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Lactoferrin is responsible for the fungistatic effect of human milk

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Abstract

Human milk has recognized anti-microbial effects and it has been repeatedly shown that breast-fed infants have fewer and less severe infections than formula-fed infants. While most studies have focused on anti-bacterial and anti-viral activities few have focused on the anti-fungal effect of human milk. Dermal and other infections caused by fungi are common in very low birth weight (VLBW) infants. Using a liquid culturing method and *Candida albicans* and *Rhodotorula rubra* as representative fungi, we studied the anti-fungal effect of human milk and certain human milk proteins. In vitro, human milk showed potent inhibitory effect on fungal growth. Most, if not all of this effect was caused by lactoferrin via its iron-binding capacity; increasing the iron content of the incubation medium abolished the inhibitory effect. In contrast, other human milk proteins with known or suggested anti-microbial effects rather increased fungal growth. Viability test and electron microscopy revealed that the growth inhibitory effect of human milk, i.e. mediated by lactoferrin, is fungistatic rather than fungicidal.

Keywords: Human milk; Lactoferrin; Skin care; Neonatal care; Fungal infections

1. Introduction

Due to immature immune and barrier functions preterm infants, and particularly very low birth weight (VLBW) infants, are more susceptible to infections than term infants. Preterm infants have a large surface area relative to total body mass. Thin, translucent and nonkeratininized skin is prone to infections and, thus, septicemia resulting from a compromised dermal barrier may occur. Besides being a natural barrier towards microbes, the skin functions as an important regulator of temperature,

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water and electrolytes [1]. Thus, skin integrity is essential for the well being of the infant. Staff at neonatal intensive care units (NICU) are becoming increasingly aware of the importance of careful skin care to prevent dermal lesions. Special skin care programs for VLBW infants have been developed because programs used for adults may not be effective, or even harmful; precautions must be taken that substances applied to the skin do not place the infant at risk for systemic toxic effects or dermal derangement [2]. Thus, most NICU have skin care routines, although they are not always scientifically founded. At our unit it has been a custom to sometimes lubricate the VLBW infants with breast milk. The reason being empiric experience that this reduces the number of skin infections. Anecdotally, it is old practice in many cultures to apply human milk topically to cure abscesses, exfoliative eczema and conjunctivitis. In the 18th century London Pharmacopoeia breast milk is described as 'emolient and cool and cureth red eye immediatley' [3].

The anti-microbial effect of human milk is multifactorial; several milk components, e.g. secretory IgA, oligosaccarides, lysozyme, lactoperoxidase, lactoferrin, glycopeptides, glycolipids, fatty acids, monoglycerides and leukocytes have all been shown to have effect in vitro. The isolated effect of each of these compounds in vivo are however, so far, less well documented and understood, although it has been repeatedly shown that breast-fed infants have fewer infections, and less severe infections than formula-fed infants. This is particularly true for respiratory and gastrointestinal infections [4–6].

While many studies have focused on the anti-bacterial and anti-viral effects of milk or milk constituents, there are only few studies concerning possible anti-fungal effects. Since dermal infections caused by fungi are a common problem in VLBW infants and also results in significant morbidity and, even mortality [7–9], our aim was to explore if human milk has anti-fungal effect, and, if so, if a distinct anti-fungal milk component could be identified.

To study the anti-fungal effect of milk we used *Candida albicans*, a human pathogen and the most common cause of fungal infection in infants, and *Rhodotorula rubra*, a fungus prevalent in the normal skin flora and considered a non-pathogen to individuals with non-compromised immune system. However, when the immune system is compromised, as in VLBW infants, *R. rubra* may be pathogenic. Furthermore, *R. rubra* has been reported to be transmitted via indwelling central venous catheters, to which many VLBW infants are exposed [10,11].

2. Material and methods

2.1. Assay of fungal growth

Inhibition of fungal growth in response to milk or purified proteins was determined as follows: *C. albicans* or *R. rubra* were grown in RPMI 1640 medium supplemented with L-glutamine without serum and antibiotics (Life Technologies Ltd., Paisley, UK). Incubations were carried out in 24-well dishes, Nunclon™, (nunc a/s, Roskilde, Denmark). The wells contained various concentrations of either milk or a milk

constituent, 50 000 fungi and medium in a total volume of 1 ml. The dishes were subsequently incubated at 37°C in the presence of 4% CO_2. After various times of incubation, the number of fungi in each well was determined by counting in a Bürker chamber. All incubations were performed in duplicate. Inhibition of growth was expressed as number of fungi/volume at a certain time point when compared to control incubation without inhibiting factor.

2.2. Preparation of skim milk

100 ml of fresh human milk, collected from a mother 6 weeks after delivery, was centrifuged at 10 000 *g* for 30 min. The top fat layer was discarded, the skim milk collected and sterilized by filtering trough a 0.22 μm filter (Millipore S.A., Molsheim, France) whereafter it was stored at −20°C until analysis. Human milk from two other donors, at the same lactational stage, were used to confirm the results.

Pooled fresh, raw bovine milk was collected from a local farm and stored frozen until used. Before analysis the skim milk was prepared as described for human milk. Dog milk from a Spaniel was collected 3 weeks after delivery. The skim milk was prepared as human milk, and analysed fresh.

2.3. Purified human proteins

Human holo-lactoferrin (iron-saturated), apo-lactoferrin (iron-unsaturated), holo-transferrin (iron-saturated), apo-transferrin (iron-unsaturated), transferrin (80% iron-saturated), casein (containing both β- and κ-casein) and α-lactalbumin were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Human β and κ-casein were purified as previously described [12,13]. Lactoferrin and transferrin were added to the medium at final concentration of 0.15 μg to 150 μg/ml. Concentrations of α-lactalbumin ranged from 1 μg to 200 μg/ml and of caseins from 0.1 μg to 500 μg/ml.

2.4. Incubations with iron

In a set of experiments the effect of iron was tested. Iron as $Fe_2(SO_4)_3$ (Mallinckrodt Inc., Kentucky, USA), in concentrations varying from 0 to 300 μg/ml were added to incubations containing 10% skim milk, and growth assessed after various time of incubation. Incubations without added skim milk served as controls.

2.5. Inhibitory effect of milk

2.5.1. Effect of relieving inhibitor

In an attempt to clarify if the observed anti-fungal effect was fungicidal or fungistatic, fungi were grown in medium containing 0.05, 0.1, 1 or 10% skim milk, respectively. After 48 h of incubation, cultures were transferred to sterile tubes and centrifuged at 600 *g* for 10 min. Supernatants were discarded, and 10 ml of fresh medium (without milk) was added. After suspension, the tubes were again centrifuged

at 600 *g* for 10 min. This washing procedure was repeated twice whereafter washed fungi, 50 000/ml medium, were incubated in new wells and the growth assessed after 24 h.

2.5.2. Test of viability

To explore if the inhibitory effect was mainly fungistatic or fungicidal we used a fluorescence probe for yeast viability, FUN-1™ (Molecular Probes Europe, PoortGebouw, Leiden, The Netherlands). The test was performed according to the manufacturer's instruction with minor modification. Fungi were grown as described in presence of various concentrations of skim milk. After 72 h of incubation all fungi in each well were transferred to a microfuge tube and centrifuged at 10 000 *g* for 5 min. The supernatant was discarded and the pellet resuspended in 2% D-glucose with 10 mM Hepes pH 7.2. The wash was repeated once and the fungi finally suspended in 0.5 ml of the same solution. Five μl of FUN-1 was added and samples incubated in the dark at 30°C for 60 min. Number of fungi with or without fluorescence were counted in a Bürker chamber under a fluorescence microscope (Olympus Optical Co. Ltd., Shibuya-ky, Tokyo, Japan).

2.5.3. Visualisation of membrane structure

After incubation, medium with fungi were centrifuged at 10 000 *g* for 5 min and fixed over night with 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 at 4°C, rinsed in buffer and postfixed for 2 h in 1% osmium tetraoxide in 0.1 M phosphate buffer pH 7.4. After dehydration in graded alcohol and embedded in Poly/Bed (Polysciences Inc., Worrington, PA, USA), ultrathin sections were cut by use of a LKB ultratome and placed on gold grids, stained with uranylacetate and lead citrate. Observations were performed using a Jeol 100 CX transmission electron microscope.

3. Results

3.1. Human skim milk inhibits fungal growth

In our standard experimental system *C. albicans* grew with a generation time of approx. 2.5 h. When human milk, independent of concentration, was added to the incubation medium a slight increase of growth was observed after 5 h of incubation (Fig. 1a). In contrast, after 24 h of incubation the presence of skim milk caused a significant dose-dependent inhibition of fungal growth (Fig. 1b). The inhibitory effect remained after 48 h and 72 h of incubation. Similarly, growth of *R. rubra* was inhibited after addition of human milk to the medium (Fig. 2). However, *R. rubra* seemed to be more sensitive to human skim milk since inhibition of growth was achieved at even lower milk concentrations. Having confirmed this, *C. albicans* were used for further studies.

Bovine skim milk, in the same concentrations as human milk, showed no inhibitory effect on fungal growth (data not shown). Similarly, no inhibition of fungal growth was observed when dog milk, in the same concentrations as human milk, was added

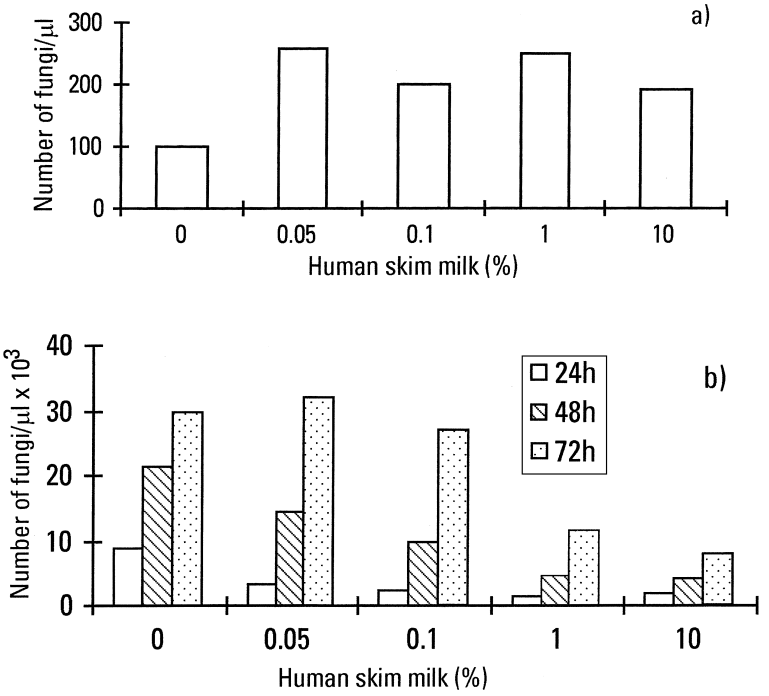

Fig. 1. Effect of skim milk concentration on growth of *C. albicans*. Fungi were incubated for 5 h (a) or 24, 48 and 72 h, respectively (b). Incubation was carried out as described in Material and methods.

to the medium (data not shown). In contrast, at all concentrations tested, both bovine and dog skim milk increased the number of fungi as compared to controls when assessed after various time of incubation.

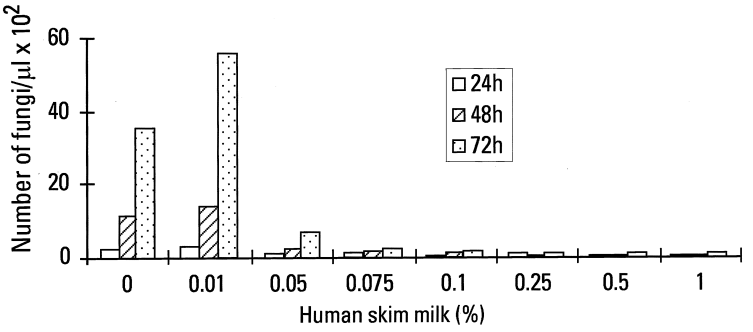

Fig. 2. Effect of concentration of skim milk on growth of *R. rubra* after 24, 48 and 72 h incubation. Incubations were done as described in Material and methods.

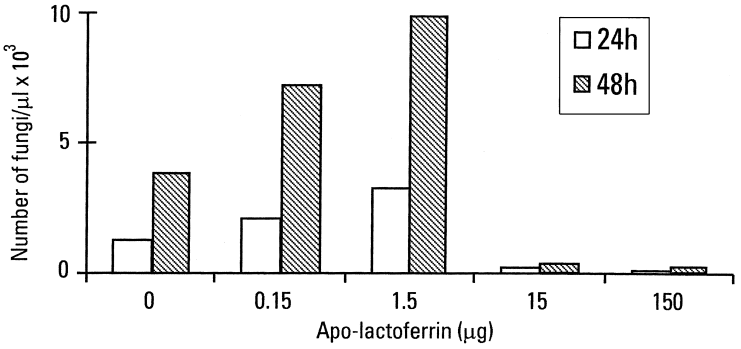

Fig. 3. Effect of apo-lactoferrin on growth of *C. albicans* after 24 and 48 h incubation. Incubation was done as described in Material and methods.

3.2. Iron binding proteins affect fungal growth

Addition of 15 μg/ml or more of purified human apo-lactoferrin to the growth medium significantly inhibited growth of *C. albicans* (Fig. 3). In contrast, under the same incubation conditions, holo-lactoferrin did not inhibit growth, rather there was a slight increase in growth.

The effect of purified human apo-transferrin gave comparable results with apo-lactoferrin, i.e. a decrease of fungal growth except at the lowest concentrations tested, which resulted in increased growth (data not shown). Also in accordance with the effect of lactoferrin, iron saturated transferrin increased growth. With transferrin, partly saturated with iron, a decrease in fungal growth was observed at all concentrations tested (data not shown).

Neither at 24 h, nor at 48 h incubation did human casein, even at the highest concentration used (500 μg/ml), inhibit growth of *C. albicans*. In fact, with increasing concentration and time of incubation casein increased the number of fungi. When purified human β- and κ-casein were tested we found no difference between the two and both shared the effect of unfractionated casein. Similar to casein human α-lactalbumin increased the growth of *C. albicans* during 24 h incubation. Maximal effect was found already at the lowest concentration (1 μg/ml) tested (data not shown).

3.3. Effect of iron in fungal growth

When iron as $Fe_2(SO_4)_3$ was added to incubations with 10% skim milk the inhibition of fungal growth caused by the milk was abolished with increasing iron concentration. Addition of 0.3 μg of iron/ml completely restored fungal growth (Fig. 4).

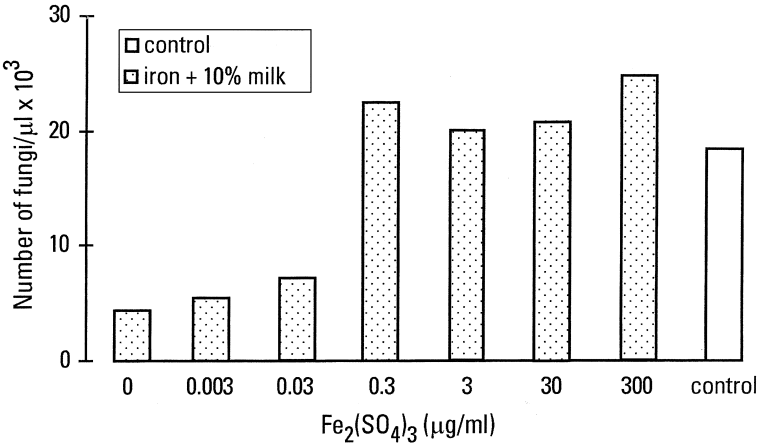

Fig. 4. Relief by iron sulphate of growth inhibition caused by human milk. Incubation was done as described in Material and methods.

3.4. The effect of human milk is fungistatic

When fungi had been incubated for 48 h with varying concentration of human skim milk, washed in fresh medium and incubated again similar fungal growth as in controls without skim milk was recorded (data not shown) suggesting a fungistatic rather than fungicidal effect of human milk. This was supported by data on yeast viability. The conversion of FUN-1 to intravacuolar orange-red structures require both plasma membrane integrity and metabolic competence of the fungus. After 72 h incubation we found 90% live fungi in control wells. Incubations with 1% skim milk showed a decrease of live fungi to 84%, but with 10% skim milk the number of live fungi decreased to only 80%. Hence, these results are in accord with human milk having mainly a fungistatic effect on fungal growth.

Transmission electron microscopy revealed no membrane derangement of fungi after 48 h incubation with 10% skim milk or 150 μg lactoferrin per ml. Even at 10 times higher lactoferrin concentration (1500 μg/ml), no membrane derangement was observed (Fig. 5a−c).

4. Discussion

To explore the anti-fungal effect of human milk a liquid culturing method was developed. The effect of skim milk, and of the purified human milk proteins; lactoferrin, β-casein, κ-casein and α-lactalbumin as well as that of human transferrin were studied. We chose to study skim milk rather than whole milk for several reasons. Lipids, e.g. free fatty acids released during hydrolysis of milk triglycerides during

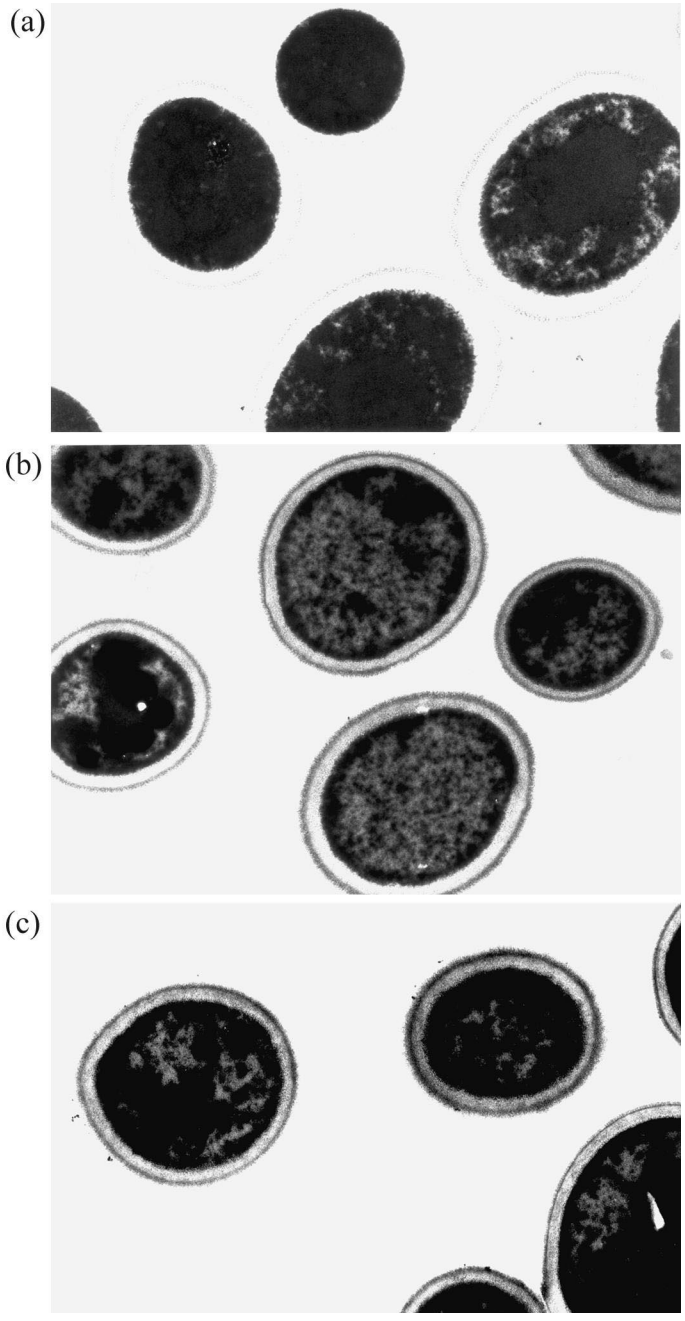

Fig. 5. Ultra structural visualisation of fungi after 48 h of incubation in growth medium without milk (a), 10% skim milk (b), and 1500 μg lactoferrin (c). For details see in Material and methods.

storage, mediate killing of microorganisms [14]. Further, by using skim milk the confounding effect of phagocytosis and killing of fungi by cells in the milk were avoided [15].

Under the conditions described growth of both fungi tested, i.e. *C. albicans* and *R. rubra* was inhibited by human skim milk. *R. rubra* was more sensitive to skim milk than *C. albicans*. When assay conditions affecting fungal growth were scrutinised, it turned out that incubation time was crucial. After 5 h incubation fungal growth had increased in presence of skim milk, whilst inhibition was evident after 24 h, and at higher concentration of skim milk even more pronounced after 72 h of incubation. Despite the inhibiting effect compared to control incubations the number of fungi increased with time. Even in incubations with 10% skim milk the number of fungi was higher after 72 h compared to 24 h of incubation. The growth promoting effect is likely explained by skim milk being also a source of nutrients promoting fungal growth and that the fungistatic effect is not complete, allowing growth, albeit at reduced rate. This effect is most evident at the lowest milk concentrations (Fig. 1b). At higher milk concentrations virtually all iron is bound by lactoferrin (see below) and the fungistatic effect is more complete.

The finding that skim milk affected fungal growth also at low concentration suggested that one of the major skim milk proteins, reported to have anti-microbial effects might be responsible. Lactoferrin exerts its anti-microbial effect via at least two completely different mechanisms; one, being dependent on the iron binding capacity of lactoferrin and the other on lactoferricin, the 18 amino acid N-terminal peptide of lactoferrin cleaved off by pepsin. Lactoferricin does not bind iron but mediates killing of *C. albicans* and other microbes by causing loss of colony-forming capability, due to disruption of ultrastructural features, and, ultimately cell damage [16,17]. By using transmission electron microscopy, we found no evidence that human milk or purified lactoferrin mediates alterations of fungi membranes, and viability test showed high percentage of viable microbes after 72 h incubation. This is compatible with lactoferrin having a static effect on fungal growth. In fact, not even after extended incubation and high concentration of milk or lactoferrin did we find evidence of a fungicidal effect. These results are in contrast to Soukka et al. [18], who found a fungicidal effect. However, these authors used completely different experimental conditions and also considerably shorter incubation times why comparisons are different to make.

Lactoferrin at a concentration ≥ 15 μg/ml showed strong inhibition of fungal growth. In contrast, lactoferrin at 0.15 and 1.5 μg/ml concentrations increased fungal growth at both 24 h and 48 h incubation. A reasonable explanation was the latter concentrations were too low to allow binding of sufficient amount of the iron in the incubation medium to reduce fungal growth. Under these conditions lactoferrin promoted growth by being used as energy substrate. Indeed, we confirmed that the amount of iron available in the growth environment was a crucial factor for the effect of milk on fungal growth. High iron concentration abolished the growth inhibiting effect of skim milk or required higher concentration of milk for inhibition. In fact, during the course of the study there were difficulties to reproduce the effect of milk

with different batches of medium. It turned out that the reason was that the iron content varied considerably from batch to batch as delivered by the manufacturer. Medium with an iron concentration above 0.5 μmol/l abolished the fungal growth inhibiting effect of skim milk, even at 10% concentration. It was recently shown that the iron concentration in the growth medium of *C. albicans* is important for the regulation of the iron-uptake mechanism of the fungus, which in turn affects its virulence [19].

The effect of cow milk and dog milk was different from that of human milk. Although pure transferrin, the iron-binding protein in dog milk [20] had comparable effect to lactoferrin on fungal growth, dog milk, at all concentrations tested, increased fungal growth. This may be explained by the fact that the transferrin concentration in dog milk is about 30 times lower than the lactoferrin concentration in human milk and the iron concentration 12–65 times higher (6–13 μg/ml and 0.2–0.5 μg/ml, respectively) [21]. Hence, there should be sufficient amount of unbound iron in dog milk to promote fungal growth.

Casein has been suggested to have anti-microbial effects, and casein is a major protein fraction in skim milk [22]. Purified human β- and κ-caseins, as well as α-lactalbumin, one of the major whey proteins in human milk, having an apoptotic effect on cancer cells [23], all had the same principal effect, namely to promote fungal growth. A likely explanation could be that these proteins like holo-lactoferrin merely served as nutritional proteins increasing the nutrient density of the growth medium. However, lactoferrin has been shown to have a direct growth promoting effect on cells in culture [24].

In conclusion, we found that human skim milk, by the method used, at as low concentration as 0.1%, inhibits growth of *C. albicans* while inhibition of *R. rubra* is seen at 0.05% concentration Most, if not all, of this effect was caused by lactoferrin through its iron-binding capacity. The effect of milk was only observed when the iron content in the growth medium was less than 0.5 μmol/l. When the iron-binding capacity of lactoferrin was exceeded the inhibiting effect was abolished. In contrast, neither other human milk proteins, nor bovine or dog milk had an inhibitory effect. Rather, these proteins increased fungal growth, possibly by serving as nutrient for growing fungi. These results give some support to the clinical observation that lubricating VLBW infants with human milk may prevent skin infections. However, the effect should be evaluated in a prospective clinical randomised study, and further, our data show that milk could be replaced by purified apo- but not holo-lactoferrin.

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