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Possible labile inhibition of the growth of *Streptococcus uberis* in milk from cows free from mastitis

By KIRSTY E. KLIEM* and J. ERIC HILLERTON
Institute for Animal Health, Newbury, Berkshire, RG20 7NN, UK

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Summary. Milk from dairy cows never known to have had an intramammary infection with *Streptococcus uberis* can inhibit growth of *Str. uberis* for up to 7 h. This inhibition is abolished if milk is heated to 80 °C. Inhibition appears not to be related to immune defences as it occurs in skimmed milk (cell free), is unrelated to the concentration of immunoglobulin and survives heating to 56 °C. The effect is partly overcome by addition of selected amino acids and vitamins. It is suggested that the inhibition is caused by a restriction in the supply of essential nutrients part of which may require the conversion of plasminogen to plasmin.

Keywords: *Streptococcus uberis*, milk, mastitis, cow.

The most successful means of mastitis control in the dairy cow is prevention and yet bacterial invasion of the mammary gland is still extremely common. There are several levels of resistance to this. These include physical barriers such as the teat duct whose role has been partly described (Lacy-Hulbert & Hillerton, 1995). Bacterial survival after invasion depends on resisting immune defences and on growing fast enough to survive being milked out. That mastitis occurs shows this is achievable. It is obviously beneficial for a potential pathogen to grow in milk, yet one successful organism, *Streptococcus uberis*, grows poorly in milk unless it can obtain essential nutrients, especially amino acids (Leigh, 1999). Most of the experiments to describe its growth have been conducted in laboratory media or *in vivo*. The ability of the bacteria to grow in milk *in vitro* is poorly understood.

Routine monitoring of udder health of Institute for Animal Health (IAH) cows reveals that a number of cows appear never to have suffered any mastitis, shown by no isolation of bacteria in quarter milk or a raised monthly cell count, at any time. It seems unlikely that bacteria never penetrate the mammary gland, hence any 'resistance' must be at a different level. Leigh (1999) showed the potential for preventing mastitis by preventing growth of *Str. uberis*. Therefore, it was decided to examine milk from cows that had never had a clinical infection due to *Str. uberis* to determine its ability to sustain bacterial growth and to determine any ability to inhibit or restrict growth of *Str. uberis*.

The approach was to analyse the ability of milk, in the absence of leucocytes, to sustain growth and then to determine whether any inhibition was related to known inhibitory mechanisms such as complement-neutralizing antibody, the lacto-peroxidase system or any shortage of essential nutrients.

* For correspondence; e-mail: kirsty.kliem@bbsrc.ac.uk

materials and methods

Preparation of milks

Nine cows were selected from the IAH dairy herds. These were cows that had never had a recognized intramammary infection by *Str. uberis* and were free of any intramammary infection.

Milk samples were taken from the right fore quarter of each cow after it had been shown that the effect seen after heat treatment was similar for all four quarters (Kliem & Hillerton, 2000). Aliquots were spread on aesculin blood agar and incubated at 37 °C for 48 h to confirm that the milks were free of bacteria. 'Clean' milks, stored at 4 °C, were then skimmed by centrifugation at 1800 ***g*** at 4 °C for 15 min. Each batch of skimmed milk was cell-counted by the Fossomatic method to show that it was free of leucocytes. Skimmed milks were stored frozen at −20 °C in aliquots.

Heat treatment of skimmed milks was by holding them in a waterbath at 80 °C for 4 min (referred to later as heat-treated) as this temperature/time combination abolishes enzymic action, or at 56 °C for 30 min, as this is known to denature complement. These milks were then cooled to room temperature before inoculation of bacteria.

Bacterial growth

Fresh aliquots of *Str. uberis* strain 0140J from a single supply stored at −20 °C in Todd Hewitt Broth (THB) containing 25% (v/v) glycerol, were used to determine the ability of milks to sustain growth. Alternative bacteria used were *Str. uberis* EF20 and C197C, *Escherichia coli* and *Str. dysgalactiae* A17. All isolates were obtained from clinical cases of bovine mastitis of lactating cows in the UK.

Bacteria were grown overnight at 37 °C in THB then 1 ml was centrifuged at 5000 ***g*** for 5 min to recover a bacterial pellet. The pellet was washed three times in phosphate-buffered saline (PBS). A preparation of approximately 2×10^4 cfu/ml was made. Bacteria were inoculated into 5 ml skimmed milk, making a bacterial concentration at time 0 of approximately 10^3 cfu/ml. Milks were incubated at 37 °C. Duplicate 50 μl samples were taken at 0, 1·5, 3, 4, 5, 6, 7 and 24 h, spread on aesculin blood agar plates and incubated at 37 °C for 24 h to determine bacterial concentration. Results were analysed statistically, where appropriate, by comparing the growth difference between 0 and 7 h for each curve, and then using a paired Student's *t* test (MINITAB, 1998).

Measurement of specific antibody by ELISA

Skimmed milk samples from different cows were analysed by ELISA for antibody to cellular extract plus cell walls of *Str. uberis* after Leigh & Field (1994). *Str. uberis* were grown up overnight in 4000 ml THB at 37 °C. The broth was then centrifuged at 5000 ***g*** for 15 min and a bacterial pellet was prepared by repeated washing with PBS centrifugation. A cellular extract was prepared from the concentrated bacterial pellet using a cell disrupter (''X-Press''). The resulting extract contained cell walls and cell contents. This extract was aliquoted in 100-μl amounts and stored at −20 °C, to be used as the antigen. Antigen was thawed and diluted 10000-fold in carbonate coating buffer (0·05 M, pH 9·6). To each well of a microtitre plate, 100 μl diluted antigen was added before being kept at 4 °C overnight. Plates were emptied, 200 μl blocking agent (3% w/v 'Marvel' dried skimmed-milk powder [Premier Brands, Merseyside, UK] in ELISA buffer [PBS containing 0·5 ml/l Tween 20) added

to each well, and left for 30 min at room temperature. After washing with ELISA buffer, pooled bovine serum (1/500 dilution with ELISA buffer) was added to the first three columns (therefore in triplicate) to act as a positive control. This was double-diluted down the plate with ELISA buffer. To the rest of the plate, test samples were added in triplicate. The plate was then left for 2 h at room temperature. After washing, 100 μl conjugate (horseradish peroxidase conjugated rabbit anti-bovine IgG at a concentration of 1/50000) was added to each well. A response was developed by adding 100 μl tetra-methyl benzidine (TMB) solution (1 : 10 : 1000, 30 % H_2O_2 : 42 mM-TMB in dimethyl sulphoxide : 0·1 M-sodium acetate buffer adjusted to pH 6·0 with 0·1 M-citric acid). Concentrated H_2SO_4 was used to stop the reaction, and the response was expressed as absorbance at 450 nm. An arbitrary standard curve was prepared. Results were analysed by comparing the value obtained with the 'inhibition' value for each sample (growth at 7 h of the heat-treated milk minus growth at 7 h of the non heat-treated milk) and finding Pearson's correlation coefficient (MINITAB, 1998).

Measurement of lactoperoxidase (*LP*) *activity*

A modification of the method of Marshall *et al.* (1986) was used. Skimmed milk samples were diluted 1 : 4 with acetate buffer, and 3 μl added to wells in a microtitre plate, in triplicate, then 294 μl 1 mM-2,2-Azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) in acetate buffer was added. Absorbance was read at 405 nm. Next, 3 μl 10 mM-H_2O_2 (in acetate buffer) was added and mixed. After 5 min the absorbance was read and LP activity calculated using the principle that LP activity (U/ml milk) is equal to change in absorbance per min/32·4 × 400 (dilution factor). Results were analysed as were the IgG levels (Pearson's correlation coefficient).

Added nutrients

Nutrients essential for growth of *Str. uberis* in chemically defined medium (CDM) (Leigh & Field, 1991) were investigated. These were specific amounts of the 21 amino acids, 8 vitamins and ionic magnesium, iron and manganese. Individual solutions were made up as per Leigh & Field (1991) (amino acid solution, vitamin solution, and mineral solution). The amino acid solution was made up at 2·5 × CDM concentration, and was added to 5 ml skimmed milk so that the concentration of each amino acid in the milk was 50 % that of CDM. The same principle was followed with the other solutions, so that each constituent was 50 % of its concentration in CDM. Milk was then adjusted to pH 7·6 to compensate for any pH fluctuations after the addition of the nutrients.

RESULTS AND DISCUSSION

Rate of growth of *Str. uberis* 0140J has been determined numerous times in skimmed milk prepared from a number of different cows. A slight variation between cows and within milk samples in the precise rate of growth was usual. An apparent slower rate of growth occurred in skimmed milk from some cows, usually for at least 4 h, and this slow growth phase lasted for as long as 7 h (Fig. 1). Rate of growth between 0 and 7 h, for any single milk sample showing a slow rate of growth, was significantly greater ($P < 0{\cdot}001$) when the skimmed milk had been heated to 80 °C compared with no treatment (Fig. 1). This suggests some inhibition of growth in the unheated milks. When milks were heated to 56 °C for 30 min prior to inoculation of bacteria then there was no significant effect ($P = 1{\cdot}000$) on the growth curve (Fig. 2).

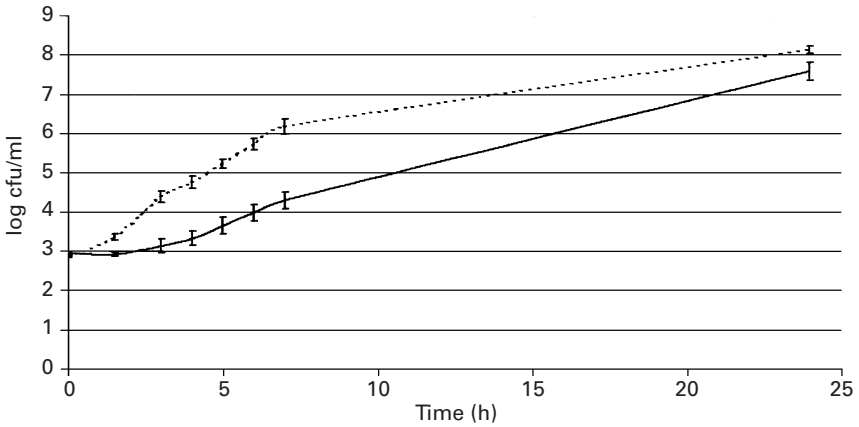

Fig. 1. Growth (mean ± SEM) of *Streptococcus uberis* in skimmed milk (solid line) or skimmed milk heated to 80 °C (broken line) from cows ($n = 9$) believed never to have had an intramammary infection.

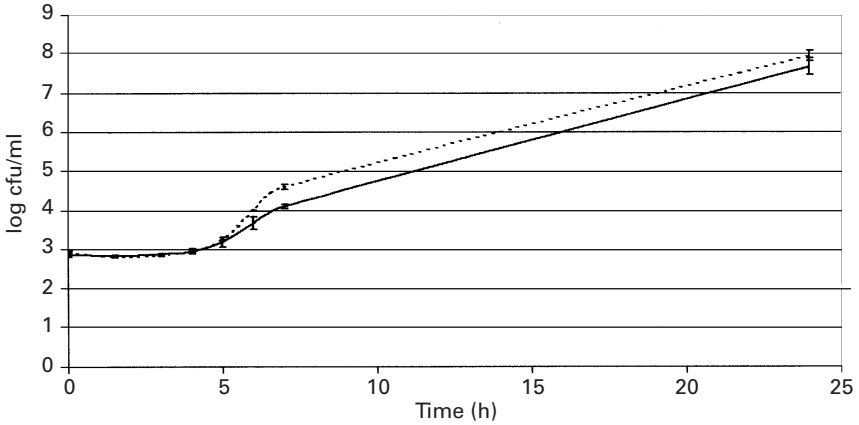

Fig. 2. Growth (mean ± SEM) of *Streptococcus uberis* in skimmed milk (solid line) or skimmed milk heated to 56 °C (dotted line) from cows ($n = 2$) believed never to have had an intramammary infection.

Heat inactivation of the inhibitory effect suggests that it may be protein-related but is not complement-related.

The extent of the inhibitory effect varied slightly with the strain of *Str. uberis* used (Fig. 3). This is similar to the variation in rate of growth of these strains in THB. There was complete abolition of the inhibitory activity against all strains when the milk had been previously heated to 80 °C. When other mastitis pathogens were grown in skimmed milk, there was also a heat-labile inhibition of growth in unheated milk. With *Esch. coli* there was less inhibition of growth than with *Str. uberis* (Fig. 4). With *Str. dysgalactiae* there was only a 100-fold difference in growth between heat-treated and unheated milk, than the more usual 1000-fold effect found for *Str. uberis*.

Inhibitory milks from nine different cows were assayed to determine the concentration of *Str. uberis*-specific IgG. Resulting IgG concentrations were compared with the degree of inhibition found for each milk sample. The difference in the amount of bacterial growth over 7 h was compared with the concentration of immunoglobulin. There was no significant correlation ($P = 0{\cdot}55$) (Table 1). It is unlikely that inhibition of bacterial growth is due to IgG concentration alone.

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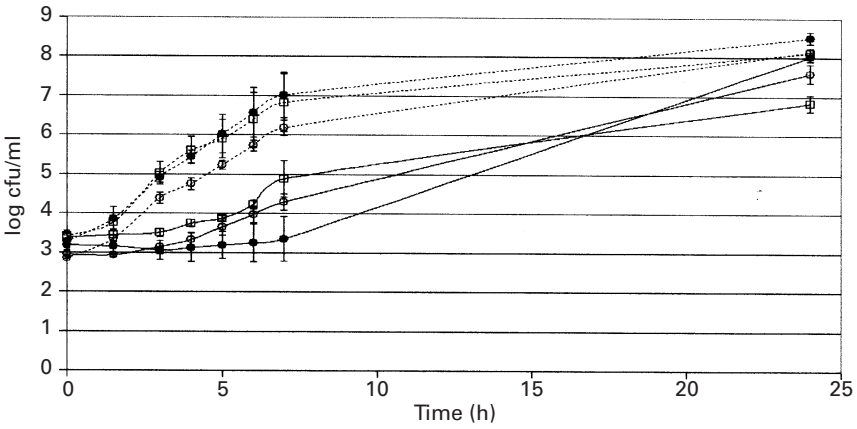

Fig. 3. Growth (mean ± SEM) of three different strains of *Streptococcus uberis* (○ – 0140J [$n = 9$], ● – EF20 [$n = 3$], □ – C197C [$n = 3$]) in skimmed milk (solid line) and heat-treated skimmed milk (broken line) from one cow.

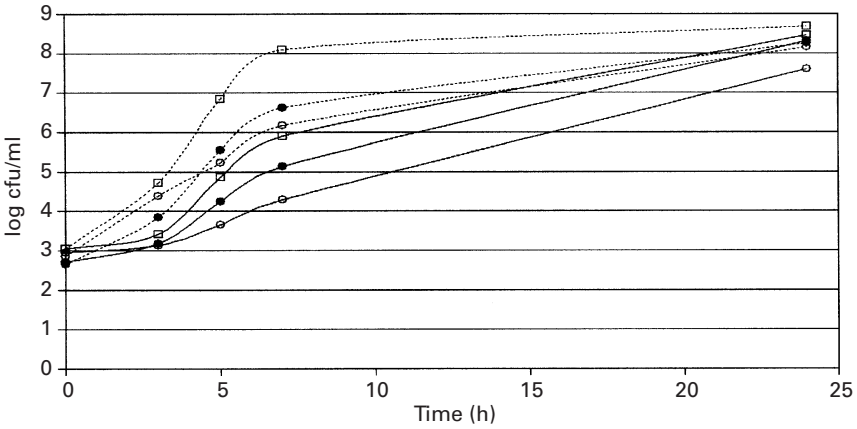

Fig. 4. Growth of three different species of mastitis-causing bacteria (○ – *Streptococcus uberis* 0140J, ● – *Streptococcus dysgalactiae* A17, □ – *Escherichia coli* from a clinical case) in skimmed milk (solid line) and heat-treated skimmed milk (broken line) from one cow.

Table 1. *The relationship between degree of inhibition of bacterial growth at* 7 *h, IgG concentration and lactoperoxidase (LP) activity in skim milk from nine cows*

Cow No.	Degree of inhibition of growth at 7 h	IgG conc. (U/ml milk)	LP activity (U/ml milk)
5392	0·9	81	0·4
5138	1·3	80	1·0
5324	1·3	102	0·5
5287	1·7	79	0·5
4397	2·0	83	0·8
6092	2·3	54	0·7
5387	2·4	101	0·5
6098	2·4	72	0·5
5326	2·8	81	0·3

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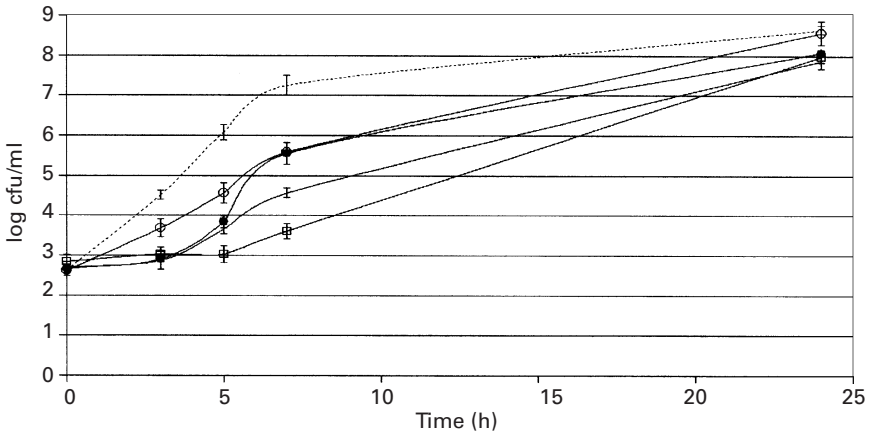

Fig. 5. Growth (mean ± SEM) of *Streptococcus uberis* in skimmed milk (solid line) and heat-treated skimmed milk (broken line) from one cow, with the addition of ○ – amino acids ($n = 4$), ● – vitamins ($n = 4$) and □ – minerals ($n = 2$).

LP activity in milks was comparable to that reported by Marshall *et al.* (1986). LP activities showed no significant correlation ($P = 0{\cdot}52$) with degree of growth inhibition by the milks (Table 1). This suggests that perhaps LP does not cause the inhibition.

Addition of various CDM nutrients to skimmed milk produced variable effects on bacterial growth but always produced a rate of growth lower than in the heated milk control (Fig. 5). When rate of bacterial growth in skimmed milk from one cow was measured it became clear that there was no significant ($P = 0{\cdot}125$) reversal of growth inhibition over 7 h when the milk was supplemented with the CDM minerals (Fig. 5). When skimmed milk was supplemented with vitamins, exponential growth started sooner and there was a partial but significant ($P = 0{\cdot}017$) reversal of inhibition after 7 h (Fig. 5). A significant reversal ($P = 0{\cdot}03$), but still partial, of the inhibition of the initial growth rate of bacteria in skimmed milk also occurred when key amino acids were added. However, when the growth rates after 5 h were compared it was apparent that the addition of amino acids had a significant effect ($P = 0{\cdot}008$) but that the addition of vitamins did not ($P = 0{\cdot}515$).

The impact of several endogenous antibacterial mechanisms on the keeping quality of milk has been described (Barrett *et al.* 1999). Recently, the limited ability of *Str. uberis* to grow unless essential nutrients, especially amino acids, are supplied has been described (Leigh, 1993). Until now the ability of *Str. uberis* to grow in milk, as influenced by any antibacterial mechanism and the possible variation between milks, has not been described. The observation that a proportion of cows may never suffer intramammary infection may be explained by any of a number of limiting factors. This report shows that natural limitation of bacterial growth in milk is one, possibly significant, factor.

Large differences in initial rate of growth of *Str. uberis* in untreated and heat-treated skimmed milk, with a difference in effect with the temperature used, indicate a labile inhibitory mechanism. Inhibition is usually overcome after 4–7 h suggesting that alternative growth mechanisms can be induced in *Str. uberis*, or that the inhibition is overcome by limited growth.

It is possible that the inhibitory agent(s) directly inhibit or kill bacteria. Several possible mechanisms have been investigated and shown to have little or no influence, including complement and immunoglobulins.

Removal of growth inhibition by heating milk to 80 °C is suggestive of an inhibitory protein that is denatured. Enzymic inhibitors occur in milk. The LP system is active against Gram-negative bacteria (Bjorck *et al.* 1975) and *Str. agalactiae* (Brown & Mickelson, 1979). However, it appears not to be involved, as there was no correlation of LP activity with degree of inhibition.

Other antimicrobial enzymes in milk include lactoferrin and lysozyme. Streptococci are more resistant to lactoferrin action than are other organisms such as Gram-negative bacteria (Rainard, 1987) because of their low iron requirements. It has been shown that increasing concentrations of lactoferrin do not appear to play an important role in inhibiting growth of *Str. uberis* (Fang *et al.* 1998). Lysozyme concentration of bovine milk is low (average 13 μg/100 ml; Reiter, 1978) compared with that of milk from other species (the average in human milk is 39 mg/100 ml; Reiter, 1978). Therefore it seems unlikely that lysozyme is the protective heat-labile protein present in cows that have never had infections.

Given that Leigh (1993) showed that *Str. uberis* requires certain essential nutrients for growth, the effect of these on growth in milk was examined. Kitt (1998) showed that 11 amino acids, five out of the eight vitamins present in the CDM and three minerals are essential for the growth of *Str. uberis*. It might be that the heat-labile factor is involved in the acquisition and/or uptake of these selected nutrients. Heat treatment might also induce breakdown of complex peptides into smaller peptides and even individual amino acids, without involving enzymes. Therefore the mere increase in available amino acids might be the reason for increased initial growth rate in heat-treated milk.

Mineral supplementation had no effect on growth suggesting that this is not the restriction in skimmed milk. After addition of vitamins to skimmed milk, the exponential phase of growth commenced slightly earlier, and after addition of amino acids, the exponential growth phase started at the same time as in heat-treated milk. Less of a lag phase was needed. However, neither supplementation allowed growth as fast as heat treatment. It may be that the effects are different because the vitamins allowed growth but there was still a lag phase, whilst slower growth occurred after addition of amino acids. It is possible that *Str. uberis* in some skimmed milks is unable to release or take up amino acids.

Limited heat treatment is known not to inactivate plasmin; pasteurization has been found to increase plasmin activity (Richardson, 1983). As plasmin action is the primary method of peptide release in milk, perhaps heat-treating the milk denatures a protein associated with control of the plasminogen/plasmin system. Plasminogen activators and activator inhibitors are present in milk, and several endogenous proteins are known to affect plasmin activity (Politis *et al.* 1990; Fattal *et al.* 1992). Given the proposed role of plasminogen activation in pathogenesis of *Str. uberis* mastitis (Leigh, 1993) presence of a proteinaceous inhibitor in milk is one hypothesis.

Some heat-labile factor, variable between cows, affects the rate of growth of *Str. uberis* in milk and presumably its establishment in the bovine mammary gland. Enough of this factor to delay bacterial growth for several hours may affect the incidence of new *Str. uberis* infections and hence clinical mastitis. Identification of the factor will allow cows with some natural resistance to one form of mastitis to be selected.

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