to centrifugation at 45,000×g at 4˚C for 60min and translucent supernatant was collected, followed by filter-sterilization using pore size 0.22μm (Fang et al 1995a).

For preparation of boiled whey, whey was heated to 100˚C for 30min in a water bath, followed by centrifugation at 8,000×g for 10min at 4˚C to remove the precipitate.

Comparison of pH indicator-based technique and the plate counting method in evaluation of the bacterial growth in milk

50mL raw milk was 0.1% inoculated with overnight culture of *E.coli K12* and *S. aureus*

ATCC 6538 respectively. The inoculated samples were incubated at 37˚C for 25h. Aliquot samples were taken immediately at different time intervals for parallel pH measurement and plate counting.

Fluorescence microscopy

Staining of bacteria by DAPI (Wako, Japan) was performed as described previously with some modification (Miyanaga et al. 2007). Briefly, Overnight culture of *E.coli K12* and *S. aureus ATCC 6538* mixed with same volume of DAPI (final concentration: 0.05mg/mL), and incubated in the dark at room temperature for 5min. The stained cells were observed through fluorescent microscope with a cooled charge coupled device camera (DP70, Olympus, Japan) at UV light (330-385nm). All images were captured on the identical conditions (magnification: 100×objective, sensitivity: ISO200, exposure time: 10^{-1} s)

Broth microdilution assay for antimicrobial susceptibility

Susceptibility test was carried out in parallel in Muller Hinton broth (Becton Dickinson, USA), raw milk, skim, whey or boiled whey using standard broth microdilution method. Firstly, tetracycline-HCl (Wako, Japan) and penicillin G (Nacalai tesque Ltd. Japan) stock solutions were prepared in saline, filter-sterilized and stored in -20˚C. They were diluted 2-fold serially in saline to cover the possible susceptibility range on test day. Secondly, single colonies of *E.coli* and *S.aureus* isolates were subcultured in LB and brain-heart medium respectively (37˚C for 12-14h), then bacterial solution was adjusted by saline to

achieve turbidity equivalent to 0.5 McFarland standard which is an index of bacterial concentration approximately 10^8CFU/mL (NCCLS, 2002). The bacterial suspension was further diluted 10-fold in saline to reach a final inoculum concentration of 10^7 CFU/ml. 25µl of the inoculum were added to individual wells within a sterile 96-well polyethlene plate (Becton Dickinson, USA), and mixed with 200µl of MH broth or 1% Bromocresol purple (Nacalai tesque Ltd. Japan) supplemented milk, skim, whey or boiled whey, finally 25µl of serially diluted antibiotics were added to each well. A negative control column was included in the plate that included 250 µl of medium alone or with 1% Bromocresol purple only. The plates were incubated for 18 h at 37˚C with ambient air circulation and without shaking. The MIC was determined as the lowest antibacterial concentration at which no cloudiness or no color change in a given well. All assays were replicated in three times.

HPLC analysis

Antibiotic concentrations were analyzed by a reversed phase HPLC method utilizing a Shimazu name series (Shimazu Co., Japan). The analysis column was a 4.6×75mm Zorbax SB-C18 3.5µm (Agilent technology Co., USA) with a reliance cartridge guard column. Injection of sample (5µl) onto the HPLC was by the use of autosampler. All solvents were of HPLC grade and/or prepared with doubly deionized ultra-high purity (18.2 MΩ) water. Mobile phase buffers were filtered through 0.22µm filters and degassed by sonication prior to use.

The optimal HPLC-UV/Vis condition for tetracycline analysis was to use a mobile phase consisted of 0.025M aqueous KH_2PO_4 (pH 3.0): acetonitrile (85:15 by volume) with a flow rate of 1ml/min. Detector wavelength and column compartment temperature was adjusted to 350nm and 25˚C respectively.

For analyses of penicillin G, the mobile phase used was 0.025M aqueous KH_2PO_4 (pH 3.0): acetonitrile (70:30 by volume) with a flow rate of 1mL/min. Detector wavelength and column compartment temperature was adjusted to 204nm and 40˚C respectively.

The limit of quantitation for each antibiotics was 1μg/mL and the standard curve was linear within the range from 1 to 10μg/mL (Coefficient of correlation was 0.9989 for tetracycline and 0.9958 for penicillin G).

Dialysis model

The study was performed in two stages: (1) Transfer of antibiotic from MH broth to saline (0.85% Nacl solution); (2) transfer of antibiotic from raw milk to saline. The dialysis system utilized dialysis tube (Spectrum Laboratories Inc., USA) with a cut-off point at molecular weight of 5,000Da. All the conditions were the same, except that 2mL of MH broth or raw milk containing 1mg/mL antibacterial were introduced into each dialysis tube. Then the tube was placed in a beaker containing 200mL saline and dialyses were undertaken at room temperatures. A sample (0.5mL) from the outer fluid was taken at a given time intervals. Antibiotic concentrations in the samples were determined by HPLC

method described above. Each stage was replicated three times.

Relative surface hydrophobicity measurements

The relative surface hydrophobicity which was defined by the partition between aqueous solution and organic solvent was investigated. The organic solvents used in this study were hexadecane (Wako, Japan) and chloroform (Naccalai tesque. Ltd, Japan). Briefly, antibiotic solutions (10μg/mL) were prepared in distilled water. 1mL of organic solvent was added to 1mL of aqueous solution, mixed by votex 30s, centrifuge for 10min at 8000×g. The antibiotics concentration of the aqueous layer was measured by HPLC method. The partition value was calculated from the change of antibiotic's concentration in aqueous solution as follows (C_0: concentration of antibiotics in aqueuous solution before treatment. C_1: concentration of antibiotics in aqueous solution after treatment)

$$\text{Partition } (\%) = \frac{C_0 - C_1}{C_0} \times 100\%$$

Results

pH indicator-based assay for analysis of the bacterial growth in milk

To compare the ability of pH-indicator based method and plate counting method to estimate the bacterial growth in milk, both the pH and cell counts were monitored by using two reference strain *E.coli K12* and *S. aureus* ATCC 6538 (Fig. 1). With the growth of each bacterium, the progressively reduction of pH in raw milk from 6.81 to 5.69 or 5.61 was observed as the incubation time going, which is within the transition pH range of

Bromocresol purple. On the other hand, the plate-count technique shows that the number of *E.coli K12* increased, however, *S. aureus ATCC 6538* cell counts decreased at certain time. Examination of *S. aureus ATCC 6538* by fluorescent staining method clearly indicated cell agglutination occurred in raw milk (Fig. 2e and f)

Effect of raw milk on the antibacterial activity of tetracycline and penicillin G

6 *S.aureus* and 6 *E.coli* strains, both of which were major pathogens associated with mastitis, were isolated from mastitic milk of infected cows from Hokkaido. The broth microdilution susceptibility assay was used to compare the activity of two widely used antibiotics against these pathogens in raw milk and ordinary MH broth. Of the 14 isolates together with references strains, 13 were sensitive to tetracycline in MH broth. The tetracycline's MIC value was estimated to be ≤0.5μg/mL for all the gram-positive *S.aurues*, and ≤1μg/mL for six of seven gram-negative *E.coli.* However, the MIC values of tetracycline on all the test organisms were 4-32 times those in MH (Table 1), indicating the inhibition effect of milk toward the interaction between tetracycline and tested organisms.

In the case of penicillin G, no significant differences were observed between the MICs measured in MH and those measured in raw milk (Table 1). However, it had the demonstrable growth inhibitory effect toward only 6 strains. All the gram-negative *E.coli* exhibited resistance to penicillin G., with MIC ≥16μg/mL. What's more, even within the group of gram-positive *S. aureus* isolates, there was also strain-related variation in their

susceptibility to penicillin G., for example, *S. aureus* isolate 26 was shown to be quite resistant to penicillin G.

Binding of tetracycline to components of raw milk

To further understand the inhibition effect of milk toward the interaction of tetracycline and tested organisms, a series of experiments were performed by focusing on the interaction between antibiotics and milk while no bacterium was involved in. In the first experiment, diffusion of tetracycline or penicillin G through the dialysis membrane in two different dialysis systems: MH to saline and raw milk to saline are depicted in Fig. 3. In the MH system, the tetracycline concentration outside reached 9.5μg/mL after incubated for 31h and stayed constant then. This value was also comparable with that calculated according to noninteracting equilibrium dialysis theory which is nearly 9.9μg/mL. However, in the presence of raw milk, the diffusion rate of tetracycline across the membrane markedly decreased. At 31h, when the steady-state attained in MH system, the tetracycline concentration in the outer fluid was 5.53μg/mL, only 58.2% of the concentration measured in MH system. And even after 45h of incubation, at which the steady-state reached, less than 70% of the tetracycline introduced into the inner dialysis tube had diffused across the membrane. A comparison of the two diffusion patterns indicates a rapid and high degree binding of tetracycline to components with large molecules in raw milk by the irreversible way.

On contrary, the equilibrium dialysis patterns of penicillin G in two systems did not demonstrate big difference, although the diffusion rate in raw milk system was a little slower, which might due to lesser extent of binding of penicillin G to milk components. However, it was observed that concentration of penicillin G in MH system stabilized at 9.9μg/mL by 25h, and the same concentration reached in raw milk system 5h later, indicating this binding might be reversible if it happened.

The physical-chemical properties of antibiotics are generally considered the major determinants for the extent of their interactions with other substances. The relative surface hydrophobicity of tetracycline and penicillin G, one of important physical-chemical parameters, was also evaluated by examining the ratio of antibiotics absorbed by organic solvent respectively in current study. The partition of tetracycline and penicillin G between organic solvent and water in terms of partition value was shown in Table 2. Penicillin G possessed very poor solubility in organic solvent, while tetracycline has 7 times or 10 times higher solubility than that of penicillin G., meaning that relative surface hydrophobicity of tetracycline is higher. This difference observed might account partly for the different behavior of two antibacterial in raw milk, and the adhesion of tetracycline to large molecules in milk might due to hydrophobic bindings.

Inhibitors to the antibacterial activity of tetracycline in raw milk

To determine which components of milk might be responsible for the irreversible binding

and thus inhibit tetracycline bioavailability. A reference strain *E.coli K12* was chosen, and susceptibility of it toward tetracycline in skim and whey was investigated (Table 3). A 16-fold increase in tetracycline concentration was required to eliminate the growth of *E.coli K12* in skim, which is devoid of fat. The level of inhibition exhibited by skim was the same as that by raw milk. However, whey had less inhibition level by removing the casein, the activity of tetracycline in whey increased by 8 times than that in raw milk, and further treatment of the whey by boiling increased bioavailability of tetracycline to the levels comparable with those found in MH broth.

Discussion

The problem of antibiotic therapy toward bovine mastitis has been recognized for some time (Craven 1987), there is suspicion that antimicrobial susceptibility testing of mastitis pathogens has not been adequately validated for most mastitis pathogens and antibiotics. This has led to a search for alternative explanations for failure mechanisms (Sandholm et al. 1990). Our results confirm the problem of artificial susceptibility test in bovine mastitis. We found direct analysis of bacterial growth in milk is very difficult and inaccurate by plating count method, because some bacteria such as *S. aureus* tend to aggregate in raw milk as observed in this study, and also demonstrated by other group (Korhonen et al. 2000). While pH indicator-based method was clearly shown to be sensitive and reliable for antibacterial susceptibility testing in milk. Since most mastitis pathogens are

lactose-fermentation bacteria, the growth of these bacteria will ferment the lactose in milk to lactic acid, with an accompanying decrease in pH (George et al. 1993). As a consequence, the color change from purple to yellow will occur, which can be used to determine the MIC. In this study, the pH-indicator-based techniques showed good agreement with the turbid-metric method in testing bacterial susceptibility in MH (data not show).

Penicillin G and tetracycline are both long-established antibacterial agents and have been widely used in the treatment of bovine mastitis. For penicillin G, on the condition that intramammary was infected with penicillin-sensitive strains, it is generally considered a first-line antibiotic due to its therapeutic advantages compared with β-lactamase-stable penicillins (Grave et al. 1999). However, the appearances of mastitis-causing Staphylococci resistant cells have been reported repeatedly. In some country, the percentage of resistant isolates reached to even 50% (Gentilini et al. 2000, Erskine et al. 2002). Our study also detected the existence of staphylococci isolates from bovine mastitis in Hokkaido of Japan which is resistant to penicillin G. This result together with the fact that penicillin G has narrowed spectrum activity might limit its future application. For tetracyclines, because of their broad spectrum activity and low cost, they have emerged as a second choice of antibiotics to beta-lactams for the management of mastitis in dairy herds in certain regions of the world,

However, our results showed the activity of tetracycline, one of the most-widely used antibiotic in tetracycline group, decreased markedly when presented in milk. About 4-16 fold higher concentration of tetracycline was required in milk compared with MH broth to inhibit growth of isolated pathogens. A possible explanation might be that bacterial growth rate is lowered in milk since fast growing bacteria was reported to be more susceptible to the slow growing ones (Fang 1996), or the aggregation of bacteria in milk. But in this experiment, milk didn't impaired activity of penicillin G which was tested in parallel. This led to our assumption that the cause of the inhibition might be largely due to physical-chemical and pharmacological properties of tetracycline itself. As demonstrated by our results, tetracycline exhibited a higher hydropobicity compared to penicillin G and a strong irreversible binding took place between it and large molecules in milk. As a result, the concentration of free tetracycline molecules in milk which is accessible to bacteria reduced to a high degree, rendering more tetracycline are required to be added in milk to achieve the same inhibition effect as that in MH broth.

Currently, only the pharmokinetics of tetracycline which are based solely on achievable serum and interstitial fluid concentrations in humans after oral administration are available (Kenneth et al. 2006). Neuvonen PJ (1976) had shown tetracyclines can form insoluble complexes with calcium, magnesium, iron and aluminum, which markedly reduces its absorption in serum. Peter G et al (1977) reported that protein, fat and carbohydrate meals

in food reduce the absorption of tetracycline by about 50% in human serum. Since bovine milk is a complex medium, composed of water, proteins (caseins, β-lactoglobulins, α-lactoglobulins, immunoglobulins, bovine serum albumin and various enzymes), lipids, lactose, metal ions, minerals, vitamins, acid and gases (O`Flaherty et al. 2005), a parallel mechanism may be at work in the case of tetracycline in milk. By comparing the MIC of tetracycline against *E.coli K12* in different fractions prepared from raw milk, we found that fat was not the possible factor for impairing effect because remove fat alone did not influence the MIC value as compared with that in milk, while the strongest reduction of MIC value was observed after removing the casein, implying it might be the major inhibitor in milk. Casein, which account for nearly 80% of milk protein is organized in micelles and has very porous and hydrophobic structures. It is very likely that tetracycline was tightly entrapped into porous casein micelles through hydrophobic interaction, thus reducing its antibacterial activity. In addition to potential inhibitory roles of casein to tetracycline, the existing 2-fold difference of MIC in whey and MH broth revealed the presence of other inhibitors. Though the detailed information of these inhibitors is unknown, the sensitivity of them to heat indicates it is probably mediated by a protein or a group of proteins. Further characterization of these inhibitors is deserved.

Results

It is clear that tetracycline behaves quite differently in raw milk as compared with MH

broth, an irreversible binding between tetracycline and milk components which might due to the hydrophobic interaction was observed and thus might account for the tetracycline decreased bioavailability in raw milk. Removing the casein and heat-sensitive substances in raw milk, the activity of tetracycline can be recovered as that in MH broth, suggesting the role of these components in decreasing the activity of tetracycline in raw milk.

Acknowledgments

We would like to thank Mr.Aidan Synnott (Tokyo Institute of Technology, Japan), who provided critical opinions and helpful English checking for this paper. We are also grateful to Dr. Kazuhiko Miyanaga (Tokyo Institute of Technology, Japan) for his helpful advice.

References

Agwuh KN, MacGowan A (2006) Pharmacokinetics and pharmacodynamics of the tetracyclines including glycylcyclines. J Antimicrob Chemoth 58: 256-265

Ali-vehmas T, Westphalen P, Myllys V et al (1997) Binding of Staphylococcus aureus to milk fat globules increases resistance to penicillin-G. J Dairy Res 64: 253-260

Al-Nazawi MH (2006) Resistance and residues of antibacterial in dairy farm and diary production in Al-Hassa region, Saudi Arabia. J Med Sci 6:198-202

Constable PD, Morin DE (2003) Treatment of clinical mastitis using antimicrobial susceptibility profiles for treatment decisions. Vet Clin Food Anim 19:139-155

Craven N (1987) Efficacy and financial value of antibiotic treatment of bovine clinical

mastitis during lactation-a review. Br Vet J 143: 410-422

Erskine RJ, Walker RD, Bolin CA, Bartlett PC, White DG (2002) Trends in antibacterial susceptibility of mastitis pathogens during a seven-year period. J Dairy Sci 85: 1111–1118

Fang W, Vikerpuur M, Sandholm M (1995a) A fluorometric β-glucuronidase assay for analysis of bacterial growth in milk. Vet Microbiol 46: 361-367

Fang W, Vikerpuur M (1995b) Potency of antibacterial drugs in milk as analysed by β-glucuronidase-based fluorometry. J Vet Pharmacol Therap 18: 422-428

Fang W, Pyörälä S (1996) Mastitis-causing *Escherichia coli*: serum sensitivity and susceptibility to selected antibacterials in milk. J Dairy Sci 79:76-82

Gentilini E, Denamiel G, Betancor A et al (2000) Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Argentina. J Dairy Sci 83, 1224–1227

George S (1993) Clinical applications of an antibiotic susceptibility test for mastitis. Agri practice 14: 25-28

Grave T, Greko C, Nilsson L et al (1999) The usage of veterinary antibacterial drugs for mastitis in cattle in Norway and Sweden during 1990-1997. Prev Vet Med 42:45-55

Korhonen H, Marnila P, Gill HS (2000) Milk immunoglobulin and complement factors. Br J Nutr 84: S75-S80

Kutter E, Sulakyelidze A (2005) Bacteriophages: Biology and Applications, CRC Press, pp 288

Miyanaga K, Takano S, Morono Y et al (2007) Optimization of distinction between viable and dead cellsby fluorescent staining method and itsapplication to bacterial consortia. Biochem Eng J 37: 56-61

NCCLS (2002) The National Committee for Clinical Laboratory Standards (NCCLS): Performance Standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals: approved standard, 2nd edn. Wayne, PA, pp 86

Neuvonen PJ (1976) Interactions with the absorption of tetracyclines. Drugs 11(1):45-54

Owens WE, Watts JL (1986) Effects of milk on activity of antimicrobics against *Staphylococcus aureus* isolated from bovine udders. J Dairy Sci 70:1946—1951

O` Flaherty S, Coffey A, Meaney WJ et al (2005) Inhibition of bacteriophage K proliferation on Staphylococcus aureus in raw bovine milk. Lett Appl Microbiol 41: 274-279

Sandholm M, Kaartinen L, Pyörälä S (1990) Bovine mastitis-why dose antibiotic therapy not always work? An overview. J Vet Pharmacol Ther 13: 248-260

Welling PG, Koch PA, LAU CC, Craig WA (1977) Bioavailability of tetracycline and doxycycline in fasted and nonfasted subjects. Antimicrob Agents Ch, 11(3): 462-469

Table 1 Antimicrobial activity of penicillin G and tetracycline in MH broth and raw milk

	Penicillin G MIC (μg/mL)		Tetracycline MIC (μg/mL)	
	MH broth	Raw milk	MH broth	Raw milk
Staphylococcus. aureus				
isolate 2	0.015125	0.015125	0.5	2
isolate 3	0.03125	0.03125	0.25	2
isolate 19	0.015125	0.015125	0.25	1
isolate 20	0.015125	0.015125	0.25	2
isolate 21	1	1	0.25	2
isolate 26	16	16	0.25	2
ATCC 6538	0.03125	0.03125	0.25	2
Escherichia coli				
isolate 1	16	16	0.25	8
isolate 2	128	128	8	128
isolate 12	32	32	0.5	8
isolate 14	32	32	0.5	8
isolate 15	64	64	0.5	8
isolate 20	32	32	0.5	8
K12 (W3110)	1024	1024	1	16

Table 2 The partition values of penicillin G and tetracycline in two organic solvent/water combinations

	Partition value (%)	
	Tetracycline	Penicillin G
Hexadecane/H_2O	8%	0.6%
Chloroform/ H_2O	35%	4%

Table 3 Antimicrobial activity of tetracycline against *E.coli K12* in different fraction of raw milk

	MH broth	raw milk	skim	whey	heated whey
MIC (μg/mL)	1	16	16	2	1

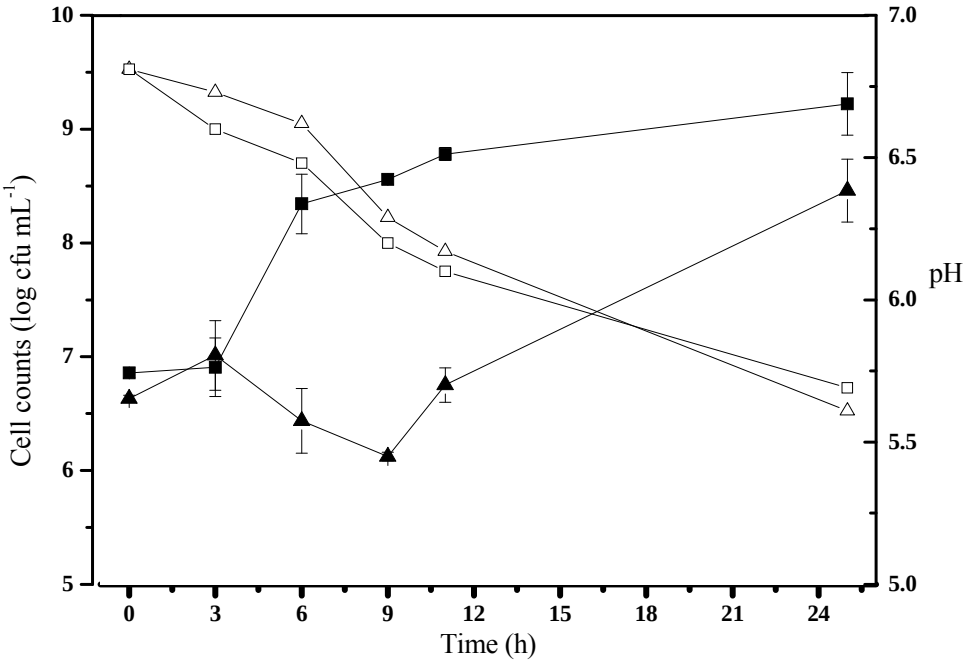


Fig. 1 Change of pH and cell counts in the raw milk cultures. Δ pH of raw milk inoculated with *S.aureus ATCC 6538*, □ pH of raw milk inoculated with *E.coli K12*, ▲ Cell counts of *S.aureus ATCC 6538* in raw milk, ■ Cell counts of *E.coli K12* in raw milk. Data reported are mean±standard deviations.

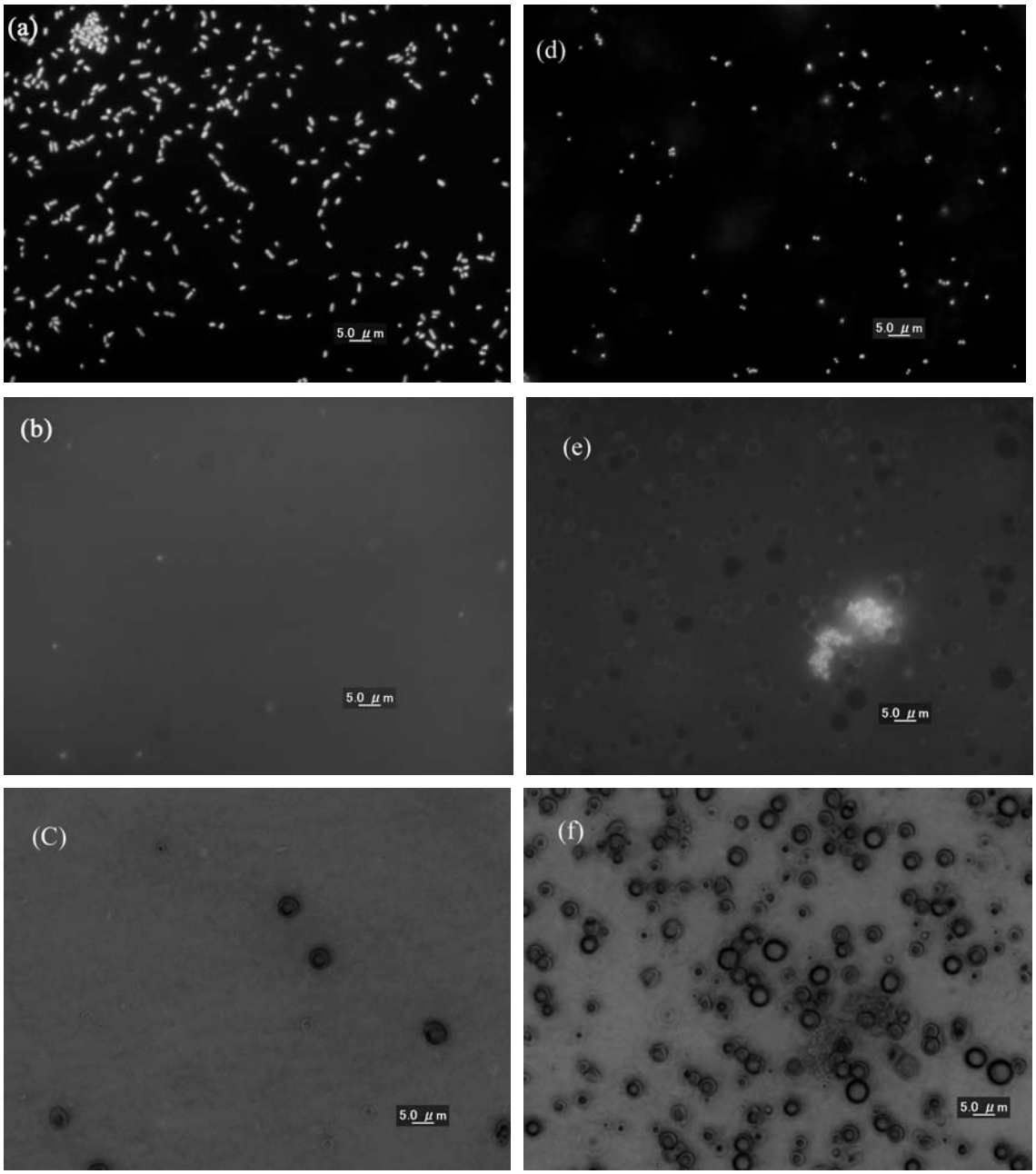
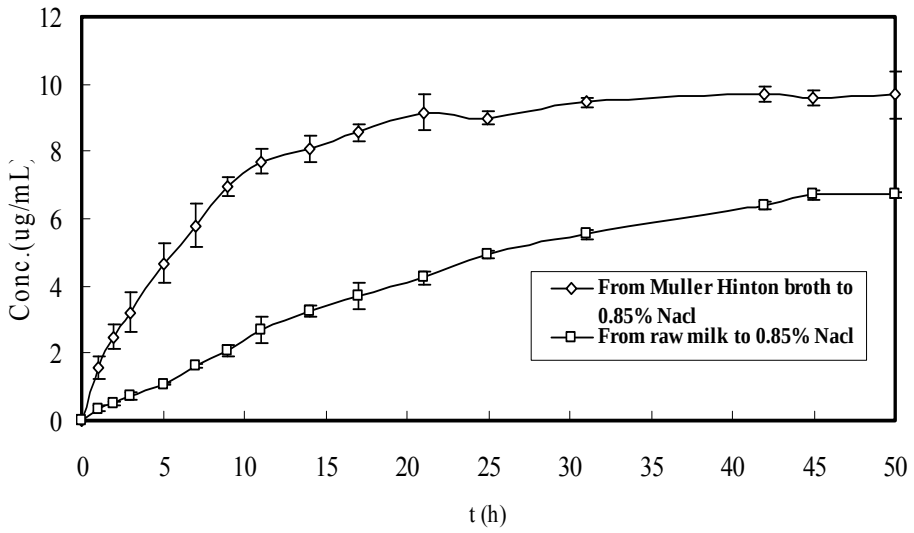
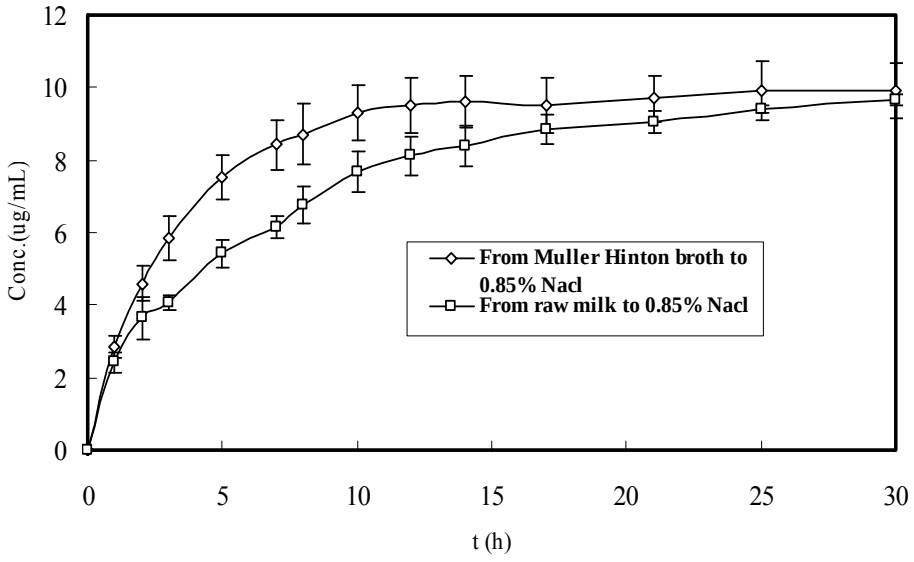


Fig. 2 Observation of *E.coli K12* and *S.aureus ATCC 6538* grown in LB and raw milk. (a) fluorescent image of *E.coli K12* in LB, (b) fluorescent image of *E.coli K12* in raw milk (c) light field image of *E.coli K12* in raw milk (d) fluorescent image of *S.aureus ATCC 6538* in LB, (e) fluorescent image of *S.aureus ATCC 6538* in raw milk, (f) light field image of *S.aureus ATCC 6538* in raw milk (The scale bar under pictures represents 5μm).



(A)



(B)

Fig. 3 Tetracycline (A) or penicillin G (B) concentration in the dialysis fluid (out fluid) as a function of time (mean of 3 replicates) in two different dialysis systems: MH broth to saline solution and raw milk to saline solution.