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Original article

The effect of glycative stress on human symbiotic bacterium *Staphylococcus epidermidis*.

Kyle Haasbroek, Wakako Takabe, Masayuki Yagi, Yoshikazu Yonei

Anti-Aging Medical Research Center and Glycative Stress Research Center,
Graduate School of Life and Medical Sciences, Doshisha University, Kyoto, Japan

Abstract

Glycative stress is a contributing factor to senescence and the pathogenesis of diabetes. Advanced glycation end products (AGEs) form from the reactions between reducing sugars and amino acids, lipids, and nucleic acids, and cause protein disfunction, aggregation, and inflammation throughout various tissues within the body. While non-enzymatic glycation has been observed to occur within bacterial cells, the effects of glycative stress on bacteria have not yet been thoroughly explored. Speculatively, AGE buildup within the body associated with aging, in this case the skin, could potentially impact the bacteria of the human microbiome. *Staphylococcus epidermidis*, a ubiquitous inhabitant of the skin with numerous protective effects, was chosen for testing. *S. epidermidis* was subjected to glycative stress *in vitro* by culturing in a growth media with added glucose or glycated proteins. Glucose concentrations of 5.0 – 10 mg/mL were observed to cause a significant decline in viable cell counts after 72 – 96 hours of incubation. Glycated type I collagen and keratin significantly inhibited growth as early as 24 hours into incubation, with glycated keratin culture colony forming units reaching only 34% of control at a keratin concentration as low as 0.5 mg/mL after 72 hours. Concentrations at 1.0 mg/mL and above had a dramatic effect, reducing *S. epidermidis* cultures to less than 5% of the control by the end of the observation period. At the same concentrations, glycated keratin was observed to cause biofilm-like cellular aggregation in planktonic cultures. While the mechanism of this effect of AGEs is not yet known, these results suggest that glycative stress can have a strong impact on the growth and physiology of bacteria. The influence of glycative stress may alter the balance of other bacteria in the human microbiome, with implications for health and infection.

KEY WORDS: *Staphylococcus epidermidis*, skin microbiome, glycative stress, advanced glycation end products (AGEs), agglutination

Introduction

Glycation is the non-enzymatic reaction between reducing sugars and amino acids that produces Schiff bases, which then form into Amadori products before ultimately becoming advanced glycation end products (AGEs). Glycative stress that results from the presence of AGEs is a contributing factor to the pathology of diabetes [1)] and age-related complications, such as macular degeneration and renal failure [2)]. As AGEs develop in the body, they cause protein dysfunction and aggregation, as well as promotion of the inflammatory response, via activation of RAGE (receptor for AGEs). Glycative stress is also significant in skin health [3)], disrupting dermal homeostasis by promoting inflammation and inhibiting the proliferation of keratinocyte and fibroblast cells. AGEs form cross-link bridges in collagen, resulting in reduced skin elasticity, color changes, and increased wrinkling.

However, the effects of AGEs on bacteria have yet to be examined. As glycation is a non-enzymatic process, the reaction to produce AGEs occurs relatively slowly at normal body temperature in food; in humans, however, the effects of glycative stress can take a variety of time duration, from minutes to years, to accumulate, depending on the half-life of protein types; the reaction is especially rapid once aldehydes are involved. As such, bacteria have been overlooked for study into glycation as their short lifetimes, fast generation time,

Contact Address: Professor Yoshikazu Yonei, MD, PhD
Anti-Aging Medical Research Center/Glycative Stress Research Center,
Graduate School of Life and Medical Sciences, Doshisha University
1-3 Tatara Miyakodani, Kyotanabe, Kyoto, 610-0394 Japan
Phone/Fax: +81-774-65-6394 Email: yyonei@mail.doshisha.ac.jp
Co-authors: Haasbroek K, cygd2001@mail.doshisha.ac.jp;
Takabe W, wtakabe@mail.doshisha.ac.jp; Yagi M, myagi@mail.doshisha.ac.jp;
Yonei Y, yyonei@mail.doshisha.ac.jp

and quick protein turnover have been thought to be inadequate for significant amounts of glycation to take place. However, glycation does, in fact, occur in bacteria: non-enzymatic glycation was first observed in *E. coli* [4, 5]. Since then, there has been growing evidence that glycative stress plays a physiologically significant role in bacteria. Glycative stress induced by a high glucose environment has been found [6] to lead to significant cell mortality in *E. coli*, which can be ameliorated by the presence of known anti-glycative agents, such as carnosine. In these cases, the agents act as inhibitors to prevent the reactions that ultimately lead to the production of AGEs.

Glycative stress of bacteria may be relevant to human health. The human body is inhabited by an enormous variety of symbiotic bacteria that make up the human microbiomes. Metabolic disorders such as diabetes mellites and advanced age are associated with increased glycative stress and AGE accumulation; physiological levels of AGEs within the body could reach high enough levels to impact these resident bacteria in ways that could alter microbial communities, leading to dysfunction.

One such bacteria is *Staphylococcus epidermidis* (*S. epidermidis*), a ubiquitous commensal colonizer of the human skin. It produces anti-microbial peptides (AMPs) that have been observed to inhibit the growth and colonization of potentially pathogenic bacteria, such as Group A Streptococcus [7] and Propionibacterium acnes [8]. There is also evidence that *S. epidermidis* primes the immune response of keratinocytes [9], and thereby promotes improved immune defense in the host. While it can occasionally be an opportunistic pathogen in those with compromised immune systems or on surgical implants, in normal circumstances, it can be thought of as a probiotic member of the skin community. Among the diabetic foot flora, Staphylococcus species (excluding *S. aureus*) are diminished [10]; as diabetes is associated with high levels of glycative stress, AGEs in the skin could plausibly contribute to this imbalance in the microbial community.

This study was carried out to determine if exposure to AGEs has any effect on the survival of *S. epidermidis*. Glycative stress was applied to *S. epidermidis* by adding higher-than-normal concentrations of glucose or glycated proteins to active cultures of the bacterium and observing its growth. If bacteria are similarly vulnerable to the effects of AGE exposure as animal cells, then they could serve a useful role as a model organism for the study of glycative stress and its molecular mechanisms with greater ease of care and manipulation than animal models, such as mice. Some microbial organisms may even possess defense mechanisms against glycative stress, such as *E. coli* [11], which produces a metallo-protease capable of breaking down intracellular AGEs before secretion. The biodiversity of bacteria contains within it an enormous potential source of novel mechanisms to combat glycative stress.

Materials and methods

Bacterial strains

S. epidermidis of an unknown strain was isolated from healthy human skin. The *S. epidermidis* strain NBRC100911 was purchased from the National Institute of Technology and Evaluation Biological Resource Center.

Glycation model

Glycated Protein Solutions used in testing were produced by incubating proteins with glycating agents in accordance with the recipes outlined by the previous report [12]. Glycated protein solutions consisted of 50 mM phosphate buffer (pH 7.4), 0.60 mg/mL keratin or type I collagen, and 40 mM glucose. Solutions were incubated at 60 °C for 10 days to accelerate the glycation process. After incubation, centrifugal ultrafiltration was performed to remove unreacted glucose, and the >10 kDa fraction was collected and added to a Tryptic Soy Broth (TSB) growth medium to meet the designated concentration of each condition.

Growth conditions

Tryptic Soy Broth was prepared according to the manufacturer's specifications, and supplemented with glucose or glycated protein. Overnight cultures were prepared from frozen stock and used to inoculate prepared growth media in a 1 : 40 volume ratio (overnight stock to fresh media). Each culture was grown in 6 mL of TSB in a 12 mL test tube (2 : 1, flask to volume ratio) at 37 °C, shaking at an rpm of 250. Every 24 hours post-inoculation, tubes containing the growing cultures were vortexed, and 100 µL aliquots were collected and serially diluted for plating and colony counting. Colonies on plates of TSB agar were counted after 24 hours of incubation at 37 °C.

Fluorescent AGE measurement

Fluorescent AGE content in liquid solutions was measured using a Varioskan Flash Multimode Microplate Reader (Thermo Fisher Scientific, MA, USA). Aliquots of 200 µL of solution were measured in triplicate for emissions at 440 nm after excitation at 370 nm, the peak of Emission/Excitation for fluorescent AGEs [13]. Samples were normalized using Quinine Sulphate as a reference, with the following formula:

> Normalized Emission Value = (Fluorescence Intensity of Sample/Fluorescence Intensity of Quinine Sulphate) * 1,000.

Final values are presented as normalized to quinine sulphate. Background values were determined by collecting a 1 mL aliquot of prepared test solutions prior to bacterial inoculation, and kept at 4 °C until the final measurement (typically, no longer than one week). Post incubation samples were prepared by centrifuging 1 mL aliquots of solutions at 6,000 g for 15 minutes to pellet bacterial cells before collecting the supernatant for measurement alongside the preserved background samples.

Results

Glucose toxicity

The first test was a simplified replication of the *E. coli* glycative stress experiment [6], using *S. epidermidis* isolated from human skin. TSB with increasing glucose concentration (2.5 mg/L – 10 mg/L) was prepared and inoculated with *S. epidermidis*, then incubated for four days. Beyond 24 hours after inoculation, increasing glucose content was

associated with a decline in viability (***Fig. 1***). By the 96-hour mark, cell concentrations had fallen to roughly 44% and 11% of control in the 5.0 mg/mL and 7.5 mg/mL glucose conditions, respectively, while the highest condition of 10 mg/mL proved fatal as the culture fell below the limit of detection at less than 1% of the control. After 24 hours of seemingly normal growth, *S. epidermidis* was succumbing to the toxic effects of the excess glucose. The lag time in the effect is likely due to the time necessary for the glucose to be taken up and to react with lipids and proteins within the cells, and before damage could be caused by the resulting formation of intracellular AGEs.

Test runs with unknown strains of S. epidermidis

Using *S. epidermidis* of unknown strain isolated from healthy human skin, a number of tests with type I collagen or keratin with glucose or fructose as the glycating agent were carried out (***Fig. 2*** and ***Fig. 3***). AGEs produced from both protein types were found to inhibit *S. epidermidis* growth and resulted in a loss of cell viability. No significant differences were observed between fructose and glucose as the glycating agent. Fluorescent AGE content in each growth culture were measured and compared to the peak of growth achieved by each condition, revealing a negative correlation between AGE concentration and peak cell concentration (***Fig. 4***).

Fluorescent AGE content in the growth media was observed to decrease after incubation with *S. epidermidis* under all testing conditions. The phenomenon did not occur when sterile TSB was incubated alone or with heat-deactivated *S. epidermidis* (***Fig. 5***).

Concentrations of glycated protein are calculated based on the initial recipe to be 1 mg/mL for the initial test and 2 mg/mL for the double dosage test; however, there was significant protein loss during preparation unaccounted for which results in actual concentrations being lower than shown. The actual value of the concentrations based on later measured sample loss are likely to be roughly 0.8 mg/mL and 1.6 mg/mL for the initial and double dosage tests, respectively. For the test with NBRC100911, fully-prepared glycated protein samples were assayed directly to measure protein content more accurately.

Glucose-keratin toxicity

Keratin was glycated using glucose to model AGEs that could be found on the skin surface, and added to TSB in concentrations of 0.5, 1.0, and 1.5 mg/mL before inoculation with NBRC100911. Heated keratin without glucose was tested at 1.0 mg/mL, and the control consisted of standard growth medium. No changes in pH were observed after addition of glycated-keratin. Although there was a slightly higher in-cell count, heated keratin had no significant effect on *S. epidermidis* growth over three days of incubation, while glycated-keratin conditions all achieved less than half of the control's peak growth at 24 hours (***Fig. 6-a***). The final cell counts after 72 hours of AGE exposure were reduced in a dose-dependent manner, reaching 34%, 4%, and 0.8% of the control, with an increasing concentration of glycated keratin (***Fig. 6-b***). Compared to the results of the test run with the unknown strain of *S. epidermidis* collected from healthy human skin, NBRC100911 suffered greater growth inhibition at relatively lower dosages of glycated protein.

Despite constant shaking during incubation, a large mass of bacteria settled at the bottom of the culture tubes in only the glycated keratin conditions; their volume increased

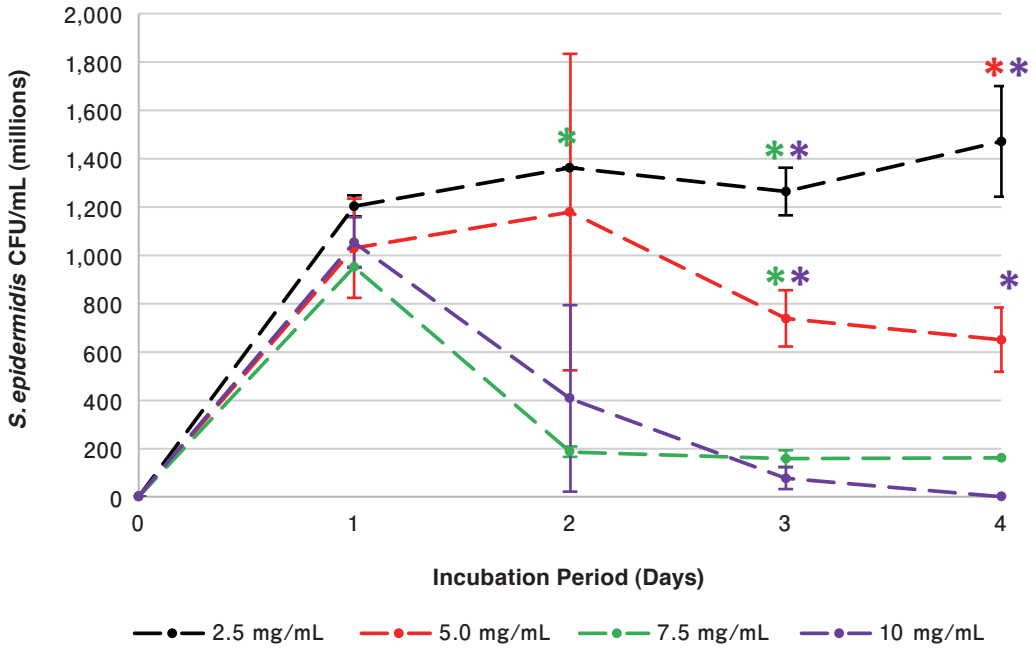


Fig. 1. **Altered *S. epidermidis* growth: Comparison of high glucose concentrations**

S. epidermidis cell count over time in solutions with incrementally increasing glucose concentration. Standard TSB solution contains 2.5 mg/mL glucose. Glucose content was increased in each sample condition in intervals of 2.5 mg/mL to a maximum of 10 mg/mL. On day 4 the 10 mg/mL condition falls below the limit of detection at < 10 million CFU/mL. Asterisks indicate p-value of < 0.05 according to Student's t-test, n = 3. Bars indicate standard error mean. CFU, colony forming unit; TSB, tryptic soy broth.

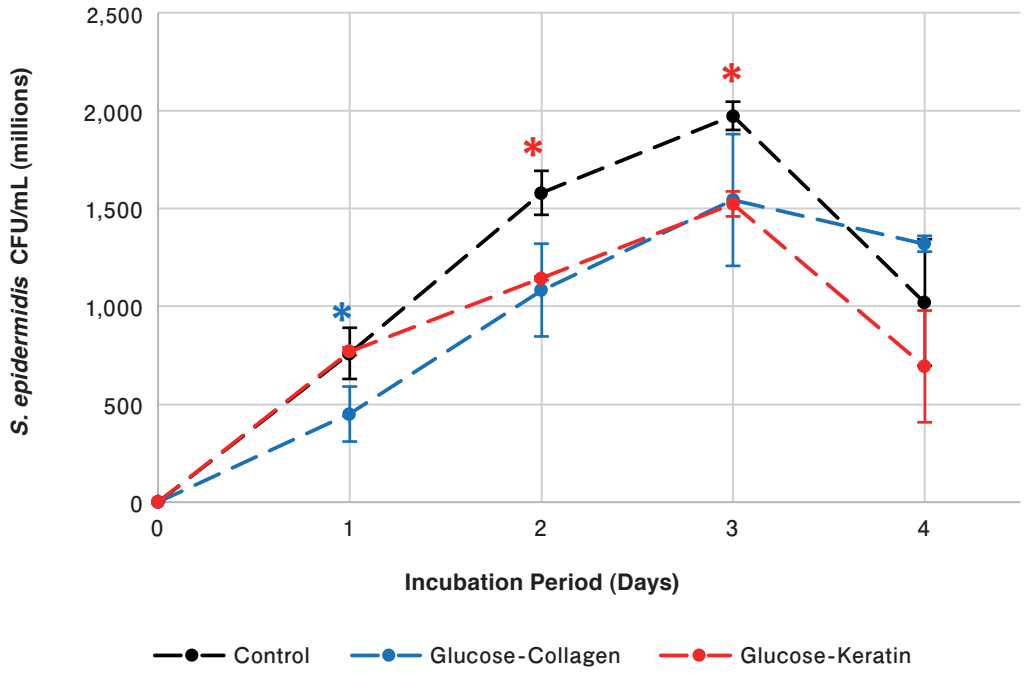


Fig. 2. **Altered *S. epidermidis* growth: Comparison of glucose-glycated type I collagen and keratin**

S. epidermidis cell count over time in solutions with added 0.8 mg/mL glucose-glycated keratin or collagen type I. Control consists of standard recipe TSB. Asterisks indicate p-value of < 0.05 according to Student's t-test, n = 3. Bars indicate standard error mean. CFU, colony forming unit; TSB, tryptic soy broth.

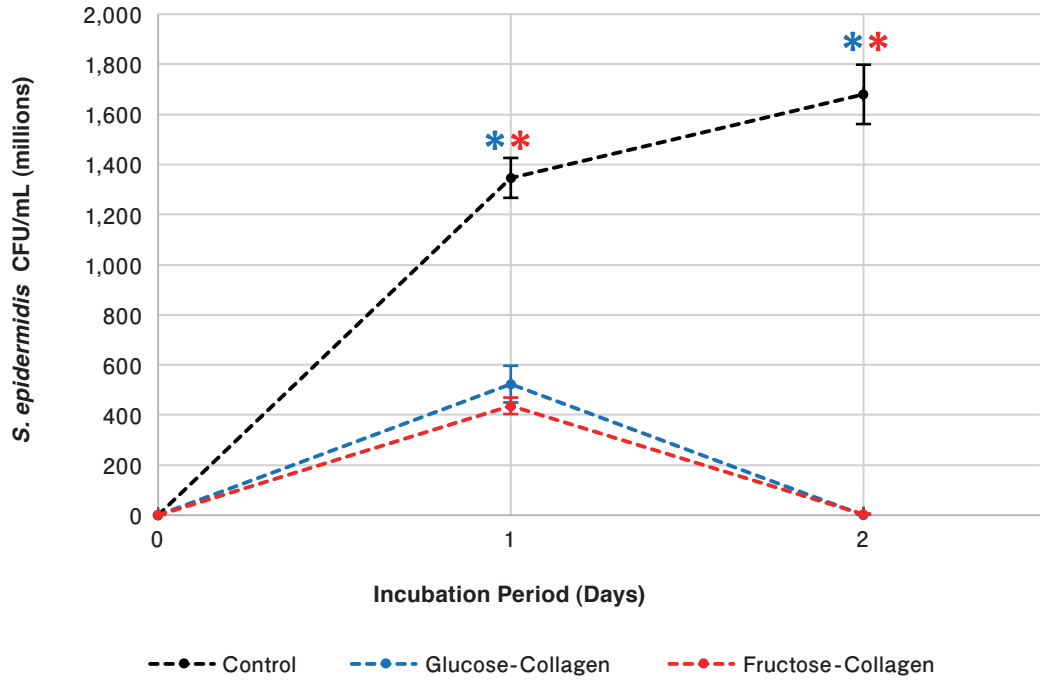


Fig. 3. **Altered *S. epidermidis* growth: Comparison of fructose and glucose-glycated type I collagen at double dosage**

S. epidermidis cell count over time in solutions with added 1.6 mg/mL glucose or fructose glycated collagen type I. Control consists of standard recipe TSB. On day 2 both glycated collagen conditions fall below the limit of detection at < 10 million CFU/mL. Asterisks indicate p-value of < 0.05 according to Student's t-test, n = 3. Bars indicate standard error mean. CFU, colony forming unit; TSB, tryptic soy broth.

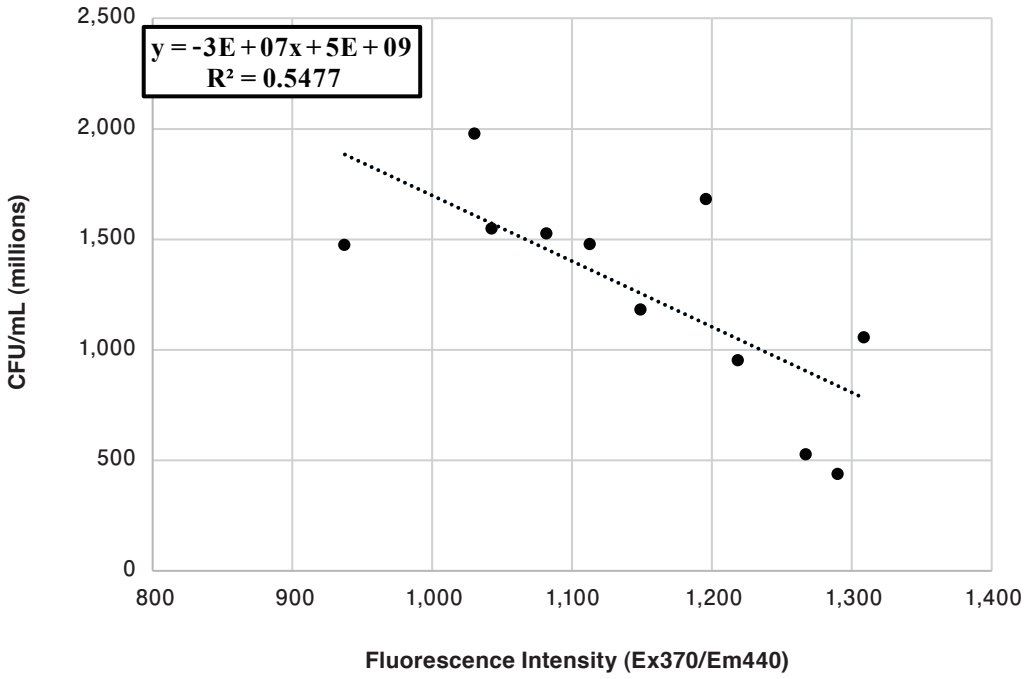


Fig. 4. **Relationship of *S. epidermidis* peak growth and fluorescent AGE content in growth medium**

Linear relationship between peak cell count and fluorescence intensity of growth medium. Data collected from all unknown strain *in vitro* tests. Linear line of best fit, n = 11. CFU, colony forming unit; AGE, advanced glycation end product.

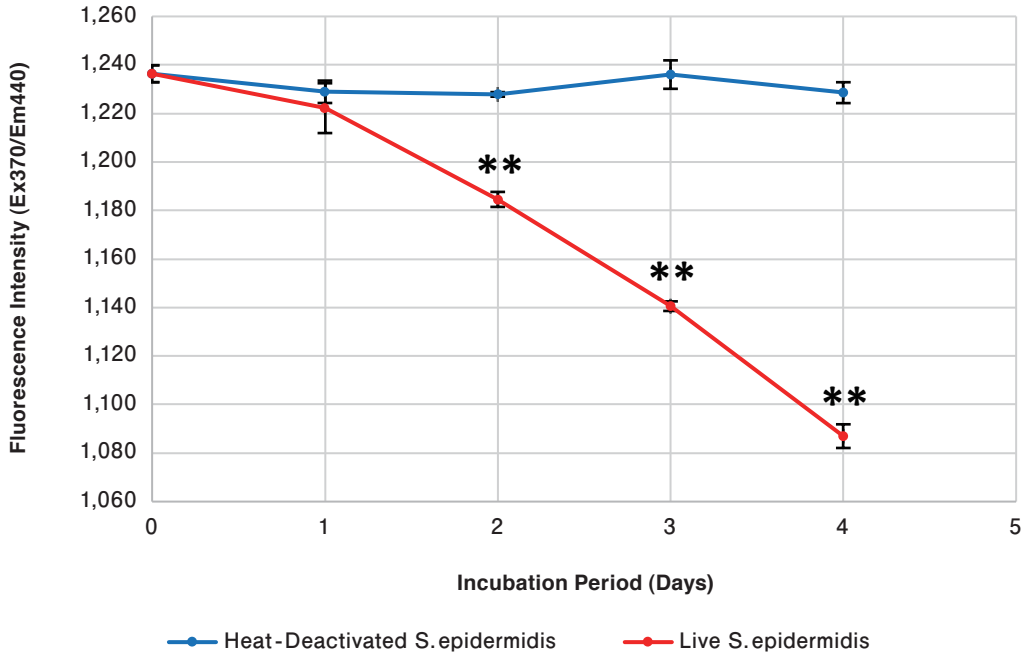
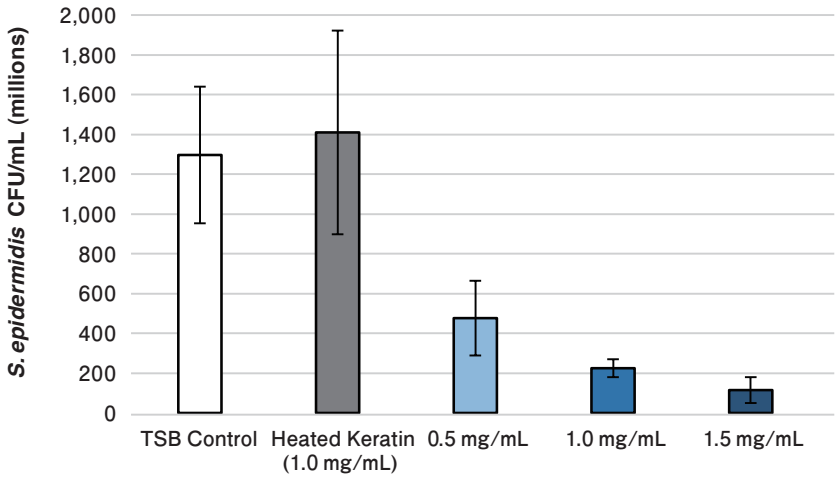


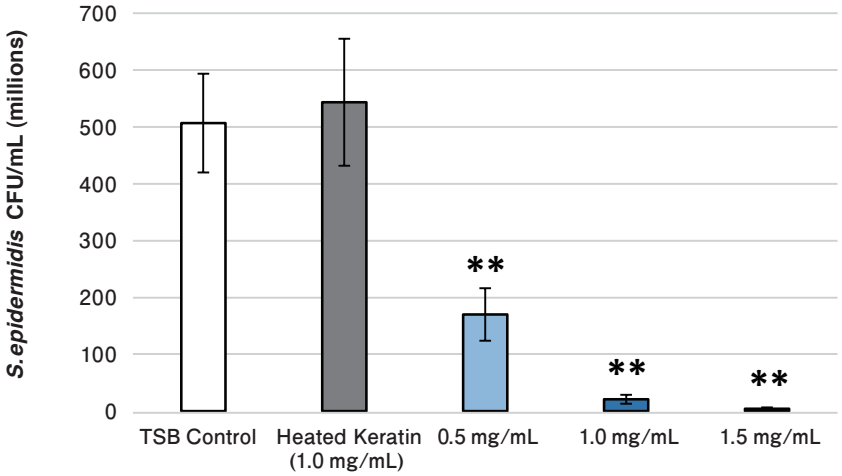
Fig. 5. **Reduction of fluorescent AGE content after incubation with *S. epidermidis***

Change in fluorescence intensity over time of live and heat-deactivated cultures of *S. epidermidis*. Measurements taken from supernatant of growth medium. Fluorescence value standardized to the value of quinine sulphate as 1000. Asterisks indicate p-value of < 0.05 according to Student's t-test, n = 3. Bars indicate standard error mean. AGE, advanced glycation end product.

a) 24 hours



b) 72 hours



c) Growth curve

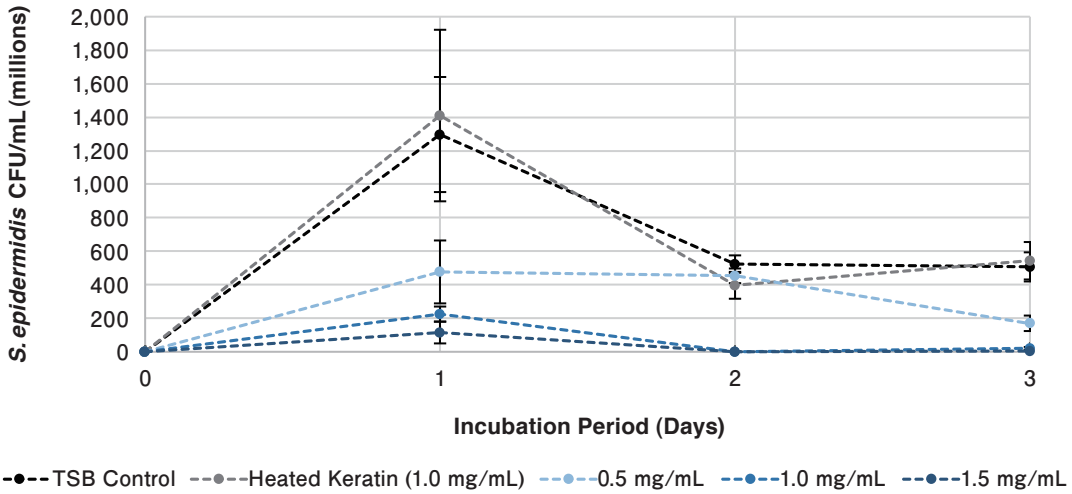


Fig. 6. **Reduction of colony forming units of *S. epidermidis* strain NBRC100911 after incubation with glucose-glycated keratin**

S. epidermidis strain 100911 cell counts at 24 hours **(a)** and 72 hours **(b)** of incubation, followed by the overall growth curve **(c)**. Note that on day 2 both 1.0 and 1.5 mg/mL conditions fall below the limit of detection at < 10 million CFU/mL. Control consists of standard recipe TSB. Double asterisks < 0.01 according to Student's t-test, n = 3. Bars indicate standard error mean. CFU, colony forming unit; TSB, tryptic soy broth.

with higher concentration of glycated protein. By the end of the incubation period, clumps were also seen forming along the length of the sides of the sample tube. Microscopic examination of liquid cultures revealed a significant clumping of cells in the 1.5 mg/mL condition compared to the control, which consisted of mainly solitary or paired cells (***Fig. 7***).

Discussion

Glucose toxicity

The results of the test show a successful replication of the effect observed by Pepper ED *et al.*[6] in *S. epidermidis*. It is likely that glycative stress is a concern for organisms among all of the domains of life.

While not examined in detail specifically in this study, the mechanism for the toxic effect of glucose seems to likely be due to glycation and AGE formation occurring within the bacterial cell after the uptake of high amounts of glucose. Mironova R *et al.*[4, 5] demonstrated the formation of AGEs and glycation of nucleic acids within *E. coli*, which would likely disrupt normal functioning of the proteins and other molecules involved. The lag time observed between normal growth and cell mortality fits well with this explanation, as some time would be necessary for the reactions involved in glycation to take place. Pepper ED *et al.*[6] notes formation of CML occurring only with active *E. coli*. Incubation of TSB alone with added glucose at testing conditions did not lead to any significant production of fluorescent AGEs within the growth media (data not shown).

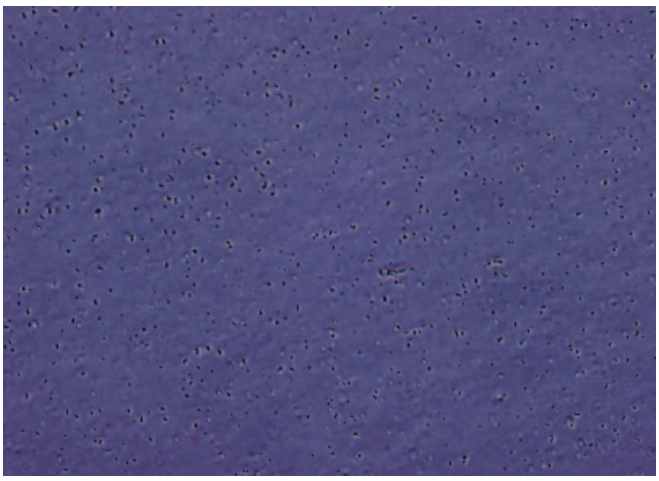
AGE toxicity

In contrast to the high glucose conditions, the inhibitory effects of AGEs on *S. epidermidis* growth were observable much earlier in incubation, being apparent even at 24 hours. As mentioned previously, this is likely due to the AGEs being able to act on the bacteria immediately, rather than taking time to react and form from glucose. In the early tests with only rough estimates of concentration of 50% inhibition was achieved between 1 – 2 mg/mL of glycated protein; however, the precise measurements carried out when testing strain NBRC100911 revealed a peak growth of 34% compared to the control at only 0.5 mg/mL of glycated keratin, suggesting that the half-maximal inhibition concentration is even lower.

The next relevant question to ask is whether physiological levels of AGEs within the skin actually reach significant levels to exert such a strong effect in reality. As well, the skin environment is a solid surface, not exactly comparable to the experimental growth in liquid media, which may, additionally, alter the dynamics. Nonetheless, in the clinical data (not yet published), a small number of elderly participants hosted no *S. epidermidis* at all, and *S. epidermidis* concentrations did not significantly change with age in absolute terms; rather, the balance between *S. epidermidis* and *S. aureus* was characterized by greater domination by *S. aureus*. AGE concentrations in the skin do not need to reach levels that would kill most of the *S. epidermidis* inhabitants, but only enough to shift the balance essening their competitiveness. A relatively small level of inhibition could create more room for *S. aureus* to expand its colonization, which could snowball into total dominance of the staphylococcus biomass.

Some of the reduction in colony forming units (CFUs) in the higher concentration AGE conditions may be due to aggregation, as clumps of cells would likely produce single colonies rather than a number equivalent to the amount of constituent cells. However, the reduction in cell count in high glucose conditions and lower AGE dosages (*i.e.* the test runs) were not accompanied by aggregation, suggesting there is an actual loss of cell viability occurring, as well. Optical density was also used to measure cell concentration, but results were inconsistent beyond the exponential growth phase and abandoned in favor of CFU counting.

a)



b)

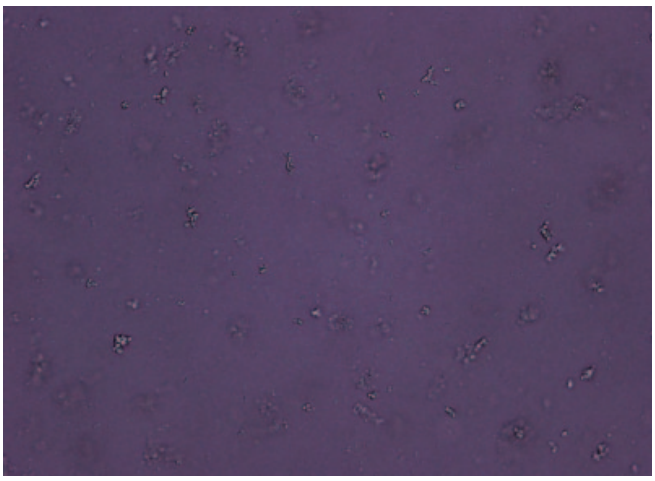


Fig. 7. **Biofilm-like cellular aggregation in *S. epidermidis* NBRC100911 exposed to high concentrations of glucose-glycated keratin**

Liquid cultures of *S. epidermidis* strain 100911 were sampled after 72 hours of incubation from TSB control culture **(a)** and 1.5 mg/mL glucose-keratin culture **(b)** for visualization via bright-field microscopy at 400X magnification. Contrast and sharpness of images were altered digitally to improve visibility. TSB, tryptic soy broth.

Cellular aggregation

The high dosage glucose-keratin condition (1.5 mg/mL) displayed a significant macroscopic clumping of cells visible during incubation. There are numerous adhesion and aggregation mechanisms within Staphylococcus [14]. Clumping is regulated by the ArlRS/MgrA cascade; activation of ArIRS (whose ligand is unknown) is a potential receptor for some AGE(s) in that case. Another method of aggregating in *S. aureus* has been noted to involve polysaccharide intercellular adhesin (PIA), which provides another potential target for study [15].

A similar biofilm-like aggregation in planktonic *S. aureus* has been observed when it is grown in synovial fluid [16]. The phenomenon has been replicated in *S. epidermidis*, as well [17]. Unfortunately, in these studies, the clumping occurred after only a few hours, and produced much larger aggregates of cells than were observed when incubated with glycated keratin. Planktonic aggregates of Staphylococcus share some similarities with biofilms, including increased antibiotic resistance. The bacteria may be aggregating together in order to protect themselves from environmental stressors, in this case AGEs. In the clinical study (not yet published) *S. aureus* collected from elderly participants was often characterized by abnormal colony morphology due to biofilm production. This phenotype may have been triggered by prolonged exposure to glycated proteins on the surface of aged skin.

Mechanism of the Effect

The mechanisms for the effects of AGEs on *S. epidermidis* are unknown, although there are a few plausible avenues of investigation that should be further investigated.

Hypothesis 1: AGE receptor

In humans, RAGE binds its titular ligand, triggering the inflammatory response and other regulatory mechanisms. RAGE itself is a member of the immunoglobulin superfamily of receptors [18]. With few possible exceptions, bacteria do not generally possess immunoglobulin-like receptors with sequence similarity to Eukaryotes [19]. As such it would be very unlikely for any bacteria to possess this receptor barring some significant lateral gene transfer between domains of life. When using the Basic Local Alignment Search Tool (BLAST) to test amino acid sequences against the UniProt [20] bacterial protein database, no results are found with any significant similarity to human RAGE. This suggests that no known homologous RAGE receptor exists in bacteria. However, one could imagine that an analogous bacterial receptor exists which is capable of detecting AGEs; for example, *E. coli* which possess a fimbria capable of binding to AGEs on the cell surface of bladder epithelial cells [21]. General cellular stress sensors may also react to the disturbance caused by AGEs. Protein misfolding caused by glycation could induce folding stress (analogous to endoplasmic reticulum [ER] stress in eukaryotes).

The cellular clumping behavior observed during the experiment could involve cellular adhesion pathways involved in biofilm formation, which may be activated by the stress of AGE exposure or direct detection of AGEs themselves. This could be tested by targeting the effects of AGE exposure on various adhesin expressions [22, 23]. It is also possible that glycated proteins do not directly increase expression of these factors, but act as ligands for cell-surface anchors, causing the cells to adhere to each other via aggregates of glycated proteins.

Hypothesis 2: Glycated protein digestion

Another possible mechanism for the effect of AGEs is intake of the glycated proteins into the bacterial cell; once inside, AGEs are free to react with native proteins and cause misfolding and aggregation. If this occurs, a possible pathway through peptide metabolism may be used. Bacteria consume protein by releasing proteases into their environment to break down peptides into consumable sizes, which are then transported into the cells [24]. If AGEs are left intact by the proteolytic process, then they could smuggle themselves inside the cells connected to smaller segments of peptides. This is in line with the observations of decreasing fluorescent AGE content in the growth medium over time; although, it is not yet clear whether the AGEs themselves are being broken or simply accumulating within *S. epidermidis* cells. Further testing is required to examine the effect in detail, including testing of specific free AGEs unattached to larger proteins.

If *S. epidermidis* does possess a protease that is capable of breaking down AGEs themselves rather than the protein chains they are attached to, such a protease could have practical use as an AGE breaking compound if it can be isolated and mass produced.

Relationship with Decrease of S. epidermidis Carriage Observed on Elderly Skin

In the clinical study (not yet published), while average abundance of Staphylococcus CFU on the skin of the volar forearm tended to increase in the elderly, the carriage rates of *staphylococcus epidermidis* (whether or not a participant was colonized by the bacterium) were significantly decreased compared to the young. As reported by Redel H *et al.* [10], *S. epidermidis* is also decreased on the foot of diabetic men. As both diabetes and old age are associated with increased accumulation of AGEs in the skin, and considering the inhibition of growth produced by AGEs *in vitro* in this study, AGEs in the skin could be a contributing factor to this phenomenon. AGEs may accumulate to a high enough level to inhibit normal growth of *S. epidermidis* on the skin.

Conclusion

Based on the outcome of these experiments, it is apparent that AGEs can interfere with the physiological functioning of bacteria, significantly inhibiting their growth *in vitro*. A corresponding reduction in beneficial bacteria upon the human body and its impact on health are tempting targets for future research. Additionally, the observed biofilm-like aggregation caused by exposure to AGEs may have implications in the pathogenicity of biofilms during infection. The mechanism of this effect is still unknown, but may provide insight into all of these factors.

Conflict of interest statement

The authors claim no conflict of interest in this study.

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