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Original article

Glycated keratin promotes cellular aggregation and biofilm formation in *Staphylococcus aureus*

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Abstract

Glycation is a non-enzymatic reaction that occurs under physiological conditions which contributes to the pathogenesis aging and related diseases. *Staphylococcus aureus* is a frequent cause of infection, whose overabundance on the skin is linked to conditions involving elevated glycative stress such as atopic dermatitis and diabetes mellitus. The influence of glycative stress on host-pathogen interactions is still poorly understood. Therefore, we conducted a small-scale observational study of the human skin, alongside *in vitro* experiments to determine the effects on *S. aureus* of exposure to advanced glycation endproducts (AGEs). In contrast to that of young adults, the elderly skin was characterized by significantly increased fluorescent AGE content, a 10-fold increase in *S. aureus* abundance, and a 20% reduction in coagulase-negative *Staphylococcus* carriage. Glycated keratin triggered the formation of large multicellular *S. aureus* aggregates *in vitro*. Under static conditions, glycated keratin promoted biofilm formation in a dose-dependent manner (ED_{50} = 0.64 mg/mL). AGE accumulation in the skin may play a crucial role in dysbiosis and recurring *S. aureus* infection. Preventative treatments to reduce glycative stress could reduce the risk of disease and avoid overuse of antibiotics.

KEY WORDS: *Staphylococcus aureus*, glycative stress, biofilm, aggregation, advanced glycation endproducts (AGEs), skin microbiome.

Introduction

Glycation, also known as the Maillard reaction, is a non-enzymatic process that occurs between reducing sugars and protein or lipids. In addition to the well-known browning reaction that occurs during the cooking process, glycation also occurs under physiological conditions in living organisms. The resulting products of these reactions are called Advanced Glycation Endproducts (AGEs). AGEs contribute to the aging process and the pathogenesis of various age-associated illnesses such as diabetes mellitus[1,2] and neurodegenerative disease[3]. When AGEs form in association with proteins, they disrupt protein function and may cause aggregation[4]. AGEs also activate the Receptor for AGEs (RAGE), triggering an inflammatory response[5]. AGEs accumulate in the body over time and may interfere with a variety of processes from bone homeostasis[6] to the functioning of the immune system.

Aging of the skin also involves glycative stress[7,8]. AGEs form crosslinks between collagen fibers, reducing the skin's elasticity and forming wrinkles. The accumulation of AGEs can also lead to discolouration[9], further contributing to the physical signs of aging. Recently, beauty products and dietary supplements are sold which aim to inhibit glycation and breakdown AGE cross-links and aggregates in the skin and throughout the body in order to maintain a healthy and youthful appearance. However, skin aging is not all about looks. The thinning and stiffening of the epidermidis that comes with age weakens the skin's barrier function, increasing the risk of injury and infection.

S. aureus is a gram-positive Firmicute that frequently inhabits the skin and nasal epithelia of humans, and is a major pathogen that is a frequent cause of skin infections in hospital and community settings, including antibiotic

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resistant varieties (*i.e.*, MRSA). Carriage of the bacterium is a risk factor for post-surgery infection [10, 11]. *S. aureus* can also cause bacterial pneumonia, particularly in co-infection with viruses such as Influenza A [12, 13] and SARS-CoV-2 [14].

During infection *S. aureus* often forms biofilms: communities of bacteria that live in a viscous extracellular matrix consisting of polysaccharides, proteins, and eDNA. The viscosity of the biofilm, the density of cells, presence of protective enzymes, and changes in metabolic state of resident bacteria all contribute to increased resistance to antibiotics and host immune response [15]. *S. aureus* readily forms biofilms on catheters and medical implants which can lead to serious infection [16].

In addition to medical plastics, *S. zaureus* is also known to form biofilms on the skin itself. Skin lesions resulting from poorly treated diabetes mellitus often form on extremities where they are readily colonized, leading to staph infection. *S. aureus* abundance increases while the diversity of commensal and/or mutualistic *Staphylococcus* species is lost on the feet of diabetic patients [17]. Staphopain and other proteases are secreted which further breakdown the skin barrier, worsening the risk of systemic infection. Other skin conditions associated with elevated glycative stress, such as atopic dermatitis [18, 19] and psoriasis [20, 21] are also characterized by colonization with *S. aureus* [22, 23], which forms biofilms in skin lesions and exacerbates the severity of accompanying symptoms.

While the effects of glycative stress on aging and the pathogenesis of various diseases has come to be better understood in the recent years, its interactions with bacteria are still poorly understood. While it has been known for some time that AGEs or Maillard Reaction Products in culture media may influence the growth of some microorganisms under laboratory conditions [24], the implications for human health in more complex systems are largely unknown. Among the research that has been published, there are indications that glycative stress can be a factor in infectious disease. Glycation of the bladder epithelium increases the binding affinity of *E. coli* [25], facilitating bacterial adhesion and infection, partially explaining the increased frequency of UTIs in diabetes. Dietary AGEs may impact the gut microbiome [26], and coffee melanoidins have been reported to modulate adhesion by *H. pylori* in the stomach [27].

In our previous research [28] we found that AGEs formed from glucose, fructose, and proteins found in skin (collagen I and keratin) exhibited growth inhibitory effects on planktonic cultures of *Staphylococcus epidermidis*, a ubiquitous skin inhabitant with a variety of beneficial effects on skin health and immunity. We noted the formation of cellular aggregates following exposure to AGEs, and hypothesized that this may be related to biofilm formation. Considering the association of *S. aureus* skin colonization and disorders involving elevated glycative stress, we hypothesized that AGEs may modulate the pathogenicity of *S. aureus* by promoting biofilm formation. The aim of the present study was to examine the effects of glycative stress on *S. aureus*. We conducted a small-scale sampling of the elderly skin in order to observe age-related changes in abundance and carriage of *S. aureus*, and examined the effects of AGEs on biofilm formation *in vitro*.

Materials and Methods

Human Study Design

A human study was carried out to examine the coagulase-negative *Staphylococcus* vs. *Staphylococcus aureus* makeup of the skin microbiome of healthy young adults and the elderly. Volunteers were recruited into two age groups: a young cohort of 20-30 years of age, and an elderly cohort of 65 or greater years of age. Participants with diagnosed skin conditions or use of topical steroids or antibiotics within 30 days of testing were excluded to eliminate potential confounding effects. Participants were asked to refrain from the use of skin creams or sunscreen lotion on the days of testing, and to avoid the use of antimicrobial soaps. Testing consisted of bacterial sampling via swabbing and skin autofluorescence measurement of the volar forearm.

Doshisha University Ethics Review Committee on Research involving Human Subjects approved of the study (application number #17085). Participants provided written informed consent prior to taking part in the study.

Bacterial Sampling

Bacterial skin samples were collected by swabbing using sterile cotton swabs. Sterile swabs were dipped in extraction buffer (0.15 M NaCl and 0.01% Tween20) then rubbed against a 4 cm^2 section of the volar forearm for 15 seconds. Samples were immediately plated onto mannitol salt agar and incubated at 37 °C for up to 72 hours. Control swabs were exposed to the air in the sampling room and then applied to sample plates to test for contamination.

Skin Autofluorescence

Fluorescent AGE content in the skin was measured using a TruAGE Scanner (Morinda, UT, USA). Participants rested their arms on the device, exposing the volar forearm to the sensor, and the mean value of three readings was recorded. Fluorescent AGE readings were taken after swabbing to prevent potential cross-contamination of skin bacteria by contact with the device.

Bacterial Strains

S. epidermidis NBRC 100911 (ATCC 14990) and *S. aureus* NBRC 100910 (ATCC 12600) were purchased from the National Institute of Technology and Evaluation Biological Resource Center (Tokyo, Japan).

Growth Conditions

Planktonic bacterial cultures were grown in Tryptic Soy Broth (TSB) at 37 °C, with 250 rpm shaking. Solid media cultures were grown on either Tryptic Soy Agar or Mannitol Salt Agar at 37 °C. All growth media used was purchased from Becton, Dickinson, and Company (MD, USA).

Bacterial viability of planktonic cultures was determined by plate-counting after serial dilution; Tryptic Soy Agar plates were inoculated in triplicate and incubated at 37 °C for up to 72 hours with daily colony counting. Turbidity assay (OD_{600}) was found to poorly correlate with

viable cell concentration in post-logarithmic growth phase cultures containing glycated keratin, and was omitted from the results.

Glycation Model

Glycated keratin solutions used in testing were produced by incubating proteins with glycating agents in accordance with the recipes outlined by [29]. The final concentration of the solution consisted of 50 mM phosphate buffer (pH 7.4), 0.60 mg/mL keratin (Nacalai Tesque, Kyoto, Japan), and 40 mM glucose. Solutions were incubated at 60 °C for 10 days to accelerate the glycation process. After incubation, ultrafiltration was performed using Amicon Ultra Centrifugal Filters according to the manufacturer's instructions (Merck Millipore Ltd, Cork, Ireland) to remove unreacted glucose. Protein concentration was measured by BCA protein Assay (Thermo Fisher Scientific, MA, USA).

Quantification of Cellular Aggregation

Planktonic cultures were supplemented with either milliQ (control) or 0.5 mg/mL glycated keratin (AGE+) and incubated for 24 hours. After incubation, samples were vortexed and 2 µL aliquots were collected, transferred to microscope slides, and flame fixed. Slides were observed via brightfield microscopy, and images were captured at a maximum magnification of 400X. Image analysis was performed using ImageJ. Briefly, images were converted to black and white and size of cells and cellular aggregates was measured (size represented by area in μm^2).

Biofilm Assay

Biofilm assay was performed as previously reported [30]. 1 mL solutions of TSB were prepared, with the desired amount of glycated keratin added per sample type. Each solution was inoculated with 1:100 volume (10 µL) of *Staphylococcus* overnight stock and thoroughly vortexed before pipetting 100 µL aliquots of each sample into the wells of a sterile un-treated 96-well plate (8 replications per sample). The outer wells of each plate were filled with purified water to prevent evaporation of test wells. Plates were then sealed and incubated at 37 °C for 48 hours before staining with 1.0 mg/mL crystal violet solution for 10 minutes. After staining, plates were gently washed with water twice and left to dry overnight. Each dried and stained biofilm well was solubilized using 200 µL of 30 % glacial acetic acid solution. Finally, the absorbance of 125 µL aliquots were measured at 587 nm using a Varioskan Flash Multimode Microplate Reader (Thermo Fisher Scientific, MA, USA).

Dose-Response Curve Fitting

Logistic curve-fitting was performed in R [31] using the Dose Response Curve package [32].

Statistical Analysis

Simple descriptive statistical analysis was performed using Excel (Microsoft). Between group statistical difference was calculated using Student's T-test. For multiple comparisons, One-way ANOVA was performed with an α of 0.05, followed by Tukey's test using SPSS Statistics (IBM).

Results

S. aureus Abundance on Elderly Skin

For the purposes of comparative analysis, participants were divided into two cohorts by age: one group for those 20-30 years of age (hereafter Young Cohort) and one for those aged 65 years or older (hereafter Elderly Cohort). As outlined in ***Table 1***, the groups were characterized by the following observations:

Table 1. Skin sampling data.

AGE compound	Young Cohort (20-30 years)	Elderly Cohort (65+ years)	p value
Age (years)	23.5 ± 2.1	79.0 ± 5.7	2.02E-26 ***
Skin Autofluorescence (units)	1.6 ± 0.2	2.8 ± 0.5	3.24E-09 ***
	Abundance (CFU/Sample)		
Coagulase-Negative *Staphylococcus*	50.8 ± 73.0	61.4 ± 94.6	7.35E-01
S. aureus	5.5 ± 6.6	55.9 ± 71.6	1.70E-02 *
Total Colony Forming Units	56.3 ± 77.3	117.3 ± 132.2	1.40E-01
S. aureus Proportion (%)	12.0 ± 14.9	50.0 ± 36.8	1.30E-03 **
	Detection Rate (%)		
Coagulase-Negative *Staphylococcus*	100	81	4.97E-02 *
S. aureus	69	81	4.49E-01

Coagulase-negative *Staphylococcus* was differentiated from *S. aureus* by colour after growth on Mannitol Salt Agar selective media. The young cohort consisted of 13 volunteers, and the elderly 21. Mean ± standard deviation. Statistical difference calculated by Student's t-test. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$.

The Young Cohort consisted of 13 volunteers with a mean age of 23.5 ± 2.07 years, and a mean fluorescent AGE score of 1.60 ± 0.16. The Elderly Cohort consisted of 21 volunteers with a mean age of 79.0 ± 5.75 years and a mean fluorescent AGE score of 2.77 ± 0.47. General bacterial abundance was estimated by a total count of colony forming units in each sample. The mean bacterial abundance in the young cohort was 56.31 ± 77.30 CFU, while in the elderly cohort it was 117.30 ± 132.20 CFU. Although nearly a two-fold increase, the difference was not statistically significant due to the large amount of variation between individuals. Coagulase-negative *Staphylococcus* mean abundance was 50.85 ± 73.04 and 55.94 ± 71.59 in the young and elderly cohorts respectively, not significantly different. However, the mean *S. aureus* abundance represents the main between-group difference, with mean abundances of 5.46 ± 6.64 CFU in the young cohort which were significantly less compared to 55.94 ± 71.59 CFU in the elderly cohort ($p = 0.017$).

The relative abundance of *S. aureus* as a proportion of total CFUs was calculated to normalize the data, with the results given as a percentage of *S. aureus* colonies per sample. Using these criteria, the relative abundance of *S. aureus* was 12.01 ± 14.87% and 49.97 ± 36.83% in the young and elderly samples respectively. This difference was also statistically significant ($p = 0.001$).

The *S. epidermidis* carriage rate was 100% in the Young Cohort and 81.0% in the Elderly Cohort, a difference with borderline significance ($p = 0.049$). *S. aureus* carriage rates were 69.2% and 81.0% respectively, but the difference observed was not significant ($p = 0.449$).

Growth Inhibitory Effect of Glycated Keratin

As *S. aureus* is increased on the elderly skin in comparison to *S. epidermidis*, we initially hypothesized that differential responses to AGE exposure between the species could be responsible for the observed difference in abundance on the aged skin; if *S. aureus* has greater resistance to glycative stress, it could outcompete *S. epidermidis* in a glycated skin environment. As we have previously reported [28], *S. epidermidis* growth was inhibited by exposure to exogenous glycated protein *in vitro*. Here we replicated the previous *S. epidermidis* test using *S. aureus* strain NBRC100910, observing the effects of glucose-keratin exposure. As shown in ***Fig. 1***, addition of glycated keratin to growth media results in a 0.9 log reduction of *S. epidermidis* compared to a 1.1 log reduction of *S. aureus* after 24 hours. Both species are similarly vulnerable to glycative stress from exogenous glycated proteins. Thus, the differences observed on the human skin are likely not due a direct difference in vulnerability to glycative stress by *S. aureus*.

Cellular Aggregation

During growth of *S. aureus* in media containing glycated proteins, the formation of large macroscopic cellular aggregates was observed, settling in a large cloudy mass at the bottom of culture tubes. Microscope images of cellular aggregates of *S. epidermidis* and *S. aureus* can be seen in ***Fig. 2-a***. When observed microscopically, the initial

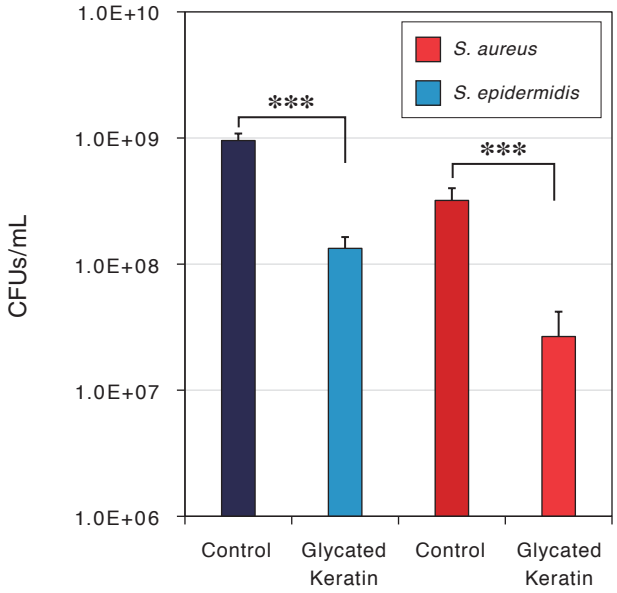

Fig. 1. **Growth inhibitory effect of glycated keratin.**

24-hour cell viability of planktonic cultures of S. epidermidis and S. aureus in media with 1.0 mg/mL glycated keratin. n = 3. Mean ± standard deviation. Statistical difference calculated by Student's t-test. *** $p < 0.001$.

formation of small clumps of up to a dozen cells differentiated glycated keratin cultures from controls in the logarithmic growth phase, roughly 5 hours after inoculation. Image analysis conducted on samples collected after 24 hours of growth (***Fig. 2-b***) showed aggregates in glycated keratin cultures were significantly larger than those in control cultures: roughly 3-fold larger for *S. epidermidis*, and 8-fold for *S. aureus* ($p < 0.001$). Aggregate size was substantially smaller in *S. epidermidis* samples, and was not apparent macroscopically.

Biofilm Production

Suspecting biofilm formation from the observance of cellular aggregates, we performed photometric assays in order to quantitatively measure the amount of *S. aureus* biofilm formation in response to glycated keratin AGEs. Biofilm absorbance was measured at 587 nm after 48 hours of stationary incubation.

Fig. 3-a demonstrates the biofilm response of *S. aureus* to glycated and un-glycated keratin at a concentration of 0.5 mg/mL. Sterile TSB was also tested to ensure there was no interference during the crystal violet staining, producing negligible absorbance values (not shown). For glucose conditions, an additional 2.5mg/mL of glucose was added to TSB, resulting in a final glucose concentration of 5.0 mg/mL. Heated keratin, produced following the same procedure as glycated keratin, but without glucose, did not differ significantly from control. All sample varieties resulted in significantly less biofilm formation than glycated keratin, which produced the strongest response at 1.9. Exposure to

glycated keratin resulted in a roughly 10-fold increase in absorbance compared to heated, un-glycated keratin ($p < 0.001$).

The biofilm response of *S. epidermidis* and *S. aureus* are compared in ***Fig. 3-b***. While glycated keratin produced a significant increase in biofilm formation in both species, *S. aureus* produced significantly more biofilm than *S. epidermidis* at the same dosage ($p < 0.001$).

Biofilm Dose-Response

The dose-response curve of *S. aureus* biofilm production in response to glucose-keratin exposure was also examined. The resulting curve, shown in ***Fig. 4***, reveals a classic sigmoidal relationship with a midpoint of 0.64 mg/mL of glucose-keratin ($R^2 = 0.9998$). Biofilm abundance rises rapidly at increasing dosage below 1 mg/mL, before beginning to plateau at an absorbance value of roughly 3.0 above baseline between a dosage of 2–5 mg/mL.

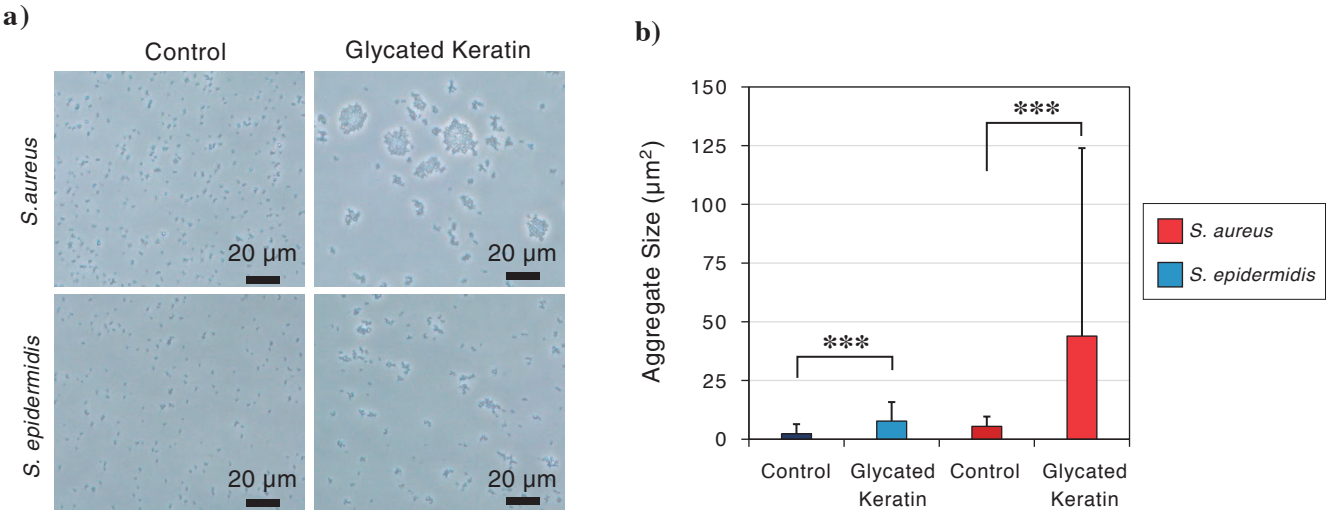

Fig. 2. **Glycated keratin induced cellular aggregation.**

a: Representative bright-field microscopy images of *S. aureus* and *S. epidermidis* cellular aggregation after 24 hours under planktonic conditions. Images captured at 400X magnification. **b:** Mean bacterial aggregate size. Samples were vortexed and 2 µL inoculates were transferred to microscope slides. n = 10. Mean ± standard deviation. Statistical difference determined by Student's t-test. *** $p < 0.001$.

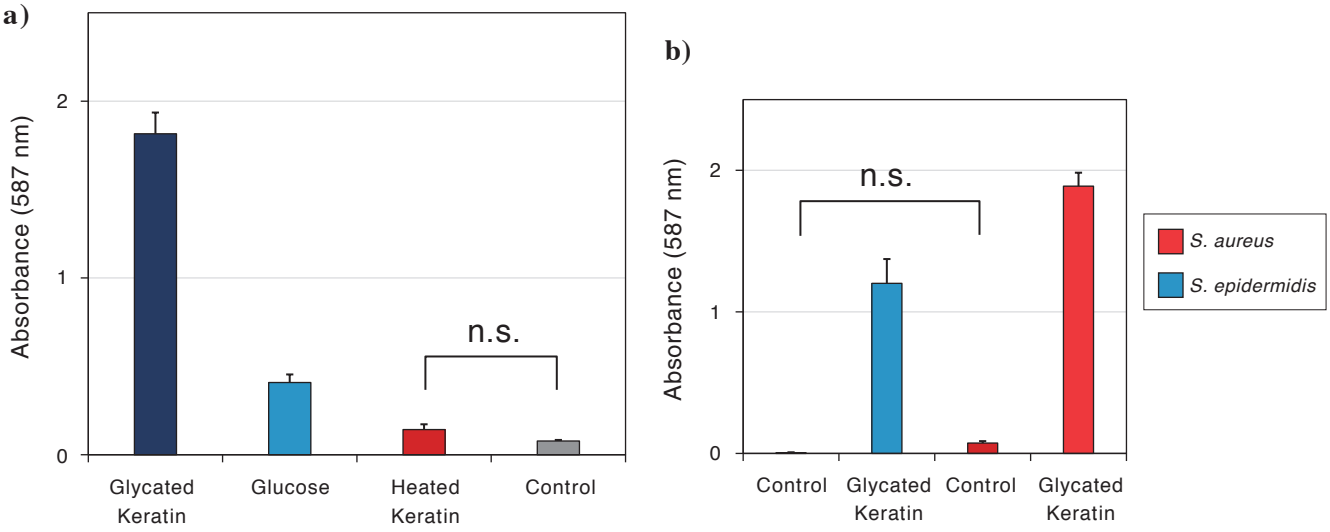

Fig. 3. **Glycated keratin induced biofilm formation.**

a: *S. aureus* biofilm formation in response to 0.5 mg/mL of keratin. Glucose conditions contain 5.0 mg/mL of glucose, double the concentration of standard TSB. **b:** Difference in biofilm formation between *S. epidermidis* and *S. aureus*. n = 8. Mean ± standard deviation. Bars indicate standard deviation. Statistical difference was calculated via One-way ANOVA followed by Tukey's test. $p < 0.001$.

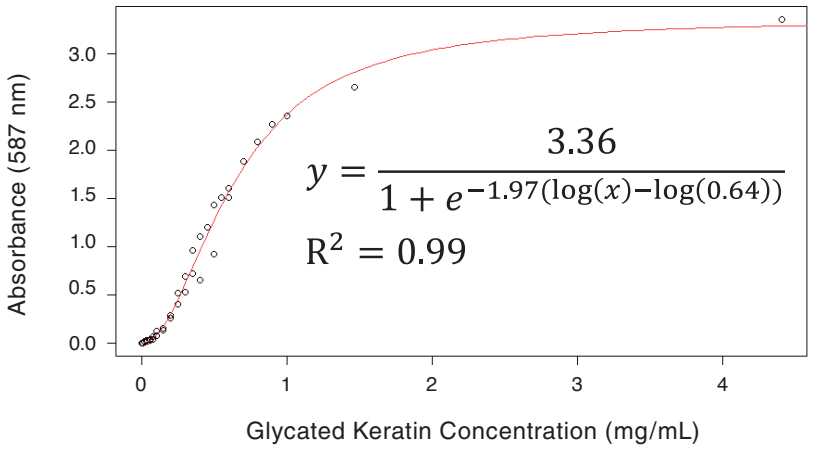

Fig. 4. **Glycated keratin dose-response relationship**

Dose-response relationship of *S. aureus* biofilm formation and >10 kDa glucose-keratin concentration. Biofilms were stained with crystal violet and absorbance was measured at 587 nm after 48 hours of growth under static conditions at 37 °C. Each data point represents the mean of eight samples. Midpoint = 0.64 ± 0.03. Upper limit = 3.36 ± 0.12. R2 = 0.99.

Discussion

Human Study

S. aureus carriage was higher in our participants than is reported among the general population in other studies [33], although the trend of increased carriage in the elderly was replicated. These differences may be due to this study's focus on the skin. Skin sampling may be more likely to capture transient carriage, overestimating *S. aureus* carriage rates in comparison to the more common nasal sampling methods. The sampling location of the volar forearm may also have resulted in relatively low overall bacterial abundance compared to more sebaceous regions. The inner forearm was used to measure skin autofluorescence as it is less exposed to UV damage; the same location was thus used for the bacterial sampling. Healthy participants with no diagnosed skin disorders were selected to remove the potential confounding effects and focus on the difference in fluorescent AGE skin content between the age groups.

The participants were not evenly distributed by sex, and female participants outnumbered male. Regardless, there was no statistically significant difference observed in bacterial abundance or carriage between male and female participants. While we were able to obtain statistically significant data points, a larger sampling including the full age range of the population would provide further evidence for an age-related shift in skin *S. aureus* abundance.

Cellular Aggregation

Cellular aggregation can occur for a variety of reasons. A similar response has been reportedly observed when cultivating *Staphylococcus* in synovial fluid[34], which occurs due to the binding of fibrin/fibrinogen to cell surface receptors which trigger cellular aggregation and biofilm response. Aggregation may also occur in response to toxic stimuli, such as exposure to sub-lethal concentrations of antibiotics. The formation of cellular aggregates protects constituent cells from antibiotics [35] and immune response by physically shielding cells in the interior of aggregates, as well as by decreasing the surface area-to-volume ratio of the structure. We speculate that the glycative stress exerted by exogenous AGEs triggers an aggregation response rather than acting as a binding substrate for the nucleation of bacterial aggregates, as aggregation did not occur when *Staphylococcus* was resuspended in carbon free non-growth media and exposed to glycated keratin. However, we have not ruled out the possibility that glycated keratin upregulates the expression of cell surface binding receptors that are not expressed under standard culture conditions.

When both *Staphylococcus* species were compared, *S. aureus* demonstrated a stronger aggregation response compared to *S. epidermidis* at the same glycated keratin dosage. A similar difference was also observed in the capacity for biofilm formation. This may indicate that *S. aureus* is better able to respond to the effects of glycative stress and protect itself, allowing it to better outcompete *S. epidermidis* in environments under high levels of glycative stress, such as the skin of those with diabetes or atopic dermatitis.

Potential Biofilm Formation Mechanisms

In *S. aureus* there are several physiological processes that govern biofilm formation. As a natural process of their life-cycle, *S. aureus* may form biofilms from the presence of suitable binding substrates (*e.g.*, fibronectin [36], collagen [37], and keratin [38] depending on strain) for cell-surface adhesins or high concentrations of neighboring cells.

Extracellular DNA can also act as a trigger of *S. aureus* biofilms [39, 40]. The eDNA released from damaged or autolysed cells becomes a major part of the biofilm itself, and upregulates biofilm formation in surviving cells. Membrane damage by AGEs could lead to an increase in mortality and biofilm formation.

In response to cellular stress, many bacteria form biofilms to protect themselves from adverse environmental

conditions or host immune response. *S. aureus* is known to modulate its biofilm production in response to conditions such as hyperoxia [41], anoxia and hypoxia [42, 43], pH [44], high salt and suboptimal temperature [45], and sub-lethal concentrations of alcohol [46]. Based on our observations, we believe it is likely that glycative stress is also a factor in *S.aureus* biofilm formation and regulation. Further investigation is necessary to uncover the precise mechanism(s) by which glycated proteins exert this stress. In future, we plan to examine the effect of exposure to glycated proteins on the expression of genes related to biofilm formation, such as the *iac* locus which regulates polysaccharide intercellular adhesin production [47].

Conclusion

The aging microbiome of the skin is characterized by increasing abundance, in absolute and relative terms, of *S. aureus*. *In vitro,* the addition of AGEs produced from glucose and keratin triggered cellular aggregation and biofilm production in *S. aureus*. The presence of elevated concentrations of AGEs in the skin (whether it be from general aging, metabolic syndrome/diabetes mellitus, or a skin disorder) may exacerbate the formation of *S. aureus* biofilms on the skin, and allow it to more easily outcompete its mutualistic relative *S.epidermidis*. Consequently, this may worsen the severity of skin lesions and increase the risk of staph infection. Treatments to reduce the buildup of AGEs in the skin may help to reduce the severity of skin lesions, recurring staph infections, and other risks of *S. aureus* carriage in those experiencing elevated glycative stress. Such preventative measures may also help to avoid the overuse of antibiotics and the development of further antibiotic strains of *S. aureus* and other bacteria.

Conflict of Interest

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

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