



Antibacterial activity and impact on keratinocyte cell growth of *Cutibacterium acnes* bacteriophages in a *Cutibacterium acnes* IA₁-colonized keratinocyte model

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ABSTRACT

Acne is an inflammatory disease in which microbial disbalance is represented by an augmented population of phylotype IA₁ of *Cutibacterium acnes*. Various treatments for acne can cause side effects, and it has been reported that *C. acnes* is resistant to prescribed antibiotics. Phage therapy has been proposed as an alternative treatment for acne, given its species-specificity to kill bacteria, its relative innocuity, and its potential to manage antibiotic-resistant pathogens. Moreover, bacteriophages (phages) may modulate the microbiota and immune responses. Some studies have shown the potential use of phages in the treatment of acne. Nevertheless, the capacity to specifically reduce phylotype IA₁ and the effect of phage treatment on skin cells are poorly understood. We assessed the capacity of phages to clear *C. acnes* IA₁ and their effects on cell cytotoxicity and growth in HEKa cells- *C. acnes* IA₁ co-culture. Phylotypes IA₁ and IB had similar effects on HEKa cells, causing cytotoxicity and diminishing cell growth. Nevertheless, IA₁ caused a higher impact on cell doubling time by increasing it 1.8 times more than cell growth control group. Even though there are no phages IA₁-specific, we found phages that have a diminished effect on other phylotypes not related to acne. Phage treatment in general reduced IA₁-caused cytotoxicity, with differences in efficacy among phages. In addition, phage purification was necessary to restore metabolic activity and growth of HEKa. Overall, phage evaluation as a therapeutic alternative should include phage-bacteria interactions and their impact on skin cells because of the differences that each phage can exhibit.

1. Introduction

Acne is a multifactorial disease in which the bacterium *Cutibacterium acnes* (formerly *Propionibacterium acnes*) is associated with its pathogenesis (Cong et al., 2019). This bacterium is divided into six phylotypes named IA₁, IA₂, IB, IC, II, and III, being the phylotype IA₁ the most frequently associated with acne (Dagnelie et al., 2018). It is proposed that a microbial imbalance characterized by a reduction in *C. acnes* phylotype diversity is the actual cause of the disease (Dagnelie et al., 2019). In this disbalance, the phylotype IA₁ is usually found enriched in acne patients (Rozas et al., 2021).

Compared to other phylotypes, phylotype IA₁ bears several virulence

factors that are involved in colonization, inflammation, and metabolism of sebum lipids encountered in pilosebaceous units and comedones (Mías et al., 2023). Moreover, *C. acnes* IA₁ interacts with skin cells to induce inflammatory responses (Cong et al., 2019) and can provoke other cell changes, such as deterioration of cell integrity (Spittaels et al., 2020), alterations in cellular junctions (Bolla et al., 2020), and modifications in surface glycosaminoglycans that facilitate bacterial adhesion (Martín et al., 2022).

Various treatment options are available for controlling acne symptoms, including antimicrobials, benzoyl peroxide, azelaic acid, retinol and its derivatives, salicylic acid, clascoterone, hormonal medications, phototherapy, chemical peels, and comedo extraction (Mohsin et al.,

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2022). The use of a single agent or a combination of anti-acne agents depends on acne severity and may result in different outcomes for each patient (Mavranzeouli et al., 2022). Moreover, some acne treatments have adverse effects that negatively influence the course of treatment (Tripathi et al., 2013).

Although the involvement of *C. acnes* as the sole cause of acne remains controversial, antibiotics have been extensively used to treat it (Xu & Li, 2019). Antibiotics are used because they can have a dual effect: topical antibiotics have antibacterial effects on *C. acnes*, and some oral antibiotics could have additional anti-inflammatory properties (Xu & Li, 2019). Nevertheless, antibiotic resistance in *C. acnes* has been reported to be an increasing phenomenon, particularly for topical antibiotics such as erythromycin and clindamycin (Karadag et al., 2020). In addition, antibiotic treatment affects the skin microbiota, which can have a detrimental effect on microbial balance and, therefore, skin homeostasis (Rocha & Bagatin, 2018). Through the loss of microbial balance and selection of antibiotic-resistant bacteria, the skin is prone to colonization by opportunistic pathogens that can worsen acne and other skin pathologies (Karadag et al., 2020).

Given the existence of adverse effects and the progression of antibiotic resistance, it is important to find new alternatives to treat acne. These new alternatives must be innocuous to improve patient safety and compliance with treatment and must consider the microbial balance in the skin to maintain its health (Dessinioti & Dreno, 2020). Phage therapy has been considered as a therapeutic alternative to treat acne, given the specificity of bacteriophages (phages are viruses of bacteria), potential for microbiota modulation, and possible immunomodulatory activity (Castillo et al., 2019; Dessinioti & Dreno, 2020; Farfán et al., 2022; Jończyk-Matysiak et al., 2017). Moreover, phage therapy has been proposed as an option to address antibiotic resistance (Hatfull et al., 2022). Phage therapy consists of the use of lytic phages isolated and characterized for a single bacterial species, which implies no major disturbance to healthy microbiota (Loc-Carrillo & Abedon, 2011). In addition, as bacteria-specialist viruses, phages do not infect eukaryotic cells; therefore, phage therapy is considered virtually innocuous in humans (Rehman et al., 2019).

Studies on *C. acnes* phages and their applications in the treatment of acne are scarce. The available literature on *C. acnes* phages describes how they can be isolated and characterized, typically isolated from healthy skin or susceptible to acne (Castillo et al., 2019; Jończyk-Matysiak et al., 2017; S. Kim et al., 2022). As acne is a skin disease, phage therapy for this condition is conceived as a topical treatment. Some studies have shown that *C. acnes* phages are stable in semi-solid preparations, gels, and liposomes, giving some favorable results (Brown et al. 2016; Rimon et al. 2023; Torres Di Bello et al. 2023). Currently, there is a single clinical trial testing the administration of a three-phage cocktail to patients with mild to moderate acne. This randomized, controlled clinical study showed safety, tolerability, and efficacy in reducing *C. acnes* load in the facial skin (Golembo et al., 2022). However, *in vivo* studies have been conducted in mice and rats. These studies reported a reduction in induced inflammatory lesions and inflammatory markers (Lam et al., 2021a; Rimon et al., 2023). Animal models, although significant, do not retain the physiology and anatomy of human skin.

Although phages are considered harmless to human tissues, they can interact with various human cells. These interactions are not completely understood because there is no single route or receptor that mediates phage-human cell interactions. In addition, these interactions can have varied outcomes depending on the phage and cell type (Bodner et al., 2021; Van Bellegheem et al., 2018). Keratinocytes are the most abundant cells in the epidermis (Arda et al., 2014), and share developmental stem cells with sebocytes (Najafzadeh, Esmaeilzade & Dastan, 2015). Therefore, it is important to assess whether phages can interact with these cells. Previously, our laboratory explored the innocuity of a *C. acnes* phage in HaCaT keratinocytes in free suspension and encapsulated within liposomes and observed that both phage applications were safe for HaCaT cells (Torres Di Bello et al., 2023).

To evaluate the potential application of phages in the treatment of acne, it is important to examine the interaction between phages, bacteria, and skin cells. This evaluation should include the lytic efficacy of the isolated phages against acne-related *C. acnes* strains and possible interactions between *C. acnes* and skin cells. Therefore, it is important to assess the impact of *C. acnes* on the growth and viability of skin cells, and how this impact changes with phage application.

In this study, we explored phage specificity for acne-related *C. acnes* phylotype IA₁. The effect of different *C. acnes* phylotypes on adult Human Epidermal Keratinocytes (HEKa) was assessed to corroborate the involvement of phylotypes in pathogenicity. Finally, *in vitro* phage application to *C. acnes*-inoculated HEKa cells was appraised to measure cytotoxicity and cell growth.

2. Methodology

2.1. Isolation and primary identification of *C. acnes*

C. acnes isolates were obtained from the skin swabs of 10 volunteers. The sampling procedures were approved by the Research Ethics Committee of the Universidad de Los Andes-Colombia, memorandum no. 1130 of 2020. Volunteers were notified of the procedure through informed consent, which was read and accepted prior to the sampling. Volunteers were selected between 18 and 40 years of age, without significant medical records of systemic diseases, male and female, and without acne or with mild acne. Skin swabs were obtained from the cheeks, forehead, and mouth corners using cotton swabs saturated with NaCl 0.8 % (w/v). Additionally, samples from pilosebaceous units were taken with acne strips (A.C.Derma SAS, Medellín, Colombia) and applied to the nose by each volunteer.

Samples were suspended in 9 mL NaCl 0.8 % (p/v), then vigorously shaken for 30 seconds (s). Serial dilutions (1:10) were then made until a dilution factor of 10^{-3} . 0.1 mL of each dilution was plated in a New Medium supplemented with 0.002 % (w/v) bromocresol purple. The new Medium was prepared as described by Kishishita et al. (1980) with the following components: tryptone (1.5 %), agar (1.5 %), yeast extract (0.5 %), brain-heart infusion broth (0.5 %), glycerol (1.0 %), NaCl (0.02 %), K_2HPO_4 (0.02 %), L-cysteine HCl (0.039 %), and polysorbate 80 (0.025 %). Sample dilutions were incubated for 5–7 days at 37°C in anaerobiosis generated by anaerobic gas-generating sachets (Oxoid Anaerogen, Oxoid Ltd, Basingstoke, England). Colonies with the following characteristics were selected: opaque yellow or beige, 0.5–2.5 mm diameter, round-shaped with defined edges, and convex (Kishishita et al., 1980).

Microscopic and biochemical identification were performed for the new isolates and isolates previously obtained in our laboratory. Biochemical identification involved hemolysis in blood agar, indole production, catalase activity, and ribose and sorbitol fermentation (Corvec et al., 2019; Dréno, Bissonnette, et al., 2018; Lomholt & Kilian, 2010).

2.2. Molecular identification of isolates and *C. acnes* phylotypes

Bacterial DNA was extracted using a combined protocol consisting of bacterial lysis in a boiling acidic, sodium dodecyl sulfate (SDS) suspension (Schaub & Dillard, 2017), phenol-chloroform-isoamyl alcohol, and isopropanol precipitation (Natarajan et al., 2016). Briefly, 1 mL of an overnight (ON) *C. acnes* culture grown in broth medium was centrifuged at 5000 gravities (xg) for 10 minutes (min) at 4°C to retrieve the bacterial pellet. The pellet was then washed with 125 μ L of Phosphate Buffered Saline buffer (PBS) (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM), and centrifuged again. The pellet was frozen at -80°C ON. After the freezing step, the pellet was resuspended in 100 μ L Phosphate Buffer (PB) (0.3 % [w/v] $NaH_2PO_4 \cdot H_2O$, 0.1 % [w/v] $Na_2HPO_4 \cdot 7H_2O$; pH= 6.0). Each bacterial cell suspension was then drip-incorporated into a PBS 8 % [w/v] SDS suspension at 98°C. The

incubation tubes were capped with perforated aluminum foil and incubated for 30 min. After boiling, the tubes were placed at room temperature for approximately 7 min to allow cooling. The bacterial suspensions were then centrifuged at 20000 xg for 40 min. The recovered supernatant was washed with 100 uL PBS buffer and centrifuged at 20000 xg for 30 min. Washed supernatant was then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The resulting supernatant was mixed with 0.6X volume of isopropanol and sodium acetate 0.3M (pH 5.2). DNA precipitation was carried out at 4°C for 18-24 hours (h), and the precipitated DNA was concentrated by centrifugation at 16000 xg for 20 min. DNA was finally washed with cold 70 % [v/v] ethanol, centrifuged at 14000 xg and resuspended in type I water. DNA quality was assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific).

New isolates were identified through 16S rRNA gene amplification and Sanger sequencing. The obtained sequences were analyzed using 4Peaks software and MegaBLAST algorithm of the National Center for Biotechnology Information online platform (<https://blast.ncbi.nlm.nih.gov/>).

Phylotypes were identified in all isolates using the Multiplex Polymerase Chain Reaction (PCR) protocol developed by Barnard et al. (2015). This PCR reaction amplifies six genes (16S rRNA partial, *ATPase*, *sodA*, Toxin Fic family, *atpD*, and *recA*) that are useful for identifying each *C. acnes* phylotype (Barnard et al., 2015).

2.3. Preparation of bacteriophages

Nine lytic phages, previously isolated and characterized, were used in this study (Vives Flórez et al., 2018). These phages belong to the Siphoviridae family and have small genomes, as described for most *C. acnes* phages (Castillo et al. 2019). Phages were concentrated by ultracentrifugation at 100000 xg for 2 h. Phage propagation was done using the *C. acnes* Pa6 isolate (Table 1), which was weekly thawed and recovered in agar New Medium. Phages were then propagated using a double-layer plating technique in co-culture with a ON *C. acnes* Pa6 culture (Kropinski et al., 2009). The plates were incubated for 24-36h at 37°C in a 10 % CO₂ atmosphere. Phages were recovered from the top agar, rinsed with 2 mL of SM buffer (NaCl 100mM, MgSO₄·7H₂O 8 mM, Tris-HCl 50 mM, Gelatin 0.002 % w/v), and centrifuged at 5000 xg for 20 min at 4°C. Finally, phage lysates were filtrated through 0.22 µm disposable filters (Santa Cruz Biotechnology, Inc. Dallas TX, USA) and stored at 4°C.

Phage lysates were further purified by cesium chloride (CsCl) gradient ultracentrifugation and dialysis, following the protocols developed by (Luong et al., 2020).

Absence of phage cross contamination was assessed using flow cytometry, according with the protocol developed by Brussaard (2009). Sample preparation consisted in obtaining a 10¹⁰ PFU/mL phage suspension, then fixating it with glutaraldehyde to a final concentration of 0.5 % during 20 mins at 4°C. Fixed samples were frozen at -80°C for at least 12h. Samples were stained with SYBR Green I (Sigma-Aldrich, St. Louis MO, USA), to a final dilution of 5 × 10⁻⁵, incubating at 80°C for 10 mins in dimmed light. Cytometry analysis was performed using a FACs

Canto II cytometer and FACs Diva software (BD Biosciences, San Jose, AC, USA)

2.4. Host range and plating efficiency of *C. acnes* bacteriophages on different *C. acnes* phylotypes

Phages were diluted to a titer of 10⁷ plaque forming units (PFU) per mL in SM Buffer. The host range was qualitatively evaluated using 16 *C. acnes* isolates belonging to different phylotypes. Phage specificity was tested using two bacterial strains previously obtained from skin, *Demacoccus nishinomiyanensis* and *Staphylococcus epidermidis* (Tabares Ruiz, 2012). These two bacterial species are representatives of the normal skin microbiota, as facial oily skin is mainly colonized by *Cutibacterium/Propionibacterium* spp., *S. epidermidis*, and Actinomycetes species, in which *D. nishinomiyaensis* belongs (Byrd et al., 2018; Smythe & Wilkinson, 2023). Also, is expected that *C. acnes* phages are species specific and do not infect other bacterial species present in skin microbiota, as previously reported (Jończyk-Matysiak et al., 2017).

Phage inoculation to each bacterial strain was performed using a spot technique. For this assay, each isolate was incubated in New Medium with 0.25 % agarose and plated in a Petri dish containing 15 mL of New Medium agar. Then, 10 uL of each phage suspension was inoculated directly onto the bacterium-inoculated medium. This was repeated three times for each isolate. The incubation was set at 37°C and 10 % CO₂, for 24-48 h. After incubation, the host range was determined based on the ability of phages to cause lysis zones or plaques.

Plating efficiency (PE) was performed to evaluate the differential lytic activity of *C. acnes* phages on different *C. acnes* phylotypes. For this assay, the selected phages were diluted in SM buffer to obtain a titer of 10⁶ PFU/mL. Serial dilutions with a 1/10 factor were prepared, and each dilution was inoculated in Petri dishes with different *C. acnes* isolates (Table 1). Phage inoculation was performed using the spot technique (Kutter 2009). Plating efficiency was calculated by dividing the titer of each phage obtained in phylotypes not related to acne (II and IB) by the titer obtained in the IA₁ isolates.

2.5. Keratinocyte cell line culture and preparation of bacterial suspensions

The keratinocyte cell line "Adult Human Epidermal Keratinocytes (HEKa- ATCC PCS-200-011)" was generously donated by Dr. Adriana Marcela Celis from the Universidad de Los Andes-Colombia. This cell line was cultivated in Dulbecco's Modified Eagle Medium (DMEM, MP Biomedicals, São Caetano do Sul-SP, Brazil) supplemented with 10 % decomplemented Fetal Bovine Serum (dFBS) (Microgen, Bogotá, Colombia) and 1 % penicillin-streptomycin (10000 U each, Lonza, Bend OR, USA). Cells were incubated at 37°C in a 5 % CO₂ atmosphere for approximately 72 h or until they reached >80 % confluence. Cells were harvested using trypsin 0.25 % (Lonza, Bend OR, USA) and inoculated at a density of 5000 cells per well in 96-well plates. Keratinocytes were incubated at 37°C and 5 % CO₂ for 24 h to reach cell attachment.

Bacterial suspensions were prepared separately using ON cultures of each of the three *C. acnes* isolates belonging to three phylotypes: IA₁ (Pa6), IB (8409 H2), and II (A4). These isolates were used as a representation of the predominant phylotype in acne (IA₁), a related phylotype but not involved in the disease (IB), and a phylotype usually related to healthy skin (II) (Cong et al., 2019). An aliquot of 1mL of each ON was centrifuged, and the pellet was resuspended in 1mL of PBS 1X. Cell suspensions were adjusted to a McFarland standard of 0.5, which represents a bacterial cell density of 10⁷ Colony Forming Units (CFU)/mL. Serial dilutions (1/10 dilution factor) of the adjusted bacterial cell suspension were prepared in DMEM supplemented with 10 % dFBS, without antibiotics or antimycotics.

Table 1
Isolates used to estimate plating efficiency of *C. acnes* phages.

<i>C. acnes</i> isolate	Phylotype
Pa6	IA ₁
Pa1	IA ₁
ParT2	IA ₁
Pa5	IA ₂
8409 H2	IB
C32	IB
A4	II
AleT2	II

2.6. Cytotoxicity of *C. acnes* phylotypes in keratinocytes

Keratinocytes are a major component of skin tissue, especially in the epidermis (Arda et al., 2014). Since the potential application of acne phage therapy is as a topical treatment, mainly keratinocytes would be in contact with *C. acnes* and phages, therefore its importance as a study model. The cytotoxic effect of three phylotypes (IA₁, IB, and II) on keratinocytes was measured at three different bacterial-cell densities (10⁵, 10³, and 10¹ CFU/well), by triplicate. After keratinocyte incubation in 96-well plates, the medium supernatant was discarded and 100 µL of each bacterial cell suspension (prepared as described above) was added to the corresponding wells. Inoculation of attached keratinocytes in fresh DMEM without antibiotic-antimycotic solution was used as a negative control. The inoculated keratinocytes were incubated at 37°C in a 5 % CO₂ atmosphere for 72 h. After the incubation period, the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. Briefly, the supernatant was discarded, and the wells were washed with PBS buffer 1X. MTT reagent was added, and the cells were incubated at 37°C in a 5 % CO₂ atmosphere for 2h. Subsequently, the MTT reagent was removed and formazan crystals were suspended in dimethyl sulfoxide (DMSO). The absorbance was recorded at a 595 nm wavelength. The absorbance measured for the MTT assay represents the metabolic activity of keratinocytes, given that tetrazolium bromide is metabolized by mitochondria (Kumar et al., 2018). Therefore, there is a direct relation between absorbance and metabolic activity. Consequently, we expressed absorbance results as absorbance and as a percentage of metabolic activity measured by MTT. This percentage was calculated using the negative control group (HEKa without inoculation) as a 100 % reference of metabolic activity. A treatment was considered cytotoxic when there was a reduction of more than 30 % of metabolic activity regarding control group.

Independently, the effects of bacterial preparations were also evaluated for *C. acnes* IA₁: cell lysate, bacterial cell surface, and bacterial soluble products. The cell lysate was prepared by temperature shock, heating a bacterial pellet re-suspended in PBS buffer 1X to 98°C for 10 min, and then reducing its temperature with liquid nitrogen for 5 min. This process was performed twice, and the lysate suspension was centrifuged at 6900 xg for 5 min to recover the bacterial cell pellet, which was then resuspended in 1000 µL PBS. The bacterial cell surface was prepared by fixing the bacterial pellets in pure methanol. This pellet was obtained by centrifuging at 6900 xg 1mL of an ON *C. acnes* culture. The supernatant was discarded, and the pellet was resuspended in 100 µL methanol. Methanol was washed with PBS buffer 1X and then centrifuged at 6900 xg for 5 min, conserving the pellet and resuspending it in 1000 µL PBS. Bacterial soluble products were recovered from the ON supernatant by centrifuging 1 mL of the ON *C. acnes* culture broth. Then, a ~500 µL aliquot of the supernatant was recovered in a separate centrifuge tube until use.

Bacterial preparations were separately resuspended in 1mL PBS buffer 1X and inoculated in 2mL PBS buffer 1X solutions, with the same volume used for bacterial adjustment to 0.5 McFarland standard (Absorbance: λ_{625nm} ≈ 0.1). The adjusted preparations were diluted 1/10 in DMEM without antibiotic-antimycotic and inoculated into adherent keratinocytes by triplicate.

2.7. Effect of *C. acnes* phylotypes on keratinocyte growth

Keratinocytes were harvested from cell culture flasks, as previously described. The cells were counted and inoculated into 96-well plates at a density of 2500 cells/well. After incubating for 24 h, the medium was replaced with bacterial cell suspensions of the three *C. acnes* phylotypes (IA₁, IB, and II) using the same procedure described in section 6 of this methodology. As a control, a keratinocyte growth curve was constructed and sampled under the same conditions and at the same time points. Every experimental group in this experiment was performed by triplicate.

For cell counting, the supernatant medium was discarded, and each well was washed twice with PBS buffer 1X. Cells were detached with 20 µL Accumax (1X, Sigma-Aldrich, Saint Louis MO, USA), following manufacturer instructions. Cells were re-suspended in PBS buffer 1X and stained with 0.25 % w/v trypan blue. Cell counting was performed using an automatic cell counter (Corning Cell Counter-Corning, Inc. NY, USA), which reported the number of viable and non-viable cells. Sampling was performed at 0, 24, 48, 72, and 96h post phage and bacteria inoculation.

2.8. Phage inoculation of *C. acnes*-colonized keratinocytes

Four phages were selected, based on their ability to infect and lyse IA₁ isolates, for evaluation of their potential to counteract the effects of *C. acnes* on keratinocytes. Each phage suspension was diluted to 10⁷ PFU/mL in PBS Buffer 1X and two 1/10 serial dilutions were prepared in DMEM without antibiotic-antimycotic.

To measure the effect of phages on keratinocyte cytotoxicity caused by *C. acnes* IA₁, phages and bacteria were inoculated simultaneously in adherent keratinocytes incubated in 96-well plates, by triplicate. Phages and *C. acnes* IA₁ were inoculated at different multiplicities of infections (MOIs): 1, 0.1, and 0.001, decreasing phage titers and maintaining a bacterial cell density of 10⁵ CFU/well. As phage-innocuity controls, phages were also inoculated at a titer of 10⁵ PFU/well in the absence of bacteria. Co-cultures were incubated for 72 h at 37°C in a 5 % CO₂ atmosphere to measure metabolic activity by MTT assay, as previously described.

The impact of phage inoculation on keratinocyte growth was evaluated by inoculating *C. acnes* IA₁ on adherent keratinocytes and, at the same time, the four selected phages. For this experiment, only the MOIs that yielded the best cytotoxicity diminution results for each phage in the previously mentioned experiment were used. Each experimental group was inoculated by triplicate and it was measured viable and non-viable cells at 0, 24, 48, 72, and 96 h after phage and bacterial inoculation.

3. Results

3.1. *C. acnes* phylotype identification of isolates obtained from healthy skin

According to macroscopic identification of the colonies, 22 bacterial isolates were obtained from the skin samples of volunteers; the majority (86.4 %) were isolated from acne strips. Gram-positive bacilli and catalase positivity were the criteria for selecting 10 isolates identified as *C. acnes*. Indole production was positive in all isolates obtained in this study. However, its production varied among isolates previously identified as *C. acnes*, in which 72 % of the isolates tested positive.

The isolates obtained in this study were further identified by 16S rRNA sequencing, which confirmed nine isolates as *C. acnes*. The isolate 1703 T1 was identified as *Bacillus thuringiensis*, an environmental bacterium commonly associated with insects (Beegle & Yamamoto, 1992). Therefore, its identification was classified as sample contamination and was discarded from subsequent experiments.

Hemolysis and fermentation of sorbitol and ribose were used as a screening for phylotype identification, which was confirmed by multiplex-PCR (Table 2). Only hemolysis was useful for screening I phylotypes (IA₁, IA₂, and IB), where complete hemolysis was expected (Corvec et al., 2019; Dréno, Pécastaings, et al., 2018; Lomholt & Kilian, 2010). The majority of previously and new *C. acnes* isolates were identified as phylotype IA₁ (57 %), even though the samples were collected from individuals without acne or with moderate acne. The identified phylotypes were followed by isolates belonging to IB and II (19 % of isolates for each phylotype), and one isolate (5 %) was identified as IA₂.

Table 2
Phylotype identification of *C. acnes* isolates by multiplex PCR.

Isolate	Molecular phylotype identification
1703 T1*	-
ATCC	IA ₁
Pa 1	IA ₁
Pa 6	IA ₁
Pa 7	IA ₁
1A	IA ₁
1703 H1	IA ₁
1703 T2	IA ₁
3924 T2	IA ₁
Par T2	IA ₁
8409 T1	IA ₁
8409 H1	IA ₁
Pa 5	IA ₂
C32	IB
13B	IB
4699 T1.1	IB
8409 H2	IB
Pa 3	II
A.3	II
A.4	II
Ale T2	II

*Although 1703 T1 was identified as *C. acnes* by 16S sequencing, it was confirmed as *Bacillus thuringiensis* by primary identification. Therefore, phylotype identification was not possible for this isolate and it was discarded for future experiments.

3.2. Effects of inoculation with different *C. acnes* phylotypes on keratinocytes

Absorbance was recorded for keratinocytes inoculated with three different isolates representing three phylotypes: Pa6 (IA₁), 8409H2 (IB), and A4 (II) (Fig. 1). The recorded metabolic activity did not change during the first 48 h, regardless of the inoculated phylotype. At 72h, decreased absorbance was detected in keratinocytes inoculated with phylotypes IA₁ and IB. Keratinocytes inoculated with *C. acnes* II did not show decreased absorbance.

The measurable effect of bacterial inoculation was then clear at 72 h.

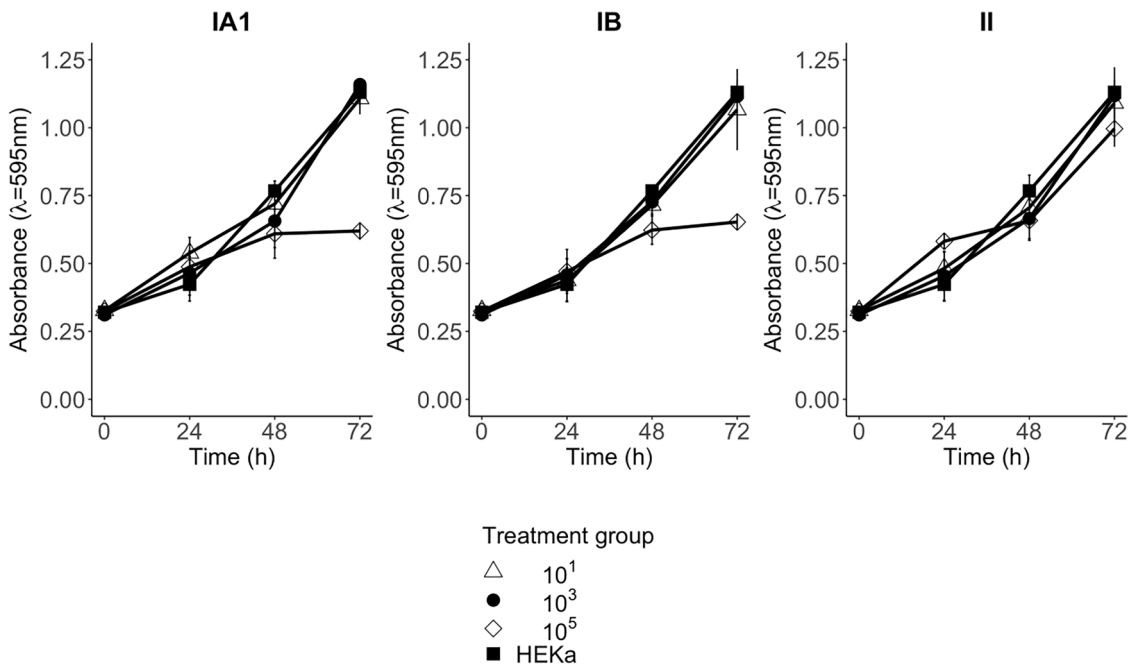


Fig. 1. Absorbance of tetrazolium salts for keratinocytes colonized by phylotypes IA₁, IB, and II. Graphs represent keratinocytes inoculated with three bacterial cell densities (10⁵, 10³, and 10¹ CFU/well) named E5, E3, and E1, respectively, and keratinocytes without bacterial inoculation (HEKa). Error bars represent interquartile ranks (25 %) for each treatment group.

Therefore, we compared the effect of the three phylotypes on keratinocyte cytotoxicity at this time point, taking tetrazolium salts absorbance in non-inoculated keratinocytes as a reference. The absorbance data is presented as a percentage of metabolic activity estimated by MTT, taking the mean absorbance for this group as a 100 % (Fig. 2). Kruskal-Wallis and Dunn tests were performed to evaluate significant differences among the groups, at 72h PI. *C. acnes* II did not cause a significant impact on keratinocytes whereas phylotypes IA₁ and IB did reduce metabolic activity percentage relative to control by 45.2 % and 42.2 % respectively (P-value<0.01). There were no significant differences between these two groups (P-value >0.05). In contrast, *C. acnes* II

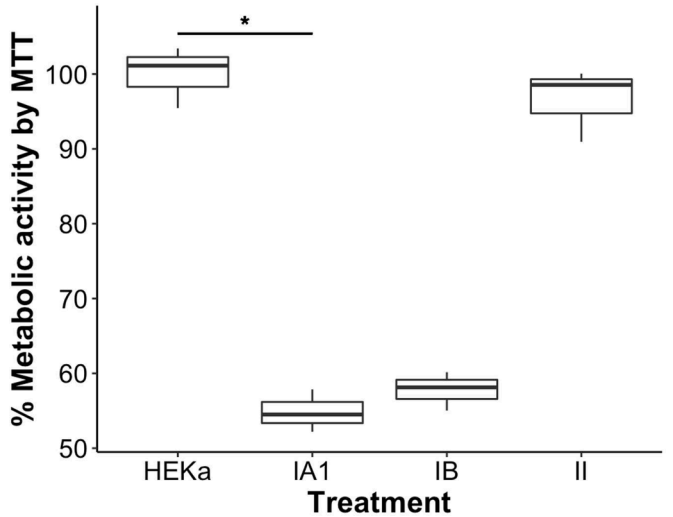


Fig. 2. Percentage of metabolic activity in keratinocytes inoculated with *C. acnes* IA₁, IB, and II, compared to non-inoculated keratinocytes (HEKa). The Kruskal-Wallis test revealed significant differences in the treatment groups (P-value <0.05), which represented differences between keratinocytes colonized with *C. acnes* IA₁ non-colonized (P-value <0.05). Error bars represent the interquartile ranks (25-75 %) of data for each treatment group.

only reduced metabolic activity by 3.5 %, which was not significantly different from that of non-inoculated keratinocytes (P-value >0.05).

Fig. 3 shows the growth curves of keratinocytes colonized with the three tested phylotypes, as well as a growth control group consisting in non-inoculated HEKa keratinocytes. Non-inoculated and inoculated keratinocytes exhibited different behaviors, showing distinct growth rates and different beginnings of the exponential phase. Non-inoculated keratinocytes started the exponential phase at 24 h post-inoculation (PI). In comparison, bacteria-inoculated keratinocytes exhibited delayed exponential phases. The calculated doubling times (DT) during 24 to 48 h for the control group was 12.166 h, in contrast to 29.706 h for IA₁ inoculated keratinocytes, 20.602 h for IB, and 64.179 h for II. After 48 h PI control keratinocytes entered the stationary phase, and the bacteria-inoculated groups showed differences among them. Between 48 to 72 h PI, phylotype IA₁ caused the greatest reduction in cell growth rate (DT_{48-72h}=20.769 h), whereas IB- and II-inoculated keratinocytes had a lower DT (DT_{48-72h}= 14.320 h and 11.018 h, respectively). It is interesting to note the behavior of HEKa cells inoculated with the phylotype II isolate. From 48 h PI, these cells had the lowest DT among all experimental groups, including the cell growth control. In the 72–96 h PI time rank, the phylotype II-inoculated cells continued to proliferate. Cell growth results agree with the MTT experiments, since IA₁ had a higher negative impact on keratinocyte growth, followed by IB. Phylotype II also reduced the growth rate of keratinocytes between 24 to 48 h. However, after 48 h phylotype II-inoculated keratinocytes have a similar DT to the control group.

3.3. Host range and plating efficiency of *C. acnes* phages in different phylotypes

Lytic phages infecting *C. acnes* collected in our laboratory were tested against 14 *C. acnes* isolates and two other skin-related bacterial species (Table 3). The lytic effect of each phage on different bacteria was evaluated as follows: complete lysis in the spot area (+), production of 1 to several isolated plaques (±), and absence of lysis or plaques (-). These results were interpreted as high, reduced, or no activity. None of the tested phages were able to infect *S. epidermidis* and *D. nishinomiyensis*, which confirmed phage species specificity and lack of activity against other skin microbiota bacteria.

The host range was intended to be the main criterion to select phages

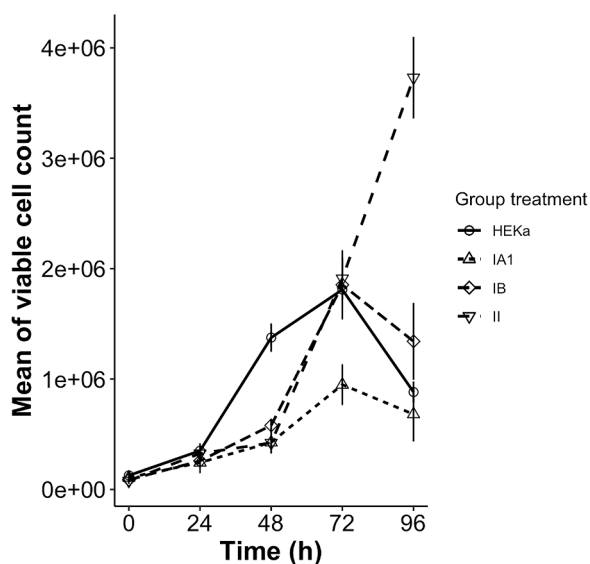


Fig. 3. Cell growth curves of keratinocytes inoculated with different *C. acnes* phylotypes. Cell count represents the mean of three replicates, each of them with four measurements. Error bars represent standard deviation for each group at each sampling point. The cell growth control group was designated as HEKa.

for subsequent experiments. Nevertheless, it was observed that no phages infected only IA₁ isolates. Consequently, the phages $\phi 5$, ϕA , ϕC , and $\phi 7.3$ were selected because of their reduced effect on the IB and II phylotypes, which are not usually related to acne (Lomholt et al., 2017).

Given that none of the tested phages were specific to IA₁ isolates, it was crucial to determine the activity of the selected phages on non-acne-related phylotypes. For this purpose, plating efficiency (PE) values were calculated for phylotypes not related to acne (IA₂, IB, and II), using three different IA₁ isolates as references (Table 4). More than half of PE values for phages ϕA and $\phi 7.3$ were less than 1. These results indicate that these phages have a higher activity against IA₁ phylotype isolates, which makes them good candidates for controlling IA₁ over other phylotypes. In contrast, most PE values for phages ϕC and $\phi 5$ were higher than 1. Therefore, these phages have higher efficacy against non-acne-related phylotypes, which is not desirable for acne treatment. Moreover, PE was also calculated among IA₁ isolates using isolate Pa6 as a reference, as this was used to isolate and characterize all phages. As it is noted, phages ϕC and ϕA have overall higher PEs for the IA₁ tested isolates.

3.4. Effect of bacteriophage inoculation on keratinocytes co-incubated with *C. acnes* IA₁

The results showed that phylotypes IA₁ and IB produced similar effects on the cytotoxicity and growth of keratinocytes. We continued the phage inoculation experiments using only *C. acnes* IA₁ isolate Pa6. To evaluate the effect of phage administration on *C. acnes* IA₁-colonized keratinocytes, we performed an experiment in which selected phages (ϕA , ϕC , $\phi 5$, and $\phi 7.3$) were inoculated to keratinocytes and to IA₁-colonized keratinocytes. The former group assessed the effects of phages on the growth and viability of keratinocytes. For the *C. acnes* IA₁-colonized groups treated with phages, we intended to detect and measure the effect of phage inoculation on bacterial-derived damage. First, the effect of phage inoculation on keratinocyte cytotoxicity was assessed using an MTT assay (Fig. 4). It was found that each phage had a different effect on keratinocytes, both in the absence and presence of bacteria. Phage $\phi 5$ appeared to be relatively innocuous for keratinocytes compared to other phages, since its inoculation into cell culture reduced keratinocyte metabolic activity by 19.63 %. In contrast, inoculation with phages ϕA , ϕC , and $\phi 7.3$, reduced the keratinocyte metabolic activity by more than 30 %. Moreover, phages ϕC and $\phi 7.3$ produced an effect like *C. acnes* IA₁ colonization (46.4 % and 46.8 % respectively).

Application of phages to bacteria-inoculated keratinocytes showed two different effect patterns. Inoculation with phages ϕC and $\phi 7.3$, was related to a higher reduction of keratinocyte cytotoxicity at lower MOIs. In contrast, inoculation with phages ϕA and $\phi 5$ did not show such a behavior in colonized keratinocytes. However, the metabolic activity of these phages slightly increased compared to ϕC and $\phi 7.3$. In all cases, lower MOIs resulted in metabolic activity resembling that of the non-inoculated keratinocytes.

3.5. Comparison between non-purified and purified phage lysates

The phage suspensions used in this study consisted of phage lysates obtained from bacterial cultures. Phage lysates had a cytotoxic effect on keratinocytes (Fig. 4). Nevertheless, when applied to bacteria-colonized keratinocytes, lower phage titers diminished observed cytotoxicity. This suggested the possible presence of bacterial residues in the lysates, given that phage suspension processing was only done by 0.22 μ m filtration. As the phage lysate is diluted to lower MOI values, bacterial cell residuals might also be diluted.

To test this hypothesis, we first evaluated the effect of inoculating keratinocytes with different bacterial preparations. Fig. 5 shows the effect of boiled bacterial lysate, methanol-fixed bacterial cell suspension, and broth supernatant from *C. acnes* IA₁ ON culture. These preparations were intended to evaluate bacterial cell residuals from whole bacterial lysates, superficial bacterial components, and extracellular

Table 3
Qualitative host range of *C. acnes* phages in isolates related to acne and healthy skin.

Bacterial isolates	Phylotype	Phage susceptibility	C3	5	D	A	B	C	7.3	ab
		aa								
1703 T2	IA ₁ *	+	+	+	+	+	+	+	+	+
3924 T2		-	+	±	±	-	±	-	-	-
Par T2		+	+	+	+	+	+	+	+	+
Pa 1		+	+	+	+	+	+	+	+	+
Pa 6		±	+	+	+	+	+	+	+	+
Pa 5	IA ₂ *	+	+	+	+	+	+	+	+	+
4699 T1.1		+	+	+	+	+	+	+	+	+
8409 H2	IB	+	+	+	+	+	+	+	+	+
C32		-	+	±	+	±	+	±	-	-
13B	II	-	+	±	±	-	+	-	-	-
Ale T2		+	+	+	+	+	+	+	+	+
Pa 3		+	+	+	+	+	+	+	+	+
A.3		*	+	+	+	+	+	+	+	+
A.4		-	+	+	+	+	+	+	+	+
<i>Staphylococcus epidermidis</i>	NA	-	-	-	-	-	-	-	-	-
<i>Demacococcus nishinomiyanensis</i>	NA	-	-	-	-	-	-	-	-	-

*Phylotypes usually associated to acne pathogenesis.

Table 4
Plating efficiency (PE) compares the number of PFUs/mL generated by phages in different tested bacterial isolates to the production of PFUs/mL in a reference isolate. In this experiment, we assessed the PE of different *C. acnes* isolates and compared them to reference *C. acnes* IA₁ isolates (ParT2, Pa 1 and Pa6). PE values > 1.00 represent higher activity of selected phages on the tested isolates. In contrast, PE values < 1.00 (highlighted in gray) indicate a better performance of the phages in the reference IA₁ isolate. Comparisons between the IA₁ reference isolates were also made using isolate Pa6 as an absolute reference.

Isolate	Phylotype	φA	φC	φ5	φ7.3
ParT2 (IA ₁)					
Pa6	IA1	0.81	1.76	0.44	0.38
PA5	IA2	0.38	1.56	3.33	0.80
8409H2	IB	1.43	0.70	3.33	1.73
4699T1.1	IB	0.02	0.03	0.28	0.12
AleT2	II	0.84	2.00	4.11	3.20
A4	II	0.18	0.12	0.28	0.16
PA1 (IA ₁)					
PA6	IA1	0.70	0.91	0.70	0.19
PA5	IA2	0.44	3.01	2.09	1.62
8409H2	IB	1.65	1.35	2.09	3.51
4699T1.1	IB	0.02	0.06	0.17	0.24
AleT2	II	0.97	3.87	2.58	6.49
A4	II	0.21	0.23	0.17	0.32
PA6 (IA ₁)					
PA5	IA2	0.31	2.75	1.46	0.30
8409H2	IB	1.16	1.24	1.46	0.65
4699T1.1	IB	0.01	0.06	0.12	0.05
AleT2	II	0.68	3.53	1.80	1.20
A4	II	0.14	0.21	0.12	0.06

products produced by *C. acnes* IA₁ while growing in broth, respectively. These preparations did not contain viable *C. acnes*. All treatments slightly caused a reduction in metabolic activity in keratinocytes: 8.58 % for the supernatant, 11.27 % for boiled lysate, and 18.60 % for the methanol-fixed cell suspension. The effect of these bacterial preparations was significantly inferior to that of viable bacteria, which induced cytotoxicity in keratinocytes by 52.3 %. These results implied that bacterial cell residuals alone do not significantly affect keratinocyte cytotoxicity. Therefore, we assessed the effects of applying purified phage suspensions to *C. acnes* IA₁-colonized keratinocytes. The results of this experiment were compared with those observed for non-purified phage suspensions (Fig. 6). It should be noted that the purified and non-purified suspensions of phage φ5 did not differ. Meanwhile, purified suspensions of phages φA, φC and φ7.3 displayed different effects on keratinocytes. For these suspensions it was recorded an enhanced reduction in the cytotoxic effect of phylotype IA₁ on keratinocytes. Phages φA and φ7.3 had significant differences between purified and

non-purified suspensions, resulting in similar attenuation (P-value<0.01). MTT assay for φA reported a similar metabolic activity to control (92.22 %) at MOI= 0.01, which represents a restauration of keratinocyte cytotoxicity of 44.52 % compared to HEKa cells colonized with *C. acnes* IA₁. Colonized HEKa cells treated with φ7.3 showed the highest metabolic activity similarity to control group (93.52 %) at MOI of 1, indicating an decrease in cytotoxicity of 45.82 % compared to that of *C. acnes* IA₁-colonized HEKa cells. The restoration of cytotoxicity displayed by φC was lower, compared to φA and φ7.3. Apparently, there were no differences between non-purified and purified lysates for 2 of 3 tested MOIs. We found that only treatment of colonized keratinocytes with purified phage at MOI= 0.1 to colonized keratinocytes was significantly different (P-value<0.01) from its non-purified counterpart. Nevertheless, treatment with purified suspension at this MOI resulted in a reduction of 43.46 % of the cytotoxicity recorded for colonized HEKa cells.

Taken together, these results indicate that only viable *C. acnes* induces cytotoxicity in keratinocytes, which can be restored by the application of purified phages. However, each phage has a different effect on keratinocyte cytotoxicity, which must be evaluated separately. Non-purified phage lysates were not effective in reducing the cytotoxicity caused by *C. acnes*.

3.6. Effect of purified phage lysates on the growth rate of *C. acnes*-colonized keratinocytes

To further evaluate how phages reduce the effect of *C. acnes* IA₁ on keratinocytes, we measured the growth of phage-treated keratinocytes. This experiment was performed using only the MOI values that had the best cytotoxicity reduction effect. Therefore, we inoculated the phages as follows: φA at MOI= 0.01; φC at MOI= 0.1; φ7.3 and φ5 at MOI= 1. Consistent with the metabolic activity results, phages had differential effects on *C. acnes* IA₁-inoculated keratinocyte growth rates. As shown in Fig. 7, inoculation with phages φC and φ7.3, resulted in cell counts like those of the growth control group. Phage φA also showed an increased cell count, although it was lower than those observed for phages φC and φ7.3. Nevertheless, these three phages resulted in similar HEKa cell counts after 96h. It is noteworthy that phage φ5 did not show an increase in HEKa cell count to the level of the other phages. Keratinocytes treated with this phage displayed a cell count similar to that of keratinocytes inoculated with *C. acnes* IA₁. However, these two groups were different, given that keratinocytes treated with φ5 began their exponential growth phase earlier. In addition, the doubling time (DT) DT_{24-72h} for the φ5-treated group is higher than the one calculated for *C. acnes* IA₁ colonized keratinocytes (24.154h and 38.412h, respectively). Overall, phage φ5

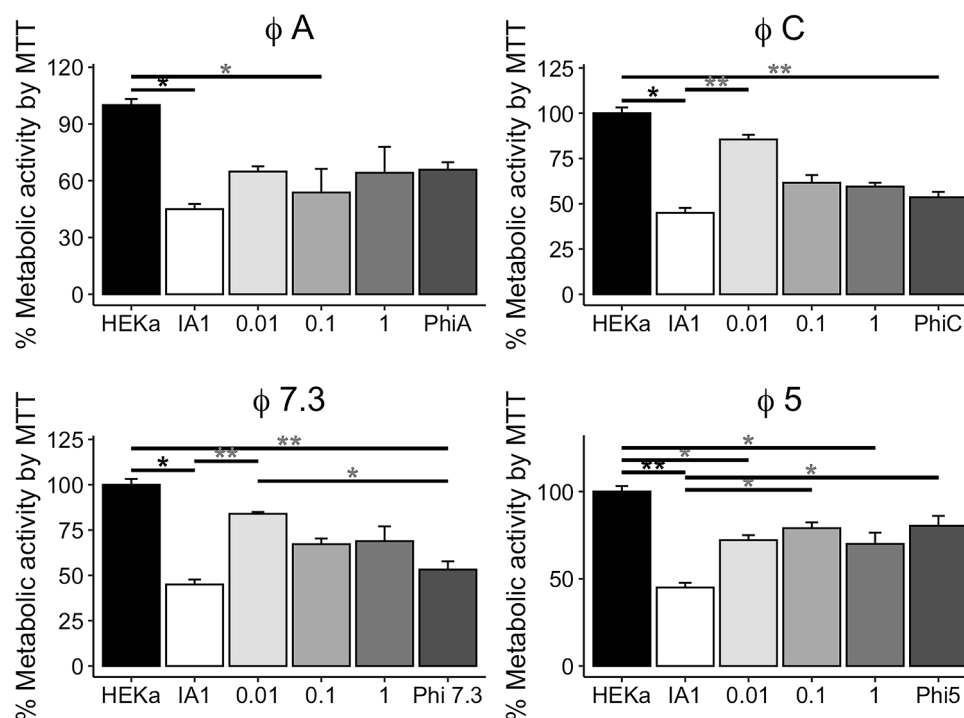


Fig. 4. Effect of phage inoculation on keratinocytes colonized with *C. acnes* IA₁ (isolate Pa6), and treated with four different phages: ϕA , ϕC , $\phi 7.3$, and $\phi 5$, respectively. Non-colonized keratinocytes (HEKa) were used as negative control. The positive control groups consisted in keratinocytes colonized with *C. acnes* IA₁ (IA1) without phage addition. Phage treatments of *C. acnes*-inoculated HEKa cells were performed by testing three different MOIs: 0.01, 0.1, and 1. Finally, the last bar in each plot (left to right) shows phage innocuity, assessed by inoculating HEKa cells with 10^5 PFU/well of each phage. Error bars represent standard deviation for each treatment group. Kruskal-Wallis and Dunn tests were performed to find differences among groups. Bonferroni method was used to calculate adjusted p-values (shown in black stars *P-value<0.05; **P-value<0.01). However, significant differences are also displayed without adjusted P-values (shown in gray stars *P-value<0.05; **P-value<0.01).

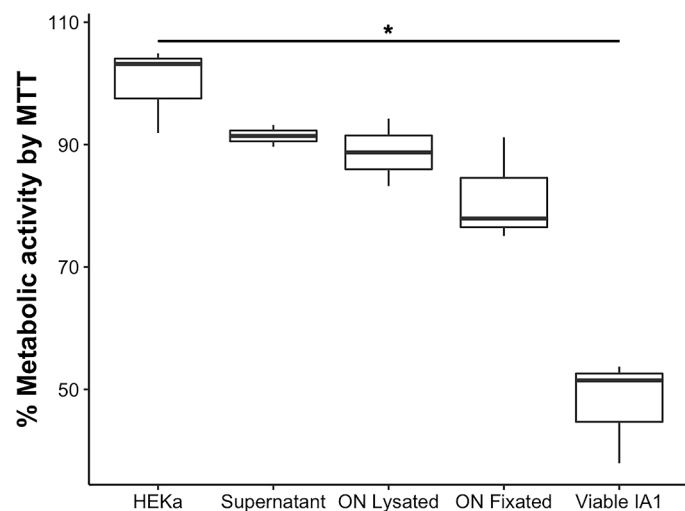


Fig. 5. Inoculation of non-viable bacterial cells and extracellular products in non-colonized keratinocytes. HEKa represents metabolic activity in HEKa keratinocytes without bacterial inoculation, hence it was used as a reference. Viable IA1 indicate HEKa inoculated with *C. acnes* IA₁ 10^5 CFU/well. Error bars in each boxplot represent interquartile ranks (25-75 %) for each treatment group. Kruskal-Wallis test followed by Dunn comparison test was conducted to determine differences between groups. (*P-value<0.05).

had the lowest improvement effect of the tested phages; the other phages presented DTs comparable to those of the negative control group: ϕA = 18.479h, ϕC = 17.490h, $\phi 7.3$ = 15.700h, and HEKa= 15.641h.

Finally, we analyzed the correlation between cytotoxicity and cell growth in phage-treated keratinocytes. According to the Spearman

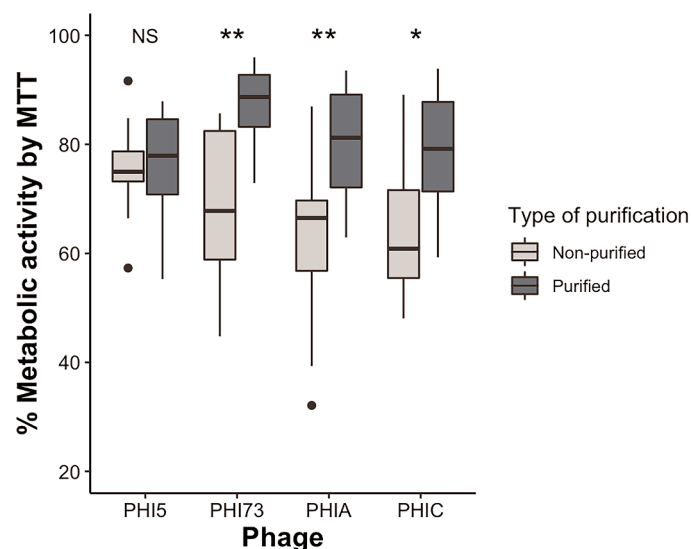


Fig. 6. Comparison of the impact of phage inoculation of non-purified and purified phage suspensions on the cytotoxicity of IA₁-colonized keratinocytes. MTT assays were performed on bacterial-colonized keratinocytes treated with each selected phage prior to purification (boxplots on the left) and after CsCl gradient purification (boxplots on the right). Error bars represent interquartile ranks (25-75 %) for each treatment group. Kruskal-Wallis and Dunn tests were carried out to find statistically significant differences (*P-value<0.05; **P-value<0.01; NS non-significant).

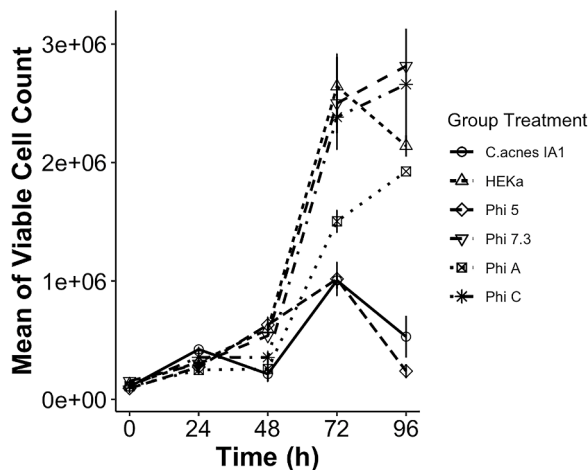


Fig. 7. Cell growth curves of keratinocytes treated with purified phage. Each point represents the mean of the three replicates. Error bars represent standard deviation for the replicates depicted in each point. The negative control group was designated HEKa, and the positive control group was inoculated with 10^5 CFU/well of *C. acnes* IA1 and is indicated as IA1.

correlation test, these two measurements were highly correlated ($\rho=0.864$; $p\text{-value}<0.01$) meaning that metabolic activity increases in treatment groups as HEKa cell number is augmented.

4. Discussion

C. acnes is a commensal bacterium typical of the skin microbiota, but its population disbalance (defined as microbial dysbiosis) is associated with acne (Dréno et al., 2020). Although we did not carry out a *C. acnes* population study, various phylotypes have been isolated from people without acne. Our sampling retrieved more IA₁ isolates, especially those from the acne strips. The strips removed the entire pilosebaceous unit content, which is the main skin niche for *C. acnes* (Fitz-Gibbon et al., 2013). The type I strains (IA₁, IA₂, IB, and IC) are more abundant in acne-affected skin than other strains, probably owing to their virulent potential (Rozas et al., 2021). This potential is represented by the capacity of type I strains to produce virulence factors, bacterial competition factors, and biofilms (Cros et al., 2023).

The ability of *C. acnes* type I isolates to produce virulence factors likely influences their negative impact on the skin and therefore contributes to acne pathogenesis. In contrast, it is expected that other phylotypes do not have such significance, as they are not involved in this skin condition. We assessed the effect of different phylotypes on human keratinocytes by measuring the cytotoxicity caused by the inoculation of type I (IA₁ and IB) strains and phylotype II as a comparison. The tested bacterial cell densities covered CFUs between 10^1 and 10^5 , given a maximum of 10^5 CFU/cm² of *C. acnes* density in the facial skin. This skin type has the highest abundance of *C. acnes* in the body (Leyden et al., 1998). In this study, we found that phylotype IA₁ caused cytotoxicity at a density of 10^5 CFU/5000 HEKa cells. Similar results were obtained for phylotype IB, although this phylotype was not widely correlated with acne. Inoculation of keratinocytes with these phylotypes at a cell density of 10^5 CFU/5000 HEKa diminished about 40–50 % of the absorbance of tetrazolium salts of non-inoculated HEKa. Moreover, an impact on keratinocyte growth and survival was detected. HEKa cells inoculated with *C. acnes* IA₁ showed a reduced growth rate compared to non-inoculated keratinocytes. Between 24 and 48h of co-incubation, *C. acnes* IA₁ inoculated keratinocytes had a DT about 1.8 times delayed compared to non-inoculated keratinocytes.

The role of phylotype IB in acne is unclear, but it could be involved, given that it also produces virulence factors associated with cutaneous disease (Dekio et al., 2021). Our results showed that phylotype IB

impacts keratinocyte growth and cytotoxicity, similar to IA₁. Nevertheless, the characteristics of each phylotype distinctively impact their relation to acne. The IA₁ and IB phylotypes produce the Christie-Atkins-Munch-Peterson (CAMP) factor, which is involved in acne pathogenesis (McLaughlin et al., 2019). However, each phylotype produces a different CAMP factor, and its production by IB varies with changes in growing conditions (it is reduced in oxygen-rich atmospheres) (Dekio et al., 2013). On the other hand, the IA₁ phylotype can produce additional factors that can lead to a higher pathogenic potential in acne. Superficial dermatan-sulfate binding adhesins are expressed in IA₁, but not in the IB phylotype. These adhesins play inflammatory signaling roles and can be linked to acne pathogenesis and recurrence (McDowell et al., 2011). These differences account for the disparities in phylogenetic relatedness and pathogenic potential. Although IA₁ and IB are classified in a single clade (type I or *C. acnes* subsp. *acnes*), IA lineages have diverged from IB, and the latter is more involved in deep-tissue opportunistic infections (Dekio et al., 2021; McDowell et al., 2012). Moreover, the difference in their pathogenic potential may be due to the low capacity of IB isolates to form biofilms (Kuehnast et al., 2018). This factor is relevant to acne pathogenesis because it can help in *C. acnes* attachment and survival, eventually leading to dysbiosis represented by augmented IA₁ richness (Coenye et al., 2022). These differences were reflected in our study; although IA₁ and IB produced similar effects on keratinocyte cytotoxicity and growth rate, the IA₁ isolate produced a sharper effect. DTs reported for IB were lower than those for IA₁-colonized keratinocytes, then keratinocytes inoculated with IB grow at a higher rate compared to cells inoculated with IA₁. This implies that the former phylotype has a lower impact on the keratinocyte growth rate. Therefore, its pathogenic potential is lower despite the recorded cytotoxicity. Moreover, this growth rate can be analogous to keratinocyte growth without bacteria, regardless of the difference at the beginning of their exponential growth phase. This study presents novel *in vitro* data regarding *C. acnes* virulence. However, microbiota interactions and the potential of developing virulence factors in future studies should be considered in a skin-like setting.

Inoculation with phylotype II showed interesting results, as increased cell growth was observed after reaching the logarithmic growth phase. In contrast, phylotypes IA₁ and IB showed reduced cell growth rates. Previous studies have found that *C. acnes* can increase keratinocyte proliferation, which can be linked to comedogenesis (Isard et al., 2011; Yu et al., 2024; Zhu et al., 2020). Cell proliferation was observed alongside pro-inflammatory cytokine expression (Zhu et al., 2020). Remarkably, the expression of interleukin 8 (IL-8) was associated with keratinocyte proliferation by activating the AKT Kinase/ Forkhead box transcription factors (FOXO1) that lead to proliferation and differentiation of keratinocytes (Yu et al., 2024). Also, inoculation of skin explants with membrane extracts of *C. acnes* induces Insulin-like Growth Factor and its receptor. The expression of these markers was also associated with an increase in Ki-67 and filaggrin, which have an inductive role in keratinocyte proliferation and differentiation (Isard et al., 2011; Jarrouse et al., 2007). None of the beforementioned studies reported the *C. acnes* phylotype used. Therefore, it is possible that phylotype II isolates were employed in these experiments. Our results indicate a potential role of *C. acnes* phylotype II in acne pathogenesis, which must be further explored.

These results suggest that the bacterial component of acne pathogenesis is more likely in the dysbiosis scenario than the sole interaction between skin cells and a given phylotype. The role of *C. acnes* in acne pathogenesis represents a particular case of Koch's postulates due to the colonization particularities of this bacterium. Healthy skin has a balanced co-existence of *C. acnes* diverse phylotypes, other commensal bacteria, and skin cells (Byrd, Belkaid & Segre 2018). However, in acne-affected skin, dysbiosis results in increased inflammation, which can be derived from the phylotype IA₁ impact on skin cells (Dagnelie et al., 2019). Particularly, cytotoxicity is linked to activating inflammatory pathways and cell damage. Binding of *C. acnes* antigens to

Toll-like receptors can lead to pyroptosis (Chen et al., 2023), and loss of cell integrity by disintegrating tight junction proteins (Bolla et al., 2020). Moreover, other phylotypes, in a dysbiosis scenario, can also trigger other pathogenic determinants, such as keratinocyte proliferation. These negative impacts on skin may be only presented in dysbiosis, in which *C. acnes* populations are increased in comparison to other bacterial species; It is important to recall that all *C. acnes* isolates induced in HEKa cells delayed the beginning of the logarithmic growth phase, at a density of 10^5 CFU/2500 cells. In homeostatic conditions, other phylotypes, particularly II and III, have important roles in maintaining skin microbial homeostasis (Rozas et al., 2021).

There are studies that have addressed phage therapy to treat acne, focusing on the ability of phages to infect *C. acnes*, possible topic applications, and *in vivo* assays in mice (Brüggemann & Lood, 2013; Castillo et al., 2019; Kim et al., 2019; Lam et al., 2021b). Nevertheless, phylotype specificity of phages in the context of phage therapy has not been extensively studied.

We hypothesized that by reducing the IA₁ population through phage infection, microbial dysbiosis would be restored and acne symptoms would be reduced. Phages may modulate the skin microbiota at the strain level, it has been reported that healthy skin has greater phage abundance compared to acne-affected skin (Barnard et al., 2016). Also, phylotype II strains have CRISPR systems that make them more resistant to phages than type I phylotypes (Knödlseider et al., 2022; Liu et al., 2015). Consequently, phage therapy to treat acne must resemble a healthy skin microbiota, by controlling phylotype IA₁ population (Liu et al., 2015). Therefore, one of the objectives of this study was to test for the presence of phylotype-specific phages with reduced or no lytic activity on other phylotypes. The host range for our collection of *C. acnes* phages did not show such specificity. Broad lytic activity was observed in the tested isolates, with a few exceptions being resistant to various phages. Isolates 3924 T2 (IA₁) and 13 B (IB) were resistant to most phages and, to a lesser extent, isolate C32 (IB). Interestingly, all phages were able to infect phylotype II isolates. This indicates that resistance to phages is not necessarily associated with phylotypes, but with specific isolates. However, the first approach to evaluate phage specificity was qualitative. Consequently, we also carried out plating efficiency for non-acne related isolates to quantitatively check phage activity in the isolated phylotypes. This assay was performed for the four selected phages that did not infect two of the four IB isolates. Despite their similarities in host range, these phages showed differences in plating efficiency. Phage ϕ A had the lowest plating efficiency in non-IA₁ isolates, followed by phage ϕ 7.3. Furthermore, ϕ A obtained higher plating efficiencies for IA₁ isolates while phage ϕ 7.3 was less effective infecting these *C. acnes* isolates. These plating efficiency results are of interest for potential phage therapy applications. Although *C. acnes* phages have little genomic and morphological diversity (Liu et al., 2015; Marinelli et al., 2012), they bear differential plating efficiencies, although this does may not depend on phylotypes. We did not study why these differences appeared, but variations in genome structure or organization that contribute to differential plating efficiency might exist (Azam & Tanji, 2019; Laanto et al., 2017). It is also plausible that *C. acnes* isolates can harbor mechanisms that prevent or reduce phage infection (Liu et al., 2015), which is beyond the scope of this study. This last hypothesis could be favored, given that we found here that phage resistance may depend on *C. acnes* isolates. These results indicate that variations in plating efficiency for IA₁ and other phylotypes should be investigated to formulate phage therapy. It might be interesting to characterize phage infectiveness for *C. acnes* isolates on a case-to-case basis to determine the benefits of a particular set of phages for each potential patient. This approach will place phage therapy as precision medicine, a set of personalized therapies that are relevant to complex and microbiota-related diseases (Annese & Annese, 2023; Park et al., 2022; Perelló-Reus et al., 2022; Ryu et al., 2021; Wu et al., 2020).

Beyond the infectious properties of phages to host bacteria, we also explored differences in phage interactions with skin cells that may

impact therapeutic outcomes. In particular, the extent to which phages reduce the impact of *C. acnes* IA₁ on keratinocytes, and whether phages are innocuous to these skin cells. It was determined that *C. acnes* IA₁ was found to have cytotoxic and antiproliferative effects on HEKa cells, which concurs with literature about virulence factors in the phylotype IA₁. Next, we explored whether phage inoculation reduced these negative cellular effects. After analyzing the results of the first experiment, we theorized that phage suspensions may contain bacterial debris that can induce negative effects in HEKa cells. Given that the tested phages are lytic, they cause complete bacterial lysis and can release toxins from the cytoplasm or surface of *C. acnes* (Mayslich et al., 2021). It was observed that purified phage lysates had overall better performance, reducing the cytotoxic effect caused by *C. acnes* IA₁ inoculation. In addition, purified phage suspensions did not induce significantly HEKa cytotoxicity, compared to their non-purified counterparts, especially phages ϕ C and ϕ 7.3. An exception to this observation was phage ϕ 5, which had a similar cytotoxic effect regardless of its purification. Furthermore, non-viable bacterial preparations had a significant cytotoxic effect on HEKa. These results indicate that certain bacterial products are likely to be produced during phage infection, which can have a potential negative effect on keratinocytes. Moreover, similar bacterial products might be produced in the *C. acnes* IA₁-HEKa co-culture. These products could be bacterial virulence factors produced under stress conditions, represented by phage attack on bacteria and/or skin cells' response to microbial colonization. However, this hypothesis remains unproven.

Treatment of *C. acnes* IA₁-inoculated HEKa cells with purified phage reduced the negative impact of bacteria. However, each phage has a distinctive behavior in terms of titer, which has the best cytotoxic-reduction activity. Similar results were obtained from the cell counting assays. Remarkably, phages ϕ C and ϕ 7.3, and to a lower extent ϕ A, ameliorated the cell growth of *C. acnes* IA₁-inoculated keratinocytes. These phages can effectively mitigate the negative effects on bacteria by reducing cytotoxicity and restoring cell growth. Phage ϕ 5 poorly reduced the bacterial effects on keratinocytes. Consequently, this phage is not suitable for future therapeutic applications. All phages were able to reduce *C. acnes* inoculum in co-culture with keratinocytes (Supplementary Information S1).

Translated to a therapeutic application, phages are not only innocuous but can also reestablish keratinocyte cell growth over the course of treatment. This restoration effect was demonstrated to be dependent on the isolated phage and bacteria-phage ratio, supporting that differences among *C. acnes* phages are more profound than their genomic and morphological similarities. These differences also account for differences in their bacteria-host cell/tissue-phage interactions, which are meaningful for phage therapy. Therefore, it is necessary to address phage therapy for acne by combining classical phage studies with its host bacterium and phage-skin cell interactions. Consequently, phage therapy studies must evaluate not only the capacity of these agents to infect and lyse bacteria, but also consider the interactions between human cells in *in vitro* studies that involve this three-partite interaction. Phages may interact with keratinocytes, and these interactions are distinct from each other. For instance, Shan et al. (2018) reported that phages can adhere to and colonize human colon cells *in vitro*, providing a niche where they can infect *Clostridium difficile* more efficiently. This phage-colon cell line interaction was described to be phage-specific, suggesting that their adhesion to cells depends on specific interactions between phage proteins and human cell receptors (Shan et al., 2018). To the best of our knowledge, the interactions of *C. acnes* phages with skin cells have not yet been studied. Therefore, this study provides the first exploration of the interactions between phages, bacteria, and skin cells in the context of phage therapy to treat acne.

5. Conclusions

In this study, we found that *C. acnes* impacts keratinocyte

cytotoxicity depending on the inoculated bacterial phylotype. Phylotypes IA₁ and IB induced cytotoxicity in HEKa keratinocytes, whereas phylotype II had no measurable impact. Despite this, all three phylotypes can affect keratinocyte growth, given the recorded delay at the beginning of the exponential growth phase when HEKa cells were inoculated with *C. acnes*. Phylotypes IA₁ and IB have important effects on cellular growth between 72 and 96h of co-culture, with IA₁ inoculation being the most detrimental for HEKa cell growth.

It is possible to select phages with higher lytic efficiency on IA₁, although the tested phages did not show phylotype specificity. Besides, phage application on *C. acnes* IA₁-inoculated keratinocytes restored cellular growth and reduced cytotoxicity compared to non-inoculated HEKa cells. This is achieved for most phages by purifying phage suspensions, which likely reduces bacterial by-products that can affect cellular growth and metabolism. This recovery effect was different for each phage at different concentrations.

In principle, the objective of phage therapy is to reduce pathogenic bacterial populations. Nevertheless, phages interact with human cells in ways that are not completely understood, and this interaction could be beneficial for successful therapy. Phage therapy to treat acne encloses the lytic effect of phages on *C. acnes* and their interactions with skin cells. Therefore, it is important to evaluate bacterial-phage-skin cell interactions to better understand the potential of phage therapy to treat acne. It is still pending to investigate the extent to which phage-derived lysis of bacteria is involved in the cellular attenuation effect, given that each phage also influences keratinocyte cytotoxicity. Additionally, the role of microbiota interaction in acne phage therapy should be considered because phylotypes may also influence keratinocyte growth without prompting cytotoxicity.

CRedit authorship contribution statement

Juan C Farfán-Esquivel: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **María Victoria Gutiérrez:** Formal analysis, Investigation. **Alejandro Ondo-Méndez:** Methodology, Resources, Supervision, Writing – review & editing. **John M González:** Conceptualization, Methodology, Resources, Supervision, Writing – review & editing. **Martha J Vives-Flórez:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Martha Vives reports a relationship with SciPhage that includes: board membership and consulting or advisory. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics statement

This study was performed in accordance with the Declaration of Helsinki and the Nuremberg Code. Human primary cell lines included in this study were approved as part of this study protocol. Collection of human samples for this study was approved as part of the study protocol. This human study was approved by Research Ethics Committee of the Universidad de Los Andes-Colombia - approval: memorandum no. 1130 of 2020. All adult participants provided written informed consent to participate in this study.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.crmicr.2025.100356](https://doi.org/10.1016/j.crmicr.2025.100356).

Data availability

Data will be made available on request.

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