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Characterization of the Pilosebaceous Microbiota and Biofilm-Forming Capacity of Bacteria Isolated from Healthy Individuals

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ABSTRACT

Background and Aim: The microbial flora colonizing the pilosebaceous unit plays a crucial role in maintaining skin health and homeostasis. This study aimed to isolate, identify, and evaluate the biofilm-forming capacity of bacterial strains derived from the pilosebaceous follicles of healthy human facial skin.

Materials and Methods: Facial sebum samples were collected from 15 healthy volunteers. Bacterial strains were isolated and cultured under anaerobic conditions. Species identification was performed using 16S rRNA gene sequencing. The biofilm-forming ability of the isolates was quantified using the crystal violet staining assay.

Results: A total of 22 bacterial strains were isolated, predominantly Gram-positive, nonmotile, and morphologically characterized as rod-shaped (20/22) or coccoid (2/22). Colonies appeared opaque and white, with diameters ranging from 0.5 to 1.5 mm. Biochemical profiling showed that most isolates were catalase positive (18/22) and gelatinase positive (20/22), while the majority were oxidase negative (18/22). Three isolates exhibited lipase activity, and eight demonstrated hemolysin production. Based on 16S rRNA gene sequencing, *Cutibacterium* was identified as the dominant genus, with *C. acnes* accounting for 68% of the isolates. Biofilm assays revealed variability among *C. acnes* strains, with isolates Hn15-2, Hn4, and Hn13 displaying strong biofilm-forming capacity.

Conclusion: Healthy human facial skin harbors a diverse bacterial community, predominantly composed of *Cutibacterium acnes*. Notably, several *C. acnes* isolates demonstrated substantial biofilm-forming ability, suggesting potential implications for skin microbiome stability and pathogenicity.

Keywords: *Acne vulgaris*, Biofilm, *Cutibacterium acnes*, Facial Skin Microbiota

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1. Introduction

The skin serves as both a physical barrier and a habitat for a diverse microbial ecosystem **(1)**. Variations in skin structure and physiology generate distinct microenvironments that influence microbial colonization. Regional differences in temperature, humidity, sebaceous gland density, and pH create unique ecological niches across the body **(1, 2)**.

Microbial colonization begins at birth, with the initial composition influenced by the mode of delivery. Over time, the skin microbiota is shaped by intrinsic factors (such as body site, interindividual and intraindividual variation, ethnicity, sex, and age) and extrinsic factors (including lifestyle, hygiene practices, cosmetic use, antibiotic exposure, geography, climate, and seasonality) **(3, 4)**. The skin microbiota interacts dynamically both within its microbial communities and with the host. Therefore, understanding and maintaining the delicate equilibrium between the skin and its microbiota are essential for elucidating mechanisms that sustain cutaneous health, where microorganisms play an active and functional role **(5, 6)**.

The skin microbiome is dominated by four major bacterial phyla: *Actinobacteria*, *Firmicutes*, *Proteobacteria*, and *Bacteroidete* **(7)**. Dry, salty, and slightly acidic regions (e.g., forearms, hands, and feet) support only extremotolerant species, whereas sebum-rich areas such as the face, back, and chest, particularly the pilosebaceous follicles, provide a lipid-rich, anaerobic environment that favors the growth of lipophilic bacteria such as *Cutibacterium acnes* **(4, 8)**.

Among these microorganisms, *C. acnes* is a commensal bacterium essential for maintaining skin homeostasis; it regulates lipid metabolism and inhibits opportunistic pathogens through nutrient competition and antimicrobial production **(9)**. However, dysbiosis or immune dysregulation can convert this commensal into an opportunistic pathogen. Notably, *C. acnes* forms biofilms that enhance persistence by evading host immune responses and reducing antibiotic susceptibility **(10-12)**.

Antimicrobial resistance further complicates management of *C. acnes*–associated infections. Skadins et al **(13)** in 2021 reported concerning resistance rates among *C. acnes* isolates from acne patients, 21.4% to clindamycin and 28.6% to erythromycin. Most existing studies focus on *C. acnes* isolated from acne lesions, with limited attention to its biological characteristics within healthy skin microbiota. Characterizing the microbial composition and biofilm potential of bacteria inhabiting nonlesional, healthy skin is therefore essential for understanding commensal equilibrium and evaluating potential reservoirs of resistance.

In this study, we isolated and identified bacterial species from the pilosebaceous follicles of healthy human facial skin, emphasizing their genetic diversity and biofilm-forming capacity. The findings aim to contribute to a better understanding of the physiological role of commensal *C. acnes* and to support strategies that preserve the stability of the cutaneous microbiome.

2. Materials and Methods

2.1 Sample Collection

2.1.1 Study Population

A total of 15 healthy volunteers with clinically normal facial skin were enrolled and provided facial sebum samples.

2.1.2 Inclusion Criteria

Participants were healthy adults whose skin condition was verified according to the diagnostic standards of *American Family Physician* and DermNet New Zealand **(14)**. At the time of sampling, all participants met the following criteria: 1) no current or recent antibiotic use (within the past three months) and 2) no history of psychiatric or behavioral disorders **(15)**.

2.1.3 Exclusion Criteria

Volunteers were excluded if they 1) had systemic medical conditions such as hepatic insufficiency, renal failure, or diabetes mellitus; 2) were receiving immunosuppressive therapy; or 3) declined to participate.

2.1.4 Sampling Approach

A nonprobability convenience sampling method was applied because recruitment was restricted to healthy volunteers within a defined geographic area. Although this approach facilitated practical sampling and baseline microbiota characterization, it may limit generalizability to other populations or age groups. The sample size (n = 15) was determined to yield at least 20 independent bacterial isolates for descriptive, rather than inferential, microbiological analysis.

2.2 Bacterial Isolation

The study aimed to isolate a minimum of 15 distinct bacterial strains from the pilosebaceous follicles of healthy volunteers. Sebum specimens were collected from each participant and transported to the laboratory under aseptic conditions. Samples were enriched in thioglycolate medium and incubated anaerobically at 37 °C for 72 h. Serial dilutions (10^{-1}, 10^{-2}, and 10^{-3}) were prepared and plated on Schaedler agar medium, followed by anaerobic incubation in gas-pack jars for 5–7 days. Representative colonies were selected based on morphology and repeatedly subcultured to obtain pure isolates. Each isolate was preserved in thioglycolate medium containing 30% (v/v) glycerol at −40 °C until further analysis.

2.3 Genetic Diversity Assessment of Bacterial Strains

The isolated bacterial strains were identified to the species level by amplification and sequencing of the 16S rRNA gene from bacteria recovered from the pilosebaceous follicles of healthy volunteers. Amplification was performed using the universal primer pair 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGCTACCTTGTTACGACTT-3'). Each 50 µL PCR mixture contained 25 µL deionized water, 20 µL MyTaq mix, 1 µL of each primer (20 µM), and 3 µL of template DNA **(16)**.

Thermal cycling conditions consisted of an initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min.

PCR products were verified by 2% agarose gel electrophoresis. Amplicons exhibiting bright, distinct bands without primer dimers were selected for bidirectional sequencing (DNA Sequencing Company Limited, Can Tho City, Vietnam). The resulting sequences were quality-checked and edited using BioEdit software and identified through BLAST analysis against the NCBI nucleotide database.

Sequence alignment was performed using the MUSCLE algorithm in MEGA X software. Redundant or low-quality sequences were excluded. Genetic distances were computed with the Distance tool in MEGA X, and phylogenetic relationships were inferred using the Neighbor-Joining (NJ) algorithm with 1,000 bootstrap replicates to evaluate branch reliability.

The 16S rRNA sequences obtained in this study have been deposited in GenBank under accession numbers PX631895–PX631916.

2.4 Evaluation of Bacterial Biofilm Formation Capacity

The biofilm-forming ability of the isolated bacterial strains was evaluated using the crystal violet staining assay **(17)**, with modifications to accommodate anaerobic growth requirements. Bacteria were initially cultured in thioglycolate medium for 72 h. Subsequently, 100 µL of bacterial suspension was inoculated into new Eppendorf tubes containing 900 µL of thioglycolate medium supplemented with 1% glucose and incubated statically under anaerobic conditions at 37 °C.

After seven days of incubation, the culture supernatant was carefully removed, and 1 mL of 1% crystal violet solution was added to each tube. The tubes were left undisturbed for 15 min, after which the staining solution was discarded. The tubes were gently rinsed with distilled water until the wash solution was clear. The biofilm adhering to the tube walls was then solubilized in 1 mL of 95% ethanol, and the optical density (OD) was measured at 600 nm.

Each isolate was tested in triplicate across three independent experiments. *Cutibacterium acnes* ATCC 6919 served as the positive control, and uninoculated sterile medium served as the negative control. The biofilm formation capacity was classified according to OD_{600} values as follows: < 0.2, non-biofilm forming; 0.2–0.4, weak; 0.4–0.7, moderate; and > 0.7, strong biofilm producers.

2.5 Statistical Analysis

All quantitative data were analyzed using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). Biofilm assay results are presented as mean ± SEM. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was performed to assess differences among isolates. A p value of < 0.05 was considered statistically significant. Each experiment included three independent biological replicates, each performed in triplicate.

3. Results

3.1 Morphological Characteristics of the Isolated Bacterial Strains

A total of 22 bacterial strains were isolated from 15 facial skin samples collected from healthy individuals. The colony and cellular morphological characteristics of these isolates are summarized in Table 1 and illustrated in Figure 1. All isolates were Gram-positive, nonmotile bacteria. Of these, 20 (90.9%) exhibited a rod-shaped morphology, while the remaining two strains (9.1%) were coccoid.

Regarding colony pigmentation, most isolates appeared opaque white, whereas a few displayed a light pink coloration. Colony diameters primarily ranged from 0.5 to 1.5 mm, although a small subset showed larger diameters of 1–2 mm or, in rare cases, 1–3 mm.

3.2 Biochemical Characteristics

The biochemical profiles of the isolated bacterial strains, including catalase, oxidase, indole, methyl red, lipase, gelatinase, and hemolytic activities—are summarized in Table 2 and illustrated in Figure 1. Among the 22 isolates, 19 were catalase positive, 18 were oxidase negative, six were indole positive, and 12 were methyl red positive. With respect to hydrolytic enzyme activity, 20 strains exhibited gelatinase activity, three strains (Hn1-1, Hn7-2, and Hn12-1) showed lipase activity, and eight strains demonstrated hemolysin production.

3.3 Genetic Diversity Assessment

In this study, 22 bacterial strains isolated from the pilosebaceous follicles of healthy human facial skin were genetically identified through 16S rRNA gene sequencing. The analysis revealed four distinct species: *Cutibacterium acnes* (15 strains, 68.2%), *Cutibacterium avidum* (3 strains), *Staphylococcus lugdunensis* (1 strain), and *Enterococcus faecalis* (2 strains) (Figure 2).

Phylogenetic analysis demonstrated that the 15 *C. acnes* strains shared 97.45–100% sequence similarity with the reference *C. acnes* ATCC 6919. The three *C. avidum* strains exhibited 99.03–99.63% similarity to *C. avidum* ATCC 25577 and DSM 4901. The single *S. lugdunensis* strain showed 99.23% similarity to *S. lugdunensis* ATCC 43809, while the two *E. faecalis* strains displayed 100% similarity to *E. faecalis* NBRC 100480 and ATCC 19433.

Given the predominance of *C. acnes*, these strains were further evaluated for their biofilm-forming capacity.

3.4 Integrated Interpretation

The *C. acnes* strains exhibited varying biofilm-forming ability, as presented in Table 3. Strain Hn15-2 demonstrated strong biofilm production with an OD value of 1.00. Several other strains, including Hn4, Hn13, and Hn2, also displayed robust biofilm formation, with OD values ranging from 0.7292 to 0.9023. In contrast, Hn14, Hn8-1, and Hn10-1 showed weak biofilm formation, with OD values between 0.18 and 0.23.

Table 1. Morphological characteristics of the isolated bacterial strains.

Clinical Source	Colony Characteristics						Cellular Characteristics		
	Color	Shape	Elevation	Margin	Surface	Diameter (mm)	Morpho-logy	Gram	Motility
Hn1-1	Opaque white	Circular	Convex	Entire	Smooth	0,5 - 1,0	Bacillus	+	–
Hn1-2	Light pink	Circular	Convex	Entire	Smooth	1,0 - 2,0	Bacillus	+	–
Hn1-3	Opaque white	Circular	Raised	Entire	Smooth	1,0 - 1,5	Bacillus	+	–
Hn2	Opaque white	Circular	Pulvinate	Entire	Smooth	1,0 - 1,5	Bacillus	+	–
Hn3-3	Light pink	Circular	Convex	Entire	Smooth	0,5 - 1,5	Bacillus	+	–
Hn4	Opaque white	Circular	Pulvinate	Entire	Smooth	1,2 - 1,5	Bacillus	+	–
Hn5-1	Opaque white	Circular	Convex	Entire	Smooth	0,5 - 1,0	Bacillus	+	–
Hn5-2	Light pink	Circular	Convex	Entire	Smooth	0,5 - 1,0	Bacillus	+	–
Hn7-2	Opaque white	Circular	Pulvinate	Entire	Smooth	1,0 - 1,5	Bacillus	+	–
Hn8-1	Opaque white	Circular	Convex	Entire	Smooth	1,2 - 1,5	Bacillus	+	–
Hn8-2	Opaque white	Circular	Pulvinate	Entire	Smooth	1,0 - 1,5	Bacillus	+	–
Hn9-2	Opaque white	Circular	Convex	Entire	Smooth	1,0 - 2,0	Bacillus	+	–
Hn9-3	Light pink	Circular	Convex	Entire	Smooth	0,5 - 2,0	Bacillus	+	–
Hn10-1	Opaque white	Circular	Pulvinate	Entire	Smooth	0,5 - 1,0	Bacillus	+	–
Hn10-2	Opaque white	Circular	Pulvinate	Entire	Smooth	0,5 - 1,0	Bacillus	+	–
Hn11-1	Opaque white	Circular	Raised	Entire	Smooth	1,0 - 1,5	Coccus	+	–
Hn12-1	opaque white	Circular	Pulvinate	Entire	Smooth	1,0 - 1,5	Bacillus	+	–
Hn12-2	Opaque white	Circular	Pulvinate	Entire	Smooth	1,0 - 3,0	Coccus	+	–
Hn13	Opaque white	Circular	Pulvinate	Entire	Smooth	1,2 - 1,5	Bacillus	+	–
Hn14	Opaque white	Circular	Convex	Entire	Smooth	0,5 - 1,0	Bacillus	+	–
Hn15-1	Opaque white	Circular	Convex	Entire	Smooth	1,0 - 2,0	Bacillus	+	–
Hn15-2	Opaque white	Circular	Pulvinate	Entire	Smooth	1,0 - 1,5	Bacillus	+	–

Note: (+) = Gram-positive; (–) = non-motile

Table 2. Biochemical characteristics of the isolated bacterial strains.

Strains	Biochemical characteristics						
	Catalase	Oxidase	Indol	Methyl red	Lipase	Gelatinase	Hemolysis
Hn1-1	+	-	+	+	+	+	-
Hn1-2	+	-	-	-	-	+	+
Hn1-3	+	-	-	-	-	+	+
Hn2	+	-	-	-	-	+	-
Hn3-3	-	-	-	-	-	-	+
Hn4	+	+	-	+	-	+	-
Hn5-1	+	-	+	+	-	+	-
Hn5-2	+	-	-	-	-	+	+
Hn7-2	+	+	+	+	+	+	-
Hn8-1	+	-	+	+	-	+	-
Hn8-2	+	-	-	+	-	+	-
Hn9-2	+	+	-	-	-	+	-
Hn9-3	-	-	-	-	-	+	+
Hn10-1	+	-	-	-	-	+	-
Hn10-2	+	-	-	+	-	+	-
Hn11-1	-	-	-	-	-	-	+
Hn12-1	+	-	+	+	+	+	-
Hn12-2	-	+	-	-	-	+	+
Hn13	+	-	-	+	-	+	-
Hn14	+	-	+	+	-	+	-
Hn15-1	+	-	-	+	-	+	+
Hn15-2	+	-	-	+	-	+	-

Note: (+) = Positive; (–) = Negative

Table 3. Biofilm formation capacity.

Strains	OD (600 nm)	Level
Hn1-1	0,36±0,11^{bcd}	++
Hn2	0,73±0,19^{abc}	+++
Hn4	0,90±0,10^{ab}	+++
Hn5-1	0,33±0,05^{bcde}	++
Hn7-2	0,24±0,02^{cde}	+
Hn8-1	0,22±0,01^{de}	+
Hn8-2	0,24±0,02^{cde}	+
Hn9-2	0,26±0,03^{cde}	+
Hn10-1	0,18±0,02^{de}	+
Hn10-2	0,25±0,01^{cde}	+
Hn12-1	0,25±0,03^{cde}	+
Hn13	0,83±0,32^{abc}	+++
Hn14	0,23±0,03^{cde}	+
Hn15-1	0,34±0,13^{bcde}	++
Hn15-2	1,00±0,04^{a}	+++
C. acnes ATCC 6919	0,32±0,03^{bcde}	+
Negative control	0,11±0,00^{e}	-

Note: (–) No biofilm formation; (+) Weak; (++) Moderate; (+++) Strong. Data represent mean ± SEM values. Different superscript letters indicate statistically significant differences ($p < 0.05$, one-way ANOVA).

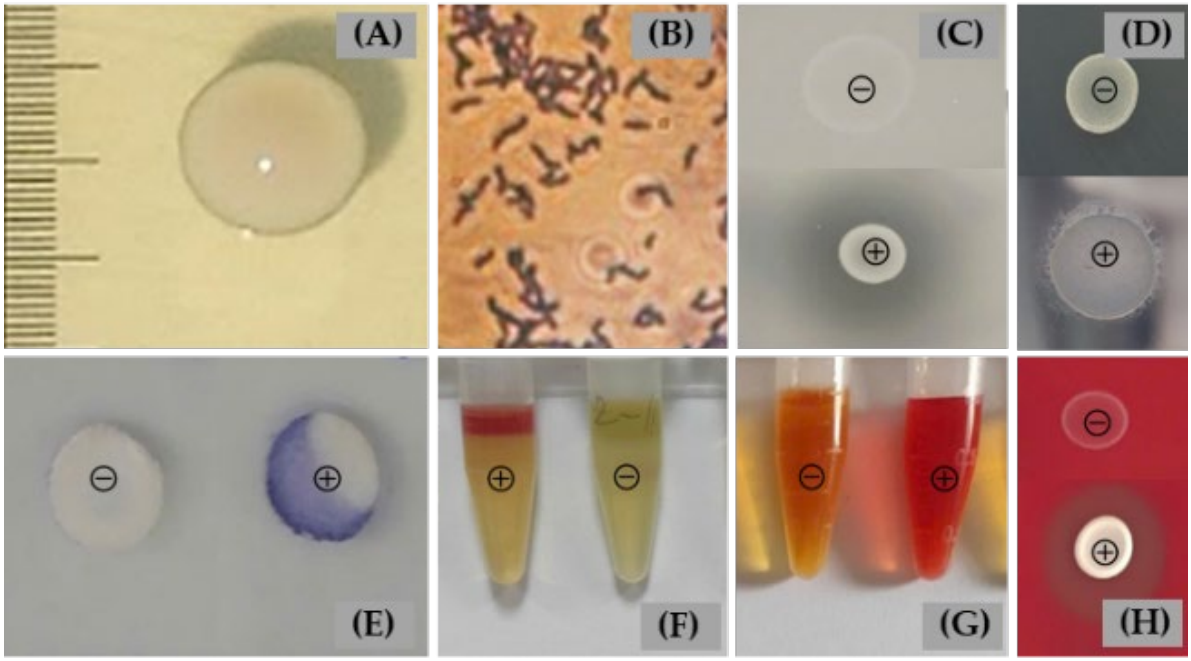

Figure 1. Colony morphology, cellular characteristics, and biochemical properties **(Prepared by Authors, 2025)**.

Note: (A) Colony morphology observed on Schaedler agar; (B) Cellular morphology after Gram staining; (C) Gelatin hydrolysis; (D) Lipid hydrolysis; (E) Catalase reaction; (F) Indole production; (G) Methyl red reaction; (H) Hemolytic activity, ⊖ Negative, ⊕ Positive.

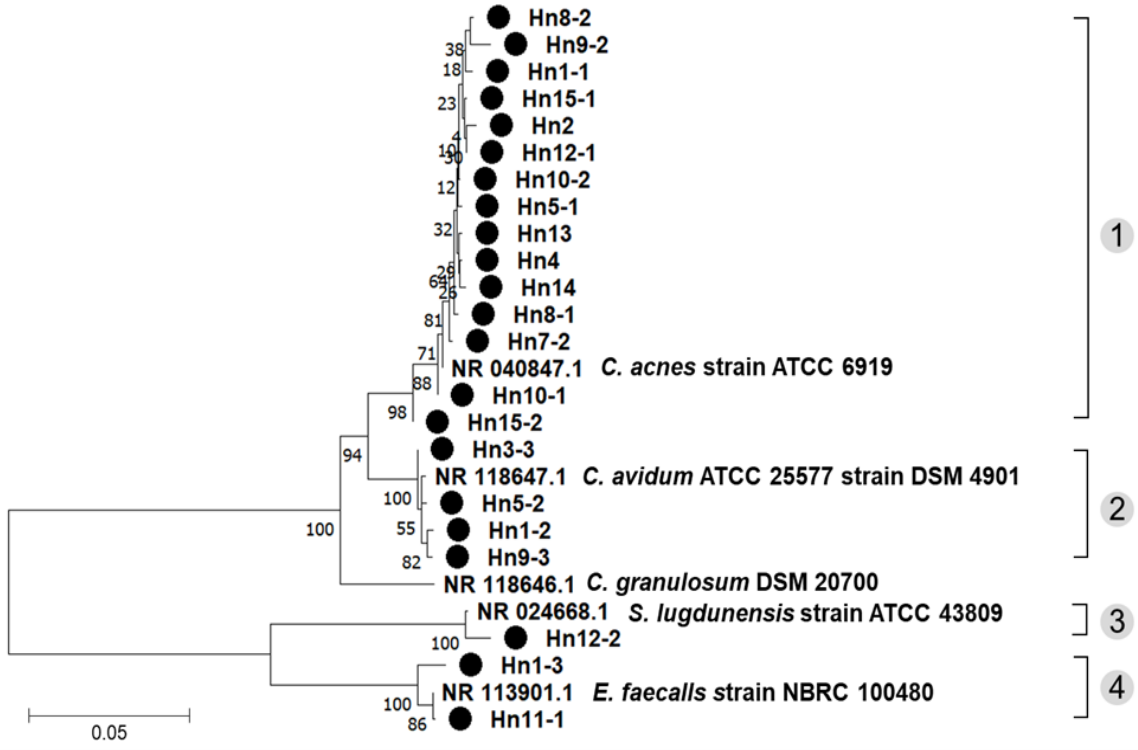

Figure 2. A phylogenetic tree illustrates the genetic relationships among the isolated bacterial strains **(Prepared by Authors, 2025)**.

4. Discussion

The facial skin of healthy individuals predominantly harbors Gram-positive, rod-shaped, nonmotile bacteria. This distribution reflects the biological adaptation of the microbial community within the pilosebaceous unit and is consistent with the features of typical resident species such as *Cutibacterium acnes*, *C. avidum*, and *Staphylococcus* spp. These microorganisms are well-documented constituents of the normal human skin microbiome **(1, 18)**.

Lipase hydrolyzes triglycerides into glycerol and free fatty acids, maintaining the skin's acidic environment, inhibiting pathogenic bacteria, and supporting commensal microbiota **(8, 19, 20)**. However, excessive lipase activity can lead to an accumulation of fatty acids, which may trigger inflammation and contribute to the pathogenesis of acne vulgaris **(21, 22)**. The low proportion of lipase-producing isolates in this study (3 of 22) suggests an effective lipid regulation mechanism in healthy skin.

Gelatinase degrades gelatin into peptides and amino acids, facilitating nutrient availability and microbial growth. However, when combined with protease and lipase activity, gelatinase can also promote tissue damage and inflammation **(23, 24)**. The observation that 20 of 22 isolates exhibited gelatinolytic activity underscores this enzyme's potential role in maintaining microbial equilibrium on healthy skin.

α-Hemolysin produced by *Staphylococcus aureus* forms pores in host cell membranes through interaction with ADAM10 receptors, leading to hemolysis, inflammation, and epithelial barrier dysfunction **(25)**. The presence of hemolytic activity in 8 of 22 isolates indicates that this virulence factor is relatively common among commensal skin microbiota but may become pathogenic under conditions of dysbiosis.

Overall, most bacterial isolates obtained from healthy facial skin exhibited low extracellular enzyme activity, particularly for lipase (13.6%) and hemolysin (36.4%), suggesting limited pathogenic potential and supporting their commensal role in the cutaneous microbiota **(8, 19)**. In contrast, the high frequency of gelatinase-positive isolates (90.9%) suggests that this enzyme plays a physiologic role in bacterial colonization and nutrient cycling, although it could contribute to pathogenicity when combined with other virulence factors **(24)**.

The uneven distribution of enzymatic activities such as lipase, gelatinase, and hemolysin among the isolates highlights substantial functional diversity within the facial skin microbiota. These findings reinforce that bacterial presence alone is insufficient to cause disease; rather, pathogenicity depends on the expression of virulence genes, the local microenvironment (e.g., pH, sebum content), and the

stability of the skin microbiome (26). Cobian et al (27) in 2021 further demonstrated that *C. acnes* strains belonging to phylotype IA_1, most strongly associated with acne vulgaris, harbor a higher number of virulence-related genes (encoding lipase, hyaluronidase, and protease) and exhibit stronger immunomodulatory effects than other phylotypes.

Genetically, *Cutibacterium acnes* is classified into three major phylotypes, type I (subgroups IA_1, IA_2, IB, and IC), type II, and type III, each exhibiting distinct biological properties and pathogenic potentials (28, 29). Ribotype analysis, which targets rRNA gene regions, enables differentiation of genetic signatures within these phylotypes. Notably, ribotypes RT4, RT5, and RT8 (phylotype IA_1) are frequently associated with inflammatory acne lesions because they harbor virulence genes encoding lipase, hemolysin, and CAMP factor, whereas ribotypes RT2 and RT6 (phylotype II) predominate on healthy skin and contribute to local immune regulation (30).

The *C. acnes* isolates obtained in this study shared 97.45–100% sequence similarity with the reference strain *C. acnes* ATCC 6919, a representative of phylotype IA_1. However, phylogenetic clustering revealed intraspecies genetic variability among isolates, which may underlie differences in biological behavior, such as biofilm-forming capacity and toxin production. Although *C. acnes* dominates the facial skin microbiota of healthy individuals, its presence alone does not indicate disease. Metagenomic studies have demonstrated comparable *C. acnes* abundance in both healthy and acne-affected skin, with pathogenicity determined by strain-specific genomic and phenotypic traits (26). Thus, the overall prevalence of *C. acnes* reflects its role as a stable commensal component of the skin microbiome rather than a direct etiologic agent of acne (26, 31). Emerging evidence suggests that acne pathogenesis is driven by phylotype imbalance rather than total bacterial load, emphasizing the importance of strain-level functional diversity in disease mechanisms.

Biofilm formation represents a crucial adaptive strategy that facilitates bacterial persistence on skin surfaces, particularly under environmental stress or antimicrobial exposure (32). In this study, *C. acnes* isolates from healthy facial skin demonstrated variable biofilm-forming capacity, ranging from weak to strong. The robust biofilm production observed in strains Hn15-2 and Hn4 indicates that biofilm formation is not restricted to acne-associated isolates but may be a common trait among commensal *C. acnes* populations. These findings are consistent with McDowell et al (29) in 2013, who reported that both acne-derived and healthy skin-derived *C. acnes* strains exhibit biofilm-forming ability, albeit with differing intensities.

Future investigations integrating metagenomic and transcriptomic profiling could further elucidate the molecular determinants of strain-specific biofilm formation in *C. acnes* and clarify its dual role in skin health and disease.

5. Conclusion

This study provides baseline insight into the pilosebaceous microbiota of healthy human facial skin, dominated by *Cutibacterium acnes* and *C. avidum*. The observed variability in biofilm-forming capacity among commensal *C. acnes* strains highlights strain-level diversity that may influence skin microbiome stability and the transition from commensalism to opportunistic pathogenicity. These findings may assist in developing standardized models for evaluating biofilm behavior in dermatologic research.

6. Declarations

6.1 Acknowledgment

We sincerely thank the Institute of Food and Biotechnology (Can Tho University, Viet Nam) for their kind support and for providing access to their laboratories. We thank Can Tho hospital of Dermato-Venereology for the great help in collecting samples.

6.2 Ethical Considerations

The protocol for this Research was approved by Ethic Committee of Biomedical Research, Can Tho University of Medicine and Pharmacy (Ref.No.24.004/PCT-HĐĐĐ). All participants were given formal consent.

6.3 Authors' Contributions

All authors contributed to the study conception, design, and production.

6.4 Conflict of Interests

The authors have no conflicts of interest to declare.

6.5 Financial Support and Sponsorship

This study received no financial support or sponsorship.

6.6 Using Artificial Intelligence Tools (AI Tools)

This study do not use artificial intelligence tools.

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