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# Effect of *Lactobacillus reuteri* on the Proliferation of *Propionibacterium acnes* and *Staphylococcus epidermidis*

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**While it is generally accepted that *Propionibacterium acnes* is involved in the development of acne, other bacteria including *Staphylococcus epidermidis* have also been isolated from the acne lesion. The interaction between *Lactobacillus reuteri*, a probiotic bacterium, and acnegenic bacteria is unclear. This study examined the effects of *L. reuteri* on the proliferation of *P. acnes* and *S. epidermidis*. Human-derived *L. reuteri* strains (KCTC 3594 and KCTC 3678) and rat-derived *L. reuteri* KCTC 3679 were used. All strains exhibited significant inhibitory effects on the growth of *P. acnes* and *S. epidermidis*. The proliferation of *P. acnes* was decreased by 2-log scales after incubation with *L. reuteri* for 24 h. In addition, the proliferation of *S. epidermidis* was decreased by 3-log scales after incubation with *L. reuteri* for 24 h, whereas the growth of *L. reuteri* was unaffected by *P. acnes* or *S. epidermidis*. Among the *L. reuteri* strains examined, *L. reuteri* KCTC 3679 had the strongest inhibitory effect on the growth of *P. acnes* and *S. epidermidis*, followed by *L. reuteri* KCTC 3594 and *L. reuteri* KCTC 3678. Interestingly, reuterin, an antimicrobial factor, was produced only by *L. reuteri* KCTC 3594. The most pronounced the antibacterial activities of *L. reuteri* were attributed to the production of organic acids. Overall, these results suggest that *L. reuteri* may be a useful probiotic agent to control the growth of bacteria involved in acne inflammation and prevent acne.**

***Keywords***: *Lactobacillus reuteri*, *Propionibacterium acnes*, *Staphylococcus epidermidis*, antibacterial

## Introduction

Acne vulgaris is one of the most common skin diseases, and is observed in approximately 80% of young adults (Johnson *et al.*, 1984). Acne vulgaris affects areas containing the largest oil glands, including the face, back, and trunk (Leyden, 1997). Acne is affected by several factors, specifically hormonal imbalances, bacterial infections, stress, food, or cosmetic application (Burkhart *et al.*, 1999). Normal skin commensals including *Propionibacterium acnes*, *Propionibacterium granulosum*, *Staphylococcus epidermidis*, and *Malassezia furfur*, proliferate rapidly during puberty and are often involved in the development of acne (Holland *et al.*, 1979). *P. acnes*, an obligate anaerobic organism, has been implicated in the development of inflammatory acne by its ability to activate complements and metabolize sebaceous triglycerides into fatty acids, which attract neutrophils chemotactically (Webster *et al.*, 1980). On the other hand, *S. epidermidis*, an aerobic organism, normally involves superficial infections within the sebaceous unit (Burkhart *et al.*, 1999).

As therapeutic agents for acne, antibiotics are normally used to inhibit inflammation or kill bacteria (Guin *et al.*, 1979). Triclosan, benzoyl peroxide, azelaic acid, retinoid, tetracycline, erythromycin, macrolide, and clindamycin are among these antibiotics (Bettley and Dale, 1976; Breathnach *et al.*, 1984; Zouboulis and Piquero-Martin, 2003). On the other hand, these antibiotics also induce side effects. Benzoyl peroxide and retinoid cream cause xerosis cutis and skin irritation if used excessively as treatments and several reports have also suggested that tetracycline, erythromycin, macrolide, and clindamycin are associated with the appearance of resistant bacteria, organ damage, and immunohypersensitivity if taken for a long time (Ochsendorf, 2006; Leyden *et al.*, 2007; Patel *et al.*, 2010). Therefore, many researchers have tried to develop therapeutic agents for acne with no side effects but high antibacterial activity (Nam *et al.*, 2003; Kang *et al.*, 2009; Niyomkam *et al.*, 2010).

Lactic acid bacteria (LAB), such as *Lactobacillus* species, have protective effects against a variety of pathogenic infections in the gastrointestinal systems of humans and animals (Ahrné *et al.*, 1998; Gill *et al.*, 2001). These strains produce a wide range of antimicrobial agents, and have been studied extensively with regard to their possible use in the field of probiotics. Probiotics may be a useful alternative for the treatment and prevention of a variety of infectious diseases caused by oral, enteric and urogenital pathogens (Shornikova *et al.*, 1997; Çaglar *et al.*, 2006; Abad and Safdar, 2009). *Lactobacillus reuteri*, an established probiotic agent, is used widely in viral diarrhea diseases (Saavedra, 2000; Szajewska and Mrukowicz, 2005).

*L. reuteri*, an obligatory heterofermentative resident in the gastrointestinal tract of humans, produces a water soluble antimicrobial compound, reuterin, which is effective over a wide pH range, and resistant to proteolytic and lipolytic

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enzymes (Talarico *et al.*, 1988; El-Ziney *et al.*, 1999). Reuterin-producing *L. reuteri* strains have inhibitory effects against many Gram-positive and Gram-negative bacteria including enteric pathogens (Talarico *et al.*, 1988; Cleusix *et al.*, 2007; Spinler *et al.*, 2008). In addition, the interaction between *L. reuteri* and a major cariogenic pathogen *S. mutans* and several periodontal pathogens have also been studied (Ishihara *et al.*, 1985; Ahola *et al.*, 2002; Nikawa *et al.*, 2004; Kang *et al.*, 2011). These studies suggest that *L. reuteri* has potential as a useful probiotic agent but there are no data on the inhibitory effects of *L. reuteri* on acne-involved microorganisms.

The purpose of this study was to examine the potential of *L. reuteri* as an effective treatment for acne, based on the previously known antibacterial effects of *L. reuteri*. Specifically, the effects of *L. reuteri* on the growth of *P. acnes* and *S. epidermidis* were examined.

## Materials and Methods

### Bacterial strains and growth conditions

*L. reuteri* KCTC 3594 and KCTC 3678 were isolated from the intestines and feces of humans, respectively. *L. reuteri* KCTC 3679 was isolated from rat. *L. reuteri* strains were grown in De Man, Rogosa, Sharpe broth (MRS broth; Difco, USA). *S. epidermidis* ATCC 12228 was grown in brain heart infusion broth (BHI broth; Difco). These bacteria were incubated at 37°C for 16 h under aerobic conditions. *P. acnes* ATCC 11828 was grown in Actinomyces broth (Difco) under anaerobic conditions (85% $N_2$, 10% $H_2$, 5% $CO_2$) at 37°C for 24 h. Bacterial cultures in the logarithmic growth phase were used in all experiments. Before being used in each experiment, they were propagated twice in the appropriate broth.

### Effect of *L. reuteri* on the proliferation of *P. acnes* and *S. epidermidis*

To determine the effects of *L. reuteri* on the proliferation of *P. acnes* and *S. epidermidis*, *P. acnes* or *S. epidermidis* and *L. reuteri* strains were incubated in a mixture of an equal volume of each growth medium at 37°C for 8 and 24 h under aerobic or anaerobic conditions. The inoculum sizes of the tested bacteria were determined by measuring the optical density at 600 nm ($OD_{600}$) and extrapolating the CFU/ml using preset standard curves. The final inoculum concentrations of *P. acnes*, *S. epidermidis*, and *L. reuteri* were $1\times10^6$, $5\times10^5$ and $5\times10^5$ CFU/ml, respectively. Each culture was diluted serially and plated on MRS agar for the *L. reuteri* strains, Actinomyces agar for *P. acnes* and BHI agar for *S. epidermidis* to determine the effects of *L. reuteri* on the proliferation of *P. acnes* and *S. epidermidis*.

### Acid and hydrogen peroxide ($H_2O_2$) production

The amount of acid produced by *L. reuteri* was determined indirectly by measuring the pH of the supernatant of a culture using a pH meter (Corning Inc., USA). Each *L. reuteri* strain was tested for its ability to generate $H_2O_2$ using a modification of the method reported by Eschenbach *et al.* (1989). *L. reuteri* was plated onto MRS agar containing 0.25 mg/ml of TMB (3,3′,5,5′-tetramethylbenzidine; Sigma) and 0.01 mg/ml of peroxidase (Sigma), and was incubated anaerobically for 48 h. Subsequently, *L. reuteri* was exposed to ambient air. Colonies showing a blue color were considered to be $H_2O_2$ producers.

### Quantification of reuterin

Reuterin was prepared as described elsewhere (Slininger *et al.*, 1983). Briefly, *L. reuteri* was incubated at 37°C for 24 h under anaerobic conditions. The cells were harvested by centrifugation at 5,000×*g* for 15 min at 20°C, washed with 50 mM sodium phosphate buffer (pH 7.4) and resuspended to a concentration of $1.5\times10^{10}$ CFU/ml in a 300 mM glycerol solution. The cells were incubated at 37°C for 3 h under anaerobic conditions to examine the production of reuterin as a function of time. Simultaneously, the samples were taken immediately after resuspension in glycerol, and the number of viable *L. reuteri* cells was determined. The reuterin-containing supernatants were collected by centrifugation (5,000×*g*, 15 min), filter-sterilized and stored at 4°C until needed. The reuterin samples were analyzed colorimetrically as described elsewhere (Lüthi-Peng *et al.*, 2002) with some modifications. Briefly, 1 ml of each reuterin sample was mixed with 0.75 ml of 10 mM tryptophan dissolved in 0.05 M HCl followed by the addition of 3 ml of 12 M HCl and incubation at 37°C for 20 min. The optical density of each reaction was measured at 560 nm. Acrolein (Sigma) in 50 mM sodium phosphate buffer was used as the calibration standard. The amount of reuterin was normalized to the initial CFU/ml of the culture.

### Antimicrobial characterization

Cell-free supernatants of *L. reuteri* were prepared and tested against *P. acnes* and *S. epidermidis* to determine the cumulative inhibitory activity, reflecting the activity of the organic acids, $H_2O_2$ or a bacteriocin-like compound (BLC). Each *L. reuteri* strain was incubated for 24 h at 37°C under aerobic conditions. After pelleting the bacterial cells (4,000×*g*, 20 min, 4°C), the supernatants were obtained and filter-sterilized (0.22 μm pore size; Millipore, USA). The inhibitory effects of organic acids on growth were determined after mixing the sterilized supernatants with proteinase K (0.1 mg/ml; Sigma) and catalase (0.5 mg/ml; Sigma). The $H_2O_2$-dependent activity was evaluated using neutralized, proteinase K-treated supernatants and the BLC-dependent activity was analyzed using catalase. Logarithmic-phase cultures of the bacteria were added to the culture medium containing the *L. reuteri* culture supernatants in the wells of microtiter plates, and their growth was assessed after a period of incubation. A one-tenth volume of each tested organism's culture was inoculated with 0.1 ml of the *L. reuteri* culture supernatants. The final inoculum concentrations of *P. acnes* and *S. epidermidis* were $1\times10^6$ and $5\times10^5$ CFU/ml, respectively. The controls consisted of cells grown in the medium only. After 24 h incubation, the $OD_{600}$ of the cell suspension was measured and the inhibition percentage was calculated.

### Statistics

Each experiment was carried out in triplicate, and the mean value was determined. Statistical analysis was carried out using SPSS version 17.0 (Statistical packages for Social Science version 17.0; SPSS Inc., USA). A Mann-Whitney test was used to determine the statistically significant differences in the experiments.

## Results

### Inhibitory effect of *L. reuteri* against *P. acnes*

The antibacterial activities of *L. reuteri* against *P. acnes* were evaluated. All three *L. reuteri* strains inhibited the growth of *P. acnes*, over the 24 h time period (Fig. 1A; $p<0.05$). The proliferation of *P. acnes* decreased by 2-log cycles ($1.0\times10^{2} \pm 7.1\times10^{1}$ CFU/ml, $p<0.05$) after incubation at 37°C for 24 h in the groups to which the *L. reuteri* strains had been added, as compared to the control group ($1.6\times10^{9} \pm 1.3\times10^{8}$ CFU/ml), whereas the growth of *L. reuteri* strains were unaffected by *P. acnes* (Fig. 1B).

### Inhibitory effect of *L. reuteri* against *S. epidermidis*

The antibacterial activities of *L. reuteri* against *S. epidermidis* were evaluated. All three *L. reuteri* strains inhibited the growth of *S. epidermidis* significantly over the 24 h time period (Fig. 2A; $p<0.05$). The concentrations of *S. epidermidis* decreased by 3-log cycles ($5.0\times10^{3} \pm 2.1\times10^{3}$ CFU/ml, $p<0.05$) after incubation at 37°C for 24 h in the groups to which the *L. reuteri* strains had been added, compared to the control group ($1.5\times10^{8} \pm 1.4\times10^{7}$ CFU/ml). On the other hand, the growth of *L. reuteri* strains was unaffected by *S. epidermidis* (Fig. 2B).

### Characterization of antimicrobial substances

*L. reuteri* strains produced antimicrobial substances including $H_2O_2$, organic acid and reuterin (Table 1). Among these, reuterin was produced only by *L. reuteri* KCTC 3594. The antimicrobial activities of *L. reuteri* were modified by pH, catalase and proteinase K (Fig. 3). The inhibitory effects of *L. reuteri* on the growth of *P. acnes* and *S. epidermidis* were not affected by the proteinase K and catalase treatment. The acid product of *L. reuteri* inhibited the growth of *P.*

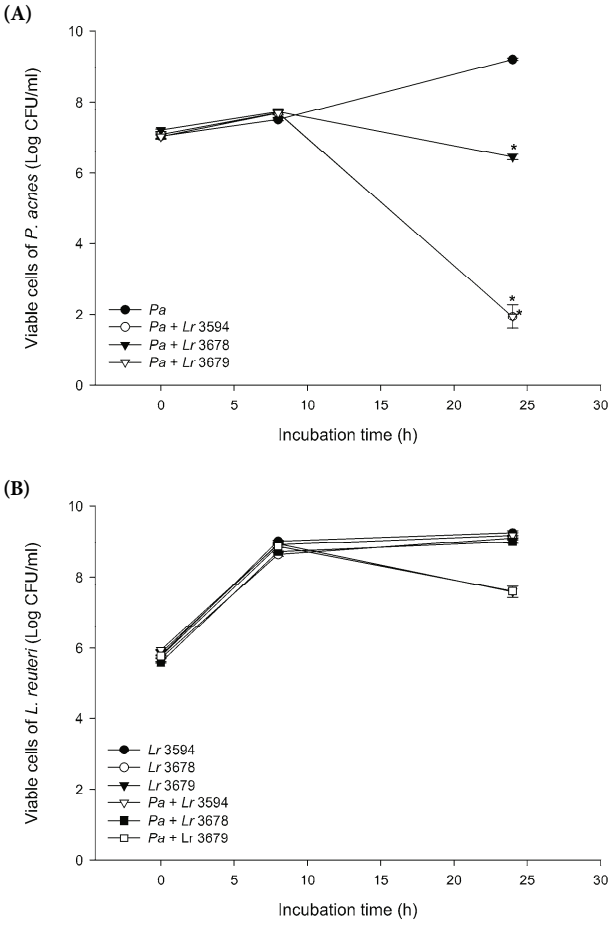


**Fig. 1. Viable cells of *P. acnes* (A) and *L. reuteri* (B) strains in the mixed cultures as a function of the incubation time.** *Pa*, *P. acnes*; *Lr*, *L. reuteri*. *=$p<0.05$ for coculture vs. monoculture. The values are reported as the mean±SD of three independent experiments.

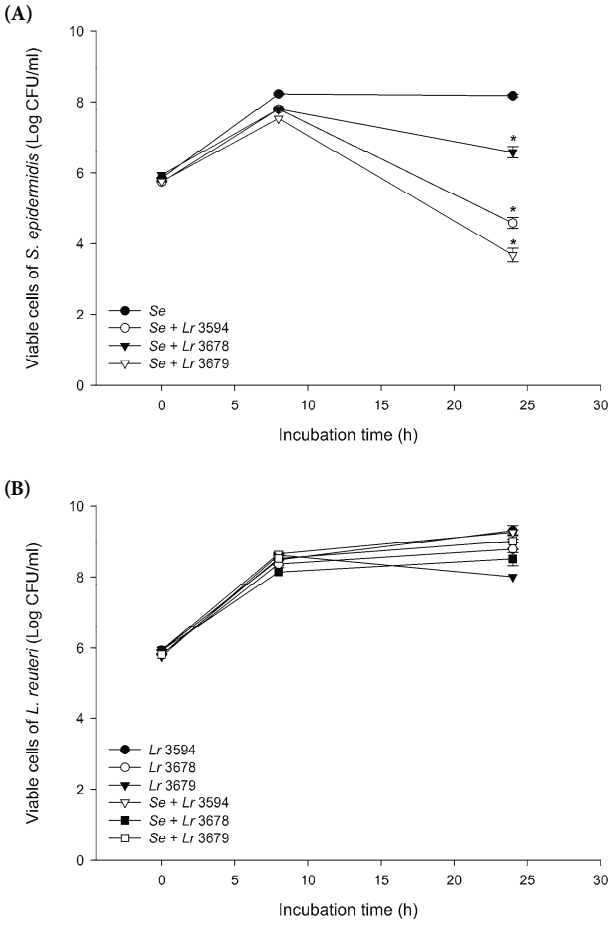


**Fig. 2. Viable cells of *S. epidermidis* (A) and *L. reuteri* (B) strains in the mixed cultures as a function of the incubation time.** *Se*, *S. epidermidis*; *Lr*, *L. reuteri*. *=$p<0.05$ for coculture vs. monoculture. The values are reported as the mean±SD of three independent experiments.

**Table 1. Production of antimicrobial substances by *L. reuteri***

| | *L. reuteri* KCTC strains | | |
|---|---|---|---|
| | 3594 | 3678 | 3679 |
| Source | Human intestine | Human feces | Rat |
| $H_2O_2$ production | Positive | Positive | Positive |
| pH of medium at the end of growth | 4.35 | 4.34 | 4.35 |
| Reuterin production (mM)[a] | 2.51 | 0.00 | 0.00 |

[a] Reuterin was assessed for each *L. reuteri* strain in 300 mM glycerol and analyzed using a quantitative method. Each concentration was determined by a colorimetric method performed in triplicate and is represented as the mean.

*acnes* and *S. epidermidis* completely. The antimicrobial activity of *L. reuteri* was reduced significantly after neutralization ($p<0.05$). On the other hand, the $H_2O_2$ or BLC of *L. reuteri* exerted 18–36% growth inhibition of *S. epidermidis* (Fig. 3B), whereas the growth of *P. acnes* was not affected by these antimicrobial substances (Fig. 3A).

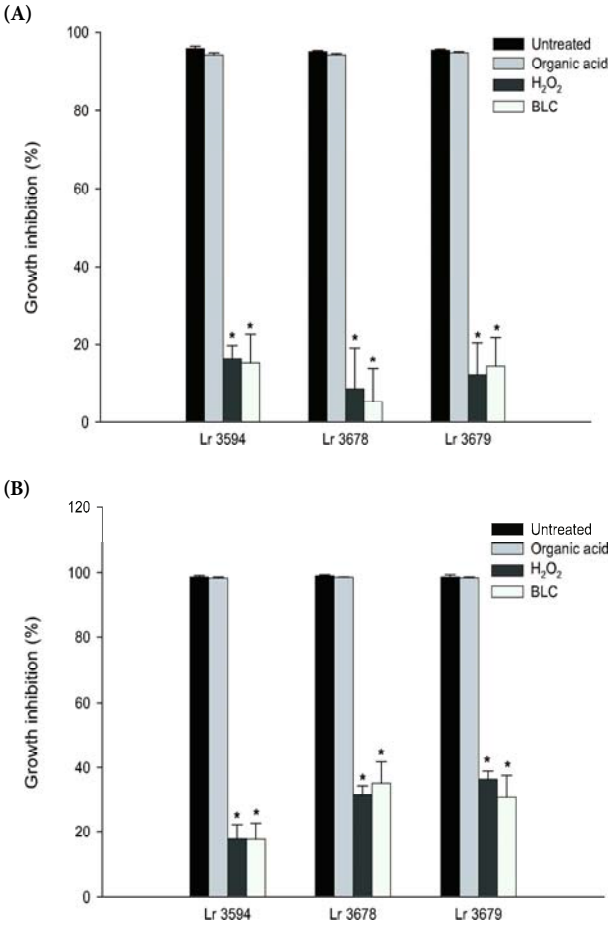


**Fig. 3. Antimicrobial activity of organic acid, hydrogen peroxide ($H_2O_2$) and a bacteriocin-like compound (BLC) in cultured supernatants of *L. reuteri* against *P. acnes* (A) and *S. epidermidis* (B).** $OD_{600}$ of the cell suspension was measured and the percentage inhibition was calculated using the following formula: Inhibition (%)=$OD_{600}$ (control - tested supernatant) / $OD_{600}$ (control)×100. Inhibition of growth by organic acid was measured after a treatment of the sterilized supernatants with proteinase K (0.1 mg/ml) and catalase (0.5 mg/ml). $H_2O_2$-dependent activity was evaluated using the neutralized and proteinase K-treated supernatants. Inhibition of growth by the BLC-dependent activity was evaluated using the neutralized and catalase-treated supernatants. *Pa*, *P. acnes*; *Se*, *S. epidermidis*; *Lr*, *L. reuteri*. *=$p<0.05$, inhibition by $H_2O_2$ or BLC vs. organic acid. The values are reported as the mean±SD of three independent experiments.

## Discussion

Acne lesions are colonized mainly by two coexisting groups of bacteria, anaerobic diphtheroids (*P. acnes* and *P. granulosum*) and staphylococci (*S. epidermidis*) (Koreck *et al.*, 2003). *S. epidermidis* is a gram-positive coccus that comprises greater than 90% of the aerobic cutaneous flora (Cogen *et al.*, 2008). *P. acnes* is a Gram-positive, non-spore forming, pleomorphic, and anaerobic bacterium that is ubiquitous in nature and found commonly as normal flora of the human gut. If the microbial flora is significant in the pathogenesis of acne, the most likely organism is *P. acnes* (Webster *et al.*, 1980). Moreover, this strain has been recognized as a bacterium causing acne and skin inflammation (Meisler and Mandelbaum, 1989), and has been implicated as contributing to the inflammatory response of acne. This strain acts as immunostimulator that can produce a variety of enzymes and biologically active molecules involved in the development of inflammatory acnes.

LAB produces lactic acid and other organic acids that can exhibit antimicrobial activity (Taniguchi *et al.*, 1998). In this context, this study examined the antibacterial activities of *L. reuteri* strains on the bacteria involved in the development of acne. The present study is the first report on the inhibitory effects of *L. reuteri* on the proliferation of *P. acnes and S. epidermidis*. These results showed that the three *L. reuteri* strains were excellent producers of organic acids as antimicrobial agents, inhibiting *P. acnes* and *S. epidermidis*.

The antimicrobial activity of the *L. reuteri* culture supernatants was not lost after a catalase and proteinase K treatment. In addition, there was a significant decrease in antimicrobial activity after adjusting the supernatants to pH 7.0. This finding suggests that the antimicrobial activity of *L. reuteri* might be due to acids. On the other hand, *L. reuteri* still showed 18–36% growth inhibition of *S. epidermidis* after pH neutralization. Therefore, organic acids are not the only antimicrobial substances produced by these *L. reuteri* strains. This observation suggests the presence of other antimicrobial substances, such as $H_2O_2$ or BLC. The present study corroborates many other studies showing that LAB can produce organic acids, $H_2O_2$, and bacteriocins (Eschenbach *et al.*, 1989; Reid *et al.*, 2004).

Many authors reported that *L. reuteri* produced reuterin (Talarico *et al.*, 1988; El-Ziney *et al.*, 1999; Cleusix *et al.*, 2007). The present study showed that *L. reuteri* KCTC 3594

produced a substantial quantity of reuterin. Although only the *L. reuteri* strain KCTC 3594 produced reuterin, the other three *L. reuteri* strains had similar ability to inhibit the proliferation of oral pathogens, as previously shown (Kang *et al.*, 2011). This result suggests that the inhibitory effect of *L. reuteri* is not completely explained by the reuterin activity.

Probiotics are live microorganisms that can benefit the host when administered in adequate amounts (Guarner *et al.*, 2005). Since the normal microbiota of the skin is likely involved in competitive exclusion of pathogens, this activity can be enhanced by the use of probiotics. The results of the current study suggest that *L. reuteri* has potential as a probiotic for the skin. Because probiotics on the skin interact with potential skin pathogens, it is expected that the production of antimicrobial substances of *L. reuteri* is indispensible for its inhibiton effect of skin pathogens. Future studies are warranted to identify and isolate specific antimicrobial components of *L. reuteri* that can be used in the management of acnes.

In conclusion, *L. reuteri* can inhibit the growth of *P. acnes* and *S. epidermidis*. Therefore, *L. reuteri* may be useful as a probiotic agent to prevent the development of acne. Further studies are needed and should focus on the assessment of *L. reuteri* strains that also exhibit activity against potential skin pathogens and will indeed persist on the skin *in vivo* and be active there.

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