Effect of Tween 60 and Tween 80 on the growth of *Malassezia* species

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Summary

Yeasts in the genus *Malassezia* are a member of the normal skin flora and also cause various skin diseases in both human and animals. Except *M. pachydermatis*, all of the other *Malassezia* species are known to be lipid-dependent fungi which require external lipid sources for growth. Fatty acid-derived Tweens are widely used in various products such as foods, medicines, cosmetics, and also laboratory culture purpose. Therefore, it is necessary to evaluate the effect of Tweens on fungal growth. Three *Malassezia* species; *M. furfur* CBS 1878^{T}, *M. pachydermatis* CBS 1879^{NT} and *M. slooffiae* CBS 7956^{NT} appeared to have different preference for Tween in that they showed optimal growth at 1% Tween 80, 2% Tween 60, and 2% Tween 80, respectively. Tween with concentration more than 0.5% showed significant growth of *Malassezia*. In addition, under

co-culture conditions, the population ratio of each *Malassezia* species maintained equilibrium regardless of initial cell ratio, implying an interspecies interaction of *Malassezia*. The results suggest a need to re-evaluate the use of Tweens in products associated with the skin.

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Tween, polysorbate, Malassezia, co-culture, growth

Introduction

The yeasts in the genus *Malassezia* are a part of cutaneous normal flora in humans and warm blood mammals.[1] Based on molecular analysis, seventeen species are recognized to date.[2-4] Except *M. pachydermatis*, all *Malassezia* species are lipid-dependent and they require external lipid sources for growth. Therefore, they commonly colonize the seborrheic part of host skin and also are associated with various skin disorders such as pityriasis versicolor, seborrheic dermatitis, *Malassezia* folliculitis, and atopic dermatitis in both normal individuals and immunocompromised patients.[5-7] Despite the fact that most of the superficial infections caused by *Malassezia* are mild cases and can be treated with typical antifungal agents, complete recovery is difficult because recurrences have also been observed within years after healing.[8] Moreover, dermatological conditions caused by the yeast such as hypopigmentation and hyperpigmentation can make patient lose self-confidence. Though the pathogenic mechanism of *Malassezia* remains unclear, an etiological model of *Malassezia* infection was recently proposed, that the yeasts may play an important role in skin disorders via their extracellular lipase activity where the enzyme produced by *Malassezia* hydrolyzes sebum triglycerides and increases the level of free fatty acids (FFAs).[9-10] Based on the evidence supporting the etiological model of dandruff and seborrheic dermatitis, some FFAs are consumed by *Malassezia* and stimulate fungal proliferation growth concurrently with the excess portion of FFAs penetrating into stratum corneum, leading to skin irritation.[11] Therefore, lipid environment and growth of the yeast are supposed to be closely related to pathogenicity.

Tweens or polysorbates are a class of nonionic emulsifiers derived from polyethoxylated sorbitan esterified with fatty acids and classified by the type of the esterified fatty acids designated as a number following polysorbate or Tween (e.g. Tween 20 refers to polyoxyethylene (20) sorbitan monolaurate). Tweens, especially those containing long chain fatty acids such as Tween 80, are widely used for solubilizing or stabilizing oil in aqueous products of foods, pharmaceuticals and cosmetics.[12-13] Due to lipid-dependency, the ability of utilizing lipids and esterified fatty acids from the environment of *Malassezia* species has been studied and the assimilation pattern of Tweens of each species has been previously described by many groups suggesting key characteristics of the yeasts for routine clinical identification.[14-16] According to the growth pattern of *Malassezia* species on media, most of *Malassezia* species have shown preference to Tweens esterified with long chain fatty acids, e.g. Tween 60 and Tween 80.[17-19] These Tweens are also contained in various products associated with skin such as cosmetics and therefore they may positively affect the growth of *Malassezia*. Recently, the effect of Tween 80 on bacterial biofilm formation was investigated and the results were different by types of bacteria.[20] Nevertheless, except of qualitative data on culture-based identification, the quantitative evaluation of the effect of Tweens on *Malassezia* growth *in vitro* or *in vivo* has not been reported thus far.

Concerning the correlation between the substances and cutaneous microflora therefore, the purpose of this study was to evaluate the effect of the concentration of polysorbates esterified with long chain fatty acids of stearic acid (Tween 60) and oleic acid (Tween 80) on the i*n vitro* growth of three *Malassezia* species namely *M. furfur, M. pachydermatis*, and *M. slooffiae*. In addition, a trial experiment of co-culture of different *Malassezia* species was conducted to investigate growth and population changes among species in the presence of Tween, which could help obtaining a better understanding of the impact of Tweens to *Malassezia*.

Materials and methods

Strains and medium

The yeast strains used in this study were *M. furfur* CBS 1878^{T}, *M. pachydermatis* CBS 1879^{NT} and *M. slooffiae* CBS 7956^{NT} (Centraalbureau voor Schimmelcultures, since 2017 renamed as Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands). The strains were routinely maintained by subculture on YPD-Tween agar [1% yeast extract, 2% bacto peptone, 2% glucose, 2% agar, 0.5% Tween 80 (Sigma, USA)] at 30ºC.

Effect of Tweens on growth of *Malassezia*

To study the effect of Tweens on the growth of *Malassezia*, the yeasts were pre-cultured in YPD-Tween broth (1% yeast extract, 2% bacto peptone, 2% glucose, 0.5% Tween 80, pH 6.4) at 30ºC, 150 rpm for 2 days in a shaking incubator. Then the cells were washed twice with distilled water and collected by centrifugation at 2000g, 4ºC for 5 min. The cell suspension with optical density (OD) 0.01 (approximately 2.5x10^{5} cells/mL) measured at 600 nm were further inoculated in 50 mL of YPD broth supplemented with Tween 60 or Tween 80 (Sigma, USA) at a concentration between 0–2% (v/v) and incubated in a shaking incubator at 30ºC, 150 rpm for 11 days. The growth of *Malassezia* was measured by spectrophotometer at 600 nm every 24 h.

Co-culture assay

According to the results described above, the treatment that the lipid-dependent species showed a sufficient growth and represented a similar growth pattern, was 0.5% Tween 80. Therefore, this condition was selected for cultivation with *M. pachydermatis* in the co-culture assay. The cells of each *Malassezia* species were pre-cultured and prepared as described above. The cell suspensions were adjusted to OD 10 (approximately 2.5 x 10^{8} cells/mL) measured at 600 nm and the mixtures of cell suspensions of *M. pachydermatis:M. furfur (Mp:Mf)* and *M. pachydermatis:M. slooffiae (Mp:Ms)* were prepared at the ratio of 1:9, 5:5, and 9:1. The main cultures were inoculated by adding 0.5 mL of the mixed *Malassezia* suspension into 49.5 mL of YPD-Tween broth (1% yeast extract, 2% bacto peptone, 2% glucose, 0.5% Tween 80, pH 6.4) (final OD = 0.1) and incubated at 30ºC, 150 rpm for 10 days. The OD values of each culture were measured spectrophotometrically every 24 h.

Viable cell count of the lipid-dependent and non-lipid-dependent Malassezia species in co-culture

Since *M. pachydermatis* is the only lipid-independent species, viable cells in co-culture mixture are distinguishable from those of lipid-dependent species based on the ability to grow on a medium without a lipid source. The cultures from the co-culture assay were collected daily and diluted 10^{4}- to 10^{6} -fold with distilled water by using a ten-fold serial dilution method. Then 100 µL of diluted samples were spread on YPD agar and YPD-Tween agar, respectively, and incubated at 30ºC for 3 days. The yeast colonies that appeared on YPD-Tween agar and YPD agar were considered as total viable yeast cells (lipid-independent and lipid-dependent species) and only viable lipid-independent *M. pachydermatis* cells, respectively..

Statistical Analysis

The results were analyzed by one-way ANOVA followed by Duncan's multiple range test. Differences were considered as statistical significance at $p < 0.05$.

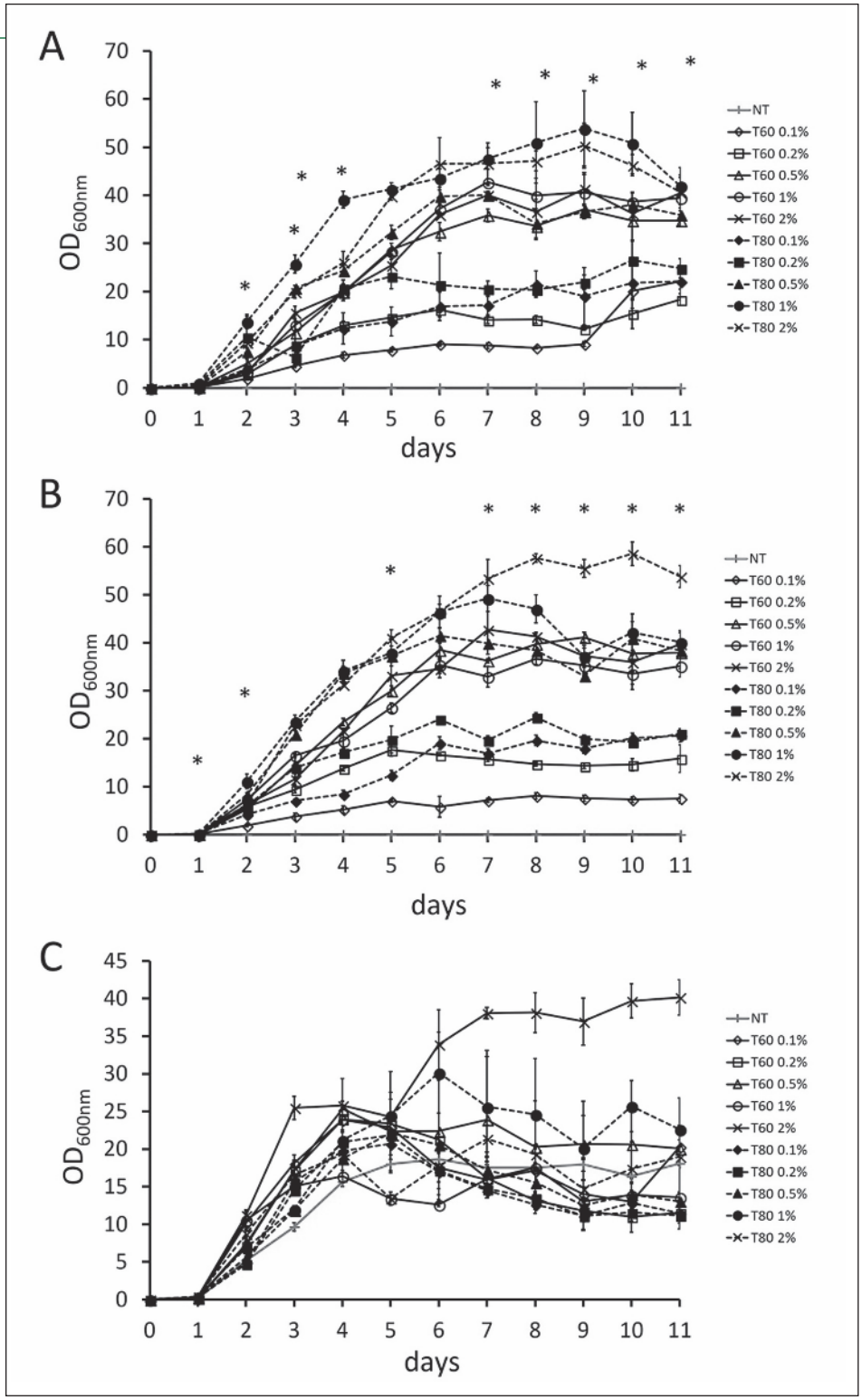
Results

Effect of Tweens on the growth of *Malassezia* species

The growth curve of all *Malassezia* strains cultured in YPD-Tween broth corresponded to a typical growth of microorganisms consisting of lag phase, log phase, and stationary phase. As expected, no growth was observed in non-Tween treatment of the lipid-dependent species of *M. furfur* and *M. slooffiae*. During day 0 to day 1, all treatment exhibited a lag phase where the growth rate was not active. The following period of log phase varied in accordance with species and Tween concentration, occurring from day 1 until day 4 to 7. During the log phase, each *Malassezia* species showed different preference for type and concentration of Tween in that the treatment of 1% Tween 80 indicated statistically dominant growth for *M. furfur,* in the same manner, 2% Tween 60 and 2% Tween 80 for *M. pachydermatis* and *M. slooffiae*, respectively (Fig. 1 A-C).

Figure 1

The growth curve of *Malassezia* species cultured in YPD broth under the presence of Tween 60 and Tween 80 at 120 rpm, 30ºC for 11 days; (A) *M. furfur* CBS 1878^{T}, (B) *M. slooffiae* CBS 7956^{NT}, and (C) *M. pachydermatis* CBS 1879^{NT}. All experiments were performed in triplicate. The red lines indicate non-Tween (NT) treatment. The values represent the mean ± SD of OD at 600 nm. The asterisks show statistical significance ($p < 0.05$).



The stationary phase in which the reproduction rate and cell death of the yeast are equal, indicated different trend of OD values in each treatment and species. In case of lipid- dependent species, interestingly, the OD values increased in treatment with higher Tween concentration; the growth was significant when the Tween concentration was over 0.2% (Fig. 1 A, B). For *M. furfur,* 1% Tween 80 showed the highest OD values in all treatments followed by 2% Tween 80, 0.5% Tween 80, 1% Tween 60, and 2% Tween 80, respectively, since day 3 of cultivation. The maximum OD was 53.89 ± 7.80 on day 9 in 1% Tween 80. However, no statistically differ-

ence was found between 1% and 2% Tween 80 during the stationary phase of *M. furfur* (Fig. 1A). For *M. slooffiae*, since day 3 of cultivation, the ODs of 0.5%, 1%, and 2% Tween 80 were significantly higher than other treatments in the middle of the log phase. The treatment of 2% Tween 80 reached the highest OD of 58.55 ± 2.44 on day 10. During the stationary phase, the ODs of 0.5% to 2% of both Tween 60 and Tween 80 showed statistical differences from Tweens with a concentration below 0.2% (Fig. 1B).

On the other hand, Tween type and concentration seemed to be unrelated to the growth of lipid-independent *M. pachydermatis* since all treatments showed similar growth patterns. However, since day 7, the 2% Tween 60 treatment showed remarkable OD values compared to other treatments followed by those of 1% Tween 80, and 0.5% Tween 60, respectively. The maximum OD was 40.16 ±2.36 on day 11 in 2% Tween 60 while that of the control was 18.65 ± 1.33 on day 6 (Fig. 1C).

The growth of co-culture of *Malassezia* species

The growth curves of co-culture *Mp:Mf* and *Mp:Ms* also indicated a pattern of normal growth curve of the microorganism. Initially, the lag phase could be seen from day 0 to day 1. The continued log phase of *Mp:Mf* and *Mp:Ms* occurred from day 1 to day 5. However, there was a variation of OD values during the stationary phase in which the maximum OD values showed a range from 30 to 50 (Fig. 2A). Intriguingly, the stationary phase was not observed and the OD values of all ratios of *Mp:Ms* decreased instantly after they reached a maximum OD on day 6 or 7 (Fig. 2B).

Changes in population of *Malassezia* species under co-culture

The proportion of viable *M. pachydermatis* in the treatment Mp:Mf (1:9) increased rapidly from 10% to 41.16 ± 7.28% on day 1 and then reached the maximum of 58.64 ± 11.25% on day 5. Afterwards, the *M. pachydermatis* population fluctuated in a range of 45–60%. Conversely, the population of *M. pachydermatis* in the treatment *Mp:Mf* = 9:1 decreased significantly to 57.81 ± 6.37% on day 1 and showed a continued decrease until day 4. Thereafter, the population of *M. pachydermatis* almost stayed unchanged at approximately 30% until the end of the culture. However, the population of *M. pachydermatis* in the treatment of *Mp:Mf* (5:5) showed a proportion between 40–60% with a minor fluctuation (Fig. 3A).

Interestingly, co-cultures of *Mp:Ms* showed similar results as those of *Mp:Mf*. The population of *M. pachy-*

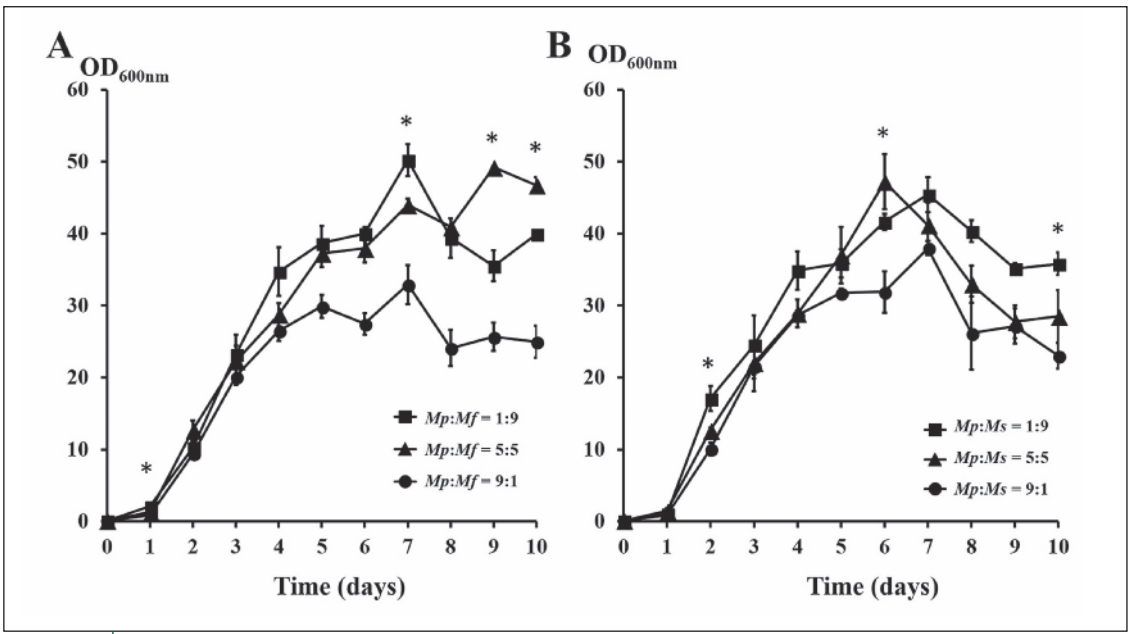


Figure 2 The growth curves of *Malassezia* species in co-culture. The yeasts were co-cultured in YPD-Tween broth at 30ºC for 10 days. (A) Co-culture of *M. pachydermatis* CBS 1879^{NT} and *M. furfur* CBS 1878^{T}, (B) co-culture of *M. pachydermatis* CBS 1879^{NT} and *M. slooffiae* CBS 7956^{NT}. All experiments were performed in triplicate. The values represent the mean ± SD of the OD at 600 nm from three independent experiments. Asterisks represent statistical significance between groups ($p < 0.05$).

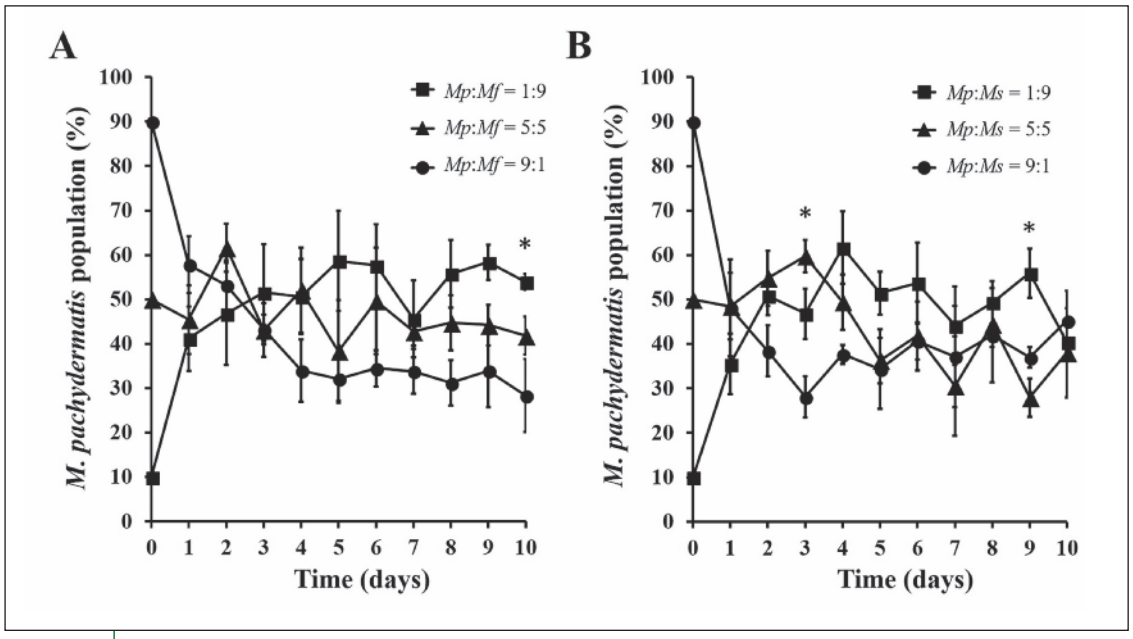


Figure 3 Population of *M. pachydermatis* CBS 1879^{NT} in co-cultures. The yeasts were co-cultured in YPD-Tween broth at 30ºC for 10 days. Total *Malassezia* yeast cells and *M. pachydermatis* cells were counted by colonies grown on YPD-tween agar and YPD agar, respectively. (A) Co-culture of *M. pachydermatis* CBS 1879^{NT} and *M. furfur* CBS 1878^{T}, (B) co-culture of *M. pachydermatis* CBS 1879^{NT} and *M. slooffiae* CBS 7956^{NT}. The values of each ratio represent the mean ± SD from three independent experiments. Asterisks represent statistical significance between groups ($p < 0.05$).

dermatis to *M. slooffiae* in the treatment *Mp:Ms* (1:9) increased rapidly on day 1 and reached the maximum value of 61.66 ± 8.15% on day 4. During the stationary phase, the population of *M. pachydermatis* fluctuated between 44–56% and decreased to 40.40 ± 4.23% on day 10. In contrast, the population of *M. pachydermatis* in the treatment *Mp:Ms* = 9:1 decreased rapidly from 90% on day 0 and reached the minimum of 28.09 ± 4.57% on day 3 and thereafter showed an upward tendency to approximately 45%. The population ratio of *M. pachydermatis* in the treatment of *Mp:Ms* = 5:5 slightly increased to the maximum value of 59.77 ± 3.64% on day 3 and was followed by a decreasing trend (Fig. 3B).

Discussion

Recently, some groups reported that *M. globosa* and *M. restricta* are found in high frequency on human skin.[21-22] However, these species are very fastidious in laboratory culture making them difficult to be used as a model microorganism in laboratory research. Therefore, *M. furfur, M. slooffiae*, and *M. pachydermatis* which are relatively easy to culture and also found on both healthy skin and affected sites of human and animals were selected to be a subject for the experiments. These species easily grew in YPD-Tween broth and showed the growth curves of a typical microorganism suggesting that the assay was quantitatively evaluable. According to typical biochemical characteristics of these species in the aspect of routine identification, *M. furfur,* and *M. pachydermatis* are able to grow on modified Dixon agar supplemented with either 0.5% Tween 60 or 0.1% Tween 80 whereas the growth of *M. slooffiae* is positive on the medium with Tween 60 but negative on Tween 80.[18] Interestingly, the present study showed that the three *Malassezia* species were all able to grow in YPD-Tween broth regardless of Tween 60 or Tween 80. Moreover, *M. furfur* and *M. slooffiae* seemed to have a preference for Tween 80 more than Tween 60 for their growth while *M. pachydermatis* preferred Tween 60, suggesting that the ability of utilizing Tween by the yeast can be readily adapted to the environment such as types of culture medium. *M. slooffiae* has hypothetically more demand for Tween 80 to proliferate, thereby it could grow in a more enriched nutritional environment in this study but could not grow on the test agar with 0.1% Tween 80 as described previously.[15,17] This hypothesis was

supported by the results, that both *M. furfur* and *M. slooffiae* showed a significant growth when the Tween concentration was over 0.5% (higher than the concentration used in common phenotypic feature testing). *M. pachydermatis* and *M. slooffiae* appeared to have a favorable growth under high Tween concentration. In contrast, the growth of *M. furfur* was statistically optimal at 1% Tween 80 and slightly decreased with the increase of Tween concentration to 2%, indicating a limitation effect on growth under high Tween concentration. Previously, a morphological alteration of the outer lipid layer has been reported in *M. furfur* cells treated with detergent mixture of Tween 80, sodium lauryl sulfate (SLS), and ammonium lauryl sulfate (ALS).[23] Presumably, the surfactant effect of Tween and its degraded products such as fatty acids may disturb the cell membrane of *M. furfur*, resulting in lower fungal growth at high concentrations. Moreover, the Tween concentration which optimally enhanced the growth of *Malassezia* in this study is similar to those used in most skin-related product formulations (e.g. moisturizing skin care, lotion, eye shadow, makeup bases, skin fresheners, and hair conditioners) which are within the range of 1-5%,[24] underlining the impact of Tween on members of cutaneous microorganisms.

As *Malassezia* species belong to the skin normal flora of both humans and animals, are acknowledged as both commensal microorganisms and opportunistic pathogens.[4,16] Some groups mention the relationship between fungal load and severity of dandruff.[25-26] Actually, commensal *Malassezia* comprises multiple species in the same site though the distribution of each species is variable among individuals.[27] It becomes questionable whether the population of each *Malassezia* species is constant or changes temporarily. As it is difficult to study straightforwardly *in vivo* and the results from both culture-based identification and molecular studies indicated only a transitory event occurring at the time of sampling, herein, a subsequent attempt to evaluate the growth and population ratio of different *Malassezia* species was conducted *in vitro* under different co-culture conditions. Interestingly, despite a trial experiment, the results from this study suggest that *Malassezia* yeasts possess the ability to maintain a certain population balance between species.

Although the pair of *Malassezia* species co-cultured in this study are generally specialized to colonize different hosts' skin,[2] the growth curves of the *Mp:Mf* and *Mp:Ms* under in vitro co-culture conditions were similar to those in the previous single-culture. When the yeast population in each experimental set was examined more closely, the massive change in the population of each species was observed in co-culture at a very early stage of the growth and the population was almost constant through the culture, regardless of the initial population ratio. Interestingly, though an intergenus interaction, a similar observation was made in an *in vitro* co-culture model of *Pseudomonas aeruginosa* and *Staphylococcus aureus*, that the population of both bacteria was kept in equilibrium which was independent from the initial ratio or whether the starting inoculum was high or low.[28] These findings support the concept of homeostatic balance maintained by the host cutaneous microbiota including *Malassezia* yeasts, that the disruption of this homeostasis may progress into skin disorders.[8-9] Therefore, the conclusive evidence to explain mechanisms of population restoration of *Malassezia* remains an open question for further investigation.

In summary, this study revealed that not only the type but also the concentration of Tween affected the growth of *Malassezia* species. Changes in population ratios of *Malassezia* under co-culture conditions were also observed. Since Tween 80 is widely used in multiple purposes, addition of excess Tween in cosmetics could affect the cutaneous microbiota. The data obtained in the present study emphasize a necessity to reevaluate the use of Tween in products associated with skin from a microbiological aspect.

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Περίληψη

Η δράση των Tween 60 και Tween 80 στην ανάπτυξη ζυμομυκήτων του γένους *Malassezia*

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Οι ζυμομύκητες του γένους *Malassezia* είναι μέλη της φυσιολογικής χλωρίδας ανθρώπων και θερμόαιμων ζώων. Εκτός από την *M. pachydermatis*, όλα τα είδη του γένους *Malassezia* spp. είναι γνωστά ως λιπόφιλα, γιατί έχουν ανάγκη από εξωτερικές πηγές λιπιδίων για την ανάπτυξή τους. Λιπαρά οξέα προερχόμενα από τα Tweens είναι ευρέως χρησιμοποιούμενα σε διάφορα προϊόντα, όπως τρόφιμα, ιατρικά, είδη κοσμετολογίας, καθώς και σε μικροβιολογικές καλλιέργειες. Σκοπός της παρούσας μελέτης ήταν η επίδραση των Tween 60 (περιέχει στεαρικά λιπαρά οξέα) και Tween 80 (περιέχει ολεϊκά λιπαρά οξέα) σε συγκεντρώσεις 0-2% (v/v) για την ανάπτυξη τριών ειδών *Malassezia* spp.: *M. furfur* CBS 1878^{T}, *M. pachydermatis* CBS 1879^{NT} και *M. slooffiae* CBS 7956^{NT}. Σύμφωνα με τα αποτελέσματα της μελέτης, όλα τα είδη είχαν καλή ανάπτυξη σε συγκεντρώσεις Tween > 0.5% και τη βέλτιστη σε 1% Tween 80 η *M. furfur*, σε 2% Tween 60 η *M. pachydermatis* και 2% Tween 80 η *M. slooffiae*. Επιπρόσθετα μελετήθηκε η επίδραση των ειδών μεταξύ τους σε συνθήκες συν-καλλιέργειας, και συγκεκριμένα *M. pachydermatis:M. furfur* και *M. pachydermatis:M. slooffiae* σε αναλογία 1:9, 5:5 και 9:1, όπου διαπιστώθηκε ότι ο πληθυσμός του κάθε είδους *Malassezia* ήρθε σε τελική ισορροπία, ανεξάρτητα της αρχικής του συγκέντρωσης, αποτέλεσμα της επίδραση του ενός είδους στο άλλο. Όλα τα παραπάνω δείχνουν την ανάγκη επαναπροσδιορισμού της χρήσης των Tweens αναφορικά τόσο με το είδος, όσο και με τις συγκεντρώσεις τους, στα διάφορα προϊόντα εξωτερικής χρήσης για το δέρμα.

Λέξεις κλειδιά
Tween, Malassezia, καλλιέργεια, ανάπτυξη

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