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MASTITIS WHEY — A GOOD MEDIUM FOR BACTERIA?

By

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MAISI, P., T. MATTILA and M. SANDHOLM: *Mastitis whey — a good medium for bacteria?* Acta vet. scand. 1984, *25*, 297—308. — Growth of mastitis pathogenic bacteria was measured in bovine whey samples by a turbidometric microtechnique. Whey from mastitis cows supported growth as compared with whey prepared from normal milk. Blood proteins leak into milk during mastitis. A study was undertaken to analyze which molecules from blood would promote bacterial growth in whey Fractions containing hemoglobin showed a distinct growth-promoting effect. An inadequate iron supply is one of the restricting growth factors for bacteria in milk. By utilizing heme-compounds the pathogens can by-pass the effect of antimicrobial iron-binding present in milk in the form of lactoferrin.

milk; bovine milk; bovine mastitis; milk bacteria; heme; hemoglobin.

Mastitis is a complex problem and agreement about its pathogenesis is not complete in all aspects. The cow is often thought of as simply the passive mastitis sufferer. However the problem includes on one hand the environment as the source of bacteria, on the other hand the udder as the growth medium of the bacteria. The present investigation focused on the host-parasite relationship in mastitis from the point of view of bacterial nutrient requirements.

It is well known that cows milk inhibits the growth of a number of bacterial species. In normal milk, massive bacterial growth is thought to be prevented by natural inhibitors such as lysozyme, peroxidase and xanthine oxidase activity, lactoferrin, complement components, antibodies and phagocytic activity (*Reiter & Oram* 1967). Bacterial growth in the udder theoretically de-

pends on the quality and quantity of nutrients supporting bacterial growth and factors inhibiting bacteria in milk. The overall balance between the efficacy of the defence mechanisms and growth-enhancing nutrient factors could be obtained by analyzing bacterial survival and growth in milk.

The predisposition of cows to mastitis seems to differ. It is possible that the defence mechanisms in the udder are poorly developed in mastitis cows. On the other hand, cows may differ in their milk which contains the nutrient factors for the infective agents. During pilot studies, mastitis bacteria were seen to grow better in mastitis lactoserum, which hinted that there might be a difference considering milk as the medium of bacterial growth. In mastitis the permeability between the blood and the milk compartment increases and blood components are known to enter the milk (*Honkanen-Buzalski* 1982). Therefore the growth-enhancing factors for bacteria in mastitis milk could originate from blood. The present study was undertaken to find out which blood components would promote bacterial growth in milk. This could give an answer to why bacteria grow better in mastitis milk.

MATERIAL AND METHODS

Bacterial growth curves in whey cultures were produced turbidometrically and the effect of added blood factors was studied in this system.

Micro-organisms

Bacteria isolated from 24 milk samples were collected from the daily material delivered for bacterial identification to the Mastitis Laboratory at the State Veterinary Institute, Helsinki. One strain of Staphylococcus aureus and one of Escherichia coli were selected for further studies concerning growth-promoting effects of blood components.

Whey samples

In the pilot study, 33 milk samples from healthy cows and 22 samples from cows suffering from mastitis, were collected by the Ambulatory Clinic of the College of Veterinary Medicine and the Department of Agriculture, University of Helsinki, Finland. The criteria for normal milk were negative CMT, negative bacteriology, BSA-level below 0.2 mg/ml and antitrypsin content

below 0.5 % of the respective serum value (*Honkanen-Buzalski & Sandholm* 1981). Mastitis samples were selected among milk samples having all these parameters at increased levels.

Bacterial growth was followed by turbidometry. As the turbidometric method requires clear base solutions, the culturing was carried out in whey rather than whole milk. Acid precipitation at pH 4.5 (adjusted by 1 mol/l HCl) was selected as the method of decaseination and the samples were cleared by centrifugation (39 000xg/1 h). The pH of the supernate was adjusted back to 6.8. The whey samples were sterile filtered (Millipore filter type HA, pore size 0.45 μm). The sterility of the whey media before bacterial inoculation was assured by γ-ray-sterilization (Cs^{137}).

Analysis of bacterial growth

The isolated micro-organisms were harvested from blood agar plates and suspended in 0.9 % saline to produce a turbidity of 0.15 as measured at 620 nm using a 0.5 cm light path. Bacterial growth was followed turbidometrically on flat bottomed microtitration plates (8x12 wells á 300 μl). Each well included whey (100 μl), bacterial inoculation (50 μl) and the addition under investigation (50 μl). Each analysis of the growth curve included a blank with the same additions as in the sample wells but 0.1 % sodium azide added to prevent bacterial growth. The plates were incubated at 37°C for 50 h with intermittant shaking for 15 min each hour. The turbidity was measured at 0, 3, 10, 24 and 50 h by a Titertek Multiskan instrument at 620 nm. The turbidity of each test well on the tested hour was adjusted by subtraction of the turbidity of the same well before inoculation. To obtain an automatic blanking and readout the Multiskan instrument was connected to a desk computer (HP 9815A). The bacterial growth curves were drawn from the absorbance differences. The length of the lag phase, tangential increase of the turbidity at the loghasritmic growth phase and maximum turbidity were used as criteria of growth-promotion. The first generation time was calculated by adding the lag phase period to the generation time as calculated from the tangential growth. The correlation of the turbidity values with actual bacterial counts were determined by serial dilutions of each bacterial suspension.

Determination of the growth promoting-effect of the blood and plasma fractions

Hemolyzed blood and plasma samples (4 ml) were subjected to gel-filtration chromatography on Sephacryl S-300 (Pharmacia Fine Chemicals, Sweden), column size 2.6 cm × 94 cm (flow rate 25 ml/h) eluted by 0.9 % saline at + 4°C, 5 ml fractions were collected. The function of the gel-filtration was assured by internal molecular weight standardization. The elution pattern of BSA was determined by analyzing the BSA content of each fraction by quantitative radial immunodiffusion (*Mancini et al.* 1965). The concentration of hemoglobin of each fraction was determined by the Drabkin cyanmethemoglobin method (*Davidsohn & Henry* 1974). The positions of IgG_1, IgG_2 and IgM were determined by analyzing the fractions by double immunodiffusion against specific antisera. The location of fibrinogen in blood and plasma eluates was determined by mixing 10 NIH units of thrombin (Topostasin, Roche) with 0.5 ml of each fraction followed by the observation of any coagulation.

The effect of each fraction on bacterial growth was analyzed by following bacterial growth curves by turbidometry. Fifty µl of each fraction (100 fractions from both plasma and whole blood) was added to the whey cultures of S. aureus and E. coli (the whey was obtained from a healthy cow). The growth curves of both strains of bacteria were followed using eluate fractions as additives.

Other growth factors

The main growth-promoting effect became localized in the hemoglobin peak (Fig. 2) which co-eluted with albumin. Therefore, the effect of pure BSA was tested for its growth-promoting effects in the same system as used for testing blood and plasma fractions.

BSA (bovine serum albumin) (Sigma A-4378) was dissolved in saline. The final concentration in the well was adjusted to correspond with the protein content of a hemoglobin concentration 2.8 mg/ml.

Pure bovine hemoglobin (Merck art. 4300) was tested in the same system. Hemoglobin was dissolved in 0.9 % saline so that the final concentration in the test cuvette came out to be 2.8 mg/ml. A double step dilution series was prepared in 0.9 % saline.

The presence of hemoglobin in milk

An antiserum against bovine hemoglobin was produced in a rabbit by injecting 2 mg of hemoglobin in complete Freund's adjuvant intracutaneously at multiple sites 5 times at 2 weeks intervals. The γ-globulin fraction of this antiserum was used in

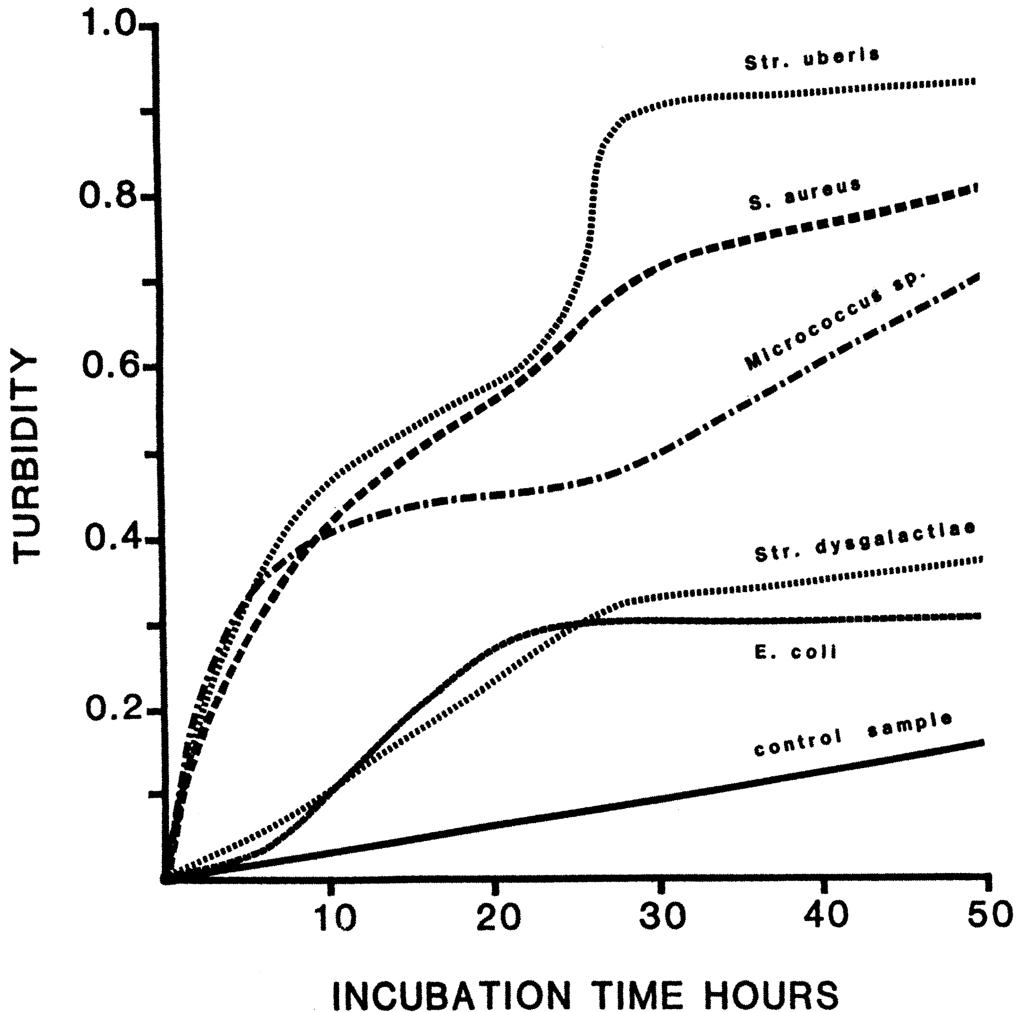

Figure 1. Growth of S. aureus in a few selected whey samples. The dotted lines represent whey samples from chronical mastitis quarters (the type of bacteria isolated from the whey indicated). S. aureus was inoculated into sterilized whey samples and the growth curves were obtained by turbidometry. Note that S. aureus shows a better growth in whey samples from mastitis cows.

gel-diffusion studies to analyze for the precence of Hb in milk samples. A selection of 22 mastitic milk samples and 22 normal milk samples were analyzed by double immunodiffusion for their hemoglobin content.

RESULTS

Bacterial growth in whey

The generation times of S. aureus and E. coli in the control whey were 42 and 10 h respectively. When mastitis whey samples (n = 22) were compared with healthy whey (n = 33) as a growth medium for bacteria, both S. aureus and E. coli grew significantly better in whey samples from infected quarters (Fig. 1).

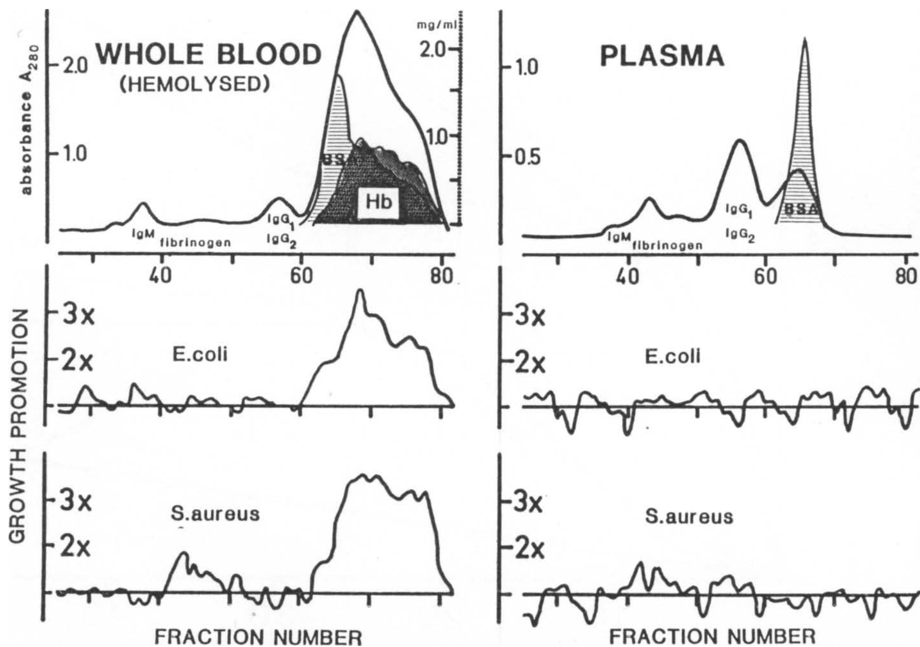

Figure 2. Enhancement of bacterial growth at 50 h in whey by blood fractions. Hemolyzed whole blood and plasma were subjected to gel-filtration chromatography on Sephacryl S-300 and the growth-promoting effect of each fraction on whey cultures of E. coli and S. aureus was determined turbidometrically. Note that no significant effect was obtained by plasma fractions (right panel) but an clear enhancement of bacterial growth was seen in blood fraction co-chromatographed with hemoglobin (left panel).

Blood fractions as growth factors

The analysis of hemolyzed blood and plasma fractions as growth factors showed that the growth-promoting fractions were co-eluted with hemoglobin. None of the plasma fractions significantly promoted growth (Fig. 2).

Effects of hemoglobin and albumin on bacterial growth

Because the growth-promoting effect was pinpointed to hemoglobin, a dilution series of hemoglobin was prepared and tested for growth-promoting effect. The best promoting effect was seen at a concentration of 2.8 mg hemoglobin per ml in the test well. At this concentration the generation time of S. aureus was 11.9 h (average of 4 strains) and that of E. coli 5.9 h (average of 3 strains). Stronger solutions inhibited growth whereas more diluted solutions were less effective in promoting growth (Table 1 and Table 2).

Table 1. Effect of hemoglobin addition on growth of S. aureus in normal whey.

Hemoglobin (mg/ml)	Generation time (h)	Growth promotion of bacterial growth of 50 h
0	20.9	1.0 ×
0.4	17.2	1.2 ×
0.7	12.6	1.6 ×
1.4	10.5	3.6 ×
2.8	10.1	9.1 ×

All the bacterial strains tested (24 strains) showed a marked growth stimulation (at least 100 % increase of bacterial growth at 50 h or the generation time halved) C. ulcerans showed the lowest response (Table 2).

There was no correlation between the type of hemolysis and the growth promotion of the tested bacteria.

A dilution series of albumin was prepared as well (the protein concentrations corresponding the dilutions of hemoglobin). None of the albumin concentrations were able to promote bacterial growth.

Table 2. Growth promotion of added hemoglobin (2.8 mg/ml) on whey cultures of bacteria isolated from bovine milk samples.

Strain	Number of strains tested	Average generation (hours)		Average promotion of bacterial growth by hemoglobin at 50 h
		control	hemoglobin added	
Gram positive				
S. aureus	4	18.0	11.9	6.2 ×
Micrococcus sp.	5	23.5	11.6	2.5 ×
Str. agalactiae	1	17.2	5.4	13.5 ×
Str. dysgalactiae	4	18.4	11.0	7.6 ×
Str. faecalis	4	8.4	6.4	3.9 ×
Str. uberis	2	24.2	8.6	1.2 ×
Bacillus sp.	1	3.2	2.2	9.5 ×
C. ulcerans	1	12.0	10.5	1.2 ×
Gram negative				
E. coli	3	7.9	5.9	7.2 ×
Klebsiella sp.	1	2.3	1.8	6.4 ×
Ps. aeruginosa	1	4.7	4.7	8.2 ×

Presence of hemoglobin in the milk

Of the mastitis milk samples 27 % gave a precipitate by the hemoglobin antiserum, but only 3 % of the normal milk samples gave the precipitation band. The detection limit of the immunodiffusion technique was about 5 μg hemoglobin/ml as determined by serial dilutions of pure hemoglobin.

DISCUSSION

The pilot studies indicated that bacteria show an enhanced growth rate in whey from mastitis cows as compared with whey from healthy cows (Fig. 1). The aim was to investigate which factors in mastitis milk made it a better medium for bacteria. One of the main characteristics in inflammatory response is alternation in permeability of micro-vasculature vessels. The endothelial cells respond to inflammatory mediators by contraction which leads to a fenestration due to the separation of tight junctions connecting the endothelial cells. In mastitis the permeability between blood and milk compartments is known to increase. In general terms it has been established that blood proteins enter the milk during mastitis (*Honkanen-Buzalski* 1981). Therefore it seemed logical to study whether some blood proteins could have an enhancing effect on bacterial growth in whey.

In order to be able to test hundreds of blood fraction parallelly, a new microtechnique was developed to produce bacterial growth curves. A Titertek Multiskan instrument was used. This apparatus measures vertical absorbance of wells of microtitration plates (8 $\times$ 12 wells). The medium in which the bacterial growth takes place should be clear and for that reason whey was used rather than whole milk. Uniform turbidity was ascertained by shaking the plates during incubation. Sterile microtitration plates were used. Azide was used to assure the sterility of the blank row. Before inoculation with bacteria each plate was sterilized once more with γ-ray-sterilization (plates with whey and additives). After irradiation, the bacterial inoculum was added to the test wells.

When studying the effect of blood fractions on growth-promotion of bacterial whey cultures, the growth-promoting factors appeared to become eluted with hemoglobin and BSA (Fig. 2). Plasma fractions did not promote growth neither did BSA; therefore hemoglobin was the growth-promoting factor in hemolyzed whole blood. A slight growth-promoting effect on S. aureus cultures was co-eluted with fibrinogen (Fig. 2). However this turbidity was not due to bacterial growth but due to staphylococcal coagulase. The wells containing fibrinogen became turbid before any noticeble bacterial growth. As the growth-promoting effect of whole blood was pinpointed to hemoglobin, it seemed important to study whether the growth-enhancement in mastitis whey really originates from hemoglobin. Mastitis milk was shown to contain hemoglobin.

The explanation why the whey from healthy cows was a poor substrate for bacteria is most probably the high iron-binding capacity of milk. In vitro, the bacteriostatic effect of lactoferrin can be reversed by saturation of lactoferrin by inorganic iron or by addition of heme-containing molecules to the medium (*Marcelis* 1980). Bacterial virulence can be enhanced in experimental infections by injecting iron-compounds into the experimental animals at the time of infection (*Bornside et al.* 1968, *Bullen* 1981). This enhancement has been clearly demonstrated by injecting hemoglobin and hemolyzed blood into animals.

In vitro, iron is one of the most important growth factors for bacteria. Iron is important for microbial metabolism. The primary function of iron in microbe species is in respiration. Iron is an essential component in the cytochrome chain which is

used to provide chemical energy for the bacteria (*Marcelis* 1980). Iron is also an essential constituent of other metabolic enzymes, such as catalase and peroxidase.

The total iron-concentration in the body fluids is about 2×10^{-5} mol/l of which almost all is tightly accociated with special iron-binding proteins. The concentration of free iron is about 10^{-18} mol/. Most aerobic bacteria require 10^{-6} mol/ iron for growth (*Eaton et al.* 1982). Therefore it has been suggested that the availability of iron may be the major nutritional limitation of many pathogenic bacteria in vivo (*Weinberg* 1978). The iron-binding protein in serum is transferrin and in milk lactoferrin. Normal milk has been seen to inhibit the growth of various gram-positive and -negative bacteria (*Reiter & Oram* 1967). This inhibiting effect comes partly from lactoferrin although other inhibitors such as peroxidases, lysozyme, complement components and immunoglobulins may be partly involved.

Hemoglobin is not bound by lactoferrin which may be the reason why hemoglobin was such a strong growth-promoter in whey. It can be noted that haptoglobin, the hemoglobin binding protein in plasma, was shown to be a natural bacteriostat like transferrin (*Eaton et al.* 1982). Bacteria may aquire hemoglobin by breaking up the erythrocyte membrane by their hemolysins. Most strains of bacteria generally regarded as mastitis pathogens, are hemolytic. The molecular weight of these hemolysins are below the MW of blood proteins, which mean that they could pass outside the milk alveoli and reach local blood cells. The fact that hemoglobin was present in mastitis milk could indicate that local hemolysis was going on and hemoglobin leaked into the milk. It is also possible that mastitis pathogens derive some iron from damaged epithelial cells or phagocytes.

Some bacteria have developed their own iron-chelating systems to obtain iron from its bound forms. The determinant for increased bacterial virulence might be the ability of the strain to detach iron from transferrin or lactoferrin (*Cowart* 1980).

The present study clearly indicates that hemoglobin can be an important nutrient factor for mastitis pathogens. It should be noted that besides hemoglobin, mastitis milk may contain other heme-proteins, such as catalase lactoperoxidase, myeloperoxidase and cytochrome proteins. Only 27 % of the mastitis milk samples showed detectable hemoglobin, therefore, hemoglobin alone cannot be responsible for the observed growth-enhance-

men in all cases. Another possible explanation of heme-compounds promoting bacterial growth could be in their function of inactivating toxic oxygen products produced by the one-electron reduction pathway of molecular oxygen.

It seems important to re-evaluate the host-parasite relationship in mastitis from the perspective of iron supply for bacteria. How do mastitis sensitive and resistant cows differ as far as the iron supply is concerned and is it possible to develop new pharmacological principles to treat mastitis by blocking the leakage of heme-proteins into milk?

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SAMMANDRAG

Ökad tillväxt av bakterier i vassla från mastitkor.

Tillväxt av mastitpatogena bakterier framkallades i prover av kovassla med turbidometrisk mikroteknik. Jämfört med vassla från normal mjölk förbättrade vassla från mastitkor bakterietillväxten. Vid mastit läcker blodproteiner ut i mjölken. Man analyserade vilka molekyler från blodet hade en tillväxtfrämjande effekt i vasslan. Fraktioner innehållande hemoglobin hade en markant tillväxtbefrämjande effekt. En inadekvat tillgång på järn är en av de tillväxthämmande faktorerna för bakterier i mjölk. Genom att använda hem-föreningar kan patogenerna kringgå den antimikrobiella järnbindande effekten i mjölk i form av laktoferrin.

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