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## ***“A Novel Microbiome in-vitro Assessment Test Model Utilizing Metagenome Analysis”***

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### **1. Introduction**

The microbiome field has made significant advancements over the past few decades, largely due to improvements in analytical technologies and the ability to process large datasets [1,2]. Studies have revealed diverse roles for the microbiome in the human gut and oral cavity—and, increasingly, on the skin[3,4].

In the field of skin research, particular attention has been given to analyzing the microbiome by age, focusing on aging-related indicators such as wrinkles, moisture, and sebum levels [5-7]. Additionally, studies have examined how the skin microbiome correlates with atopic dermatitis, acne, and psoriasis[4],[8,9],[15,16].

Moreover, beyond basic microbial community analysis, technologies such as metagenomic profiling and metabolomics have been employed to explore the interactions of metabolites produced by skin microorganisms, as well as the interactions between the skin and microorganisms and their effects on skin condition[10,11]. Based on these studies, skin microbiome analysis has gained increasing attention due to its accessibility, ecological complexity, and relevance to skin and other diseases.

Recent microbiome research has highlighted various diseases caused by the dominance of specific microorganisms resulting from microbiome imbalance, shifting the focus away from solely beneficial or harmful bacteria[4],[8,9]. It has been suggested that rebalancing the microbiome may have effects such as improving atopic dermatitis and wound healing[8,12]. Consequently, microbiome research has made significant contributions to industries such as therapeutics, cosmetics, and diagnostic kits[8,9],[13].

However, despite advancements in various technologies and research, microbiome analysis and the evaluation of raw materials and products must be conducted through clinical trials, which poses certain limitations. Significant time and cost are required for microbiome-related research because clinical validation is both time-consuming and costly[14]. Additionally, ethical constraints in clinical trials mean that only materials and products with completed safety reviews can be tested, making it impossible to assess the toxicity of material and products about the microbiome. Furthermore, the inability to precisely control environmental conditions during clinical sampling limits the reproducibility and scalability of microbiome research. These

constraints also make it challenging to continuously observe interactions between microorganisms or between microorganisms and cells.

To overcome these limitations in microbiome research, the development of *in-vitro* test models is essential. By controlling the uncontrollable environmental factors that arise during clinical skin microbiome analysis, more objective results can be obtained. In addition, *in-vitro* models enable the cultivation of specific microbial communities under controlled conditions, facilitating detailed investigations into host-microbe interactions, antimicrobial activities within microbial communities, and the effects of external factors such as prebiotics. This approach is expected to provide valuable insights into microbiome research. In this study aims to overcome these limitations by identifying conditions that allow each strain to establish dominance independently, rather than simply mixing microbial suspensions into a cocktail. Through the analysis of microbial metabolites produced during the dominance formation process, we seek to identify biomarkers associated with changes in microbial dominance, thereby establishing criteria for efficacy evaluation. Furthermore, we aim to develop a model that can be linked to *in-vitro* skin efficacy by also identifying biomarkers related to interactions with cells.

## 2. Materials and Methods

### 2.1 Bacterial Strain

The *Staphylococcus aureus* ATCC 6538 strain was purchased from ATCC through KORAM BIOTECH. CORP. (Seoul, Korea). *Staphylococcus epidermidis* KCCM 35494 (ATCC 12228) was purchased from KCCM (Korean Culture Center of Microorganism, Seoul, Korea), *Cutibacterium acnes* KCCM 41747 (ATCC 6919) was purchased from KCCM (Korean Culture Center of Microorganism, Seoul, Korea). The strains used in this study were selected based on previous research. A preliminary study revealed that individuals with androgenetic alopecia (AGA) harbored a higher prevalence of *Staphylococcus aureus*, whereas healthy controls exhibited a comparatively greater abundance of *Cutibacterium acnes* and *Staphylococcus epidermidis*. Accordingly, in our co-culture model, *S. aureus* was designated the alopecia-associated strain, and *S. epidermidis* and *C. acnes* were defined as commensal strains to modulate microbial predominance[17].

### 2.2 Preparation of bacterial suspension

To prepared bacterial suspensions, *S.aureus* and *S.epidermidis* were inoculated in Tryptic Soy Broth (TSB, BD Difco), cultured at 37°C with shaking at 100 rpm (IST-4075, JEIO TECH.CO.,LTD. Korea) in Erlenmeyer flask, (SPL Life Sciences Co.,Ltd. Korea ) *C.acnes* were inoculated in Reinforced Clostridial Medium (RCM, BD Difco), cultured at 37°C without shaking. (MIR-554, PHCbi. Co.,Ltd.) After incubation, bacterial broth was centrifuged at 6,000 rpm for 15 minutes. (5430R Centrifuge, Eppendorf, Germany)

The obtained pellet was resuspended in 0.85% NaCl (Thermo Fisher Scientific Inc., USA). Each bacterial suspension was measured for absorbance at 600nm using UV spectrophotometer (10S UV-Vis Spectrophotometer, Thermo Scientific™) and adjusted to a bacterial cell density of  $1.0 \times 10^6$  CFU/mL by dilution using 0.85% NaCl (w/v).

### 2.3 Growth in Single-culture

The prepared bacterial suspensions were inoculated at 1% (v/v) into tryptic soy broth (TSB) for both *Staphylococcus aureus* and *Staphylococcus epidermidis*, and incubated under static conditions at 37 °C. For *Cutibacterium acnes*, the suspension was similarly inoculated at 1% (v/v) into reinforced clostridial medium (RCM) and incubated statically at 37 °C. Absorbance at 600 nm (OD<sub>600</sub>) was measured every 3 hours for the first 12 hours, and then every 12 hours

thereafter, to determine the time to entry into the logarithmic phase and the onset of the death phase.

## 2.4 Modified Inoculating Time

Reinforced Clostridial Medium (RCM)—the optimal growth medium for *C. acnes*, which exhibits the slowest entry into the exponential phase—was used to establish the appropriate inoculation sequence of the three strains in the co-culture model. The strains were simultaneously inoculated into RCM, and bacterial counts were measured at defined time points (Table 1). After confirming conditions that favored *C. acnes* predominance, tryptic soy broth (TSB) was added to the co-culture to observe subsequent shifts in strain dominance.

**Table 1. Inoculation method for co-culture groups**

To examine shifts in strain dominance, inoculation time points were staggered to induce and monitor changes in predominance. ‘Inoculation Time<sup>1</sup>’ refers to the interval between the initial inoculation of *C. acnes* and the subsequent inoculation of the other strain. ‘Culture Duration<sup>2</sup>’ denotes the total incubation period for each strain. ‘Measurement Point<sup>3</sup>’ indicates the specific time points after each strain’s inoculation at which bacterial counts were determined.

Inoculation Time <sup>1</sup> (h)	Culture Duration <sup>2</sup> (h)	Measurement Point <sup>3</sup> (h)	Figure Panel
<i>C.acnes</i> : 0, <i>S.epidermidis</i> : 0 <i>S.aureus</i> : 0	72	P <sub>1</sub> -P <sub>4</sub> (0, 24, 48, 72)	Fig.2 (a)
<i>C.acnes</i> : 0 <i>S.epidermidis</i> : 6 <i>S.aureus</i> : 12	72 66 63	P <sub>1</sub> -P <sub>5</sub> (0, 24, 36, 48, 72) P <sub>1</sub> -P <sub>6</sub> (0, 3, 21, 30, 42, 66) P <sub>1</sub> -P <sub>6</sub> (0, 18, 27, 39, 63)	Fig.2 (b)
<i>C.acnes</i> : 0 <i>S.epidermidis</i> : 14 <i>S.aureus</i> : 17	72 58 55	P <sub>1</sub> -P <sub>5</sub> (0, 24, 36, 48, 72) P <sub>1</sub> -P <sub>6</sub> (0, 3, 10, 22, 34, 58) P <sub>1</sub> -P <sub>5</sub> (0, 7, 19, 31, 55)	Fig.2 (c)

## 2.5 Growth in co-culture

Co-cultures were established by inoculating bacterial suspensions into the appropriate media at a fixed ratio (Table 2), in order of increasing time to enter the logarithmic phase. To synchronize log-phase onset, *Cutibacterium acnes* was inoculated first; *Staphylococcus epidermidis* was added 14 h later, and *Staphylococcus aureus* was introduced 17 h after the initial *C. acnes* inoculation (i.e., 3 h after *S. epidermidis*), using an inoculum volume equivalent to that of the other strains. Based on single-culture growth profiles, bacterial counts were measured at 12-hour intervals, beginning when each strain entered the stationary phase.

## 2.6 Bacterial Count in single and co-culture

At each time point, 1 mL of culture broth was collected and diluted with 9 mL of 0.85% (w/v) NaCl. The samples were then serially diluted to 10<sup>-5</sup>, 10<sup>-6</sup>, and 10<sup>-7</sup>, and 0.1 mL of each dilution

was plated onto tryptic soy agar (TSA) and reinforced clostridial medium agar (RCMA). RCMA plates were incubated anaerobically at 37 °C for 3 days in a BD GasPak™ EZ Anaerobe Container System with BD BBL™ GasPak™ CO<sub>2</sub> generators and anaerobic/CO<sub>2</sub> indicator strips, then transferred to aerobic conditions at 37 °C for an additional 3 days. TSA plates were incubated aerobically at 37 °C for 24 hours.

**Table 2. Co-culture Medium Ratio**

To evaluate how medium composition affects strain dominance, three experimental groups were defined: a) RCM/TSB Ratio Variation Group, in which the proportions of both RCM and TSB were simultaneously adjusted. b) Fixed-TSB, Variable-RCM Ratio Group, in which the TSB proportion was held constant at 10 and the RCM proportion was incrementally increased. c) Fixed-RCM, Variable-TSB Ratio Group, in which the RCM proportion was held constant at 10 and the TSB proportion was varied.

a) RCM/TSB Ratio Variation Group

Medium	Ratio1	Ratio2	Ratio3	Ratio4	Ratio5
Tryptic Soy Broth	0	2	5	8	10
Reinforced Clostridial Medium	10	8	5	2	0

b) Fixed-TSB, Variable-RCM Ratio Group

Medium	Ratio1	Ratio2	Ratio3
Tryptic Soy Broth	10	10	10
Reinforced Clostridial Medium	2	5	8

c) Fixed-RCM, Variable-TSB Ratio Group

Medium	Ratio1	Ratio2	Ratio3
Tryptic Soy Broth	2	5	8
Reinforced Clostridial Medium	10	10	10

## 2.7 Colony Count on Agar Plate in co-culture

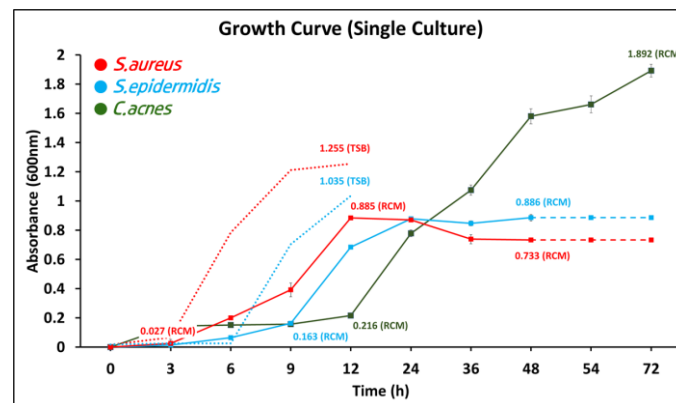
Colony counts were obtained using a Scan 4000 automatic color colony counter and inhibition zone reader (Interscience, France), which differentiates colonies by color and elevation. *Staphylococcus aureus* colonies appeared yellow, *Staphylococcus epidermidis* colonies white, and *Cutibacterium acnes* colonies were distinguished by their greater height using the instrument's light detection system. The counted colonies were multiplied by the reciprocal of the dilution factor to determine colony-forming units per milliliter (CFU/mL), and these CFU/mL values were subsequently log-transformed.

## 3. Results

### 3.1 Growth in Single Culture

When the three strains were cultured individually in TSB and RCM media, *S. aureus* entered the log phase 3 hours after inoculation in both media. *S. epidermidis* entered the log phase 6

hours after inoculation in TSB and 9 hours after inoculation in RCM. *C. acnes* showed no growth in TSB, but entered the log phase 12 hours after inoculation in RCM. Regarding the stationary phase, *S. aureus* entered the stationary phase 9 hours after inoculation in TSB and 12 hours in RCM. *S. epidermidis* entered the stationary phase 12 hours after inoculation in TSB and 24 hours in RCM, while *C.acnes* did not enter the stationary phase even after 72 hours (Figure 1). These results confirmed that *S.aureus* and *S.epidermidis* exhibited slower growth in RCM medium, while *C.acnes* was unable to grow in TSB medium.



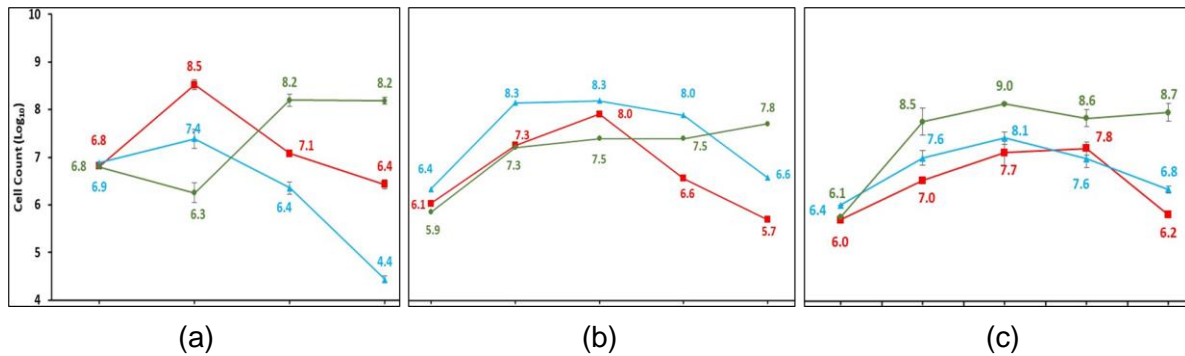
**Figure 1. Growth Curve in Single Culture.** The three strains were inoculated at 1%(v/v) into TSB and RCM, respectively, and the absorbance at 600 nm was measured at regular time intervals.

### 3.2 Modified Inoculating Time

In RCM—the optimal growth medium for *C. acnes*—the three strains were inoculated either simultaneously or at staggered time points. In simultaneous inoculation, *S. aureus*, which exhibits the shortest lag phase, displayed the fastest exponential growth but also reached the death phase earliest. *S. epidermidis* entered exponential growth the second-fastest and, for reasons not yet understood, progressed directly to the death phase without a discernible stationary phase. In contrast, *C. acnes*, the slowest-growing strain, entered exponential growth only after 24 h of incubation and then transitioned into a stationary phase (Figure 2a).

In the co-culture experiment, *C. acnes* was inoculated at  $t = 0$  h, followed by *S. epidermidis* at  $t = 6$  h and *S. aureus* at  $t = 12$  h. Except for differences in the onset of the death phase, all three strains exhibited essentially identical growth curves, and no single strain achieved predominance under these conditions (Figure 2b).

In the co-culture experiment depicted in Figure 3, *C. acnes* was inoculated at  $t = 0$  h, followed by *S. epidermidis* at  $t = 14$  h and *S. aureus* at  $t = 20$  h. Under these conditions, *C. acnes* established predominance by 24 h post-inoculation, with both *S. epidermidis* and *S. aureus* exhibiting lower cell counts relative to *C. acnes*. Thus, the inoculation regimen used in Figure 3 confirmed that *C. acnes* became the dominant strain (Figure 2c).



**Figure 2. Effect of Inoculation Order on Strain Dominance**

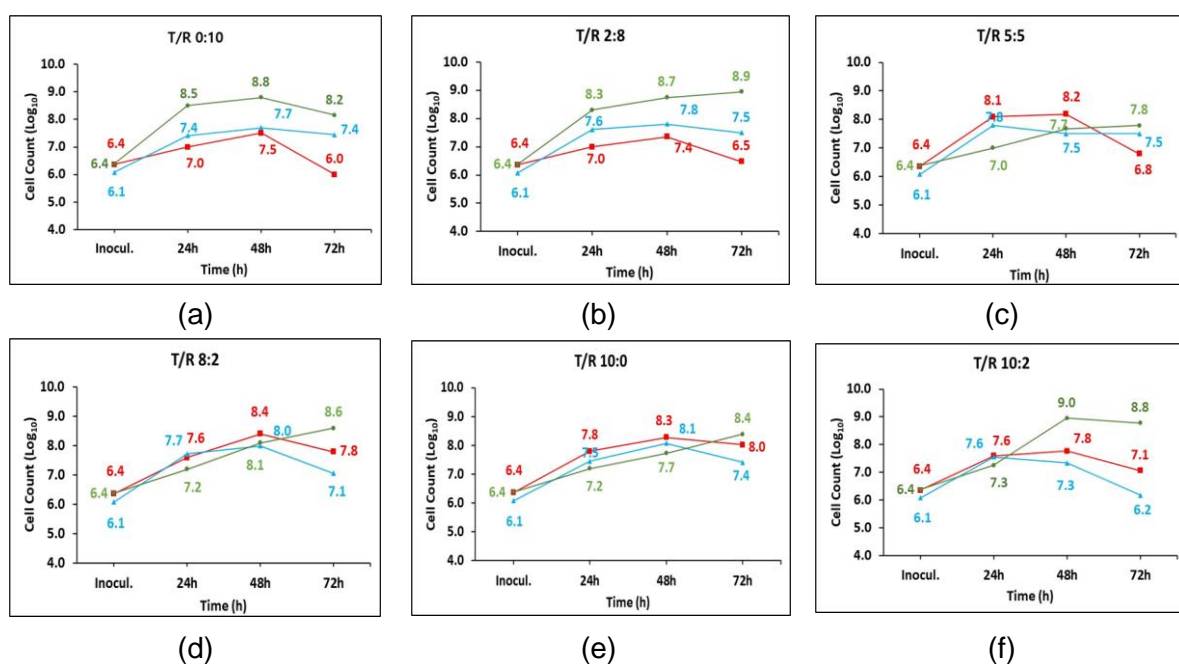
The inoculation sequence of each strain in RCM medium was varied, and bacterial counts were measured at each sampling point. The incubation durations corresponding to each time point are presented in Table 1.

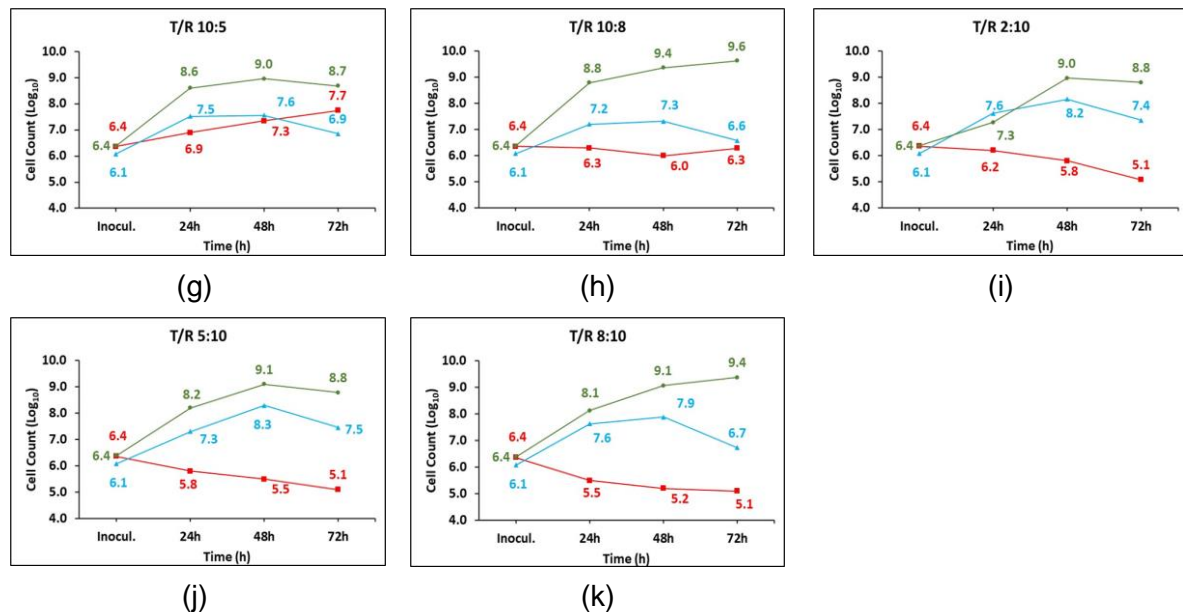
### 3.3 Results of Co-culture Condition Experiments

Figure 2. (a) shows the results for the group in which both TSB and RCM proportions were varied simultaneously according to inoculation sequence. When the death-phase interval is excluded, all three strains co-predominate in Figure (d) and (e). Under conditions (a) and (b), *C. acnes* predominance is evident, whereas condition (c) displays no single-strain dominance.

In the Fixed-TSB, Variable-RCM Ratio Group (TSB fixed at 10; RCM varied), *C. acnes* predominance is observed in panels (g) and (h), with no co-predominance of all three strains. Under condition (f), co-predominance appears to emerge but is accompanied by an extended lag phase of *C. acnes*. Notably, in Figure (h), growth of *S. epidermidis* and *S. aureus* is nearly undetectable, and *S. aureus* fails to exceed the inoculum level.

In the Fixed-RCM, Variable-TSB Ratio Group (RCM fixed at 10; TSB varied), *C. acnes* growth dominates. Cell counts measured immediately prior to the death phase Figure (j) and (k) reveal co-predominance of *C. acnes* and *S. epidermidis*. In this group, *S. aureus* growth is absent under all conditions and instead declines below the initial inoculum level.





**Figure 3. Strain Dominance under Different Medium Compositions**

These results depict the outcomes observed when strain inoculation times were adjusted according to the medium composition.

In Figure 2 (a), where medium composition was varied according to inoculation sequence, all three strains exhibited co-predominance in Figures 3. (d) and (e) when the death phase was excluded. Conditions Figure 3. (a) and (b) showed clear *C. acnes* predominance, whereas under condition (c), no single strain achieved dominance.

#### 4. Discussion

In this study, we developed a flexible *In vitro* co-culture model for a defined three-species skin microbiome by precisely controlling inoculation timing and medium composition. We found that rapid simultaneous inoculation induces transient *S. aureus* overgrowth, moderate delays synchronize growth without permitting any single species to dominate, and prolonged delays lead to *C. acnes* predominance. Adjusting the RCM/TSB ratio further modulated competitive fitness: joint adjustment of both media promoted co-predominance, whereas fixing one component at high concentration selectively reinforced or suppressed individual strains.

Importantly, our platform overcomes the high cost, lengthy timelines, and environmental variability of clinical microbiome studies by enabling systematic dissection of temporal and nutritional drivers of strain dominance. Future work will enhance enumeration accuracy via next-generation sequencing (NGS), expand the range of strains in the model, and integrate skin organoids or reconstructed human skin equivalents to better mimic the *In vivo* microenvironment.

Additionally, we plan to compare and validate the similarity between previously analyzed hair loss microbiome data and *in-vitro* data. Through this comparative analysis, the study aims to enhance the accuracy of *in-vitro* test data and establish an evaluation model that reduces cost and time by ensuring compatibility with clinical results. This research will continue with the goal of developing a more efficient and reliable assessment model.

#### 5. Conclusion

In summary, we have developed a versatile *In vitro* co-culture model that allows precise manipulation of both inoculation timing and medium composition to control strain-level dominance

within a defined three-species skin microbiome. Our findings demonstrate that rapid simultaneous inoculation promotes transient *S. aureus* overgrowth, moderate staggering of inoculation synchronizes multi-species growth without dominance, and prolonged delays shift the competitive balance in favor of *C. acnes*. Moreover, simple adjustments in RCM/TSB ratios further modulate competitive fitness, enabling selective reinforcement or suppression of individual strains. This platform overcomes key limitations of clinical microbiome studies namely cost, time, and environmental variability and provides a powerful tool for screening microbiome-modulating interventions. Future implementation of high-resolution community profiling (e.g., NGS) and integration with skin organoid or reconstructed skin models will enhance the predictive capacity and translational relevance of our system for efficacy, cosmetic, and diagnostic applications.

## 6. Reference

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