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ORIGINAL ARTICLE

Characterization of a live *Cutibacterium acnes* subspecies *defendens* strain XYCM42 and clinical assessment as a topical regimen for general skin health and cosmesis

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Abstract

Background: When formulating topical products to treat skin diseases and addressing general skin health and cosmesis, most of the focus has traditionally been placed on how any given ingredient may impact the structure, function, and health of human skin elements. However, recent research is beginning to highlight the importance of the skin microbiome in relation to certain skin conditions and general cosmesis. *Cutibacterium acnes* is one of the most prolific skin-specific bacterial species. Research has shown that the species is divided into subspecies, some of which are thought to be beneficial to the skin. This paper aims to determine the efficacy of strainXYCM42, a *C. acnes* subspecies *defendens* derived strain designed to improve the health and appearance of the skin.

Methods: In vitro studies were performed on human keratinocyte and fibroblast monolayers, human peripheral blood mononuclear cells (PBMC), and skin explants to elucidate the effects of live XYCM42 cells and their ferment on human skin cells and tissues. Subsequently, clinical studies were performed using XYCM42-based topical regimens designed to deliver and support the engraftment of live XYCM42 cells onto subjects' skin. Two studies were performed, a 3-week pilot study ($n = 10$) and a 8-week pivotal study ($n = 121$). In the latter, 32 subjects were enrolled for an in-clinic portion for efficacy evaluation, with clinic visits occurring at Baseline, Week 1, Week 4, and Week 8.

Results: In vitro data suggest that XYCM42 and its ferment filtrate have potential to provide benefits to the skin via antioxidant, anti-inflammatory, and select antimicrobial activities. Clinical observation demonstrated that a XYCM42-containing regimen supports a healthy skin environment, promotes increased skin hydration, decreases erythema, calms the skin, and regulates sebum production.

Conclusion: These studies provide further evidence that specific strains of *C. acnes*, such as XYCM42, have a more beneficial function regarding skin health and

Mun Su Rhee and Mona L. Alqam contributed equally to the manuscript and share first authorship.

appearance than was previously thought. Appropriate use of formulations derived from symbiotic strains within the skin microbiome can support the development of novel, beneficial topicals.

KEYWORDS
Cutibacterium acnes, *defendens*, microbiome, skin probiotic, topical skin products

1 | INTRODUCTION

When formulating topical products to treat skin diseases and to address general skin health and cosmesis, most of the focus has traditionally been placed on how any given ingredient may impact the structure, function, and overall health of human skin elements. This includes both cellular (keratinocytes, fibroblasts, dendritic cells, etc.) and structural (extracellular makeup, barrier function, etc.) components of the skin.[1] While these are important considerations when formulating topical skin products, what has been relatively unappreciated until recently is the effects of said ingredients and formulations on the skin microbiome and how they may affect skin microflora which may then in turn impact overall skin health both acutely and chronically.[2] Formulation strategies have slowly begun to shift recently as the way in which topical ingredients may indirectly affect skin via the microbiome has become an area of more interest. Such a shift is due in part to a growing body of evidence reinforcing the idea that the human body depends on symbiosis with its skin microflora. As human skin houses many millions of microbes per square centimeter,[2] and copious amounts of small molecules, carbohydrates, peptides, and enzymes that they secrete constitutively have been observed to directly affect skin health in both positive and negative ways; the importance of impacting the overall skin biome (i.e., the combination of the human host cells, the microbiome, and their environment) becomes much more salient for consideration when formulating topical skin products.

For this reason, scientists have begun to focus not only on the composition of any given skin microbiome and its association with skin health or disease but also on the modulation of the microbiome constituents via topical ingredients and how this too might affect skin health.[3] While no agreed-upon specific composition of microbes constitutes a "healthy microbiome," research shows strong associations between skin health or disease and levels of skin microbe diversity, richness, and the presence or absence of select microbial strains.[4] It should be noted, however, that despite such associations there is still variability due to the diversity of human genetics between individuals. Interestingly, while it was previously assumed that some skin disease states were associated with the presence of certain pathogenic microbial species, it is now known that the effects of microbes on the human host can be subspecies/strain specific. For example, some *Staphylococcus aureus* strains populate atopic dermatitis lesions and cause inflammation in animal models. In contrast, genetically distinct strains of the same species do not exhibit the same pathogenic properties on non-lesioned skin.[5]

Another microbial species with strain-specific effects is *Cutibacterium acnes* (*C. acnes*, formerly *Propionibacterium acnes*),[6] one of the most abundant microbes in the human skin microbiome. This species accounts on average for 89% of the bacteria in sebaceous skin follicles, depending on the skin area sampled.[7] There are many genetically distinct strains of *C. acnes*, some of which are more associated with skin disease, some more associated with skin health, and some equally associated with both disease and health.[8] Because the differences genetically and phenotypically have been observed to be so significant between groupings of *C. acnes* strains, the species has been divided into three main phylotypes: phylotype I as *C. acnes* subsp. *acnes*, phylotype II as *C. acnes* subsp. *defendens*, and phylotype III as *C. acnes* subsp. *elongatum*. Recent research has suggested that strains more associated with health come from the *C. acnes* subsp. *defendens* phylotype.[8–10]

While *C. acnes* as a species tend to have some universal characteristics (e.g., propionic acid [PA] production), health-associated *C. acnes* subsp. *defendens* strains have been observed to have distinct protective characteristics that set them apart from their disease-associated relatives. These include differences in their genetic makeup (e.g., CRISPR elements in some health-associated strains), genes or gene expression (e.g., differences in the expression of the CAMP genes and types/existence of bacteriocin genes), and how they interact with human cells (e.g., select strains modulate $CD4^+$ cells to produce antimicrobial peptides).[11,12] As such, it would be reductive to assume that all strains within the species would share equal culpability as to skin disease or credit as to skin health. While the role of the *C. acnes* species in skin health and disease is still being elucidated, many still consider the species to play a pivotal role in the pathology of acne. However, while this may be the case for some disease-associated strains, there is growing evidence that the colonization of protective strains of *C. acnes* on the skin is not only not associated with inflammatory issues like acne but can actually reduce acneic symptoms.[10] Additionally, certain protective strains of *C. acnes* subsp. *defendens* can confer significant benefits to the skin through modulation of skin and immune cell activity, secretion of protective substances (such as unique bacteriocins, antioxidants, anti-inflammatory cytokines, and short-chain fatty acids [SCFAs]), and secretion of little-to-none of pathogenic molecules that their disease-associated counterparts may produce (e.g., porphyrins).[6]

As any given permutation of the many pathogenic, commensal, or protective strains of the *C. acnes* species may inhabit the skin, select protective strains of *C. acnes* subsp. *defendens* could be a

viable candidate for legitimate skin probiotics to (1) help outcompete pathogenic microbes, (2) help mitigate inflammatory skin issues, and (3) act as an effective conservator of general skin cosmesis.[7] Here, we describe the characterization of a modified *C. acnes* subsp. *defendens* strain, XYCM42, and its ferment as well as clinical assessment of topical application of both the live strain and ferment for improving skin health and appearance.

2 | MATERIALS AND METHODS

2.1 | Strain isolation and characterization

2.1.1 | Materials

Biochemicals, organic, and inorganic chemicals were acquired from Sigma Chemical Co. and Fisher Scientific. L-Ascorbic acid and all trans retinoic acid were obtained from Millipore Sigma. Adult human dermal fibroblasts (HDFa), fibroblast growth medium, and supplement kit were purchased from Promocell GmbH. Glassware and plasticware were from VWR and Fisher Scientific.

2.1.2 | Microbe isolation and culture

Cutibacterium acnes subspecies *defendens* strains were isolated from human skin via swab and anaerobic cell culture. Strain ribotype was determined by 16S sequencing. All *C. acnes* strains were cultured anaerobically in brain heart infusion broth (BHI) at 37°C until the late log phase. Candidate RT6 strains were cultured and transformed according to the procedure described by Rhee with minor changes.[11] Creation of a derivative strain from wild-type RT6 *C. acnes* subsp. *defendens* strain (XYCW1) was done by inserting a genetic switch via an inducible promoter in front of the *ftsAZ* operon, upon obtaining a stable transformant the derivative strain XYCM41 allowed for control of bacterial cell division (Figure 1). No antibiotic resistance gene was used as a selection marker for the manufacturing of clinical products via the same methods (XYCM42). We used a modified reinforced clostridial medium to culture bacteria after strain isolation/transformation.

2.1.3 | Biopsy preparation and culture

Obtained human skin biopsies from skin explants post facelift surgery (Faceplus) were prepared using a 3 mm biopsy punch (Miltex). Punched biopsies were inserted into transwells (0.4 μm pore size; Corning Incorporated) and cultured with keratinocyte medium (Promocell) in 12-well plates at 37°C.

2.1.4 | PBMC isolation and culture

Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy individual whole blood (Pooled; Zenbio) and acne patient blood (Faceplus) using a density gradient (Ficoll-plaque; GE Healthcare Life Science). Isolated PBMCs were cultured in RPMI 1640 medium supplemented with penicillin–streptomycin, and 10% fetal bovine serum for 24 h, then changed to antibiotics free RPMI 1640 medium. PBMCs were exposed to *C. acnes* strains for 24 h following an initial treatment with peptidogylcan (PGN, 10 μg/ml) for 3 h.

2.1.5 | Detection of antioxidant activity

To assess the potency of antioxidant activity in the ferment of *C. acnes* subsp. *defendens* strains, cells were grown in BHI for 7 days.

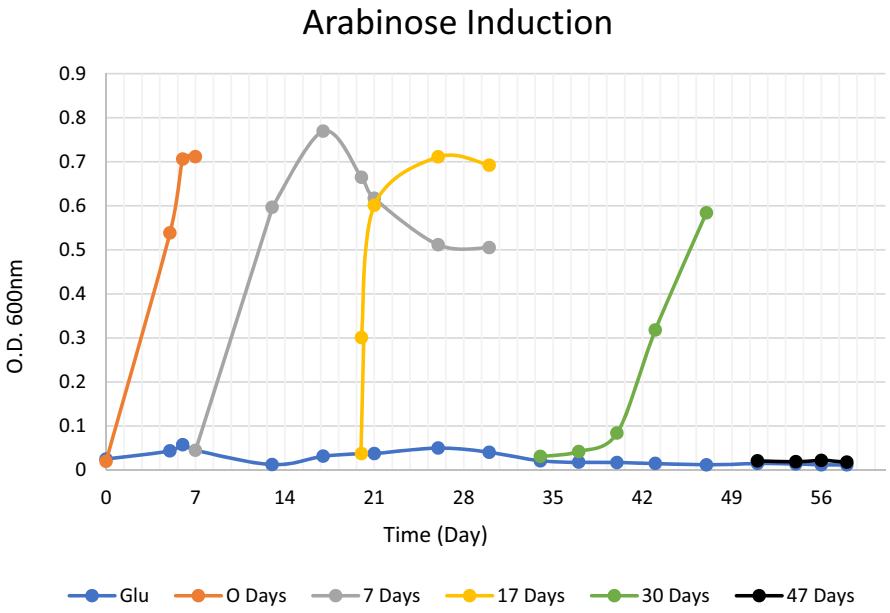

FIGURE 1 Growth-arrest strain XYCM41 was inoculated into mRCM with Glucose (•) or Arabinose after 0 (•), 7 (•), 17 (•), 30 (•), and 47 (•) days of incubation in mRCM. When cells were inoculated into mRCM with arabinose, more typical growth was observed through Day 30. Cells did not respond when given arabinose on Day 47. This demonstrates that the growth-arrested cells remain metabolically viable without cell division for at least 30 days. mRCM, modified reinforced clostridial medium

Since components of the growth media had antioxidant properties, 5.7×10^7 colony forming unit (CFU)/ml cells were washed twice in phosphate-buffered saline (PBS) and incubated in 1 ml of PBS at 37°C for 90 min. The antioxidant activity of the PBS in which cells were resuspended was then measured (Figure 2) via the OxiSelect Trolox Equivalent Antioxidant Capacity (TEAC) Assay. The TEAC assay kit measures the amount of antioxidants against a Trolox standard, a vitamin E-analogous antioxidant. The TEAC kit is standardized by adding oxidized 2,2′-azinobis[3-ethyl benzothiazoline-6-sulfonic acid] substrate to Trolox and measuring the absorbance. After measuring the antioxidant activity of *C. acnes* subsp. *defendens* against the Trolox standard, we calculated the equivalent antioxidant activity of ascorbic acid by using the following formula.

$$\left[\left(A_{(\text{ascorbic acidO.D.})} / A_{(\text{TroloxO.D})}\right) \times C.\,acnes_{\text{antioxidant activity}}\right].$$

Comparing gene expression in HDFa cell lines

The effects of XYCM42 cells (10^7 CFU/ml) on the human dermal fibroblasts cell lines were compared to that of L-ascorbic acid (100 μM) and All trans retinoic acid (8 μM) after 6 and 24 h of treatment at 37°C. This was determined by evaluating changes in gene expression of COL1A1, COL3A1, TNF-α, IGF-1, and SIRT-1 via qRT-PCR.

2.1.6 | Cytokines enzyme linked immunosorbent assay

The supernatant from control and XYCM42-treated PBMCs were collected and stored at −80°C. For the assay, samples were thawed and centrifuged, and each cytokine level was measured using an ELISA kit (R&D Systems) per the manufacturer's instructions. All experiments were performed on at least two separate occasions.

2.2 | Topical formula development

All formulas were individually tested for effects on strain XYCM42 by mixing with liquid cultures, plating on agar and incubating in anaerobic conditions at 37°C for 7 days to observe growth. Water-based formulas are pH balanced to ensure the skin barrier is not disturbed.

[Correction added on 24 November 2022 after first online publication: The section heading '2.2 Topiformula development' has been updated to '2.2 Topical formula development'.]

2.3 | Clinical (8-week study)

2.3.1 | Study design

This 8-week claims substantiation study was conducted at four locations; WI (Milwaukee), NY (Harrison), California (LA), and TX (Dallas), and the in-clinical efficacy evaluation was conducted at the Crown Laboratories Research Clinic in Dallas, TX. A commercial institutional review board approved the protocol and informed consent form (IntegReview). Subjects were enrolled in the clinical study if they were generally healthy women between the ages of 18 and 70, and if they were willing to discontinue all other facial skincare and topical products 3 days before the start and during the study duration. Pregnant or nursing women were excluded. Subjects were required to withhold the use of any medications or

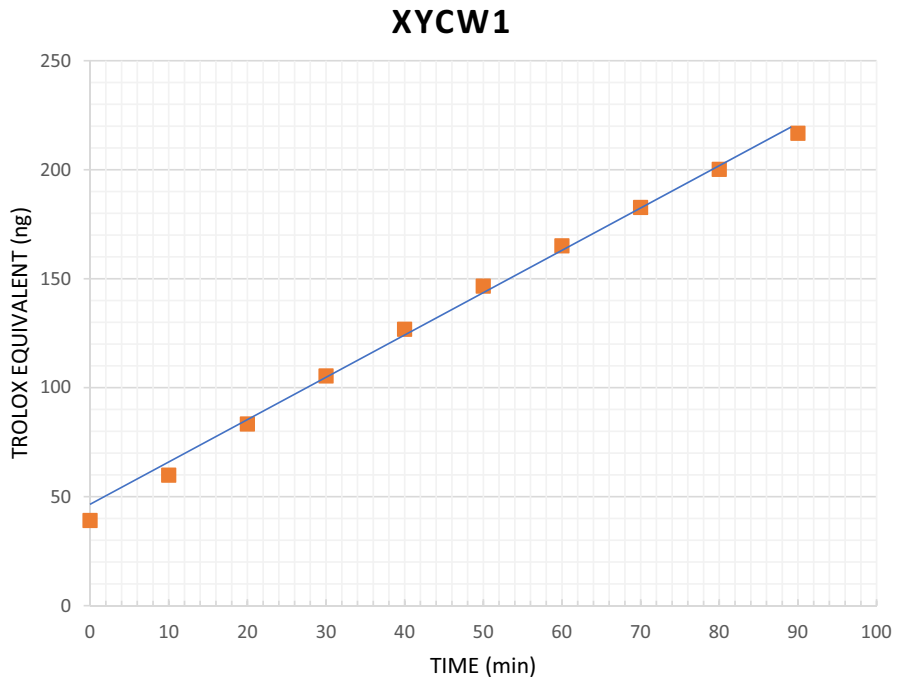

FIGURE 2 Time-dependent antioxidant activities of XYCW1 ferment in phosphate-buffered saline up to 100 min.

over the counter products that may interfere with the study results (e.g., anti-inflammatory drug, retinoids, or topical benzoyl peroxide) throughout the study. Individuals with a history of having cosmetic treatments within 12 months prior to enrollment (such as injectable dermal fillers, neurotoxins, or glycolic acid peels) in the area(s) to be treated or had planned on treating during participation in the study were excluded from participation. Each in-clinic enrolled subject signed an IRB approved photography release form.

2.3.2 | Participants

A total of 121 subjects in generally good health and met the eligibility criteria were enrolled to robustly assess the benefits of daily application of XYCM42, 108 of which completed the study. Of the 121 subjects enrolled, a subset total of 32 TX (Dallas) subjects were enrolled for an in-clinic portion for efficacy evaluation with clinic visits occurring at Baseline, Week 1, Week 4, and Week 8. Twelve subjects were discontinued from the study as they withdrew consent for personal reasons, none of which were due to product related adverse events.

2.3.3 | Regimen application

Subjects received individual regimen kits and instructions on properly applying products for a.m. and p.m. use. In the mornings, subjects were first instructed to use the Prebiotic Cleanser, followed by XYCM42 Ferment Based Serum, Moisturizer, and Sunscreen. At night, subjects used Makeup Remover (if needed), followed by Prebiotic Cleanser, Live XYCM42 Gel, and Prebiotic Activator. The Moisturizer had two versions: an extra hydrating for dry skin and an oil control formulation for normal, combo, and oily skin. Subjects were instructed to return products upon study completion for product accountability purposes.

2.3.4 | Imaging

Color standard photographs using a Canon EOS 80D camera were taken at every site visit. Three views were taken on each subject: the left profile, right profile, and full-face image. Subjects were instructed to adopt neutral, non-smiling expressions. Before imaging procedures, study personnel ensured the face and neck were free of makeup, and jewelry was removed from the treatment area. Subjects were provided with a black headband to keep hair away from the neck, and a black cloth was draped over the subjects' clothing.

2.3.5 | Subject-reported outcomes

At Weeks 1, 4, and 8, subjects completed online, claim-substantiating questionnaires about the regimen as a whole and each product individually. In addition, the study investigator noted any new adverse events from the current use of test products by grading erythema, scaling/peeling, edema, burning/stinging, and itching.

2.3.6 | Clinical grading

At baseline and Weeks 1, 4, and 8, the investigator performed clinical grading for each subject and assessed texture, clarity (lack of), even skin tone, discrete pigment, mottled pigment, fine wrinkling, coarse wrinkling, laxity, undereye dark circles, turgor (lack of), undereye puffiness, and overall photodamage at each grading visit.

2.3.7 | Bioinstrumentation

A multi-parameter skin analysis system (DermaLab Combo; Cortex Technology) was used to obtain the following parameters: transepidermal water loss (TEWL), elasticity, hydration, sebum, pH, and skin color (melanin and erythema). These skin parameters were measured at four clinic visits (Baseline, Week 1, Week 4, and Week 8). To determine the improvement in each parameter that was measured with bioinstrumentation, we calculated the percentage change between each Weeks 1, 4, and 8 and baseline and carried out t-tests.

3 | RESULTS

3.1 | Strain isolation, development, and in vitro characterization

3.1.1 | Strain isolation and development

Although it is now being seen that certain strains of *C. acnes* can be protective in nature, there has been some concern as to the activity of these strains in other tissues other than skin.[4] To investigate topical applications of XYCW1 as a legitimate skin probiotic strain, a genetic switch was added to the FTZ operon to allow for cell viability and metabolism without cell division (data on file). This would allow for topical application while mitigating any potential issues that may be caused by over-proliferation or significant migration. The derivative strain (XYCM42) was viable for at least 39 days and up to 45 days in culture, maintaining log-phase metabolism without cell division. Turning on the genetic switch via application of arabinose (inducer) allows for cell replication and growth to recommence, albeit at a more moderate rate than wild type (Figure 1).

3.1.2 | XYCW1 and XYCM42 secrete multiple antioxidant metabolites

The existence of a gene unique to *C. acnes* species that codes for the potent antioxidant enzyme RoxP has been previously reported.[13]

Since the expression of RoxP was verified in XYCW1 and XYCM42, it was expected that the ferments of the strains when grown in specific conditions would have overall antioxidant properties. Upon testing for antioxidant activity within the cell ferments, it was observed that antioxidant activity increased in proportion to the number of CFU (Figure 2) with an increase in production over time even when nutrients were not immediately available to the microbes. Based on these results, the activity of one cell per unit of time was calculated using the Trolox equivalent as a standard. Using this method, it was calculated that in nutrient poor conditions 3×10^9 CFU of XYCW1 would generate 15.05 mg of Trolox equivalent or 12.72 mg of ascorbic acid equivalent antioxidant activity in a 24-h period.

To determine whether there were any additional antioxidant molecules other than RoxP within the strains' secretomes, the cultured PBS was then fractionated using a 3 kD filter to segregate the protein fractions based on size. Comparable antioxidant activity was observed in both fractions (Table 1). Additionally, when the ferments were fractionated using fast protein liquid chromatography (FPLC), two peaks of strong antioxidant activity were observed, one peak of which contained proteins of which size corresponds to RoxP and another containing protein of a smaller size (data not shown). This suggests that there are multiple sources of antioxidant activity secreted by XYCW1 and XYCM42.

3.1.3 | XYCM42 ferment contains significant levels of PA levels

It is well known that *C. acnes* strains can produce short-chain fatty acids, largely PA, and thus why the species was previously under the genus *Propionibacterium*.[8] However, as PA and its derivatives that exist within the skin have been studied, research shows significant skin benefits of this particular short-chain fatty acid metabolite. One noteworthy observation is that PA has been found to have significant tyrosinase inhibitory activity within skin melanocytes.[15] This benefit was seen as 4 mM PA concentration. To determine the potential benefit of PA produced by XYCM42 during fermentation, the levels of PA were detected using high performance liquid chromatography at intervals during 3 days of fermentation. It was observed that XYCM42 produced 18.6 mM of PA during fermentation (Figure 3).

TABLE 1 Antioxidant activity of XYCW1 culture supernatant fractionated using a 3 kD filter to determine whether there were small molecules contributing to antioxidant activity of XYCW1 ferment.

Sample	Antioxidant activity (μM) (ascorbic acid equivalent)
Supernatant	44.68
Fraction <3 kD	39.90

Note: Both fractions showed similar antioxidant activity, suggesting that molecules other than RoxP contribute to the antioxidants secreted by XYCW1 and XYCM42.

3.1.4 | XYCM42 cells and ferment induced upregulation of structural genes

It has been reported previously that exposure of *C. acnes* cells to skin induces genes involved in skin barrier and proliferation, such as IGF-1 and Ki-67.[14] Here, we have corroborated that XYCM42 can also upregulate IGF-1, as well as downstream upregulation of Col1A and Col3A in human dermal fibroblasts (Figure 4). Additionally, when fibroblasts were treated with XYCM42 cells over 6 and 24 h, trends toward marginal increases in upregulation of these genes were observed to increase over that time (Figure 5).

3.1.5 | XYCM42 cells and ferment induced gene regulation of inflammation

It has been documented that protective strains of *C. acnes* can interact with the immune cells within the skin and modulate their activity to a more balanced and anti-inflammatory state.[16] When XYCM42 cells were added to cultures containing either keratinocyte or fibroblast monolayers, reductions in the gene expression of several inflammatory cytokines were observed, including IL-1a, IL-6, IL-8, and TNF-a (Figure 6). This was also observed with treatment using ferment of XYCM42, both ferment as well as a 50× concentrate filtrate (Figure 4). Given the known interaction of *C. acnes* with the immune cells surrounding hair follicles in skin, we also added XYCM42 cells to cultured peripheral blood mononuclear cells and observed a trend toward reduced expression of inflammatory genes IL-1β, IL-6, and IFN-γ (Figure 7). Additionally, skin explants were tested and showed a similar trend for IL-6 and IL-8 (Figure 8).

3.2 | Clinical results

3.2.1 | Three-week clinical evaluation

As in vitro tests suggested protective properties of XYCM42, a small pilot study of 10 subjects was conducted where 3×10^9 CFU of XYCM42 was applied daily to the face using a simple 1% hyaluronic acid hydrogel vehicle. After 3 weeks of daily application, we observed a marginal increase in *C. acnes* species on the facial skin and a reduction in *Staphylococcus* species (data now shown). No evidence of adverse events or inflammatory issues was observed for any study participants during the pilot study (Figure 9).

3.2.2 | Two-month clinical evaluation

A larger clinical study ($n = 121$) was performed to assess the benefits of daily application of XYCM42 and its ferment filtrate. Here, we asked subjects to apply a daily regimen consisting of an anhydrous gel containing live XYCM42, a serum containing XYCM42 ferment, and adjunct products that helped to support XYCM42 engraftment and function

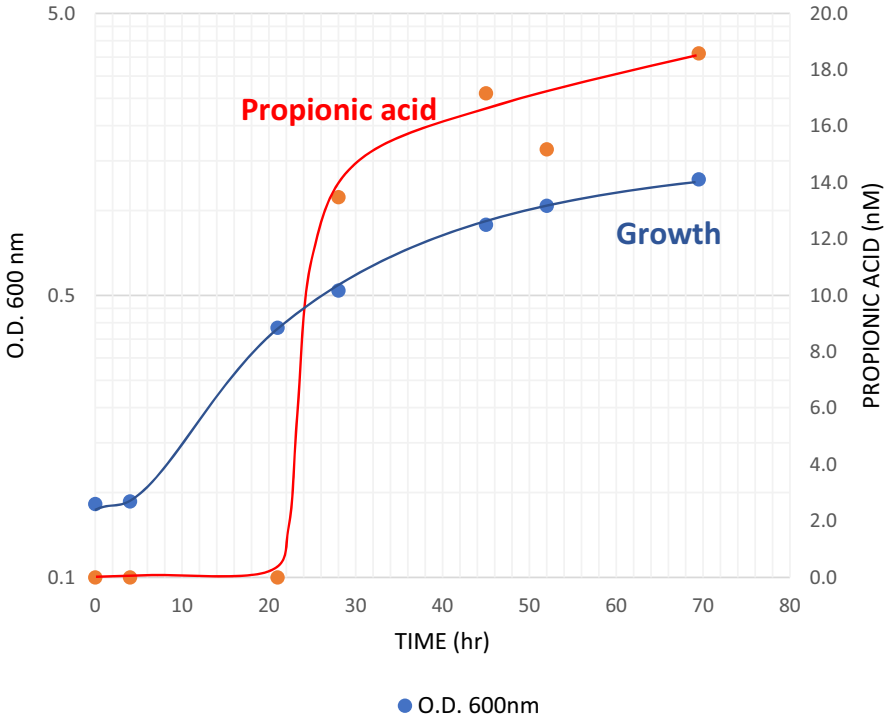

FIGURE 3 Propionic acid production after using arabinose as a carbon source of XYCM42 during large scale fermentation.

(microbe-gentle probiotic cleanser, microbe-friendly moisturizer, and sunscreen SPF30). Consumer questions using a 5- or 7-point Likert-scale (agree/neither/disagree) showed high agreement for product benefits and attributes at Weeks 1, 4, and 8. Agreement responses at the 90th percentile or higher level included skin texture, tone, healthy, natural appearance, felt conditioned, radiant, and vibrant (data not shown).

A subpopulation of this group was evaluated in a clinical setting. Results demonstrate that the XYCM42-based regimen did not adversely affect any measured skin properties. The study results assessing skin health parameters indicated that the regimen continues to support a healthy environment as it maintained normal physiologic pH and TEWL ranges, increased skin hydration levels, reduced erythema, increased elasticity, and an apparent reduction of surface sebum on both the forehead and nose regions (Figure 10). Clinical grading showed visible improvement in all assessed parameters beginning at Week 1 and showed increased benefits throughout the length of the study at Weeks 4 and 8 (Figure 11).

During the study, the most significant improvements observed were in skin texture, evenness of skin tone, under-eye dark circles and puffiness, laxity, pigmentation, and wrinkles (Figures 12–16). Subjects showed dramatic and unexpected levels of wrinkle and texture improvements within a relatively short, two-month use period (Figures 12–14). The regimen demonstrated a strong safety profile as continual use over the 2-month clinical study did not reveal any significant adverse events, and the product regimen was well tolerated.

4 | DISCUSSION

For the last 50 or so years, innovations in topical skin care have come from research into how certain ingredients affect the health and beauty of human skin. Molecules such as alpha hydroxy acids, beta hydroxy acids, retinoids, hyaluronic acid, and ascorbic acid derivatives have proven quite effective and become staples in daily topical regimens for cosmesis. However, what has not been elucidated to date is how these ingredients, some of which are antimicrobial in nature, affect the overall skin biome via modulation of its microbiome and how such may unintentionally negate some of the beneficial effects of skincare ingredients via creation of a dysbiotic skin state.

Considering the makeup of the skin microbiome, it has been reported that the major bacterial constituents of young, healthy skin are comprised largely of a mixture of both *C. acnes* and *S. epidermidis* strains.[17] While some strains within these species have been shown to be protective in nature, others have been associated with disease, corroborating the fact that the benefits seen by even commensal microbe species can be specific to both the strain and an individual's skin environment.[4,7,18] Since protective strains within both these species are (1) native to the skin's environment, (2) have been shown to provide benefit to the skin, and (3) can engraft on the skin; they make prime candidates for what should be considered legitimate skin probiotics.[19] However, one major difference between these two species is their location on, and within the skin. While *S. epidermidis* is found almost exclusively on the skin surface of healthy individuals, *C. acne* can be found on both the surface and within the pilosebaceous units (PSU)/follicles composing around 89% of the follicular microbiome in adult sebaceous skin.[7] Given that the surface area of the collective PSU of the skin is estimated to be roughly somewhere around 10 times greater than that of the surface, and that immune cells are particularly numerous in the areas surrounding PSUs, it can be argued that *C. acnes* has greater access to the epithelial and immune cells and thus a relatively greater opportunity to provide more

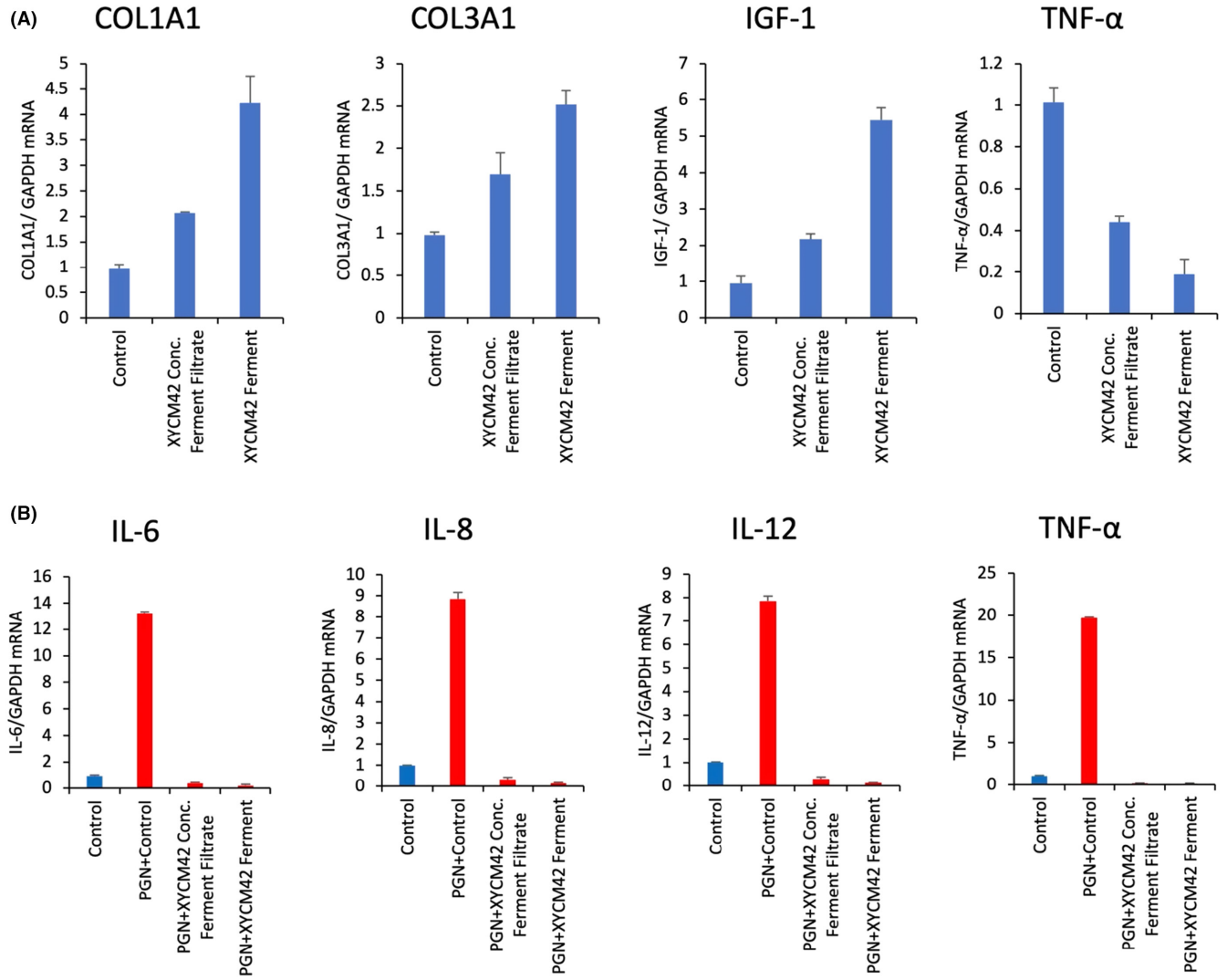

FIGURE 4 (A) Expression of mRNA for COL1A1, COL3A1, IGF-1, and TNF-α in human dermal fibroblast cells after treating with either XYCM42 ferment filtrate (50×), or XYCM42 ferment (1×). (B) Induction of IL-6, IL-8, IL-12, and TNF-α after treatment with either peptidoglycan (PGN), PGN + XYCM42 ferment filtrate (50×), or PGN + XYCM42 ferment (1×).

significant influence to the entirety of the skin, especially the more sebaceous areas of skin such as the face.[20]

The selection of a *C. acnes* strain that would be the best candidate for use as a skin probiotic was based on several factors: (1) The strain must be health-associated, (2) it must exhibit protective attributes and lack pathogenic attributes in culture, (3) the beneficial attributes must be characterized in vitro, and (4) observed clinically. We identified a strain, XYCW1, and its growth arrested derivative, XYCM42, that fit these criteria. These strains fall under the *C. acnes* subsp. *defendens* phylotype and are of the ribotype 6 designation, a ribotype reported to be almost exclusively associated with healthy skin.[7]

As the species *C. acnes* is a facultative anaerobe, it is logical that the organisms possess antioxidant mechanisms to modify the oxygen-rich skin surface environment to facilitate colonization on both the surface of the skin and within the oxygen-poor PSU. The enzyme RoxP is unique to *C. acnes* with no known homologs in other bacteria.[14] This enzyme is highly expressed and is the most abundant protein secreted by *C. acnes*.[24] This benefits both the bacteria and host by protecting them from free radical damage and oxidation by environmental and internal stressors.[13] Interestingly, we observed that RoxP is not the only potent antioxidant substance in the secretome of XYCW1/XYCM42. While the other substance(s)' have not yet been identified or characterized, we know that they are molecules smaller than RoxP (<3 kD) which may allow for further benefits as the smaller size would potentially allow for further penetration into surrounding tissues. This may allow for a multifactorial benefit for human skin rich in protective strains of *C. acnes*.

Antioxidant ingredients are typically found in skincare products, one of the most well studied of these being vitamin C (ascorbic acid) and its derivatives (sodium ascorbyl phosphate, magnesium ascorbyl phosphate, etc.). Vitamin C derivatives are popular antioxidant ingredients as they are well studied and have additional benefits beyond their antioxidant properties, namely pigmentation modulating

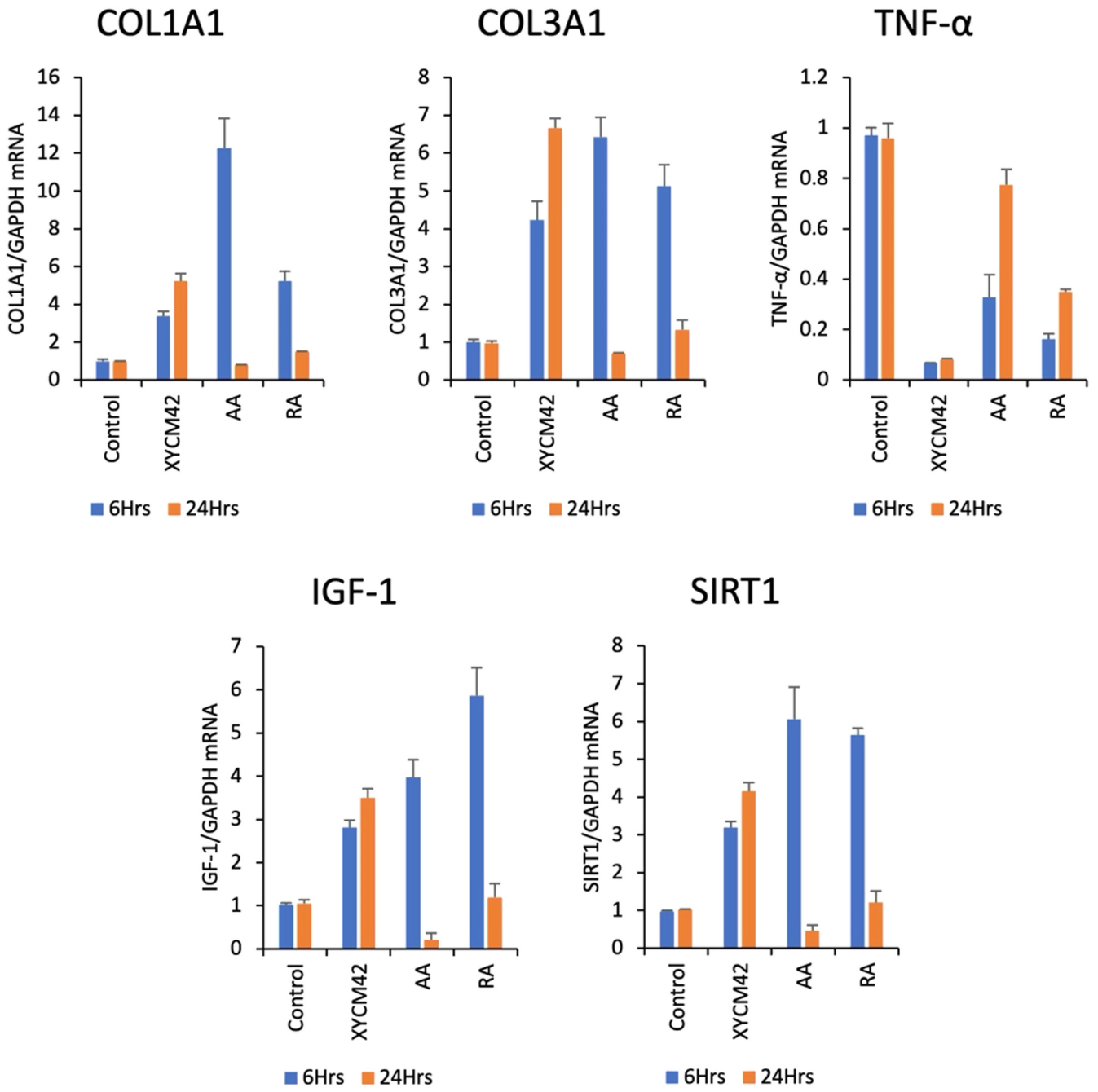

FIGURE 5 Comparison of gene expression of COL1A1, COL3A1, TNF-α, IGF-1, and SIRT1 in human dermal fibroblast cells treated with either live XYCM42 cells (colony forming unit 10^6–10^7), L-ascorbic acid at 100 μM (AA), or retinoic acid at 8 μM (RA) at 6 and 24 h.

and wound healing properties.[21] However, vitamin C is not a very stable molecule, often limiting protective benefits and reducing formulation shelf life and effectiveness.[21] Thus, application of antioxidant topical ingredients like vitamin C has many benefits that are only evident during times of initial application and some ambiguous time thereafter, resulting in dosage spikes not uncommon to many medicinal molecules. Since XYCM42 has been shown to produce multiple antioxidant molecules, constitutively this allows for antioxidant benefits for the skin to be present consistently as long as there are sufficient CFUs present. In our tests on the effect duration of L-ascorbic acid in comparison with XYCM42 cells on human fibroblast monolayer cultures, we observed that while L-ascorbic acid showed higher upregulation of COL1A, COL3A, IGF-1, and SIRT1 and downregulation of TNF-α initially at 6 h, at 24 h any differences in gene regulation from baseline induced by L-ascorbic acid was attenuated while the benefits seen with XYCM42 treatment had not only sustained but increased (Figure 5). This trend was also observed when comparing retinoic acid with XYCM42 at 6 and 24 h. These data suggest that treatment with XYCM42 can provide similar benefits at more consistent lower levels, rather than at short, higher bursts as seen via most labile topical ingredients like retinoids, L-ascorbic acid, and their derivatives.

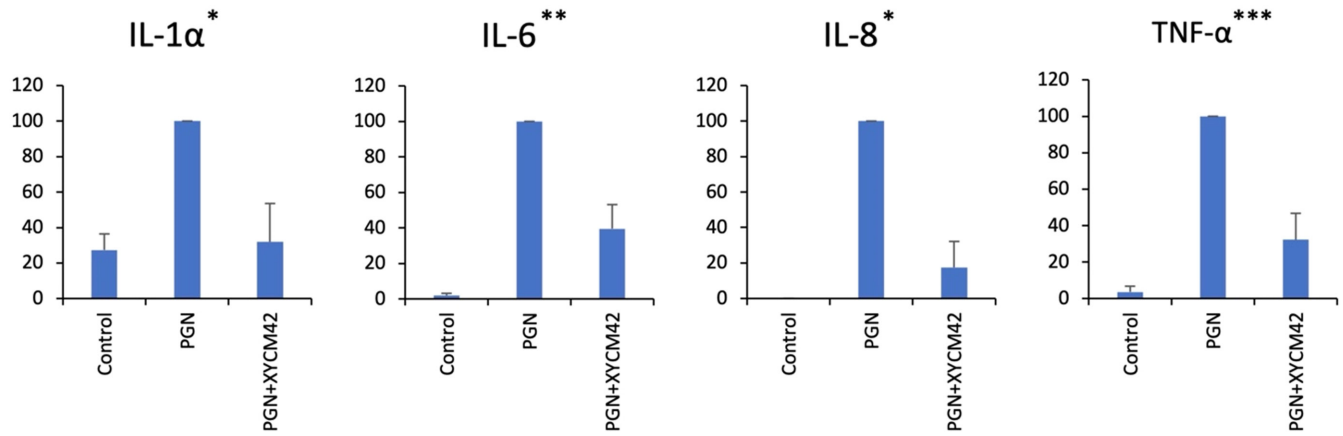

FIGURE 6 Changes in gene expression after PGN-induced inflammation (PGN normalized to 100) and subsequent treatment with XYCM42 in keratinocytes (*$n = 5$, **$n = 4$, and ***$n = 3$).

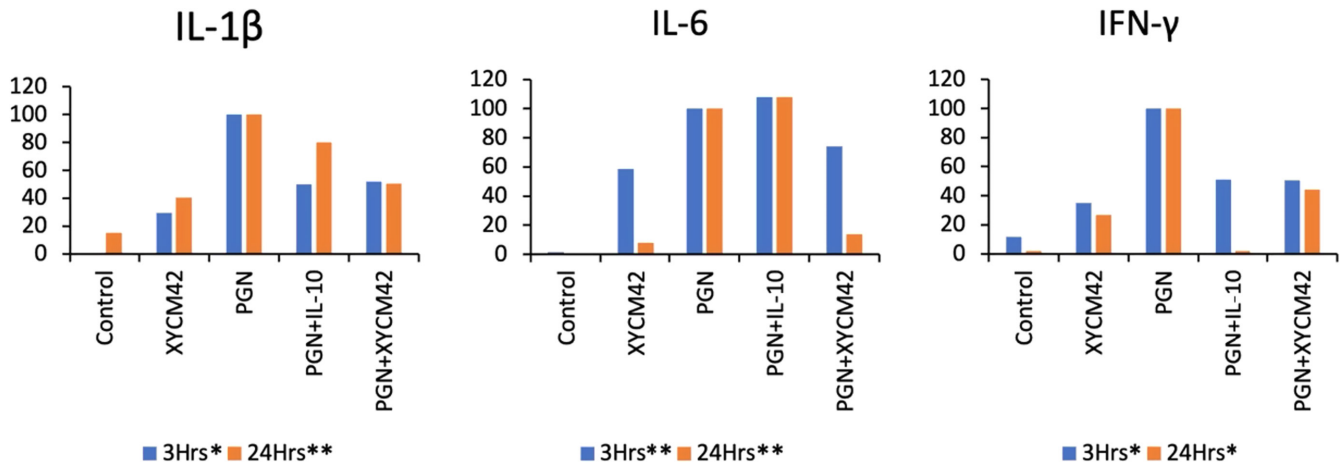

FIGURE 7 Gene expression of IL-1β, IL-6, and IFN-γ after treatment with either PGN, PGN + IL-10, or PGN + XYCM42 in cultured human peripheral blood mononuclear cells of healthy subjects (*$n = 1$ and **$n = 2$).

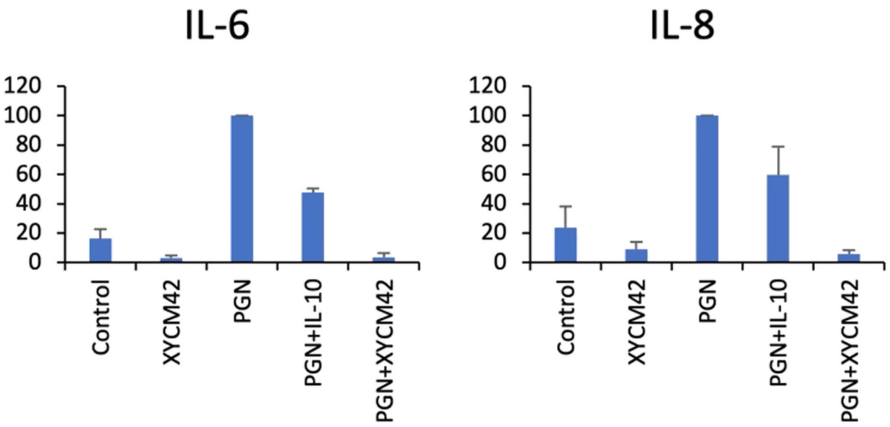

FIGURE 8 Gene expression of IL-6 and IL-8 after treatment with either XYCM42, PGN, PGN + IL-10, or PGN + XYCM42 in skin explants ($n = 3$).

Vitamin C application has also been observed to also benefit hyperpigmented skin by reduction of melanogenesis via tyrosinase inhibition.[21] Interestingly, tyrosinase inhibition has also been observed in human in vitro and mouse in vivo studies upon application of *C. acnes* ferment containing propionic acid.[15] This may explain why a continuing reduction of mottled and discrete dyspigmentation was observed starting at 1-week post-initiation of topicals containing live XYCM42 and its ferment with continuing improvements even at 8 weeks (Figure 13). It should be noted that this was observed even though there was an overall seasonal increase in overall melanin (tanning) observed via bioinstrumentation. Additionally, the production of SCFAs such as propionic acid and butyric acid by *C. acnes* has

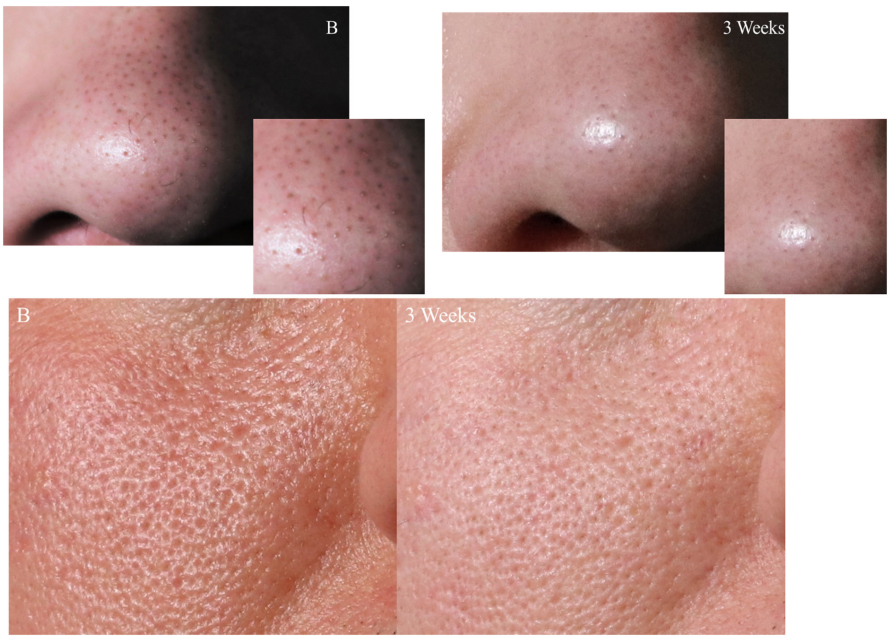

FIGURE 9 Facial skin after topical application with live XYCM42 culture in 0.1% HA serum at 3×10^9 CFU. Frontal nose of a male subject and right cheek of another male subject at baseline and 3 weeks. Noticeable reduction of sebum production, open comedone oxidation, and texture after 3 weeks.

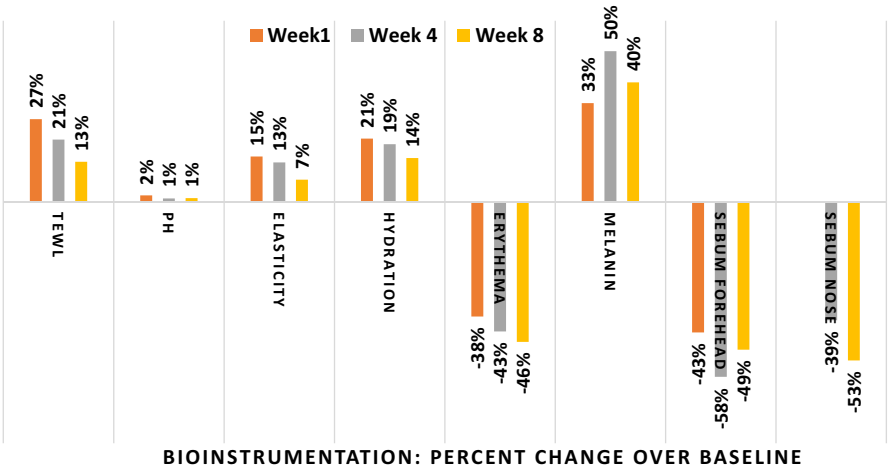

FIGURE 10 Clinical bioinstrumentation measurements demonstrated that the regimen continues to support a healthy skin environment (e.g., low pH, low transepidermal water loss) and promotes increased skin hydration, melanin, decreases erythema, and downregulates surface sebum on both the forehead and nose area.

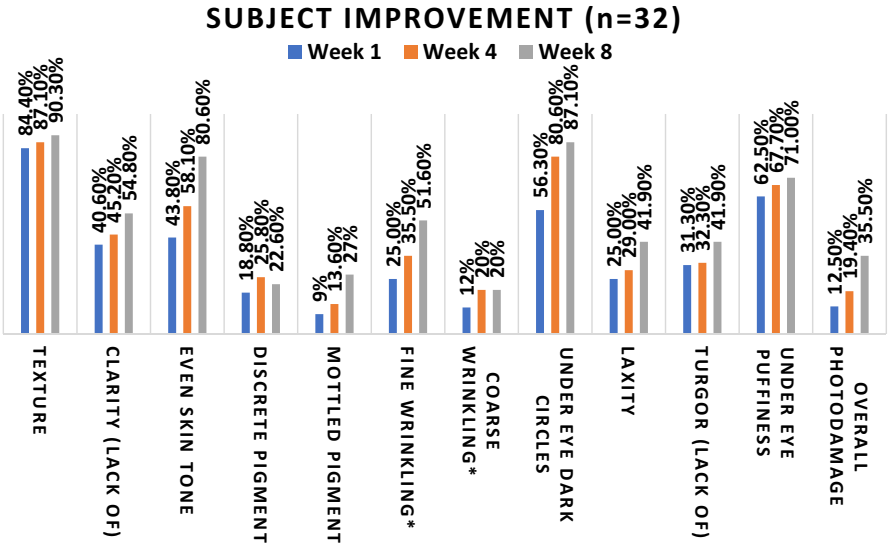

FIGURE 11 Results for Clinical Grading and percentage of subject improvement over baseline at Weeks 1, 4, and 8. ($n = 31$; *$n = 9$).

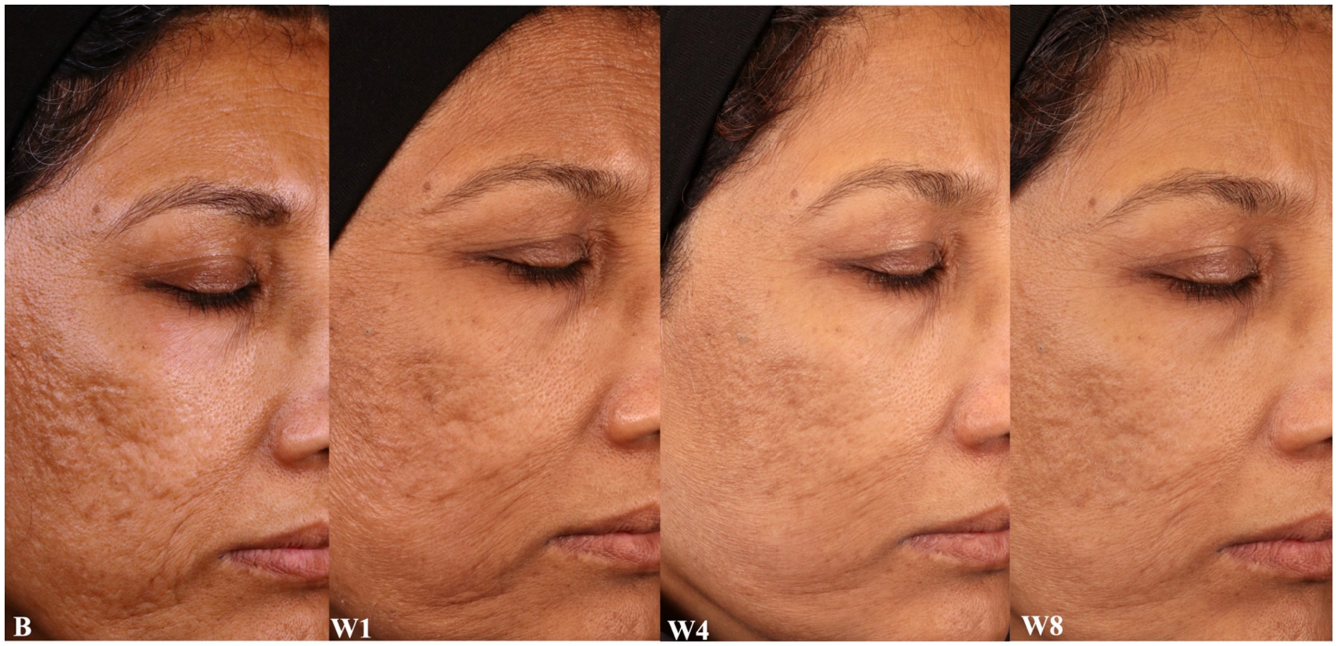

FIGURE 12 Right side facial image taken at baseline (B) and Weeks 1, 4, and 8 (W1, W4, and W8) of live XYCM42-based regimen use. Clinically significant improvements in texture, laxity, pore appearance, wrinkles, and sebum regulation were observed.

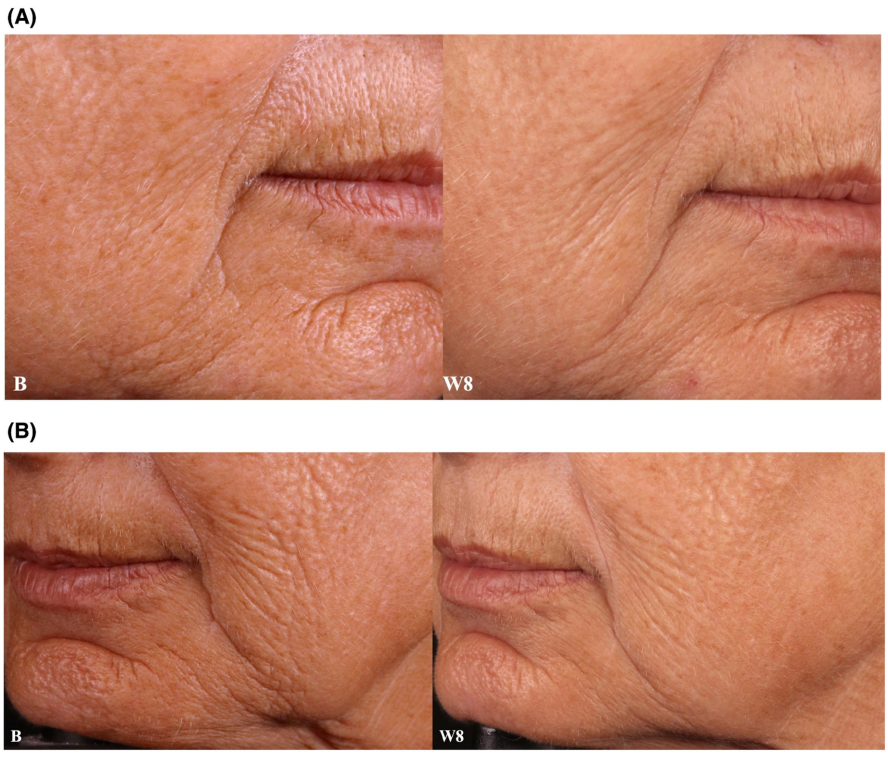

FIGURE 13 (A) Right side facial images were taken at baseline (B) and Week 8 (W8) of XYCM42 regimen use. A visual "mattifying" effect was noted, reduction in wrinkles, improved texture, and pore appearance were observed. (B) Left side facial images taken at Baseline (B) and Week 8 (W8). Noticeable improvements in skin texture, clarity, tone, wrinkles, and laxity in photodamaged skin were observed.

been reported to have other potential benefits, such as reduction of skin inflammation via activation of immune T reg cells,[22] inhibition of pathogens like that of the *Staphylococcus* species,[17] and reduction of skin pH. Therefore, SCFA produced by certain protective *C. acnes* strains, such as XYCM42, could contribute to the homeostasis of healthy skin and corroborate the clinical responses observed in our clinical studies.

Other research has reported that topical application of protective *C. acnes* strains can ameliorate acneic lesions in vivo, which is contrary to general belief regarding *C. acnes*.[23] This reinforces the significant differences between phylotypes of the species, and how skin may be affected should be considered strain specific.[10] To explore the anti-inflammatory properties of strain XYCM42, we performed in vitro tests using human keratinocyte and fibroblast monocultures,

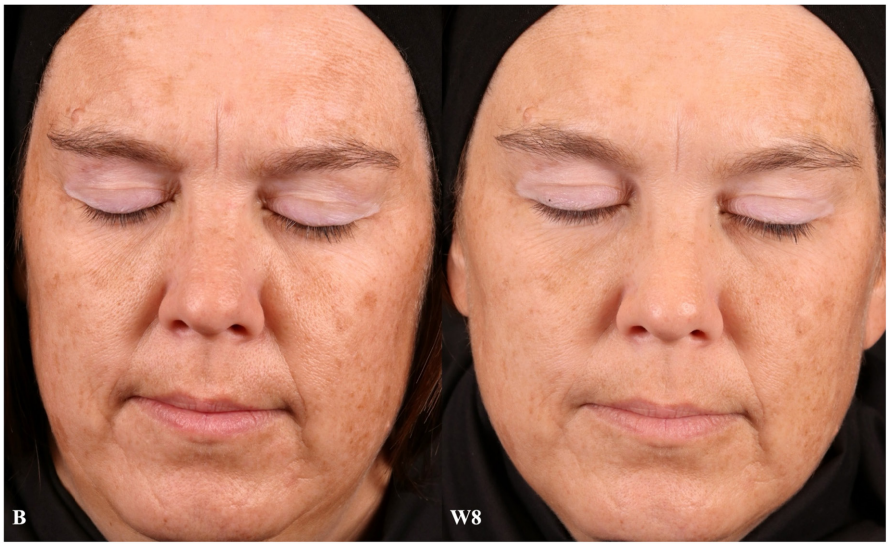

FIGURE 14 Frontal side facial images were taken at baseline (B) and Week 8 (W8) with XYCM42 regimen use, demonstrating improvement in pigmented lesions, overall skin tone, texture, clarity, and wrinkles.

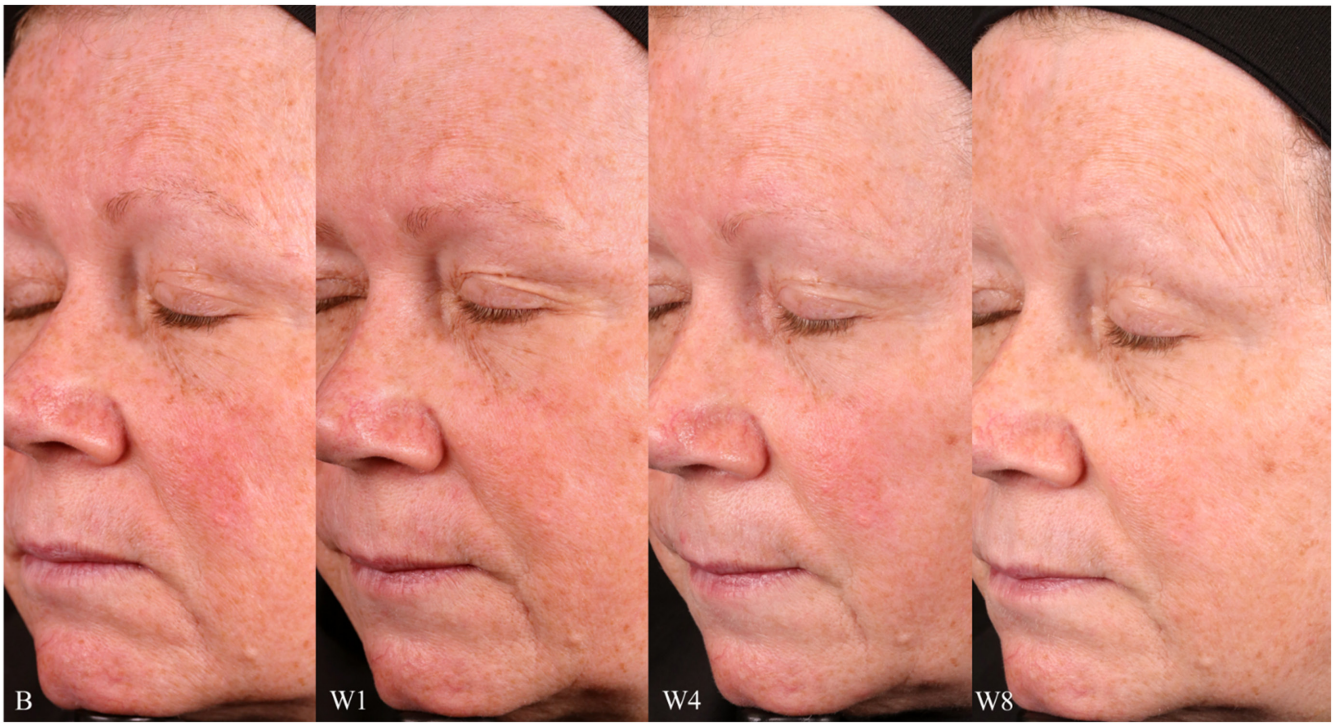

FIGURE 15 Left side facial images at baseline (B) and Weeks 1, 4, and 8 (W1, W4, and W8) of XYCM42 regimen use. Clinically visible significant improvement in erythema was observed.

skin explants, and PBMC $CD4^+$ cell cultures. These test results have shown that the application of live XYCM42 can prevent the upregulation of key proinflammatory cytokines, potentially dampening the inflammatory cascade (Figures 5 and 6). Additionally, we observed this anti-inflammatory effect as well in PBMC cells obtained from acneic subjects, which has been reported to be predisposed to inflammation.[24] The anti-inflammatory effect, while significant, was not as robust as in cells obtained from normative individuals (data not shown).

To further substantiate these data through clinical use, we asked subjects to apply a daily regimen consisting of an anhydrous gel containing live XYCM42, a serum containing XYCM42 ferment filtrate, and adjunct products that helped to support XYCM42 engraftment and function (microbe-gentle probiotic cleanser, microbe-friendly moisturizer, and sunscreen SPF30). Study subjects presenting with erythematous skin showed significantly reduced redness and swelling (Figures 10, 11, and 14–16). In addition, a reduction in sebum was measured (Figure 10) in both the forehead and nose region and corroborated by the progressive mattified appearance of the subjects' skin over time. However, no subjects presented with dry skin following the course of treatment with XYCM42 (Figures 12 and 13). A reduction in sebum after application of *C. acnes* to the skin is contrary to some past literature using animal sebocyte models, which has reported that *C. acnes* strains trigger lipogenesis through the corticotropin-releasing

FIGURE 16 Frontal facial views at baseline (B) and Week 8 (W8) demonstrated a visual decrease in undereye puffiness and dark circles with XYCM42 regimen use.

hormone (CRH)/CRH receptor pathway.[6] However, our clinical data may corroborate a recent in vitro study suggesting that lipogenesis is downregulated in cultured immortalized human SZ95 sebocytes when formalin-killed *C. acnes* are applied.[25] It may also be that the apparent reduction in sebum detected may be attributed, at least in part, to increased metabolic conversion of the sebum by the XYCM42 cells into SCFA, such as propionic and butyric acids.

Additionally, significant improvements in texture were measured and reported. This included improvements in the appearance of pore size and in significantly reduced fine lines and wrinkles. This may be due in part to the upregulation of collagen genes (Figure 4) as well as keratinocyte proliferation and turnover via IGF-1/Ki-67 upregulation as has been reported previously after application of live *C. acnes* strains, specifically here XYCM42 (Figure 4).

5 | CONCLUSION

We are in many ways still in the adolescence of understanding the skin biome and the relationship between the microbes that live on and in the skin with health and disease of both the skin and the body as a whole. This research and other research like it are helping to elucidate the importance and power of the skin biome. Such research is relevant and important to dermatology as it suggests how working to balance the microorganisms that live on and in the skin in very specific ways can have important positive impacts on skin health and aging.

For decades, *C. acnes* has been regarded by most as a skin pathogen or, at best, a skin commensal. However, now it is known that there are significant differences between the many strains of the species, some of which are being shown to be protective or even symbiotic in nature. In this paper, we have reported on in vitro characterization as well as clinical data suggesting the benefits of topical application of a XYCM42-based topical regimen, including both legitimate skin probiotic cells and their ferment filtrate, for the health and beauty of human facial skin. While these studies were limited, and further study and characterization should be performed to substantiate the findings, these studies provide further evidence that certain strains of the *C. acnes* species may have a more significantly beneficial influence on our skin health and appearance than was previously thought. Thus, appropriate formulations derived from specific strains of the skin microbiome can support the development of novel, beneficial topical products that may be more appropriately referred to as "skin biome care" (rather than skin care) as they may address the skin health of the holobiont more comprehensively than the status quo.

AUTHOR CONTRIBUTIONS

Mun Su Rhee, PhD and Thomas Hitchcock, PhD were responsible for the planning and execution of all pre-clinical data. Mona L. Alqam, MD; Brian C. Jones, PhD; Doris Day, MD and Thomas Hitchcock, PhD were responsible for clinical study protocols development and Mona Alqam, MD and Thomas Hitchcock, PhD was responsible with execution of the clinicals (131 subject study and 10 subject study, respectively). Study topicals were formulated by Sasima Phadungpojna PhD under the direction of Thomas Hitchcock, PhD. Manuscript was written by Thomas M. Hitchcock, PhD with significant contributions by Doris Day, MD, Mun Su Rhee, PhD, Mona Alqam, MD, and Brian C. Jones, PhD.

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CONFLICT OF INTEREST

Mun Su Rhee, PhD, Mona L. Alqam, MD, Brian C. Jones, PhD, Sasima Phadungpojna PhD, and Thomas M. Hitchcock, PhD are employees of Crown Laboratories, which funded the study presented in the manuscript. Doris Day, MD served in an advisory position for the initial clinical study and is paid consultant of Crown Laboratories.

DATA AVAILABILITY STATEMENT

Research data are not shared.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. Our protocol to use human subjects for our original study was approved by our Institutional Review Board (IRB) for the clinical portion of the study to use human subjects under IRB study numbers CL-XYC-20-03 and XYC-001.

PHOTO CONSENT STATEMENT

All subjects that participated in the clinical portion of the study signed a photo release form and consented to the use of their photographs for publication purposes in peer review journals.

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Notes:

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