Glycerol monolaurate inhibits lipase production by clinical ocular isolates without affecting bacterial cell viability

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Abstract

PURPOSE. We sought to determine the relative lipase production of a range of ocular bacterial isolates and to assess the efficacy of glycerol monolaurate (GML) in inhibiting this lipase production in high lipase-producing bacteria without affecting bacterial cell growth. METHODS. Staphylococcus aureus, Staphylococcus epidermidis, Propionibacterium acnes, and Corynebacterium spp. were inoculated at a density of 106/mL in varying concentrations of GML up to 25 μg/mL for 24 hours at 378C with constant shaking. Bacterial suspensions were centrifuged, bacterial cell density was determined, and production of bacterial lipase was quantified using a commercial lipase assay kit. RESULTS. Staphylococcus spp. produced high levels of lipase activity compared with P. acnes and Corynebacterium spp. GML inhibited lipase production by Staphylococcal spp. in a dosedependent manner, with S. epidermidis lipase production consistently more sensitive to GML than S. aureus. Glycerol monolaurate showed significant ($P < 0.05$) lipase inhibition above concentrations of 15 μg /mL in S. aureus and was not cytotoxic up to 25 μg /mL. For S. epidermidis, GML showed significant ($P < 0.05$) lipase inhibition above 7.5 μg /mL. CONCLUSIONS. Lipase activity varied between species and between strains. Staphylococcal spp. produced higher lipase activity compared with P. acnes and Corynebacterium spp. Glycerol monolaurate inhibited lipase production by S. aureus and S. epidermidis at concentrations that did not adversely affect bacterial cell growth. GML can be used to inhibit ocular bacterial lipase production without proving detrimental to commensal bacteria viability.

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Glycerol Monolaurate Inhibits Lipase Production by Clinical Ocular Isolates Without Affecting Bacterial Cell Viability

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Purpose. We sought to determine the relative lipase production of a range of ocular bacterial isolates and to assess the efficacy of glycerol monolaurate (GML) in inhibiting this lipase production in high lipase–producing bacteria without affecting bacterial cell growth.

Methods. *Staphylococcus aureus, Staphylococcus epidermidis, Propionibacterium acnes,* and *Corynebacterium* spp. were inoculated at a density of 10^6/mL in varying concentrations of GML up to 25 µg/mL for 24 hours at 37°C with constant shaking. Bacterial suspensions were centrifuged, bacterial cell density was determined, and production of bacterial lipase was quantified using a commercial lipase assay kit.

Results. *Staphylococcus* spp. produced high levels of lipase activity compared with *P. acnes* and *Corynebacterium* spp. GML inhibited lipase production by *Staphylococcal* spp. in a dose-dependent manner, with *S. epidermidis* lipase production consistently more sensitive to GML than *S. aureus*. Glycerol monolaurate showed significant ($P < 0.05$) lipase inhibition above concentrations of 15 µg/mL in *S. aureus* and was not cytotoxic up to 25 µg/mL. For *S. epidermidis*, GML showed significant ($P < 0.05$) lipase inhibition above 7.5 µg/mL.

Conclusions. Lipase activity varied between species and between strains. *Staphylococcal* spp. produced higher lipase activity compared with *P. acnes* and *Corynebacterium* spp. Glycerol monolaurate inhibited lipase production by *S. aureus* and *S. epidermidis* at concentrations that did not adversely affect bacterial cell growth. GML can be used to inhibit ocular bacterial lipase production without proving detrimental to commensal bacteria viability.

Keywords: bacterial lipase, dry eye, microbiome

Dry eye is one of the least well-defined disorders of the ocular surface, with characterization, prevalence, and etiology constantly reassessed in the literature. In 2007, the International Workshop on Dry Eye adopted the concept of dry eye disease as a multifactorial disease of the tears and ocular surface accompanied by increased osmolarity of the tear film and inflammation of the ocular surface.[1] Meibomian gland dysfunction (MGD) is believed to play a leading role in the development of dry eye and has been suggested as the leading cause of this disorder.[2] The consensus definition of MGD encompasses terminal gland obstruction and alterations of the tear film, clinically apparent inflammation, and ocular surface disease.[2]

The pathophysiology of MGD is complex, and the role of bacteria or parasites in the etiology of MGD remains controversial. It is well known that commensal bacteria such as *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Corynebacterium* spp., and *Propionibacterium acnes* are associated with, and contribute to, the pathologic course of chronic blepharitis, but to date no reports implicate bacteria in the primary pathologic process of MGD.[3] Paralleling the rising interest in the human microbiome in health and disease,[4] the role of microbial flora in ocular health and disease is beginning to be appreciated.[5] Quorum sensing has been implicated in perturbation of the ocular microbiome (as cause or effect of an altered eye lid microbial environment), leading to a pathologic imbalance of commensal bacteria.[6] This imbalance can trigger release of exotoxins, lipopolysaccharides, and lipase activation, causing eyelid inflammation and lipidic changes (including the release of free fatty acids), leading to reduced tear film stability.[6]

Bacterial lipases can alter tear lipids, leading to increased cytokine expression and inflammation associated with dry eye disease. Numerous studies[7–10] have reported the benefit of tetracycline and its analogues in treating meibomianitis and blepharitis, demonstrating a persistent reduction in *S. aureus* and *S. epidermidis* colonization after 3 months. Tetracyclines are primarily used in MGD treatment for their anti-inflammatory and lipid-regulating effects.[11] However, long-term use of antibiotics has deleterious side effects including emergence of resistance and elimination of commensal bacteria that can lead to undesirable side effects resulting from the collapse of the

eye's own immunodefense system.[12] The present study explores the potential of a compound that, in the absence of biocidal activity, inhibits bacterial lipases that can alter tear lipids.

Generally recognized as safe by the US Food and Drug Administration for topical use at doses up to 100 mg/mL,[13] glycerol monolaurate (GML) is a fatty acid monoester that has long been used in the food and cosmetics industries as an emulsifier and preservative.[14] At concentrations lower than those required for biocidal action, GML blocks bacterial release of virulence factors including bacterial lipase.[15]

We sought to determine lipase production in a range of *Staphylococcus* spp., *P. acnes*, and *Corynebacterium* spp. ocular isolates and to characterize the inhibitory actions of GML on bacterial lipase production in the absence of biocidal activity.[16] We found that lipase production varies in a species- and strain-specific manner and that GML can inhibit lipase production without affecting bacterial cell viability.

MATERIALS AND METHODS

Chemicals

Glycerol monolaurate was purchased from (Corden Pharma Pty Ltd., Liestal, Switzerland) and dissolved in 100% ethanol to a final stock concentration of 200 mg/mL and stored at −20°C.

Growth of Bacterial Strains

In total, clinical isolates primarily comprising ocular commensal species were examined. These included 37 isolates of *Staphylococcus aureus,* 31 of *Staphylococcus epidermidis*, 25 isolates of *Propionibacteria acnes*, and 8 of *Corynebacterium* spp. regrown from frozen stocks on chocolate agar for 24 hours in an ambient air incubator or, for *P. acnes,* an anaerobic jar at 37°C.

Production of Bacterial Lipases

Single colonies were inoculated into 10 mL tryptone soy broth (TSB; Oxiod, Thermo Fisher Scientific Australia Pty Ltd., Adelaide SA, Australia) and incubated under the appropriate conditions. After 24 hours, OD_{660nm} was measured to assess bacterial growth. Extracellular lipase enzymes in the supernatant were separated from bacterial cells by centrifugation (956g, 10 minutes). Supernatants were collected and filtered through a 0.22-μm filter to remove any residual bacterial cells and stored at −20°C till further use.

Analyzing Lipase Activity

Lipase activity in culture supernatant (as a measure of lipase production) was quantified using a commercially available kit (QuantiChrom Lipase Assay Kit; BioAssay Systems, Hayward, CA, USA). The colorimetric assay was performed as per manufacturer's instructions. Lipase activity was measured in units per liter.

Lipase Enzymic Activity in the Presence of GML

Staphylococcus aureus 020 was streaked on chocolate agar plates and incubated at 37°C for 24 hours. Resulting colonies were inoculated into 3 mL TSB and incubated at 37°C for 24 hours. Resulting bacterial suspensions were centrifuged to collect supernatant (containing lipases). These fractions were then filtered through a 0.22-μm filter to remove contaminating cell debris. To this "conditioned" supernatant was added GML up to 500 μg/mL. These supernatant + GML fractions were

incubated 24 hours at 37°C. Lipase assay detection was performed as described above. Control comprised filtered bacterial supernatant from *S. aureus* 020 was grown overnight in the presence of GML (20 μg/mL) and similarly assayed for lipase activity.

GML Inhibition of Bacterial Lipase Production

Two *S. aureus* and two *S. epidermidis* isolates with the highest lipase activities were selected and grown for 24 hours at 37°C in the presence or absence of GML (0–25 μg/mL) diluted in TSB. Lipase production (measured as lipase activity) was quantitated as above.

Statistical Analysis

The Kolmogorov-Smirnov test and the Shapiro-Wilk test were used to test for normality of lipase production between isolates of *S. aureus, S. epidermidis, P. acnes*, and *Corynebacterium* spp. To overcome limitations due to determined nonnormality or small sample size, bootstrapping (Statistical Package for Social Sciences; http://www-01.ibm.com/software/analytics/spss/products/statistics/) was used to obtain unbiased estimates of 95% confidence intervals for the population median. Significant differences in lipase production of each of the four groups was determined using the nonparametric test of Oyeka and Okeh.[17] This test is based on the statistic Q calculated from the ranks of items in the combined sample of size $n = n1 + n2$ (where $n1$ and $n2$ are ≥ 5)

$$Q = \frac{12}{n(n+1)} \sum_{i=1}^{2} \frac{R_i^2}{n_i} - n(n+1),$$

where R_i is the sum of ranks in the combined sample of items from sample i, and Q is asymptotically χ^2 distributed with one degree of freedom.

Lipase inhibition results are presented as mean ± SD. The Student's *t*-test was performed for comparison of data comprising two groups at the $P < 0.05$ level of significance.

RESULTS

Bacterial Lipase Production

Levels of bacterial cell lipase production varied between species and within strains (Fig. 1). Because the distribution of lipase activities is positively skewed (Fig. 1) as determined by the Kolmogorov-Smirnov and Shapiro-Wilk tests, to determine significant differences in lipase production, we compared median lipase production (lower and upper 95% confidence limits, respectively; Fig. 2). Potential outliers as identified in the boxplot were included in our analysis as a more conservative test for significant differences because, as discussed by Hubert and Vandervieren,[18] the SPSS Boxplot procedure is based on the tacit assumption that the data are drawn from a normal distribution and, in the case of positively skewed distributions (as our data are), typically misclassifies approximately 8% of the higher end values as possible outliers.

Median lipase production for *S. aureus* was significantly different to medians of *S. epidermidis, P. acnes*, and *Corynebacterium* spp. Median lipase production for *S. epidermidis* was similarly significantly different to median production for each of the other bacterial groups tested (Fig. 2). The difference in median lipase production for *P. acnes* and *Corynebacterium* spp. did not reach significance. Similarly, median lipase production was highest in *S. aureus,* followed by *S. epidermidis* isolates, *P. acnes*, and *Corynebacterium* spp.,
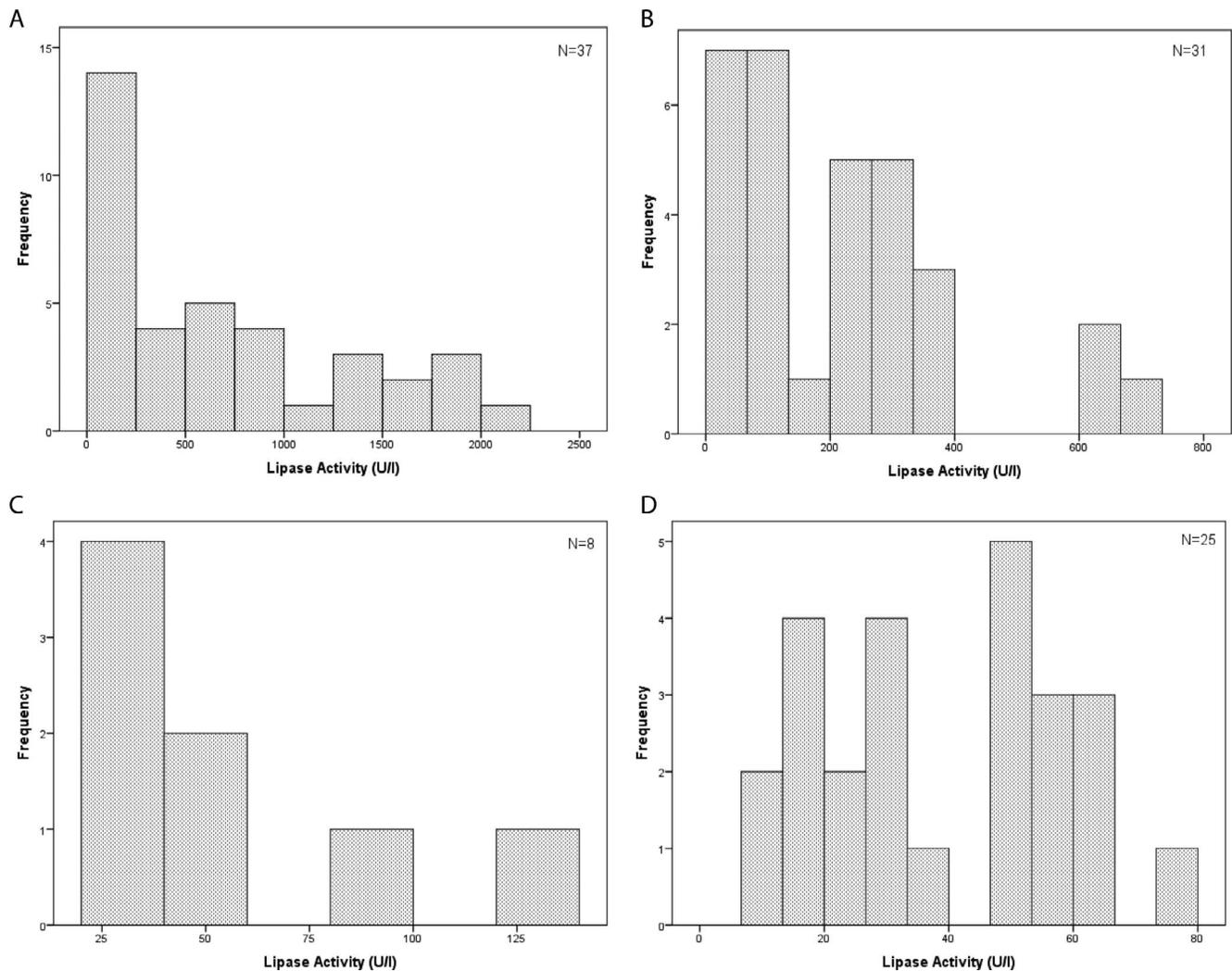
FIGURE 1. Lipase activity histograms showing distribution of lipase production in clinical isolates (**A**) *S. aureus*, (**B**) *S. epidermidis*, (**C**) *P. acnes*, and (**D**) *Corynebacterium* spp.

respectively. Lipase production by *Staphylococcus* spp. was generally much higher than that of *P. acnes* and *Corynebacterium* spp. isolates. Because lipase production by *P. acnes* and *Cornyebacterium* spp. was minimal, none of these isolates were tested further.

### Determination of Lipase Enzyme Stability in the Presence of GML

We sought to determine the mode of action of GML: whether inhibition of lipase production or inhibition of lipase activity (Fig. 3). In the presence of conditioned supernatant containing bacterially expressed lipase from overnight cultures (*S. aureus* 020), increasing concentrations of GML had no inhibitory effect on lipase activity. This is in stark contrast to the inhibition of lipase production evident in the bacteria control in which *S. aureus* was grown overnight in the presence of GML (20 μg/mL). That there was little lipase activity detected when *S. aureus* was grown in the presence of GML compared with the lipase activity remaining in the supernatants containing expressed lipase incubated with GML illustrates the mechanism of action of GML resides in inhibition of production rather than inhibition of lipase activity per se.

GML Inhibition of Bacterial Growth

Bacterial toxicity of GML was titrated to determine the bacteriocidal threshold. We chose to monitor the bacteriocidal activity of increasing concentrations of GML on the two highest lipase producing isolates from *S. aureus* and *S. epidermidis*, respectively: *S. aureus* 020 and 134, and *S. epidermidis* 001 and 024. We observed that growth of both clinical isolates of *S. aureus* remained unaffected in the presence of GML up to 25 μg/mL (Fig. 4A). *Staphylococcus epidermidis* exhibited a strain-dependent sensitivity to the growth inhibition of GML, with *S. epidermidis* 024 exhibiting a slight decline in growth when incubated in concentrations of GML greater than 15 μg/mL (Fig. 4B).

Effect of Varying Concentrations of GML on Bacterial Lipase Production

Glycerol monolaurate exerted a dose-dependent inhibition of bacterial lipase production by *S. aureus* 020 and 134 (Fig. 4A) and *S. epidermidis* 001 and 024 (Fig. 4B). Glycerol monolaurate concentrations of 15 μg/mL and above induced significant ($P < 0.05$) inhibition of lipase production by both
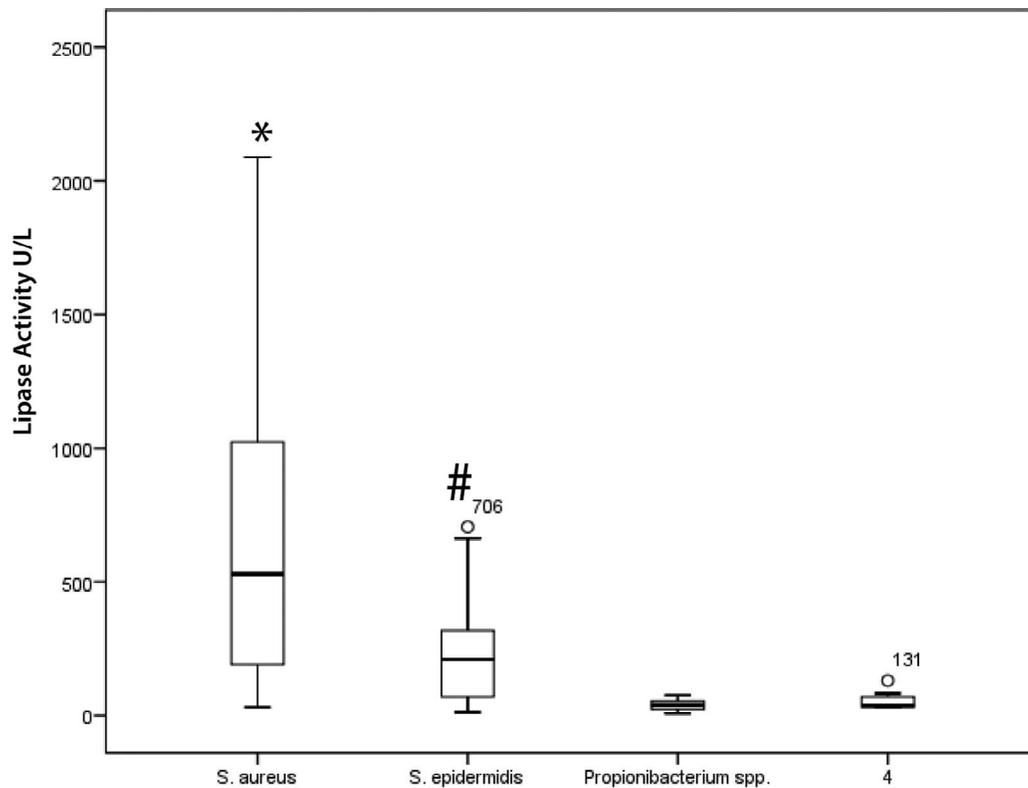
Species	Sample Median	LCL95% for Median*	UCL95% for Median*
S. aureus.	359	223	834
S. epidermidis	210	100	252
P. acnes	31	26	48
Corynebacterium spp.	38	31	83

FIGURE 2. Lipase activity of clinical isolates. Box plot showing relative lipase activities of *S. aureus, S. epidermidis, P. acnes*, and *Corynebacterium* spp. Lipase activity was determined as in Materials and Methods. * and # indicate significant difference $P < 0.05$ from each of the other groups.

S. aureus strains; lipases were reduced by approximately 60% in the presence of GML at 15 µg/mL and almost 90% at 25 µg/mL GML.

   Staphylococcus epidermidis lipase production was consistently more sensitive to GML than were *S. aureus* isolates. Glycerol monolaurate at 10 µg/mL for *S. epidermidis* 001 and as low as 5 µg/mL for *S. epidermidis* 024 significantly ($P < 0.05$) reduced bacterial lipase production relative to no GML. The lipase inhibition was concentration dependent for both strains.

DISCUSSION

Lipase production activities were determined for *S. aureus,* coagulase-negative staphylococci, *P. acnes,* and *Corynebacterium* spp. The level of bacterial lipase activity varied widely between the ocular species and strains examined: *P. acnes* and *Corynebacterium* spp. produced relatively low lipase activity, whereas the *Staphylococcus* spp. produced relatively high lipase activities.

   The finding of low lipase production by *P. acnes* and *Corynebacterium* spp. is interesting in light of evidence from Lee et al.[19] that, in blepharitis subjects, the relative abundance of *P. acnes* in the ocular microbiome was decreased compared with healthy subjects, whereas that of *Staphylococcus* spp. was increased. In an earlier study,[20] *Corynebacterium* spp.

were more likely to be detected in patients suffering from blepharitis who presented without dry eye than those blepharitis patients with dry eye. The species we examined in the present study are the most frequently isolated from blepharitis patients.[21]

   Our observations are consistent with the idea that a higher proportion of low lipase producing bacteria might be protective against tear film lipid degradation. Conversely, an overrepresentation of high lipase–producing bacteria might destabilize the tear film. In the present study, *S. aureus* and *S. epidermidis* showed a divergent range of lipase activities, with *S. aureus* generally exhibiting higher lipase activities than the *S. epidermidis* isolates. It has been proposed that *S. epidermidis* might have evolved not to cause disease but to maintain a benign relationship with the host[22] and further, might play a probiotic role in preventing colonization by more severe pathogens, such as *S. aureus*.[23]

   Culture independent analyses of the ocular microbiome—in contrast to the relatively low culturable bioburden on the eye—are reporting a robust community of complex dynamics.[19] As with other regions of the body, microbial dysbiosis characterized by shifts in populations and a loss of species diversity—a feature of chronic disease[24]—can occur in the ocular microbiome. How these communities shift in chronic dry eye is a matter of great interest. Zegans and Van Gelder[25]
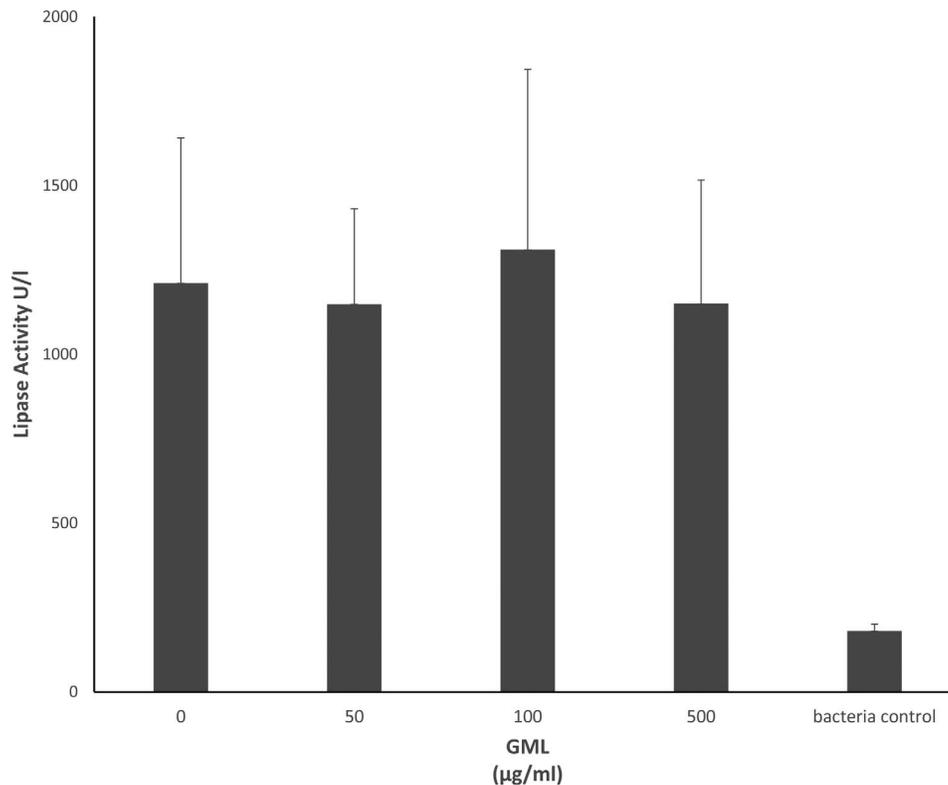
FIGURE 3. Stability of bacterial lipase enzyme activity in the presence of GML. Filtered supernatant from overnight cultures of *S. aureus* 020 grown in the absence of GML was incubated with increasing concentrations of GML, and lipase activity was assessed. Control culture *S. aureus* 020 was grown in the presence of GML (20 μg/mL), and lipase activity was similarly assessed.

recently suggested that many idiopathic ocular disorders, including dry eye disease, that are characterized by an inflammatory component might be in part due to dysregulation of the ocular microbiota and the concomitant release of specific toxins or the triggering of a large immune response. This is consistent with our hypothesis of the contribution of dysregulation of lipase-producing commensal bacteria to the etiology of dry eye disease. *Staphylococcus epidermidis* (a ubiquitous constituent of commensal microflora[26]), along with the more virulent *S. aureus,*[22] can become opportunistic under the appropriate conditions. Exploring dry eye disease in terms of a tipping point in the ecologic balance of the ocular microbiota might shed new light on this disorder. We previously reported (Zhu H, et al. *IOVS* 2013;54:ARVO E-Abstract 926) that in normal subjects (without dry eye or other ocular disease), the average number of bacteria recovered from eyes of younger females was lower than that of older females or males and that this reduced bacterial burden correlated with clinical measures of increased meibum quality and function.

Lipase inhibitors exert a specific bacteriostatic action on Gram-positive bacteria through a delay in growth initiation and a decrease in maximum growth achieved.[27,28] Concentrations at which GML was observed to inhibit growth are in keeping with previous reports of minimal inhibitory concentrations (between 10 and 63 μg/mL) of GML against *S. aureus* strains; the magnitude of the range was ascribed to inherent strain variation, inoculum size, and culture conditions.[13,16,28,29]

Bacterial lipases in growing culture can hydrolyze GML to lauric acid.[14] Lauric acid is more stable in the presence of bacterial lipases and similarly inhibits staphylococcal exoprotein production.[14] In the skin, lauric acid is released from sebaceous triglycerides through bacterial lipase action,[30] acting to protect against pathogens and drying. We speculate that a similar action

might occur in the tear film and meibomian triglycerides once the meibum has been exposed to the ocular microbiota and that a balanced presence of lipase producing *S. epidermidis* is part of a healthy ocular environment. Bacteriostasis could check overgrowth of opportunistic pathogens and help rebalance the commensal microflora, producing sufficient lipase to effect a steady concentration of lauric acid (as a product of hydrolysis of meibum) while limiting deleterious lipase action on tear film stability. In support of this, in 1982, Doughtery and McCulley[31] reported significantly reduced amounts of C12:0 fatty acids in mixed seborrheic/staphylococcal blepharitis and meibomian seborrheic blepharitis compared with normals, whereas recently, Arita et al.[32] reported the presence of C12:0 fatty acids in meibum of dry eye patients and indicated a continual decrease of this fatty acid species as meibum moved from clear to cloudy to yellow, suggesting that healthier meibum is associated with increased concentration of C12:0 fatty acids.

Tetracycline analogues have been used to treat dry eye symptoms.[8] The mechanism of action of these analogues in treating meibomianitis is not yet fully elucidated; however, tetracycline analogues are known to inhibit bacterial lipase activity, inducing a concomitant reduction in free fatty acids generated from hydrolysis of wax and sterol esters.[10,33,34] As it is well known that long-term use of antibiotics has deleterious consequences including increased resistance and a potentially catastrophic shift in the commensal flora, lipase inhibition in the absence of antimicrobial action is preferable.

Use of GML to reduce virulence factors in toxic shock syndrome has shown GML does not alter mucosal or microflora integrity and stabilizes mammalian cell membranes leading to reduced production of proinflammatory cytokines from epithelial cells.[35,36] As GML is safe for use on skin and mucosal surfaces[13] and is considered to be an ocular nonirritant,[37] it
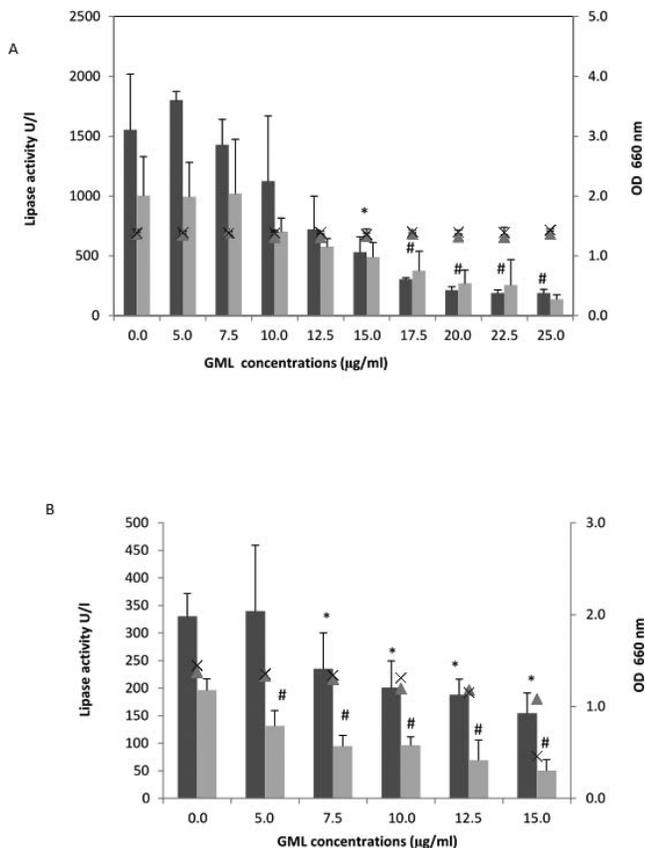
FIGURE 4. Effect of increasing concentrations of GML on bacterial cell viability and lipase production. (A) Glycerol monolaurate proved nontoxic to *S. aureus* 020 and *S. aureus* 134 up to 25 μg/mL GML and inhibited the bacterial lipases in a dose-dependent manner. * and # indicate significant differences ($P < 0.05$) in the lipase production in the presence of GML by *S. aureus* 020 and *S. aureus* 134, respectively, compared with their lipase production in the absence of GML. ■ *S. aureus* 020 ■ *S. aureus* 134 ▲ *S. aureus* 020 growth ✕ *S. aureus* 134 growth. (B) Glycerol monolaurate was nontoxic to *S. epidermidis* 001 and 024 up to a concentration of 15 μg/mL. Glycerol monolaurate inhibited the bacterial lipases in a dose-dependent manner. * and # show significant differences ($P < 0.05$) in the lipase production in the presence of GML by *S. epidermidis* 001 and *S. epidermidis* 024, respectively, compared with their lipase production in the absence of GML. ■ *S. epidermidis* 001 ■ *S. epidermidis* 024 ▲ *S. epidermidis* 001 growth ✕ *S. epidermidis* 024 growth.

could potentially play a role in protecting against bacterially mediated tear film instability.

The role of lipases in bacterial pathogenesis is incompletely understood; however, these enzymes are thought to contribute to overall fitness of the pathogens by providing a nutrition source and releasing fatty acids to which the bacteria can adhere.[26,38] Additionally, *S. epidermidis* lipases appear to play a role in limiting colonization by *S. aureus*, and *S. aureus* lipases interfere with phagocytosis of *S. aureus* by granulocytes.[39] Controlling lipase production that results from overgrowth of opportunistic pathogens could reduce overall fitness of these bacteria and assist in rebalancing the ocular microbiome.

Interestingly, Dave et al.[40] provide evidence that efficacy of azithromycin administration for blepharitis might not be due to its antibacterial properties but more from the outcompeting of *S. aureus* by *S. epidermidis*, suggesting that azithromycin renders *S. aureus* "less fit" relative to *S. epidermidis*. Application of GML to the ocular surface might similarly affect

the fitness of the higher lipase producer (*S. aureus*) relative to that of *S. epidermidis*.

As eloquently expressed by Andersen et al.: "To study bacterial cells, we must remove them from the host environment...which may release them from selective pressures that we wish to understand."[41] Bacterial virulence factors (including *S. aureus* lipases) are regulated largely through quorum sensing.[42] As such, expression of virulence determinants in isolated strains could differ substantially compared with behaviors within the context of community.

Hence, a limitation of the present work is the determination of bacterial lipase production by isolated strains. Because more than 600 genera[43] have been identified from conjunctiva of healthy subjects, in vitro modeling of community interactions would be extremely complex. Whole community shotgun genomics or proteomics with in silico analysis of potential community interactions could offer a powerful alternative.[44,45] In addition, studying lipase production and the influence of GML in the context of the dynamics of the in vivo bacterial community is a natural extension of these investigations.

The significant finding in our study is that GML inhibits lipase production by *S. aureus* and *S. epidermidis* at low concentrations without adversely affecting bacterial cell growth and thus potentially could be used in vivo to inhibit lipases produced by ocular isolates without proving detrimental to commensal bacteria, leading to a more stable tear film. Judicious choice of a bacterial lipase inhibitor that protects the commensal ocular flora could potentially reduce the progression of dry eye disease. Improved understanding of the dynamics of the ocular microbiome in health and disease, including the role of bacterial lipases and the action of GML in vivo, might add considerably to our understanding of dry eye disease.

References

1. Lemp MA, Baudouin C, Baum J, et al. The definition and classification of dry eye disease: Report of the Definition and Classification Subcommittee of the International Dry Eye Workshop (2007). *Ocul Surf*. 2007;5:75–92.
2. Nichols KK, Foulks GN, Bron AJ, et al. The International Workshop on Meibomian Gland Dysfunction: Executive summary. *Invest Ophthalmol Vis Sci*. 2011;52:1922–1929.
3. Geerling G, Tauber J, Baudouin C, et al. The International Workshop on Meibomian Gland Dysfunction: Report of the Subcommittee on Management and Treatment of Meibomian Gland Dysfunction. *Invest Ophthalmol Vis Sci*. 2011;52:2050–2064.
4. Cho I, Blaser MJ. The human microbiome: At the interface of health and disease. *Nat Rev Genet*. 2012;13:260–270.
5. Miller D, Iovieno A. The role of microbial flora on the ocular surface. *Curr Opin Allergy Clin Immunol*. 2009;9:466–470.
6. Veldman P, Colby K. Current evidence for topical azithromycin 1% ophthalmic solution in the treatment of blepharitis and blepharitis-associated ocular dryness. *Int Ophthalmol Clinics*. 2011;51:43–52.
7. Aronowicz JD, Shine WE, Oral D, Vargas JM, McCulley JP. Short term oral minocycline treatment of meibomianitis. *Br J Ophthalmol*. 2006;90:856–860.

8. Dougherty JM, Mcculley JP, Silvany RE, Meyer DR. The role of tetracycline in chronic blepharitis: Inhibition of lipase production in *Staphylococci*. *Invest Ophthalmol Vis Sci*. 1991;32:2970–2975.

9. Shine WE, McCulley JP, Pandya AG. Minocycline effect on meibomian gland lipids in meibomianitis patients. *Exp Eye Res*. 2003;76:417–420.

10. Ta CN, Shine WE, McCulley JP, Pandya A, Trattler W, Norbury JW. Effects of minocycline on the ocular flora of patients with acne rosacea or seborrheic blepharitis. *Cornea*. 2003;22:545–548.

11. Srinivasan S, Menzies KL, Sorbara L, Jones LW. Imaging meibomian glands on a patient with chalazia in the upper and lower lids: A case report. *Contact Lens Anterior Eye*. 2013;36:199–203.

12. Vanderlaan DG, Schultz CL, Brown-Skrobot SK, inventors; Johnson & Johnson Vision Products, Inc., assignee. Ophthalmic Lens with Anti-Toxin Agent. US patent 5,472,703. December 5, 1995.

13. Lin YC, Schlievert PM, Anderson MJ, et al. Glycerol monolaurate and dodecylglycerol effects on *Staphylococcus aureus* and toxic shock syndrome toxin-1 in vitro and in vivo. *PloS One*. 2009;4:e7499.

14. Ruzin A, Novick RP. Equivalence of lauric acid and glycerol monolaurate as inhibitors of signal transduction in *Staphylococcus aureus*. *J Bacteriol*. 2000;182:2668–2671.

15. Schlievert PM, Deringer JR, Kim MH, Projan SJ, Novick RP. Effect of glycerol monolaurate on bacterial growth and toxin production. *Antimicrob Agents Chemother*. 1992;36:626–631.

16. Holland KT, Taylor D, Farrell AM. The effect of glycerol monolaurate on growth of, and production of toxic shock syndrome toxin-1 and lipase by, *Staphylococcus aureus*. *J Antimicrob Chemother*. 1994;33:41–55.

17. Oyeka ICA, Okeh UMW. Statistic: Ties adjusted two sample test. *Open Access Sci. Rep*. 2013;2:639.

18. Hubert M, Vandervieren E. An adjusted boxplot for skewed distributions. *Comput Stat Data Anal*. 2008;52:5186–5201.

19. Lee SH, Oh DH, Jung JY, Kim JC, Jeon CO. Comparative ocular microbial communities in humans with and without blepharitis. *Invest Ophthalmol Vis Sci*. 2012;53:5585–5593.

20. Groden LR, Murphy B, Rodnite J, Genvert GI. Lid flora in blepharitis. *Cornea*. 1991;10:50–53.

21. Dougety JM, McCulley JP. Comparative bacteriology of chronic blepharitis. *Br J Ophthalmol*. 1984;68:524–528.

22. Otto M. *Staphylococcus epidermidis*: The ''accidental'' pathogen. *Nature Rev Microbiol*. 2009;7:555–567.

23. Lina G, Boutite F, Tristan A, Bes M, Etienne J, Vandenesch F. Bacterial competition for human nasal cavity colonization: Role of staphylococcal Agr alleles. *Appl Environ Microbiol*. 2003;69:18–23.

24. Kerr CA, Grice DM, Tran CD, et al. Early life events influence whole-of-life metabolic health via gut microflora and gut permeability. *Crit Rev Microbiol*. 2015;41:326–340.

25. Zegans ME, Van Gelder RN. Considerations in understanding the ocular surface microbiome. *Am J Ophthalmol*. 2014;158:3.

26. Grice EA, Segre JA. The skin microbiome. *Nat Rev Microbiol*. 2011;9:244–253.

27. Kabara JJ. Lipids as host-resistance factors of human-milk. *Nutr Rev*. 1980;38:65–73.

28. Kelsey JA, Bayles KW, Shafii B, McGuire MA. Fatty acids and monoacylglycerols inhibit growth of *Staphylococcus aureus*. *Lipids*. 2006;41:951–961.

29. Preuss HG, Echard B, Enig M, Brook I, Elliott TB. Minimum inhibitory concentrations of herbal essential oils and monolaurin for gram-positive and gram-negative bacteria. *Molec Cell Biochem*. 2005;272:29–34.

30. Drake DR, Brogden KA, Dawson DV, Wertz PW. Thematic review series: Skin lipids—Antimicrobial lipids at the skin surface. *J Lipid Res*. 2008;49:4–11.

31. Dougherty JM, McCulley JP. Analysis of the free fatty acid component of meibomian secretions in chronic blepharitis. *Invest Ophthalmol Vis Sci*. 1986;27:52–56.

32. Arita R, Mori N, Shirakawa R, et al. Meibum color and free fatty acid composition in patients with meibomian gland dysfunction. *Invest Ophthalmol Vis Sci*. 2015;56:4403–4412.

33. Mates A. Inhibition by tetracycline in the occurrence of extracellular lipase from *Staphylococcus aureus*. *Microbios*. 1973;7:25–29.

34. Mates A. Effect of eight tetracyclines on growth and lipase formation of *Staphylococcus aureus*. *Microbios*. 1974; 10A(suppl):105–110.

35. Schlievert PM, Strandberg KL, Brosnahan AJ, et al. Glycerol monolaurate does not alter Rhesus macaque (*Macaca mulatta*) vaginal lactobacilli and is safe for chronic use. *Antimicrob Agents Chemother*. 2008;52:4448–4454.

36. Peterson ML, Schlievert PM. Glycerol monolaurate inhibits the effects of gram-positive select agents on eukaryotic cells. *Biochemistry*. 2006;45:2387–2397.

37. Johnson W. Final report of the amended safety assessment of glyceryl laurate, glyceryl laurate se, glyceryl laurate/oleate, glyceryl adipate, glyceryl alginate, glyceryl arachidate, glyceryl arachidonate, glyceryl behenate, glyceryl caprate, glyceryl caprylate, glyceryl caprylate/caprate, glyceryl citrate/lactate/ linoleate/oleate, glyceryl cocoate, glyceryl collagenate, glyceryl erucate, glyceryl hydrogenated rosinate, glyceryl hydrogenated soyate, glyceryl hydroxystearate, glyceryl isopalmitate, glyceryl isostearate, glyceryl isostearate/myristate, glyceryl isostearates, glyceryl lanolate, glyceryl linoleate, glyceryl linolenate, glyceryl montanate, glyceryl myristate, glyceryl isotridecanoate/stearate/adipate, glyceryl oleate se, glyceryl oleate/elaidate, glyceryl palmitate, glyceryl palmitate/stearate, glyceryl palmitoleate, glyceryl pentadecanoate, glyceryl polyacrylate, glyceryl rosinate, glyceryl sesquioleate, glyceryl/ sorbitol oleate/hydroxystearate, glyceryl stearate/acetate, glyceryl stearate/maleate, glyceryl tallowate, glyceryl thiopropionate, and glyceryl undecylenate. *Int J Toxicol*. 2004;23(suppl 2):55–94.

38. Longshaw CM, Farrell AM, Wright JD, Holland KT. Identification of a second lipase gene, *Gehd*, in *Staphylococcus epidermidis*: Comparison of sequence with those of other staphylococcal lipases. *Microbiology*. 2000;146(Pt 6):1419–1427.

39. Rollof J, Braconier JH, Soderstrom C, Nilsson-Ehle P. Interference of *Staphylococcus aureus* lipase with human granulocyte function. *Eur J Clin Microbiol Infect Dis*. 1988;7:505–510.

40. Dave SB, Toma HS, Kim SJ. Changes in ocular flora in eyes exposed to ophthalmic antibiotics. *Ophthalmology*. 2013;120:937–941.

41. Andersen SB, Marvig RL, Molin S, Krogh Johansen H, Griffin AS. long-term social dynamics drive loss of function in pathogenic bacteria. *Proc Natl Acad Sci U S A*. 2015;112:10756–10761.

42. Cheung GY, Wang R, Khan BA, Sturdevant DE, Otto M. Role of the accessory gene regulator Agr in community-associated methicillin-resistant *Staphylococcus aureus* pathogenesis. *Infect Immun*. 2011;79:1927–1935.

43. Zhou Y, Holland MJ, Makalo P, et al. The conjunctival microbiome in health and trachomatous disease: A case control study. *Genome Med*. 2014;6:99.

44. Young JC, Pan C, Adams RM, et al. metaproteomics reveals functional shifts in microbial and human proteins during a preterm infant gut colonization case. *Proteomics*. 2015;15:3463–3473.

45. Morowitz MJ, Denef VJ, Costello EK, et al. strain-resolved community genomic analysis of gut microbial colonization in a premature infant. *Proc Natl Acad Sci U S A*. 2011;108:1128–1133.