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Application of Osmometry in Evaluating Effects of Antimicrobial Agents on Milk Fermentation

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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. Since, lactic acid from fermentation normally raises milk osmolarity, the objective of this study was to assess the validity of osmometry in evaluating effects of antimicrobial agents on milk fermentation. The 10 test tubes each containing ultra high temperature treated milk+starter culture (*Lactococcus lactis* sp. *lactis*) were prepared. Using serial double dilution, cloxacillin benzathine (dry cow) was added to 5 test tubes at 75-1200 mg L^{-1} and erythromycin and trimethoprim (lactating cow) to the remaining 5 test tubes at 62.5-1000 mg L^{-1}. A negative control with neither starter culture nor antimicrobial agent and a positive control with starter culture but no antimicrobial agent was included. All samples were incubated at 30°C for 2 days and osmolarity monitored by a cryoscopic osmometer. From a starting value of 301±1.72 mOsmol L^{-1} (mean±SD), maximum osmolarity values after 2 days were 315±7.07 for non-inoculated antimicrobial-free milk, 463±19.91 for inoculated antimicrobial-free milk, 328±9.07 for inoculated milk containing erythromycin and trimethoprim and 507±35.53 for inoculated milk containing cloxacillin benzathine (n = 6). Results from negative and positive controls were consistent with absence and presence of normal fermentation, respectively. Erythromycin and trimethoprim significantly ($p<0.05$) inhibited fermentation at all concentrations throughout the incubation period. Cloxacillin benzathine exhibited significant inhibition at higher concentrations on day 1 of incubation but none on day 2.

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 cowdriosis. Use of antimicrobial agents in veterinary medicine may result in appearance of drug residues in milk if withdrawal periods are not strictly adhered by McEwen *et al*. (1992). A survey of antimicrobial residues in milk 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 (Allison, 1985). Apart from posing a health risk to consumers (Mitchell *et al*., 1998), the antimicrobial agents potentially suppress production of fermented dairy products, such as yoghurt (Grunwald and Petz, 2003) and cheese (Keceli and Robinson, 1997). In addition, prolonged exposure of LAB to even low levels of antimicrobial agents may lead to development of antibiotic resistance in these organisms (Zhou *et al*., 2012). For these reasons, a number of techniques have been developed for assessing the effects of antimicrobial agents on fermentation of milk. Inhibition of milk acidification (Cogan, 1972), changes in morphology of lactobacilli and colorimetry (Bozo and Andel, 2008) have all been employed in the past. The objective of the present study was to assess the validity of osmometry in evaluating effects of intra-mammary antimicrobial agents on fermentation of milk. Osmometry is the measurement of osmolarity, i.e., the total concentration of solutes in a solution expressed in mOsmol L^{-1}, equivalent to mMol L^{-1}. The rationale of the method is that as fermentation progresses, production of lactic acid and other soluble products normally raises the osmolarity of milk. Therefore, any changes in fermentation patterns resulting from presence of antimicrobial agents would be reflected by parallel changes in osmolarity of the milk. Osmometry is already being used for detection of extraneous water in milk in the dairy sector, therefore its application in evaluation of antimicrobial effects on fermentation patterns would broaden the industrial application of the technique at no additional cost.

MATERIALS AND METHODS

The procedure employed in the present investigation was similar to the Intertest, as previously described for detection of antimicrobial agents in cow's milk (Mohsenzadeh and Bahrainipour, 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* sp. *lactis* culture) were randomly acquired from commercial sources. The minimum count of *Lactococcus lactis* sp. *lactis* in the sour milk was 6.5×10^3 cfu mL^{-1} and the composition of the UHT milk samples used as indicated in Table 1.

In the laboratory, cloxacillin benzathine DC (Dry Cow) or erythromycin-trimethoprim LA (Lactating Cow) were mixed with the UHT milk in test tubes using the serial double dilution method, giving final antimicrobial concentrations shown in Table 2. Concentrations of antimicrobial agents were selected, so as to encompass the therapeutic ranges encountered in mastitis treatment. Altogether, 5 test tubes containing 4.5 mL UHT milk + 0.5 mL starter culture+cloxacillin benzathine DC and another 5 containing 4.5 mL UHT milk + 0.5 mL starter culture+erythromycin-trimethoprim LA were prepared from each sample. The 2 control test tubes were included in each experiment; a negative control composed of 5 mL of UHT milk with no antimicrobial agent and no starter culture as well as a positive control comprising 4.5 mL of UHT milk with 0.5 mL starter culture but no antimicrobial agent as shown in Table 2.

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 previously indicated (Baati *et al*., 2000; Pitt *et al*., 2000). At the beginning of the experiment (day 0) and after 24 (day 1) and 48 h (day 2) of incubation, 50 μL of milk were withdrawn from each test tube using an Eppendorf micropipette, transferred into Eppendorf tubes and the osmolarity measured by freezing point depression

Table 1: Composition of standard UHT milk

Constituents	Content (g/100 g milk)
Protein	2.9
Fat	3.3
Lactose	5.1
Ash	0.8

Table 2: Concentrations (mg L^{-1} milk) of antimicrobial agents in UHT milk samples

Antimicrobial agent	
Cloxacillin benzathine DC	Erythromycin and Trimethoprim LA
1200	1000
600	500
300	250
150	125
75	62.5
0	0
Un-inoculated	Inoculated

*(Pfizer Limited, Kent CT139NJ, USA); **(Interchemie, Venray, Holland)

(Osmomat 030 osmometer®, Gonotech, Germany) in accordance with contemporary practices in osmometry (Lord, 1999).

Data were processed on Microsoft Excel 2007 and results given as mean±standard deviation. 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.

RESULTS AND DISCUSSION

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

The effect of erythromycin-trimethoprim LA on osmolarity of inoculated UHT milk during 2 days of fermentation at 30°C is illustrated in Fig. 3 significant increase in milk osmolarity occurred at all concentrations on both day 1 and 2 of incubation. Maximum values of osmolarity which coincided with the minimum antimicrobial concentration were 315±4.45 mOsmol L^{-1} on day 0, 323±7.00 mOsmol L^{-1} on day 1 and 328±9.07 mOsmol L^{-1} on day 2 of incubation, respectively. On day 1 of fermentation, cloxacillin benzathine DC had significant inhibitory activity only at concentrations of 300, 600 and 1200 mg L^{-1} with no inhibitory effect at 75 and 150 mg L^{-1} (Fig. 4). On day 2, no inhibitory effect was observed at all concentrations. From a starting average of 311±5.57 mOsmol L^{-1} on day 0, maximum osmolarity values recorded on day 1 and 2 of incubation were 432±33.65 and 507±35.53 mOsmol L^{-1}, respectively. The increase in mean osmolarity was almost linear over the 2 days of incubation.

Interpretation of the results is with reference to the positive control, i.e., inoculated milk with no antimicrobial agents. In the absence of LAB the osmolarity of milk did not show any significant elevation over time, even though incubation temperature was optimal for fermentation (Fig. 1). The small increments in osmolarity recorded

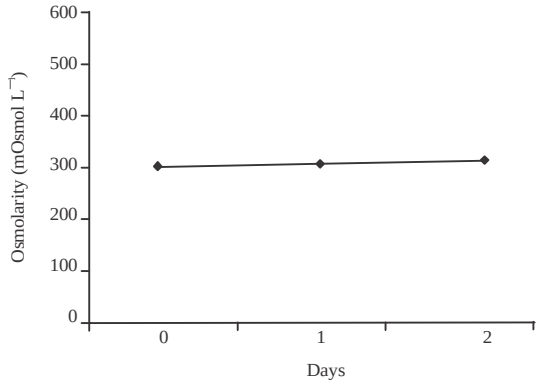

Fig. 1: Osmolarity of non-inoculated UHT milk during 2 days of fermentation at 30°C

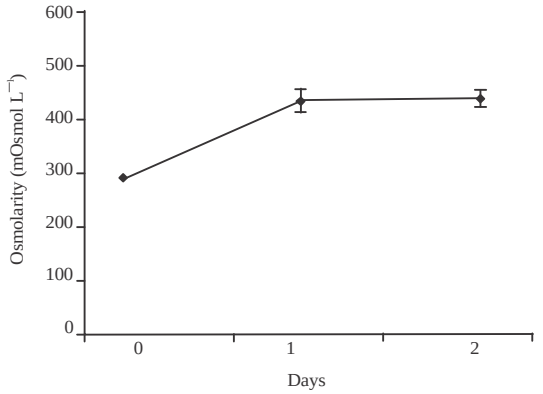

Fig. 2: Osmolarity of inoculated UHT milk during 2 days of fermentation at 30°C

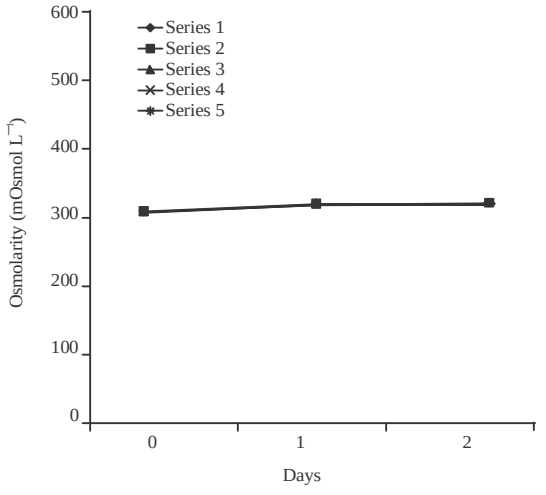

Fig. 3: Effect of erythromycin and trimethoprim LA on osmolarity of inoculated UHT milk during 2 days of fermentation at 30°C. Series 1-5 denote antimicrobial concentrations of 62.5, 125, 250, 500 and 1000 mg L^{-1}, respectively

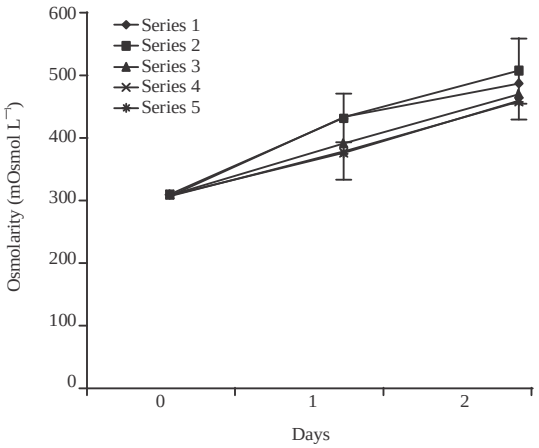

Fig. 4: Effect of cloxacillin benzathine DC on osmolarity of inoculated UHT milk during 2 days of fermentation at 30°C. Series 1-5 correspond to antimicrobial concentrations of 75, 150, 300, 600 and 1200 mg L^{-1}, respectively

over the 2 days of incubation denoted a background level of fermentation activity from natural deterioration of milk upon exposure to air and ideal temperature. In the presence of *Lactococcus lactis* sp. *lactis*, fermentation of lactose yielded water-soluble products, primarily lactic acid and a variety of small organic compounds which impart aesthetic flavors to fermented milk and milk products. The products of fermentation resulted in an increase in total concentration of solutes in the milk which was reflected as an increase in milk osmolarity (Fig. 2).

Since, the osmolarity of fermented milk is directly proportional to the degree of fermentation no significant elevation in the osmolarity of the milk occurs when fermentation is inhibited, as revealed with erythromycin-trimethoprim LA in Fig. 3. Clinically, the combination of erythromycin and trimethoprim (Megalon-M®) 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. 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 (Pieterse and Todorov, 2010). Thus, the 2 antimicrobial agents had an additive bacteriostatic effect on *Lactococcus lactis* sp. *lactis*. 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.

Cloxacillin benzathine DC (Orbenin-Extra®) is a bactericidal antimicrobial agent which prevents active multiplication of susceptible bacteria by inhibiting synthesis of the cell wall (Pierterse and Todorov, 2010). In veterinary practice it is used for the treatment of bovine mastitis caused by *Staphylococcus aureus* and *Streptococcus agalactiae* after drying off. Dry cow intra-mammary antimicrobial formulas are prepared in an oil base to ensure slow release and a prolonged therapeutic effect. Therefore, there was limited admixture of the antimicrobial agent with the aqueous phase of milk which contains the fermentation substrate lactose, particularly at lower concentrations (Fig. 4). As normal fermentation activity was recorded on the 2nd day on incubation, it is possible that some *Lactococcus lactis* sp. *lactis* variants have developed resistance to the drug since cloxacillin benzathine is one of the oldest antimicrobial agents used in the treatment of mastitis. This is in consonant with previous studies on fermentation of a mixture of colostrum and milk from cows treated for mastitis with antibiotics in which there were consistent fermentation patterns but with predominantly *Lactococcus lactis* (then known as *Streptococcus lactis*) and *Streptococcus faecalis* organisms (Loveland *et al*., 1983).

The prevalence of antimicrobial residues 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 (Aning *et al*., 2007; Shitandi and Sternesjo, 2001; Kurwijila *et al*., 2006). Not all dairy industries carry out routine tests for antimicrobial agents in all incoming milk, partly due to the high input costs of contemporary methods. Therefore, the need to monitor presence or absence of antimicrobial effects on fermentation of milk still exists. Osmometry provides a valid and affordable technique for evaluating the effects of therapeutic levels of intra-mammary antimicrobial agents on fermentation of milk.

CONCLUSION

In conclusion, osmometry is a valid tool for evaluating effects of antimicrobial agents on milk fermentation.

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