nature portfolio

Peer Review File

Microecology in vitro model replicates the human skin microbiome interactions

Corresponding Author: Professor Qing Huang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors present an interesting scaffold for hosting microbes. The ability to host nonviable cells in a hydrogel-based matrix enables creating a wide range of substrates. The manuscript also presents results from microbial metabolism of α-arbutin and niacinamide by P. acnes. The methodology used for this analysis is sound. The manuscript has been well presented, there are some minor grammatical errors but is easy to read. Their attempt to create an in-vitro model to study skin-microbiome should be commended as such a model will be very useful to both the academic and clinical community. However, there is a major issue:
The data presented in the manuscript shows that the substrate can host a microbiome seeded either from a skin swab or a simplified human skin microbiome. This colony is monitored over four days and the relative abundance at the level of genus is reported. The colony requires to be reinoculated to maintain a diversity like that of the seeding population. This only allows me to infer that the scaffold can host a microbial population, but it does not allow me to conclude that this population is characteristic of what will be observed on the skin. To support the claim that the microbiome hosted by the scaffold closely mimics that of the volunteer's skin and consequently the skin microbiome, the temporal variation of the microbiome on the volunteer's skin needs to be presented and shown to temporally exhibit similar relative abundances. This data can be further strengthened by monitoring the dynamics of the simplified human skin microbiome on the scaffold as well as an excised skin sample.
The metabolism of α-arbutin and niacinamide on the scaffold to P.acnes only reiterates the known function of P.acnes; and this can be replicated without the scaffold.
The authors should also justify their use of skin swabbing as compared to tape stripping to collect microbiome samples. While many bacteria on the skin can form biofilms, swabbing mostly picks up those that are in planktonic state. Such bacteria would form biofilms under appropriate conditions; however, they may not characterize the metabolic and virulence state of the bacteria in skin biofilm.
This a major issue and the authors claim that their substrate can host the skin microbiome is not substantiated by the data that is presented.

Reviewer #2

(Remarks to the Author)
See attached file

[Editorial Note: The attachment is displayed on the final four pages of this file]

Reviewer #3

(Remarks to the Author)

The authors describe the fabrication and functional validation of a novel engineered platform for the culture of the skin

microbiome that simulates the stratum corneum of the epidermis. By providing devitalized epithelial cell as a nutrient source, and an irregular porous scaffold to create oxygen gradients to recapitulate both aerobic and anaerobic conditions, the authors were able to culture over several days simple and complex microbiota associations that effectively recapitulate skin microbiome. The functional validation with drug metabolism analysis demonstrates the platform suitability for pharmacological research.
Although several attempts of establishing a skin-microbiome in vitro platform are reported in literature, here the lack of high nutrient medium to support the viability of human cells allowed to achieve a stable and diverse colonization, preventing the overgrowth and monopoly of single strains.
The work support most of the conclusions and claims, but despite the quality of the data presented and the appropriateness of the analysis, major and minor changes are required for publication.
Major Critiques
- Line 120: “The morphology of the hydrogel as presented does not resemble the morphology of the skin and its appendages.” Provide further evidence or remove this statement.

- Line 121. “which are vital to maintain a stable state of the microbiota in vitro, especially in the bacterial and fungal communities in the sebaceous gland area22. Not clear what is vital to bacterial communities. Is the porosity and morphology of the substrate or the follicles and glands? If the former, the cited reference does not suggests that; and the authors should demonstrate how porosity/morphology affects the communities. If the latter, hair follicles and sebaceous glands are not included in the model, so it becomes less relevant.

- Line 145-147: The meaning of this statement is not clear. What is shown in Figure 1e suggests that the final bacteria density is directly proportional to the amount of initial inoculation.

- Figure 1. It would be interesting to know what design parameters positively/negatively affect the microbiome culture. How far is the model from optimized conditions? There is no comparison of the effect of parameters, such as hydrogel type (e.g., would this work with gelatin methacrylate, GELMA), hydrogel stiffness, pH, porosity, density of cells (nutrients) etc. These comparisons are important to appreciate the success of the model, the mechanism behind its superiority over the previous model systems, and for its further optimization.

- Figure 1. The author says “Another important factor in judging the success of a microbial model is whether it can support the bacterial diversity at a steady-state level.”, but the data shown in Figure 1 and Supp Fig 2-3 do not show bacterial diversity over time. The introduction to this section looks misplaced and misleading. If the authors claim stability of these 2 strain over time, the data should include multiple time points for distribution of each strain.

- Figure 2. There is no justification of why day 2 and day 3 were chosen to make the comparison of bacterial diversity and stability. The data is encouraging that within 24 hours the colonies can say stable. It is equally interesting and important to know how long such stability lasts. I recommend conducting a longer term experiment (e.g., 7 days as in Figure 1).

- Figure 2a: why the microbiome was not analyzed before inoculation (while it was in Figure 3)? Provide a justification.

- Line 217-220: Please discuss in the discussion section the reason why multiple inoculations are required. Does the preecolonization with certain strain is required for the successful inoculation of other strains? Could you identify pathogens among the strains that required a second inoculation to successfully colonize the platform?

- In the methods the authors define dry skin only based on the low sebum content. Although the classification of skin in normal, oily, and dry used to be associated with the amount of sebum secretion, dry skin is primarily characterized by a low water content that can be caused by multiple factors (such as filaggrin mutations), not exclusively by reduced sebum secretion, to the extent that dry skin is often associated with hyperseborrhea (e.g. in seborrheic dermatitis). The criteria used for the classification and selection of the skin microbiome donor area should be better described in the results section and further discussed. To further understand the variable outcome of microbiome culture isolated in from different skin type, it would be important to determine the level of hydration of the hydrogel using perhaps confocal Raman spectroscopy or a simple corneometer, and investigate whether modulating the water content the culture conditions for the dry skin microbiome would improve.

- Figure 3b. “…the microbiome composition of normal and oily skin returned to the pre-inoculation (day 0) level”. Since there is no perfect overlap, this should be represented as percentage of similarity.

- Figure 3. “observed previously, the bacterial population and community diversity on the day 2 were very different from the original (day 0), as the bacteria needed time to adapt to their new environment” There is no data shown for day 2? If the authors meant day 1, what is evidence for the claim of bacterial adaptation? What would happen if the authors did not reinoculate on day 1, and only left those inoculated on day 0 for 4 days? Would the bacteria adapt and return to normal diversity? These experiments are necessary to understand the bacterial adaptation hypothesis? If it is true, than it means the strains showed a similar level of adaptation to the new environment, likelihood of which should be discussed in detail.
Minor critiques

- Line 7: “This kind of balance is achieved by the multifactorial interactions” since no specific interacting factors were discussed, either describe or remove “the” before “multifactorial”.
- Line 33: reference 3. The reference only refers to acne. Provide additional references for atopic dermatitis and sensitive skin.

- Line 46: reference 8 is a review. It would be more appropriate to cite the original articles mentioned in the review (Dunbar, J., Barns, S. M., Ticknor, L. O., and Kuske, C. R. (2002). Appl Environ Microbiol 68, 3035-45, Bowler, P. G., Duerden, B. I., and Armstrong, D. G. (2001). Clin Microbiol Rev 14, 244-69, Davies, C. E., Wilson, M. J., Hill, K. E., Stephens, P., Hill, C. M., Harding, K. G., and Thomas, D. W. (2001). Wound Repair Regen 9, 332-40.)
- Line 54: References for clinical trials are missing.
- Line 57: Reference 14 is the same as reference 5.
- Line 68: Reference 19 is the same as reference 17.
- Line 75: “and nonviable HaCaT, sebaceous cells as nutrients” rephrase at this sounds like HaCaT cells are sebaceous cells.
- Line 77: The reference provided does not report pH values for the polymerized hydrogel.
- Figure 1b: indicate what kind of inverted microscope was used.
- Line 117: “Scmic” should be “SCmic”
- Line 124: skin pH averages between 5 and 6. The pH of 6.8 reported for the hydrogel is well above skin physiological values, please include in the discussion.
- Line 130: The official name of what was formerly known as “Propionibacterium acnes” is now “Cutibacterium acnes”. Update name in the text.
- Line 132: The reference included makes does not mention endogenous infections. Although C. acnes is an opportunistic pathogen, it is not a frequent source of endogenous infections when compared to other genus that also inhabit skin such as Staphylococcus and Streptococcus. Also, the author should cite the original source supporting the statement “Anaerobic bacteria, such as P. acnes, constitute a significant portion of the human facial skin microbiota” (McGinley et al PMCID PMC242779).
- Line 158: replace “thinness” with “thickness”.
- Line 164-165: how did the authors compensate for the loss of signal due to light scatter? Please analyze a cross-section of the hydrogel to support your statement.
- Line 169: replace the word “significantly” since no statistical analysis is shown.
- Figure 2a: Why the genus Prevotella is reported twice? What is the meaning of the square brackets?
- Line 180: “health” should be “healthy”.
- Figure 3c: moving the graph legend next to figure 3c would help make it more visible for a faster understanding of the data.
- Line 204: “As observed previously, the bacterial population and community diversity on the day 2 were very different from the original (day 0), as the bacteria needed time to adapt to their new environment.” the authors refer to data not shown. Why were these data not shown in figure 2?
- Line 256: Before the period the authors should refer Figure 4a.
- Line 263: please explain in the text the experimental setup of Figure 4d.
- Line 273: “verity” should perhaps be “variety”?
- Line 293: “which was relatively variation” please correct.
- Line 305: was the olive oil composition defined? Olive oil contains over six different kind of fatty acids, including linoleic acid, and other different lipids. It does not seem a valid option to demonstrate “an excessive increase in specific sebum components”.
- Line 350: Reference 31 is the same as reference 1.
- Reference 33 is missing authors. Please revise the whole references list and use a cohesive style.
- Line 358: Reference 34 is the same as reference 2.
- Line 371-371: “The hydrogels replicate the intricate topography marked by skin depressions and pockets.” No evidence of this is provided. What is shown is that the scaffold is porous, unlike the human skin.
- Line 452: please provide the source for HaCaT and sebaceous cells.
- Including the manufacturer and catalog number of the reagents employed would increase reproducibility.
- Line 75 HaCaT or sebaceous cells
- Line 82 rather than
- Line 87 remove well
- Line 120 morphological
- Legend of Figure 1 h is missing
- Line 307 “oily” to oils”

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have presented an in-vitro scaffold for hosting skin microbes. The methodology used for this analysis is sound and presented in detail. The model will be very useful to both the academic and clinical community.

The authors have addressed the major issues that I had raised during the initial review. The revised manuscript provides sufficient data that supports the claims on the representativeness and stability of the in-vitro setup to mimic non-dry skin.

There are few grammatical issues and a figure legend that is incomplete, these should be addressed before publication.

I also recommend that the title be made more specific "Mimic the skin microecology- a novel in vitro model replicates the microbiome world", the study only looked into the skin microbiome and it is not representative of the microbiome world.

I recommend that the paper be published subject to these minor corrections.

Reviewer #2

(Remarks to the Author)
The authors did an excellent job addressing my concerns.

Reviewer #3

(Remarks to the Author)
The authors addressed the majority of my comments satisfactorily. Below are my remaining concerns:
Major:
To my comment “It would be interesting to know what design parameters positively/negatively affect the microbiome culture…..”, the authors responded with adding discussions, and suggesting future work. However, I think it is critical to test at least several conditions (not a full optimization), such as different pH and stiffness to fully appreciate the superiority of the model and get an insight into its mechanism. Without such comparison and mechanistic data, it is difficult to trust the robustness of the model and data overall.
Minor:
Missing a word after “morphological”: The hydrogel exhibits porous properties and a morphological that mimics the invaginations and depressions found in the skin (Fig. 1b), which are important for creating niche-specific microenvironments.” (lines 118120)

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The authors addressed my comments.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Point-to-point response to reviewers' comments

Reviewer #1 (Remarks to the Author):

The authors present an interesting scaffold for hosting microbes. The ability to host nonviable cells in a hydrogel-based matrix enables creating a wide range of substrates. The manuscript also presents results from microbial metabolism of α-arbutin and niacinamide by P. acnes. The methodology used for this analysis is sound. The manuscript has been well presented, there are some minor grammatical errors but is easy to read. Their attempt to create an in-vitro model to study skin-microbiome should be commended as such a model will be very useful to both the academic and clinical community. However, there is a major issue:

Authors' response: Thank you very much for your time involved in reviewing the manuscript and your very encouraging comments on the merits. We sincerely appreciate your positive evaluation and insightful comments.

The data presented in the manuscript shows that the substrate can host a microbiome seeded either from a skin swab or a simplified human skin microbiome. This colony is monitored over four days and the relative abundance at the level of genus is reported. The colony requires to be reinoculated to maintain a diversity like that of the seeding population. This only allows me to infer that the scaffold can host a microbial population, but it does not allow me to conclude that this population is characteristic of what will be observed on the skin. To support the claim that the microbiome hosted by the scaffold closely mimics that of the volunteer's skin and consequently the skin microbiome, the temporal variation of the microbiome on the volunteer's skin needs to be presented and shown to temporally exhibit similar relative abundances. This data can be further strengthened by monitoring the dynamics of the simplified human skin microbiome on the scaffold as well as an excised skin sample.

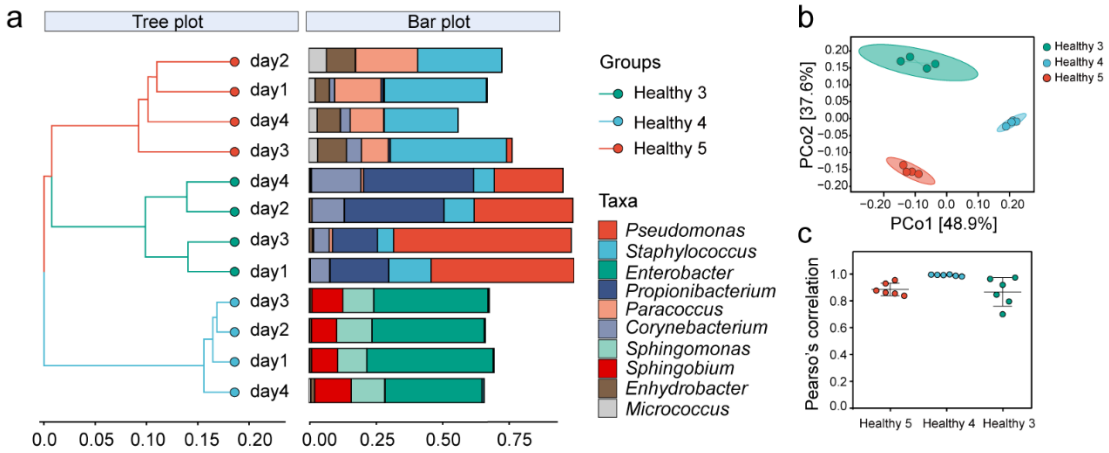
Authors' response: Thank you for your valuable feedback. We acknowledge the necessity of presenting data that demonstrate the temporal variation of the microbiome on the volunteer's skin to show similar relative abundances over time. This comparison is essential to support the claim that the microbiome hosted by the scaffold closely mimics that of the volunteer's skin, thus reflecting the characteristics of a typical skin microbiome. While we did refer to the study by *Oh et al.*[1], which found that the skin microbiota of healthy individuals typically remains stable for up to two years under normal conditions, we realize that it is crucial to present our own data to substantiate these claims more robustly. The study by *Elizabeth K. et al.*[2] also supports that skin microbiome similarity is significantly higher in the short term (24 hours) than in the long term (3 months).

In our experiment, to address this, we included a control group that consisted of microbiota samples collected from the subject's face over four consecutive days. As illustrated in the Responded Fig. 1 (new Supplementary Fig. 4), our results indicate that the microbiome derived from the skin of healthy individuals remains relatively stable over short periods. This finding was assessed using a phylogeny-based metric, weighted

UniFrac[3], where smaller Unifrac distances suggest a higher similarity between microbial communities (Responded Fig. 1a - new Supplementary Fig. 4a). The bar plot showing the stacked abundance of the top 10 genera (Responded Fig. 1a - new Supplementary Fig. 4a) also supports the stability of these communities.

Further, we utilized PCoA (Responded Fig. 1b - new Supplementary Fig. 4b) and Pearson correlation analysis (Responded Fig. 1c - new Supplementary Fig. 4c, $r > 0.7$) to demonstrate the stability of the skin microbiota on the volunteer's skin over the short term. These methods provided strong evidence that the bacterial community on the skin did not significantly change during the experiment's timeframe, affirming the scaffold's capability to mimic the temporal dynamics observed in natural skin environments.

We have revised the manuscript to more clearly present these findings in the results (lines 221-226). These revisions aim to provide a more comprehensive understanding of the scaffold's ability to maintain a representative skin microbiome short time.



Responded Figure 1 (new Supplementary Fig. 4 in manuscript) The skin microbiome in healthy individuals remains stable over short periods. **a** UPGMA phylogenetic tree constructed based on weighted Unifrac distances; bar plot shows top 10 genera abundance. **b** Principal Coordinate Analysis (PCoA) plots (based on weighted Unifrac) analyzing changes in microbiota over four days on the skin. **c** Pearson's correlation coefficient (r) between the skin microbiota on each day over four days and the average community of day one.

The metabolism of α-arbutin and niacinamide on the scaffold to P.acnes only reiterates the known function of P.acnes; and this can be replicated without the scaffold.

Authors' response: We acknowledge the point that the metabolism of *α*-arbutin and niacinamide by *P. acnes* reflects the known function of this bacterium and that these metabolic activities could potentially occur without the use of our scaffold.

While previous studies have demonstrated that certain human skin microbiota[6] (*S. epidermidis* and *S. aureus*) and gut microbiota[7] (*B. stercoris* and *B. longum*) can hydrolyze *β*-arbutin into hydroquinone, and research by *Shats et al.*[8] and *Feng et al.* [9] has shown that murine gut microbiota can metabolize niacinamide into niacin, with reports from the NCBI database suggesting that bacteria containing amidases, including *P. acnes* and *S. epidermidis*, have the potential to metabolize niacinamide, our study provides novel insights into the metabolic capabilities of a more comprehensive human

skin microbial community. Specifically, our research is the first to confirm that a human skin microbiome community composed of seven strains—*P. acnes*, *C. granulosum*, *S. epidermidis*, *S. capitis*, *C. simulans*, *S. aureus*, and *M. furfur*—is capable of metabolizing both *α*-arbutin and niacinamide. These compounds are widely used and effective skin care agents, and our findings suggest that under the influence of the skin microbiome, these substances can be converted into metabolites potentially harmful to the skin, which might explain why some consumers experience adverse reactions when using skin-care products containing these ingredients.

We recognize, however, that the metabolic activity observed could also occur outside of the scaffold. The primary contribution of our study is to illustrate how this model supports the metabolic functions of controlled microbiota in vitro. It not only allows us to observe these known metabolic pathways but also lays the groundwork for exploring more complex interactions and applications in the future.

The authors should also justify their use of skin swabbing as compared to tape stripping to collect microbiome samples. While many bacteria on the skin can form biofilms, swabbing mostly picks up those that are in planktonic state. Such bacteria would form biofilms under appropriate conditions; however, they may not characterize the metabolic and virulence state of the bacteria in skin biofilm.

Authors' response: Thank you for raising this important point regarding the choice of sampling method. We agree that the method of microbiome sampling can influence the type of bacteria collected and, consequently, the interpretation of microbial diversity and community composition.

In our study, we opted for skin swabbing instead of tape stripping primarily due to its suitability as a non-invasive and practical method for assessing microbiome diversity. Swabbing is less disruptive to the skin's surface than tape stripping and can be consistently performed with minimal discomfort to the volunteers. It effectively collects bacteria from the skin surface, including those in the planktonic state, which are likely to form biofilms under suitable conditions. While we acknowledge that tape stripping may capture more bacteria embedded within biofilms and provide a closer representation of the bacteria's metabolic and virulence state in skin biofilms, our primary focus was on capturing a broad overview of the skin microbiome that could be maintained in our scaffold model. According to studies comparing sampling techniques, swabbing is considered a suitable alternative to scraping for evaluating microbial diversity[4]. This approach has been validated in other research as an appropriate and reliable method for assessing the skin microbiome[5].

To address this in our manuscript, we have included a discussion (lines 454-459) about the rationale for using swabbing and its implications for capturing microbial diversity. This addition has helped clarify our methodological choices and the scope of our findings.

This a major issue and the authors claim that their substrate can host the skin microbiome is not substantiated by the data that is presented.

Authors' response: Thank you for highlighting this critical issue. We acknowledge that

strong evidence is required to substantiate our claim that the substrate can effectively host and reflect the skin microbiome. To address this concern, we have made two significant revisions to our manuscript:

1. Inclusion of Temporal Stability Data: We have added data demonstrating the temporal variation of the microbiome on the volunteers' skin (lines 221-226), showing that the skin microbiome remains stable over four consecutive days. This temporal stability is crucial for supporting our claim that the microbiome hosted by the scaffold exhibits similar characteristics to the natural skin microbiome. By showing that the scaffold can maintain a stable microbiome over time, analogous to what is observed on the skin, we provide evidence that supports the scaffold's capacity to host skin-representative microbial communities.

2. Discussion of Sampling Methods: We have also revised the manuscript to include a detailed discussion on the rationale and appropriateness of using swab sampling (lines 454-459).

These revisions and supplementary data are intended to provide a more comprehensive and convincing argument that the scaffold can effectively support a microbiome representative of the skin microbiome. We hope these updates will address your concerns and clarify the functionality of the scaffold in hosting a skin-like microbial community.

We appreciate your valuable feedback, which has been instrumental in enhancing the rigor and clarity of our study.

References

1. Oh, J., Byrd, Allyson L., Park, M., Kong, Heidi H. & Segre, Julia A. Temporal Stability of the Human Skin Microbiome. *Cell* **165**, 854-866 (2016).
2. Costello, E. K. et al. Bacterial Community Variation in Human Body Habitats Across Space and Time. *Science* **326**, 1694-1697 (2009).
3. Lozupone, C. & Knight, R. UniFrac: a new phylogenetic method for comparing microbial communities. *Applied and environmental microbiology* **71**, 8228-8235 (2005).
4. Grice, E. A. et al. A diversity profile of the human skin microbiota. *Genome research* **18**, 1043-1050 (2008).
5. Grice, E. A. et al. Topographical and Temporal Diversity of the Human Skin Microbiome. *Science* **324**, 1190-1192 (2009).
6. Bang, S. H., Han, S. J. & Kim, D. H. Hydrolysis of arbutin to hydroquinone by human skin bacteria and its effect on antioxidant activity. *Journal of cosmetic dermatology* **7**, 189-193 (2008).
7. Blaut, M. et al. Mutagenicity of arbutin in mammalian cells after activation by human intestinal bacteria. *Food and Chemical Toxicology* **44**, 1940-1947 (2006).
8. Shats, I. et al. Bacteria Boost Mammalian Host NAD Metabolism by Engaging the Deamidated Biosynthesis Pathway. *Cell metabolism* **31**, 564-579 (2020).
9. Feng, S., Guo, L., Wang, H., Yang, S. & Liu, H. Bacterial PncA improves diet-induced NAFLD in mice by enabling the transition from nicotinamide to nicotinic acid. *Communications biology* **6**, 235 (2023)

Reviewer #2 (Remarks to the Author):

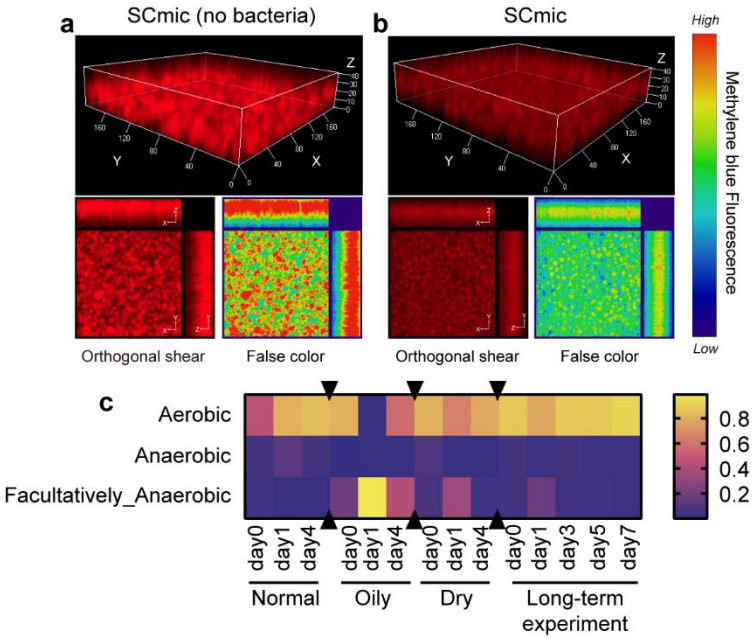
In this manuscript, Wang et al. describe the development of a highly physiologically relevant in vitro skin tissue model that successfully supports the culture of diverse skin microbiota. This complex and innovative approach has the potential to transform the study of host-microbe interactions in the skin. This reviewer is excited about the potential of this work. However, I believe there are some major issues left to be addressed to convincingly demonstrate the utility and potential of the engineered model.
Authors' response: Thank you for your thorough review of the manuscript and the kind words regarding its strengths. We truly value your positive assessment and thoughtful suggestions.

- A major concern for me is the lack of description of a formal ethics committee approval to collect human donor samples and conduct these experiments. The authors only describe the Declaration of Helsinki but do not describe going through an institutional review process.

Authors' response: Thank you for raising this important point. We apologize for the oversight in not explicitly mentioning the institutional review process in the manuscript. We confirm that all experiments involving human microbiome samples were conducted following the approval of the ethics committee at the Third Affiliated Hospital of Nanjing Medical University. The study protocol was reviewed and approved under the approval number 2024-SR-056, and all procedures adhered to the ethical standards outlined in the Declaration of Helsinki. We have updated the manuscript to include this information in the Methods section to ensure compliance with ethical standards.

- Generally speaking, throughout the paper, the authors emphasize multiple times that their model is successful at supporting the culture of diverse bacteria over time. However, their data repeatedly shows that their model is much better at supporting the culture of anaerobic than aerobic bacteria. I think this is an incredibly interesting finding that could address a major limitation of most existing models. I encourage the authors to discuss in more detail why they think this is the case and to pay attention to the comparison of aerobes versus anaerobes across all experiments. There might be interesting underlying biology there.

Authors' response: Thank you for highlighting this interesting aspect of our findings. We have agreed that our model has been more successful in supporting the culture of anaerobic bacteria, which may be due to the non-uniform oxygen content naturally present in the model (Responded Figure 2 - new Supplementary Fig. 1 in manuscript). This gradient has likely created microenvironments that favor the growth of anaerobes, mimicking the relatively low-oxygen conditions they encounter on the skin. We have further explored this in the results and discussion (lines 120-124, lines 430-432) and have examined the comparison of aerobes versus anaerobes across all experiments to better understand the underlying biological mechanisms at play (Responded Figure 2c - new Supplementary Fig. 6 in manuscript).



Responded Figure 2 (new Supplementary Fig. 1 in manuscript) Formation of oxygen gradients in SCmic. **a** Fluorescence imaging of SCmic model without bacteria culture. **b** Fluorescence imaging of SCmic model after 24 hours of bacteria culture. Methylene blue produces high fluorescence intensity at high oxygen levels. Methylene blue exhibits high fluorescence intensity in regions with elevated oxygen levels. **c** BugBase prediction of oxygen utilization for microbiota across all experiments.

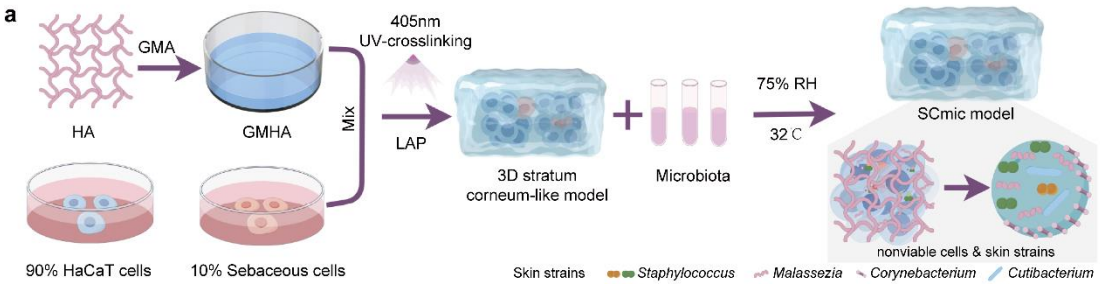
- Given the noted differences in stability and abundance between anaerobes and aerobes in this model, it is surprising that the authors do not describe the exact composition of the air in which the models were cultured. I know it is 75% relative humidity but what about %CO_2 and %O_2? Both of these gases could have a significant impact on the composition of the microbiota.

Authors' response: The models were cultured in normal atmospheric air conditions, without any specific adjustments to the CO_2 or O_2 levels. However, due to the properties of the hydrogel used, an oxygen gradient naturally forms within the model (Responded Figure 2a - new Supplementary Fig. 1a in manuscript). After the microbiota is inoculated, the oxygen content decreases, but the gradient remains present (Responded Figure 2b - new Supplementary Fig. 1b in manuscript). This oxygen gradient may explain why some anaerobic bacteria were able to grow well despite being cultured in ambient air. The presence of this gradient could provide microenvironments with lower oxygen concentrations suitable for anaerobes.

- **Figure 1** – This approach is quite innovative and intriguing, particularly due to the use of dead cells that mimic the epidermis. I have a few questions on the representative schematic for the process of generating the model in Figure 1a that were not answered in the methods section:
  - The schematic implies that the HaCaT and sebaceous cells are added in layers to the model. However, the methods do not seem to imply the same.

Could the authors please clarify this in both the figure and methods section?

Authors' response: We apologize for the confusion caused by the schematic. The cells are indeed mixed and not layered in our model. We have corrected the figure to accurately represent the method used, where HaCaT and sebaceous cells are mixed before being added. We have also updated the methods section to clearly state this procedure (lines 541-545). The revised figure was provided below for your reference.



Responded Figure 3 (Figure 1a in manuscript) a Workflow for the establishment of the SCmic model.

- The authors also should specify the ratio of HaCaT:sebaceous cells employed in the model.

Authors' response: We agree that specifying the ratio of HaCaT to sebaceous cells is important for the clarity and reproducibility of our model. We have now included this information in the manuscript. The ratio of HaCaT to sebaceous cells employed in our model is 9:1. Additionally, we chose this ratio based on literature reports indicating that keratinocyte cells comprise up to 90% of the stratum corneum[1]. This ratio ensures that our model accurately reflects the cellular composition of the epidermal layer. We have added this ratio in the methods and results section and explained the rationale for this choice (lines 130-132, line 543).

- Similarly, the right hand side of the figure depicts skin strains layered on the model. The figure seems to imply that certain types of bacteria aggregate to a particular part of the model. If that is the intended effect, please add legends to the bacteria so the reader can understand your interpretation of the data.

Authors' response: The intention of the figure was to illustrate that skin microbes utilize inactive cells as nutrients and are able to colonize and grow on these cells, rather than to show specific bacteria aggregating in particular locations. We've revised the legend and accompanying text to make it clearer. We appreciate your feedback and have made the necessary adjustments to better convey our intended effect (Fig. 1a).

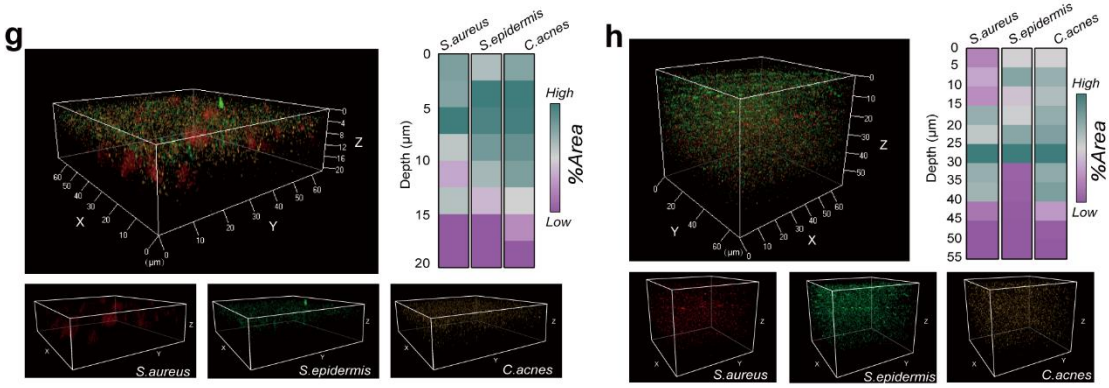
- **Figure 1e** – no legend present describing the changes in color presented in the figure

Authors' response: We have added a legend to the Figure 1e.

- **Figure 1g** – I found it really difficult to truly understand what was

happening in this figure. It was challenging to distinguish between orange and red-labeled bacteria. Also, is it possible to quantify the penetration of these bacteria along the thickness of the model in any way? As is, it is almost impossible for the reader to independently evaluate the images and thus, we are left to rely on the authors' interpretation of the data.

Authors' response: Thank you for your valuable feedback. We recognize that Figure 1g was challenging to interpret due to the difficulty in distinguishing between the orange and red-labeled bacteria. To address this, we have added individual channels for each of the three colors used in the figure, which should enhance clarity. Additionally, we quantified the relative changes in bacterial distribution along the thickness of the model by analyzing the fluorescence distribution area for each color. This quantification provides a more objective way to evaluate bacterial penetration, allowing readers to independently assess the data rather than solely relying on our interpretation. We hope these revisions will make the figure more accessible and understandable.



Responded Figure 4 (Figure 1g, h in manuscript) g 3D reconstructed CLSM Z-stacks of triple species biofilm images containing *S. epidermidis*, *S. aureus* and *C. acnes*. Bacteria were detected by FISH using *S. epidermidis*-specific (green), *S. aureus*-specific (red) and *C. acnes*-specific (orange) probes. SCmic model with a thickness of 20 μm. The heatmap represents the distribution changes of bacteria at different depths. **h** SCmic model with a thickness of 50 μm. 3D reconstructed CLSM Z-stacks of triple species biofilm images containing *S. epidermidis*, *S. aureus* and *C. acnes*. Bacteria were detected by FISH using *S. epidermidis*-specific (green), *S. aureus*-specific (red) and *C. acnes*-specific (orange) probes. The heatmap represents the distribution changes of bacteria at different depths.

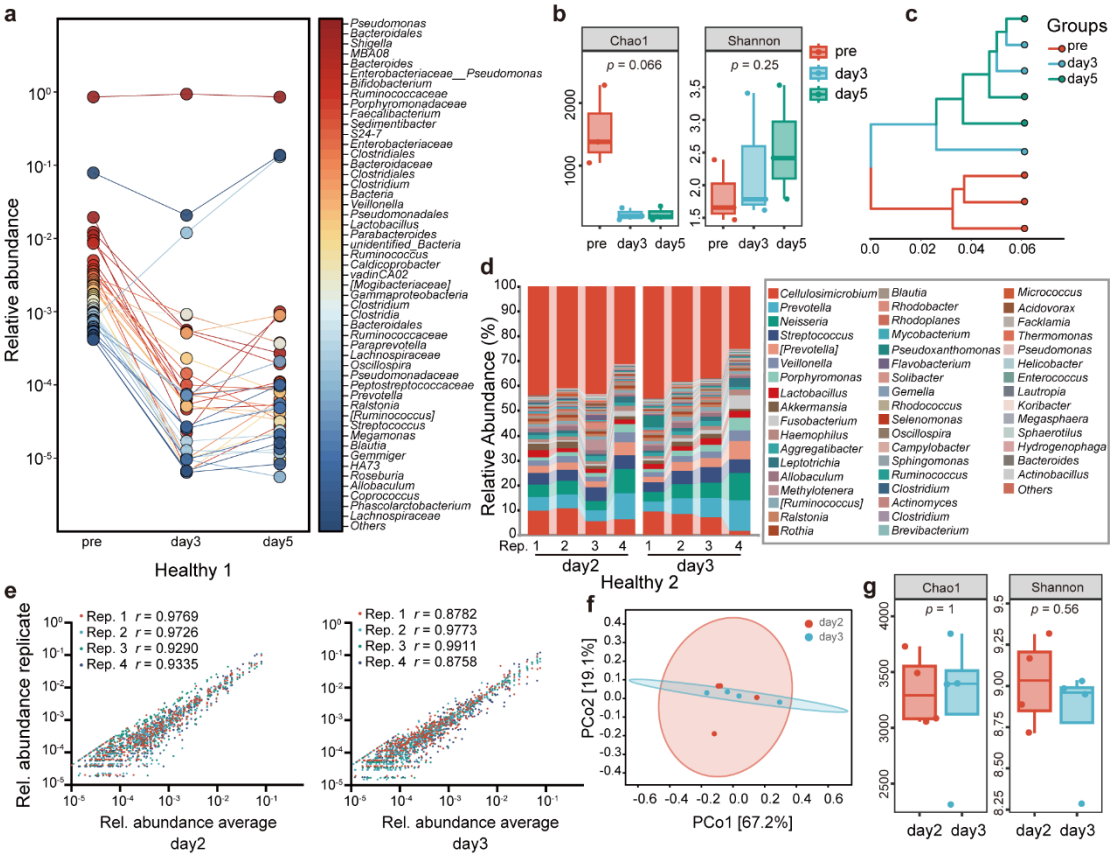
- **Figure 2** – I agree that the data nicely highlights that the model can maintain ecological stability. Nonetheless, a major hole in the presented data is the lack of information of the inoculum used for these experiments. What did the community look like prior to inoculation at day 0? Without this information, it is not possible to determine what bacteria colonized the model.

Authors' response: Thank you for your question, which was also raised by Reviewer 3. We apologize for any confusion regarding the presentation of the inoculum data in Figure 2. Our initial focus was on assessing the stability and diversity of the microbiota following inoculation on the scaffold. Based on our previous research (not initially

included in the manuscript), we observed that when the microbiota is inoculated on day 0 and left for five days, microbiota diversity decreases, and low-abundance communities diminish, leading to changes in community structure. However, we found that microbiota communities tend to stabilize between days 3 and 5. Research by Elizabeth K. et al.[2] also indicates that human skin microbiota remains relatively stable over time, with 24-hour variation being significantly smaller than variation over three months. Thus, we focused our investigation on days 2 and 3 to examine whether the skin-derived microbiota can maintain stability after being inoculated and colonized onto the model within this shorter time frame.

We apologize for any confusion caused by not initially presenting this data, and we have now included it in the revised manuscript (Responded Fig. 5a – new Fig. 2a in manuscript). In Responded Fig. 5a, solid bar graphs that add up to 100% are the standard practice; however, due to the relative abundances of the microbial community spanning several orders of magnitude, using solid bar graphs would obscure the information on less abundant strains (Responded Fig. 6). Therefore, we followed the approach used by Cheng et al. (Responded Fig. 6b), representing the community composition with scatter plots.

We recognize the importance of including the initial microbiota analysis for a comprehensive understanding. Therefore, we have added the baseline microbiota analysis before inoculation in the supplementary long-term stability experiment. The updated figure and corresponding details have now been included in the revised manuscript for your review (Responded Fig. 8 - new Supplementary Fig. 5 in manuscript).



Responded Figure 5 (Figure 2 in manuscript) The skin microbiome can maintain community ecological stability on the model. **a** The bubble chart shows the distribution of the top fifty genera biomass in relative abundance in the model for pre-culture, and after 3 and 5 days of culture. Each dot is an individual strain, the collection of dots in a column represents the community at a single group (Healthy 1, n=3). **b** Chao1 and Shannon indices of alpha diversity. The Kruskal-Wallis rank-sum test and Dunn's test were used as post-hoc tests to verify the significance of the difference. **c** UPGMA phylogenetic tree constructed based on weighted Unifrac distances. **d** The composition bar plot shows the distribution of microbiome genera-level biomass in the model for 2 and 3 days of culture (Healthy 2, n=4). **e** Communities generated from the same inoculum (technical replicates) have a nearly identical composition at day2 and day3, the color of each circle represents the corresponding genus, Pearson's correlation coefficient r of corresponding genus between average and technical replicate relative abundance (log10 rel. ab.). **f** Principal coordinate analysis (PCoA) plot based on weighted Unifrac. **g** Boxplots of pairwise comparison of the treatment alpha diversity indices using Wilcoxon's non-parametric test.

- Figure 2b – this graph does not have any meaningful biological meaning in my opinion. It is not particularly informative to now the number of taxa and plot them across all taxonomic levels. I recommend removing it.

Authors' response: We appreciate your perspective on Figure 2b and agree that the graph may not provide meaningful biological insights in the context of our study. Based on your recommendation, we have removed Figure 2b from the manuscript. We believe this change will enhance the overall clarity and focus of the results presented.

- There are several recurring issues related to the analysis and depiction of sequencing data:
  - In line 182, the authors report that PMA-Illumina sequencing was used to evaluate the microbiome. Yet, they do not specify whether the 16S rRNA or shotgun sequencing was used to profile the microbiome. This needs to be specified here in addition to in the results section.

Authors' response: Thank you for highlighting the need for clarification regarding the sequencing method used. We have revised line 182 to specify the type of sequencing employed (line 198). Additionally, we have reviewed the entire manuscript to ensure that the sequencing method is consistently and accurately described throughout, including in the Results section.

- Insufficient details are provided in the results section to describe the analysis of the obtained 16S rRNA data. For example, no description of the reference database used for taxonomic profiling is included. Additionally, there is no description of quality control or data processing. Similarly, there is no describtion of the NMDS analysis used in Figure 3. Thus, it is difficult to determine whether the data was analyzed correctly.

Authors' response: We acknowledge the importance of providing sufficient details

regarding the analysis of the 16S rRNA data. In response, we have added more comprehensive descriptions in both the Results and Methods sections to clarify the process used for analyzing the obtained 16S rRNA data. (Lines 676-714)

To provide transparency, we have specified the reference database used for taxonomic profiling. We employed the Greengenes Database 13.8 for assigning taxonomy, which is widely regarded as a reliable and comprehensive reference for microbial 16S rRNA sequences. This addition should help clarify how the microbial communities were classified.

Furthermore, we have included additional information regarding quality control and data processing. The raw sequence data was processed using the DADA2 pipeline, which encompasses filtering, trimming, denoising, and chimera detection. These steps were implemented to ensure high-quality sequence data and robust identification of amplicon sequence variants (ASVs). The quality control measures ensure that the data is reliable and ready for further analysis.

And we have thoroughly rechecked all the figures in the manuscript and provided clear descriptions for all analyses.

These updates in the manuscript ensure a more transparent and detailed account of the data analysis procedures, enabling a more comprehensive evaluation of the correctness of the methods employ.

- The analysis of beta diversity throughout the manuscript was confusing – the methods section describes using Unweighted Unifrac distances, yet several figures report using Bray Curtis distances instead – which one is it? This should probably be consistent throughout the paper or both distances should be reported leveraging supplementary figures.

Authors' response: Thank you for your observation regarding the consistency of the beta diversity analysis in our manuscript. We acknowledge the confusion caused by the use of different distance metrics in various sections. To ensure consistency and clarity, we have revised the manuscript to use only weighted Unifrac distances throughout all analyses and figures. Although the p-values changed, the conclusion did not change in all the experiments. The revised manuscript reflects this adjustment, and we have ensured that all relevant figures and descriptions align with the use of weighted Unifrac distances.

- I understand the authors chose to exclusively plot bar graphs of distances between groups to highlight similarities (or lack thereof) between experimental groups. Standard practice in the microbiome field is to also include PCoA plots of the beta diversity matrix. This would allow the reader to visually understand the data better and observe any clustering happening between samples of the same group and across different groups.

Authors' response: Thank you for your insightful suggestion regarding the inclusion of PCoA plots to enhance the visualization of beta diversity data. We acknowledge that PCoA plots are a standard and valuable tool in microbiome research for illustrating sample clustering and relationships across different experimental groups. To address

this, we have included PCoA plots of the beta diversity matrix in the revised manuscript. These plots provide a clearer visual representation of the clustering patterns and similarities among the samples, enabling readers to better understand the relationships within and between experimental groups. These additions have been incorporated into the results section and corresponding figures (Figure 2, 3 and 4), ensuring a more comprehensive presentation of the date.

- Figure 3 – The comparison across different skin types is an excellent proof of concept on the utility of this model. However, several points need to be addressed:
    - It was difficult for me to identify exactly how many individuals were recruited to represent each condition. It seems to me it was only 1 individual per group. If so, that would temper my excitement, as it is difficult to make broad conclusions about groups of individuals with only 1 biological representative sample per group.

Authors' response: We acknowledge the importance of having a sufficient number of biological replicates to draw robust conclusions. In this study, the initial proof-of-concept phase included only one individual per group, but we collected four microbiota samples from each individual. The variation in skin microbiota between individuals is greater than the variation within an individual. Therefore, we obtained four biological samples from the same individual, with the primary aim of determining the feasibility of our method.

We agree that this limitation constrains the generalizability of the findings. However, it serves as a foundational step toward broader studies, which will include a larger cohort of individuals for each condition. These future studies will address inter-individual variability and enable us to draw more conclusive results about group-specific trends, such as atopic dermatitis, psoriasis, acne, and seborrheic dermatitis, each of which is associated with distinct skin characteristics.

We appreciate your suggestion and understand that the inclusion of additional biological replicates will be necessary for future work to solidify the observations made in this preliminary study.

    - Figure 3a – in the schematic for this figure, it suggest that multiple samples were obtained from the donors every 24 hours. I did not find a description of this process in the methods. Please clarify what is meant here.

Authors' response: Thank you for highlighting the need to clarify the sampling process described in Figure 3a. The schematic aimed to represent the collection of microbiome samples from the donor's skin over a 24-hour period. Specifically, we collected skin swabs daily from the same site on the donor's skin to monitor temporal stability and changes in the skin microbiome. This approach aligns with findings from Elizabeth K. et al.[2], which demonstrated that the skin microbiome of healthy individuals remains stable over a period of three months, with higher similarity observed over shorter intervals (24 hours).

Our repeated sampling method was designed to assess the short-term variations in the microbiome, providing a reference point for evaluating the stability of the

microbiome cultured on our scaffold. To ensure this process is clearly understood, we have added detailed descriptions to the methods section of the manuscript. The revised text now specifies that multiple swabs were taken from the same location on the donor's skin every 24 hours throughout the study (lines 221-226). These samples were then analyzed to monitor the stability of the microbiome and to serve as a control for assessing the performance of the microbiome on our scaffold. We hope this addition provides the necessary clarification regarding the sampling process.
lines 221-226 "*To further demonstrate that skin microbiota can remain stable on the model, we included a control group with microbiota samples collected from the volunteer's face over four consecutive days (Fig. 3a). The results of 16S rRNA sequencing, shown in Supplementary Fig. 4, indicate that healthy human facial microbiota maintains a stable composition without large fluctuations.*"

- The results of the re-inoculation process are quite intriguing! I recommend the authors expand on why they think reinoculation leads to such a stark recovery in the discussion.

Authors' response: Thank you for your suggestion. This question was also raised by Reviewer 3. We have added a detailed discussion in the discussion section. (lines 413-441)

- Figures 3g-h – the results from the dry skin donor are missing from these graphs. I urge the authors to include these data, as it is clear it was processed and obtained in the process of generating the rest of this Figure.

Authors' response: We have now included the results from the dry skin donor in Figures 3g-h to provide a complete and comprehensive representation of our findings. The updated figures are included in the revised manuscript.

- **Figure 4** – the labels used for the conditions and experimental groups used in this study are quite confusing. Is there a different way the authors could label and plot the data? Perhaps by using grouped columns?

Authors' response: We apologize for any confusion caused by the current labeling of conditions and experimental groups in Figure 4. To improve clarity, we have re-labeled and re-plotted the data using grouped columns, making it easier to distinguish between the different conditions and experimental groups. The revised figure has been included in the updated manuscript for your review.

- Figure 4e: I am not sure I understand what the authors are trying to showcase with this panel.

Authors' response: Thank you for raising the question regarding Figure 4e. The panel is intended to compare the microbial composition between two groups across different taxonomic levels. Although there was no significant overall change in the community composition following the rinsing challenge, this does not imply that all species showed no differences. To highlight taxa with significant differences, we used LEfSe analysis, which identifies taxa that are significantly different between groups. The results

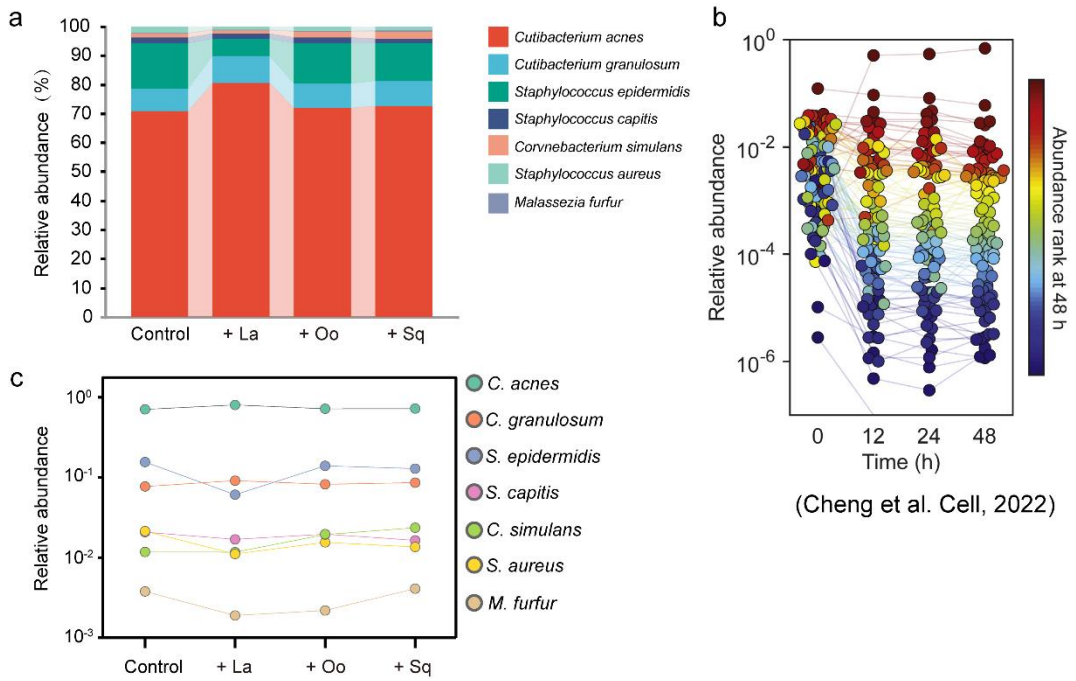
demonstrated that altering the environment from dry to wet in the model led to a significant increase in *Staphylococcus spp* (Fig. 4e), which is consistent with previous studies[3]. This finding suggests that the composition of the microbiota on the model is not static but changes dynamically in response to new environmental conditions.

The taxonomic clade in Figure 4e provides a clear and intuitive representation of these differences at various taxonomic levels, from phylum to genus (moving from the inner to the outer circles). The size of each node reflects the average relative abundance of the respective taxon. Hollow nodes represent taxa where there is no significant difference between the groups, while colored nodes (blue or red) highlight taxa that show statistically significant differences (Wilcoxon test, $P < 0.05$), with the color indicating higher abundance in the respective group. Letters are used to label the specific taxa that exhibit significant inter-group differences.

This panel is designed to visually showcase the abundance differences at each taxonomic level, providing a comprehensive overview of how the microbial composition changes post-rinsing. We hope this explanation clarifies the intention behind Figure 4e.

Figure 4 and 5g: I do not understand the choice to represent relative abundance data in this way. It is almost impossible for the reader to parse through all these points. Standard practice in the field is to use solid bar graphs that add up to 100%

Authors' response: We agree that using solid bar graphs that add up to 100% is the standard practice, we have replaced the bar graphs in Figure 4 with solid bar graphs to represent relative abundance data. However, in Figure 5g, due to the relative abundances of the microbial community spanning several orders of magnitude, using solid bar graphs would obscure the information of less abundant strains (Responded Fig. 6a). Therefore, we followed the approach used by Cheng et al. (Responded Fig. 6b), representing the community composition with scatter plots. To facilitate readers' understanding, we have added a detailed description in the manuscript: "*Each dot represents an individual strain, the collection of dots in a column represents the community at a single group.*" (Lines 318-320)



Responded Figure 6 a The composition bar plot shows the distribution of microbiome genera-level biomass. **b** Scatter plot of community composition from the literature. **c** Scatter plot shows the distribution of microbiome genera-level biomass.

- **Figure 5** – Why did the authors decide to combine the 7 bacterial species in those exact proportions? Also, could you use RT-PCR rather than 16S sequencing/relative abundance to determine absolute numbers of each bacteria and thus get a better idea of the stability of each of these species over time?

Authors' response: The decision to combine the 7 bacterial species in the specific proportions used in our study was based on the goal of creating a simplified yet representative human skin microbiome model. Our objective was to develop a conserved human skin microbiota (Hcm) model that effectively simulates a significant portion of the natural skin microbiome while minimizing the complexity associated with individual variability. We referenced the study by Zhiming Li et al., which analyzed the skin microbiome of a large sample of healthy Han Chinese individuals. The study indicated that *C. acnes*, *S. epidermidis*, *C. granulosum*, *S. aureus*, *S. capitis*, and *C. simulans* are commonly present bacteria, and *Malassezia* is a major skin fungus. We selected these species and created a model that captures the essential characteristics of the skin microbiome based on their average abundance proportions in the study.

By focusing on these seven species, we aimed to reduce the potential impact of different skin states on the microbiome, ensuring consistency and reproducibility across experiments. Although this conserved microbiota model does not fully represent the entire skin ecological community, it provides a robust platform for studying microbial interactions and skin-related microbiological processes in a controlled environment. We hope this explanation clarifies the rationale behind the specific bacterial species and their proportions used in our model.

We apologize for the oversight in not providing a detailed method description in the

Results section. In Figure 5, we used Real-time PCR rather than 16S sequencing for detection and determined the absolute abundance of each bacterial species (Figure 5b). We have provided a detailed explanation of the Real-time PCR method and its application in determining bacterial absolute abundance in the Methods section of the revised manuscript (lines 648-650, lines 665-667). We regret any confusion caused by the previous description and have thoroughly rechecked the entire manuscript to ensure that the Results section contains the necessary detailed descriptions.

- **Figure 6** – Quite interesting application of the model. However, I found the description of these results a little rushed and confusing. For example, I completely missed the niacinamide results after my first read of the paper.

Authors' response: We apologize if the description of the results, particularly regarding niacinamide, appeared rushed or unclear. We recognize the importance of clearly presenting all findings to ensure that key outcomes, such as the effects of niacinamide, are not overlooked. We have revised the manuscript to provide a more detailed and structured explanation, emphasizing the significance of the niacinamide findings (lines 365-370). These improvements aim to clarify the narrative and enhance the flow of the Results section.

- There seem to be references missing in the discussion. Several statements are given as facts without citations. See, for example Lines 385 – 390.

Authors' response: Thank you for pointing this out. We have reviewed the discussion and added the necessary references to support the statements made, including those on lines 385–390. The updated manuscript now includes these citations, and we have thoroughly reviewed the entire manuscript to ensure that all factual statements are properly supported (lines 461-462).

- For me, personally, the introduction described the results in too much detail, interrupting the flow of the paper. Similarly, I found it odd that random Figure panels were referenced here.

Authors' response: Thank you for your feedback. We have revised the introduction to focus more on the background and objectives, while minimizing detailed descriptions of the results.

- The authors employ the terms "microbiome" and "microbiota" interchangeably. I believe the authors usually mean "microbiota" as they are growing bacterial communities in this model. I recommend going through the manuscript and ensuring the terms are used properly.

Authors' response: We appreciate your recommendation and recognize the importance of using these terms accurately. We have gone through the manuscript and ensured that "microbiota" is used appropriately to refer specifically to the bacterial communities being cultivated in our model, while reserving "microbiome" for contexts that encompass both the microbial communities and their associated environments.

- The authors should consider going back through the manuscript and proofreading it to make sure all sentences are complete and there are no typos. Here is a small (not comprehensive) subset of issues I noticed:

Authors' response: Thank you for highlighting these issues. We have corrected all the sentences, and have asked native English-speaking authors to have the whole manuscript carefully read and edited.

  - Line 38-40: this is not a complete sentence.

Authors' response: We have revised the sentence in the manuscript to improve completeness.
(Lines 38-40) "The diversity of bacterial communities is greatly influenced by the ecologically distinct microenvironments present on the skin, such as sebaceous, moist, and dry areas."

  - Lines 81-83: this is not a complete sentence.

Authors' response: We have revised the sentence in the manuscript to improve completeness.
(Lines 81-83) "In this study, we sought to establish a reproducible and cost-effective in vitro skin microbiome model focusing on skin microbiology-related assays, rather than on evaluating the microbiota-skin interactions."

  - Lines 166-168: this is not a complete sentence.

Authors' response: We have revised the sentence in the manuscript to improve completeness.
(Lines 167-170) "In the 50 μm SCmic model (Fig. 1h), it was observed that both orange (*C. acnes*) and green fluorescent (*S. epidermidis*) bacteria exhibited a comparable distribution at 0~40 μm, interspersed with a small number of red fluorescent (*S. aureus*) bacteria."

  - Lines: 251-253: incorrect grammar – difficult to establish what the sentence means to say.

Authors' response: We appreciate your feedback regarding the grammatical issues in that section. We have revised the sentence for clarity and correctness to ensure the intended meaning is clear and easily understood.
(Lines 276-278) "To test whether the skin microbiota colonized on the SCmic model exhibits resistance to some external influences, we rinsed the model with PBS to simulate daily face washing."

  - Lines 504-506: mixed use of past and present tense.

Authors' response: We have revised the sentence in the manuscript.
(Lines 592-594) "After incubating *C. acnes* inoculated at about 1×10^7 CFU/cm² at 32℃ for 48 hours, we freeze-dried the SCmic model and took images of microbial colonization on the model using SEM."

- Line 152: what is meant by co-growth of bacterial habitats?

Authors' response: We have revised the term "habitats" to "communities" in line 152 for greater clarity. The phrase now accurately reflects our intention to describe the co-growth of bacterial communities within the context of our study. We appreciate your feedback, which has helped us improve the precision of our manuscript. (Line 155)

References

1. Eckert, R. L. Structure, function, and differentiation of the keratinocyte. *Physiological Reviews* **69**, 1316, (1989).
2. Costello, E. K. et al. Bacterial Community Variation in Human Body Habitats Across Space and Time. *Science* **326**, 1694-1697, (2009).
3. Grice, E. A. et al. Topographical and temporal diversity of the human skin microbiome. Science 324, 1190-1192 (2009).

Reviewer #3 (Remarks to the Author):

The authors describe the fabrication and functional validation of a novel engineered platform for the culture of the skin microbiome that simulates the stratum corneum of the epidermis. By providing devitalized epithelial cell as a nutrient source, and an irregular porous scaffold to create oxygen gradients to recapitulate both aerobic and anaerobic conditions, the authors were able to culture over several days simple and complex microbiota associations that effectively recapitulate skin microbiome. The functional validation with drug metabolism analysis demonstrates the platform suitability for pharmacological research.

Although several attempts of establishing a skin-microbiome in vitro platform are reported in literature, here the lack of high nutrient medium to support the viability of human cells allowed to achieve a stable and diverse colonization, preventing the overgrowth and monopoly of single strains.

The work support most of the conclusions and claims, but despite the quality of the data presented and the appropriateness of the analysis, major and minor changes are required for publication.

Authors' response: We are grateful for the time and effort you put into reviewing the manuscript and for your encouraging remarks on its merits. Your constructive feedback and insightful comments are greatly appreciated.

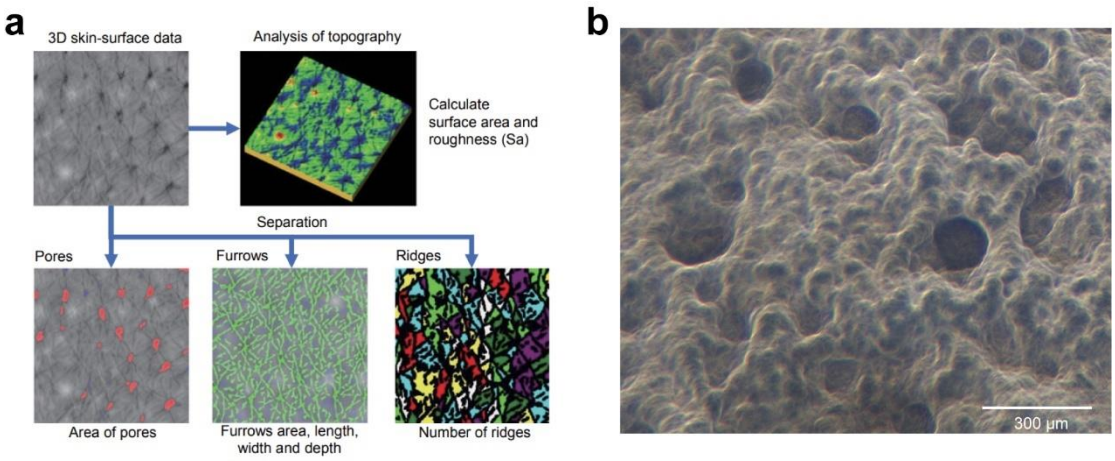
Major Critiques

- Line 120: "The morphology of the hydrogel as presented does not resemble the morphology of the skin and its appendages." Provide further evidence or remove this statement.

Authors' response: Thank you for pointing out this issue. We have revised the statement in the manuscript to more accurately reflect the intended meaning. Our original aim was to convey that the hydrogel is capable of forming a complex topography similar to the invaginations and depressions found on the skin. As Responded Fig. 7a illustrated, from a macroscopic perspective, the skin is a complex terrain with numerous invaginations, pockets, and niches[1]. Each anatomical niche provides a unique microenvironment, which the resident microbial communities of the skin are adapted to. Studies have demonstrated that such topography plays a crucial role in the uniqueness of the skin microbiome. In response to your feedback, we have adjusted the text to emphasize that our model can mimic the complex terrain of invaginations and depressions rather than directly resembling the morphology of the skin and its appendages.

The revised sentence now reads: "*The hydrogel exhibits porous properties and a morphological that mimics the invaginations and depressions found in the skin (Fig. 1b), which are important for creating niche-specific microenvironments.*" (lines 118-120)

We appreciate your feedback, which has improved the clarity and accuracy of our manuscript.



Y. Masuda et al. Skin Research and Technology, 2014 Fig. 1b

Responded Figure 7 The literature illustrates the macroscopic perspective of the skin. The Fig. 7a illustrate that, from a macroscopic perspective, presents a complex terrain characterized by numerous invaginations, pockets, and niches.

- Line 121. "which are vital to maintain a stable state of the microbiota in vitro, especially in the bacterial and fungal communities in the sebaceous gland area22. Not clear what is vital to bacterial communities. Is the porosity and morphology of the substrate or the follicles and glands? If the former, the cited reference does not suggests that; and the authors should demonstrate how porosity/morphology affects the communities. If the latter, hair follicles and sebaceous glands are not included in the model, so it becomes less relevant.

Authors' response: Thank you for highlighting this issue. We recognize that the original text was unclear, and we have revised line 121 for clarity. The revised sentence now reads: (lines 118 - 124)

"The hydrogel exhibits porous properties and a morphological that mimics the invaginations and depressions found in skin (Fig. 1b), which are important for creating niche-specific microenvironments. These properties are essential for maintaining the steady state of the microbiota in vitro, as they create a non-uniform oxygen content in the model (Responded Fig. 2 - new Supplementary Fig. 1 in manuscript) that provides suitable conditions for the co-growth of microbiota, including bacteria found in relatively anaerobic environments."

This revision clarifies that it is the porosity and morphology of the hydrogel, rather than actual hair follicles or sebaceous glands, that are crucial for maintaining stable microbial communities in our model. We constructed the model using mildly acidic GMHA hydrogel with controlled environmental temperature and humidity. Beyond these factors, we believe that the topography of the hydrogel—featuring invaginations and depressions formed after loading with non-viable cells—also plays a significant role. We have provided additional evidence demonstrating that the porosity and morphology of the hydrogel create relatively anaerobic conditions, as shown in Responded Fig. 2 (new Supplementary Fig. 1 in manuscript).

We hope this modification addresses the concerns raised and provides a clearer

understanding of our approach.

- Line 145-147: The meaning of this statement is not clear. What is shown in Figure 1e suggests that the final bacteria density is directly proportional to the amount of initial inoculation.

Authors' response:

Thank you for pointing this out. We acknowledge that the statement could be clearer. We have revised this sentence in the manuscript. The revised sentence should read: "*However, the bacterial numbers stabilized within a certain range from day 1 to day 7 (Fig. 1e).*" (lines 149 - 150)

- Figure 1. It would be interesting to know what design parameters positively/negatively affect the microbiome culture. How far is the model from optimized conditions? There is no comparison of the effect of parameters, such as hydrogel type (e.g., would this work with gelatin methacrylate, GELMA), hydrogel stiffness, pH, porosity, density of cells (nutrients) etc. These comparisons are important to appreciate the success of the model, the mechanism behind its superiority over the previous model systems, and for its further optimization.

Authors' response: Thank you for your insightful feedback. We acknowledge the importance of understanding the design parameters that affect the microbiome culture. Our current study focused on establishing the feasibility and basic functionality of our model. We chose glycidyl methacrylate hyaluronate for its slightly acidic nature (pH ~6 before bacterial inoculation, Supplementary Fig. 2) and its similarity to the hyaluronic acid found in human skin (0.5-1 mg per gram of wet tissue weight[2]). Additionally, we selected a lower gel stiffness and ensured an adequate cell density. Different ecological niches on human skin significantly influence the microbiome, and individual differences in skin microbiota are substantial. Currently, we have focused on one infrastructural condition to validate the feasibility of establishing a skin microecology model.

Future work will explore the effects of various parameters such as hydrogel type, hydrogel stiffness, pH, porosity, and nutrient density on the microbiome culture. These comparisons will help us optimize the model and elucidate the mechanisms behind its performance. As you mentioned in your subsequent question, improving the growth of microbiota from dry skin by adjusting the model's moisture content is an inspiring idea. Given that certain skin conditions, like atopic dermatitis, psoriasis, acne, and seborrheic dermatitis, often occur in specific skin areas with unique features, optimizing the model to sustain microbiota from pathological skin sources would be highly valuable.

We have added this discussion to the manuscript to highlight the potential for further optimization and the comparative analysis of different parameters. (line 444-453)

- Figure 1. The author says "Another important factor in judging the success of a microbial model is whether it can support the bacterial diversity at a steady-state level.", but the data shown in Figure 1 and Supp Fig 2-3 do not show bacterial diversity over

time. The introduction to this section looks misplaced and misleading. If the authors claim stability of these 2 strain over time, the data should include multiple time points for distribution of each strain.

Authors' response: Thank you for highlighting this issue. We recognize that the initial statement may have caused confusion, and we apologize for its misplaced placement in the manuscript. To address this, we have removed the statement to avoid any potential misunderstandings.

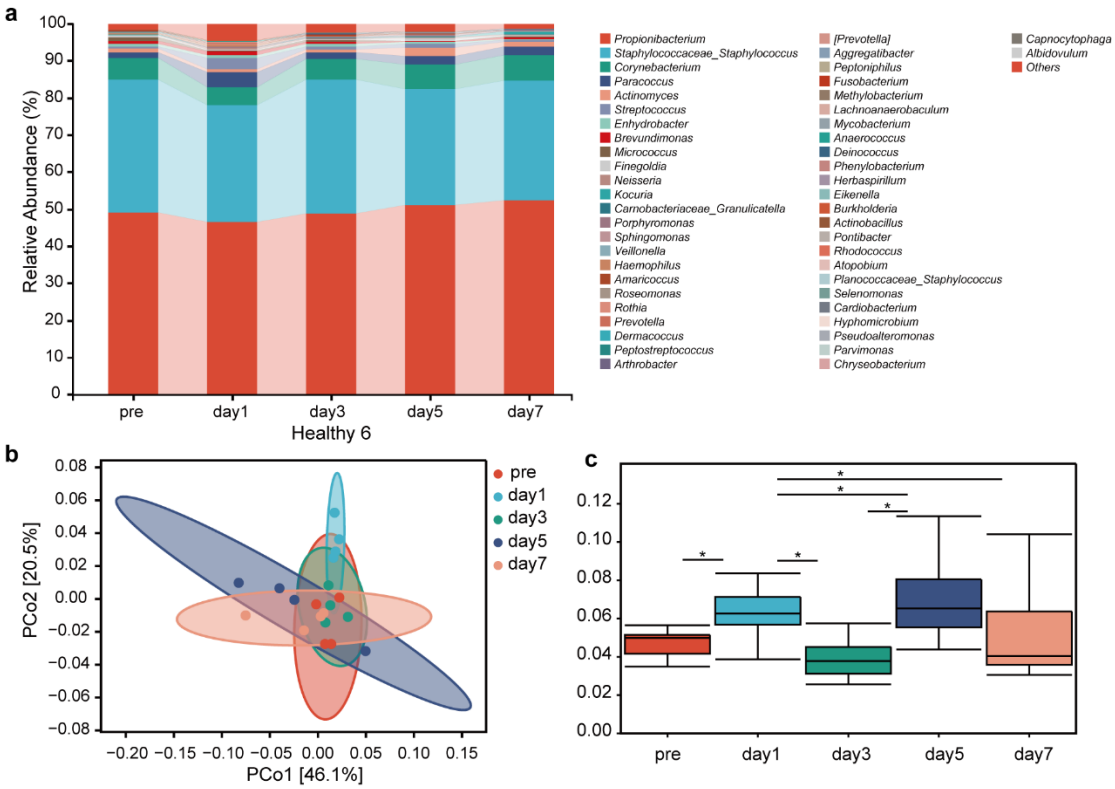
We appreciate your feedback, which has helped improve the clarity of our work. Additionally, we conducted a thorough review of the entire manuscript to ensure that no similar issues remain. Thank you once again for your valuable input.

- Figure 2. There is no justification of why day 2 and day 3 were chosen to make the comparison of bacterial diversity and stability. The data is encouraging that within 24 hours the colonies can say stable. It is equally interesting and important to know how long such stability lasts. I recommend conducting a longer term experiment (e.g., 7 days as in Figure 1).

Authors' response: Thank you for raising this important point. Based on our previous research results (not initially included in the manuscript, Responded Fig. 5a – new Fig. 2a in manuscript), we observed that when the microbiota is inoculated on day 0 and left for five days, microbial diversity decreases, and low-abundance communities diminish, leading to changes in community structure. However, we found that bacterial communities tend to stabilize between days 3 and 5.

We hypothesize that after the microbiome is first transplanted onto the model, high-abundance strains dominate, while low-abundance strains are initially disadvantaged but eventually reach a stable state after some time. Research by Elizabeth K. et al.[3] also indicates that human skin microbiota remains relatively stable over time, with 24-hour variation being significantly smaller than variation over three months. Thus, we chose to compare bacterial diversity and stability on days 2 and 3 to investigate whether the microbiota derived from the skin can maintain stability after being inoculated and colonized onto the model. Additionally, we aimed to demonstrate technical stability by assessing whether the microbiota from the same individual, with identical community composition, could retain high similarity after being cultured on the model. Our data show good temporal and technical stability within the 24-hour period.

During the revision process, we removed this portion of the data to present our findings more clearly and logically, but we overlooked the mention in line 204. We apologize for any confusion caused by not initially presenting this data, and we have now included it in the revised manuscript (Responded Fig. 5a – new Fig. 2a in manuscript). We also appreciate your suggestion to conduct longer-term experiments, as this is indeed crucial. We have supplemented our study with long-term stability experiments and included the analysis of microbiota before inoculation. The results are shown in the updated manuscript (Responded Fig. 8 - new Supplementary Fig. 5 in manuscript).



Responded Figure 8 (new Supplementary Fig. 5 in manuscript) The skin microbiome remains stable on the model over time. **a** The composition bar plot shows the distribution of microbiome genera-level biomass in the model over 0-7 days of culture (Healthy 6, n=4). **b** Principal coordinate analysis (PCoA) plot (based on weighted Unifrac) of normal microbiota cultured for 0-7 days. **c** Analysis of similarities (Anosim) based on weighted Unifrac distance matrix shows differences between the microbiota of normal for pre, day1, day3, day5 and day7. Anosim, $^{*}P < 0.05$.

- Figure 2a: why the microbiome was not analyzed before inoculation (while it was in Figure 3)? Provide a justification.

Authors' response: Thank you for your question. This question was also raised by Reviewer 2. We apologize for any confusion. The microbiome was indeed analyzed before inoculation in Figure 3 to provide a baseline for comparison and demonstrate the initial state of the microbiome. However, for Figure 2a, our primary focus was on assessing the stability and diversity of the microbiome after inoculation on the scaffold. We recognize the importance of including the initial microbiome analysis for a comprehensive understanding. Therefore, we have added the baseline microbiome analysis before inoculation in the supplementary long-term stability experiment. The updated figure and corresponding details have been included in the revised manuscript for your review (Responded Fig. 8 - new Supplementary Fig. 5 in manuscript).
We hope this addresses your concern, and we appreciate your feedback.

- Line 217-220: Please discuss in the discussion section the reason why multiple inoculations are required. Does the preecolonization with certain strain is required for the successful inoculation of other strains? Could you identify pathogens among the

strains that required a second inoculation to successfully colonize the platform?

Authors' response: Thank you for your insightful question. We have now included a detailed discussion on the necessity of multiple inoculations in the revised manuscript. The primary reason for the need for re-inoculation is to maintain microbial diversity on the scaffold that closely resembles the initial seeding population. In our study, we did not specifically analyze whether pre-colonization with certain strains is required for the successful inoculation of other strains, nor did we identify pathogens among the strains that required a second inoculation for successful colonization. We chose to re-inoculate because a single inoculation may lead to greater microbial community changes, especially due to the presence of hard-to-colonize or low-abundance microbes. Re-inoculation can help mitigate these changes. And our re-inoculation approach was inspired by common practices in microbiome transplantation, as referenced in the studies by Aranda-Díaz et al.[4], Cheng et al.[5], and Desai et al.[6], all of which employed multiple inoculations in germ-free mice.

A detailed discussion has been added to the manuscript, as shown below: (line 413-441)

"Based on the results from Fig. 2a, we decided to reinoculate on day 1. In the Fig. 2a experiments, we observed that when microbiota was inoculated only on day 0 and left for 5 days, microbial diversity declined and community structure changed (anosim, $p = 0.102$). However, the bacterial communities stabilized between days 3 and 5 (anosim, $p = 0.903$), though with reduced diversity compared to the original community. In addition, we observed that the majority of microbiota derived from human skin could colonize the model by day 5, with 88% of the top 50 most abundant genera establishing themselves. We speculate that the changes in community structure may be due to the loss of low-abundance genera or those that are more difficult to colonize (Fig. 2a). For example, low-abundance genera (<1%) generally decreased from day 0 to day 3. Similar to previously reported results, when gut microbiota was introduced into germ-free mice, low-abundance members were more likely to disappear[4]. In such cases, a single inoculation of hard-to-colonize microbes could lead to more significant microbial community changes, and multiple inoculations in germ-free mice have been shown to improve this issue[7].

In addition, the SCmic model environment is not identical to the natural skin environment, and the initial inoculation may not provide optimal colonization conditions for all strains, which could lead to reduced diversity (Fig. 2b, 3h). After the initial inoculation of skin microbiota onto the model, changes in the model's pH and oxygen levels likely create an environment favorable for a second inoculation. The model's pH increases after inoculation (Supplementary Fig. 2), and a neutral pH is more conducive to the colonization of commensal bacteria compared to an acidic pH. Although the skin surface is often acidic, recent research by Fukuda et al.[8] found a pH gradient in the skin's stratum corneum, with the middle layers being acidic to serve as a barrier against pathogens, while the upper layers, influenced by microbial growth, tend to be neutral. The pH of the SCmic model shows a similar trend to the upper layers of the skin's stratum corneum. Furthermore, we also found that oxygen levels decreased and a non-uniform oxygen content existed within the SCmic model after the initial

inoculation, supporting the coexistence of a diverse microbial community."

This addition aims to clarify the rationale behind our methodology and the importance of multiple inoculations in maintaining a stable and diverse microbiota within our model.

- In the methods the authors define dry skin only based on the low sebum content. Although the classification of skin in normal, oily, and dry used to be associated with the amount of sebum secretion, dry skin is primarily characterized by a low water content that can be caused by multiple factors (such as filaggrin mutations), not exclusively by reduced sebum secretion, to the extent that dry skin is often associated with hyperseborrhea (e.g. in seborrheic dermatitis). The criteria used for the classification and selection of the skin microbiome donor area should be better described in the results section and further discussed. To further understand the variable outcome of microbiome culture isolated in from different skin type, it would be important to determine the level of hydration of the hydrogel using perhaps confocal Raman spectroscopy or a simple corneometer, and investigate whether modulating the water content the culture conditions for the dry skin microbiome would improve.

Authors' response: Thank you for your insightful comments. We acknowledge the importance of defining dry skin based on both sebum secretion and water content, as skin hydration plays a critical role in characterizing different skin types. In response to your suggestions, we have revised our approach and updated the methods and results sections to include a more comprehensive description of the criteria used for classifying and selecting the skin microbiome donor areas.

During the previous selection process, we measured the sebum secretion and skin water content of the participants' facial skin, and the specific data for the participants have now been included in the supplementary materials. The definition of dry skin in the Methods section has been revised to incorporate the criterion of low skin water content, better reflecting the complex characteristics of dry skin (lines 578-584). These updates ensure more accurate classification and provide clearer criteria for selecting donor areas.

Furthermore, we recognize the importance of optimizing the model parameters to improve the culture conditions for microbiomes isolated from different skin types. As illustrated in the Responded Fig. 9a, we quantified the hydration level of the model using a corneometer, finding that the water content was relatively high (c. u. > 115). We attempted to modulate the hydrogel's water content by increasing the hydrogel concentration (up to 30 mg/mL) and testing alternative hydrogel materials such as GELMA. However, these adjustments did not result in significant improvements.

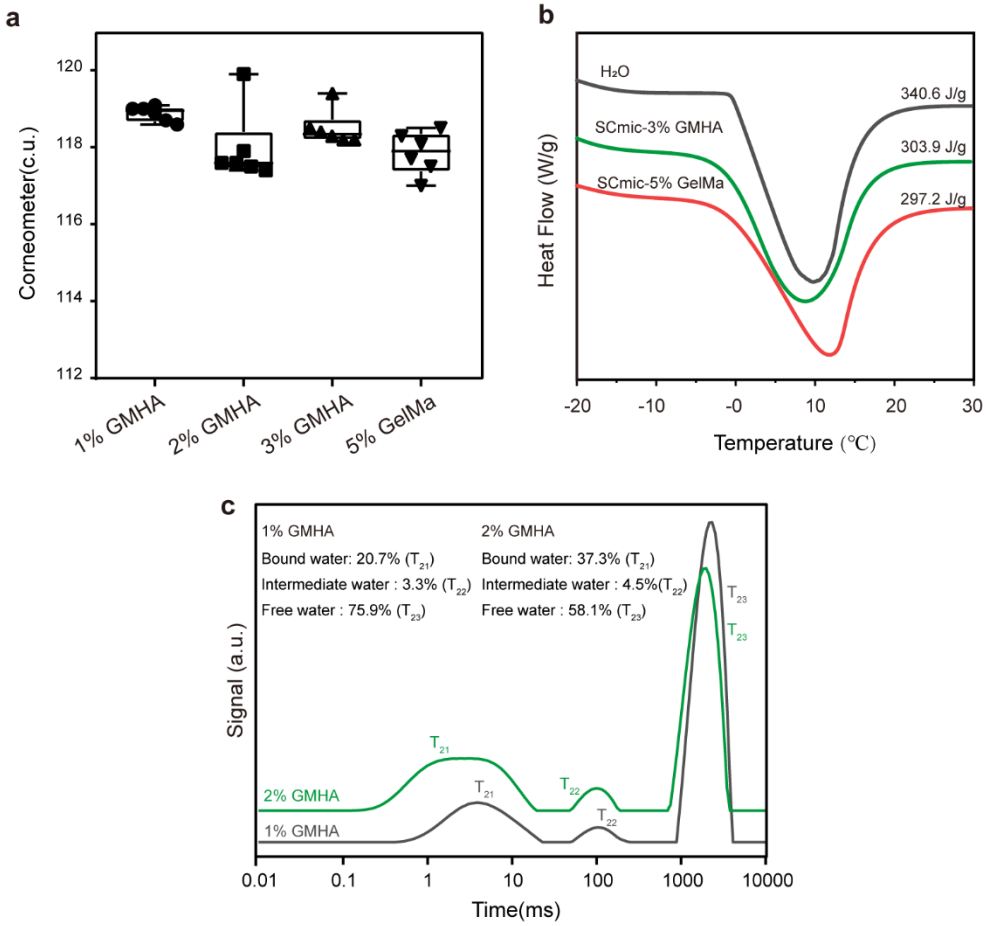
Differential Scanning Calorimetry (DSC) analysis showed a distinct melting peak around 10°C, indicating that most of the water within the model is free water (Responded Fig. 9b). To further analyze the mobility and distribution of water molecules within the gel system, we employed low-field nuclear magnetic resonance (LF-NMR), which effectively determined water distribution within the gel matrix (Responded Fig. 9c). Factors such as gel type, porosity, and crosslinking degree affect the free water content, making the optimization of culture parameters for microbiomes

from different skin types complex[9]. In the future, we plan to reduce the free water content in the gel by increasing the crosslinking degree or using a combination of gels. Additionally, we will investigate whether adjusting the water content could improve the cultivation of microbiomes from dry skin.

Although our study focused on dry skin, we are also interested in exploring the application of our model to other skin conditions, such as atopic dermatitis, psoriasis, acne, and seborrheic dermatitis, each associated with distinct skin characteristics. We plan to investigate how adjusting the model parameters could enable the culture of microbiomes from a broader range of skin types in future studies. These points have been discussed in the revised discussion section to provide a more comprehensive understanding of the model's capabilities and the challenges associated with culturing microbiomes from diverse skin types. (lines 442-453)

lines 578-584: "*Skin type classification was based on sebum and water measurements[52], categorized as follows: oily type (Sebumeter: >66 μg/cm^2, Corneometer: >40 c.u.); normal type (Sebumeter: 33-66 μg/cm^2, Corneometer: >40 c.u.); dry type (Sebumeter: <33 μg/cm^2, Corneometer: <30 c.u.). The amount of facial sebum secretion was measured using the Sebumeter® (cat# SM 815, Courage + Khazaka Electronic), and water content was measured using the Corneometer® (cat# CM 825, Courage + Khazaka Electronic).*"

lines 442-453: "*However, microbiota obtained from dry skin show some variation in replication. Dry skin is characterized by lower levels of sebum and moisture, whereas the SCmic model uses a hydrogel scaffold with high water content, predominantly free water. Further adjustments in humidity and other factors needs to be considered to establish a microecological environment suitable for the colonization of dry skin microbiota. Factors such as gel type, porosity, and crosslinking degree influence the free water content, which can be reduced by increasing the crosslinking degree or using a combination of gels. Besides dry skin, various pathological skin conditions, such as atopic dermatitis, psoriasis, acne, and seborrheic dermatitis, are often associated with specific skin areas with unique characteristics. Future exploration of adjusting the model parameters to alter the physical state of the model may make it suitable for culturing microbiota from a broader range of skin types.*"



Responded Figure 9 Detection of water content in SCmic models prepared with different gels. **a** The bar graph illustrates the water content of SCmic models prepared with 1% GMHA, 2% GMHA, 3% GMHA, and 5% GelMa as measured using a corneometer. **b** Differential scanning calorimetry (DSC) thermogram of SCmic models prepared with 3% GMHA and 5% GelMa. **c** T_2 transverse relaxation time distribution of LF-NMR for 1% GMHA and 2% GMHA.

- Figure 3b. "…the microbiome composition of normal and oily skin returned to the pre-inoculation (day 0) level". Since there is no perfect overlap, this should be represented as percentage of similarity.

Authors' response: Thank you for your valuable comment. We agree that representing the microbiome composition as a percentage of similarity would provide a clearer understanding of the overlap. We have revised the manuscript and now express the similarity in microbiome composition as a percentage using the Pearson correlation coefficient. This adjustment more accurately reflects the extent to which the microbiome of normal and oily skin returned to the pre-inoculation (day 0) level.

- Figure 3. "observed previously, the bacterial population and community diversity on the day 2 were very different from the original (day 0), as the bacteria needed time to adapt to their new environment" There is no data shown for day 2? If the authors meant day 1, what is evidence for the claim of bacterial adaptation? What would happen if the

authors did not reinoculate on day 1, and only left those inoculated on day 0 for 4 days? Would the bacteria adapt and return to normal diversity? These experiments are necessary to understand the bacterial adaptation hypothesis? If it is true, than it means the strains showed a similar level of adaptation to the new environment, likelihood of which should be discussed in detail.

Authors' response: Thank you for raising this important point. We apologize for the oversight regarding the reference to day 2 data. The statement should indeed refer to day 3 rather than day 2. The data referenced in line 204 is derived from our previous research. During the revision process, we removed this portion of the data to present our findings more clearly and logically, but we overlooked the mention in line 204. We apologize for any confusion this may have caused. We have now added this data back into the manuscript to ensure it is clearly presented alongside the other findings (Responded Fig. 5a – new Fig. 2a in manuscript, Line 219).

The basis for our claim that bacteria adapt to new environments comes from our experimental observations. We found that if microbiota were inoculated only on day 0 and left for 5 days, microbial diversity decreased, and the community structure changed (anosim, weighted Unifrac, $p = 0.102$). However, by days 3 to 5, the bacterial community stabilized, although its diversity remained lower than the original population (anosim, weighted Unifrac, $p = 0.903$). Additionally, by day 5, most skin-derived bacteria successfully colonized the model, with 88% of the top 50 genera establishing colonization. We hypothesize that this change may be due to the difficulty of colonizing low-abundance or hard-to-colonize strains, which is consistent with previous studies indicating that some microbes require multiple inoculations to adapt effectively. Furthermore, the SCmic model altered the pH and oxygen levels post-inoculation, providing a favorable environment for bacterial adaptation. These findings are consistent with studies by Gan et al.[7] and Aranda-Díaz et al.[4], which also demonstrated that multiple inoculations help improve the community structure of microbes in germ-free models. A detailed discussion has been added to the manuscript (lines 406- 441).

lines 406- 441: "*During our study, we observed that the composition of the microflora changed considerably when the microflora obtained from human facial skin was inoculated onto the model within the first 24 hours, as the microflora needed time to adapt to the new environment. To aid and accelerate the colonization of the microbiota on the model, we inoculated the microbiota twice in succession and successfully replicated the skin microbiota on the model, especially replicating the microbiota from normal and oily skin.*

Based on the results from Fig. 2a, we decided to reinoculate on day 1. In the Fig. 2a experiments, we observed that when microbiota was inoculated only on day 0 and left for 5 days, microbial diversity declined and community structure changed (anosim, $p = 0.102$). However, the bacterial communities stabilized between days 3 and 5 (anosim, $p = 0.903$), though with reduced diversity compared to the original community. In addition, we observed that the majority of microbiota derived from human skin could colonize the model by day 5, with 88% of the top 50 most abundant genera establishing

themselves. We speculate that the changes in community structure may be due to the loss of low-abundance genera or those that are more difficult to colonize (Fig. 2a). For example, low-abundance genera (<1%) generally decreased from day 0 to day 3. Similar to previously reported results, when gut microbiota was introduced into germ-free mice, low-abundance members were more likely to disappear[4]. In such cases, a single inoculation of hard-to-colonize microbes could lead to more significant microbial community changes, and multiple inoculations in germ-free mice have been shown to improve this issue[7].

In addition, the SCmic model environment is not identical to the natural skin environment, and the initial inoculation may not provide optimal colonization conditions for all strains, which could lead to reduced diversity after community colonization (Fig. 2b, 3h). After the initial inoculation of skin microbiota onto the model, changes in the model's pH and oxygen levels likely create an environment favorable for a second inoculation. The model's pH increases after inoculation (Supplementary Fig. 2), and a neutral pH is more conducive to the colonization of commensal bacteria compared to an acidic pH. Although the skin surface is often acidic, recent research by Fukuda et al.[8] found a pH gradient in the skin's stratum corneum, with the middle layers being acidic to serve as a barrier against pathogens, while the upper layers, influenced by microbial growth, tend to be neutral. The pH of the SCmic model shows a similar trend to the upper layers of the skin's stratum corneum. Furthermore, we also found that oxygen levels decreased and a non-uniform oxygen content existed within the SCmic model after the initial inoculation, supporting the coexistence of a diverse microbial community."

Minor critiques

- Line 7: "This kind of balance is achieved by the multifactorial interactions" since no specific interacting factors were discussed, either describe or remove "the" before "multifactorial".

Authors' response: Thank you for the suggestion; we have revised line 7 by removing "the" to improve clarity.

- Line 33: reference 3. The reference only refers to acne. Provide additional references for atopic dermatitis and sensitive skin.

Authors' response: We have now included additional references that specifically address atopic dermatitis and sensitive skin.

- Line 46: reference 8 is a review. It would be more appropriate to cite the original articles mentioned in the review (Dunbar, J., Barns, S. M., Ticknor, L. O., and Kuske, C. R. (2002). Appl Environ Microbiol 68, 3035-45, Bowler, P. G., Duerden, B. I., and Armstrong, D. G. (2001). Clin Microbiol Rev 14, 244-69, Davies, C. E., Wilson, M. J., Hill, K. E., Stephens, P., Hill, C. M., Harding, K. G., and Thomas, D. W. (2001). Wound Repair Regen 9, 332-40.)

Authors' response: We have now replaced reference 8 with the original articles by

Davies et al. (2001) as more appropriate citations.

- Line 54: References for clinical trials are missing.
Authors' response: Thank you for your comment regarding the missing references for clinical trials. We have conducted a thorough review of the relevant literature and added appropriate references for the clinical trials discussed in the manuscript. These updates will ensure that the clinical context of our research is well-supported. (Line 54)

- Line 57: Reference 14 is the same as reference 5.
Authors' response: Thank you for pointing out the duplication. We apologize for the oversight regarding references 14 and 5. We have corrected the reference list to ensure that each citation is unique and properly formatted. Your attention to detail helps enhance the accuracy of our manuscript. Thank you for your valuable feedback.

- Line 68: Reference 19 is the same as reference 17.
Authors' response: We have corrected the reference list to ensure that each citation is unique and properly formatted.

- Line 75: "and nonviable HaCaT, sebaceous cells as nutrients" rephrase at this sounds like HaCaT cells are sebaceous cells.
Authors' response: To clarify the statement, we have rephrased it. The revised sentence now reads: (Lines 73-76) "*along with nonviable HaCaT and sebaceous cells as nutrients.*"
We appreciate your feedback, which helps improve the clarity of our manuscript.

- Line 77: The reference provided does not report pH values for the polymerized hydrogel.
Authors' response: We apologize for the oversight regarding the pH values of the polymerized hydrogel. In light of your feedback, we have removed the statements related to pH from the manuscript to ensure accuracy (line 77).

- Figure 1b: indicate what kind of inverted microscope was used.
Authors' response: We have indicated the specific type of inverted microscope used in the methods section of the manuscript. (Line 586)

- Line 117: "Scmic" should be "SCmic"
Authors' response: We have carefully reviewed the entire manuscript to correct all similar instances of this error to ensure consistency in terminology. (Line 116)

- Line 124: skin pH averages between 5 and 6. The pH of 6.8 reported for the hydrogel is well above skin physiological values, please include in the discussion.
Authors' response: Thank you for your valuable feedback. We acknowledge that the average pH of the skin typically ranges between 5 and 6. We have discussed the pH of the hydrogel reported as 6.8, pointing out the reasons why this value exceeds the

physiological range of the skin. This discussion helps clarify the context of our findings and addresses any potential impacts regarding the model's relevance to skin conditions. We appreciate your suggestion and have ensured that this is clearly articulated in the revised manuscript.
line 430-439 "*After the initial inoculation of skin microbiota onto the model, changes in the model's pH and oxygen levels likely create an environment favorable for a second inoculation. The model's pH increases after inoculation (Supplementary Fig. 2), and a neutral pH is more conducive to the colonization of commensal bacteria compared to an acidic pH. Although the skin surface is often acidic, recent research by Fukuda et al. found a pH gradient in the skin's stratum corneum, with the middle layers being acidic to serve as a barrier against pathogens, while the upper layers, influenced by microbial growth, tend to be neutral. The pH of the SCmic model shows a similar trend to the upper layers of the skin's stratum corneum.*"

- Line 130: The official name of what was formerly known as "Propionibacterium acnes" is now "Cutibacterium acnes". Update name in the text.
Authors' response: We have updated the text to reflect the current official name, "Cutibacterium acnes" in place of "Propionibacterium acnes" as suggested.

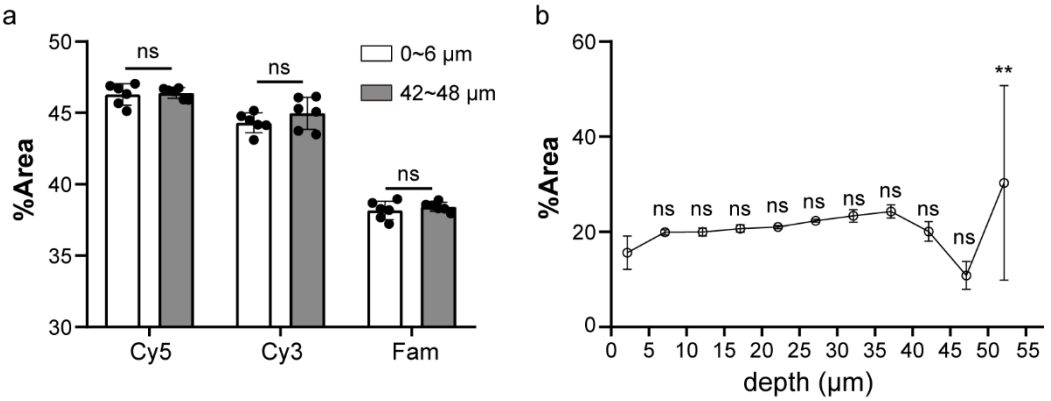
- Line 132: The reference included makes does not mention endogenous infections. Although C. acnes is an opportunistic pathogen, it is not a frequent source of endogenous infections when compared to other genus that also inhabit skin such as Staphylococcus and Streptococcus. Also, the author should cite the original source supporting the statement "Anaerobic bacteria, such as P. acnes, constitute a significant portion of the human facial skin microbiota" (McGinley et al PMCID PMC242779).
Authors' response: We have removed the reference to endogenous infections related to *P. acnes* and instead classified it correctly as an opportunistic pathogen. Additionally, we have cited the original source (McGinley et al., PMCID PMC242779) to support the statement that anaerobic bacteria, such as *P. acnes*, constitute a significant portion of the human facial skin microbiota. (Line 135-136)

- Line 158: replace "thinness" with "thickness".
Authors' response: We appreciate your careful review. We agree that "thinness" should be replaced with "thickness" to accurately describe the context. We have revised the manuscript accordingly to ensure the correct terminology is used.

- Line 164-165: how did the authors compensate for the loss of signal due to light scatter? Please analyze a cross-section of the hydrogel to support your statement.
Authors' response: We appreciate your important question regarding light scatter compensation. We are aware that using confocal laser scanning microscopy (LSM800, Zeiss, Germany) on thicker samples can lead to fluorescence signal loss due to light scatter, often addressed by Z compensation through laser gain adjustment. To address this issue, we introduced 1 μM Cy3, Cy5, and Fam-labeled probes into the GMHA gel and captured two sets of XY-plane images using a higher master gain (800 v). First, we

captured six XY-plane images at 1 μm Z intervals, followed by another set of six images at the same Z intervals but with a 42 μm height difference. Using ImageJ, we measured the percentage of the area occupied by fluorescent probes. As shown in Responded Figure 10a, there was no significant difference in signal intensity between the two groups, suggesting that light scatter did not result in notable signal loss at this sample thickness.

Additionally, in our experiments shown in Fig. 1g, h, we used DAPI staining simultaneously. As indicated in Responded Figure 10b, the fluorescence signal of DAPI did not show a significant decrease with increasing thickness, further supporting that light scatter did not substantially affect the fluorescence signal in this model.



Responded Figure 10 a Percentage area occupied by Cy5, Cy3, and FAM at depths of 0~6 μm and 42~48 μm. **b** Percentage area occupied by DAPI from Figures 1g and 1h. Anova test, $^{ns}P > 0.05$, $^{**}P < 0.01$

- Line 169: replace the word "significantly" since no statistical analysis is shown.

Authors' response: Thank you for pointing this out; we have replaced the word "significantly" with "relatively" to reflect that no statistical analysis was shown.

- Figure 2a: Why the genus Prevotella is reported twice? What is the meaning of the square brackets?

Authors' response: The distinction between [Prevotella] and Prevotella is that the name in square brackets indicates that the taxonomic unit is either unconfirmed or contentious. In the analysis at the genus level, names enclosed in square brackets may be used to substitute for strains that could not be accurately identified at the genus level. For example, [Prevotella] refers to unidentified strains within the community that are possibly associated with the genus Prevotella.

We hope this clarifies the reason for the duplicate reporting and the meaning of the square brackets. Thank you for bringing this to our attention.

- Line 180: "health" should be "healthy".

Authors' response: We have corrected "health" to "healthy" throughout the manuscript to ensure proper usage and improve the readability of the text. And we have asked native English-speaking authors to have the whole manuscript carefully read and edited.

- Figure 3c: moving the graph legend next to figure 3c would help make it more visible for a faster understanding of the data.
Authors' response: We have moved the graph legend next to Figure 3c to enhance visibility and facilitate quicker data interpretation.

- Line 204: "As observed previously, the bacterial population and community diversity on the day 2 were very different from the original (day 0), as the bacteria needed time to adapt to their new environment." the authors refer to data not shown. Why were these data not shown in figure 2?
Authors' response: Thank you for your question. The data referenced in line 204 is derived from our previous research. During the revision process, we removed this portion of the data to present our findings more clearly and logically, but we overlooked the mention in line 204. We apologize for any confusion this may have caused. We have now added this data back into the manuscript to ensure it is clearly presented alongside the other findings (Responded Fig. 5a – new Fig. 2a in manuscript).
Thank you for your understanding and for bringing this to our attention.

- Line 256: Before the period the authors should refer Figure 4a.
Authors' response: We have revised the sentence to include a reference to Figure 4a before the period, ensuring the reader can easily connect the statement with the corresponding data.

- Line 263: please explain in the text the experimental setup of Figure 4d.
Authors' response: We have revised the manuscript to provide a clearer explanation of the experimental setup shown in Figure 4 and correspondingly revised Figure 4a to offer a more intuitive representation of the setup.

- Line 273: "verity" should perhaps be "variety"?
Authors' response: We agree that "verity" was incorrect and should indeed be "variety" (line 298). We have revised the manuscript to correct this error, ensuring that the intended meaning is clearly conveyed. Your attention to detail is much appreciated, and we have carefully reviewed the manuscript to eliminate similar errors.

- Line 293: "which was relatively variation" please correct.
Authors' response: We have corrected the phrase to "which showed relatively more variation" in the revised manuscript. (line 321)
line 321 "*which showed relatively more variation*".

- Line 305: was the olive oil composition defined? Olive oil contains over six different kind of fatty acids, including linoleic acid, and other different lipids. It does not seem a valid option to demonstrate "an excessive increase in specific sebum components".
Authors' response: Thank you for your insightful comment. We have removed the term "specific" from the text (Line 335). Triglycerides are important component of sebum, and we chose olive oil to represent the excessive increase in triglycerides

because it contains more than 98% triglycerides[10], which are a group of glycerol esters with varying fatty acids commonly used in artificial sebum preparations[11]. However, we recognize that this choice may not adequately illustrate a particular component of sebum. We have made the necessary revisions in the manuscript to clarify this point.

- Line 350: Reference 31 is the same as reference 1.
Authors' response: We have corrected the citation in the manuscript.

- Reference 33 is missing authors. Please revise the whole references list and use a cohesive style.
Authors' response: We have corrected the citation in the manuscript.

- Line 358: Reference 34 is the same as reference 2.
Authors' response: We have corrected the citation in the manuscript.

- Line 371-371: "The hydrogels replicate the intricate topography marked by skin depressions and pockets." No evidence of this is provided. What is shown is that the scaffold is porous, unlike the human skin.
Authors' response: Thank you for pointing out this issue. We have revised the statement in the manuscript to more accurately reflect the intended meaning. As Responded Fig. 7a illustrated, from a macroscopic perspective, the skin is a complex terrain with numerous invaginations, pockets, and niches. In response to your feedback, we have adjusted the text to emphasize that our model can macroscopically simulate the complex topography of skin surface depressions and indentations. (Lines 400-401)
Lines 400-401 "*This hydrogel can macroscopically simulate the complex topography of skin surface depressions and indentations.*"

- Line 452: please provide the source for HaCaT and sebaceous cells.
Authors' response: We have included this information in the revised manuscript for clarity. (Lines 530-532)

- Including the manufacturer and catalog number of the reagents employed would increase reproducibility.
Authors' response: We have updated the methods section of the manuscript to include the manufacturer and catalog numbers for all key reagents.

- Line 75 HaCaT or sebaceous cells
Authors' response: We have revised that sentence accordingly. (Line 75)
Line 75 "*Unlike living skin models, our model consists solely of a composition of photocuring crosslinked hydrogels (glycidyl methacrylate hyaluronic acid, GMHA) used as the scaffold, along with nonviable HaCaT and sebaceous cells serving as nutrients.*"

- Line 82 rather than

Authors' response: The sentence has been revised for clarity. (Line 82)

Lines 81-83 *"In this study, we sought to establish a reproducible and cost-effective in vitro skin microbiome model focusing on skin microbiology-related assays, rather than on evaluating the microbiota-skin interactions."*

- Line 87 remove well

Authors' response: The word "well" has been removed from the text. (Line 87)

- Line 120 morphological

Authors' response: We have revised that sentence accordingly.

Lines 118-120 "*The hydrogel exhibits porous properties and a morphological that mimics the invaginations and depressions found in skin (Fig. 1b), which are important for creating niche-specific microenvironments*[23,24]."

- Legend of Figure 1 h is missing

Authors' response: We have ensured that the legend for Figure 1h has been added to the manuscript for clarity.

- Line 307 "oily" to oils"

Authors' response: Thank you for pointing that out. We have corrected "oily" to "oils" to improve clarity. (Line 336)

References:

1. Masuda, Y. et al. Three-dimensional morphological characterization of the skin surface micro-topography using a skin replica and changes with age. *Skin research and technology: official journal of International Society for Bioengineering and the Skin (ISBS) [and] International Society for Digital Imaging of Skin (ISDIS) [and] International Society for Skin Imaging (ISSI)* **20**, 299-306, (2014).
2. Sakai, S. et al. Hyaluronan exists in the normal stratum corneum. *The Journal of investigative dermatology* **114**, 1184-1187, (2000).
3. Costello, E. K. et al. Bacterial Community Variation in Human Body Habitats Across Space and Time. *Science* **326**, 1694-1697, (2009).
4. Aranda-Díaz, A. et al. Establishment and characterization of stable, diverse, fecal-derived in vitro microbial communities that model the intestinal microbiota. *Cell host & microbe* **30**, 260-272, (2022).
5. Cheng, A. G. et al. Design, construction, and in vivo augmentation of a complex gut microbiome. *Cell* **185**, 3617-3636, (2022).
6. Desai, M. S. et al. A Dietary Fiber-Deprived Gut Microbiota Degrades the Colonic Mucus Barrier and Enhances Pathogen Susceptibility. *Cell* **167**, 1339-1353, (2016).
7. Gan, B. et al. Dynamic Monitoring of Changes in Fecal Flora of Giant Pandas in Mice: Co-Occurrence Network Reconstruction. *Microbiology spectrum* **11**, e0199122, (2022).
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Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Authors' response: Thank you for your valuable feedback and for your involvement in the co-review process as part of the Nature Communications initiative. We appreciate the opportunity to contribute to the training of Early Career Researchers and recognize the importance of collaborative peer review in enhancing manuscript quality. Your input has been instrumental in helping us refine our work.

Point-to-point response to reviewers' comments

Reviewer #1 (Remarks to the Author):

The authors have presented an in-vitro scaffold for hosting skin microbes. The methodology used for this analysis is sound and presented in detail. The model will be very useful to both the academic and clinical community.
The authors have addressed the major issues that I had raised during the initial review. The revised manuscript provides sufficient data that supports the claims on the representativeness and stability of the in-vitro setup to mimic non-dry skin.
There are few grammatical issues and a figure legend that is incomplete, these should be addressed before publication.
I also recommend that the title be made more specific "Mimic the skin microecology- a novel in vitro model replicates the microbiome world", the study only looked into the skin microbiome and it is not representative of the microbiome world.
I recommend that the paper be published subject to these minor corrections.
Authors' response: Thank you for your thoughtful feedback. We have greatly appreciated your positive evaluation of our methodology and the utility of the in-vitro model for the academic and clinical communities.

We have promptly made minor revisions to address some grammatical issues and the figure legend based on your suggestions, ensuring clarity and completeness. (line 1, line 146, line 208, line 347, line 368, lines 375-376, line 575, line 633, line 657, line 658, line 722)

Additionally, we have acknowledged your suggestion regarding the title. We agree that the current title could be more specific. Therefore, we have revised the title to better reflect the scope of our study, focusing on the skin microbiome, as follows: "Mimic the skin microecology- a novel in vitro model replicates the human skin microbiome world"

Thank you again for your constructive feedback.

Reviewer #2 (Remarks to the Author):

The authors did an excellent job addressing my concerns.
Authors' response: Thank you for your kind feedback. We are glad to hear that the revisions we made have successfully addressed your concerns. We appreciate your thoughtful review and the opportunity to improve the manuscript.

Reviewer #3 (Remarks to the Author):

The authors addressed the majority of my comments satisfactorily. Below are my remaining concerns:
Major:
To my comment "It would be interesting to know what design parameters positively/negatively affect the microbiome culture…..", the authors responded with adding discussions, and suggesting future work. However, I think it is critical to test at

least several conditions (not a full optimization), such as different pH and stiffness to fully appreciate the superiority of the model and get an insight into its mechanism. Without such comparison and mechanistic data, it is difficult to trust the robustness of the model and data overall.

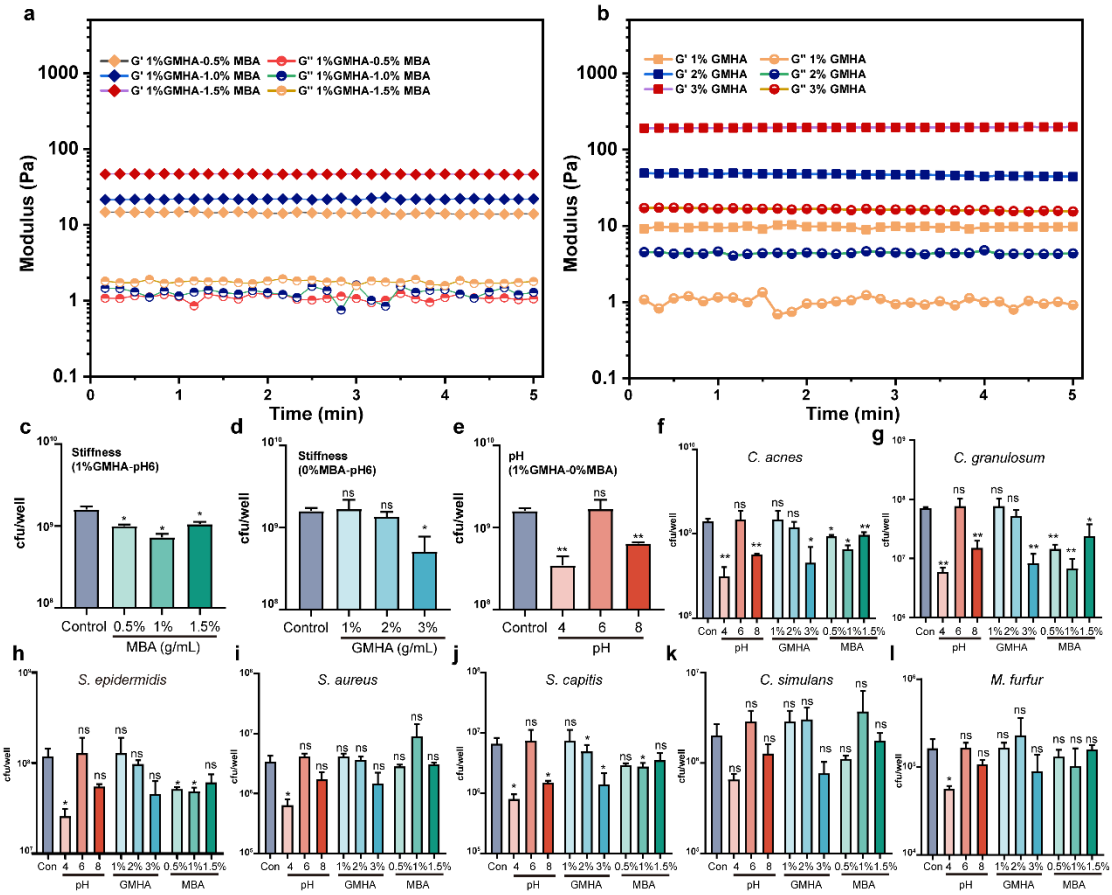
Authors' response:

Thank you for your valuable feedback and for pointing out the importance of testing additional conditions to further validate the model. In response, we have conducted additional experiments to explore the impact of design parameters, such as stiffness and pH, on the microbiome culture. The conserved human skin microbiota (Hcm) was utilized in this study. The microbiota was assessed using the PMA-qPCR method. Specifically, we measured the G' and G" to evaluate the stiffness of the model by adding the crosslinking agent N, N'-methylene diacrylamide (MBA) and increasing the GMHA gel concentration as shown in Responded Figure 1 a and b. Moreover, we found that increasing MBA had a significant impact on the microbiome (Responded Figure 1c), while increasing the gel concentration (from 1% to 2%) did not have a significant impact on the microbial community so far compared with the pre-culture microbiota (Responded Figure 1d). Only when the concentration reached 3% did we see a notable change in the microbiome.

In terms of pH, we built the model with buffer solutions (pH 4, pH 6 and pH 8). We found that both excessively acidic and alkaline environments caused a significant impact on the microbial community (Responded Figure 1e). These findings suggest that the model is sensitive to both gel stiffness and pH conditions, which could significantly impact its performance. We have added these findings to the manuscript (lines 452-456), highlighting that extreme pH values and increased cross-linking with MBA significantly affect bacterial colonization, while increasing gel concentration is an effective way to change the gel physical properties and influence the bacterial colonization.

We hope this additional data addresses your concerns and strengthens the overall validity of the model.

Lines 452-456: We have found that extreme pH conditions and varying the amount of the cross-linking agent MBA can significantly affect bacterial colonization. Additionally, increasing the concentration of GMHA enhances gel stiffness, which can influence bacterial colonization. (Supplementary Fig. 7).



Responded Figure 1 (new Supplementary Fig. 7 in manuscript) Influence of gel characteristics on microbiota in the SCmic model. **a** Energy storage modulus (G') and loss modulus (G") of hydrogels with varying N, N'-methylene diacrylamide (MBA) crosslinker concentrations. **b** Energy storage modulus (G') and loss modulus (G") of hydrogels with varying GMHA concentrations. **c** Effect of gel stiffness on total microbiota counts with 1% GMHA and varying MBA concentrations from 05-1.5%. d Effect of gel stiffness on total microbiota counts with varying GMHA concentrations from 1-3%. **e** Effect of gel on total microbiota under different pH conditions. **f-l** Effect of different pH values and gel stiffness on seven bacterial species in the microbiota: *C. acnes*, *C. granulosum*, *S. epidermidis*, *S. aureus*, *S. capitis*, *C. simulans*, and *M. furfur*. T- test, $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs control, n=3 per group. Control: Hcm microbiota pre-culture. All experimental groups: Hcm microbiota were cultured on the model for 3 days at 32°C and 75%RH.

Minor:

Missing a word after "morphological": The hydrogel exhibits porous properties and a morphological that mimics the invaginations and depressions found in the skin (Fig. 1b), which are important for creating niche-specific microenvironments." (lines 118120)

Authors' response:

Thank you for pointing that out. The correct wording should be: "The hydrogel exhibits porous properties and a morphological structure that mimics the invaginations and

depressions found in skin (Fig. 1b), which are important for creating niche-specific microenvironments." We have updated this in the manuscript accordingly

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Authors' response: Thank you again for your involvement in the co-review process as part of the Nature Communications initiative. Your input has been instrumental in helping us refine our work, and we greatly appreciate your valuable feedback.

In this manuscript, Wang *et al.* describe the development of a highly physiologically relevant *in vitro* skin tissue model that successfully supports the culture of diverse skin microbiota. This complex and innovative approach has the potential to transform the study of host-microbe interactions in the skin. This reviewer is excited about the potential of this work. However, I believe there are some major issues left to be addressed to convincingly demonstrate the utility and potential of the engineered model.

- A major concern for me is the lack of description of a formal ethics committee approval to collect human donor samples and conduct these experiments. The authors only describe the Declaration of Helsinki but do not describe going through an institutional review process.

- Generally speaking, throughout the paper, the authors emphasize multiple times that their model is successful at supporting the culture of diverse bacteria over time. However, their data repeatedly shows that their model is much better at supporting the culture of anaerobic than aerobic bacteria. I think this is an incredibly interesting finding that could address a major limitation of most existing models. I encourage the authors to discuss in more detail why they think this is the case and to pay attention to the comparison of aerobes versus anaerobes across all experiments. There might be interesting underlying biology there.
    - Given the noted differences in stability and abundance between anaerobes and aerobes in this model, it is surprising that the authors do not describe the exact composition of the air in which the models were cultured. I know it is 75% relative humidity but what about %CO_2 and % O_2? Both of these gases could have a significant impact on the composition of the microbiota.

- **Figure 1** – This approach is quite innovative and intriguing, particularly due to the use of dead cells that mimic the epidermis. I have a few questions on the representative schematic for the process of generating the model in Figure 1a that were not answered in the methods section:
    - The schematic implies that the HaCaT and sebaceous cells are added in layers to the model. However, the methods do not seem to imply the same. Could the authors please clarify this in both the figure and methods section?
    - The authors also should specify the ratio of HaCaT:sebaceous cells employed in the model.
    - Similarly, the right hand side of the figure depicts skin strains layered on the model. The figure seems to imply that certain types of bacteria aggregate to a particular part of the model. If that is the intended effect, please add legends to the bacteria so the reader can understand your interpretation of the data.
    - **Figure 1e –** no legend present describing the changes in color presented in the figure
    - **Figure 1g –** I found it really difficult to truly understand what was happening in this figure. It was challenging to distinguish between orange and red-labeled bacteria. Also, is it possible to quantify the penetration of these bacteria along the thickness

of the model in any way? As is, it is almost impossible for the reader to independently evaluate the images and thus, we are left to rely on the authors' interpretation of the data.

- **Figure 2 –** I agree that the data nicely highlights that the model can maintain ecological stability. Nonetheless, a major hole in the presented data is the lack of information of the inoculum used for these experiments. What did the community look like prior to inoculation at day 0? Without this information, it is not possible to determine what bacteria colonized the model.
    - Figure 2b – this graph does not have any meaningful biological meaning in my opinion. It is not particularly informative to now the number of taxa and plot them across all taxonomic levels. I recommend removing it

- There are several recurring issues related to the analysis and depiction of sequencing data:
    - In line 182, the authors report that PMA-Illumina sequencing was used to evaluate the microbiome. Yet, they do not specify whether 16S rRNA or shotgun sequencing was used to profile the microbiome. This needs to be specified here in addition to in the results section.
    - Insufficient details are provided in the results section to describe the analysis of the obtained 16S rRNA data. For example, no description of the reference database used for taxonomic profiling is included. Additionally, there is no description of quality control or data processing. Similarly, there is no describtion of the NMDS analysis used in Figure 3. Thus, it is difficult to determine whether the data was analyzed correctly.
    - The analysis of beta diversity throughout the manuscript was confusing – the methods section describes using Unweighted Unifrac distances, yet several figures report using Bray Curtis distances instead – which one is it? This should probably be consistent throughout the paper or both distances should be reported leveraging supplementary figures.
    - I understand the authors chose to exclusively plot bar graphs of distances between groups to highlight similarities (or lack thereof) between experimental groups. Standard practice in the microbiome field is to also include PCoA plots of the beta diversity matrix. This would allow the reader to visually understand the data better and observe any clustering happening between samples of the same group and across different groups.

- **Figure 3 –** The comparison across different skin types is an excellent proof of concept on the utility of this model. However, several points need to be addressed:
    - It was difficult for me to identify exactly how many individuals were recruited to represent each condition. It seems to me it was only 1 individual per group. If so, that would temper my excitement, as it is difficult to make broad conclusions about groups of individuals with only 1 biological representative sample per group.

    - Figure 3a – in the schematic for this figure, it suggest that multiple samples were obtained from the donors every 24 hours. I did not find a description of this process in the methods. Please clarify what is meant here.
    - The results of the re-inoculation process are quite intriguing! I recommend the authors expand on why they think reinoculation leads to such a stark recovery in the discussion.
    - Figures 3g-h – the results from the dry skin donor are missing from these graphs. I urge the authors to include these data, as it is clear it was processed and obtained in the process of generating the rest of this Figure.

- **Figure 4** – the labels used for the conditions and experimental groups used in this study are quite confusing. Is there a different way the authors could label and plot the data? Perhaps by using grouped columns?
    - Figure 4e: I am not sure I understand what the authors are trying to showcase with this panel.

- **Figure 4 and 5g**: I do not understand the choice to represent relative abundance data in this way. It is almost impossible for the reader to parse through all these points. Standard practice in the field is to use solid bar graphs that add up to 100%

- **Figure 5 –** Why did the authors decide to combine the 7 bacterial species in those exact proportions? Also, could you use RT-PCR rather than 16S sequencing/relative abundance to determine absolute numbers of each bacteria and thus get a better idea of the stability of each of these species over time?

- **Figure 6 –** Quite interesting application of the model. However, I found the description of these results a little rushed and confusing. For example, I completely missed the niacinamide results after my first read of the paper.

- There seem to be references missing in the discussion. Several statements are given as facts without citations. See, for example Lines 385 – 390.

- For me, personally, the introduction described the results in too much detail, interrupting the flow of the paper. Similarly, I found it odd that random Figure panels were referenced here.

- The authors employ the terms “microbiome” and “microbiota” interchangeably. I believe the authors usually mean “microbiota” as they are growing bacterial communities in this model. I recommend going through the manuscript and ensuring the terms are used properly.

- The authors should consider going back through the manuscript and proofreading it to make sure all sentences are complete and there are no typos. Here is a small (not comprehensive) subset of issues I noticed:
    - Line 38-40: this is not a complete sentence.
    - Lines 81-83: this is not a complete sentence.
    - Lines 166-168: this is not a complete sentence.
    - Lines: 251-253: incorrect grammar – difficult to establish what the sentence means to say.
    - Lines 504-506: mixed use of past and present tense.

- Line 152: what is meant by co-growth of bacterial habitats?