HOST RESPONSE TO INFECTION

Synergic antistaphylococcal properties of lactoferrin and lysozyme

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Staphylococcus epidermidis colonises a wide range of implanted prosthetic devices, but rarely contact lenses – despite a similarity in material composition. A conceivable explanation for this anomaly is the action of the tear defences, including the constitutive proteins lactoferrin and lysozyme. Therefore this study investigated the effect of lactoferrin, lysozyme and serum on the growth of S. epidermidis isolates in artificial tear fluid. Whether supplemented with serum alone or serum with either apolactoferrin or lysozyme, this medium induced a similar, strain-variable effect. However, simultaneous addition of these proteins induced a greater bactericidal or bacteristatic effect. Of those strains killed by the concerted action of apolactoferrin and lysozyme, the absence of serum led to a further increase in the bactericidal effect, whereas strains displaying bacteriostasis were unaffected by serum. Iron saturation of lactoferrin reversed the antimicrobial synergy of apolactoferrin and lysozyme. These results show synergy between lactoferrin and lysozyme which is dependent on the iron limitation of lactoferrin. As a bactericidal mechanism, this synergy is augmented by serum, but bacteriostasis remains unaffected by serum supplemention. Thus, the combination of lysozyme and lactoferrin may partly explain the low level of contact lens colonisation by S. epidermidis in vivo.

Introduction

Staphylococcus epidermidis is a major pathogen of implanted medical devices such as intravenous catheters [1] and intraocular lenses [2]. Despite a similarity in the material composition of implanted biomaterials and contact lenses, this organism has rarely been associated wtih contact lens-related infections [3]. We hypothesised that this anomaly was due to tear fluid defence proteins which bathe the anterior surface of the eye. As well as adaptive components such as IgA [4], tears contain an array of non-adaptive but interactive antimicrobial factors including complement [5], lactoferrin [6] and lysozyme [4]. An in-vitro examination of the individual effects of these substances has shown that *S. epidermidis* strains resist complement-mediated killing and the action of iron-limited (apo-)lactoferrin or lysozyme [7].

Previous studies have demonstrated an irreversible antimicrobial action of apolactoferrin unrelated to its iron-chelating capacity [8, 9], including a bacteriostatic effect on a clinical strain of *S. epidermidis* [10]. This action is thought to be caused by the highly cationic region near the N-terminus of lactoferrin [11] which can be isolated by proteolysis; the resulting peptide is referred to as lactoferrin [11]. The mechanism of action of lactoferrin on gram-negative bacteria has been demonstrated to involve the release of lipopolysaccharide (LPS) [12] and a decrease in the semi-permeable nature of the outer membrane [13]. The basis for the action of this protein on gram-positive bacteria is unknown.

In comparison with other body fluids, human tears contain the highest concentration of lysozyme [14]. This protein is a muramidase that cleaves peptidoglycan [14]. Also, lysozyme has been shown to activate pre-existing autolytic enzymes of *S. aureus* in acidic conditions [15]. Lysozyme activity has been shown to be additive to the action of apolactoferrin on gram-negative bacteria [16].

Functionally active complement components have been found in closed eye tears at concentrations of 1–20% of their plasma levels, depending on the individual

complement protein. The alternative pathway of complement, which can be activated directly by bacteria, was found to be present in normal human tears [5]. Previous results have indicated that strains of *S. epidermidis* are resistant to complement killing in serum 10%, higher than the in-vivo tear concentration of most complement proteins [7]. Ethylenediaminetetraacetic acid (EDTA), which has been shown to release LPS in a manner similar to that of lactoferrin, has rendered resistant gram-negative bacteria sensitive to complement [17].

The individual effects of complement, apolactoferrin and lysozyme on strains of *S. epidermidis* [7] and the synergy between apolactoferrin and lysozyme for gram-negative bacteria have been described previously [16]. This study examined the interactive effects of these defence proteins in an artificial tear fluid on various *S. epidermidis* isolates. The role of iron limitation of lactoferrin in this medium was explored.

Materials and methods

S. epidermidis strains

The following *S. epidermidis* strains were assayed: ATCC 35983 and ATCC 14990; three ocular strains isolated at the Cornea and Contact Lens Research Unit, University of New South Wales from asymptomatic subjects wearing soft contact lenses (SE3, SE4 and SE5); and three isolates from infected biomaterials (SE7 and SE13, central line tips; SE14, swan-fent tip) which were kindly supplied by the Prince of Wales Hospital, Sydney, Australia. All strains were identified as *S. epidermidis* by a previously described identification procedure [18] and stored at −70°C until required.

Artificial tear fluid

Artificial tear fluid was prepared as described previously [19], supplemented or unsupplemented with pooled normal human serum 2%, human milk lactoferrin (Sigma) 1.8 mg/ml and egg-white lysozyme (Sigma) 5.4 mg/ml. Artificial tear fluid was selected as a suitable medium to evaluate the effects of various tear antimicrobial factors on *S. epidermidis* as it contains similar salts, lipids and proteins to tears in comparable concentrations [19]. Lactoferrin and lysozyme are present in closed eye tears at a concentration of 1.8 mg/ml [20]. Chick egg-white lysozyme has approximately one-third the activity of its human counterpart [21] and the supplemented concentration of 5.4 mg/ml reflects lysozyme activity *in vivo*. Human milk lactoferrin was added at 1.8 mg/ml. The effect of serum 2% was evaluated in this study as the alternative complement pathway has been shown to be active in closed eye tears with most complement components being present at concentrations of 0.2–2.2% of their plasma level [5]. The effect of 31.25 μM EDTA and lysozyme 5.4 mg/ml on strain SE5 was assessed to confirm the absence of EDTA activity in apolactoferrin preparations.

Ferric ion content of lactoferrin

Lactoferrin was iron-limited as described previously [22]. Briefly, lactoferrin was dialysed three times against EDTA (Merck, Kilsyth, Australia) 1% in phosphate buffer (0.1 M Na_2HPO_4, pH 4.6; Bacto Laboratories, Liverpool, Australia) followed by dialysis (1 in 1000 dilution) twice against distilled H_2O. Lactoferrin was analysed for contaminating EDTA spectrophotometrically at 195 nm and the absorbance of lactoferrin preparations was found to be less than the absorbance of 31.25 μM EDTA. The amount of ferric ions bound to lactoferrin was determined by a previously published method [23] whereby reduced protein was precipitated and ferric ions detected with ferrozine and neocuproin (Sigma). This was miniaturised for use in a 96-well microtitration plate and analysed spectrophotometrically at 570 nm with a plate reader (Model 3550, BioRad, Regents Park, Australia).

Growth conditions

Each strain was inoculated into 10 ml of Tryptone Soya Broth (TSB; Oxoid, Melbourne, Australia) and grown overnight at 37°C until the stationary phase had been reached. The bacteria were then washed three times in phosphate-buffered saline (PBS; NaCl 8 g/L; $Na_2HPO_4.2H_2O$ 1.14 g/L; KCl 0.2 g/L, KH_2PO_4 0.2 g/L; pH 7.2) by centrifugation and resuspended in PBS to an optical density of 0.1 at 660 nm. Bacterial suspensions were added (1 in 10) to various artificial tear fluid types and incubated with rotation for 10 h at 37°C. Samples (20 μl) were extracted immediately on addition of bacteria and thereafter at 2.5-h intervals. These samples were diluted (1 in 100) in PBS, vortex mixed for 10 s, then sonicated at 50 Hz for 15 min with an ultrasonic cleaner (Unisonics, Sydney, Australia). Serial dilutions were performed and three 20-μl samples from each dilution were plated on nutrient agar (Oxoid) to determine the number of viable bacteria [24].

Statistical analyses

Statistical analyses were performed by Student's *t* test.

Results

Iron content of artificial tear fluid types

The average free ferric ion content of artificial tear fluid types, apolactoferrin and non-iron-limited (holo)-lactoferrin is shown in Table 1. Lactoferrin (mol. wt 80 000) was added to artificial tear fluid at a concentration of 1.8 mg/ml (22.5 μM), requiring 45 μM (2.52 μg/ml) of ferric ions to fully saturate this protein which binds two ferric ions per molecule.

Table 1. Ferric ion content in artificial tear fluid and bound to lactoferrin

Medium	Ferric ion content (μg/ml)		Medium	Ferric ion content (μg/ml)	
ATFS	0.47*	(0.00)	ATF	0.49*	(0.00)
ATFS+apoLF	0.26†	(0.07)	ATF+apoLF+LZ	0.43*	(0.00)
ATFS+LZ	0.44*	(0.00)	ATFS+holoLF	3.31†	(1.63)
ATFS+apoLF+LZ	0.28*	(0.00)	ATFS+holoLF+LZ	0.28†	(0.46)
apoLF	0.22†	(0.01)	holoLF	3.10†	(0.42)

*Results represent the mean (SD) of two experiments.
†Results represent the mean (SD) of two experiments for each of two media.

Hence apolactoferrin (0.22 μg/ml) is approximately 8.7% saturated with ferric ions and hololactoferrin (3.1 μg/ml) is fully saturated.

Sonication

Cell clustering was displayed by *S. epidermidis* strains grown in artificial tear fluid. Clustering was considered likely to affect viable counts and was reduced by sonication. Fig. 1 shows the cluster sizes of isolates sonicated for 0 or 14 min after incubation in artificial tear fluid supplemented with serum for 10 h. The average cluster size for each strain decreased after sonication, although strain SE14 still retained an average of four cells/cluster. The viability of all strains was retained after sonication.

Growth in artificial tear fluid containing serum

The difference in bacterial numbers between the initial inoculum and after growth for 10 h in artificial tear

fluid containing serum only (ATFS) is shown in Table 2. ATFS was bactericidal for strains ATCC 14990, SE4 and SE13. Bacteriostasis was maintained for 2.5 h followed by a gradual decrease in numbers for a further 5 h. This bactericidal effect was similar in ATFS containing apolactoferrin (ATFS+apoLF) or lysozyme (ATFS+LZ). Compared with these three media, there was a significant (p < 0.05) increase in bacterial killing for these strains in ATFS containing both apolactoferrin and lysozyme (ATFS+apoLF+LZ) and a greater bactericidal effect. A bacteriostatic effect was observed for strains ATCC 35983 and SE3 and there was no statistically significant difference between different media. Strains SE5, SE7 and SE14 showed a similar growth in ATFS, ATFS+apoLF and ATFS+LZ. These strains showed a typical growth curve revealing the stationary phase attained by 7.5 h. However, compared with the growth observed in these media, there was a significant decrease (p < 0.01) in growth in ATFS+apoLF+LZ. Strain SE14 showed a similar growth pattern as before, but with lower bacterial
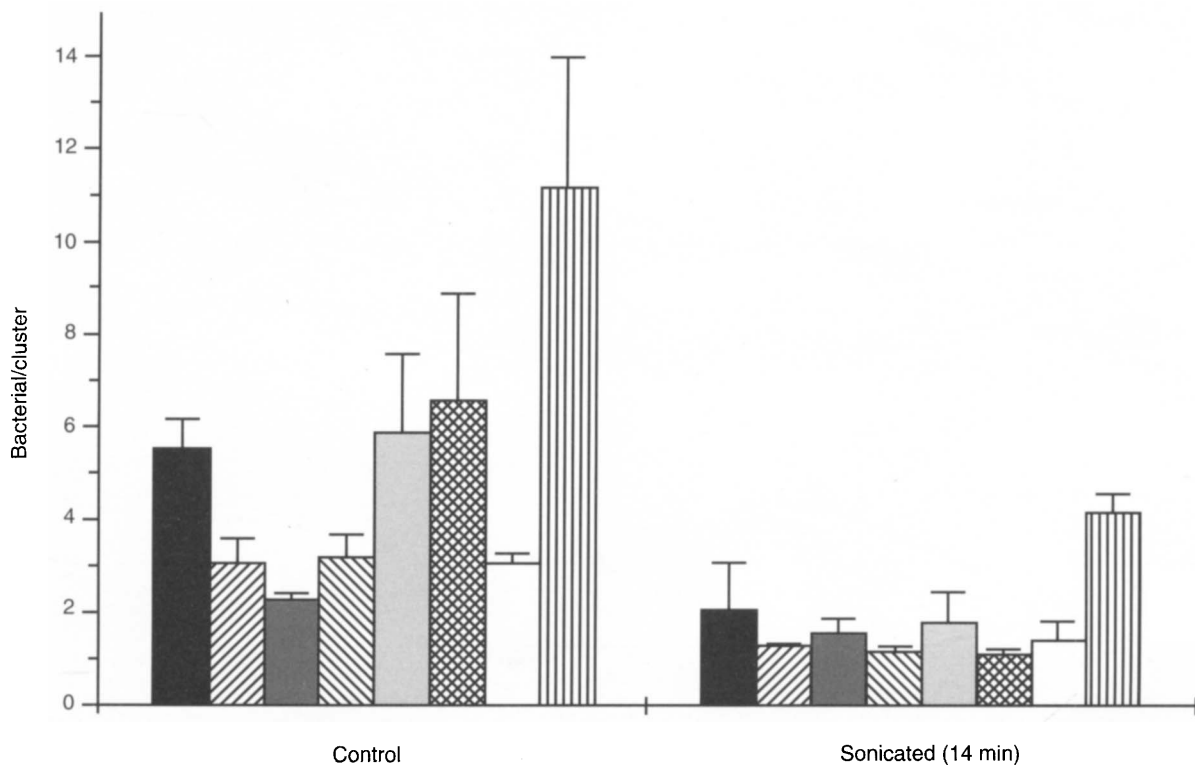

Fig. 1. Average number of bacteria/cluster after sonication for 0 or 14 min at 50 Hz after growth in artificial tear fluid plus serum. Results represent the mean (bar, SD) of 10 experiments. ■, ATCC 14990; ▨, ATCC 35983; ■, SE3; ▨, SE4; ▧, SE5; ▩, SE7; □, SE13; ▥, SE14.

Table 2. Effect of apolactoferrin and lysozyme on bacterial numbers of *S. epidermidis* strains growing in artificial tear fluid

Strain no.	Mean (SD) difference in bacterial numbers (log$_{10}$ bacteria/ml)* in				
	ATFS	ATFS+apoLF	ATFS+LZ	ATFS+apoLF+LZ	ATFS+LZ+EDTA
ATCC 14990	−0.65 (0.37)[†]	−0.35 (0.27)	−0.67 (0.50)	−1.27 (0.27)	...
ATCC 35983	+0.21 (0.24)[‡]	+0.21 (0.26)	+0.22 (0.27)	0.00 (0.06)	...
SE3	−0.15 (0.20)	−0.09 (0.34)	−0.26 (0.23)	−0.50 (0.51)	...
SE4	−0.48 (0.09)	−0.33 (0.22)	−0.71 (0.27)	−1.00 (0.06)	...
SE5	+1.02 (0.07)	+0.82 (0.23)	+1.02 (0.11)	+0.18 (0.28)	+1.03 (0.03)
SE7	+0.78 (0.09)	+0.50 (0.03)	+0.66 (0.12)	+0.16 (0.10)	...
SE13	−0.39 (0.21)	−0.40 (0.51)	−0.23 (0.28)	−0.75 (0.11)	...
SE14	+0.92 (0.07)	+0.71 (0.04)	+0.71 (0.10)	+0.46 (0.09)	...

ATFS+apoLF+LZ showed significant (p < 0.05) differences in the final bacterial numbers from all other growth conditions for all strains with the exception of ATCC 35983 and SE3.
..., not tested.
*Results represent the mean of six experiments and are expressed as the difference between the initial inoculum and the final (10 h) sample.
[†]Negative data represent a decrease in bacterial numbers.
[‡]Positive data represent an increase in bacterial numbers.

numbers attained at stationary phase; this medium was bacteriostatic for strains SE5 and SE7. Fig. 2 illustrates the growth displayed by strain SE5. The growth of strain SE5 in ATFS+LZ containing 31.25 μM of EDTA was similar to the growth of this strain in ATFS+LZ alone and significantly (p < 0.001) greater than that in ATFS+apoLF+LZ (Table 2). This indicates that EDTA potentially contaminating apolactoferrin (< 31.25 μM by spectrophotometry) was not responsible for the synergy displayed between apolactoferrin and lysozyme.

The effect of serum on growth

Table 3 shows the extent of growth in ATFS unsupplemented with serum (ATF) and ATFS +apoLF+LZ lacking serum (ATF+apoLF+LZ). Comparing growth of strains SE4 and SE13 in ATF with that in ATF+apoLF+LZ, there was a significant increase (p < 0.0001) in killing. For strains SE5 and SE7, there was a significant increase in bacterial numbers (p < 0.01) in ATF compared with that in ATF+apoLF+LZ. Growth in medium lacking serum (Table 3) was compared with that in serum-supple-
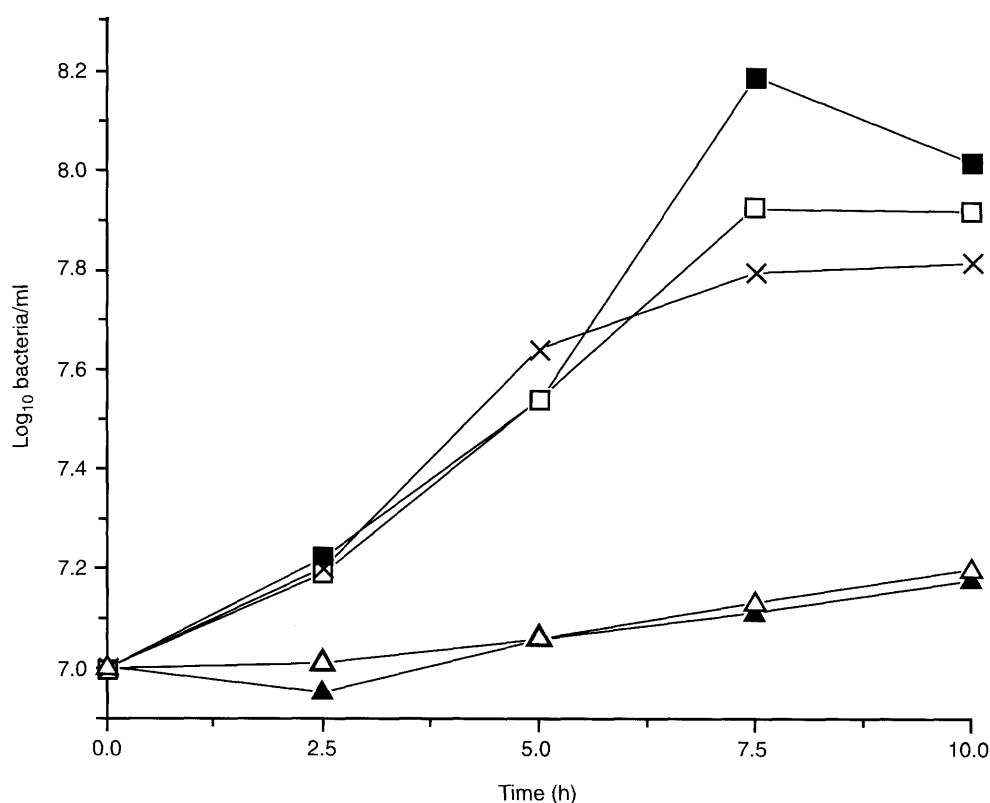

Fig. 2. Growth of strain SE5 over 10 h in artificial tear fluid supplemented with serum and lysozyme (ATFS+LZ, ■); serum, hololactoferrin and lysozyme (ATFS+holoLF+LZ, □); serum and apolactoferrin (ATFS+apoLF, X); serum, apolactoferrin and lysozyme (ATFS+apoLF+LZ, ▲); or apolactoferrin and lysozyme (ATF+apoLF+LZ, △). Results represent the mean of six experiments.

Table 3. Effect of serum depletion of artificial tear fluid, with or without apolactoferrin and lysozyme, on viability of *S. epidermidis* strains

| Strain no. | Mean (SD) difference in bacterial numbers ($\log_{10}$ bacteria/ml)* in | |
	ATF	ATF+apoLF+LZ
SE4	−0.07 (0.06)[†]	−1.78 (0.03)
SE5	+1.08 (0.29)[‡]	+0.20 (0.08)
SE7	+0.60 (0.24)	+0.11 (0.06)
SE13	−0.03 (0.03)	−1.65 (0.12)

ATF+apoLF+LZ showed a significant (p < 0.01) difference in the final bacterial numbers from ATF for all strains.
*[†‡] See footnote to Table 2.

mented medium (Table 2). Bacteriostasis was maintained for strains SE4 and SE13 in ATF, with a statistically increased (p < 0.01) survival compared with the bactericidal effect observed in ATFS. Conversely, ATF+apoLF+LZ caused a statistically increased (p < 0.0001) bactericidal effect on strains SE4 and SE13 compared with its serum-supplemented counterpart and growth curves were similar, but with continued killing after 7.5 h in the former medium. Growth of strains SE5 and SE7 was not statistically different in ATF compared with ATFS or in ATF+apoLF+LZ compared with its serum-supplemented counterpart, as illustrated in Fig. 2 for strain SE5, and similar growth curves were obtained.

The effect of hololactoferrin on growth

The effect of supplementing ATFS with hololactoferrin (holoLF) is shown in Table 4. The bactericidal effect on strains SE4 and SE13 observed in ATFS+holoLF, which was slight but gradual for 7.5 h, was further increased (p < 0.001) in ATFS+holoLF+LZ and continued for 10 h. No significant data were obtained comparing growth of strains SE5 and SE7 in ATFS+holoLF and ATFS+holoLF+LZ. Growth in medium supplemented with hololactoferrin (Table 4) was compared with medium containing apolactoferrin (Table 2). There was no statistical difference in the growth of strain SE5 in ATFS+holoLF compared with ATFS+apoLF and similar growth curves were ob-

Table 4. Effect of hololactoferrin on bacterial numbers of *S. epidermidis* strains grown in artificial tear fluid with or without lysozyme

| Strain no. | Mean (SD) difference in bacterial numbers ($\log_{10}$ bacteria/ml)* in | |
	ATFs+holoLF	ATFs+holoLF+LZ
SE4	−0.35 (0.17)[†]	−0.91 (0.05)
SE5	+0.95 (0.13)[‡]	+0.92 (0.16)
SE7	+0.81 (0.10)	+0.67 (0.16)
SE13	−0.36 (0.10)	−0.86 (0.06)

ATFs+holoLF+LZ showed a significant (p < 0.001) difference in the final bacterial numbers from ATFs+holoLF for strains SE4 and SE13.
*[†‡] See footnote to Table 2.

served. However, the growth of strain SE7 in ATFS+holoLF was increased statistically (p < 0.001) compared with growth in ATFS+apoLF. The antimicrobial effect of ATFS+apoLF+LZ on strains SE4, SE5 and SE7 was lost when holoLF was substituted for apoLF (p < 0.05) with growth curves more closely resembling those observed without lactoferrin supplementation, as depicted in Fig. 2 for strain SE5. Although a similar effect was seen for strain SE13, it was not significant.

Discussion

This study has shown that apolactoferrin and lysozyme, at concentrations found in tears, exert antimicrobial synergy against isolates of *S. epidermidis*. An additive antimicrobial effect of lactoferrin and lysozyme toward various gram-negative bacteria has been shown [16], similar to the synergy displayed in this study against some *S. epidermidis* strains. Lactoferrin is a highly cationic protein [12] and this may be essential to its mechanism of action. It has been shown that lactoferrin is able to interfere with the LPS layer of gram-negative bacteria, increasing its permeability [12], in a similar way to that of EDTA, polymyxin B [12] and defensins [25]. This might allow greater accessibility of other antimicrobial factors such as lysozyme into the bacteria [16]. It is not known how lactoferrin affects gram-positive bacteria; however, its polycationic properties suggest that it may interact with the anionic cell wall teichoic acid. Lactoferrin has been shown to have an antimicrobial effect similar to that of other polycationic peptides such as defensins [25] and polymyxin B [12]. One group of workers [11] has developed a synthetic analogue of the suspected active site of lactoferrin, which they have called lactoferricin. This peptide is also highly cationic and has been shown to have an increased antimicrobial effect *in vitro* compared with lactoferrin. The authors have speculated that the protease cleavage of lactoferrin to smaller peptide fragments may occur *in vivo*. Plasmin present in tears [26] may similarly cleave lactoferrin into these fragments.

Iron saturation has been shown to reverse the antimicrobial effects of apolactoferrin and it has been speculated that these antimicrobial properties occur independently of iron chelation [8, 9]. Similarly, the present study has determined that hololactoferrin does not induce a synergic antimicrobial effect with lysozyme for *S. epidermidis*. This might be caused by the conformational change observed with ferric ion binding of lactoferrin [27]. Previous studies have shown that *S. epidermidis* ATCC 14990 requires 0.5 μM iron (0.03 μg/ml) for growth in a defined medium [28]. This amount of free ferric ions was probably available in all artificial tear fluid types, even taking into account the amount bound in the lactoferrin molecules of ATFS+apoLF+LZ.

In-vitro studies have shown that apolactoferrin has an antimicrobial effect [8, 9] on various bacteria, including S. epidermidis [10]. The results of the present study indicated that apolactoferrin individually has no effect on strains of S. epidermidis grown in ATFS. This may be caused by a difference in growth conditions. One study [16] demonstrated that increasing the osmolality of the growth medium led to a decreased killing ability of apolactoferrin, an effect which was absent above 60 mOsm. Most body fluids have a higher osmolality than this. Tears, for example, have an osmolality of 304 mOsm [29] and the artificial tear fluid used in this study had a similar osmolality of 336 mOsm. This may explain the difference in results.

In summary, the growth of S. epidermidis strains in an artificial tear fluid was synergically reduced by the addition of apolactoferrin and lysozyme. This differed from the results of previous work in that apolactoferrin alone did not affect growth, which may reflect the type of growth medium used in the present study. This synergy was dependent on the iron limitation of lactoferrin, and serum augmented the bactericidal action for certain strains. Strain variation was evident, suggesting differences in either the type or amount of target sites expressed.

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