Malassezia furfur promoting growth of *Staphylococcus epidermidis* by increasing pH when cultured in a lipid-free environment

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To the Editor: Malassezia and *Staphylococcus epidermidis* (*S. epidermidis*) are commensal organisms found on human skin, and both display an imbalance of colonization in individuals with seborrheic dermatitis/dandruff (SD/D).[1-3] *Malassezia* have been implicated in SD/D pathogenesis, as evidenced by an imbalance in the numbers of *Malassezia* that colonize the skin lesions of people with SD/D, and the fact that antifungal treatment exerts a modifying effect.[4] Among *Malassezia* species, *Malassezia furfur* (*M. furfur*) is the predominant in Chinese seborrheic dermatitis (SD) patients.[5] Recently, large numbers of *S. epidermidis* have been found in the lesions of individuals with acquired immunodeficiency syndrome (AIDS)-related SD, classical SD, and dandruff.[1,2] A topical antibiotic cream such as fusidic acid cream can often improve the symptoms of SD, suggesting a pathological role for *S. epidermidis* in SD.[1] Whether co-colonization of *Malassezia* and *S. epidermidis* in the skin lesions of SD/D suggests their biological interactions is an interesting topic to investigate.

The SD/D lesions have decreased levels of skin surface total lipids,[6,7] and dandruff contains lower levels of triglycerides, cholesterol, and total ceramides in the corneocytes. All *Malassezia* species, except *M. pachydermatis,* require exogenous lipids, and especially triglycerides, to maintain their growth. Under SD/D conditions, a deficiency of lipids might not only limit the growth of *Malassezia* species, but also affect the metabolites produced by *Malassezia,* which can further affect the microenvironment of the skin. The growth of *S. epidermidis* is sensitive to certain environmental factors, such as changes in pH, temperature, and the concentration of glucose. Therefore, we hypothesized that under conditions of lipid deficiency, *Malassezia* yeasts would change the environment of skin and further affect the colonization of *S. epidermidis*. In this study, we revealed the impacts of *M. furfur* culture supernatant on the growth of *S. epidermidis* in a lipid-free environment

in vitro as the first step to study the interaction between *M. furfur* and *S. epidermidis*.

M. furfur (ATCC 14521) and *S. epidermidis* (ATCC 12228) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). *M. furfur* was cultured in modified Dixon medium (Qingdao Haibo Biological, China) under aerobic conditions at 30°C. *S. epidermidis* was routinely cultured on Tryptone soya agar (TSA, 236950, Becton Dickinson, Franklin Lakes, NJ, USA) under aerobic conditions at 37°C.

M. furfur were cultured on modified Dixon agar for 3 days, and the isolated colonies were inoculated into a lipid-free medium (beef extract-sodium chloride-Peptone [BSCP], 3 g of beef extract, 10 g of Peptone, and 5 g NaCl dissolved in 1 L of distilled water). BSCP is a common culture medium that does not contain lipids. The concentration of *M. furfur* was adjusted to 1.5×10^7 colony forming units (CFUs)/mL, and the adjusted suspension was maintained 30°C under aerobic conditions with shaking overnight (15 h). The yeast cells were removed from the BSCP by centrifugation at $2000 \times g$ for 5 min. Next, the culture supernatant was filtered through a 0.22 μm membrane (Advantec, Japan) to remove any remaining cells, and collected as the culture supernatant of *M. furfur* (SMF) for use in further studies. In addition, we prepared BSCP at different pH values. The pH of BSCP was adjusted to values of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 by addition of 1 mol/L HCl or 1 mol/L NaOH.

S. epidermidis was grown in TSA for 24 h and a selected colony was added to BSCP. Aliquots of bacterial suspension (90 μL, $A_{600\,nm} = 0.02$) were seeded into a U-bottom 96-well microtiter polystyrene plate (Corning, Corning NY, USA). *M. furfur* supernatant was prepared as a 5-fold dilution series (0, 5, 25, 125, and 625-fold) in BSCP. Different concentrations of *M. furfur* culture supernatant and BSCP (90 μL per well, as the control

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group) were added to the bacterial suspensions. The growth of *S. epidermidis* was measured by the hourly testing of bacterial turbidity at 600 nm with a microplate reader. The optical density (*A*) values at different time points were used to create a growth curve for each group.

A second 96-well plate was prepared as described above and seeded with bacteria. After being cultured for 15 h, the bacterial suspension was mixed by pipetting and transferred to a new tube containing 520 μL BSCP. Next, 20 μL of count bright absolute counting beads (Molecular Probes, C36950, Eugene, OR, USA) was added to each tube as an internal standard. The number of bacteria in each sample was counted by flow cytometry, and the concentration of *S. epidermidis* was calculated using the following formula: $\frac{A}{B} \times \frac{C}{D}$ = Concentration of sample as cells/μL, where: *A* = number of cell events; *B* = number of bead events; *C* = assigned bead count of the lot (beads/50 μL); *D* = volume of the sample (μL).

To investigate how pH affected the growth of *S. epidermidis*, we used BSCP of different pH values to adjust the concentrations of the bacterial suspensions (90 μL, $A_{600\,nm}$ = 0.02). Aliquots (180 μL) of bacterial suspensions with different pH values were added into a 96-well plate. The growth of *S. epidermidis* was measured by flow cytometry in conjunction with a growth curve. To explore how the pH of SMF affected the growth of *S. epidermidis*, we synchronized the pH values of BSCP and SMF, and then used the growth curve method in those studies.

The pH values of SMF and BSCP were measured using a pH reagent. The Berthelot method and a urease activity assay kit (MAK120, Sigma, St. Louis, MO, USA) were used to test the urease activity of *M. furfur* in lipid-sufficient conditions (BSCP with 2 mL of glycerol, BSCP with 2 mL of oleic acid, and BSCP with 10 mL of Tween 40) in lipid-free conditions (BSCP). Briefly, *M. furfur* was cultured in each condition for 15 h, after which the cells were collected by centrifugation and washed three times with phosphate-buffered saline. Each yeast pellet was resuspended in assay buffer to the same concentration ($A_{600\,nm}$ = 0.90), and 90 μL aliquots were transferred into a 96-well flat bottom plate. Next, 10 μL of urea was added to each well, and the plate was incubated for 1.5 h at 37°C. Next, 100 μL of reagent A was added to terminate the urease reaction, and 50 μL of reagent B was then added to each well. The reaction was then incubated for 30 min in the dark. The absorbance of each well was measured at 670 nm, and the *A* values were made proportional to the urease activity in each sample. Acetohydroxamic acid (AHA; 159034–5MG, Sigma, USA) was used as a known bacterial urease inhibitor. A 10 mg/mL solution of AHA was used for preparing the SMF. After co-cultivation for 15 h, the urease activity of *M. furfur* and the pH values of SMF plus AHA, and BSCP plus AHA growth media were measured. The *M. furfur* suspension used for detecting urease activity was adjusted to *A* value of 0.30 as measured at 600 nm.

Each experiment was performed at least three times, and results are presented as the mean ± standard error (SE). The significance of differences between groups was analyzed by one-way analysis of variance (ANOVA) and the un-paired *t*-test. Statistical analyses were performed using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). A *P* < 0.05 were considered statistically significant.

To investigate the effect of SMF on the growth of *S. epidermidis*, we treated *S. epidermidis* with different concentrations of SMF and then evaluated the bacteria's growth by growth curves and flow cytometry. The growth curves indicated that SMF promoted the growth of *S. epidermidis* when compared to *S. epidermidis* grown in the BSCP control medium. Moreover, the growth promoting effect of SMF occurred in a concentration-dependent manner [Figure 1A]. Similar results were obtained when counting the bacteria numbers by flow cytometry (FCM). When compared with *S. epidermidis* cultured with BSCP, *S. epidermidis* cultured with non-diluted SMF displayed the greatest rate of growth (*F* = 45.700, *P* < 0.001; Figure 1B).

It was previously reported that the pH value was related to the growth of *S. epidermidis* in platelet concentrates.[8] We examined the effect of pH on the growth of *S. epidermidis* in BSCP. Growth curves for *S. epidermidis* at different pH
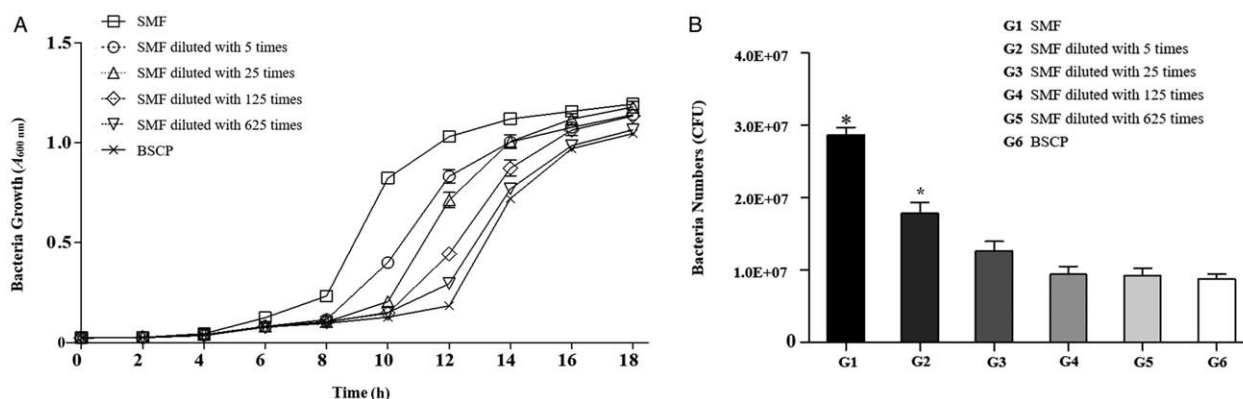

Figure 1: Effect of SMF on the growth of *S. epidermidis*. (A) Growth curves for *S. epidermidis* cultured in a five dilution series of SMF, and in BSCP (control group). (B) Flow cytometry quantification of the numbers of *S. epidermidis* after treatment with SMF or BSCP for 12 h. *Compared with G6, *F* = 45.700, *P* < 0.001. BSCP: Beef extract-sodium chloride-Peptone; SMF: Supernatant of *Malassezia furfur*.

values were constructed by examining the $A_{600\,nm}$ values of the respective groups [Supplementary Figure 1A, http://links.lww.com/CM9/A21]. We found that *S. epidermidis* growth was significantly reduced at pH values of 3.0 and 4.0, and the most favorable pH for growth in BSCP was 7.0. Similar results were obtained when we counted the bacteria numbers by FCM [Supplementary Figure 1B, http://links.lww.com/CM9/A21]. Next, we measured the pH values in the SMF and BSCP groups, and found them to be 6.23 ± 0.01 and 5.13 ± 0.01 ($F = 48.361$, $P < 0.001$), respectively [Supplementary Figure 1C, http://links.lww.com/CM9/A21], indicating that the pH value of BSCP had increased after culture with *M. furfur*. When we synchronized the pH values of BSCP and SMF, the growth rates of *S. epidermidis* in SMF and BSCP did not significantly differ [Supplementary Figure 1D and 1E, http://links.lww.com/CM9/A21]. These results suggested that an increase in the pH value of SMF promoted the growth of *S. epidermidis*.

Urease catalyzes the hydrolysis of urea into ammonia, which dissolves in water to form a weak alkaline solution, and produces an increase in pH. Urease activity was previously detected in *M. furfur*. We compared the urease activity of *M. furfur* that was cultured in lipid-sufficient and lipid-free conditions. The urease activity of *M. furfur* increased after culture in a lipid-free medium for 15 h, when compared with the urease activity of *M. furfur* that was cultured in a lipid-sufficient medium ($t = 67.540$, $P < 0.001$; Supplementary Figure 2A, http://links.lww.com/CM9/A21). We also examined the changes in medium pH that occurred during culture in lipid-sufficient and lipid-free conditions [Supplementary Figure 2B, http://links.lww.com/CM9/A21]. When *M. furfur* was cultured in a lipid-free environment, the culture supernatants showed progressive increases in pH. Whereas in the lipid-sufficient environment, the pH values of the supernatants decreased. However, after 12 h of culture, the pH values of the supernatants began to gradually increase. We speculated that the initial decrease in pH values was probably due to the generation of free fatty acids derived from decomposing lipids. After 12 h of culture, the lipids in the culture medium might be fully consumed by *M. furfur*, causing the medium to become lipid-deficient, and the pH values to increase.

Acetohydroxamic acid (AHA) is an inhibitor of urease in various bacteria and fungi. After 15 h of incubation, the urease activity of *M. furfur* cultured with AHA had decreased when compared to urease activity in the control group ($t = 15.959$, $P < 0.001$; Supplementary Figure 3A, http://links.lww.com/CM9/A21). The pH values of SMF, SMF plus AHA, BSCP, and BSCP plus AHA were 6.21 ± 0.04, 5.42 ± 0.02, 5.12 ± 0.01, and 5.13 ± 0.01, respectively [Supplementary Figure 3B, http://links.lww.com/CM9/A21]. These results indicated that AHA could inhibit the increase in pH values in the SMF group, but could not significantly change the pH values in the BSCP plus AHA group. This suggested that the urease activity of *M. furfur* contributed to the increased pH values in the SMF group. Therefore, we hypothesized that a lipid-deficient environment caused a change in *M. furfur* urease activity, which led to a subsequent change in pH that affected the growth of *S. epidermidis*.

As two members of the normal resident flora on human skin, both *Malassezia* and *S. epidermidis* display imbalanced colonization in SD/D[2,3] and are regarded as co-culprits in causing SD/D. High quantities of lipid prevent *Malassezia* from inducing inflammation in a manner consistent with its commensal status.[9] The amount of total lipid on the skin surface of SD/D patients remains controversial, and has been reported as being both higher or lower than that in healthy populations. We studied the interaction between *M. furfur* and *S. epidermidis* under lipid-free conditions. We also investigated how the culture supernatant of *M. furfur* affects *S. epidermidis*, and found that it promoted the growth of *S. epidermidis*.

The pH value needed to create favorable conditions for the growth of skin microbiota has been previously reported. For example, the growth of *P. acne in vitro* is promoted by a pH of 6.0 rather than a pH of 5.0. To further explore the mechanism by which SMF promotes the growth of *S. epidermidis*, we examined the effect of pH on the growth of *S. epidermidis* in BSCP and observed a similar effect of pH on the growth of *S. epidermidis*; that is, the growth of *S. epidermidis* in BSCP increased at a pH of 6.0, but not at a pH of 5.0. Therefore, we examined the pH values of SMF and BSCP, and found that the pH of SMF was higher than that of BSCP. When we synchronized the pH values of BSCP and SMF, the growth of *S. epidermidis* was not significantly different in the SMF and BSCP groups. Thus, we concluded that the pH level was the major factor that promoted the growth of *S. epidermidis* in SMF.

Urease has been found in a variety of bacteria and fungi, including *Helicobacter pylori*, *Proteus Mirabilis*, *Staphylococcus saprophyticus*, and *Malassezia*. Urease catalyzes the breakdown of urea into ammonia, and thereby increases the pH of an environment. Our results indicated that an inhibitor of urease (AHA) could inhibit the urease activity of *M. furfur* and prevent an increase in the pH value of SMF, suggesting that the urease activity of *M. furfur* contributes to the increase of pH values in SMF. Factors that affect urease activity in microorganisms include temperature and the presence of nickel in the growth medium of certain bacteria. In our research, we detected increased urease activity in *M. furfur* after it was cultured in a lipid-free environment when compared with a lipid sufficient environment, suggesting that the urease activity of *M. furfur* could be influenced by lipid concentrations.

Clinical studies have shown that the pH of the skin surface of individuals with SD/D slightly altered towards alkalinity (from a pH < 5 to a pH of ~6).[3] Thus, the growth of *S. epidermidis* and *P. acne* could be both promoted by this elevated pH environment. However, only *S. epidermidis* has been found in great abundance on SD/D lesions.[1] This discrepancy is probably due to the different growth cycles of bacteria. When compared with *P. acne*, the growth cycle of *S. epidermidis* was relatively short.[10] Consequently, a rapid proliferation of *S. epidermidis* would consume the available nutrients and change the environment of the skin, and thereby limit the proliferation of *P. acne*. Additionally, *S. epidermidis* was reported to inhibit the growth of *P. acne* by mediating fermentation.[11] These two reasons might

explain the imbalanced colonization of *S. epidermidis* and *P. acne* in SD/D.

In summary, the present study demonstrated that SMF could promote the growth of *S. epidermidis* in a lipid-free *in vitro* environment by raising the pH values in the culture environment. Therefore, we speculated that under lipid deficient SD/D conditions, an appropriate lipid supplement might help rebalance the colonization of *M. furfur* and *S. epidermidis* and even mitigate the SD/D. However, this hypothesis needs to be tested *in vivo*.

Funding

Conflicts of interest

None.

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