

Growth Characteristics of *Staphylococcus aureus* and *Escherichia coli* in Whey from Sequentially Infected Milk

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Mattila-Sandholm, T., T. Alivehmas, L. Kaartinen and T. Honkanen-Buzalski: Growth characteristics of *Staphylococcus aureus* and *Escherichia coli* in whey from sequentially infected milk. *Acta vet. scand.* 1990, 31, 169–174. – The growth of *Staphylococcus aureus* and *Escherichia coli* was followed in bovine whey samples which had been prepared from milk previously incubated with cultures of *S. aureus* or *E. coli*. Staphylococcal strains were divided into 2 groups according to their ability to form compact or diffuse colonies on serum soft agar, which is related to the absence or presence of capsule respectively. The growth of compact staphylococci was dependent on the bulk tank milk used whereas diffuse colony forming staphylococci grew equally well in all bulk milk, also in all inoculated milk. The growth of *E. coli* was markedly enhanced in whey samples prepared from milk preincubated with staphylococci. However, clear growth inhibition was seen with *E. coli* and *S. aureus* strains when grown in whey prepared from milk preincubated with *E. coli*. Results indicate that the growth promotion of pathogens due to compositional changes in milk are of importance during the course of infection because the growth pattern on staphylococci is dependent on these compositional changes. The growth-inhibitory effects caused by *E. coli* may explain difficulties in isolating this organism.

bacterial growth; mastitis; capsule.

Introduction

Studies concerning bacterial interactions in milk have shown that various micro-organisms can antagonize and inhibit the growth of others (Corbeil *et al.* 1985, Hogan *et al.* 1987, Woodward *et al.* 1987). This may explain the role of bacterial interaction in the course of udder infection. Mastitis pathogens are usually isolated as monocultures rather than mixed infections. Some organisms are able to inhibit the growth of others owing to competitive growth. Different inhibitory metabolites, including long-chain fatty acids, produced by patho-

gens and nonpathogens have been discussed by Hogan *et al.* (1987). Inhibitory effects have mainly been studied between pathogens and non-pathogens whereas the growth responses within various pathogenic species have received little attention (Woodward *et al.* 1987).

Other studies have shown, however, that infection and inflammation in the mammary gland lead to compositional changes in milk which enhance the growth of pathogens, although all the antibacterial factors increase (Reiter 1978, Mattila *et al.* 1984a & b, Malkamäki *et al.* 1986). Mastitic pathogens

must overcome various antibacterial factors present in milk and under adverse conditions the nutritive quality of the growth medium becomes important. Products of casein hydrolysis and heme compounds are probably the key nutrients for bacteria which are enriched in milk (Kaartinen & Sandholm 1987, Mattila et al. 1984a & b). The rise in all the antibacterial factors in milk cannot tilt the host-microbe balance in favour of the host because of increased replication rates of mastitic pathogens. Therefore the growth promotion of pathogens due to bacterial resistance and compositional changes in milk are of key importance during the course of infection and also in the infection process (Mattila et al. 1984a).

The diffuse and compact colony appearance of staphylococci has been thought to indicate capsule formation and also glycocalyx formation in vivo in the former type (Yoshida 1971, Caputy & Costerton 1982, Opdebeek et al. 1987). The bacterial capsule material and glycocalyx are known to give protection against many endogenous antibacterial factors (Christensen et al. 1982, Marrie & Costerton 1981).

The purpose of this study was to determine how the growth of one pathogenic organism in milk may affect the growth of the consecutive pathogens using compact and diffuse colony forming strains of *Staphylococcus aureus* and a single strain of *Escherichia coli*. Compact and diffuse strains of staphylococci were studied to observe the effect of the capsule.

Material and methods

Test organisms

Five strains of *S. aureus* (QLD 21, 22, 23, 24, 26) capable of forming diffuse colonies on serum soft agar and 4 strains of *S. aureus* (QLD 5, 6, 7, 9) forming compact colonies

on serum soft agar were selected for the growth experiments (Yoshida 1971). The bacteria had been isolated from clinical cases of mastitis and were kindly given to us by Dr A. Frost, University of Queensland, Australia. *E. coli* (strain EKK 64) was isolated from a cow having acute mastitis.

Quarter milk samples

Quarter milk samples from a healthy Ayrshire cow was used. The quarter milk was checked for 3 d for somatic cell count (SCC) and presence of bacteria before collection. The SCC was < 200,000/ml and bacteria were absent from samples used.

Bulk milk

Three bulk milk samples were obtained at separate time intervals from a farm with 25 Ayrshire cows having a good udder health records. The SCC of the bulk tank milk ranged from 150,000 to 200,000/ml.

In vitro inoculation of milk

Quarter milk samples and the bulk milk were inoculated and preincubated with bacteria as follows: fresh overnight bovine blood agar cultures of test bacteria were suspended in physiological saline to yield a suspension of 10^7 cfu/ml. Milk was divided into 9 ml portions and 1 ml bacterial suspension was added to each aliquot. The milk samples were then incubated at 37°C in a water bath for 4 h under continuous shaking.

Whey preparation from inoculated milk

After defatting of the preincubated milk samples, whey was prepared by centrifuging at 45,000 G for 60 min at 4°C. Whey samples were sterile-filtered (0.45 µm, Millipore) before analysing their quality as growth media for *E. coli* and *S. aureus*.

Bacterial growth in whey

Bacterial suspensions were calibrated with Multiskan microplate reader (Eflab Oy, Helsinki, Finland) to give an initial turbidity of 0.10 before inoculation (corresponds to 10^7 cfu/ml). Bacterial growth was followed by an automated growth analyser based on vertical turbidometry (Bioscreen, Labsystems Oy, Finland). The analyser used Honeycomb microplates consisting of 100 wells each and 2 plates could be included in 1 incubation run. Each well consisted of 250 μ l of the sterile-filtered whey and 50 μ l of bacterial suspension as inoculum. The control wells included the whey sample prepared from milk which had not been preincubated with bacteria. Bacterial growth was monitored every 10 min for 48 h at 37°C. The data were stored in a computer and growth curves were analysed using a special program (Bioscr-100). The area under the growth curve (AUC) within incubation of 40 h (the mean time-point for the stationary phase in all experiments) was chosen as the parameter to compare bacterial growth responses in the whey samples from milk with various preincubations. The area under the growth curve indicates both the slope and maximum turbidity of the bacterial growth curve. The experiments were performed in parallel.

Results

Growth responses of *S. aureus* in bulk milk (Table 1) showed that the growth of compact strains of staphylococci was enhanced in whey samples which had been prepared from milk inoculated with compact colony forming staphylococci. The growth of diffuse strains of staphylococci, however, was not enhanced after preincubation with staphylococci or *E. coli*. Bacterial growth responses were similar in the quarter milk samples. The growth pattern of compact and diffuse colony forming staphylococci showed slight

Table 1. Growth of strains of *S. aureus* with compact and diffuse colonies in whey prepared from bulk milk inoculated with staphylococci or *E. coli*.

	Mean area under the growth curve (AUC)	
milk inoculated with	<i>S. aureus</i> compact	<i>S. aureus</i> diffuse
<i>S. aureus</i> compact	7.1 \pm 1.0* (n=46)	5.9 \pm 0.8 (n=80)
<i>S. aureus</i> diffuse	5.9 \pm 0.5 (n=58)	4.9 \pm 0.3 (n=64)
<i>E. coli</i>	4.4 \pm 0.5 (n=28)	4.2 \pm 0.3 (n=38)
control (bulk milk 1, 2, 3)	5.9 \pm 0.4 (n=28)	5.4 \pm 0.4 (n=38)
control (bulk milk 1)	1.2 \pm 0.6 (n=10)	7.0 \pm 3.4 (n=12)

* S.E.M. = standard error of mean.

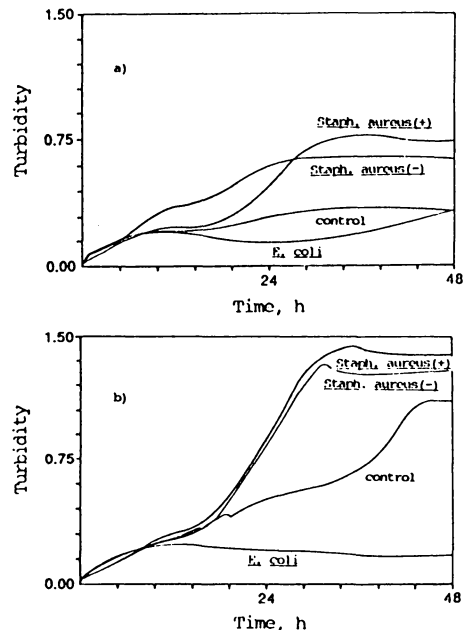


Figure 1. Growth of *E. coli* in whey prepared from milk samples preincubated with *S. aureus* or *E. coli*. Control milk was incubated without bacteria. (+) = *S. aureus* forming diffuse colonies, (-) = *S. aureus* forming compact colonies. a) Bulk milk. b) Quarter milk.

growth inhibition in milk samples preincubated with *E. coli* (Table 1).

The growth of *E. coli* (Fig. 1) was greatly enhanced in whey samples prepared from milk preincubated with staphylococci. However, the growth of *E. coli* in whey samples prepared from milk preincubated with *E. coli* was markedly inhibited to levels below those registered in the control whey samples. The coefficient of variation varied from 5–10% between the curves, which indicates that the differences were significant.

Discussion

Inflammation of the mammary gland causes compositional changes in milk which enhances the growth of mastitis pathogens (Mattila et al. 1984a & b, Malkamäki et al. 1986). This may reflect improved nutritional quality rather than suppressed antibacterial activity. Previous work has shown that the 2 groups of *S. aureus* (compact and diffuse) differ in their ability to grow in mastitic and normal milk (Mattila & Frost 1988). Those results showed that the growth of compact colony forming staphylococci was significantly enhanced in mastitic whey samples prepared from clinical cases of *S. aureus* and *E. coli*. The growth promotion by mastitic milk was not so evident for diffuse colony forming staphylococci as it was for compact strains. It was concluded that the growth factors present in milk in the mammary gland enhances the growth of the so called naked staphylococci because their metabolic energy is not used to the production of capsule matrix (Mattila & Frost 1988). The growth in normal whey however was much more evident for diffuse colony forming staphylococci than for compact ones, indicating that normal healthy whey contains less bacterial nutrients and antibacterial factors such as lactoferrin and lactoperoxidase (Reiter 1978).

The results in Table 1 do not indicate differences between the 2 groups of staphylococci if the control values from 3 bulk samples is compared with other samples. However, if the comparison is done towards 1 of the bulk tank samples (bulk tank 1) a clear difference can be seen. Hence the compact colony forming staphylococci showed marked growth promotion with non-inoculated control milks which could not be seen for the diffuse colony forming staphylococci. Otherwise no clear differences between controls of 3 bulk tanks and inoculated milks could be seen. It has to be noted that preparation of control milk in the preincubation studies might give an opportunity for normal milk contaminants to grow because bulk milk is not free of organisms and control milks are not inoculated. Bulk tank 1 contained contaminants only 4×10^1 /ml on the day of experiment whereas the other bulk milks (bulk milks 2 and 3) contained 10^3 bacteria/ml milk. The inoculation of pathogens at a level of 10^6 /ml prevents any initial contaminants of growing whereas without inoculation contaminants might grow. The growth of any microbe in milk can cause changes in its composition. Furthermore there is a possibility that microbes in bulk milk 2 and 3 may have caused composition changes in milk before the experiments, the growth of psychrotrophs in bulk milk surely affects its composition. Previous studies have shown that the compact strains of staphylococci are very sensitive to compositional changes in milk, and therefore they would react "positively" to these compositional changes by growth enhancement (Mattila & Frost 1988). This could explain why 2 of the bulk tank samples gave higher mean values for compact colony forming staphylococci.

Furthermore milk samples preincubated with *E. coli* showed growth inhibition for

both groups of staphylococci. These results seem to be in disagreement with earlier studies where experimental *E. coli* endotoxin milk stimulated the growth of staphylococci (Mattila *et al.* 1984b). It seems that inflammation of the mammary gland in response to *E. coli* results in production of factors enhancing bacterial growth whereas in the test tube only metabolites inhibiting the growth of bacteria are induced. The inhibitory metabolite could be an antibiotic, bacteriocin or antimetabolite secreted by *E. coli* interfering with the growth of staphylococci and *E. coli* itself. One further explanation could be the consumption of the key nutrient by *E. coli* during preincubation.

The growth of *E. coli* was markedly inhibited in whey prepared from milk preincubated with same *E. coli*. This could give an indication to problems in isolation of *E. coli* in clinical cases of mastitis. Previous studies, however, have shown that in mastitis, pathogenic bacteria do not inhibit their own *in vitro* growth but the reverse is the case (Mattila *et al.* 1986). The studies included streptococcal and staphylococcal strains, however *E. coli* was not included (Mattila *et al.* 1986).

Nonpathogens such as *Corynebacterium* and *Bacillus* species can inhibit the growth of pathogens such as *Staphylococci* (Woodward *et al.* 1987). Moreover, most of these studies have been carried out *in vitro* where either the bacteria have been allowed to interact directly with each other by studying the effects of the metabolites secreted by the non-pathogens (Corbeil *et al.* 1985, Hogan *et al.* 1987, Ribble 1967, Woodward *et al.* 1987). Our results show that pathogenic staphylococci may alter the milk environment to stimulate growth of other pathogens whereas *E. coli* does the reverse. This could give one answer to the unsolved problems with chronic staphylococcal infections.

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Sammandrag

Kännetecken för tillväxt av Staphylococcus aureus och Escherichia coli i vassla från infekterad mjölk
Förökning af *Staphylococcus* och *Escherichia coli* i bovin vasslapreparat framställt av mjölk, som

tidigare inkuberats med *S. aureus* eller *E. coli*, uppföljdes. Stafylokocker indelades i två grupper beroende på deras förmåga att bilda täta eller diffusa kolonier på serum soft-agar. Denna förmåga härrör sig från- resp. närvaron av kapslar. Tillväxten hos stafylokocker med täta kolonier berodde på tankmjölk medan stafylokocker med diffusa kolonier förökade sig lika bra oberoende av om man använde tankmjölk eller ympad mjölk. Förökningen av *E. coli* förbättrades märkbart i vasslepreparat framställda ur mjölk preinkuberad med stafylokocker. En klar inhibering av tillväxten kunde emellertid observeras för *E. coli* och *S. aureus* när tillväxten skedde i vasslepreparat från mjölk preinkuberad med *E. coli*. Resultaten visar att en gynnsam förökning av patogener är beroende av mjölkens sammansättning. Stafylokockerna är ytterst beroende av förändringar i sammansättningen, speciellt viktigt är detta i samband med infekteringen p. g. a. tillväxtmönstret. De tillväxtinhiberande effekterna förorsakade av *E. coli* kan eventuellt förklara svårigheterna i att isolera denna organism.

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