Differences in Sensitivity of Milk Fermentation to Lactating Cow and Dry Cow Intra-mammary Antimicrobial Agents

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Abstract

Antimicrobial agents for treatment of mastitis in dairy cows may result in drug residues in milk, suppressing production of fermented dairy products. The objective of this study was to compare the effects of lactating cow and dry cow intra-mammary antimicrobial preparations on fermentation of milk. Samples of ultra-high temperature-treated milk + starter culture (Lactococcus lactis subsp. lactis), were prepared. Using serial double dilution, erythromycin-trimethoprim lactating cow formula was added to five samples at 62.5-1000 mg/L and cloxacillin benzathine dry cow preparation to another five at 75-1200mg/L. A negative control with neither starter culture nor antimicrobial agent, and a positive control with starter culture but no antimicrobial agent were included. All samples were incubated at 30°C for two days and osmolarity (mOsmoles/L) monitored by a cryoscopic osmometer. From a starting value of 301±1.72 for fresh milk, osmolarity of non-inoculated milk did not rise beyond 314±7.07. Maximum osmolarity of inoculated milk attained in the presence of erythromycin-trimethoprim was 328±0.97, in contrast to 507±35.53 in the presence of cloxacillin benzathine. Hence hydrophilic lactating cow antimicrobial formulas exert a highly significant inhibitory effect on milk fermentation compared to hydrophobic dry cow antimicrobial preparations.

KEYWORDS: Milk; Fermentation; Antimicrobial Agents; Hydrophobic; Hydrophilic

1. Introduction

Fermentation of milk is brought about largely by lactic acid bacteria (LAB). Not surprisingly, the process of fermentation is inhibited by antimicrobial drugs. In dairy farming, antimicrobial agents are primarily used for treatment of systemic and intra-mammary bacterial infections. In addition, tetracyclines are routinely used in treatment of some rickettsial diseases such as anaplasmosis and

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cowdriosis. According to McEwen et al. (1992), use of antimicrobial agents in veterinary medicine may result in appearance of drug residues in milk if withdrawal periods are not strictly adhered to. A survey of antimicrobial residues in milk, reported by Allison (1985), attributed 61% of the residues to intra-mammary infusions in lactating cows, 31% to intra-mammary infusions at drying off, 6% to injections and 1% to other causes. Apart from posing a health risk to consumers as described by Nisha (2008), Grunwald and Petz (2003) showed that antimicrobial agents potentially suppress production of fermented dairy products such as yoghurt. This was in consonant with earlier observations on inhibitory effects of antimicrobial agents on cheese production by Albright et al. (1961). In addition, Zhou et al. (2012) demonstrated that prolonged exposure of LAB to even low levels of antimicrobial agents may lead to development of antibiotic resistance in these organisms, which may then be passed on to pathogenic bacteria with serious consequences. Clearly, presence of antimicrobial residues in milk has far reaching consequences in human health as well as in the dairy industry. The objective of the present study was to compare the inhibitory effects of lactating cow and dry cow intra-mammary antimicrobial preparations on fermentation of milk in view of the divergent physical properties between the two classes of drugs. A number of techniques have been developed for assessing the effects of antimicrobial agents on fermentation of milk ranging from the pioneering studies by Cogan (1972) on inhibition of milk acidification, to observations on changes in morphology of lactobacilli by Coeuret et al. (2003). In the present study osmometry, reviewed by Lord (1999), was used for evaluating effects of intra-mammary antimicrobial preparations on fermentation of milk. Osmometry is the measurement of osmolarity i.e. the total concentration of solutes in a solution expressed in mOsmoles/L, equivalent to mMoles/L.

2. Materials and Method

The procedure employed in the present investigation was similar to the Intertest for detection of antimicrobial agents in cow’s milk, previously described by Mohsenzadeh and Bahrainpour (2008), but with osmolarity as the measured parameter. Six samples each consisting of standard UHT (ultra-high temperature-treated) milk and sour milk (*Lactococcus lactis* subsp. *lactis* culture) were randomly acquired from commercial sources. The minimum count of *Lactococcus lactis* subsp. *lactis* in the sour milk was 6.5 x 10³ cfu/mL. In the laboratory, the lactating cow antimicrobial formula erythromycin-trimethoprim (Interchemie, Venray, Holland) or the dry cow antimicrobial preparation cloxacillin benzathine (Pfizer, Kent, USA) were mixed with the UHT milk in test tubes using the serial double dilution method, giving final antimicrobial concentrations of 62.5-1000 mg/L and 75-1200 mg/L respectively. Concentrations of antimicrobial agents were selected so as to encompass the therapeutic ranges encountered in mastitis treatment. Altogether five test tubes containing 4.5 mL UHT milk + 0.5 mL starter culture + erythromycin-trimethoprim, and another five containing 4.5 mL UHT milk + 0.5 mL starter culture + cloxacillin benzathine, were prepared from each stock sample. Two control samples were included in each experiment; a negative control composed of UHT milk with neither antimicrobial agent nor starter culture, as well as a positive control comprising UHT milk with starter culture but no antimicrobial agent. The test tubes were closed sufficiently tightly to allow fermentation gases to escape and then incubated at 30°C (Gallenkamp Thermo stirrer® 85®, Germany) as indicated earlier by Båat et al. (2000) and Wouters et al. (2000). At the beginning of the experiment (day 0) and after 24 hours (day 1) and 48 hours (day 2) of incubation, 50µL of milk were withdrawn from each test tube using an Eppendorf micropipette, transferred into Eppendorf tubes, and the osmolarity measured by freezing point.
depression (Osmomat 030 osmometer®, Gonotech, Germany) in accordance with contemporary practices in osmometry, which have been reviewed by Lord (1999).

Data were processed on Microsoft Excel 2007 and results given as mean ± standard deviation. Means were compared with reference to the negative control. The student’s t tests for comparison of means were performed on Sigma Plot for Windows version 1.02, with p<0.05 being taken as significant.

3. Results

The mean osmolarity values for the stock milk samples were 301±1.72 mOsmoles/L and 415 ±1.87 mOsmoles/L for UHT milk and sour milk respectively. Changes in osmolarity of non-inoculated UHT milk with no antimicrobial agents during two days of incubation at 30°C are shown in Fig. 1. No significant increase in osmolarity occurred over the two-day period. From an average value of 301±1.72 at the beginning of the experiment (day 0), osmolarity rose minimally to 306±3.35 mOsmoles/L on day 1 of incubation, reaching a relatively low maximum value of 314±7.07 mOsmoles/L after two days of incubation. In comparison, the osmolarity of inoculated UHT milk with no antimicrobial agents increased significantly from an average of 313±6.80 mOsmoles/L on day 0 of incubation, to 457±23.28 mOsmoles/L on day 1 before stabilizing at a peak value of 463±19.91 mOsmoles/L on day 2. Most of the increase in osmolarity occurred during the first day of incubation.

![Fig. 1. Osmolarity of UHT milk without (series 1) and with (series 2) Lactococcus lactis subsp. lactis culture during two days of fermentation at 30°C.](image-url)
The effect of erythromycin-trimethoprim on osmolarity of inoculated UHT milk during two days of fermentation at 30°C is illustrated in Fig. 2. No significant increase in milk osmolarity occurred at all concentrations on both day 1 and day 2 of incubation. Maximum values of osmolarity, which coincided with the minimum antimicrobial concentration, were 315±4.45 mOsmoles/L on day 0, 323±7.00 mOsmoles/L on day 1 and 328±9.07 mOsmoles/L on day 2 of incubation respectively.

In contrast, cloxacillin benzathine had virtually no inhibitory effect on fermentation of inoculated UHT milk at all concentrations throughout incubation (Fig. 3). From a starting average of 311±5.57 mOsmoles/L on day 0, maximum osmolarity values recorded on day 1 and day 2 of incubation were 432±33.65 mOsmoles/L and 507±35.53 mOsmoles/L respectively. The rate of increase in mean osmolarity was almost linear over the two days of incubation.
Fig. 3. Effect of cloxacillin benzathine on osmolarity of UHT milk inoculated with *Lactococcus lactis* subsp. *lactis* culture during two days of fermentation at 30°C (series 1-5 correspond to antimicrobial concentrations of 75, 150, 300, 600 and 1200 mg/L respectively).

4. Discussion

Studies by Aning et al. (2007) and Shitandi and Sternesjö (2007) indicate that the prevalence of antimicrobial residues in milk is relatively high in small scale dairy units where there is no adequate monitoring of withdrawal periods following use of antimicrobial agents in dairy cows. Therefore the need to monitor antimicrobial effects on fermentation of milk still exists. Although chemical effects of antimicrobial agents on LAB can routinely be evaluated using disc diffusion methods, osmometry provided an opportunity to study subtle effects arising from physical properties of antimicrobial preparations as well. As expected of a negative control, osmolarity of milk did not show any significant elevation over time in the absence of LAB. On the other hand, addition of *Lactococcus lactis* subsp. *lactis* to milk in the positive control resulted in fermentation of lactose. The products of fermentation, primarily lactic acid, and a variety of small organic compounds which impart aesthetic flavors to fermented milk and milk products, account for the increase in the total concentration of solutes in the milk, which was reflected as an increase in milk osmolarity. Taken together, the negative and positive controls served as the basis upon which the effects of lactating cow and dry cow intra-mammary antimicrobial preparations could be evaluated. Since osmolarity of fermented milk is directly proportional to the degree of fermentation, any changes in fermentation patterns arising from presence of antimicrobial preparations in milk would be paralleled by corresponding changes in milk osmolarity.
Clinically, the combination of erythromycin and trimethoprim is a relatively new formula used in the treatment of mastitis caused by *E. coli* as well as *Mycoplasma, Staphylococcus* and *Streptococcus* species in lactating cows. According to Pieterse and Todorov (2010), erythromycin interferes with bacterial protein synthesis by binding to bacterial rRNA whereas trimethoprim inhibits synthesis of bacterial proteins and nucleic acids by impairing production of tetrahydrofolic acid. Thus the two antimicrobial agents had an additive bacteriostatic effect on *Lactococcus lactis* subsp. *lactis*. As with all antimicrobial preparations used in treatment of cows in lactation, erythromycin-trimethoprim is hydrophilic. Owing to its free solubility in milk, the antimicrobial preparation exerted a significant inhibitory effect on lactose fermentation at all concentrations within the therapeutic range.

Pieterse and Todorov (2010) classified cloxacillin as a bactericidal antimicrobial agent which prevents active multiplication of susceptible bacteria by inhibiting synthesis of the cell wall. In veterinary practice cloxacillin benzathine is used for the treatment of bovine mastitis caused by *Staphylococcus aureus* and *Streptococcus agalactiae* at drying off. In contrast to erythromycin-trimethoprim, cloxacillin benzathine is hydrophobic. Compared to lactating cow antimicrobial preparations, all dry cow antimicrobial agents are prepared as sparingly soluble formulas in a slow release base to achieve a prolonged therapeutic effect spanning from the end of a lactation period to the beginning of the next. As a result of limited admixture with the aqueous phase of milk containing the fermentation substrate lactose, the magnitude and duration of inhibition of milk fermentation by cloxacillin benzathine were not significant. The present results cannot be attributed to antimicrobial resistance as there is no record of resistance to cloxacillin in *Lactococcus lactis* subsp. *lactis* strains currently in use in the dairy industry, as reported by Devirgiliis et al. (2013).

5. Conclusion

Historically the influence of antimicrobial residues in milk on fermentation processes has been considered without taking into account the heterogeneity in physical properties of the drugs. The present findings indicate that lactating cow antimicrobial preparations have highly significant inhibitory effects on milk fermentation in comparison to dry cow antimicrobial formulas, whose effects are blunted by their limited solubility in the aqueous phase of milk.

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Conflict of Interest

None
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