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Staphylococcus epidermidis in the human skin microbiome mediates fermentation to inhibit the growth of *Propionibacterium acnes*: Implications of probiotics in acne vulgaris

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Abstract

Increasing evidence demonstrates that commensal microorganisms in the human skin microbiome help fight pathogens and maintain homeostasis of the microbiome. However, it is unclear how these microorganisms maintain biological balance when one of them overgrows. The overgrowth of *Propionibacterium acnes* (*P. acnes*), a commensal skin bacterium, has been associated with the progression of acne vulgaris. Our results demonstrate that skin microorganisms can mediate fermentation of glycerol, which is naturally produced in skin, to enhance their inhibitory effects on *P. acnes* growth. The skin microorganisms, most of which have been identified as *Staphylococcus epidermidis* (*S. epidermidis*), in the microbiome of human fingerprints can ferment glycerol and create inhibition zones to repel a colony of overgrown *P. acnes*. Succinic acid, one of four short-chain fatty acids (SCFAs) detected in fermented media by nuclear magnetic resonance (NMR) analysis, effectively inhibits the growth of *P. acnes in vitro* and *in vivo*. Both intralesional injection and topical application of succinic acid to *P. acnes*-induced lesions markedly suppress the *P. acnes*-induced inflammation in mice. We demonstrate for the first time that bacterial members in the skin microbiome can undergo fermentation to rein in the overgrowth of *P. acnes*. The concept of bacterial interference between *P. acnes* and *S. epidermidis* via fermentation can be applied to develop probiotics against acne vulgaris and other skin diseases. In addition, it will open up an entirely new area of study for the biological function of the skin microbiome in promoting human health.

Keywords

Acne; Fermentation; *P. acnes*; Probiotic; *S. epidermidis*; Skin Microbiome

INTRODUCTION

Fermentation of milk with gut-friendly bacteria, i.e. yogurt, which is the best example of bacterial interference through fermentation, is an excellent aid to balance the bacteriological

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ecosystem in the human intestine. Bacterial interference via fermentation occurs in natural ecosystems as well. For example, microorganisms both on and inside fruits consume sugars that were converted from starch during ripening to produce fermentation products including ethanol and short-chain fatty acids (SCFAs). Production of SCFAs and ethanol by fermentative yeasts is, in fact, part of an evolved strategy to compete with other microbes for access to sugars (Dudley 2004). It is not fully clear whether friendly microbes in human skin possess the fermentation activity and whether ferments including SCFAs of these microbes have probiotic activities to maintain the homeostasis of the skin microbiome. Reports show that SCFAs produced by fermentation of microorganisms have been detected in pus from a deep-seated abscess, an anaerobic microenvironment in the context of human bacterial infection (Demaerel et al. 1994; Gorbach et al. 1976; Menon et al. 2007). Like a ripening fruit, an acne lesion, particularly a closed comedone or deep-seated abscess in an open comedone, creates an anaerobic microenvironment which facilitates overgrowth of *Propionibacterium acnes* (*P. acnes*). It has been reported that *P. acnes*, *Staphylococcus epidermidis* (*S. epidermidis*), and other skin microflora co-exist in acne lesions (Nishijima et al. 2000). We thus envision that the anaerobic acne microenvironment triggers human skin microflora to undergo fermentation, and that these skin microflora utilize fermentation to rein in the overgrowth of *P. acnes* within acne lesions.

A number of SCFAs are naturally produced by skin cells and commensal bacteria in relatively low concentrations (Burtenshaw 1942). It has been reported that SCFAs exert antimicrobial activities (Ryssel et al. 2009; Ushijima et al. 1984). Several SCFAs have been approved by the United States Environmental Protection Agency (EPA) as active ingredients for use as fungicides and bactericides on stored grains, poultry litter, and drinking water for poultry and livestock (Sebastian et al. 1996). The Food and Drug Administration (FDA) has approved succinic acid ($C_4H_6O_4$), one of the SCFAs, as a flavor enhancer, miscellaneous and general purpose food chemical, neutralizing agent, and pH control agent (2011-10-27). SCFAs (e.g. lactic acid) and glycerol are ingredients in many skin care products, where they serve as moisturizers or anti-inflammatory agents.

Acne vulgaris is an inflammatory skin disease associated with the overgrowth of *P. acnes*. Around 40 to 50 million Americans suffer from acne vulgaris each year (Imahiyerobo-Ip and Dinulos 2011). Many treatment options are available for acne, however none of them completely cure the disease in all patients, and many of them also have significant side effects. Antibiotics, for example, have been used for treating acne vulgaris, but these are non-specific and have a risk of creating antibiotic-resistant bacteria (Haider and Shaw 2004). The oxidizing agent benzoyl peroxide (BPO) is one of the most frequently used topical medications for acne treatment. It is available over-the-counter and is generally well-tolerated by patients. Isotretinoin, another acne treatment option, is a powerful and effective medication derived from vitamin A (Layton et al. 2006). However, it is strictly regulated due to the induction of serious side effects including congenital anomalies. Finally, intralesional corticosteroid injections are an important adjunct in the treatment of painful nodulocystic acne lesions. However, the injection can cause local side effects including linear hypopigmentation and atrophy (Levine and Rasmussen 1983). None of these treatments use a person’s endogenous molecules to treat acne despite the fact that these molecules may have fewer side-effects and a less of a chance of developing antibiotic-resistant microbes. Acne vaccines selectively targeting *P. acnes*-induced inflammation, not *P. acnes* bacterial particles, are actively being developed in our laboratory (Huang et al. 2008; Liu et al. 2011; Lo et al. 2011; Nakatsuji et al. 2008a; Nakatsuji et al. 2008b; Nakatsuji et al. 2008c; Nakatsuji et al. 2011). However, the vaccine may be mainly for preventive treatments. Here we introduce the concept of treating acne with probiotics or prebiotics, which include three main products: 1) anti-*P. acnes* SCFAs, 2) glycerol, which is known as a fermentation inducer and a healing enhancer (Fluhr et al. 2008), and 3) live fermenting microorganisms

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with the ability to inhibit the growth of *P. acnes*. The use of probiotic skin microorganisms or their fermentation products as innate anti-*P. acnes* therapeutics is in compliance with evolutionary medicine. It may also have a lower risk of inducing resistant strains of *P. acnes* and causing side-effects since *P. acnes*/skin commensal interference may occur naturally within lesions of acne vulgaris.

MATERIALS AND METHODS

Ethics statement

Experiments involving mice were performed at the University of California, San Diego (UCSD). The UCSD ethics committee specifically approved this study under an approved Institutional Animal Care and Use Committee (IACUC) protocol (no. S10058). The Institutional Review Board (IRB) at UCSD approved the consent procedure and bacterial sampling under approved protocols (no. 100473 and 121230). The written consents from all participants were obtained before conducting bacterial sampling.

Culture of microorganisms

P. acnes (ATCC6919) was cultured in Reinforced Clostridium Medium (RCM, Oxford, Hampshire, England) under anaerobic conditions using Gas-Pak (BD, Sparks, MD, USA) at 37°C as previously described (N akatsuji et al. 2008a). Human skin microorganisms were isolated by moving a sterile inoculating loop (Fisher Scientific, San Diego, CA, USA) along the surface of the nose of a male subject without acne vulgaris. The isolated skin microorganisms containing a mixture of various microbes were cultured in tryptic soy broth (TSB) (Sigma, St. Louis, MO, USA). Overnight cultures were diluted 1:100 and cultured to an absorbance at 600 nm [optical density $(OD)_{600}$]=1.0. Microorganisms were harvested by centrifugation at 5,000 g for 10 min, washed with phosphate buffered saline (PBS), and suspended in PBS.

P. acnes growth in a homogeneous microbial lawn

The skin microorganisms or *P. acnes* [10^5 colony forming unit (CFU] were mixed with 1% molten (w/v) agar (Oxoid. Ltd., London, UK) with/without glycerol (20 g/l) in TSB. The microbial suspension/agar was poured into plates to produce a homogeneous lawn of microbes. *P. acnes* or skin microorganisms with a serial dilution (5×10^6– 5×10^1 CFU in 5 μl in PBS) were spotted on top of the microbial lawn under anaerobic conditions at 30°C. CFUs were counted on day 6 after spotting.

Bacterial interference in the fermented skin fingerprints

Fingerprints of index, middle, and ring fingers were pressed onto the surfaces of agar plates composed of rich medium (10 ml) [10 g/l yeast extract (Biokar Diagnostics, Beauvais, France), 5 g/l TSB, 2.5 g/l K_2HPO_4 and 1.5 g/l KH_2PO_4] supplemented with/without glycerol (20 g/l). To mimic the overgrowth of *P. acnes* in lesions of acne vulgaris, a high dose of *P. acnes* (10^7 CFU in 5 μl PBS) was spotted on the central portion of fingerprints and grown for six days at 30°C under anaerobic conditions using Gas-Paks. Three subjects (2 males and 1 female) participated in fingerprinting on agar plates. All subjects were asked not to wash their hands before pressing their fingerprints. Fingers in the right hand were pressed on the glycerol-containing plates and fingers in the left hand were pressed on glycerol-free plates. The sequence analysis of 16S rRNA genes (Lindh et al. 2005) was performed to identify the microorganisms in fingerprints.

Nine single colonies of microorganisms, which created inhibition zones in three glycerol-containing plates derived from three subjects, were picked up by sterile toothpicks and

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boiled at 100°C for DNA extraction. The polymerase chain reaction (PCR) with 16S rRNA 27F and 534R primers in addition to sequencing of PCR products were conducted as previously described (Lindh et al. 2005). The 16S rRNA gene sequences were analyzed using the basic local alignment search tool (BLASTn).

Fermentation of microorganisms

The skin microorganisms (10^5 CFU/ml) isolated from the surface of the human nose by tape stripping using a D-Squame Standard Sampling Discs adhesive tape strip (CuDerm Corporation, Dallas, TX, USA) with a diameter of 2.0 cm were incubated in rich medium in the absence and presence of 20 g/l glycerol under anaerobic conditions at 30°C. Rich medium plus 20 g /l glycerol without microorganisms was included as a control. The 0.001% (w/v) phenol red (Sigma, St. Louis, MO, USA) in rich medium with 20 g/l glycerol served as an indicator, converting from red-orange to yellow when fermentation occurs.

Identification of SCFAs in the fermented media of microorganisms by nuclear magnetic resonance (NMR) analysis

The skin microorganisms isolated from the surface of the human nose were incubated in phenol red-free rich medium with $^{13}C_3$-glycerol (20 g/l) (Cambridge Isotope Laboratories, Andover, MA, USA) for six days. After that, microorganisms were discarded by centrifugation at 5,000 g for 30 min. Fermented media were then passed through 0.2-μm-pore-size filters. SCFAs and other metabolites in the microorganism-free media were identified by NMR analysis. The one-dimensional (1-D) NMR spectra were measured on a JEOL-ECS NMR spectrometer operating at a resonance frequency of 400 MHz with a repetition delay of 3 sec for both ^1H and ^{13}C. The 2-D ^1H-^{13}C heteronuclear single quantum correlation (HSQC) NMR spectra were acquired on a Bruker Avance 600 MHz NMR spectrometer with a triple resonance inverse (TCI) cryo-probe and recorded as 2048 × 256 complex points with 32 scans and 1 sec repetition time. Newly appearing peaks belonged to the intermediate or final products resulting from $^{13}C_3$-glycerol fermentation by microorganisms (Chitarra et al. 2000).

Minimal bactericidal concentration (MBC) assays

To determine the MBC values of SCFAs, *P. acnes* (10 μl; 10^8 CFU/ml in PBS) was incubated overnight with SCFAs at various concentrations (10 μl; 2.5–100 mM in PBS) as indicated in each individual experiment in media on a 96-well microplate (100 μl per well). The control received only 10 μl PBS. After incubation, the bacteria were diluted 1:10–1:10^6 with PBS. MBC was defined as a 99.9% killing level and determined by spotting the dilution (5 μl) on an agar plate supplemented with media for CFU counting. To determine the effect of pH on its survival, *P. acnes* in PBS was incubated overnight with 5 mM succinic acid (10 μl) on a 96-well microplate (100 μl per well) before spotting on an agar plate. As controls, *P. acnes* was incubated with 10 μl of PBS (pH 7.4) alone, PBS (pH 5.5; a pH value corresponding to the MBC of succinic acid in PBS), or buffered succinic acid (5 mM succinic acid, pH 7.4 buffered with ammonium hydroxide) on a 96-well microplate (100 μl per well). To determine the inhibitory constant (*K*i), the growth of *P. acnes* (ATCC6919) in PBS or succinic acid (1–50 mM) for 0, 6 12, 24, 30 and 36 h was measured by reading OD_{600}. The *K*i for succinic acid was calculated using the software Curve Expert 1.4™ via the Haldane equation as previously described (Rigo and Alegre 2004).

Measurement of intracellular pH

Measurement of intracellular pH of *P. acnes* using a carboxyfluorescein succinimidyl ester (cFSE) florescence probe (Life Technologies, Grand Island, NY) was previously described (Chitarra *et al.,* 2000). Briefly, bacteria were loaded with cFSE (5 μM) for 30 min at 37°C in

50 mM 4-(2-hydroxyethyl)-1 -piperazineethanesulfonic acid (HEPES) and 5 mM ethylenediaminetetraacetic acid (EDTA). To eliminate unbound probe, bacteria were incubated with glucose (10 mM) for an additional 30 min, washed twice in PBS with 10 mM $MgCl_2$, pH 7.0, and then re-suspended in PBS. The cFSE-loaded bacteria (3×10^4 CFU) were dispensed on a 96-well microplate containing 100 μl/well of PBS or succinic acid (5 mM). Fluorescence intensities were measured immediately and every min for 5 min using an excitation wavelength of 490 nm and emission wavelength of 520 nm. A drop in relative fluorescence indicates a decrease in intracellular pH. Fluorescence of the bacteria-free filtrate (background fluorescence) was measured after the 5-min assay. In this case, treated suspensions were centrifuged at 5,000 g for 5 min. The fluorescence of the bacteria-free supernatant was measured and deducted from values for the treated suspensions. Calibration curves were obtained by incubation of un-treated, cFSE-loaded bacteria in buffers of various pHs. The buffer containing glycine (50 mM), citric acid (50 mM), $Na_2HPO_4.2H_2O$ (50 mM), and KCl (50 mM) was adjusted to various pH values ranging from 4 to 10. Equilibration of the intracellular and extracellular pH was conducted by addition of 1 μM valinomycin and nigericin (Sigma, St. Louis, MO).

In vivo effects of succinic acid on *P. acnes* colonization and *P. acnes*-induced inflammation

The Institute for Cancer Research (ICR) mice (2–3 month-old females; Harlan Labs, Placentia, CA, USA) were anesthetized by isoflurane. Five mice per group were used in each experiment. The ears of ICR mice were injected intradermally with *P. acnes* (10^7 CFU in 10 μl PBS) or PBS (10 μl) using a 28-gauge needle. One day after injection, succinic acid (5 mM; 10 μl) or PBS was injected into inflamed lesions for an additional two days. For topical application, succinic acid (100 mM; 10 μl) or PBS was applied topically on the surface of inflamed lesions once per day every day for three days. Succinic acid or PBS was applied topically to mouse ears away from the needle injection sites to avoid it entering the dermis via a hole created by the needle injection. Ears were excised, weighed and homogenized for cytokine detection and bacterial counts. The macrophage-inflammatory protein-2 (MIP-2) in supernatants was measured by an enzyme-linked immunosorbent assay (ELISA) kit as directed by the manufacturer (BD Biosciences, San Diego, CA). The level of MIP-2 was normalized to the total amount of protein per gram of excised ear. To determine the bacterial counts in *P. acnes*-inoculated ears, mouse ears were excised and homogenized in 200 μl of sterile PBS with a tissue grinder. Bacterial CFUs in the mouse ears were enumerated by plating serial dilutions ($1{:}10^1$ –$1{:}10^6$) of the homogenate on Brucella broth agar plates (BD, Sparks, MD) supplemented with 5% (v/v) defibrinated sheep blood (LAMPIRE Biological Laboratories, Pipersville, PA), vitamin K (5 mg/ml, Remel, Lenexa, KS), and hemin (50 mg/ml, Remel, Lenexa, KS). The plates were incubated for 3 days at 37°C under anaerobic conditions using Gas-Pak (BD, Sparks, MD) to count colonies. The bacterial numbers (CFUs) per gram of excised ear were calculated. A Student's *t*-test was used to determine the significance of the differences between groups. Data represent a 95% confidence interval (95% CI) of the mean from three independent experiments. All experiments using mice were conducted in a biosafety level 2 (BSL-2) facility and in accordance with institutional guidelines for animal experiments.

Statistical analysis

To determine significances between groups, comparisons were made using the two-tailed *t*-test. For all statistical tests, the *P*-values of <0.05 (*), <0.01 (**), and <0.001 (***) were accepted for statistical significance.

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GenBank accession numbers of the 16S rRNA gene sequences

Nine colonies of skin microorganisms that created inhibition zones at the boundary of a *P. acnes* were selected for 16S rRNA gene sequencing. The 16S rRNA genes derived from eight of these colonies, namely EH-1, EH-2, EH-3, EH-4, EH-5, EH-6, EH-7, and EH-8, shared 97–99% identity with the 16S rRNA genes in *S. epidermidis* ATCC12228 or *S. epidermidis* RP62A. The 16S rRNA genes derived from one colony, namely EH-9, had 96% homology to the 16S rRNA genes in *Paenibacillus sp.* Y412MC10. The bankit numbers, sequenceID, and GenBank accession numbers of 16S rRNA sequences of nine bacterial colonies (EH-1 to EH-9) are: BankIt1661928, Seq1, KF683955; BankIt1661902, Seq2, KF683947; BankIt1661903, Seq3, KF683948; BankIt1661906, Seq4 , KF683949; BankIt1661907, Seq5, KF683950; BankIt1661908, Seq6 , KF683951; BankIt1661908, Seq6, KF683951; BankIt1661908, Seq6, KF683951; BankIt1661909, Seq7, KF683952; BankIt1661910, Seq8, KF683953; BankIt1661911, Seq9, KF683954. To obtain the phylogenetic relationships of EH-1 to EH-9, multiple sequence alignment was performed by ClustalX (ver 1.83) using the default parameters. The neighbor-joining (NJ) phylogenetic tree was constructed using Kimura’s 2-parameter model (Kimura 1980).

RESULTS

Bacterial interference enhanced by fermentation

Serial dilutions of *P. acnes* were spotted on the top of a homogeneous lawn of skin microorganisms. As shown in Figure 1a, the colonies of *P. acnes* were significantly reduced ($2.1 \pm 0.8 \times 10^5$ CFU) (without glycerol) and $1.7 \pm 0.3 \times 10^4$ CFU (with glycerol)] when they were grown on the top of a microbial lawn in the presence of glycerol under anaerobic conditions for 3 days (Figure 1a, c). Glycerol was specifically chosen for fermentation over other carbon sources because it is a naturally produced metabolite found in human skin (Fluhr et al. 2008) and has been approved by FDA for skin care products. Although *P. acnes* is an obligate anaerobic organism, it is capable of growing under both aerobic and anaerobic conditions (Cove et al. 1983; Uckay et al. 2010). The reduction in the colonies of *P. acnes* was not observed when *P. acnes*/skin microorganisms were grown on an agar plate and incubated under aerobic conditions (data not shown). There was no significant difference in the number of *P. acnes* colonies grown on regular agar plates (without a microbial lawn on the bottom) in the absence ($1.2 \pm 0.2 \times 10^8$ CFU) or presence ($1.0 \pm 0.5 \times 10^8$ CFU) of glycerol (Figure 1b, c). However, in the absence of glycerol, the number of *P. acnes* colonies that grew with microorganisms on a microbial lawn was more than three logs lower than the number of *P. acnes* colonies that grew without microorganisms (Figure 1a, b, c). The results suggest that bacterial interference occurred between *P. acnes* and skin microorganisms, and interference of *P. acnes* growth by skin microorganisms was enhanced by glycerol fermentation.

Bacterial interference in the fermented skin fingerprints

Although the isolated skin microorganisms (Figure 1) exerted anti-*P. acnes* action, these microorganisms may not fully represent the diversity of microbial populations on human skin since some skin bacteria are not cultivable. In addition, it is possible that only a few dominant microorganisms in a mixture of microorganisms may survive after multiple passages. To obtain skin microorganisms without multiple passages, fingerprints of index, middle, and ring fingers were pressed onto the surface of agar plates supplemented with or without glycerol. Since the overgrowth of *P. acnes* has been linked to acne vulgaris, we dropped a high dose of *P. acnes* (ATCC6919; 10^7 CFU) on the central portion of the fingerprints. The interaction between *P. acnes* and skin microorganisms on the fingerprints was observed daily. Under anaerobic conditions, *P. acnes* grew into a larger colony and was surrounded by skin microorganisms six days after incubation (Figure 2). On the glycerol-

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free agar plates, the colonies of *P. acnes* and skin microorganisms grew close to each other without developing inhibition zones (Figure 2a, c, d). Some microorganisms can grow within a large *P. acnes* colony. However, on the glycerol-containing agar plates, inhibition zones, the areas on the agar plate that remain free from microbial growth, were detected at the boundary of the *P. acnes* colonies and the skin microorganisms (Figure 2b, e, f). Some skin microorganisms created bubble-like competition territories within a colony of *P. acnes*. The bubble-like competition territories were not due to the gas production during fermentation because they were not formed within a large *P. acnes* colony that grew on the same agar plate, but instead formed far away from skin microorganisms (Figure 2b, insert panel). These results suggest that skin microorganisms can interfere with the growth of *P. acnes* through glycerol fermentation.

Identification of *S. epidermidis* as a skin probiotic microorganism against *P. acnes*

The sequence analysis of 16S rRNA genes (Lindh et al. 2005) was performed to identify skin microorganisms. Single colonies of nine skin microorganisms that created inhibition zones at the boundary of a *P. acnes* colony, were picked up for 16S rRNA gene sequencing. The combination of PCR using isolated DNA with 16S rRNA 27F and 534R primers with DNA sequencing was conducted as previously described (Lindh et al. 2005). The 16S rRNA genes derived from eight of these colonies shared 97–99% identity with the 16S rRNA genes in *S. epidermidis* ATCC12228 or *S. epidermidis* RP62A (data not shown). Phylogenetic analysis revealed that most bacterial colonies were clustered together with *S. epidermidis* RP62A and *S. epidermidis* ATCC12228 (Supplementary Figure S1). The 16S rRNA genes derived from one of the colonies had 96% homology to the 16S rRNA genes in *Paenibacillus sp.* Y412MC10. *S. epidermidis* is a common skin bacterium (Cogen et al. 2010). While *S. epidermidis* is a facultative bacterium, it has been reported that it can undergo fermentation under anaerobic conditions (Sivakanesan and Dawes 1980). *Paenibacillus* is a genus of facultative anaerobic, Gram-positive bacteria and can be detected in a variety of environments including soil and water as well as in clinical samples (Chow et al. 2012). Although *Paenibacillus sp.* is not a skin permanent bacterium, it has been reported that the strain of *Paenibacillus sp.* JDR-2 can ferment pyruvate to propionic acid (Chow et al. 2012). Taken together, these results demonstrate that 1) the human skin microbiome contains both short-term and long-term resident microorganisms (Lemon et al. 2012), and 2) *S. epidermidis* may control overgrowth of *P. acnes* via fermentation. To validate the anti-*P. acnes* activity of fermenting *S. epidermidis*, one of the colonies identified as *S. epidermidis* (10^5 CFU in 100 μl) was re-streaked on new plates containing glycerol. A high dose of *P. acnes* (ATCC6919; 10^7 CFU) was then spotted on the plate. Many inhibition zones developed within a large *P. acnes* colony (Fig. 2g, f), demonstrating that *S. epidermidis* exerts probiotic activity against *P. acnes*. Although selected colonies shared 97–99% identity with *S. epidermidis*, an ATCC (12228) *S. epidermidis* strain was chosen to confirm its inhibitory activity against *P. acnes* (Supplementary Figure S2). *P. acnes* was spotted on the top of a homogeneous lawn of *S. epidermdis* in the absence or presence of glycerol under anaerobic conditions for 3 days. A zone of inhibition between *P. acnes* and *S. epidermidis* colonies was observed only on the *S. epidermdis* lawn containing glycerol. This result indicates that glycerol fermentation is indispensable for *S. epidermidis* to repel *P. acnes*.

SCFAs in fermented media of skin microorganisms

To examine their fermentation activity, skin microorganisms were incubated in rich medium under anaerobic conditions in the presence of glycerol. Rich media plus either glycerol or skin microorganisms were used as controls. To monitor the fermentation process, cultures were tested with phenol red, a fermentation indicator, to assess SCFA production as a result of glycerol fermentation. Only media in the culture of skin microorganisms with glycerol turned yellow (more acidic) after six days of incubation (Figure 3a), indicating fermentation

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of skin microorganisms. This finding was further validated quantitatively by measuring the pH values of rich media. The pH values of rich media containing glycerol, microorganisms and glycerol plus microorganisms were 6.5, 6.4, and 6.0, respectively, following 6 days of incubation. To identify the SCFAs in the ferments, the skin microorganisms were incubated in rich medium under anaerobic conditions in the presence of $^{13}C_3$-glycerol (20 g/l) for six days. Supernatants of microbial fermentation in 10% deuterium oxide (D_2O) were subjected to 1-D (Figure 3b, c) and 2-D (Figure 3d) ^{13}C and ^{1}H NMR analysis. In addition to ethanol and alanine, four SCFAs [acetic acid, butyric acid, lactic acid, and succinic acid] were detected in the fermented media of skin microorganisms. These four SCFAs, but not ethanol or alanine, were also detectable in the $^{13}C_3$-glycerol fermented media of a selected re-streaked colony (Fig. 2g, f) of *S. epidermidis* (data not shown). These results demonstrate that skin microorganisms including *S. epidermidis* fermentatively metabolized $^{13}C_3$-glycerol into SCFAs.

Succinic acid decreases the survival of *P. acnes* via reduction of intracellular pH of *P. acnes*

MBC assays were performed to determine if SCFAs exert antimicrobial activities against *P. acnes*. Bacteria were incubated with acetic acid, butyric acid, lactic acid, and succinic acid at various concentrations in media for 24 h. After incubation, the bacteria were diluted with PBS and spotted on an agar plate to count CFUs. The MBC values of acetic acid, butyric acid, lactic acid, and succinic acid against *P. acnes* were 7.5, 10, 10, and 5 mM, respectively (Figure 4a and Supplementary Figure S3). Since succinic acid had the lowest MBC value, we determined the *K*i of the growth of *P. acnes* for 0–36 h in the presence of succinic acid. The *K*i for succinic acid was 0.97 mM (Supplementary Figure S4). This acid was selected for evaluation of its anti-*P. acnes* activity *in vivo* (Figure 5). Succinic acid effectively suppressed the survival of *P. acnes* at concentrations 5 and 7.5 mM, and completely killed *P. acnes* at a concentration 10 mM (Figure 4a). To assess the acidity (pH 5.5) of 5 mM succinic acid in affecting survival of *P. acnes*, bacteria were incubated with PBS (pH 5.5) or ammonium hydroxide-buffered succinic acid (pH 7.4). Incubation of *P. acnes* with PBS (pH 5.5) did not alter the survival of *P. acnes*. The antimicrobial activity of succinic acid persisted even after buffering 5 mM succinic acid with ammonium hydroxide (Figure 4b), suggesting that the ability to suppress the survival of *P. acnes* by succinic acid was unrelated to direct killing by extracellular acidification.

The antimicrobial effects of SCFAs are caused mainly by the undissociated form of SCFAs (Ostling and Lindgren 1993; Ricke 2003). Non-dissociated SCFAs can passively diffuse through the cell wall of microorganisms and, once internalized into the neutral pH of the cell cytoplasm, can dissociate into anions and protons. Generation of both anions and protons presents potential problems for microorganisms that must maintain their cytoplasm at a near-neutral pH in order to sustain functional macromolecules. Export of excess protons requires consumption of cellular adenosine triphosphate (ATP) and may result in depletion of cellular energy (Ostling and Lindgren 1993). To determine the mechanism of action of succinic acid against *P. acnes*, we loaded *P. acnes* with cFSE, an internally conjugated fluorescent pH probe. As shown in Figure 4c, succinic acid significantly lowered the intracellular pH of *P. acnes*, in agreement with previous findings that a lowered intracellular pH of microbe is a lethal mechanism of SCFA (Ricke 2003).

In vivo efficacy of succinic acid against *P. acnes*

To examine the effectiveness of succinic acid as an intralesional injection therapy against *P. acnes*, mouse ears were injected with a single intradermal injection of *P. acnes*. An intradermal injection was used as an animal model for the granulomatous type of acne inflammation that follows follicular rupture based on previous publications demonstrating

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that intradermal injection of *P. acnes* into mouse ears induces a remarkable granulomatous response (Liu et al. 2011; Nakatsuji et al. 2008a; Nakatsuji et al. 2008b), as well as the fact that *P. acnes* can enter the dermis after follicular wall rupture in severe acne (Kligman 1974; Nakatsuji et al. 2008a; Nakatsuji et al. 2008b). Furthermore, the outbred ICR mice were used for this study because they are polymorphic at a significant number of loci and have a complex genetic history similar to a human population, potentially making these results more applicable to the human population. After the intradermal injection of *P. acnes*, succinic acid (10 μl; 5 mM, a MBC concentration) or PBS control was injected into the same sites previously injected with *P. acnes* (Figure 5). Injection of succinic acid reduced *P. acnes*-induced redness compared with injection of an equal amount of PBS (Figure 5a). It has been reported that *P. acnes* can induce the production of interleukin (IL)-8 via activation of toll-like receptor 2 (TLR-2) (Kim 2005; Nagy et al. 2005). To determine whether succinic acid can reduce the production of *P. acnes*-induced inflammation, ears were homogenized two days after injection with succinic acid or PBS. The level of MIP-2, a murine counterpart of IL-8, was measured by an ELISA. MIP-2 production in the ear injected with succinic acid was approximately 50% less than that detected in the ear injected with PBS (Figure 5b). To determine the intensity of bacterial colonization, ears injected with succinic acid or PBS were homogenized to estimate the CFU. The *P. acnes* numbers in ears injected with PBS and succinic acid were $4.7 \pm 1.3 \times 10^5$ and $2.9 \pm 1.3 \times 10^4$ CFU, respectively, suggesting that succinic acid considerably decreased the growth of *P. acnes* in the lesions (Figure 5c).

SCFAs can penetrate human skin and have even been used as skin penetration enhancers (Kanikkannan et al. 2000). Since topical anti-acne agents can be designed as both over-the-counter and prescription medications, the potency of topical application of succinic acid against *P. acnes* was evaluated. One day after *P. acnes* injection, the surface of the *P. acnes*-inoculated mouse ear was treated with 100 mM topical succinic acid or PBS once per day. Both *P. acnes*-induced redness (Figure 5d) and MIP-2 production (Figure 5e) were significantly attenuated in succinic acid-treated ears compared to PBS-treated ears. The *P. acnes* numbers in succinic acid- and PBS-treated ears were $7.5 \pm 1.5 \times 10^4$ and $1.5 \pm 0.4 \times 10^4$ CFU, respectively (Figure 5f). Results in Figure 5 demonstrate the effective use of intralesional and topical succinic acid for the suppression of inflammation and *P. acnes* growth *in vivo*.

DISCUSSION

The human body is home to ten times more bacteria than human cells (Fujimura et al. 2010). The skin is the human body's largest organ, colonized by a diverse milieu of microorganisms (the skin microbiome), most of which are commensal organisms since they are harmless or sometimes even beneficial to their host (Grice and Segre 2011). SCFAs in the skin play a key role in influencing the composition of bacteria on normal human skin (Ushijima et al. 1984). It has been documented that *P. acnes* can undergo glycerol fermentation to produce SCFAs (Moss et al. 1967). Thus, application of glycerol on acne lesions may trigger the fermentation of *P. acnes*, which influences the growth of other skin commensals. As shown in Supplementary Figure S5, *P. acnes* suspension/agar was poured into agar plates to produce a homogeneous *P. acnes* lawn. Glycerol was used to trigger the fermentation of *P. acnes*. Serial dilutions of skin microorganisms were spotted on the top of a homogeneous *P. acnes* lawn. The colony numbers of skin microorganisms were no different on agar plates with/without glycerol, suggesting that the fermentation of *P. acnes* did not significantly disrupt the growth of skin microorganisms.

Bacterial interference in which friendly bacteria are used to prevent colonization of the host by pathogens has been shown to be a promising modality for preventing and/or treating infections (Frank et al. 2010; Ji et al. 1997; Nicoll and Jensen 1987; Otto 2009; Wei et al.

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2006; Whitehead et al. 1993; Wilkinson and Jensen 1987). Therapeutic application of bacterial interference by active colonization using a human commensal bacterial strain, *S. epidermidis*, was successful in counteracting the infection of *Staphylococcus aureus* (*S. aureus*) (Iwase et al. 2010; Shinefield et al. 1971). Results from a previous study have shown that *S. epidermidis* secretes a serine protease to inhibit the colonization of *S. aureus* (Iwase et al. 2010). A previous publication from our laboratory has demonstrated that *P. acnes* can exploit glycerol fermentation to suppress the growth of pathogenic USA300, the most prevalent community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) (Shu et al. 2013). All of the above studies demonstrated the ability of commensal bacteria to prevent colonization by pathogens. Little is known about the interactions among commensal bacteria. A condition with microbial imbalances on or inside the human body has been termed dysbiosis (Scanlan et al. 2012). Our results indicate that *S. epidermidis* is a probiotic bacterium that may employ glycerol fermentation to rein in the overgrowth of *P. acnes* and therefore control the dysbiosis that manifests as acne vulgaris.

As shown in Figure 1, we demonstrated that skin microorganisms inhibited the growth of *P. acnes*. Although this inhibition may result from antimicrobial proteins/peptides and/or nutrient competition, it was significantly augmented by glycerol fermentation. Eight colonies selected from skin microorganisms that were identified as *S. epidermidis* developed inhibition zones when they were co-cultured with *P. acnes* (Figure 2). We speculate that, under normal physiologic conditions, *S. epidermidis* and *P. acnes* may co-exist on the skin surface without counteracting each other. *S. epidermidis* may later enter acne lesions when acne comedones are created by the overgrowth of *P. acnes*. Human hosts may take advantage of *S. epidermidis* fermentation within an anaerobic acne lesion to combat the overgrowth of *P. acnes*. Thus, future studies will include detecting bacterial survival after co-injection of *S. epidermidis* and *P. acnes* with glycerol in mice. In addition, given that some papers report that the two bacteria do not coexist to a great extent (Fitz-Gibbon et al. 2013; Moon et al. 2012), it is possible that the abundance of *P. acnes* and *S. epidermidis* in an acne lesion may depend on the stage of acne vulgaris. If glycerol fermentation of *S. epidermidis* against *P. acnes* occurs in an acne lesion, it is worth determining if the ratio of *P. acnes* to *S. epidermidis* correlates with the severity of acne vulgaris.

The bacterial interference defined by the formation of inhibition zones and bubble-like territories of competition between *P. acnes* and other skin microorganisms was detectable in the microbiome of fingerprints. Although the composition of the human skin microbiome varies from individual to individual and is dynamic over time in every individual (Grice and Segre 2011), *S. epidermidis*, a long-term resident microorganism in the skin, appeared to strongly compete against *P. acnes* in three different subjects. *Paenibacillus sp.* EH-9 are mainly found in the environment and thus recognized as a short-term skin resident microorganism. Our observation above supports an ecological theory that each person can be viewed as an island-like "patch" of habitat occupied by both long-term and short-term microbial assemblages (Costello et al. 2012). Skin microorganisms collected from both the surface of the nose (Figure 1) and the fingertips (Figure 2) can mediate glycerol fermentation to interfere with the growth of *P. acnes*. Due to different locations and culture media, the composition of skin microorganisms cultured from fingertips may be distinct from that of skin microorganisms cultured from the surface of the nose. Although *S. epidermdis* on the fingertips was identified as a fermenting bacterium against *P. acnes*, it is unclear whether *S. epidermidis* contributes to the bacterial interference between *P. acnes* and skin microorganisms cultured from the surface of the nose. To address this issue, skin microorganisms cultured from nasal surface were streaked on agar plates supplemented with or without glycerol followed by spotting *P. acnes* on the top of bacterial streaks. A zone of inhibition of bacterial growth developed exclusively on the agar plates containing glycerol (Supplementary Figure S6). A colony of skin microorganisms that created an inhibition zone

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within a *P. acnes* colony was identified as *S. epidermdis* by 16S rRNA gene sequencing, demonstrating that glycerol fermentation of *S. epidermidis* in skin microorganisms cultured from the surface of the nose played a role in outcompeting *P. acnes*.

Both intralesional and topical application of succinic acid significantly neutralize *P. acnes*-induced inflammation (Figure 5). The *P. acnes*-induced MIP-2 production in the ear injected intralesionally with PBS or succinic acid (Figure 5b) was markedly higher than that in the ear treated with topical PBS or succinic acid (Figure 5d). The higher production of MIP-2 may result from the two consecutive needle injections with *P. acnes* or PBS/succinic acid. It has been reported that SCFAs have anti-inflammatory activities (Vinolo et al. 2011). Succinic acid can activate a G-protein coupled receptor (GPR) to prevent inflammation (Karaki et al. 2006). SCFAs can regulate several leukocyte functions including production of cytokines [tumor necrosis factor alpha (TNF)-α, IL-2, IL-6 and IL-10]. The ability of leukocytes to migrate to foci of inflammation and destroy microbial pathogens can be affected by the SCFAs (Vinolo et al. 2011). In addition, SCFAs, most notably butyrate, significantly reduced expression of lipopolysaccharide (LPS)-induced interferon (IFN)-γ, TNF-α, and IL-12 (Chakravortty et al. 2000), and *S. aureus*-induced IL-2 and IFN-γ (Park et al. 2007). Aquaporin-3 functions as a glycerol transporter in mammalian skin (Zheng and Bollinger Bollag 2003). It has been known that glycerol helps maintain healthy skin integrity (Fluhr et al. 2008). Aquaporin 3-deficient mice exhibit skin defects, including impairment of water holding capacity, barrier recovery, and wound healing (Zheng and Bollinger Bollag 2003). The above results suggest that acne probiotics containing SCFAs and glycerol may be bi-functional therapeutics targeting both *P. acnes* and skin cells.

Results in Figure 2 and Supplementary Figure S1 indicated that glycerol fermentation of *S. epidermidis* was essential in counteracting *P. acnes*. Succinic acid exerted efficient effects against *P. acnes* (Figure 5), however, it still remains unclear which SCFA in the products of *S. epidermidis* glycerol fermentation primarily contributes to the anti-*P. acnes* effect. It is also undetermined whether SCFAs act together with other antimicrobial molecules in fermentation products to display their anti-*P. acnes* activities. Our recent results have demonstrated that the fermented media of *S. epidermidis* ATCC12228 significantly suppressed the growth of *P. acnes* (Supplementary Figure S7). The anti-*P. acnes* activity of the fermented media persisted after boiling the fermented media, suggesting that the antimicrobial proteins/peptides may be not the major contributors to the anti-*P. acnes* activity of fermented media. A higher dose of SCFAs may be required to achieve *in vivo* efficacy due to its rapid metabolism by skin cells (Schroder et al. 2000; Stein et al. 2000). The pro-drugs of SCFAs such as pivaloylomethyl butyrate (AN-9) (Blank-Porat et al. 2007)have been developed to achieve pharmacologic concentrations of SCFAs *in vivo*. *S. epidermidis* grown on rich medium agar plates without glycerol failed to develop inhibition zones against *P. acnes* (Figure 2 and Supplementary Figure S1). In fact, TSB in rich media contains 2.5 g/l glucose. Thus, in the absence of 20 g/l glycerol, *S. epidermidis* may produce insufficient amounts of SCFAs via glucose fermentation.

Although ferments (SCFAs) were used in this study as anti-*P. acnes* agents, live *S. epidermidis* can potentially be used as an active component in acne probiotics for bacteriotherapy against acne vulgaris. Future studies will include an injection of *S. epidermidis* along with *P. acnes* into mouse ears in the absence or presence of glycerol. Ear homogenates will be spotted on an agar plate supplemented with furazolidone (furoxone), a culture medium selective for *P. acnes* to determine the presence of the organism in individuals with and without acne vulgaris (Marino and Stoughton 1982). Since *S. epidermidis* does not grow on this medium, only the colonies of *P. acnes* can be seen on a plate spotted with ear homogenates containing both *S. epidermidis* and *P. acnes*. The interference of *S. epidermidis* with *P. acnes in vivo* can be thus quantified using

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furazolidone-supplemented agar plates. SCFAs are normal human metabolites and theoretically less toxic, but SCFAs at high doses may create an extremely acidic solution that may be toxic to skin cells. Thus, buffered SCFAs or pro-drugs of SCFAs may serve as alternative anti-*P. acnes* agents. Application of succinic acid notably, but not completely, suppressed the *P. acnes*-induced inflammation (Figure 5). Thus, application with an acne probiotic composed of more than one SCFA (Martin-Pelaez et al. 2010) or multiple beneficial microorganisms may be needed for full potency. *S. epidermidis* that interferes with the growth of *P. acnes* via fermentation was isolated from the human skin microbiome in an attempt to develop acne probiotics. We believe that various skin microorganisms have the specific ability to antagonize different (non-)pathogens using fermentation. Thus, besides acne probiotics, other "skin probiotics" using fermentation of skin microorganisms to treat various skin conditions can potentially be achieved.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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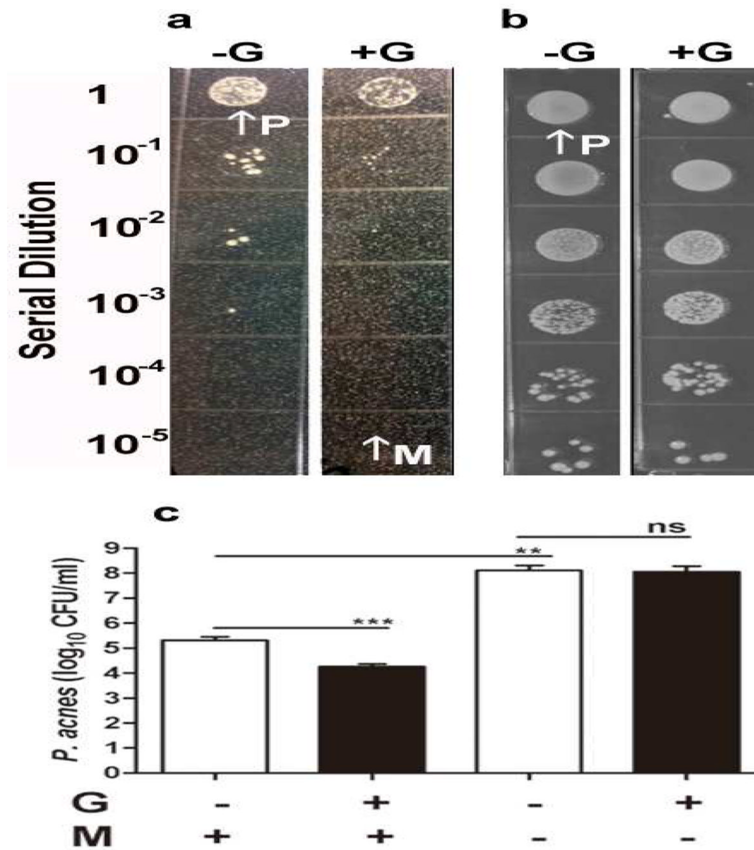

Figure 1. Inhibition of the growth of *P. acnes* by skin microorganisms in a homogeneous microbial lawn

(a) A homogeneous lawn of microbes was created by pouring the skin microorganisms (M, arrow; 10^5 CFU) that were pre-mixed with 1% agar with/without glycerol (+G/-G; 20 g/l) in TSB. *P. acnes* (P, arrow) bacteria with a serial dilution (5×10^6- 5×10^1 CFU in 5 μl PBS) were spotted on the top of microbial lawn for three days for CFU counts. (b) The serially diluted *P. acnes* was spotted on the regular plates (without pouring skin microorganisms) with/without glycerol. (c) The CFU counts of *P. acnes* were presented as 95% CI of the means of three independent experiments. **$P<0.01$; ***$P<0.001$ was obtained by two-tailed *t*-tests. ns, not significant.

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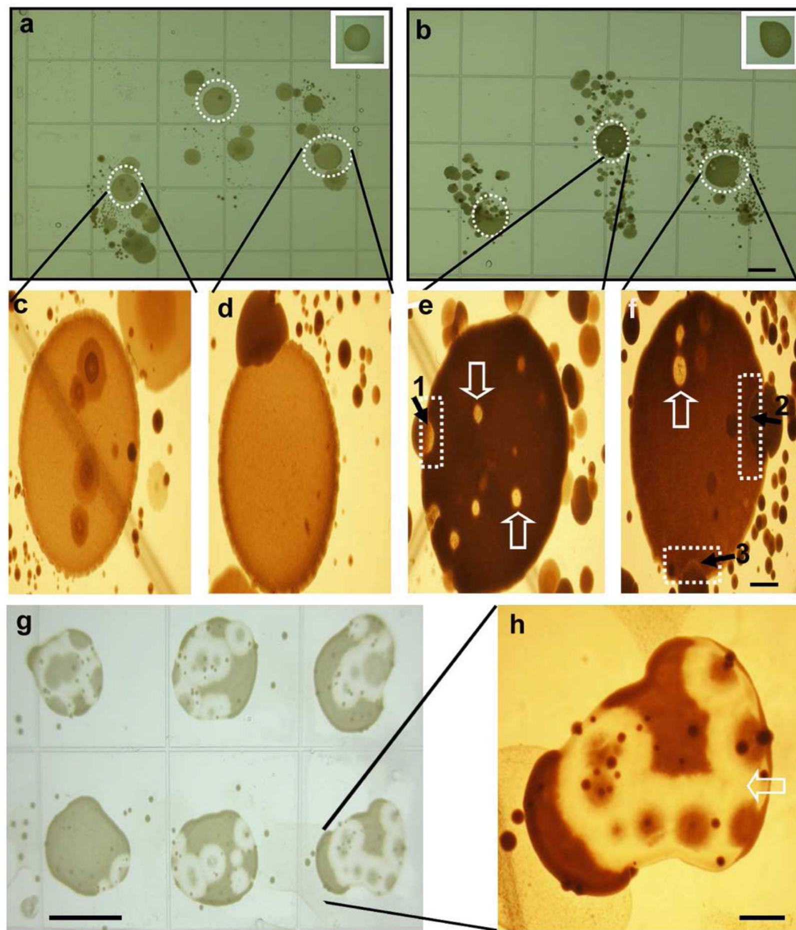

Figure 2. Skin fingerprint analysis of glycerol fermentation of skin microorganisms against *P. acnes*

The fingerprints of index, middle, and ring fingers were pressed onto the surface of rich medium agar plates in the absence (a) or presence (b) of 20 g/l glycerol at 30°C unde r anaerobic conditions using Gas-Paks. *P. acnes* (10^7 CFU in 5 μl PBS) was spotted on the central portion of each fingerprint. Spotting *P. acnes* away from fingerprints served as controls (inserts). The high magnitude photos of (a) and (b) were displayed in (c, d) and (e, f), respectively. The inhibition zones (dash squares in e and f) were detected at the boundary between colonies of *P. acnes* and skin microorganisms. The bubble-like territories of competition (open arrows) were found within *P. acnes* colonies. In a representative plate, single colonies labeled 1 and 2 (solid arrows) were identified as *S. epidermidis*. A single colony labeled 3 was identified as *Paenibacillus sp*. Y412MC1. Six additional colonies from fingerprint bacteria of two different subjects were identified as *S. epidermidis. S. epidermidis* (10^5 CFU in 100μl PBS) from colony 1 was re-streaked on an agar plate containing glycerol followed by spotting six separate drops of *P. acnes* (10^7 CFU in 5 μl PBS) on the top of a *S. epidermidis* streak (g). A high magnitude photo of one of *P. acnes* colonies (g) was displayed in (h). Bars (a-b, g)=0.5 cm; (c-f, h)=0.1 cm.

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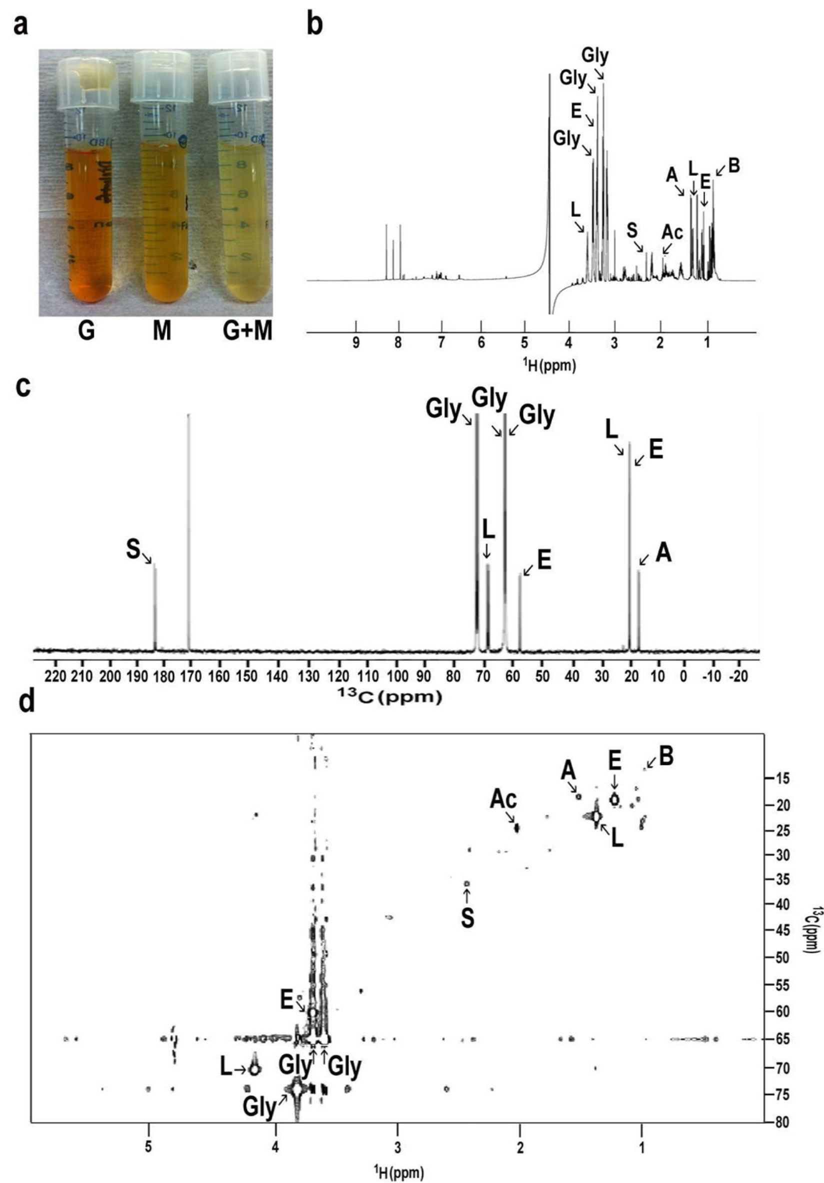

Figure 3. Identification of SCFAs in the fermented media of skin microorganisms

(a) Skin microorganisms (10^5 CFU/ml) were incubated in rich medium in the absence (M) and presence (G+M) of glycerol for six days under anaerobic conditions. Rich medium plus glycerol without skin microorganisms (G) was included as a control. Fermented media of skin microorganisms were centrifuged and passed through a 0.2 μm filter. Supernatants were then mixed with 10% D_2O and analyzed by NMR spectrometers. Representative 1-D 1H- (b) and ^{13}C- (c) NMR spectra (400 MHz) that reveal the principal SCFAs in the fermented media six days after addition of $^{13}C_3$-glycerol. (d) A 2-D ^1H-^{13}C HSQC NMR spectrum (600 MHz) was displayed. In addition to glycerol (Gly), ethanol (E), alanine (A), four SCFAs [acetic acid (Ac), butyric acid (B), lactic acid (L), and succinic acid (S)] were detected in the ferments of skin microorganisms.

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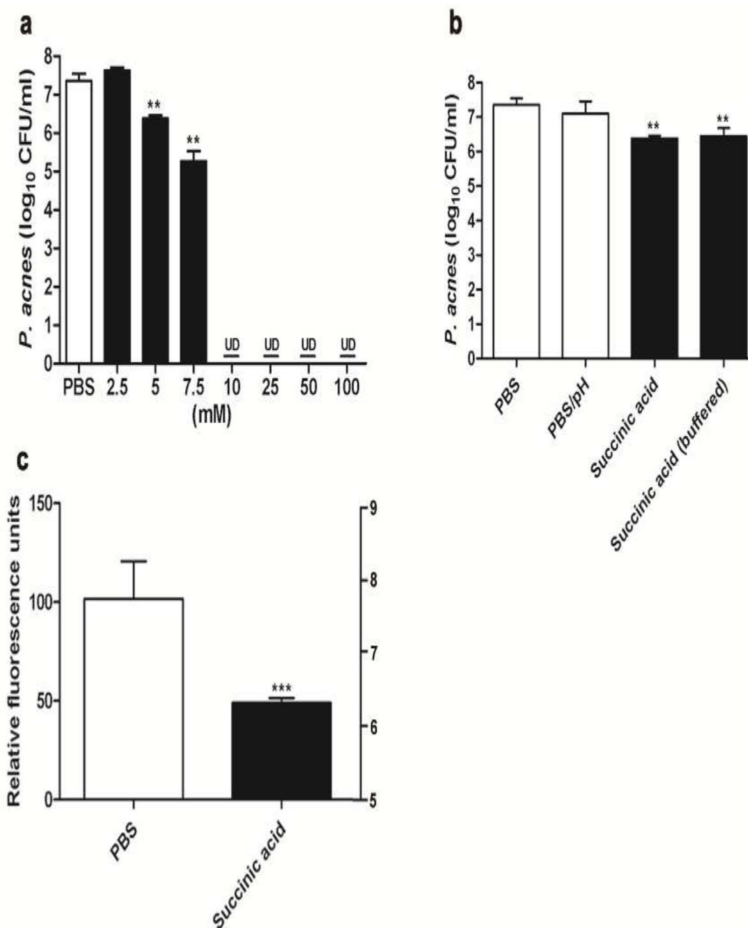

Figure 4. The MBC of succinic acid against *P. acnes*, the effect of pH on the anti-*P. acnes* activity of succinic acid, and the decrease in intracellular pH of *P. acnes* by succinic acid

(a) *P. acnes* (10^8 CFU/ml) was incubated with succinic acid (2.5–100 mM in PBS) on a 96-well microplate overnight. Bacteria incubated with PBS alone as a control. (b) *P. acnes* was incubated with PBS (pH 7.4), PBS (PBS/pH; pH 5.5), 5 mM succinic acid (pH 5.5) or ammonium hydroxide-buffered succinic acid (pH 7.4) to determine if the acidity of 5 mM succinic acid affects the survival of *P. acnes*. After incubation, *P. acnes* was diluted 1:10–1:10^6 with PBS, and 5 μl of the dilutions were spotted on an agar plate for CFU counts. (c) The cFSE-loaded *P. acnes* (3×10^4 CFU) was treated with 5 mM succinic acid or PBS. The change in the relative fluorescence units corresponding to intracellular pH of *P. acnes* was measured 5 min after treatment. **$P<0.01$; ***$P<0.001$ (two-tailed *t*-tests). Data are 95% CI of the means of three individual experiments. UD, undetectable.

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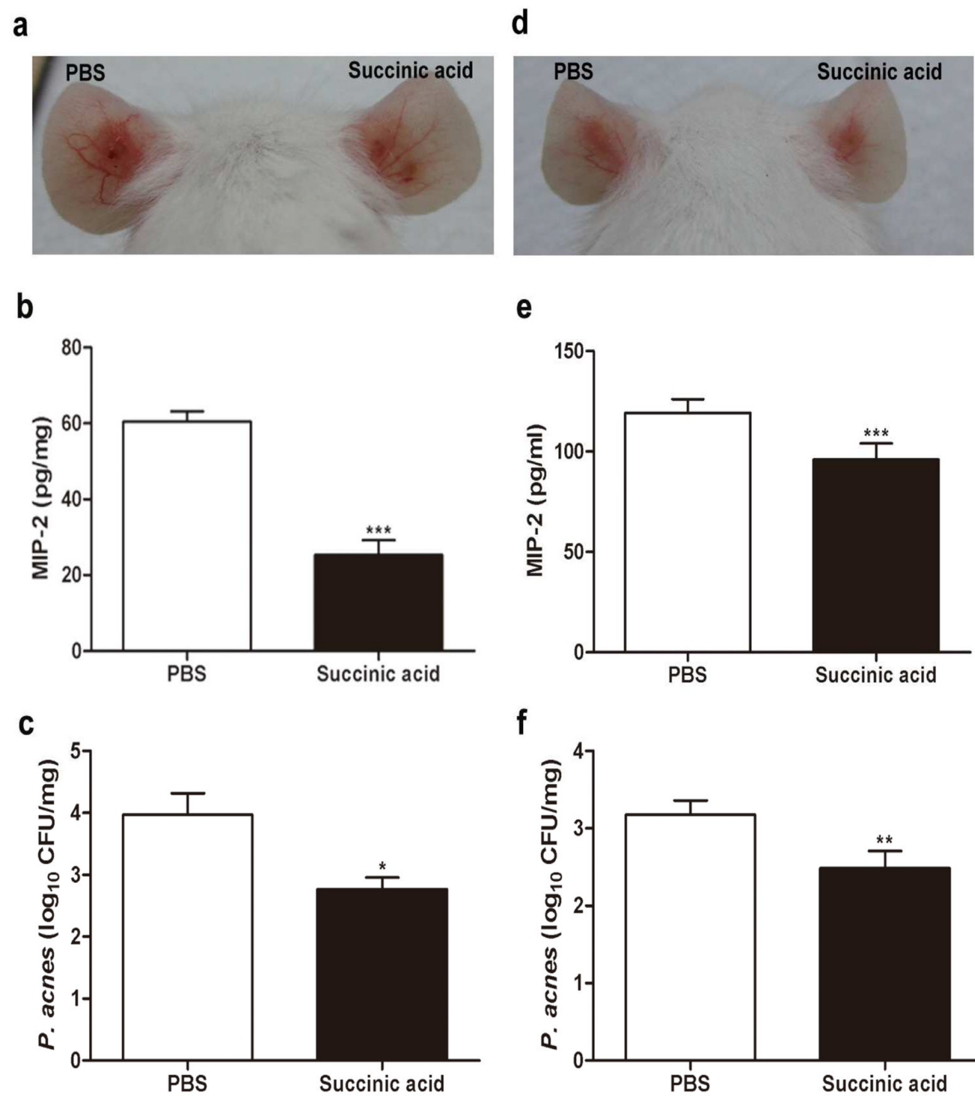

Figure 5. Succinic acid suppresses *P. acnes*-induced inflammation and decreases bacterial colonization *in vivo*

The ears of ICR mice were injected intradermally with *P. acnes* (10^7 CFU in 10 μl PBS) or PBS (10 μl). One day after injection of *P. acnes* or PBS, succinic acid or PBS was intralesionally injected into inflamed lesions (a-c) or topically applied on the surface of inflamed lesions (d-f). Photos of ear inflammation were taken three (a) or four (d) days after *P. acnes* injection. The levels of MIP-2 cytokines (b, e) in the homogenates of succinic acid- or PBS-treated ears were measured by an ELISA kit. The CFUs (c, f) in the ears treated with succinic acid or PBS were enumerated by plating serial dilutions ($1{:}10^1$–$1{:}10^6$) of the homogenate on an agar plate. ***P<0.001; ***P<0.01; *P<0.05. P-values were evaluated using two-tailed t-tests. Data are 95% CI of the means of three separate experiments using five mice per group.