

The Effects of Changes in Sodium and Potassium Concentration on the Growth of Mastitogenic Bacteria In Vitro

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ABSTRACT

Mastitis is characterized by increased sodium and decreased potassium in milk, and the effect of this on bacterial growth was examined. Colonies of *Staphylococcus aureus*, *Streptococcus uberis*, and *Escherichia coli* were grown in tryptone broth containing 0–75 mM Na or 0–40 mM K. For *S. aureus*, the total viable count (TVC) growth rate decreased as Na increased from the normal level to that typical of mastitic milk. When Na was increased, time to peak TVC and lowest pH both increased, suggesting that the fatty acid utilization phase was retarded, and final pH decreased while the time to reach it increased, suggesting that the protein utilization phase was also retarded. For *E. coli* and *S. uberis*, there were less clear effects of varying Na or K. It is concluded that an increase in milk Na, such as normally occurs during a mastitic event, reduces the growth of one mastitogenic bacteria, *S. aureus*, but not *E. coli* or *S. uberis*.

INTRODUCTION

Normally, there is a stepwise gradient of declining sodium concentration from the epithelial cells of the mammary gland to the milk.¹ During a mastitic event, damage to the epithelial tissue, and, in particular, an increase in permeability of the ‘paracellular’ junctions allows Na to diffuse into the mammary gland and milk Na concentration increases.^{2,3} At the same time, in an attempt to achieve osmotic equilibrium, milk potassium concentration declines, proportionally less than Na,⁴ but still potentially depriving bacteria of an important nutrient.⁵

Little is known about the response of mastitogenic bacteria to these changes in the major cation concentrations. *Staphylococcus aureus* survives well under Na stress,⁶ but may experience reduced growth rates and increased cell turgor.⁷ It is possible that some mastitogenic bacteria species may be susceptible to osmotic damage caused by high Na concentrations. Although these have not been investigated in mastitogenic bacteria, some responses to undernutrition have been studied, for example, *Escherichia coli* increases unit length when nutritionally stressed.⁸

An experiment was conducted to investigate the response of *S. aureus*, *Streptococcus uberis*, and *E. coli* to changes in concentrations of Na and K ions typical of a mastitic event in a dairy cow.

MATERIALS AND METHODS

Experimental Method

Mastitogenic strains of *S. aureus*, *S. uberis*, and *E. coli* were obtained from a government veterinary center (Veterinary Investigation Centre, Bury St Edmunds, Suffolk, United Kingdom) and cultured under aerobic conditions for 18 hours at 37°C in 0.1 mL of 1% tryptone broth, diluted 1/10 with distilled water. Tryptone was used as the culture medium as it is a digest of the milk protein casein.

Samples of 0.1 mL of cultured tryptone broth were added to each of 6 universal bottles containing 10 mL of a 1% tryptone solution in distilled water. The first experiment investigated the effect of Na concentration, and analytical reagent grade NaCl was added to 5 of the bottles to create solutions at 15, 30, 45, 60, and 75 mM Na, thus representing the range of values previously determined for normal (15 mM Na) to severely mastitic milk (75 mM Na).^{4,9,10} The sixth bottle acted as a control. For the experiment investigating K concentrations, the same procedure was adopted, except that analytical grade KCl was added at concentrations of 50, 40, 30, 20, and 10 mM K. Both alkali metals represented a range of typical values from normal milk to severely mastitic milk. In both experiments, 3 samples were established at each concentration, 2 for total bacterial count (TBC) and total viable count (TVC) measurements and 1 for pH (determined with a pH meter, Hanna Instruments model 8417).

The TBC, TVC, and pH of the samples were measured after 4, 6.5, 24, 30, and 48 hours on duplicate samples. Total bacterial counts were determined using a phase microscope (Opiphot, Nikon, Japan) and Improved Neubauer counting chamber

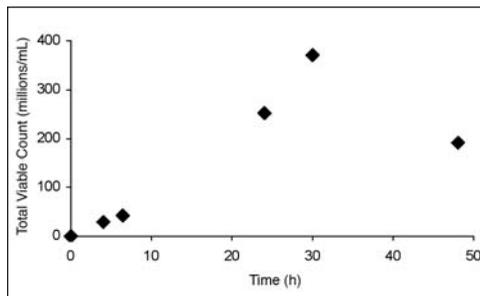


Figure 1. Typical growth curve for bacterial units—the response of *S. aureus* to a sodium concentration of 30 mM—approximating to a cubic curve.

(Weber Scientific International Ltd, United Kingdom), counting 16 squares per sample. Total viable counts were determined by culturing colonies on milk yeast extract agar (3.7% amino-nitrogen, 0.4% NaCl, 0.4% K, 0.43% total lipid concentration, pH 7.3; Oxoid Ltd, Basingstoke, United Kingdom). Samples (0.1 mL) were added to 9.9 mL phosphate buffered saline, progressively diluted by factors of 10 to increase precision of counting. Replicate 0.1 mL samples of each dilution were mixed with milk yeast extract agar and allowed to set on 90-mm Petri dishes. After inverting to prevent condensation on the agar surface, they were incubated for 18 hours at 37°C, following which the number of colonies was determined (Anderman Colony Counter, Kinston-on-Thames, United Kingdom).

S. uberis oscillates between single and paired forms and a less pathogenic chain form, which predominates when the culture medium is inadequate.¹¹⁻¹³ Therefore, during TBC analysis, it was recorded whether the bacteria were single, paired, or in the chain form, and the ratio of single and paired forms to the chain form was calculated.

Statistical Analyses

Initial exploration of lines of best fit determined that changes in the 3 parameters measured, TBC, TVC, and pH, were best described by a cubic curve, with the equation $y = at^3 + bt^2 + ct + d$, where y = the

Table 1. The Linear Relationships Between Sodium and Potassium Concentrations (\pm Standard Errors) and the Total Viable Count, Total Bacterial Count, and pH of *S. aureus*, *S. uberis*, and *E. coli* Cultures

<i>S. aureus</i>		
Sodium	r²	Probability
Time to TVC (h) = 29.5 (± 2.98) + 0.21 (± 0.068) Na conc. (mM)	0.50	0.01
Lowest pH = 6.9 (± 0.016) - 0.003 (± 0.0003) Na conc. (mM)	0.92	0.002
Time to lowest pH (h) = 11.1 (± 0.85) + 0.05 (± 0.019) Na conc. (mM)	0.54	0.06
Highest pH = 7.3 (± 0.059) - 0.004 (± 0.0013) Na conc. (mM)	0.63	0.04
Time to highest pH (h) = 37.4 (± 0.57) + 0.07 (± 0.012) Na conc. (mM)	0.84	0.006
Potassium		
Lowest pH = 7.1 (± 0.047) - 0.004 (± 0.0016) K conc. (mM)	0.53	0.06
<i>E. coli</i>		
Sodium	r²	Probability
Time to peak TBC (h) = 36.2 (± 2.02) + 0.10 (± 0.044) Na conc. (mM)	0.45	0.08
Time to lowest pH (h) = 12.3 (± 0.86) - 0.06 (± 0.019) Na conc. (mM)	0.62	0.04
Potassium		
Time to lowest pH (h) = 13.0 (± 0.24) - 0.03 (± 0.008) K conc. (mM)	0.64	0.03
<i>S. uberis</i>		
Potassium	r²	Probability
Time to peak TVC (h) = 9.8 (± 0.28) - 0.02 (± 0.009) K conc. (mM)	0.41	0.03
Time to highest pH (h) = 37.1 (± 1.93) + 0.13 (± 0.064) K conc. (mM)	0.40	0.10

population of bacterial units or pH, t = time and a , b , c , and d = constants (example in Figure 1). For TBC and TVC, the curve described a lag phase, followed by an exponential increase to a peak and subsequent decline during apoptosis. For pH, the curve described an initial decline, followed by an increase to a peak and subsequent decline. Differentiating the cubic equations for each treatment produced quadratic expressions ($dy/dt = 3at^2 + 2bt + c$). Peak and nadir populations were determined by substituting zero for the gradients, and solving the expression using the standard quadratic equation of $x = (-b \pm \sqrt{[b^2 - 4ac]}) / 2a$. Substituting the differentiated values into this quadratic equation, $t = (-2b \pm \sqrt{[4b^2 - 12ac]}) / 6a$, determined the peak value of t , or the nadir in the case of pH. By substituting these values into the cubic equation, the maximum number of bacterial units was calculated. Linear regression of peak populations and time to peak for the different Na/K treatments was conducted

using SAS software,¹⁴ where linear trends were detected; otherwise, results are presented in graph format. Growth rate was determined by dividing the peak number of bacterial units by the time taken to reach that peak. To determine treatment effects on the ratio of chains to single and double *S. uberis*, comparisons between control and all treatments with added Na or K were made by analysis of variance. Data for this comparison were normally distributed.

RESULTS

S. aureus

Sodium: Compared with no Na, the growth rate of TVC initially increased and then decreased as Na concentration increased (Figure 2). Therefore, assuming that Na concentration increases from approximately 15 to 45 mM as a result of mastitis,¹ TVC growth rate would be decreased by approximately one third by the simulated mastitis. Peak TVC was also greater at 15 mM (55×10^6 /mL) than at zero (26.5×10^6 /mL) or

Table 2. Comparison of the Ratios of the Number of Bacterial Units in Chains to the Combined Numbers of Both Single and Paired Units of *S. uberis*, Over Time, Observed During the TBC Counts

Treatment	Time (hours)					Standard Error	P-Value
	4	6.5	24	30	48		
Control	0.32	0.49	0.40	0.16	0.29	0.166	0.78
K	0.49	0.35	0.37	0.30	0.47	0.182	0.64
Na	0.28	0.49	0.62	0.45	0.22	0.118	0.04

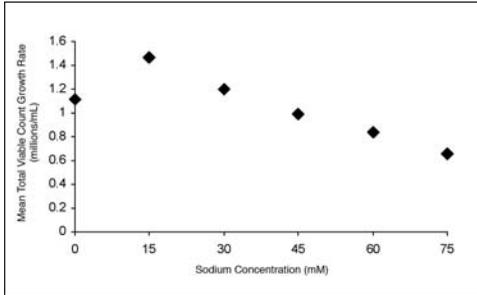


Figure 2. Effect of sodium concentration on the total viable count growth rate for *S. aureus*.

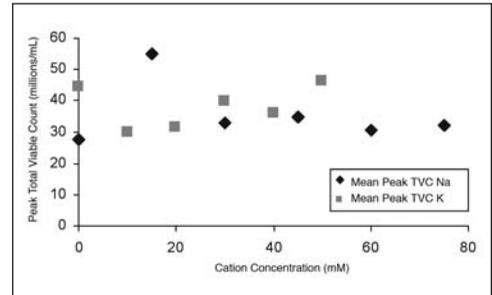


Figure 3. Effect of sodium and potassium on the mean peak total viable count (TVC) for *S. aureus*.

greater (mean $32 \times 10^6/\text{mL}$) Na concentrations (Figure 3), and the time to reach it increased with Na concentration (Table 1). There was a decrease in lowest TBC and highest pH and increase in the time taken to reach them with increasing Na concentration (Table 1).

Potassium: The response in TVC growth rate was the reverse of that observed for Na—growth rate slightly declined from 0 to 10 mM K, and then increased as K concentration increased (Figure 4). The influence of cation concentration was much less than Na. A reduction in peak TVC from 0 to $\times 10$ mM K was also evident (Figure 3). Changes in TBC were inconsistent (Figure 4). The lowest pH decreased with K concentration (Table 1).

E. coli

Sodium: The growth rate of TVC was greatest when no Na was added (Figure 5). The time to peak TBC tended to increase with Na concentration (Table 1), but the time to peak TVC increased initially to 15 mM Na, then declined with further increases in Na concentration (Figure 6). Changes in TBC were

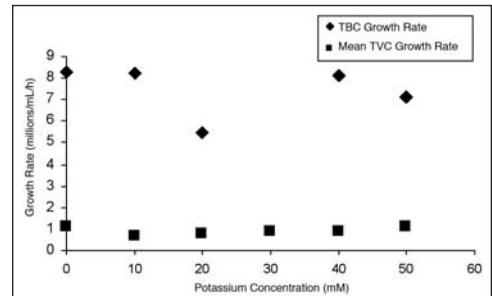


Figure 4. Effect of potassium concentration on the growth rate of total viable counts (TVC) and total bacterial counts (TBC) of *S. aureus*.

inconsistent. The time to lowest pH decreased with Na concentration (Table 1).

Potassium: The peak TVC (Figure 7) and TVC growth rate (Figure 8) were greatest when no K was added, but the time to peak TVC increased to 20 mM K and then declined (Figure 6). Changes in TBC were inconsistent.

S. uberis

Sodium: Changes in TBC were inconsistent, but there was a small increase in the time to peak TBC up to 45–60 mM Na and then a reduction (Figure 9). There were no

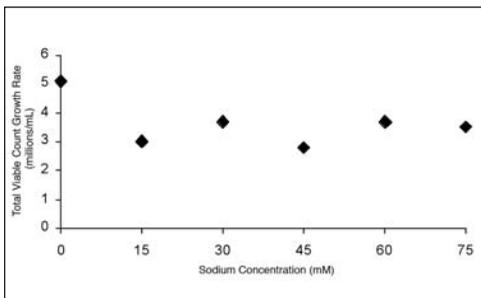


Figure 5. Effect of changes in sodium concentration on the total viable count growth rate of *E. coli*.

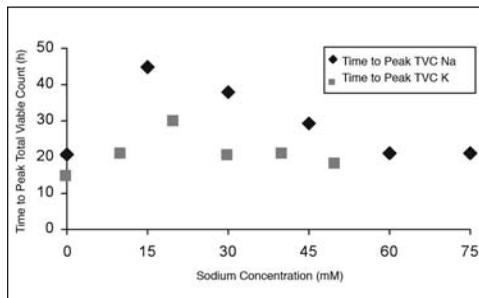


Figure 6. Effect of sodium and potassium concentration on mean time to peak total viable count (TVC) for *E. coli*.

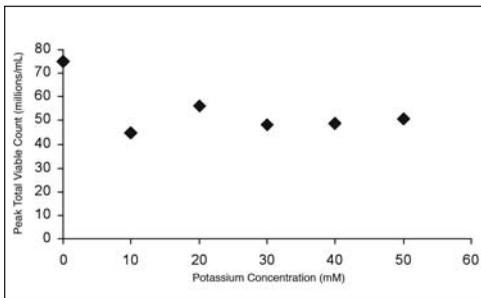


Figure 7. Effect of potassium concentration on the mean peak total viable count for *E. coli*.

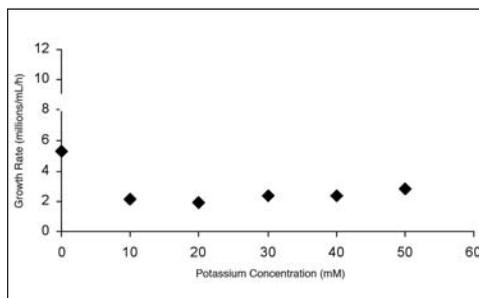


Figure 8. Effect of potassium concentration on the mean total viable count growth rate of *E. coli*.

consistent changes in TVC or pH.

Potassium: There was a small increase in the time to peak TBC with K (Figure 9), however; the time to peak TVC declined with increasing K concentration (Table 1). There was a tendency for the time to highest pH to increase with K concentration (Table 1).

In the Na treatments, there was an increase in the proportion of bacterial units in chains up to 24 hours, then a decline to 48 hours (Table 2). This was not evident for the control or K treatments.

DISCUSSION

The 3 species responded differently to the challenge of varying Na and K in their growth media. Significant effects were recorded on the growth patterns of *S. aureus* to increased Na concentration in the media. These effects were not observed on recorded TBC, which are a less accurate measure as they include both dead individuals and, depending on the skill of the observer, non-

living debris. The TVC is a more sensitive measure of the living and viable numbers in a population than the TBC.¹⁵ The experiment indicated that while Na has no effect on the ultimate potential population of *S. aureus* in a milk-derived growth medium, the rate of growth in that medium in both the acid-product metabolism phase and the base-product metabolism phase was impaired by increasing Na concentration. Because extra-cellular Na⁺ concentration increases the Na-K gradient that the Na pump must overcome, the energy requirement of the cell could be increased sufficiently to retard growth rate. A similar response to *S. aureus* species has been observed previously in ultra-high temperature milk.¹⁶ Furthermore, species known to remain viable at high concentrations of NaCl, such as *Clostridium botulinum*, still exhibit reduced growth rates with increasing NaCl concentrations.¹⁷ It is possible that strategies adopted by halotolerant bacteria to remain viable in very high

NaCl conditions, such as adopting a larger cell size⁸ and reducing the rate of cell division,⁷ may also inhibit growth rates at reduced concentrations of NaCl, while other species without this osmotic defense mechanism show less affected rates of growth.

The increase of Na and the decrease in K in the mammary gland following *S. aureus* challenge will therefore reduce the potency of that challenge. The release of Na⁺ ions from inflamed tissue may be a response that inhibits the proliferation of infective agents in other areas of the body. Previously, it has been speculated that the effect of Na in reducing milk somatic cell counts may derive from improved magnesium status.¹⁸ Sodium reduces the K inhibition of magnesium absorption in cattle¹⁹ and magnesium has an important role in the immune system.²⁰ However, the effect of Na on growth of some mastitogenic bacteria observed in this experiment suggests that the observed reduction in somatic cell count when Na status of dairy cows is enhanced may be a direct effect rather than via magnesium.

Evidence from the *E. coli* cultures was less clear than that from the *S. aureus* cultures and followed the opposite pattern. Since both time to peak acidity and time to reach peak TBC were both reduced with increasing Na, Na appears to have a beneficial effect on *E. coli* growth rates in milk-derived culture media, at least in the acid-product energy metabolism phase. These results were repeated in K-supplemented media, where again time to reach peak acidity was reduced with increasing concentration of the cation, suggesting a common Na/K effect on promoting acid-product metabolism in *E. coli*.

In the Na cultures, there was an increasing proportion of *S. uberis* chains up to about 24 hours, followed by a decrease, whereas there was no discernible pattern in K-enriched media or the control. This suggests that while the potential for growth of the population of *S. uberis* in a milk-derived medium is unaffected by the addition of Na to that medium, its potential for pathogenicity is significantly reduced.

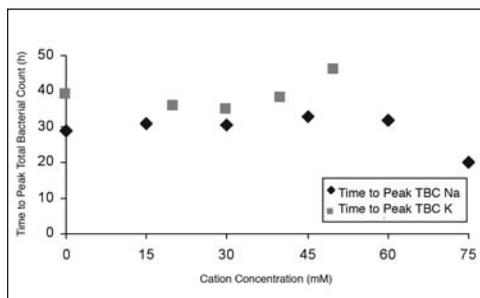


Figure 9. Effect of sodium and potassium concentration on time to peak total bacterial count (TBC) of *S. uberis*.

CONCLUSIONS

Evidence from 3 different measured parameters suggested that growth rate is inhibited by increasing the concentration of Na. However, *E. coli* populations exhibited higher growth rates in the presence of additional Na or K, and there was no effect on growth rates of *S. uberis*, although pathogenicity may be reduced with supplementary Na. The implications are that excretion of excess Na into the mammary gland during mastitis can reduce the potential of infection from *S. aureus* and may reduce the pathogenicity of *S. uberis*, while the effect on *E. coli* may be to increase its potential for growth. Since somatic cell counts are increased during *S. aureus* mastitis infection, this is a credible mechanism for the reductions in somatic cell counts recorded in animals supplemented with NaCl.

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