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Effect of plant extract spray on axillary microbiome using 3D skin cell model

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1. Introduction

Axillary odor, a common and often socially distressing condition, arises primarily from the microbial biotransformation of odorless secretions into volatile, malodorous compounds [1-2]. These secretions originate from the eccrine, apocrine, and sebaceous glands in the axillary region, where a diverse and stable microbiota thrives [3]. The resident microbial community, dominated by Gram-positive bacteria such as *Staphylococcus*, *Micrococcus*, *Corynebacterium*, and *Propionibacterium*, plays a central role in odor production. Among these, *Corynebacterium* species are particularly implicated as primary causal agents of axillary malodor, as they metabolize substrates derived from apocrine gland secretions into volatile compounds.

The chemical composition of axillary odor is complex and includes several classes of molecules, such as short- and medium-chain volatile fatty acids (e.g., isovaleric acid), thioalcohols (e.g., 3-methyl-3-sulfanylhexan-1-ol), and 16-androstene steroids. These compounds are generated through the enzymatic activity of bacteria, particularly those capable of hydrolyzing odorless precursors like (N-α-3-hydroxy-3-methylhexanoyl-L)-glutamic acid and (S-[1-(2-hydroxyethyl) -1-methylbutyl]-L)-cysteinylglycine. For example, thioalcohols are produced through the action of bacterial cysteine-thiol lyases, while fatty acids result from the breakdown of triglycerides by lipases and subsequent fermentation processes.

Recent studies have highlighted the importance of microbial community structure in determining odor intensity. Quantitative bacteriological surveys of axillary flora reveal significant differences in bacterial composition between individuals with pungent versus acid odor profiles. Notably, higher bacterial loads are associated with stronger malodor, particularly when *Corynebacterium* and *Propionibacterium* species dominate the microbiota. Gender-specific differences in axillary microbiota have also been observed, with *Propionibacterium* species being more prevalent in males, potentially contributing to gender-related variations in odor intensity [4-5].

3D skin cell models are advanced in vitro systems designed to mimic the structure and function of human skin. These models typically consist of multiple layers, including the epidermis and dermis, and are constructed using primary human keratinocytes, fibroblasts, and other relevant cell types. Unlike traditional 2D cell cultures, 3D models replicate the skin's

natural architecture, enabling more accurate studies of skin physiology, barrier function, and responses to external stimuli.

In cosmetic research, 3D skin models are used for safety assessment, efficacy testing, and mechanism-of-action studies [6]. They allow researchers to evaluate the permeability, irritation potential, and toxicity of cosmetic ingredients, providing insights into how products interact with skin cells. Additionally, these models are valuable for studying anti-aging effects, wound healing, and skin barrier repair [7-8]. By reducing reliance on animal testing, 3D skin models offer an ethical and efficient alternative for advancing cosmetic formulations. Their ability to simulate real-world skin conditions makes them indispensable tools for modern cosmetic research and development [9].

Various plant extracts have shown potential in modulating skin microbiota by inhibiting pathogenic bacteria and promoting beneficial microbial balance. *Ocimum gratissimum* leaf extract exhibits antimicrobial activity against pathogens like *Staphylococcus aureus* and *Escherichia coli*, which can disrupt skin microbiota balance. *Curcuma longa* rhizome extract, rich in curcumin, has anti-inflammatory and antimicrobial properties, which may help reduce skin infections and promote a balanced microbiota. *Tinospora cordifolia* root/stem extract demonstrates broad-spectrum antimicrobial activity against bacteria such as *Staphylococcus* and *Klebsiella pneumoniae*, potentially aiding in maintaining skin microbial homeostasis. *Piper umbellatum* and *Lavandula stoechas* extracts also show antimicrobial and anti-inflammatory effects, which may indirectly support skin microbiota health.

To address axillary odor, strategies focusing on modulating the microbiota have gained attention. Plant extract sprays, enriched with antimicrobial compounds such as phenolic acids, flavonoids, and essential oils, have shown promise in suppressing odor-causing bacteria while promoting the growth of beneficial or neutral microorganisms [10-12]. These extracts often target specific bacterial enzymes involved in odor production, such as lipases and thiol lyases, thereby reducing the formation of volatile compounds. Additionally, their antimicrobial activity helps restore microbial balance, creating a more favorable environment for non-odorous species.

2. Materials and Methods

Botanical extracts spray rich in *Ocimum gratissimum*, *Curcuma longa*, *Tinospora cordifolia*, *Piper umbellatum*, and *Lavandula stoechas* were utilized in four 3D skin models that mimic the axillary microbiome, including typical bacteria and eukarya such as *Propionibacterium acnes*, *Staphylococcus aureus*, *Corynebacterium tuberculostearicum*, *Staphylococcus hominis*, *Staphylococcus epidermidis*, *Malassezia restricta*, and *Staphylococcus haemolyticus*. These models were initiated with a bacterial colony count of 10^6 to 10^8 CFU/cm^2 and maintained at a temperature of 37±0.5°C. The plant extract spray was administered three times daily for a period of 14 days. The microbial colonies were quantified using a 16S rRNA gene sequencing method on the 7th and 14th days. The axillary odor was evaluated by a trained inspector, who conducted the assessment with their vision obscured by a cloth, assigning an odor score ranging from 1 to 5. The skin's pH levels were monitored using a skin pH meter.

3D skin model construction

Cells separation: Primary human keratinocytes were isolated from axillary skin samples obtained from healthy donors following ethical approval. Tissues were minced and digested with 0.25% trypsin for 15 min at 37°C. Keratinocytes were then separated by centrifugation and cultured in Keratinocyte-SFM medium supplemented with 0.05 ng/mL human recombinant epidermal growth factor (hEGF) and 50 µg/mL bovine pituitary extract. Human dermal fibroblasts were isolated from the same skin samples using collagenase type I (1 mg/mL,

Sigma-Aldrich) digestion for 1 h at 37°C. Fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 10% fetal bovine serum and 1% penicillin-streptomycin (P/S). Apocrine and eccrine gland cells were isolated from axillary skin using laser capture microdissection (Leica Microsystems) and cultured in specific media as previously described.

Scaffold fabrication: A biomimetic scaffold was constructed using a mixture of collagen type I (3 mg/mL) and fibrinogen (1 mg/mL) in a 3:1 ratio. The scaffold was polymerized by adding thrombin (10 U/mL, Sigma-Aldrich) and allowed to solidify at 37°C for 30 min. Fibroblasts (1 × 10^6 cells/mL) were embedded within the collagen-fibrinogen scaffold to form the dermal layer. Keratinocytes (5 × 10^5 cells/mL) were seeded onto the surface of the dermal scaffold and cultured at an air-liquid interface for 14 days to promote stratification and differentiation. Apocrine and eccrine gland cells (2 × 10^5 cells/mL) were embedded within specific regions of the dermal layer to simulate glandular structures.

Microbiota Inoculation: Axillary bacteria and eukarya, including *Propionibacterium acnes*, *Staphylococcus aureus*, *Corynebacterium tuberculostearicum*, *Staphylococcus hominis*, *Staphylococcus epidermidis*, *Malassezia restricta*, and *Staphylococcus haemolyticu*s., were cultured on selective media. Bacterial and eukarya suspensions (1 × 10^8 CFU/mL) were inoculated onto the surface of the 3D model. Amino acids (L-leucine, 1 mM), fatty acids (isovaleric acid, 0.5 mM), and cysteine conjugates (S-hydroxyalkyl-L-cysteine, 0.1 mM) were added to the culture medium to simulate the biochemical environment of the axilla.

Culture Conditions: The 3D model was cultured in a dynamic system with an air-liquid interface (ALI) to mimic natural skin physiology. The medium was changed every 2 days. The culture medium consisted of a 1:1 mixture of Keratinocyte-SFM and DMEM, supplemented with 2% FBS, 0.05 ng/mL hEGF, 50 µg/mL BPE, and 1% P/S.

Botanical extracts spray with *Ocimum gratissimum* leaf extract 0.5%, *Curcuma longa* rhizome extract 0.5%, *Tinospora cordifolia* root/stem extract 0.4%, *Piper umbellatum* root/stem/leaf extract 0.3%, and *Lavandula stoechas* extract 0.45%. The spray contains other moisturizers, skin conditioners, and pH buffers, and does not contain any antibiotics or preservatives.

The spray were utilized in four 3D skin models that mimic the axillary microbiome, including typical bacteria and eukarya such as *Propionibacterium acnes*, *Staphylococcus aureus*, *Corynebacterium tuberculostearicum*, *Staphylococcus hominis*, *Staphylococcus epidermidis*, *Malassezia restricta*, and *Staphylococcus haemolyticu*s. These models were initiated with a bacterial colony count of 10^6 to 10^8 CFU/cm^2 and maintained at a temperature of 37±0.5°C. The plant extract spray was administered three times daily for a period of 14 days. The microbial colonies were quantified using a 16S rRNA gene sequencing method on the 7th and 14th days. The axillary odor was evaluated by a trained inspector, who conducted the assessment with their vision obscured by a cloth, assigning an odor score ranging from 1 to 5. The skin's pH levels were monitored using a skin pH meter.

Microbiota 16s RNA identification

Sample Preparation: A loopful of bacterial cells was suspended in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). The optical density at 600 nm was adjusted to 4.0, and 245 µL of the suspension was transferred to a sterile microfuge tube. Lysozyme (50 mg/mL, 5 µL) was added and incubated at 56°C for 45 min. Subsequent reagents added included TE (196.2 µL), DTT (1 M, 5 µL), EDTA (0.25 M, 20 µL), SDS (10% w/v, 25 µL), and Proteinase K (20 mg/mL, 3.8 µL). Samples were incubated at 37°C for 1 h. PrepMan Ultra solution (500 µL) was added, mixed by inversion, and incubated at 56°C for 30 min. After vortexed, samples were heated at 100°C for 8 min and centrifuged at 8500 g for 2 min. The supernatant was diluted 1:10 with

deionized water, and 10 µL was analyzed on a 1% agarose gel to confirm genomic DNA presence.

PCR Amplification: PCR was performed on diluted samples using the MicroSeq 500 16S rDNA Bacterial Sequencer Kit (ThermoFisher Scientific) following the manufacturer's protocol. The primer sequences for PCR were table 1. Reactions were run on ThermoFisher PCR System ABI9600. PCR products were verified on a 1% agarose gel for a 500-bp fragment and stored at -20°C if necessary.

Table 1 Primer sequences of microbe strain identification

Strain	Primer Name	Primer Sequence
Propionibacterium acnes	P.acnes-F	5'-AGAGTTTGATCCTGGCTCAG-3'
Propionibacterium acnes	P.acnes-R	5'-GGCTACCTTGTTACGACTT-3'
Staphylococcus aureus	SA-F	5'-TTGATCCTGGCTCAGATGC-3'
Staphylococcus aureus	SA-R	5'-TAAGGCGATGGTTGTGCG-3'
Corynebacterium tuberculostearicum	C.t-F	5'-AGAGTTTGATCCTGGCTCAG-3'
Corynebacterium tuberculostearicum	C.t-R	5'-GGCTACCTTGTTACGACTT-3'
Staphylococcus hominis	S.h-F	5'-AGAGTTTGATCCTGGCTCAG-3'
Staphylococcus hominis	S.h-R	5'-GGCTACCTTGTTACGACTT-3'
Staphylococcus epidermidis	S.e-F	5'-AGAGTTTGATCCTGGCTCAG-3'
Staphylococcus epidermidis	S.e-R	5'-GGCTACCTTGTTACGACTT-3'
Malassezia restricta	M.r-F	5'-AGAGTTTGATCCTGGCTCAG-3'
Malassezia restricta	M.r-R	5'-GGCTACCTTGTTACGACTT-3'
Staphylococcus haemolyticus	S.ha-F	5'-AGAGTTTGATCCTGGCTCAG-3'
Staphylococcus haemolyticus	S.ha-R	5'-GGCTACCTTGTTACGACTT-3'

Purification and Sequencing: PCR products were purified using Microcon-100 columns. Cycle sequencing reactions were prepared with purified PCR products, deionized water, and sequencing mixes. Extension products were purified using Centri-Sep spin columns and dried in a vacuum centrifuge. Electrophoretic sequencing was performed using an Applied Biosystems 3100 Genetic Analyzer with Big Dye Terminator Version 2 chemistry.

3. Results

After 7 days and 14 days, the logarithm value changes of 7 microorganisms were: *P. acnes* -0.35, -0.56, *S. aureus* -0.76, -1.40, *C. tuberculostearicum* -0.20, -0.33, *S. hominis* -0.08, +0.35, *S. epidermidis* +0.12,+0.27, *M. restricta* -0.33,-0.60, *S. haemolyticus* -0.17, -0.51. Five microbes decreased and two microbes increased (Table 2).

Table 2. Seven micro-organisms quantity change after use plant extract spray

Microbe	Logarithm value of microorganism quantity		
	0 day	7 days	14 days
Propionibacterium acnes	5.33	4.98	4.77
Staphylococcus aureus	5.26	4.50	3.86
Corynebacterium tuberculostearicum	5.48	5.28	5.15
Staphylococcus hominis	5.30	5.22	5.65

Staphylococcus epidermidis	5.11	5.23	5.38
Malassezia restricta	5.20	4.87	4.60
Staphylococcus haemolyticus	4.96	4.79	4.45

Skin model surface pH was tested, the pH rise from 5.30 to 5.66 and 6.15 on week 1 and week 2, added 0.36 and 0.85 separately (Figure 1).

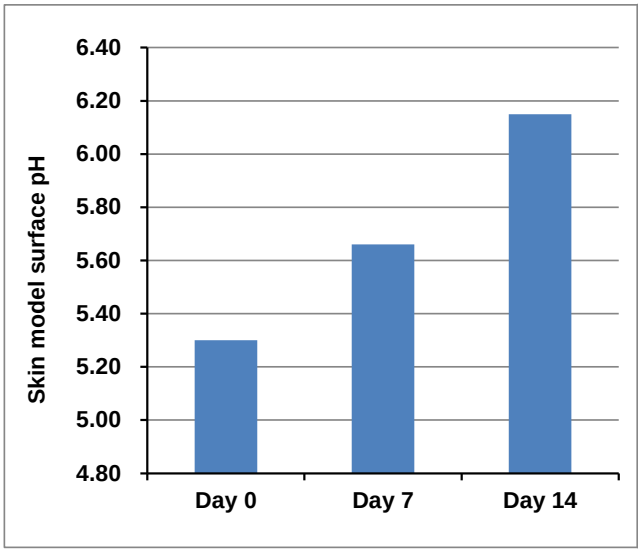

Figure 1. Skin model surface pH after 7 and 14 days

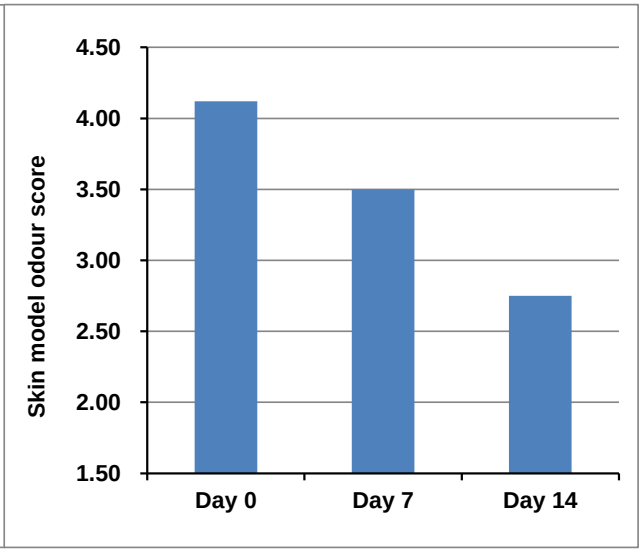

Figure 2. Skin model odor score after 7 and 14 days

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4. Discussion

Plant extracts like *Ocimum gratissimum* leaf extract, *Curcuma longa* rhizome extract, *Tinospora cordifolia* root/stem extract, *Piper umbellatum* root/stem/leaf extract, and *Lavandula stoechas* extract have shown potential in modulating skin microbiota, including the regulation of specific bacterial strains such as *P. acnes*, *S. aureus, C. tuberculostearicum*, *S. hominis*, *S. epidermidis*, *M. restricta*, and *S. haemolyticus*.

The plant extracts used in this study have ingredients such as curcumin,terpene, flavonoids, lead the over expression of the anti-oxidant enzymes, enhance the production of metabolites that are beneficial for skin health, such as short-chain fatty acids (SCFAs) and vitamins. They have antimicrobial activity, could help in maintaining a healthy skin microbiota by inhibiting the growth of harmful bacteria like *P. acnes* and promoting the growth of beneficial bacteria. They also have anti-inflammatory and antioxidant effects to balance the immune system.

The observed changes in the number of specific bacterial strains, such as a decrease in *P. acnes* (-0.56), *S. aureus* (-1.40), *C. tuberculostearicum* (-0.33), *M. restricta* (-0.60), and *S. haemolyticus* (-0.51), and an increase in *S. hominis* (+0.35) and *S. epidermidis* (+0.27), may be attributed to the combined effects of these plant extracts. The antimicrobial properties of the extracts can directly inhibit the growth of harmful bacteria, while their anti-inflammatory and antioxidant effects can create a more favorable environment for beneficial bacteria. Additionally, the modulation of the skin's immune response and the promotion of a balanced microbiota can further contribute to the observed changes in bacterial populations. However,

further research is needed to fully understand the specific mechanisms and interactions between these plant extracts and the skin microbiota.

The changes in the number of specific bacterial strains in the armpit can directly influence the intensity and type of body odor. For example, a decrease in *Corynebacterium* and *Anaerococcus* populations has been shown to reduce body odor, as these bacteria are known to produce volatile fatty acids and other odor-causing compounds. Conversely, an increase in the population of certain bacteria, such as *Staphylococcus hominis* and *Staphylococcus epidermidis*, may not necessarily increase odor intensity, as their metabolic byproducts may not be as pungent or may be less involved in odor production.

The balance of the armpit microbiota is crucial for maintaining a healthy skin environment and minimizing body odor. When the microbiota is disrupted, it can lead to an overgrowth of odor-causing bacteria, resulting in increased body odor. Therefore, maintaining a balanced microbiota through proper hygiene and the use of products that promote beneficial bacteria can help control body odor.

In this study, the skin model surface pH rise accompanied by a decrease in odor. This is due to the decrease in the concentration of volatile acidic substances, especially the decrease of thiol components, which leads to a change in pH and affects the concentration of odor.

5. Conclusion

The armpit is characterized by numerous folds, creating an environment conducive to the proliferation of a diverse array of microorganisms, further enhanced by the enclosed and humid conditions fostered by the presence of armpit hair. In this study, we employed a 3D skin model inoculated with a spectrum of typical axillary microbes to assess the impact of plant extract spray on the microbiome. Notably, the population of *Staphylococcus aureus*, a primary contributor to odor, was observed to decrease significantly, whereas the counts of *Staphylococcus hominis* and *Staphylococcus epidermidis* increased. The plant extract spray demonstrated the capacity to suppress harmful bacteria like *Staphylococcus aureus*, without adversely affecting beneficial bacteria. Consequently, the reduction in *Staphylococcus aureus* levels presents an opportunity for the expansion of *Staphylococcus hominis* and *Staphylococcus epidermidis*. The reduction in odor scores, as compared to the initial testing phase, is a clear indication of an enhanced axillary microbiome.

In conclusion, axillary odor is a multifaceted issue driven by the interplay between microbial metabolism and chemical transformations. Understanding the specific roles of bacterial species and their metabolic pathways provides a foundation for developing targeted interventions, such as plant-based antimicrobial agents, to effectively manage this common condition. Further research into the ecological dynamics of axillary microbiota and the precise mechanisms of odor formation may lead to more personalized and sustainable solutions.

6. References

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