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"Targeting Acne Dysbiosis Through An Advanced Microbial Co-Culture Model"

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1. Introduction

Acne vulgaris is a prevalent dermatological condition characterized by the overproduction of sebum, hyperkeratinization and the formation of comedones and inflamed follicles. Given the growing recognition and prevalence of acne, the anti-acne cosmetic market, valued at approximately $5.9 billion in 2023, is projected to expand at a rate of 5% annually, underscoring the significant economic importance of addressing this widespread dermatological condition (Global Market Insights, Anti-acne Cosmetics Market, 2024 - 2032). The pilosebaceous unit, the skin structure affected by acne, harbors a distinct microbiome that plays an important role for skin health. Recent research emphasizes the role of the microbiome in the pathogenesis of acne [1], [2]. The lipid-rich anaerobic conditions of the pilosebaceous unit offer a microenvironment for the proliferation of specific lipophilic bacterial species. Among these, *Cutibacterium acnes*, *Staphylococcus epidermidis*, *Corynebacterium tuberculostaericum*, and *Staphylococcus capitis* are the most frequently identified species from sebaceous skin regions [3], [4].

C. acnes is particularly notable for its genomic diversity, classified into six primary phylotypes: IA1, IA2, IB, IC, II, and III [5]. This bacterium exhibits a dual role in skin health; while phylotypes II and III are associated with beneficial commensal functions, the microbiome of acne-prone skin is typified by a reduction in phylotype diversity and a predominance of virulent *C. acnes* strains, specifically IA1 and IA2. The different phylotypes were recently grouped into three subspecies with distinct genetic and morphological characteristics, namely *C. acnes* subsp. *acnes* (phylotype I), *C. acnes* subsp. *defendans* (phylotype II), and *C. acnes* subsp. *elongatum* (phylotype III) [6]. The virulence of these strains is primarily attributed to two key factors: (1) biofilm formation, which facilitates adhesion to host cells, impedes sebum flow and increases drug resistance, and (2) the enzymatic lysis of sebum triglycerides by lipases, leading to the release of free fatty acids that are known to induce inflammation and promote comedone formation and pore blockage through hypercornification [7] - [8-12].

Recent investigations emphasize the complex interactions within the skin microbiome, highlighting phylotype-specific coexistence and exclusion dynamics among its resident species. In particular, *S. epidermidis* has been shown to selectively exclude acne-associated phylotypes of *C. acnes* while coexisting with those strains more commonly associated with healthy skin [13]. Similarly, *S. capitis* has been shown to inhibit the growth of specifically *C. acnes*, highlighting its potential role as a natural antagonist [14]. These findings emphasize the critical role of microbial interactions in maintaining skin homeostasis, suggesting that the

balance between commensal and virulent strains may significantly influence the pathogenesis of acne.

Current *in vitro* approaches to studying the acne-prone microbiome predominantly utilize single-strain assays under conditions that fail to accurately replicate this complex environment of sebaceous skin and the interactions between bacteria taking place. Recently, synthetic communities are becoming more and more state-of-the-art methodologies for *in vitro* investigation of the healthy skin microbiome [15] [16]. However so far, no synthetic skin community simulating the dynamics of pathogenic and commensal *C. acnes* and resident coagulase negative *Staphylococci* as potential antagonists under pilosebaceous unit conditions were described before. In response to these limitations, we have developed an advanced microbial co-culture model that effectively simulates the acne-prone skin microbiome. This model incorporates both commensal and virulent strains of *C. acnes*, along with the lipophilic species *S. epidermidis* and *S. capitis*. Microbial cultivation occurs under high-throughput conditions that closely mimic the physiological environment of the pilosebaceous unit, employing an artificial human sebum as matrix for biofilm growth. The model enables the quantification of biofilm formation through DNA extraction from biofilm-associated cells, coupled with the application of the qPCR techniques mentioned before. Classical anti-acne drugs and cosmetic ingredients were tested as reference substances on the model. Utilizing a novel quantitative polymerase chain reaction (qPCR) method, this model allows for the precise differentiation and quantification of virulent and commensal *C. acnes* strains, as well as the total counts of *S. epidermidis* and *S. capitis*.

2. Materials and Methods

Bacterial strains

The bacterial strains, applied in the co-culture were retrieved from Leibniz Institute DSMZ, Germany and BEI Resources Repository, United States and are listed in Table 1.

Table 1 Bacterial strains applied in this study

Species	**Strain**	***C. acnes* Phylotype**	***C. acnes* pathogenicity**
C. acnes	HL072PA1	IA1	Highly pathogenic [5]
C. acnes	HL110PA4	II	Non pathogenic [5]
S. epidermidis	DSM1798		
S. capitis	DSM20326		

Preparation of medium simulating the pilosebaceous unit

The medium simulating the pilosebaceous unit was composed of two parts: an artificial solid human sebum equivalent, coated to the bottom of the cultivation plate wells and a liquid nutrient medium on top. A modified version of the artificial human sebum based on Spittaels et al. (2018) [17] was prepared by 2.74 g mixing tripalmitin, 0.41 mg palmitic acid, 0.14 mg cholesterol, 68 µg tocopherol acetate, 0.75 mg triolein, 1.97 mg jojoba oil, and 0.06 g artificial dandruff (BZ293, Biochemazone Canada) and heating in a water bath at 85° C for 10 minutes. Artificial human sebum was prepared freshly on the day of the co-cultivation start. 100 µl of the warm, liquid artificial human sebum were added into each well of a hydrophobic, untreated polystyrene 48-well plate (Product No. 150787, ThermoFischer Scientific, USA). Afterwards, the plates were warmed again to 80° C on a MATRIX Orbital Delta FP heater (Ika, Germany) to distribute the sebum equally in each well. The liquid medium VK-1021 was prepared by mixing 30 mg/kg Caso-Bouillon (Merck, Germany), 2.14 g/L dipotassium hydrogen phosphate and 11.93 mg/l potassium dihydrogen phosphate in A. demin. (pH 6). 2 mL/L Tween-80 and 0.23 µl/L tocopherol acetate were added after steam sterilization.

Pre-Culture and co-culture of bacteria

The four strains (Table 1) were cultured as individual pre-cultures before being transferred into the shared co-culture. The two *C. acnes* strains were initially cultured anaerobically at 37°C on CBA and incubated for 5 days. After that, cell material was rinsed off, and 50 µl was transferred into 12 ml VK-1021 in a 15-ml Falcon tube and incubated standing at 37°C for 24 hours. The two *Staphylococci* were cultured directly with 50 µl from the biobank standing for 16 hours at 37°C in 10 ml VK-1021 in a 15 ml Falcon tube.

For starting the co-culture, the optical density at 600 nm of the pre-cultures was set to 0.5 using a photometer with VK-1021 dilution. The adjusted cultures were mixed to generate the inoculum suspension. The prepared wells of the sebum plate were filled with 0.5 ml VK-1021 and inoculated with 50 µl of the inoculum suspension. Additionally, three separate controls were prepared in Eppendorf tubes, filled with 50 µl of inoculum suspension and 0.5 ml VK-1021, from which 250µl were then added to 750 µl of lysis buffer from the ScreenFloX® Nucleic Acid Extraction Kit (Evonik, Germany) and used for quantification of the starting culture at time point 0 (t0). The inoculated plate was then cultivated anaerobically at 37 °C for 3 days using the ThermoFisher Scientific Oxoid™ AnaeroGen™ system.

Application of test substances

Different substances commonly used as ingredients in cosmetic formulations, most with anti-acne claims and two widely used antibiotics as drugs for treating acne were tested in the microbiome model (Table 2).

Table 2 Drugs and cosmetic ingredients tested in the microbiome model for acne prone skin

Substance	Tested concentration [%]	Solvent
Niacinamide	5	Medium
Zinc Sulfate	0.5	Medium
Epigallocatechin-3-Gallate	0.50	Medium
Adapalen	0.1	DMSO
Erythromycin	0.5	Ethanol
Clindamycin	0.5	Medium
Capryloyl Salicylic Acid	0.01	Ethanol
Cholesterol	0.5	Ethanol

Each substance was tested in triplicates. Substances were dissolved directly in VK-1021 medium or in ethanol, isopropanol, or DMSO, respectively. The effect of each substance was compared to the control containing either pure VK-1021 medium or medium with the corresponding amount of solvent.

Extraction of bacterial DNA

For harvesting the planktonically grown liquid culture, 250 µl of resuspended culture were mixed with 750 µl of lysis buffer from the ScreenFloX® Nucleic Acid Extraction Kit (Evonik, Germany). The remaining liquid culture was discarded, and if possible, the sebum is washed twice with A. demin. If test substances dissolved fats, washing was not possible without destroying the sebum layer, and the washing steps were skipped. The plate containing the washed sebum was heated on a MATRIX Orbital Delta FP heater (Ika, Germany) to 80°C until the sebum completely liquified. A mixture of lysis buffer and water (5:1, lysis buffer from the ScreenFloX® Nucleic Acid Extraction Kit) was preheated to 80°C. The liquid sebum of each well was suspended in 1 mL of warm buffer. 500 µL of this suspension were transferred to an Eppendorf tube, and 500 µL of the lysis buffer-water was added. Both sample types are lysed

for 20 minutes at 60°C before being isolated according to the manufacturer's instructions using the ScreenFloX® Nucleic Acid Extraction Kit on the KingFisher Flex Deep Well (ThermoFisher Scientific, USA).

Quantification of bacterial DNA by qPCR

For quantification of the bacterial species and strains, qPCR was used with the primers and probes listed i*n Table 3.* For total and specific pathogenic phylotype quantification of *C. acnes*, the target genes were based on the publication by Barnard, E. et al. (2015) [18]. The genes for identifying the two *Staphylococci* were selected based on Kim, J. et al. (2018) [19].

Table 3 Primers and probes used for qPCR quantification of bacterial strains

Target microorganism	Target gene	Oligo	Sequence	Amplicon size [bp]
C. acnes (all phylotypes)	16s rDNA	Forward	GTGAGTGACGGTAATGGGTAAAG	149
		Reverse	GTTAAGCCCCAAGATTACACTTCC	
		Probe	AGCACCGGCTAACTACGTGCCAGCAG	
C. acnes (IA1, IA2 and IC)	*ATPase*	Forward	CAACCCCATCCTCAAACTGTC	164
		Reverse	TGCCACCTGAAAGCTGGAAG	
		Probe	CGCGCCCATGATCCCTCGTTGGCAGAGAA	
S. epidermidis	*sodA*	Forward	TGGTGGATATATGAGTGG	116
		Reverse	CTGAAGGATGGACACTAA	
		Probe	CAGGTGTTGTTGTAGGTGAAAA	
S. capitis	*gseA*	Forward	CAATGGGGTTCTTTAGATG	135
		Reverse	TGGGTTATCTTGGTTTGG	
		Probe	TGGATCTGGTTGGGCATGG	

Promega GoTaq® Probe qPCR Mix (Promega, USA) was used to assemble the master mix for two duplex qPCR reactions according to Table 4. All assays were performed according to the manufacturer's instructions. QPCRs were run on a CFX Touch Deepwell Cycler (BioRad, Germany) with the following cycling protocol: Initial denaturation 95° C, 2 min denaturation 95° C, 15 s, annealing 60° C, 60 s, repeat for 39 cycles.

Table 4 Mastermix setup for the two duplex qPCRs

***C. acnes* Duplex qPCR**		***Staphylococcus* Duplex qPCR**	
Component	**Vol. per reaction [µL]**	**Component**	**Vol. per reaction [µL]**
GoTaq® Probe qPCR Mix	10	GoTaq® Probe qPCR Mix	10
16s For (100 µM)	0.1	S. epi for (100 µM)	0.04
16s Rev (100 µM)	0.1	S. epi rev (100 µM)	0.04
16s Probe (100 µM) Cy5	0.06	S. epi Probe (100 µM) FAM	0.01
ATPase For (100 µM)	0.06	S. cap for (100 µM)	0.1
ATPase Rev (100 µM)	0.06	S. cap rev (100 µM)	0.1
ATPase Probe (100 µM) FAM	0.02	S. cap Probe (100 µM) Cy5	0.05
Template	5	Template	5
Water	4.6	Water	4.66
Total	20	Total	20

The standard curve for qPCR quantification was prepared by using plasmids containing the target fragment with additional 100 bp up- and downstream from the primer binding sites. pEX-

A128 plasmids with the target genes were ordered from Eurofins Genomics (Germany). Plasmids were diluted to 10-10^5 copies/ml and applied as standards to every qPCR plate.

Analysis

Standard curves were used to calculate the starting quantity (SQ) for each species per well based on the volume of each sample type (550 µl liquid medium or 100 µl artificial sebum). The number of *C. acnes* type II was calculated by subtracting the SQ of the *C. acnes* type IA1 specific ATPase qPCR from the total *C. acnes* 16s qPCR.

The effect of substances on bacterial growth of each species in the community was evaluated by considering the growth reached by the untreated control. As the bacterial growth data followed a logarithmic distribution, log-transformation was performed with the SQ data. The total growth of the control was calculated by

$$total\ growth\ (\frac{SQ}{well}) = \log(SQ_{control}) - \log\ (SQ_{t0})$$

The percentage change of growth was calculated by

$$\%growth\ change = \frac{log(SQ_{Substance}) - \log\ (SQ_{control})}{total\ growth\ (\frac{SQ}{well})}$$

3. Results

In this study we used a co-culture of *C. acnes* Type II and type IA1, *S. epidermidis*, and *S. capitis* growing under pilosebaceous-unit like conditions. The application of the adapted artificial sebum on untreated polystyrene plates enabled a stable formation of a human sebum coating over the full time of cultivation providing surface for biofilm formation (Figure 1).

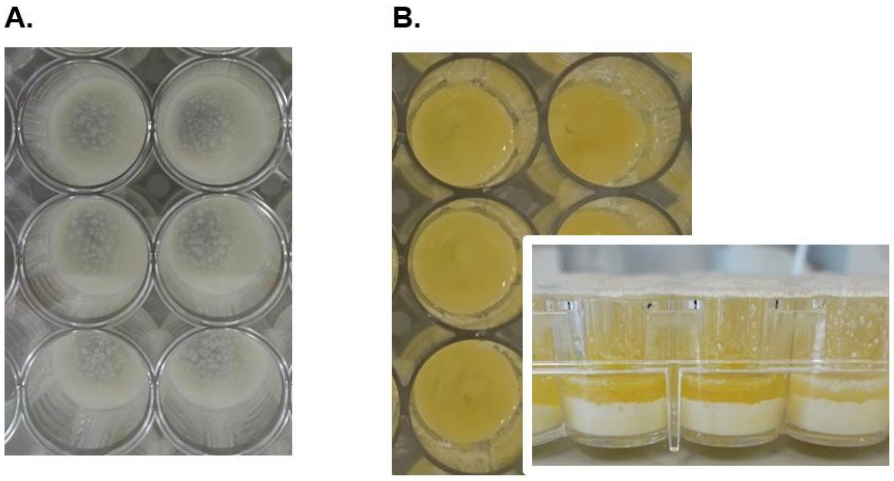
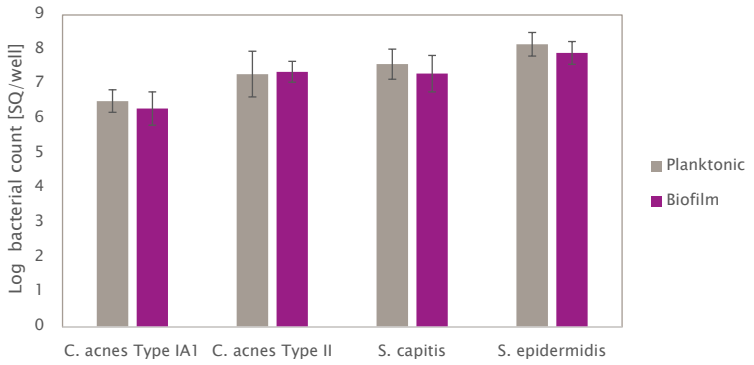


Figure 1 Artificial human sebum and dandruff coated to untreated polystyrene 48-well plate A) before addition of liquid medium and B) with liquid medium and after co-cultivating the microbial community for 72 h.

The reproducibility of the co-culture was tested by performing 15 individual experiments in total and calculating average total growth and the coefficient of variation (CV) (Figure 2). The different bacterial strains reached average densities of 6.5 to 8.1 log SQ/well in planktonic and 6.3 to 7.9 log SW/well for biofilm growth with a CV of maximum 8%. The

two *Staphylococcus* species reached higher densities than the two *C. acnes* strains. All strains grew at least 1 log/well considering the inoculum concentration (data not shown).



	C. acnes IA1	*C. acnes II*	*S. capitis*	*S. epidermidis*
Planktonic CV	4.95%	8.12%	5.71%	4.16%
Biofilm CV	7.62%	4.08%	7.19%	4.17%

Figure 2 Average logarithmic bacterial growth after 72 h of co-cultivating the four different strains and coefficient of variation over 15 independent experiments

The percentage growth change of three exemplary substances is depicted in Figure 3. Substances can increase the growth of bacteria in comparison to the control leading to a positive change or decrease growth, leading to a negative change up to -100%, which equals total growth inhibition. Erythromycin, an antibiotic commonly used for treating acne, increased the growth of *C. acnes* type IA1 while strongly inhibiting growth of all other strains in both growth states. Capryloyl Salicylic Acid, also widely used against acne, had inhibitory activity against all strains but showed lower inhibition towards *C. acnes* type IA1 planktonic and *C. acnes* type II biofilm growth state. Cholesterol as an ingredient without known anti acne activity led to increased planktonic and biofilm growth of *C. acnes* type IA1 and biofilm growth of *C. acnes* type II, while it did not change the proliferation of the other species and growth states.

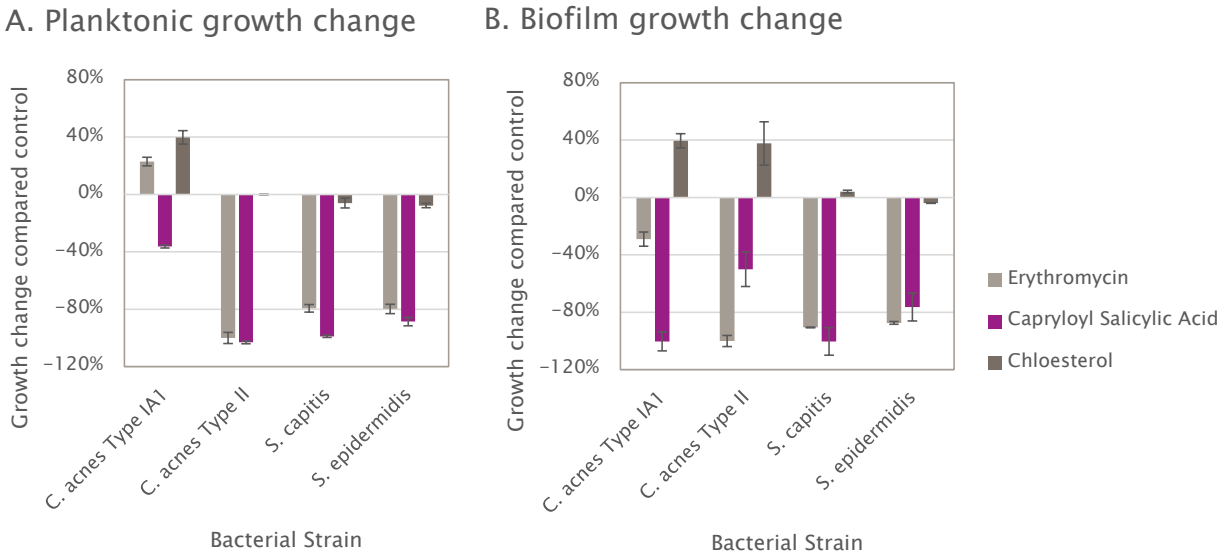


Figure 3 Growth change of A) planktonic and B) biofilm growth of the co-culture treated with three example substances erythromycin 0.5% in culture medium, capryloyl salicylic acid 0.01% and cholesterol 0.5% in comparison to the corresponding untreated control.

Table 5 summarizes the substances tested according to their growth change in comparison to the control also including the substances from Figure 3. Zinc sulfate, epigallocathecin-3-gallate (EGCG) and clindamycin showed medium to strong inhibitory activity towards all members of the microbiome community similar to capryloyl salicylic acid. Niacinamide exposed strong antimicrobial effects at 5% but did not change biofilm growth of the two *Staphylococci*. Adapalene was the only ingredient used in anti-acne formulations without antimicrobial activity.

Table 5 Summarized growth change of applied anti-acne reference substances in comparison to the corresponding control for planktonic and biofilm growth species in the co-culture model, the concentration is given as percentage in culture medium.

		Planktonic				Biofilm			
Substance	Concentration [%]	C. acnes Type IA1	C. acnes Type II	S. capitis	S. epidermidis	Type IA1	Type II	S. capitis	S. epidermidis
Niacinamide	5.00	-81%	-100%	-74%	-69%	-88%	-100%	4%	4%
Zinc sulfate	0.50	-36%	-80%	-74%	-34%	-100%	-100%	-96%	-51%
Epigallocatechin-3-Gallate	0.50	-100%	-100%	-100%	-100%	-100%	-100%	-100%	-98%
Adapalen	0.10	-9%	-3%	-8%	0%	-2%	-13%	-6%	-1%
Clindamycin	0.50	-54%	-100%	-77%	-92%	-69%	-100%	-85%	-93%
Erythromycin	0.50	23%	-100%	-79%	-80%	-29%	-100%	-91%	-87%
Capryloyl Salicylic Acid	0.01	-36%	-100%	-99%	-89%	-100%	-50%	-100%	-76%
Chloesterol	0.50	40%	0%	-6%	-8%	39%	38%	4%	-4%

Growth of strain increased Growth of strain decreased

4. Discussion

In this study we developed a microbial community model simulating acne prone skin. To our knowledge this is the first co-culture considering both pathogenic and commensal phylotypes of *C. acnes* with a differentiated quantification based on qPCR. The model does not only allow the interaction of pathogenic bacteria with commensal antagonists but also simulates the conditions within the pilosebaceous unit by using artificial human sebum under anaerobic conditions. By this, our model enables screening for substances with specific effects against pathogenic phylotypes while leaving the important commensals unaffected.

Niacinamide is a well-known cosmetic ingredient used for anti-aging, skin lightening but also acne treatment [20] [21]. It possesses antimicrobial activity that was verified by our study. Also, recent research has demonstrated that niacinamide exhibits significant antibiofilm activity against *C. acnes* but also other microbes, effectively decreasing biofilm formation and enhancing degradation [22, 23]. Our findings confirm both anti-bacterial and anti-biofilm effects of niacinamide at 5%. The antimicrobial activity against *C. acnes* appears to be stronger than that against *S. epidermis* and *S. capitis*, especially for biofilm grown bacteria, making it an interesting molecule for more specific effects.

EGCG, the major polyphenol in green tea, is known for its anti-inflammatory and antimicrobial activities and of interest as an active ingredient against acne [24, 25]. For both *C. acnes* and *S. epidermidis* a strong antimicrobial effect was already reported, which was confirmed by our model. The effect of ECGC on *S. capitis* was not reported before, however, our community model shows strong antibacterial effects against this species as well. EGCG was reported to inhibit biofilm formation by interfering with the assembly of amyloid fibers before [26]. This strong anti-biofilm activity was clearly visible in our microbial community model.

Zinc in the form of zinc gluconate or zinc sulfate is a commonly used ingredient in topical formulations against acne. It is well known for its activity against a wide range of microorganisms including the bacteria of the genus *Staphylococcus* and *Streptococcus* but

also yeast of the genus *Candida* and *Malassezia* [27]. Iinuma et al. showed antibacterial effects against *C. acnes*, also including strains with clindamycin resistance [28]. Our model confirmed this general antimicrobial activity of zinc sulfate at a concentration of 0.5% with higher activity against biofilm grown bacteria.

Topical retinoids such as adapalene are commonly applied in treating acne due to their anti-follicular hyper keratinization and anti-inflammatory activities [29]. Most retinoids do not exhibit direct inhibitory activity towards gram-positive or gram-negative bacteria and recent research indicates that antimicrobial activity is only observed for retinaldehyde [30]. Consistent with the literature, our findings indicate that adapalene does not exhibit antimicrobial or anti-biofilm activity against *C. acnes* or *S. epidermidis* and *S. capitis*, emphasizing that retinoids directly act on follicular keratinocytes to prevent hyper hornification and follicular blockage.

Salicylic acid is used in anti-acne formulations for its regulation of sebum production and its anti-inflammatory activity and is also known for its antimicrobial effects [31] [32]. Also, anti-biofilm effects against *S. epidermidis* and *C. acnes* were proposed before [7, 33]. Our results confirm this broad anti-microbial and anti-biofilm activity, not limited to *C. acnes* and *S. epidermidis* but also particularly pronounced against *S. capitis*.

C. acnes strain HL072PA1, used as type IA1 strain in this study, is known to be resistant against erythromycin (MIC > 0.5 µg/mL = 0.0005%), which was clearly validated within the microbial community model, all stains except HL072PA1 were strongly inhibited by erythromycin at 0.5% [34]. The planktonic growth of HL072PA1 was even promoted, most probably due to less competition for nutrients with the other strains in the community. Also, clindamycin at 0.5% showed the lowest inhibitory activity towards HL072PA1. Woodburn et al. showed that HL072PA1 is resistant towards clindamycin with a MIC > 32 µg/ml (0.0032 %) confirming the results. These findings align with the growing evidence that antibiotics do not promote a favorable shift in the skin microbiome towards beneficial species such as *S. epidermidis*, *S. capitis*, and commensal *C. acnes*. Instead, the selective pressure exerted by antibiotics tends to favor the proliferation of pathogenic *C. acnes* strains, which have developed resistance mechanisms, thereby exacerbating dysbiosis and highlighting the need for alternative therapeutic approaches in acne management [4].

Cholesterol is a natural lipid occurring in human sebum that is applied as an emollient and moisturizer in cosmetic formulations. In our study it specifically increased the growth of planktonic *C. acnes* type IA1 and the biofilm growth of both *C. acnes* phylotypes. Increased plasma but also sebum cholesterol in acne patients is reported in several studies indicating a connection between altered cholesterol concentration and acne pathogenesis [35-37]. Our study first found that Cholesterol specifically boosts the planktonic growth of an acne-related *C. acnes* phylotype, indicating that increased sebum cholesterol could promote the overgrowth of pathogenic *C. acnes* in acne prone skin and that cholesterol should be avoided as an ingredient in anti-acne formulations.

In summary, none of the tested ingredients were able to specifically inhibit phylotype IA1 *C. acnes*, tackling the cause of acne rather than disturbing the commensal microbiome by unspecific antibacterial effects. Some substances, such as erythromycin and cholesterol, have even been shown to specifically increase *C. acnes* phylotype IA1, raising questions about their suitability in anti-acne formulations. Such ingredients fail to rebalance the acne-prone microbiome and are often accompanied by side effects such as skin irritation or scaling.

5. Conclusion

In conclusion, our study presents a novel microbial co-culture model that effectively simulates the complex dynamics of the acne-prone skin microbiome, allowing for the differentiated quantification of both pathogenic and commensal strains of *C. acnes* in co-cultivation with *S. epidermidis* and *S. capitis* as potential antagonists. This model not only considers the importance of microbial interactions in acne pathogenesis but also underscores the limitations of traditional acne treatments, particularly antibiotics, which may exacerbate dysbiosis and favor pathogenic strains. By advancing our understanding of the skin microbiome and its role in acne, this work paves the way for the development of more targeted and effective treatments that preserve beneficial microbial communities. Finally, our research emphasizes the need for innovative approaches in acne management that prioritize microbial balance and minimize the risk of resistance.

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