Original Research

Antimicrobial activity of *Lactiplantibacillus plantarum* APsulloc 331261 and APsulloc 331266 against pathogenic skin microbiota

Minjung Chae[1,*], Beom Joon Kim[2,3,*], Jungtae Na[2,4,†], Su-Young Kim[2,3,†], Jung Ok Lee[2,†], Yu-jin Kim[2], Esther Lee[2,3], Donghyun Cho[1], Jonghwa Roh[1], Wangi Kim[1]

[1]*AMOREPACIFIC R&D Center, AMOREPACIFIC, 17074 Yongin, Republic of Korea,* [2]*Department of Dermatology, College of Medicine, Chung-Ang University, 06974 Seoul, Republic of Korea,* [3]*Department of Medicine, Graduate School, Chung-Ang University, 06973 Seoul, Republic of Korea,* [4]*Department of Life Science, Sogang University, 04107 Seoul, Republic of Korea*

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1. Abstract

Balanced skin microbiota is crucial for maintaining healthy normal skin function; however, disruption of the balance in skin microbiota is linked with skin diseases such as atopic dermatitis, acne vulgaris, dandruff, and candidiasis. *Lactoplantibacillus* species with proved with health benefits are probiotics that improve the balance of microbiome in skin and gut. In the present study, we investigated the potential antimicrobial activity of *Lactiplantibacillus plantarum* APsulloc 331261 (APsulloc 331261) and *Lactiplantibacillus plantarum* APsulloc 331266 (APsulloc 331266) derived from green tea, in inhibiting five skin pathogenic strains (*Staphylococcus aureus* (*S. aureus*), *Cutibacterium acnes* (*C. acnes*), *Candia albicans* (*C. albicans*), *Malassezia globosa* (*M. globose*), and *Malassezia restricta* (*M. restricta*)) associated with skin infection. Viability of *S. aureus*, *C. acnes*, *C. albicans*, *M. globosa*, *and M. restricta* was inhibited by indirect co-culture with APsulloc 331261 or APsulloc 331266 at various ratios. Different concentrations of the cell-free conditioned media (CM) derived from APsulloc 331261 or APsulloc 331266 inhibited the vaibility of *S. aureus*, *C. acnes*, *C. albicans*, *M. globosa* and *M. restricta* in a dose dependent manner. Moreover,

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susceptibility of *S. aureus*, *C. acnes*, and *C. albicans* against APsulloc 331261 or APsulloc 331266 was confirmed following agar overlay methods. Results of the agar overlay confirmed that various concentrations of APsulloc 331261 and APsulloc 331266 exhibited low to high inhibitory activity on the growth of *S. aureus* (ZDI 20.3 ± 2.1–32.3 ± 2.1 mm, R value 5.7 ± 0.8–7.8 ± 1.3 mm), *C. acnes* (ZDI 15.0 ± 1.7–22.2 ± 1.7 mm, R value 3.2 ± 1.3–5.5 ± 1.3 mm) and *C. albicans* (ZDI 13.3 ± 4.0–27.0 ± 3.6 mm, R value 2.8 ± 1.9–5.5 ± 1.7 mm). Finally, standard PCR analysis identified the presence of the of plantaricin genes encoding antimicrobial peptides in APsulloc 331261 and APsulloc 331266. These results suggest that APsulloc 331261 and APsulloc 331266 has a potential effect in the improvement of the balance of skin microbiota by inhibiting skin pathogenic strains.

2. Introduction

Probiotics are defined as living organisms that provide a health benefit on the host when administered in adequate amounts [1]. Probiotics are gaining immense attention in scientific research, suggesting potential health benefits to host organism [2, 3]. Lactic acid bacteria (LAB) are the main bacteria that produce lactic acid as a major fermentation product using carbohydrate, and *Lactoplantibacillus* (formerly *Lactobacillus*) [4] species abundantly being presented in the intestines of vertebrates form the major part of this group [5]. *Lactiplantibacillus plantarum* (*L. plantarum*) with proved health benefit based on well-designed clinical studies are used probiotics in the food and nutraceutical industries [6, 7]. *L. plantarum* are known to produce various antimicrobial compounds such as bacteriocin, hydrogen peroxide, and acids [8]. Bacteriocins are ribosomally synthesized antimicrobial peptides that inhibit or kill the bacterial strains closely related or non-related to produced bacteria without causing any harm to bacteria themselves owing to the presence of self-defensive proteins [9, 10].

The skin possesses diverse microbiome, which provides protection against invading pathogens by the production of protein complex antibiotics [11, 12]. Change in the homeostasis between the host and microorganism is associated with skin diseases or infections [12]. A variety of key microbial genera are associated with skin health, including bacterial species *Staphylococcus* and *Cutibacterium* (formerly *Propionibacterium*), and fungal species *Candida* and *Malassezia*. *Staphylococcus aureus* (*S. aureus*) is a gram-positive and facultative anaerobic bacteria [13]. The colonizationof *S. aureus* in the skin have an important role in the pathogenesis of atopic dermatitis (AD) by disrupting the epidermal barrier and contributing to flare and itching by inducing degranulation of mast cells [14]. *Cutibacterium acnes* (*C. acnes*), a gram-positive and aerotolerant anaerobe bacterium, is the primary trigger for opportunistic infection, aggravating acne lesions [15]. The breakout of acne occurs in the sebaceous gland in the skin where increased sebum secretion promotes abnormal keratinisation, resulting in follicular clogging and proliferation of *C. acnes*. Subsequently, it induces inflammatory cytokine responses, leading to redness and oedema, and exacerbates acne symptoms [16, 17]. Candidiasis is fungal infection caused by *Candida* and occurs in the skin, fingernails, mucous membranes throughout the body. *Candida* is a part of fungal commensals in the human body; however, when the immune system of the body becomes compromised or the skin is broken, *Candida* can cause infections in the skin, fingernails, mucous membranes throughout the body [18–20]. *Malassezia* is a lipophilic fungal genus resident on normal skin and observed in approximately 75%–98% of healthy adults [21]. *Malassezia* generated free fatty acids, particularly lipase mediated breakdown of sebum triglycerides, can induce flaking in dandruff-susceptible patients [22].

Antibiotics administration to treat skin diseases may result in negative effects, such as dose-dependent toxicity, and the emergence of resistant pathogenic strains [21, 23, 24]. Infections caused by antibiotic resistant bacteria are serious threat to global health. The pathogens causing these infections can obtain the antibiotic resistance genes from proliferation of multi-drug-resistant bacteria, which, in turn, abrogate the effect of antibiotics due to persistence of the resistance genes in the microbiota [25]. Topical or oral administration of probiotics, such as *Lactiplantibacillus* species, could ameliorate skin health by inhibiting colonisation of skin pathogens and maintaining the balance of the gut flora [26–28]. Therefore, there is an urgent need to develop innovative strains of probiotics for alternative antimicrobial therapy against infectious diseases.

The safety and beneficial effect of *Lactiplantibacillus plantarum* APsulloc 331261 (APsulloc 331261) and *Lactiplantibacillus plantarum* APsulloc 331266 (APsulloc 331266) isolated from green tea (*Camellia sinensis*) have also been described [29]. APsulloc 331261 alleviates gastric inflammation, which might be attributed to the production of propionate derived from the modulation of gut microbiome in an alcohol-dependent gastric ulcer [30]. APsulloc 331261 significantly boosts the abundance of various intestinal beneficial bacteria such as *Bifidobacterium* spp. and *Clostridium butyricum*. Moreover, APsulloc 331261-derived extracellular vesicles exerts beneficial effects on human skin by inducing anti-inflammatory cytokine-mediated by M2 macrophage polarisation [31]. In the present study,we examined the antimicrobial activity of APsulloc 331261 and APsulloc 331266 against five skin pathogenic strains (*S. aureus*, *C. acnes*, *C. albicans*, *M. globosa* and *M. restricta*). To determine antimicrobial activity of APsulloc 331261 and APsulloc 331266, we validated the viability of five strains implicated in skin disease by indirect co-culture with APsulloc 331261 or APsulloc 331266, and cell-free conditioned media (CM)-derived from APsul-

Table 1. Bacterial and fungal strains used in the present study.

Strain	ATCC no.
Staphylococcus aureus	ATCC 6538
Cutibacterium acnes	ATCC 6919
Candida albicans	ATCC 90028
Malassezia globosa	MYA-4612
Malassezia restricta	MYA-4611

loc 331261 or APsulloc 331266. Antimicrobial activity of APsulloc 331261 or APsulloc 331266 was further evaluated by plate agar overlay assay against three strains. Finally, the profile of PCR amplicon products for plantaricin genes in APsulloc 331261 and APsulloc 331266 were identified by standard PCR.

3. Materials and methods

3.1 Bacteria and fungi strains, and culture conditions

APsulloc 331261 (KCCM11179P) and APsulloc 331266 (KCCM11180P) isolated from green tea derived from the leaves of *Camellia sinensis* (Osulloc farm, Jeju island, South Korea) [29] were cultured in MRS broth at 37 °C for 24 h (Becton, Dickinson and Company, Sparks, MD, USA). The bacterial and fungal strains were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) (listed in Table 1) and cultured according to ATCC instructions. *S. aureus* and *C. albicans* were cultured in tryptic soy (TS) broth (Becton, Dickinson and Company) and yeast malt (YM) broth (Becton, Dickinson and Company), respectively, at 37 °C, on a shaking incubator, for 24 h. *M. globosa* and *M. restricta* were cultured in modified Leeming & Notman agar (MLNA; ATCC medium 2737) without agar on a shaking incubator at 30 °C for 72 h. *C. acnes* was cultured in modified reinforced *Clostridium* (MRC) medium (ATCC medium 2107) at 37 °C for 72 h under anaerobic conditions. The cultures were diluted 1:50 or 1:100 in the indicated media, sub- cultured to mid-to-late logarithmic phase and adjusted to an OD_{600} of 0.5 before use.

3.2 Indirect co-culture assay

Five pathogenic strains (*S. aureus*, *C. acnes*, *C. albicans*, *M. globosa* and *M. restricta*) were co-cultured in APsulloc 331261 or APsulloc 331266 using a 6-well transwell chamber with a 0.4 μm porous membrane (Co-star, Corning, NY, USA) [32, 33]. The total volume of a well was consisted of 5 mL broth. 0.2% (v/v) of the pathogens (OD_{600} of 0.5) was placed in the basolateral compartment of transwell. *S. aureus* and *C. albicans* were incubated with MRS broth at 37 °C for 48. *C. acnes* was incubated in MRC broth at 37 °C for 96 h using BD GasPak™ EZ container systems (Becton, Dickinson and Company). *M. globosa* and *M. restricta* were incubated with MLNA medium without agar at 30 °C for 72 h. Various ratios (v/v) of the pathogens to APsulloc 331261 and APsulloc 331266 (1:0.001, 1:0.01, 1:0.1 and 1:1) were inoculated in the insert with the indicated media used for incubation of pathogens to determine antimicrobial activity. The pathogenic culture without inoculation of APsulloc 331261 and APsulloc 331266 was used as control. After incubation, the viability of the pathogens was measured at OD_{600} using a microplate reader (SpectraMax i3x, Molecular Devices, San Jose, CA, USA).

3.3 Cell culture with CM assay

To obtain the cell-free CM, overnight culture of APsulloc 331261 and APsulloc 331266 was sub- cultured to mid-to-late logarithmic phase and adjusted to OD_{600} of 1.0. APsulloc 331261 or APsulloc 331266-derived supernatant was obtained after two centrifugation steps at 15000 $\times$ *g* for 10 min. Supernatants were sterilized using 0.45 μm and 0.2 μm syringe filters (Whatman™, Maidstone, UK) and stored at –20 °C. Prepared CM (100%) was diluted to various concentrations (75%, 50%, 25%, 10% and 5%, (v/v)) with MRS broth and incubated with 0.2% (v/v) of five pathogenic strains in 6 well plates at 37 °C for 48 h, except on *C. acnes* for 96 h. The viability of the pathogens was measured at OD_{600} using a microplate reader.

3.4 Plate agar overlay assay

The effects of APsulloc 331261 and APsulloc 331266 on the growth of *S. aureus*, *C. acnes*, and *C. albicans* were examined on agar plates as previously described [32]. For the plate agar overlay assays, 1, 2 and 4 μL of APsulloc 331261 and APsulloc 331266 adjusted to an OD_{600} of 0.5 (ca. 1×10^4, 2×10^4, and 4×10^4 colony forming unit (CFU)) were spotted on 1.5% MRS agar plates and incubated for 48 h at 37 °C. After cooling to 40 °C in an autoclaved agar medium, *S. aureus*, *C. acnes*, and *C. albicans* suspension in 0.7% soft agar (TS, MRC, YM, respectively) were prepared and overlaid on the plates incubated with APsulloc 331261 and APsulloc 331266. After the plates were incubated at 37 °C for 24 h under aerobic (*S. aureus*, *C. albicans*) or anaerobic (*C. acnes*) incubators, the zone of diameter inhibition (ZDI) value was determined following the method adopted by Shokryazdan *et al.* [34] (ZDI <10 mm, weak inhibition; ZDI 10–20 mm, intermediate inhibition; ZDI >20 mm, strong inhibition). Moreover, 'R' (width of clear zone) values were measured and interpreted, following the method devised by Kohler *et al.* [32] as follows: R = $(d_{Inhibition} - d_{Spot})/2$ (where '$d_{Inhibition}$' is the diameter of clear zone around 'd_{Spot}' and 'd_{Spot}' refers to the diameter of the *Lactiplantibacillus* spot grown over the MRS agar plate; R <2 mm, no inhibition; R = 2–5 mm, low inhibition; R >6 mm, high inhibition.

Table 2. PCR primers used in this study.

Target gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
plnA	GTACAGTACTAATGGGAG	CTTACGCCATCTATACG
plnH	TCTTACACGATCAAGGCAAC	TGTGCCATTACTTACCTGTTTC
plnG	TGCGGTTATCAGTATGTCAAAG	CCTCGAAACAATTTCCCCC
plnEF	GGCATAGTTAAAATTCCCCCC	CAGGTTGCCGCAAAAAAAG
plnJ	TAACGACGGATTGCTCTG	AATCAAGGAATTATCACATTAGTC
plnK	AATCGCAGTGACTTCCAGAAC	AGAGCAATCCGTCGTTAATAAATG
plnS	GCCTTACCAGCGTAATGCCC	CTGGTGATGCAATCGTTAGTTT
plnW	TCACACGAAATATTCCA	GGCAAGCGTAAGAAATAAATGAG
plnN	ATTGCCGGGTTAGGTATCG	CCTAAACCATGCCATGCAC
plnNC8αβ	GGTCTGCGTATAAGCATCGC	AAATTGAACATATGGGTGCTTTAAATTCC

3.5 DNA isolation, PCR, and agarose gel electrophoresis

Total DNA from APsulloc 331261 and APsulloc 331266 was extracted using a G-spin™ Total DNA Extraction Mini Kit (iNtRON BIOTECHNOLOGY, Gyeonggi-do, South Korea), following the manufacturer's protocol. A set of primers (listed in Table 2 [35]) used to amplify plantaricin genes was designed and PCR was performed using a 2X TOPsimple™ PreMIX-nTaq (Enzynomics, Daejeon, South Korea) and SimpliAmp Thermal Cycler (Applied Biosystems, Foster City, CA, USA), as previously described [35]. The PCR conditions were as follows: initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 1 min, and a final elongation step at 72 °C for 4 min. The PCR amplicons were subjected to 3.0% (w/v) agarose gel electrophoresis at 100 V for 40 min. The gel was visualized using the ChemiDoc™ MP Imaging System Universal hood III (Bio-Rad, Hercules, CA, USA) with Image Lab™ Software Version 5.2 (Bio-Rad).

3.6 Statistical analysis

The data of this study are presented as the mean ± standard deviation (SD) from at least three independent experiments with triplicate samples. For co-cultured assay, cultivation assay, and plate agar overlay assay, multiple groups were analyzed using Bonferroni's test for multiple comparisons of one-way analysis of variance (ANOVA) using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA). *p*-values of <0.05, <0.01, and <0.001 were considered statistically significant.

4. Results

4.1 APsulloc 331261 and APsulloc 331266 inhibit the growth of *S. aureus*

The antibacterial activity of APsulloc 331261 and APsulloc 331266 against *S. aureus* was examined by a viability assay with a transwell chamber. With APsulloc 331261 or APsulloc 331266 in the apical compartment and *S. aureus* in the basolateral compartment, they can interact via metabolites in their shared medium while being physically separated by a membrane. *S. aureus* was co-cultured with APsulloc 331261 or APsulloc 331266 in *S. aureus* to APsulloc 331261 or APsulloc 331266 ratios of 1:0.001, 1:0.01, 1:0.1, 1:1. These experiments revealed that *S. aureus* growth drastically decreased even in the ratio 1:0.001 of *S. aureus* to APsulloc 331261or APsulloc 331266 (Fig. 1A). APsulloc 331261 or APsulloc 331266 at ratio 1:0.001, 1:0.01, 1:0.1 and 1:1 reduced the growth of *S. aureus* by 86.6%, 88.9%, 90.0%, and 93.2%, respectively, or 86.8%, 89.7%, 89.7%, and 93.9%, respectively. We also tested the effect of various concentrations (2.5, 5, 10, 25 and 50%) of CM-derived from APsulloc 331261 or APsulloc 331266, which is cell-free supernatant containing soluble factors,on the growth of *S. aureus*. We found that the growth of *S. aureus* was suppressed by CM-derived from APsulloc 331261 or APsulloc 331266 in a dose-dependent manner (Fig. 1B). 50% of CM-derived from APsulloc 331261 and APsulloc 331266 completely inhibited *S. aureus* growth by 97.7% and 98.6%, respectively, and 2.5–25% CM-derived from APsulloc 331261 and APsulloc 331266 resulted in 21–31% and 21–29% growth inhibition, respectively. Therefore, APsulloc 331261 and APsulloc 331266 exhibit anti-bacterial activity against pathogenic *S. aureus*, which may be mediated by APsulloc 331261 and APsulloc 331266-derived metabolites.

Antimicrobial activity of APsulloc 331261 and APsulloc 331266 against *S. aureus* was further examined by agar overlay assay. Various populations (1×10^4, 2×10^4, 4×10^4 CFU) of APsulloc 331261 or APsulloc 331266 were spot inoculated on MRS agar plates, which were overlaid with TS agar containing *S. aureus*. We determined the growth inhibition of *S. aureus* by the measurement of zone of diameter inhibition (ZDI) and the wide of clear zone (R value). APsulloc 331261 or APsulloc 331266 spotted at 1×10^4, 2×10^4 and 4×10^4 CFU inhibited the growth of *S. aureus* with ZDI of 22.7 ± 0.6, 26.2 ± 2 and 32.3 ± 2.1 mm, respectively, in a dose dependent manner or

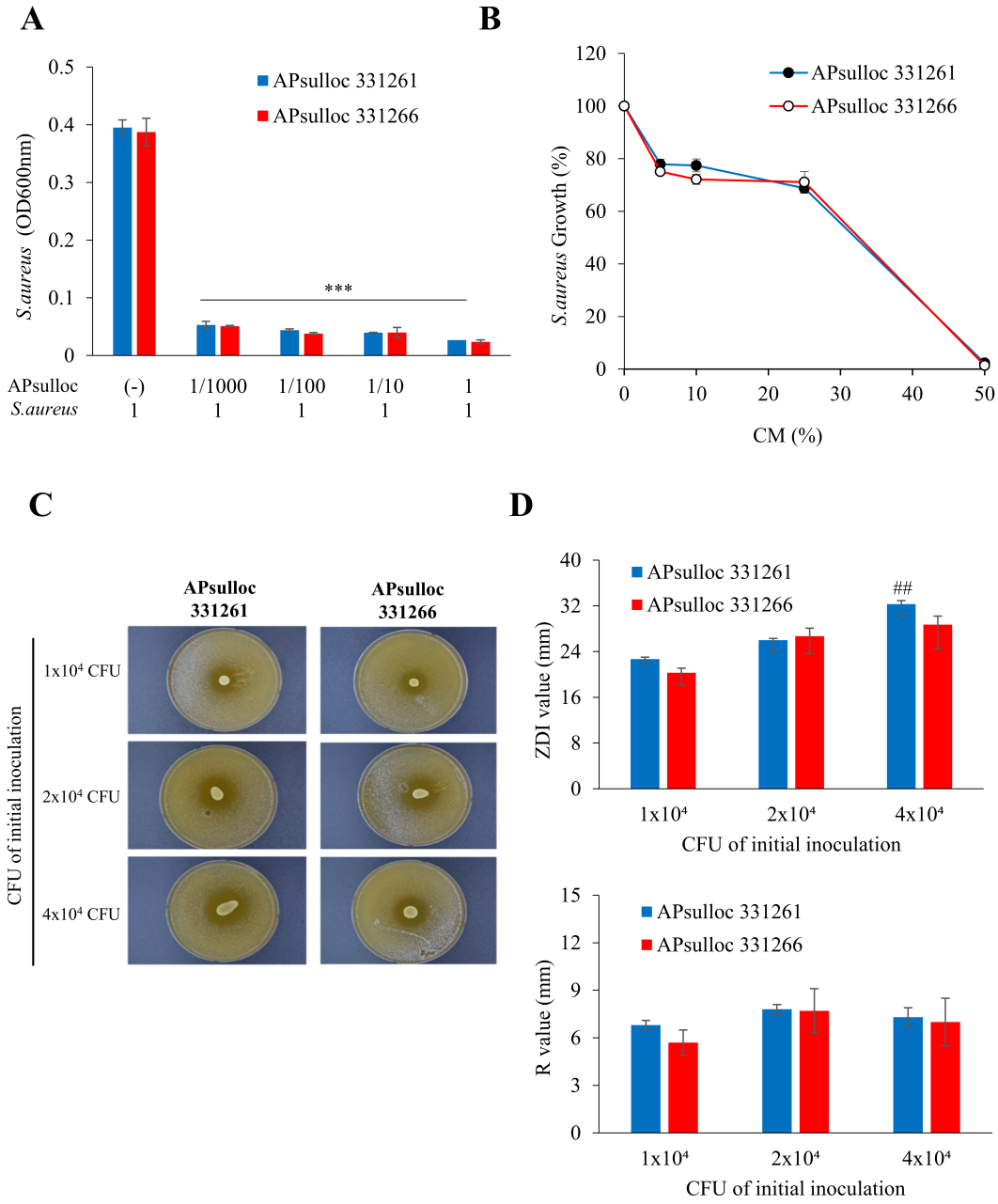

Fig. 1. Antibacterial effect of APsulloc 331261 or APsulloc 331266 against *S. aureus*. (A) *S. aureus* were co-cultured in the presence or absence of APsulloc 331261 or APsulloc 331266 in the inserts of transwells at APsulloc 331261 and APsulloc 331266 to *S. aureus* ratios of 0.001:1, 0.01:1, 0.1:1 and 1:1. (B) *S. aureus* were incubated in various concentrations of CM (0, 5, 10, 25, and 50%) derived from APsulloc 331261 or APsulloc 331266. After incubation at 37 °C for 48 h, the cell viabilities were determined at OD_{600}. (C,D) Various populations (1×10^4, 2×10^4, 4×10^4 CFU) of APsulloc 331261 or APsulloc 331266 were spot inoculated on MRS agar plates and incubated for 48 h, which were overlaid with TS agar containing *S. aureus* for 24 h. ZDI and R value were determined by the measurement of spots around APsulloc 331261 and APsulloc 331266 strains. All data represent the mean ± standard deviation (SD) from at least three independent experiments with triple samples. Statistical analysis performed by ANOVA/Bonferroni/GraphPad Prism. Significant differences: *** $p < 0.001$ vs. (-), ## $p < 0.01$ vs. 1×10^4 CFU of initial inoculation.

20.3 ± 2.1, 26.7 ± 3.1 and 28.7 ± 4.2 mm, respectively (Fig. 1C,D). Furthermore, R values for APsulloc 331261 or APsulloc 331266 against *S. aureus* ranged 6.8 ± 0.3–0.8 ± 1.3 mm or 5.7 ± 0.8–7.7 ± 1.4 mm, respectively, indicating the high inhibition capacity of APsulloc 331261 and APsulloc 331266 against *S. aureus*, but there was no dose dependent effect of APsulloc 331261 and APsulloc 331266 on R value. This might be due to dose dependent growth of APsulloc 331261 and APsulloc 331266 on the agar plate despite of its effect on dose dependent inhibition of the *S. aureus* growth. Collectively, agar overlay assay suggests that APsulloc 331261 and APsulloc 331266 exhibit strong

inhibition (ZDI >20 mm, R value >6) against *S. aureus*. Taken all together, APsulloc 331261 and APsulloc 331266 have significant antibacterial effect against skin pathogenic *S. aureus*.

4.2 APsulloc 331261 and APsulloc 331266 suppress the growth of *C. acnes*

To determine antibacterial activity of APsulloc 331261 and APsulloc 331266, the viability of *C. acnes* co-cultured with APsulloc 331261 or APsulloc 331266 in a transwell chamber was evaluated. *C. acnes* located in the basolateral compartment and APsulloc 331261 or APsulloc 331266 located in the apical compartment were co-cultured in *C. acnes* to APsulloc 331261 or APsulloc 331266 ratios of 1:0.001, 1:0.01, 1:0.1, 1:1. These experiments showed that *C. acnes* growth drastically suppressed by 98.4–99.4% even in the ratio 1:0.001 of APsulloc 331261 or APsulloc 331266 to *C. acnes* (Fig. 2A). Next, we tested various concentrations (5, 10, 25 and 50%) of CM-derived from APsulloc 331261 or APsulloc 331266 on the growth of *C. acnes*. CM-derived from APsulloc 331261 and APsulloc 331266 suppressed the *C. acnes* growth in a dose dependent manner (Fig. 2B). 50% and 25% of APsulloc 331261 or APsulloc 331266 caused significant reduction of *C. acnes* growth by 96.5% and 74.4%, respectively, or, 99.9% and 98.8%, respectively. Collectively, APsulloc 331261 and APsulloc 331266-derived soluble factors effectively inhibit the *C. acnes* growth.

Antimicrobial activity of APsulloc 331261 and APsulloc 331266 against *C. acnes* was determined by agar overlay methods. We determined antimicrobial activity of various concentrations (1×10^4, 2×10^4 and 4×10^4 CFU of APsulloc 331261 or APsulloc 331266) by the measurement of ZDI and R value. APsulloc 331261 or APsulloc 331266 spotted at 1×10^4, 2×10^4 and 4×10^4 CFU inhibited the growth of *C. acnes* with ZDI of 15.0 ± 1.7, 19.0 ± 0 and 22.2 ± 1.7 mm, respectively, or, with 15.0 ± 2.6, 17.3 ± 2.1 and 20.0 ± 2.0 mm, respectively (Fig. 2C,D). R values for APsulloc 331261 and APsulloc 331266 against *C. acnes* ranged 3.2 ± 1.3–5.5 ± 1.3 mm and 3.7 ± 1.3–4.3 ± 1.8 mm, respectively, indicating low inhibition capacity of APsulloc 331261 and APsulloc 331266 against *C. acnes*. Therefore, the results of agar overlay suggest that APsulloc 331261 and APsulloc 331266 exhibit intermediate inhibition (ZDI 10–20 mm, R value 2–5) against *C. acnes*.

4.3 The growth of *C. albicans* was inhibited by APsulloc 331261 and APsulloc 331266

The viability of *C. albicans* co-cultured with APsulloc 331261 or APsulloc 331266 in a transwell chamber was evaluated. These experiments revealed that, *C. albicans* to APsulloc 331261 or APsulloc 331266 at ratio 1:0.001, 1:0.01, 1:0.1 and 1:1 decreased *C. albicans* growth by 0, 21.4, 44.1 and 68.2%, respectively, or by 20.6, 33.0, 44.6 and 65.1%, respectively (Fig. 3A). Next, we tested various concentrations (25, 50, 75 and 100%) of CM-derived from APsulloc 331261 or APsulloc 331266 on the vaibility of *C. albicans*. The viability assays exhibited that the growth of *C. albicans* was suppressed by CM-derived from APsulloc 331261 or APsulloc 331266 in a dose-dependent manner. 100% and 75% of CM-derived from APsulloc 331261 or APsulloc 331266 caused significant reduction of *C. albicans* growth by 65.4% and 37.3%, respectively, or 75.8% and 61.9%, respectively (Fig. 3B). Collectively, APsulloc 331261 and APsulloc 331266 secreted metabolites effectively abolish the growth of pathogenic *C. albicans*.

Agar overlay assay was conducted to determine the effect of APsulloc 331261 or APsulloc 331266 on *C. albicans* growth. APsulloc 331261 or APsulloc 331266 spotted at 1×10^4, 2×10^4 and 4×10^4 CFU inhibited the growth of *C. albicans* with ZDI of 13.3 ± 4.0, 19.7 ± 1.5 or 23.0 ± 2.1 mm, respectively, or 15.0 ± 1.7, 15.0 ± 1.0 and 27.0 ± 3.6 mm, respectively, in a dose dependent manner. APsulloc 331261 or APsulloc 331266 spotted at 4×10^4 CFU exhibited strong inhibition (ZDI >20 mm) and APsulloc 331261 or APsulloc 331266 spotted at 1×10^4 and 2×10^4 CFU showed intermediate inhibition (ZDI 10–20 mm) against *C. albicans* (Fig. 3C,D). *R* values for APsulloc 331261 or APsulloc 331266 against *C. albicans* ranged 2.8 ± 1.9–4.7 ± 0.3 mm or 3.2 ± 0.8–5.5 ± 1.7 mm, respectively, against *C. albicans*, indicating intermediate inhibition capacity inhibition (R value 2–5) of APsulloc 331261 and APsulloc 331266 against the *C. albicans* growth. Therefore, APsulloc 331261 and APsulloc 331266 presented intermediate to high inhibition against the growth of *C. albicans*.

4.4 The growth of *M. globosa* and *M. restricta* was suppressed by APsulloc 331261 and APsulloc 331266

To examine antifungal activity, the viability of *M. globosa* and *M. restricta* co-cultured with APsulloc 331261 and APsulloc 331266 was evaluated in a transwell chamber. These experiments revealed that *M. globosa* and *M. restricta* growth was significantly decreased in the presence of APsulloc 331261 or APsulloc 331266 at ratio 1:0.1 and 1:1 (Fig. 4A,C). We also tested the effect of various concentrations (25, 50, 75 and 100%) of CM-derived from APsulloc 331261 or APsulloc 331266 on the viability of *M. globosa* and *M. restricta*. These experiments demonstrated that the viability of *M. globosa* and *M. restricta* was suppressed by CM obtained from APsulloc 331261 or APsulloc 331266 in a dose-dependent manner (Fig. 4B,D). APsulloc 331261 or APsulloc 331266 derived CM was effective in inhibition of *M. globosa* and *M. restricta* growth in a dose dependent manner and 100% of CM completely abolished the growth of *M. globosa* and *M. restricta*. Collectively, APsulloc 331261 and APsulloc 331266, and their CM was effective in the inhibition of *M. globosa* and *M. restricta* growth.

Plantaricin, a bacteroicin produced in *L. plantarum*, is known to have an antimicrobial effect [36]. To de-

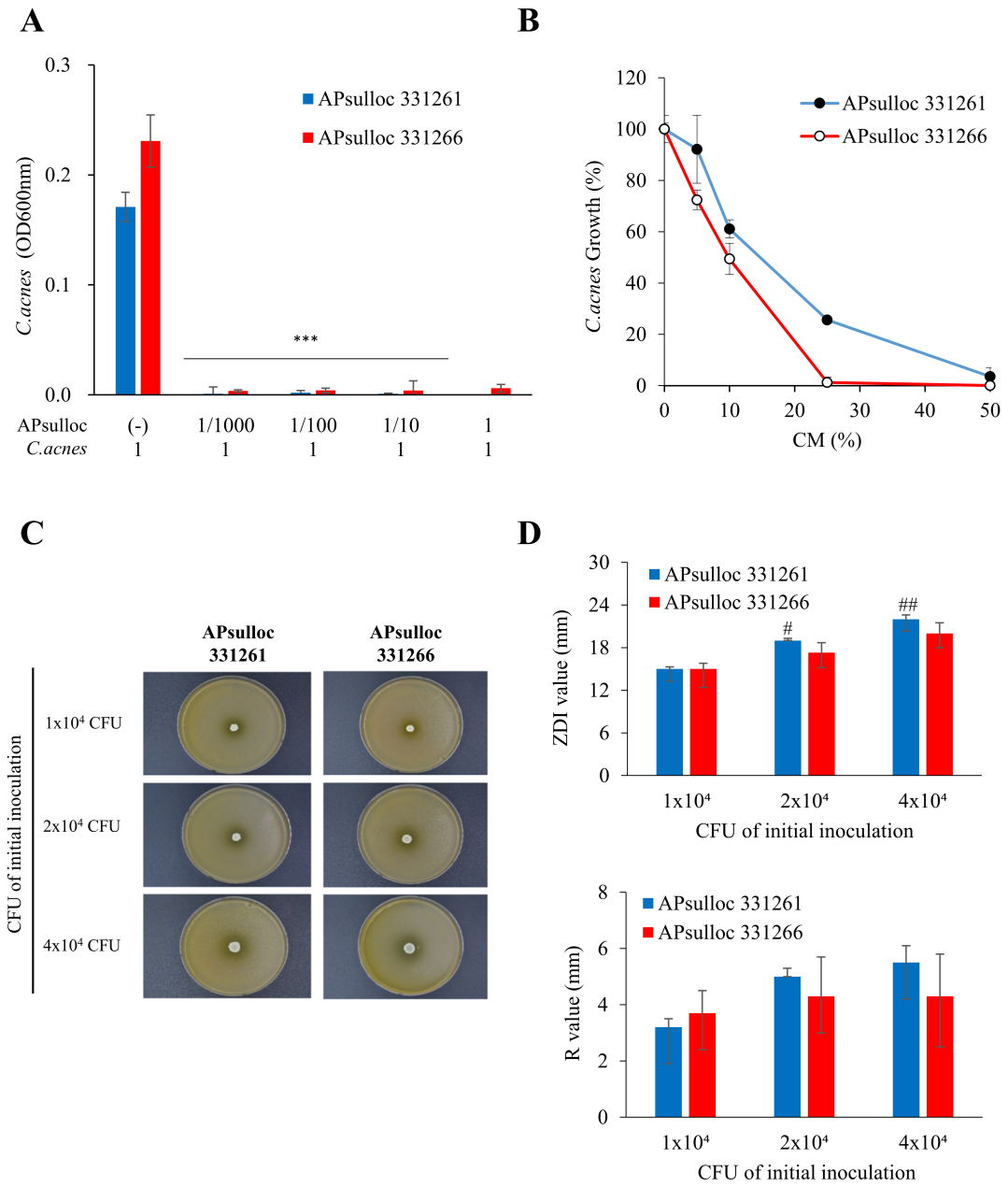

Fig. 2. Antibacterial activity of APsulloc 331261 and APsulloc 331266 against *C. acnes*. (A) Using anaerobe container systems, APsulloc 331261 or APsulloc 331266 was co-cultured with *C. acnes* at ratios of 0.001:1, 0.01:1, 0.1:1 and 1:1. (B) *C. acnes* were incubated in various concentrations of CM (0, 5, 10, 25, and 50%). After anaerobic incubation at 37 °C for 96 h, the cell viabilities were determined at OD_{600}. (C,D) ZDI and R value of plate agar overlay assay with *C. acnes* were determined. All data represent the mean ± standard deviation (SD) from at least three independent experiments with triple samples. Significant differences: *** $p < 0.001$ vs. (-), $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ vs. 1×10^4 CFU of initial inoculation.

termine whether APsulloc 331261 and APsulloc 331266 express plantaricin genes, we evaluated ten genes, which are present in the *pln* locus and are responsible for the synthesis of plantaricins. PCR analysis showed that *plnA*, *plnH*, *plnG*, *plnEF*, *plnJ*, *plnK*, and *plnN* genes exist in APsulloc 331261 and APsulloc 331266, and that *plnS*, *plnW*, and *plnNC8*$\alpha\beta$ genes do not exist (Fig. 4E,F). The plantaricin genes such as plantaricin S and plantaricin W are rarely presented in the *L. plantarum* strains [36]. Taken together, Genes *plnA*, *plnH*, *plnG*, *plnEF*, *plnJ*, *plnK*, and *plnN* were found in APsulloc 331261 and APsulloc 331266, which may render its antimicrobial activity against pathogenic skin microbes.

5. Discussion

The usage of probiotics to promote gut and immune health has recently grown, and researchers are inter-

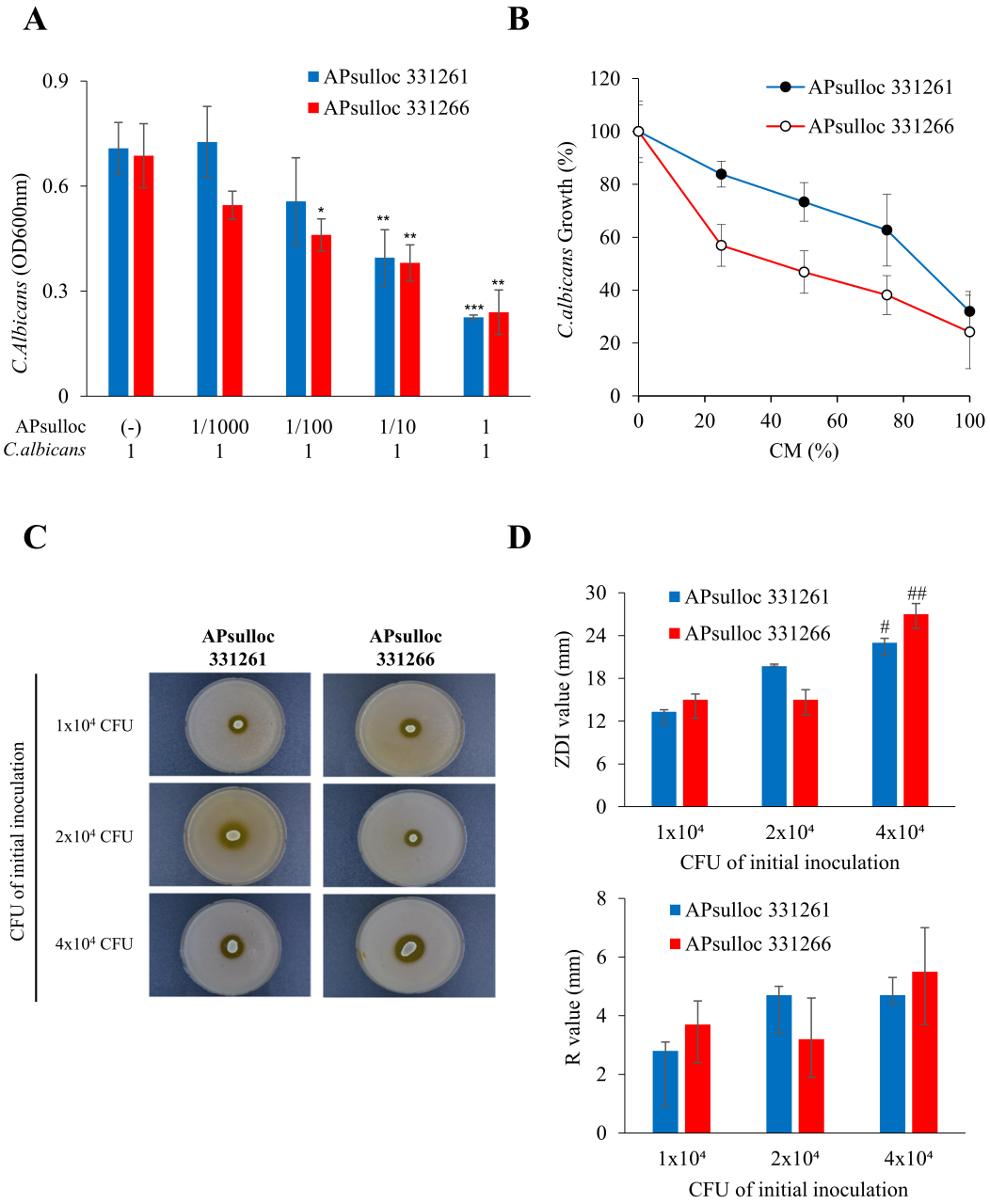

Fig. 3. Antifungal activity of APsulloc 331261 and APsulloc 331266 against *C. albicans*. (A) APsulloc 331261 or APsulloc 331266 was co-cultured with *C. albicans* at ratios of 0.001:1, 0.01:1, 0.1:1 and 1:1. (B) *C. albicans* were incubated in various concentrations of CM (0, 25, 50, 75, and 100%). After incubation at 37 °C for 48 h, the cell viabilities were determined at OD_{600}. (C,D) ZDI and R value of plate agar overlay assay with *C. albicans* were determined. All data represent the mean ± standard deviation (SD) from at least three independent experiments with triple samples. Significant differences: $^{*}p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. (-), $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ vs. 1×10^4 CFU of initial inoculation.

ested in the discovery of a variety of probiotic strains and mechanisms how probiotic microorganisms interact with the body [37]. Probiotics play an potential alternative to antibiotics in the treatment of inflammatory bowel disease (IBD) [38] as well as lower the incidence of several atopic conditions [39, 40]. Among the commercially available probiotic strains, LAB from various natural sources are being isolated and identified to explore new alternative probiotic strains [41]. Probiotic *Lactoplantibacillus* species can be considered to be candidates for alternatives to antibiotics since their various mechanisms of growth inhibition against pathogenic bacteria have been extensively documented [42–44]. *Lactiplantibacillus* species isolated from various sources have an excellent antibacterial spectrum against pathogenic bacteria [5, 45, 46]. The antagonistic activity of *Lactiplantibacillus* species has mostly been attributed to the production of lactic acid. The acidification of cytoplasm of cell by the undissociated lactic acid that

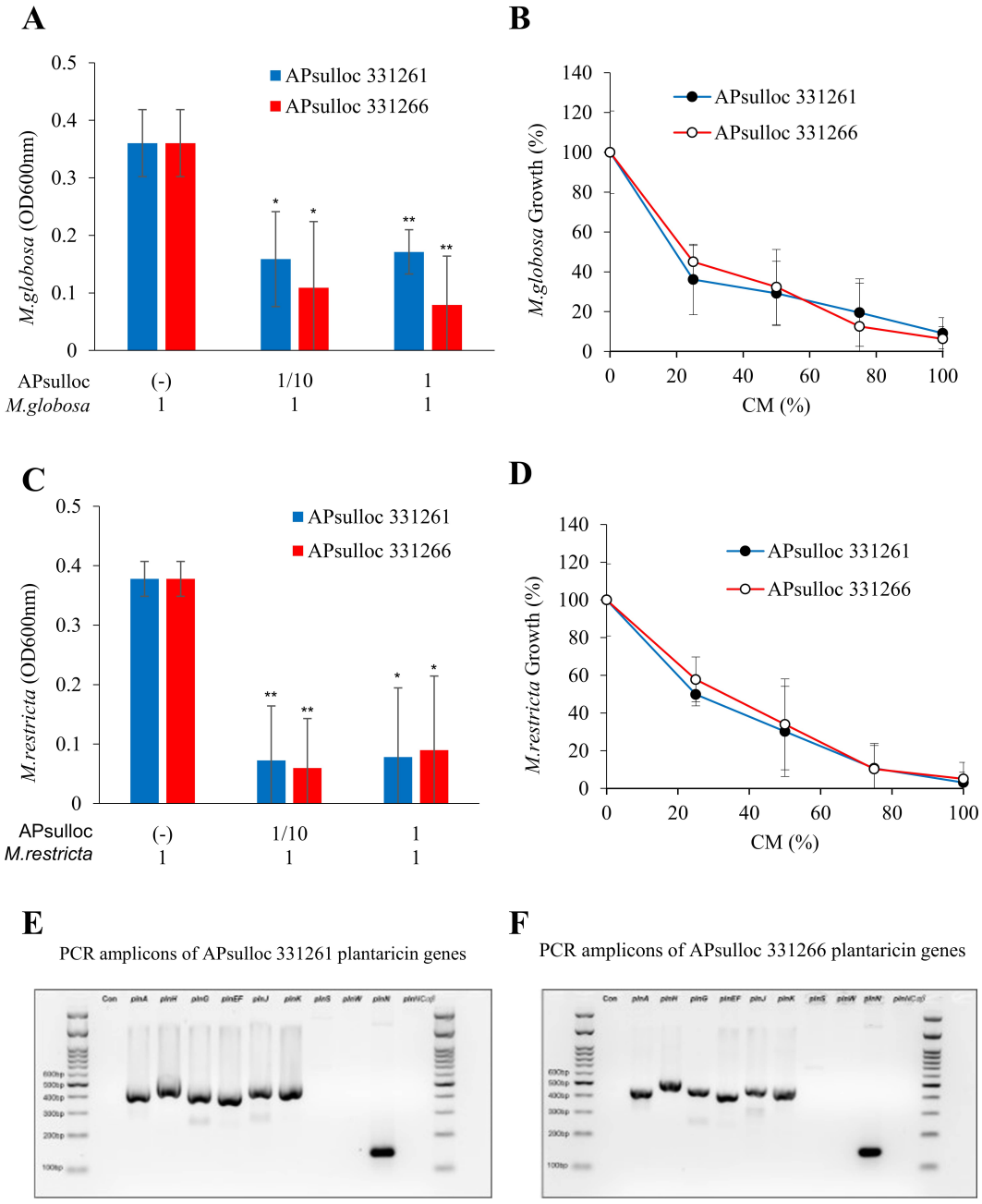

Fig. 4. Antifungal effect of APsulloc 331261 and APsulloc 331266 against *M. globosa* and *M. restricta*. (A,C) APsulloc 331261 or APsulloc 331266 was co-cultured with *M. globosa* or *M. restricta* at ratios of 0.1:1 and 1:1. (B,D) *M. globosa* or *M. restricta* were incubated in various concentrations of CM (0, 25, 50, 75, and 100%) After incubation at 30 °C for 72 h, cell viabilities were determined at OD_{600}. All data represent the mean ± standard deviation (SD) from at least three independent experiments with triple samples. Significant differences:$^{*}p < 0.05$ vs. (-). Gel electrophoresis of PCR amplicons of *pln* genes in APsulloc 331261 (E) and APsulloc 331266 (F). Lane1, ladder; Lane2, Control; Lane3-12, *pln* gene; Lanes 13, ladder.

passes through the bacterial membrane and dissociates inside the cell results in failure of proton motive forces [47]. Besides organic acids, hydrogen peroxides, bacteriocins, and short-chain fatty acids (such as acetic, propionic, and butyric acids) are antimicrobial compounds produced by *Lactiplantibacillus* species, and the compounds lead to the change of the gut redox potential and the restriction of the nutrient supply to the pathogens [48]. In particular, *L. plantarum* synthesize a variety of antimicrobial plantaricins to inhibit the growth of competing bacteria. Plantaricin A, which *plnA* is encoded, acts as an extracellular signal that induces transcription of *pln* genes as well as an antimicrobial peptides [49]. Even though plantaricin E, F, J and K, encoded by *plnE*, *plnF*, *plnJ* and *plnK*, respectively, have an

anti-microbial effect individually, two peptides are at least 1000 times more effective when cognate peptides such as PlnE and PlnF, or, PlnJ and PlnK, are combined [50]. Moreover, antifungal activity of plantaricin peptides PlnE, -F, -J, and -K is presented against pathogenic yeast, *Candida albicans* [51]. Plantaricin EF and JK form holes in the membrane of the target cell and cause the death by electronical potential and pH gradient to collapse [52, 53]. However, plantaricin EF and JK have different receptor [54]. *plnH* and *plnG* is related to ABC transport, which secrete and process pre-plantaricin E, F, J, and K to reveal the antimicrobial activity [52]. Yolanda *et al*. [55] classified the *L. plantarum* strains into seven plantaritypes, depending on the presence of the bacteriocin gene and the type of regulatory system. Both APsulloc 331261 and APsulloc 331266 do not have *plnNC8*, but *plnA* does exist, suggesting that they belong to group 1 and 2 of plantaritypes and have common characteristics of *plnABCD* regulation systems. Thus, the presence of plantaricin genes *plnA*, *plnH*, *plnG*, *plnEF*, *plnJ*, *plnK*, and *plnN* in APsulloc 331261 and APsulloc 331266 might contribute to the antimicrobial activity of APsulloc 331261 and APsulloc 331266 against skin pathogens. However, we only identified plantaricin genes, not their expression or activity. For better understanding of the mechanism of antimicrobial activity of APsulloc 331261 and APsulloc 331266, further studies are needed to confirm the plantaricin expression/activity and their antimicrobial activity against skin pathogenic microbiota.

Probiotics and prebiotics have been widely considered for the treatment and prevention of infectious diseases because of the various mechanisms by sharing genes and metabolites, helping other microbiota, and directly interacting epithelial and immune cells [56]. In this regard, several studies have investigated the effects of probiotic and prebiotics use on microbiota of other parts of human body. Therefore, probiotics and prebiotics are promising in improving skin health by modulating the cutaneous microbiota [57]. The gastrointestinal microbiota can communicate with the skin through modulation of systemic immune responses and maintain skin homeostasis [58]. In fact, beneficial effects of probiotics following topical or oral administration have been reported in skin diseases, such as acne vulgaris [27, 59, 60], atopic dermatitis [61], candida vaginitis [62], and dandruff [63]. The use of probiotics in cosmetics or nutraceuticals have been recently expanded to rebalance the microbiome for skin by inhibiting the growth of harmful pathogens while promoting the colonization of beneficial bacteria among the resident microorganisms. Thus, topical application or ingestion of APsulloc 331261 and APsulloc 331266 could improve skin health by inhibiting skin pathogens, ameliorating intestinal beneficial bacteria [30] and inducing macrophage polarisation [31]. Although we should carefully draw conclusion obtained from *in vitro* studies, our results support the hypothesis that probiotic APsulloc 331261 and APsulloc 331266 may ameliorate microbial profile and homoeostasis in the skin. Further studies are guaranteed to investigate the effectiveness of APsulloc 331261 and APsulloc 331266 as novel antibiotics or alternative therapeutics for treating skin infection.

6. Conclusions

In summary, the present study demonstrated that APsulloc 331261 and APsulloc 331266 isolated from green tea (*Camellia sinensis*) exhibited the inhibitory activity against five skin pathogenic strains (*S. aureus*, *C. acnes*, *C. albicans*, *M. globosa* and *M. restricta*) related to skin diseases. Antimicrobial effect of APsulloc 331261 and APsulloc 331266 was validated by indirect co-culture assay, CM culture assay and agar overlay assay with five pathogenic strains. These results indicate that novel APsulloc 331261 and APsulloc 331266 could serve as a potential alternative to antibiotics against infectious diseases. Further studies are guaranteed to explore the effectiveness of APsulloc 331261 and APsulloc 331266 administrated by topically or orally as alternative therapeutics in combating skin infection.

7. Author contributions

MC, BJK and JN designed the research and wrote the paper. JN, JOL and SYK performed the experiments and statistical analyses. YK, EL, DC, JR and WK prepared figures. All authors analysed and interpreted the data, revised the manuscript, and approved the final draft.

8. Ethics approval and consent to participate

Not applicable.

9. Acknowledgment

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11. Conflict of interest

The authors declare no conflict of interest.

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Abbreviations: LAB, Lactic acid bacteria; *L. plantarum*, *Lactiplantibacillus plantarum*; APsulloc 331261, *Lactiplantibacillus plantarum* APsulloc 331261; APsulloc 331266, *Lactiplantibacillus plantarum* APsulloc 331266; *S. aureus*, *Staphylococcus aureus*; *C. acnes*, *Cutibacterium acnes*; *C. albicans*, *Candida albicans*; *M. globosa*, *Malassezia globosa*; *M. restricta*, *Malassezia restricta*; ATCC, American Type Culture Collection; CM, Cell-free conditioned media; TS, Tryptic soy; MRC, Modified reinforced Clostridium; YM, Yeast malt; MLNA, Modified Leeming & Notman agar; SD, Standard deviation; ZDI, Zone diameter of inhibition.

Keywords: *Lactiplantibacillus plantarum*; APsulloc; Green tea; *Skin pathogens*; *Staphylococcus aureus*; *Cutibacterium acnes*; *Candia albicans*; *Malassezia globosa*; *Malassezia restricta*; Plantaricin

Send correspondence to:

Minjung Chae, AMOREPACIFIC R&D Center, AMOREPACIFIC, 17074 Yongin, Republic of Korea, E-mail: minjungc@amorepacific.com

Beom Joon Kim, Department of Dermatology, College of Medicine, Chung-Ang University, 06974 Seoul, Republic of Korea, Department of Medicine, Graduate School, Chung-Ang University, 06973 Seoul, Republic of Korea, E-mail: beomjoon74@gmail.com

† These authors contributed equally.