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Letter

Development of a keratin film-based assay for bacterial removal and adherence, and examination of the influence of fucoidan on skin bacteria

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ABSTRACT — The outer layer of the epidermis, called the stratum corneum (SC), comprises keratin-rich cells and intercorneal lipids. *Staphylococcus aureus* has been linked to the fragility of the stratum corneum and formation of atopic dermatitis (AD) lesions. Thus, binding of keratin to bacteria may reflect their binding to the AD-like stratum corneum. In this study, keratin films were prepared using keratin extracted from hair and their potential for bacterial removal and adhesion was investigated using chlorine dioxide, which exhibits bactericidal effects, and fucoidan, which is thought to inhibit bacterial adhesion. The results showed that chlorine dioxide was effective at the removal of *S. aureus*, whereas fucoidan effectively inhibited bacterial adhesion. Therefore, keratin films can be used to evaluate bacterial removal and inhibition of bacterial adhesion, and the results of this evaluation may reflect those of the AD-like stratum corneum. Using these methods, the effects of fucoidan on indigenous skin bacteria (*Staphylococcus epidermidis* and *Cutibacterium acnes*) and general bacteria (*Escherichia coli*) involved in moisturization were investigated. The results showed that fucoidan inhibited adhesion of *S. epidermidis* and *S. aureus*, but did not remove bacteria. Overall, the fucoidan (1700 kDa) used in this study has the potential to prevent and treat AD by inhibiting *S. aureus* adhesion and improving moisturization by maintaining *C. acnes*.

Key words: Fucoidan, Indigenous skin bacteria, Keratin film, Hair, Adhesion, Removal

INTRODUCTION

The integument of the skin comprises four tiers: the stratum corneum, stratum granulosum, stratum spinosum, and stratum basale. Most epidermal cells are keratinocytes (KC). The stratum corneum, which serves as the outermost layer of the epidermis, is composed of keratin-rich corneocytes (CC: deceased cells that have relinquished their nuclei and intracellular organelles from KC) and the intercellular lipids that envelop them (Del Rosso and Levin, 2011). *Staphylococcus aureus,* a microorganism implicated in atopic dermatitis (AD), releases alpha and other toxins that disrupt KC (Geoghegan *et al*., 2018) . Further, *S. aureus* can traverse the CC, which shows fewer intercellular lipids in AD (Lipsky *et al*., 2022). *S. aureus* is proposed to be responsible for CC fragility and the formation of AD lesions (Lipsky *et al*., 2022), thereby exposing keratin. Many cosmetic companies, including

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quasi-drug companies, have discontinued animal testing owing to concerns regarding animal welfare and costs, and have instead employed alternative methods to evaluate bacterial removal effects on the skin and inhibition of bacterial adhesion. Various methods have been used to gauge bacterial removal efficacy, including antimicrobial efficacy, the minimum inhibitory concentration method (Tamura and Ikedo, 2011), which measures the turbidity of bacteria, and the diffusion method (Stepanović *et al*., 2003), which measures the inhibition zone in an agar medium coated with bacteria on the surface. Nevertheless, because CC were not used, whether these methods reflect the effect of bacterial removal on the outer skin remains unclear. To assess the impact of bacterial adhesion, the adherence of keratin molecules to bacterial surface proteins on plates and that of bacteria to keratinocytes has been examined (Park *et al*., 2021; Trivedi *et al*., 2017). Assessment of keratin binding may also reflect the bacterial adhesion in a fragile outer skin model (resembling an AD-like stratum corneum) rather than in normal outer skin. Keratin is abundant in hair, KC, and CC in the epidermis. Compared with the epidermis, hair possesses a higher cysteine content, and keratins are tightly bound to each other (Ledford *et al*., 2022; Waters *et al*., 2018). Artificial skin and skin cells are expensive when considering substitutes that closely resemble human skin. If keratin from hair is used, the number of samples that can be tested is unlimited. Moreover, hair and skin contain several similar keratins (Langbein *et al*., 2010). This eliminates cost issues. Fujii *et al*. prepared keratin films using keratin extracted from hair (Shinshu University method) and evaluated hair damage (Fujii, 2012; Fujii *et al*., 2004). In this study, we prepared keratin films as substitutes for the AD-like stratum corneum and investigated whether these films could be employed to evaluate bacterial removal and inhibition of bacterial adhesion using chlorine dioxide (Hinenoya *et al*., 2015), which exhibits bactericidal activity, and fucoidan (Park *et al*., 2021), which is believed to exhibit bacterial removal activity. Furthermore, we evaluated the effects of fucoidan on indigenous skin bacteria and general bacteria using a keratin film. Overall, the effectiveness of fucoidan on the AD-like stratum corneum was investigated.

MATERIALS AND METHODS

Sample

Fucoidan (molecular weight: 1,700 kDa), a polysaccharide fraction (fucoidan content: 1.2%) isolated from natural mozuku (Cladosiphon novae-caledoniae) sourced from the Kingdom of Tonga (Matsuoka *et al*., 2023), was purchased from Tanglewood Co., Ltd. Cleverin, marketed as a sanitizer by Taiko Pharmaceutical Co., Ltd., is a formulation (containing surfactants, silicone-based antifoaming agents, and other components) in which chlorine dioxide (0.01% [100 ppm]) is the principal ingredient. The participants in the study were a diverse group of seven individuals (both males and females, ranging in age from their 20s to 70s).

Test strain

Three distinct species of epidermal commensal bacteria, *S. aureus* (NBRC12732), *S. epidermidis* (NBRC12993), and *Cutibacterium acnes* (NBRC107605), as well as a general bacterium, *Escherichia coli* (NBRC3972), were utilized in this study. The strains were obtained from the Biotechnology Center of the National Institute of Technology and Evaluation.

Culture medium

S. aureus and *S. epidermidis* were cultured under aerobic conditions at 37°C in a mannitic medium, composed of casein peptone, 0.5% enzymatic degradation product of animal tissue, 0.5%, meat extract, 0.1%, sodium chloride, 7.5%, D(-)-mannit, 1.0%, phenol red at 0.0025%, and 1.2% agar. *E. coli* was cultivated under aerobic conditions at 37°C in LB medium containing 1% tryptone, 0.5% yeast extract, and 1% NaCl2. *C. acnes* was propagated anaerobically at 37°C using the GAM medium from Nissui with the Anaero Pack Kenki System from Mitsubishi Gas Chemical.

Keratin extraction from hair

As reported by Fujii *et al*. (2024), hair was washed with 70% ethanol, soaked in lipid removal solution (chloroform/methanol [2:1]) for 24 hr; the dried hair (0.5 g) was treated with extraction solution (5 M urea, 2.6 M thiourea, 250 mM 2-mercaptoethanol, 25 mM tris-hydroxymethyl aminomethane), and incubated at 50°C for 48–72 hr in a shaking constant-temperature water bath (Titec Corp.) to extract keratin. The amount of protein in the extract was measured using a Nanodrop (Thermo Fisher Scientific Corporation), and keratin (approximately 40–70 kDa) and keratin-related proteins (approximately 10–30 kDa) were confirmed using electrophoresis (SDS-PAGE) (Fig. 1A, left).

Preparation of keratin film

Keratin films were prepared using a modified version of the method described by Fujii *et al*. (2004) . To each well of a 24-well plate (Azwan Corporation), 800 μL of 10% TCA was added, into which 2–3 mg/200

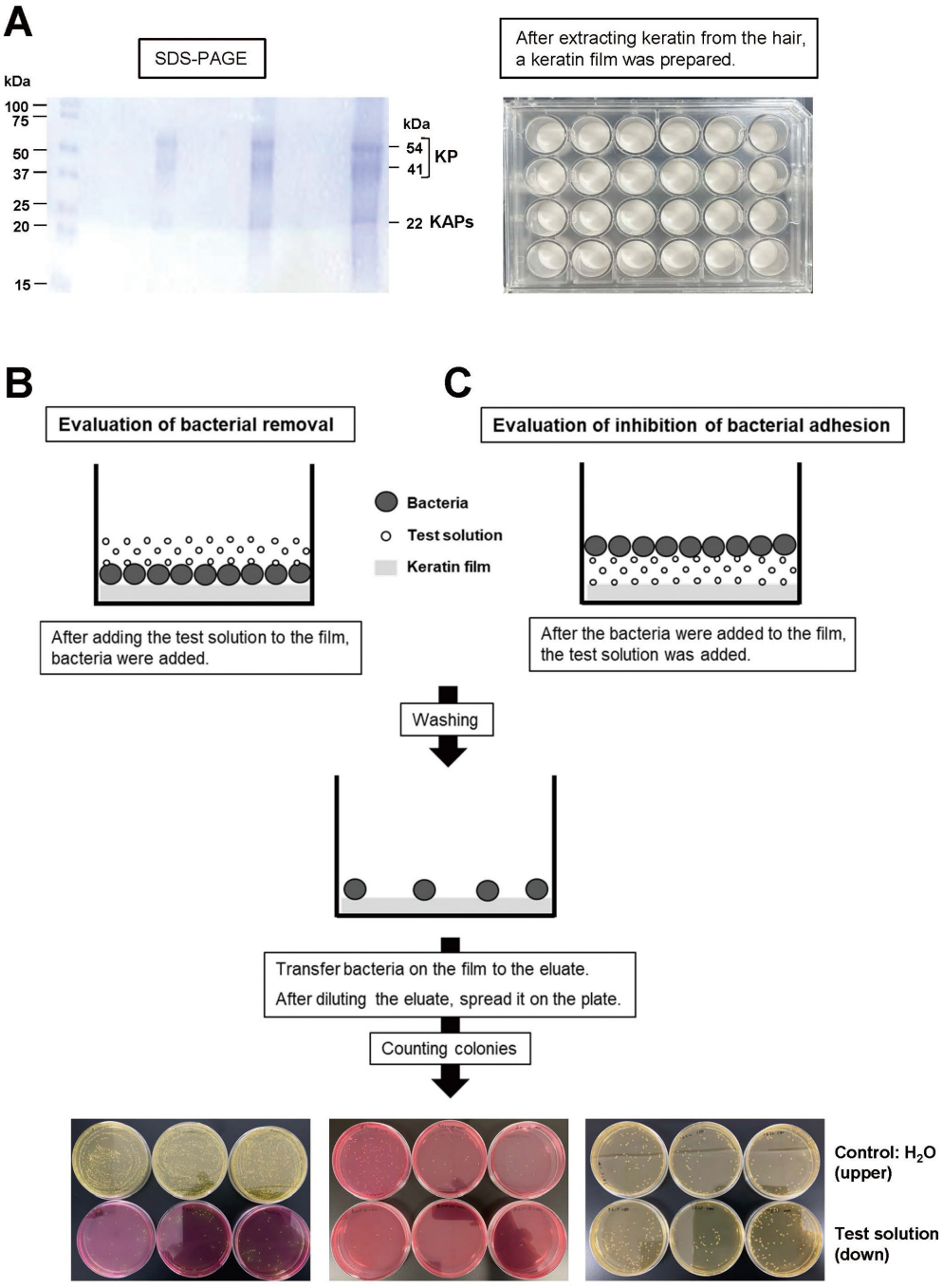

Fig. 1. Methods for assessing bacterial removal and inhibition of bacterial adhesion using keratin film. A: After keratin was extracted from hair, SDS-PAGE was performed to confirm the extraction of keratin (KP: approximately 40–70 kDa) and keratin-associated proteins (KAPs: approximately 10–30 kDa). Keratin films were prepared using 24 well plates. B: To evaluate bacterial removal, bacteria were added first, followed by the control or test solutions. C: To evaluate inhibition of bacterial adhesion, the control (sterile water) or test solution (chlorine dioxide and fucoidan) was added first, followed by the addition of bacteria. In both evaluation methods, after washing, the bacteria adsorbed on the film were transferred to a bacterial collection solution, incubated on an agar medium, and the number of colonies was determined.

μL of keratin extract warmed to 50°C was added using the post-casting method. The wells were allowed to stand for > 4 hr, immersed in distilled water, and then rinsed with running water for at least 12 hr. The wells were then immersed in distilled water for 3 hr, the liquid was removed, followed by drying for at least 12 hr at room temperature. To evaluate bacterial removal and the inhibition of bacterial adhesion, the wells were irradiated with a UV germicidal lamp for 5 min to eliminate residual bacteria (Fig. 1A, right).

Evaluation of bacterial removal

The methodology described by Park *et al*. was adapted to assess bacterial removal (Park *et al*., 2021). As shown in Fig. 1B, 150 μL of the inoculated culture (OD600 = 1.00) from a single colony was added to each well of a 24-well plate and incubated at 37°C for 1 hr. After six washes with 500 μL of sterile water, 500 μL of sterile water (control) or test solution (containing 0.16% fucoidan and 0.002% chlorine dioxide) was added and incubated at 37°C for another hour. After three washes with 500 μL of sterile water, the bacteria were swabbed thrice with 500 μL of 0.1% Tween 80 solution. Subsequently, each bacterial collection solution was diluted (*S. aureus*: 100–2000, *S. epidermidis*: 500–1000, *C. acnes*: 500–1000, and *E. coli*: 1000–3000), incubated on agar medium for at least 24 hr, and the colony count was determined. The percentage of colony-forming units with fucoidan and cleverin was calculated relative to that in the control.

Evaluation of bacterial adhesion inhibition

Inhibition of bacterial adhesion was evaluated using the method described by Park *et al*. (2021). As shown in Fig. 1B, 500 μL of sterile water (control) or the test solution (0.16% fucoidan and 0.002% chlorine dioxide) was added to each well of a 24-well plate and allowed to stand at 37°C for 30 min. An inoculated culture (150 μL of the inoculated culture from a single colony [OD600=1.00]) was added and allowed to stand at 37°C for 1 hr. After washing six times with 500 μL of sterile water, the bacteria were swabbed three times with 500 μL of 0.1% Tween 80 solution. Each bacterial collection solution was diluted (*S. aureus*: 100–2500; *S. epidermidis*: 10,000–25,000; *C. acnes*: 500–12,500; and *E. coli*: 2500–10,000), incubated on agar medium for at least 24 hr, and the colony count was determined. The percentage of colony-forming units with fucoidan and cleverin was calculated relative to that in the control.

Statistical Analysis

In determining the significant difference for the number of colonies between the control and test solutions in the evaluation of bacterial removal and inhibition of bacterial adhesion, the mean value and standard deviation were determined, followed by the Student's t-test. The significance level was set at $p < 0.05$.

Ethics review

Hair donation and keratin film preparation were performed with the approval of the Fukuyama University Research Safety and Ethics Committee (permit number R5-26).

RESULTS

Evaluation of the bacterial removal effect of cleverin or fucoidan against *S. aureus* and inhibition of bacterial adhesion using keratin film

Cleverin (0.002% chlorine dioxide) showed a significant bacterial removal effect (100%) against *S. aureus*, compared with that in the control (sterile water) (Fig. 2A). However, the results of the bacterial adhesion inhibition evaluation showed that cleverin (0.002% chlorine dioxide) was not effective at inhibiting bacterial adhesion compared to the control (Fig. 2B).

Fucoidan (0.16%) showed no bacterial removal effect against *S. aureus* compared with that in the control (Fig. 2A). However, the evaluation results for bacterial adhesion inhibition showed that 0.16% fucoidan significantly inhibited bacterial adhesion (97%) compared with that in the control (Fig. 2B). When bacterial removal and inhibition of bacterial adhesion occur, the bacteria on the keratin film are significantly eliminated. In the absence of inhibition, the bacteria remained on the keratin film to the same extent as in the control.

Evaluation of fucoidan for bacterial removal against various bacteria using a keratin film

The bacterial removal efficiency of 0.16% fucoidan against each bacterium was compared to that of the control using the keratin film; the results showed that fucoidan was not effective against any of the bacteria examined (*S. epidermidis*, *C. acnes*, and *E. coli*) (Fig. 3A).

Evaluation of fucoidan regarding inhibition of bacterial adhesion in various bacteria using keratin film

The evaluation of 0.16% fucoidan for the adhesion of

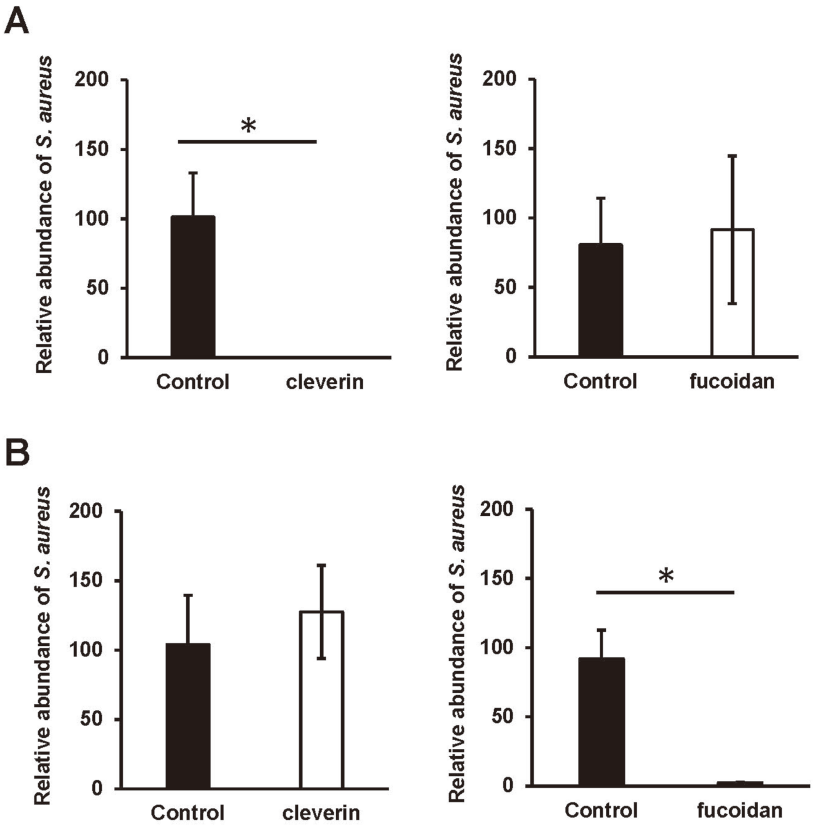

Fig. 2. Evaluation of bacterial removal and inhibition of bacterial adhesion for *S. aureus.* Bacterial removal (A) and adhesion inhibition (B) effects of cleverin (0.002% chlorine dioxide) and 0.16% fucoidan on *S. aureus*. The mean ± standard deviation in each experiment was calculated by setting the colony-forming units present in 1.0 mL of the bacterial collection solution in one of the control groups to 100%. Significant differences were determined using the Student's t-test (*$p < 0.05$, $n = 3$).

each bacterium using a keratin film compared to that of the control showed a significant inhibitory effect (96%) of fucoidan against *S. epidermidis* (Fig. 3B). However, it did not inhibit the growth of *C. acnes* or *E. coli*. Fucoidan (0.008%) also significantly inhibited (93% and 90%, respectively) *S. epidermidis* and *S. aureus* adhesion (data not shown).

DISCUSSION

We examined whether keratin films could serve as substitutes for the AD-like stratum corneum and could be utilized for bacterial removal or inhibition of bacterial adhesion inhibition. Our findings revealed that fucoidan exerted an anti-adherence effect on *S. aureus* (Fig. 2B), consistent with the results reported by Park *et al*. (2021). However, no bacterial removal was observed (Fig. 2A). Cleverin, a disinfectant used against *S. aureus*, failed to inhibit bacterial adhesion. Chlorine dioxide exerts a bactericidal effect by oxidizing tyrosine and tryptophan present in the membrane surface proteins (Ogata, 2007). When chlorine dioxide comes in contact with the keratin film, which is composed of proteins, it oxidizes the film and loses its bactericidal efficacy. Therefore, chlorine dioxide was not effective when the film was first evaluated for the inhibition of bacterial adhesion. However, chlorine dioxide was effective for sterilization when the reagent was brought in contact with the bacteria after they were uniformly spread on the film. The results obtained with chlorine dioxide and fucoidan suggest that keratin films can be used as an *in vitro* substrate to evaluate bacterial removal and bacterial adhesion and that the outcomes may reflect those on AD-like stratum corneum.

In experiments with keratinocytes, fucoidan from *Fucus versiculosus* (proprietary water-soluble extract, molecular weight unknown) was provided by Marino-

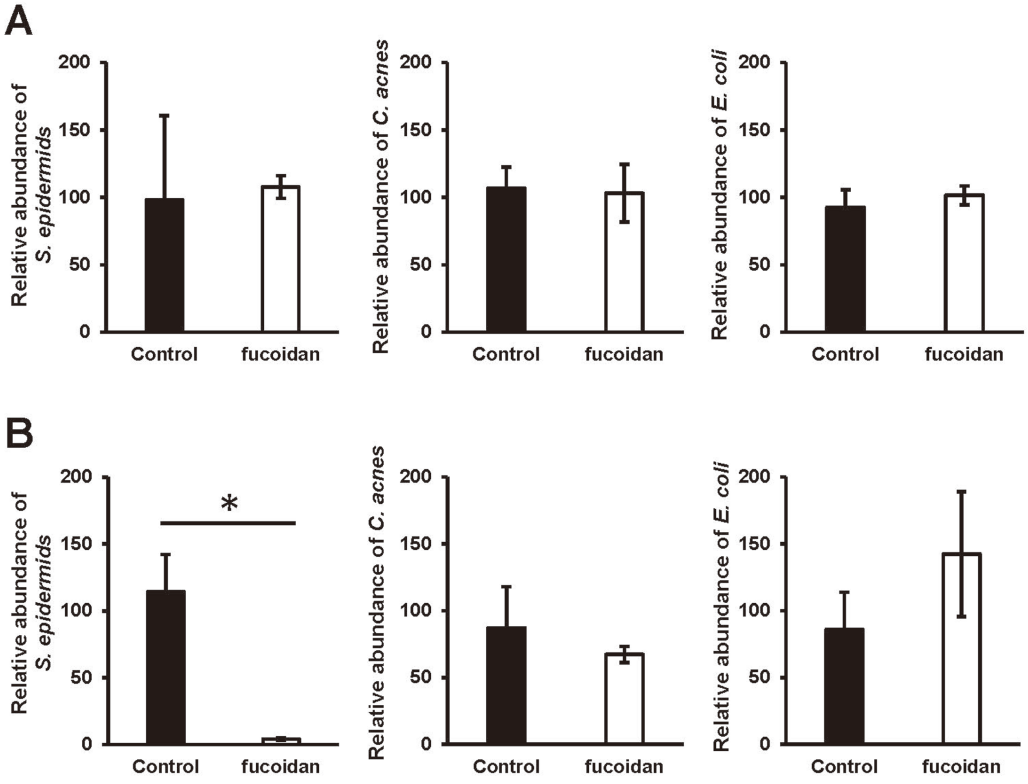

Fig. 3. Evaluation of bacterial removal and inhibition of bacterial adhesion for each bacterium. Effect of 0.16% fucoidan on the removal (A) and inhibition of adhesion (B) for each bacterium. The mean ± standard deviation in each experiment was calculated by setting the colony forming units present in 1.0 mL of bacterial collection solution in one of the control groups to 100%. Significant differences were determined using the Student's t-test (*$p < 0.05$, $n = 3$).

va Pvt., Ltd. (Cambridge, Australia) and had no effect on *S. epidermidis*. However, it has been reported to inhibit adhesion of *S. aureus* and *C. acnes* (approximately 75% and 40%, respectively) (Park *et al*., 2021). In contrast, fucoidan from Tongan mozuku, which was evaluated on keratin films in this study, showed 96% and 97% inhibition of *S. epidermidis* and *S. aureus*, respectively, but showed no effect on *C. acnes* or *E. coli* (Fig. 2B and 3 B). These variations in efficacy may be attributable to differences in the origin and molecular weight of fucoidan (Jesumani *et al*., 2020; Liu *et al*., 2017b; Ustyuzhanina *et al*., 2014), or to the discrepancies in the evaluation methods used (cells and keratin films).

Exacerbation of atopic dermatitis is believed to occur because of the inhibition of weak acidification as a result of diminished fatty acid production, and the reduction in moisture retention owing to decreased glycerol production, which is caused by a decrease in *S. epidermidis* and *C. acnes* (Grice and Segre, 2011; Nodake *et al*., 2015), leading to an increase in *S. aureus* (Duan *et al*., 2021; Liu *et al*., 2017a). *C. acnes* is the most prevalent indigenous skin bacterium, and its excessive proliferation causes acne (Tomida, 2016). Hence, fucoidan derived from *Fucus versiculosus*, which suppresses *C. acnes* without impairing *S. epidermidis* adhesion, may be effective against acne. However, considering its moisturizing properties, fucoidan derived from Tongan mozuku, which inhibits *S. epidermidis*, but not *C. acnes*, may be a more suitable option for addressing AD.

We previously reported that fucoidan from Tongan mozuku impeded the growth of *S. aureus* and *S. epidermidis* along with a modest increase in the abundance of *C. acnes* (Matsuoka *et al*., 2023). Fucoidan from Tongan mozuku, which has an ultra-high molecular weight of 1700 kDa compared to that of ordinary fucoidan (200–300 kDa), has potential to be used for AD prevention and treatment because of its moisturizing effects, ability to inhibit *S. aureus* growth and attachment, and

ability to retain moisture by maintaining *C. acnes*. However, further research is necessary to fully understand the efficacy of ultra-high-molecular-weight fucoidan on the AD-like stratum corneum.

Future Development

If we can identify active ingredients suitable for each skin disease using keratin films to eliminate bacteria and evaluate adhesion of indigenous skin bacteria that cause adverse or positive effects in skin diseases in which CC, including AD, is disrupted, we can expect to find applications in cosmetics and pharmaceuticals. We also believe that keratin films can be used as the first screening method to evaluate the inhibition of the adhesion of pathogenic microorganisms and viruses in skin diseases.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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