

피부 미생물총의 성장과 구성에 대한 화장품과 그 방부제의 영향

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Effects of Cosmetics and Their Preservatives on the Growth and Composition of Human Skin Microbiota

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요약: 화장품 및 화장품 함유 방부제에 대한 병원미생물과 사람의 피부서식세균들의 증식에 미치는 효과를 조사하였다. 파라벤, 1,2-헥산디올, 페녹시 에탄올과 같은 방부제를 함유한 토너, 에멀전, 크림, 유아용크림은 포도상구균, 대장균, 녹농균에 대해 강한 방부효과를 보였으며, 파라벤, 1,2-헥산디올, 페녹시 에탄올과 같은 방부제들은 포도상구균, 대장균, 녹농균과 같은 병원균 및 *Staphylococcus epidermidis*, *Shigella flexneri*, *Enterobacter aerogenes* 등과 같은 일부 피부서식세균에 대한 강한 항균효과를 보였다. 사람의 피부에 페녹시 에탄올 함유 크림을 도포하였을 때, 피부서식 미생물들의 구성이 바뀌었다. 문 수준에서는 Proteobacteria가 증가하였고, 종 수준에서는 *4P004125_s*가 증가하였고, *Propionibacterium humerusii* 감소하였다. 이 결과들로부터 파라벤, 1,2-헥산디올, 페녹시 에탄올과 같은 방부제는 병원균에 대한 항균효과를 가짐과 동시에 페녹시 에탄올을 함유한 화장품은 피부세균군집에 변화를 줄 수 있음을 시사하고 있다.

Abstract: We investigated the growth-inhibitory activities of cosmetics and their preservatives against pathogens and resident skin bacteria. Of the tested cosmetics, preservatives such as parabens, 1,2-hexanediol, phenoxyethanol-contained toner, emulsion, cream and baby cream exhibited potent antibacterial effects against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Parabens, 1,2-hexanediol and phenoxyethanol inhibited the growth of pathogens, as well as skin-resident bacteria such as *Staphylococcus epidermidis*, *Shigella flexneri*, *Enterobacter aerogenes* and so on. The application of a basic cream containing phenoxyethanol to human skin was shown to disturb the skin microbiota: at the phylum level, Proteobacteria increased and at species level, *4P004125_s* increased and *Propionibacterium humerusii* decreased. Based on these findings, parabens, 1,2-hexanediol and phenoxyethanol have antimicrobial activity and cosmetics containing phenoxyethanol may disturb skin microbiota.

Keywords: cosmetic, preservative, skin microbiota, growth inhibition

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1. Introduction

Newborns are born in a germ-free state, however, immediately following birth, neonates are exposed to microbes present within the parturient canal, suspended in ambient air, on the skin of the mother or nurses within the birth setting, or found on bed sheets, etc.[1,2]. These microbes establish themselves on the skin, in the respiratory tract, in the gastrointestinal tract, and in other sites within the human body, and they remain within the body through the individuals' life. The composition of human microbiota may be affected by intrinsic factors, such as genotype, age, and sex, or extrinsic factors, such as occupation, lifestyle, geographical location, and use of antibiotics[3,4]. The composition of human skin microbiota can be disturbed by treatment with drugs such as antibiotics[1,6,7]. The disruption of normal microbiota can potentially result in the formation of superinfections.

Cosmetics contain ingredients that promote microbial growth, including carbohydrates, proteins and lipids. Therefore, various preservatives are commonly used in cosmetics to control quality and to minimize health risks associated with cosmetics[8,9]. Although the antimicrobial effects of these preservatives have been investigated, the effect of the preservative on skin microbiota has not been studied.

Therefore, in this study, we measured the inhibitory effect of cosmetics and common preservatives used in cosmetic against pathogens and human skin microbes.

2. Materials and Methods

2.1. Materials

Tryptic soy broth (TSB) and agar (TSA) were purchased from DIFCO (Sparks, MD, U.S.A.). Phenoxyethanol, methyl paraben, ethyl paraben, propyl paraben and 1,2-hexanediol were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

2.2. Bacterial cells

Staphylococcus aureus (*S. aureus*) ATCC6538, *Pseudo-*

monas aeruginosae (*P. aeruginosa*) ATCC9027 and *Escherichia coli* (*E. coli*) ATCC8739 were purchased from Korean Culture Center of Microorganisms (Seoul, Korea). These pathogens and the isolated skin-resident bacteria were aerobically cultured in TSB or TSA for 20 - 24 h.

2.3. Subjects

The subjects were 5 healthy Korean females (average, 27.2 ± 1.47 yrs). Exclusion criteria included current medication, especially regular or current use of antibiotics. The recruit of subjects and collection of their skin microbiota were approved by the Committee for the Care and Use of Clinical Study in the Medical School, Kyung Hee University (IRB No KHSIRB14-007).

2.4. Assay of antibacterial activity of cosmetics and preservatives

To measure antibacterial activity of cosmetics and preservatives in Eppendorff tube, test microorganisms were precultured in TSB. The cultured bacteria (1×10^5 CFU / 0.1 mL) was inoculated into TSB (0.9 mL) containing cosmetics (Supplement data 1) and preservatives and incubated cultured for 24 h. The cultured media (0.1 mL) were periodically collected at $10,000 \times g$ for 1 min, washed with PBS (1 mL) twice, and suspended with PBS (1 mL). These suspended bacteria (0.1 mL) were inoculated into TSA plate and incubated at 37°C for 20 h and the grown colonies were counted.

For minimal inhibitory concentration (MIC) measurement, the precultured test microorganisms (1×10^5 CFU / 0.1 mL) was inoculated into TSB (0.9 mL) containing preservatives (1.6, 0.8, 0.4, 0.2, 0.1, 0.05, 0.03, 0.013 and 0 mg/mL), and cultured for 24 h and then the MIC was measured optically. Since parabens are low hydrophilicity, 1% polyethylene glycol (PEG) was added to the TSB.

For minimal bactericidal concentration (MBC) measurement, the precultured test microorganisms (1×10^5 CFU / 0.1 mL) was inoculated into TSB (0.9 mL) containing preservatives (1.6, 0.8, 0.4, 0.2, 0.1, 0.05, 0.03,

Table 1. Antimicrobial Activities of Preservatives Frequently Used in Cosmetics

	MIC (mg/mL)			MBC (mg/mL)		
	Minimal Inhibitory Concentration			Minimal Bactericidal Concentration		
	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosae</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosae</i>
Methyl paraben	0.40	0.10	0.20	0.40	0.10	0.20
Ethyl paraben	0.20	0.10	0.20	0.20	0.10	0.20
Propyl paraben	0.10	0.05	0.80	0.10	0.05	0.80
1,2-hexanediol	1.60	0.80	0.80	1.60	0.80	0.80
Phenoxyethanol	0.05	0.05	0.03	0.10	0.05	0.05

0.013 and 0 mg/mL) and incubated cultured for 24 h. The cultured media were inoculated into TSB without preservatives and then the MBC was measured optically. Since parabens are low hydrophilicity, 1% polyethylene glycol (PEG) was added to the TSB.

2.5. Isolation of normal skin bacteria

Normal skin bacteria were isolated from the face (cheek) after 12 hours of washing without using a detergent of five healthy Korean volunteers (female). Briefly, the face was swabbed by sterilized cotton buds and inoculated into brain heart infusion agar plates and incubated at 37 °C for 20 h. The grown colonies were identified to be *Staphylococcus epidermidis*, *Shigella flexneri*, *Enterobacter aerogenes*, *Kluyvera cryocrescens*, *Staphylococcus warneri*, *Bacillus safensis* *Sporosarcina luteola* and *Paenibacillus terrigena* by analyzing gram staining, sugar utilization and 16S rRNA sequencing.

2.6. DNA extraction, pyrosequencing and data analysis of skin microbiota

Genomic DNA was extracted from the face (right cheek, 5 × 5 cm) of five healthy Korean volunteers (female) using a commercial DNA isolation kit (QIAamp DNA stool mini kit, Qiagen, Hilden, Germany) by following the manufacturer’s protocol. For pyrosequencing, amplification of genomic DNA was performed using bar-coded primers, which targeted the V1 to V3 region of the bacterial 16S rRNA gene. The amplification and sequencing were performed according to the methods described by Chun *et al*[24]. and completed by Chunlab

Inc. (Seoul, Korea) using a 454 GS FLX Titanium Sequencing System (Roche, Branford, CT). Sequence reads were identified using EzTaxon-e database (<http://eztaxon-e.ezbiocloud.net/30>) on the basis of 16S rRNA sequence data. Number of sequence analyzed, observed diversity richness (operational taxonomic units, OTUs), estimated OTU richness (ACE and Chao1), and coverage in the present pyrosequencing were indicated in Table 1.

2.7. Statistics

The data are expressed as the means ± standard errors of the means. Statistical analysis of the data was performed with ANOVA and Duncan’s test. Differences with a *p* < 0.05 were considered to be statistically significant.

3. Results

3.1. Antimicrobial activities of preservatives frequently used in cosmetics

A variety of representative cosmetics were selected for this study, including toner, emulsion, cream, baby cream, powder pact, eyeliner and lip stick and we measured their antibacterial effects against representative pathogens *E. coli*, *S. aureus* and *P. aeruginosa* (Table 1). Among those evaluated, preservative-contained toner, emulsion, cream and baby cream were potent inhibition of pathogenic growth, but the eyeliner, lip stick and powder pact barely inhibited pathogen growth.

We measured the MIC and MBC of selective preserva-

Table 2. Antimicrobial Activities of Commercial Cosmetics

	Growth inhibition ^a								
	<i>S. aureus</i>			<i>E. coli</i>			<i>P. aeruginosa</i>		
	A ^b	B	C	A	B	C	A	B	C
Toner ^c	+++	+++	+++	+++	+++	+++	+++	+++	+++
Emulsion	+++	+++	+++	+++	+++	+++	+++	+++	+++
Baby cream	+++	+++	+++	+++	+++	+++	+++	+++	+++
Cream	+++	+++	+++	+++	+++	+++	+++	+++	+++
Powder pact	-	-	-	-	-	-	-	-	-
Eyeliner	-	+	-	-	-	-	+	+	+
Lip stick	-	-	+	++	+	+	+	+	+

^a +++, perfectly inhibited (100%); ++, moderately inhibited (>90%); +, weakly inhibited (>10%); -, no inhibited.

^b indicates commercial companies.

^c The concentration of tested cosmetics was 1 mg/mL.

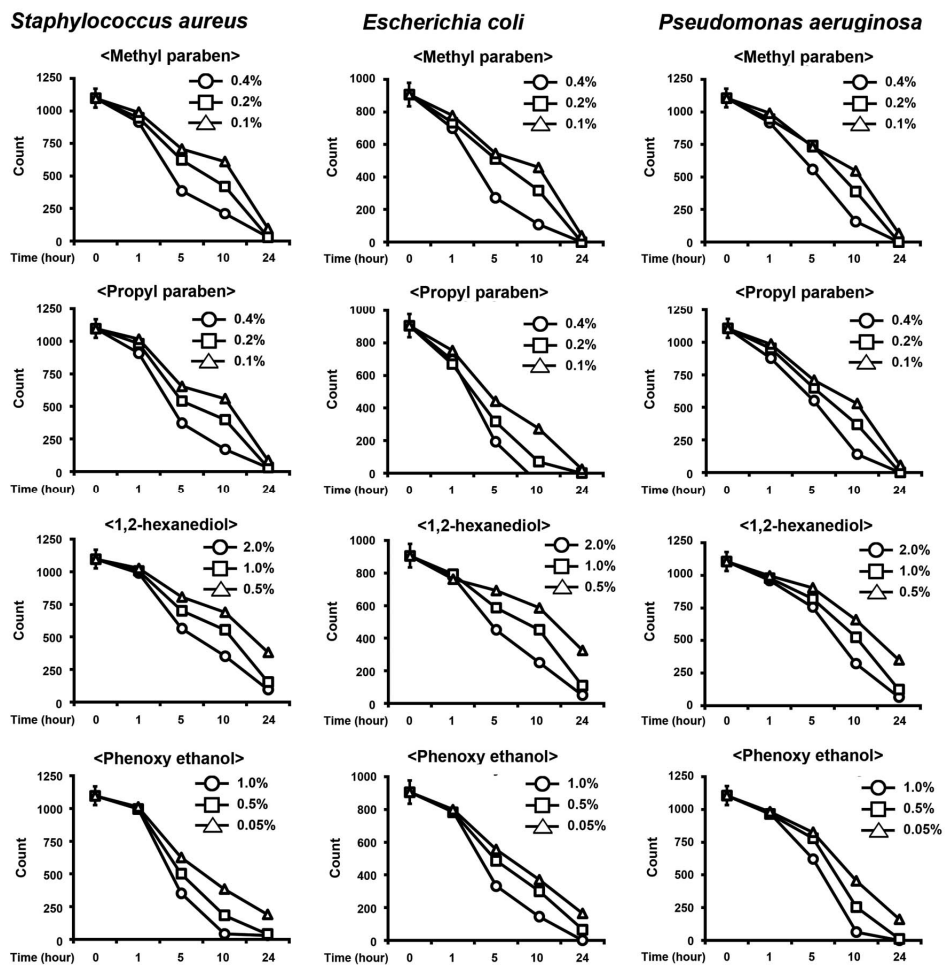


Figure 1. Antibacterial effect of preservatives against pathogens.

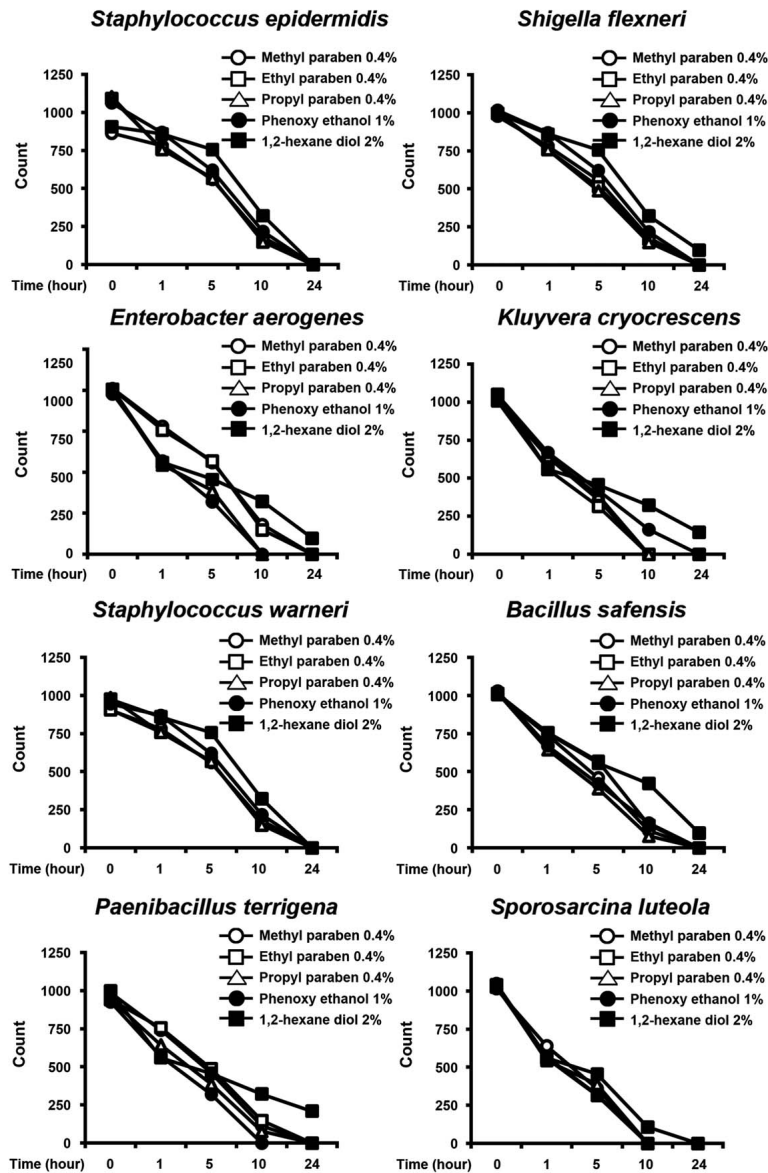


Figure 2. Antibacterial effect of preservatives against human skin bacteria. *Enterobacter aerogenes*, *Kluyvera cryocrescens*, *Staphylococcus epidermidis*, *S. warneri*, *Paenibacillus terrigena*, *Sporosarcina luteola*, *Bacillus safensis* and *Shigella flexneri* were isolated from the human skin.

tives frequently used in cosmetics against *E. coli*, *S. aureus* and *P. aeruginosa* (Table 2). Of the tested preservatives, phenoxyethanol exhibited the most potent effect against *S. aureus* and *P. aeruginosa*, followed by parabens. The most potent preservative against *E. coli* was propyl paraben, followed by phenoxyethanol.

3.2. Antimicrobial activities of commercial cosmetics

The pathogens were stored with preservatives at various concentrations and at 20 °C and their antibacterial effects were periodically measured for 24 h (Figure 1). The preservatives exhibited the potent antibacterial effects against *S. aureus*, *E. coli* and *P. aeruginosa*, in a time-dependent manner. Among the tested preservatives,

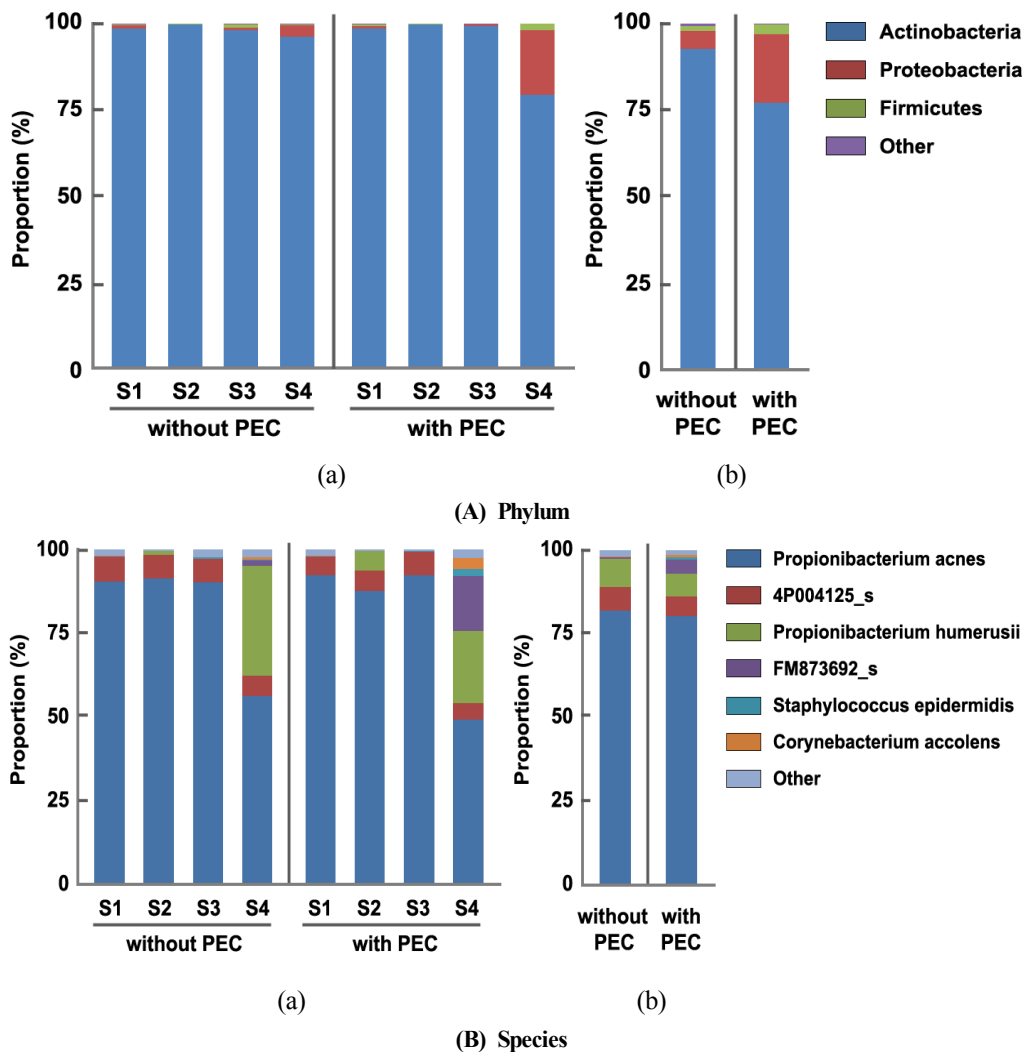


Figure 3. Effect of the phenoxyethanol-contained cream (PEC) on the composition of human skin microbiota. (A) Effect on phylum. (B) Effect on species.

parabens, particularly propylparaben, exhibited the most potent antimicrobial effects, followed by phenoxyethanol and 1,2-hexanediol.

3.3. Effect of cosmetic preservatives on the isolated human skin-resident bacteria

We also measured the growth-inhibitory effects of the preservatives against microbes isolated from the faces of human subjects (*Staphylococcus epidermidis*, *Shigella flexneri*, *Enterobacter aerogenes*, *Kluyvera cryocrescens*, *Staphylococcus warneri*, *Bacillus safensis*, *Sporosarcina luteola* and *Paenibacillus terrigena*) (Figure 2). All of

the tested preservatives, except 1,2-hexanediol, were potent inhibitors of the growth of microbes isolated from the human face. However, among the tested microbes, 1,2-hexanediol did not completely inhibit the growth of *Shigella flexneri*, *Enterobacter aerogenes*, *Kluyvera cryocrescens* and *Bacillus safensis*.

3.4. Effect of phenoxyethanol on human skin microbiota

Next we applied a basic cream, with and without a phenoxyethanol, to the right and left side of the subjects' faces, respectively, collected their skin microbiota with sterile swabs, and analyzed the microbiota by 16S rRNA

pyrosequencing (Figure 3). At the phylum level, the dominant population in human skin was Actinobacteria, followed by Propionibacteria and Firmicutes. At the species level, the dominant population was *Propionibacterium acnes*, followed by *4P004125_s*, *Propionibacterium humerusii* and *S. epidermidis*. Treatment with phenoxyethanol was shown to disturb skin microbiota such that at the phylum level, Proteobacteria was increased, and at the species level, *4P004125_s* was increased and *Propionibacterium humerusii* was decreased.

4. Discussion

The skin microbiota are consisted of transient and resident microorganism. The latter are members of the normal commensal skin microbiota, whereas the former are microbes contaminated from the environment. The composition of these skin microbiota is dependent on the intrinsic and extrinsic factors: intrinsic factors are genotype, age and sex, and extrinsic factors are occupation, lifestyle, geographical location, and use of antibiotics and cosmetics. The human skin microbiota inhibits the colonization of pathogens in the skin and can modulate the cutaneous immune system. This indicates that maintaining our skin microbiota is beneficial for our health. For example, the resident *S. aureus* inhibits *S. aureus* colonization in the skin. *S. aureus* is found on the skin in > 90% of atopic dermatitis patients and is reduced by improving it. Therefore, *S. aureus* is thought to be an important pathogen in the pathogenesis of atopic dermatitis. Additionally, preservatives have been used in cosmetics to protect the microbial contamination. There is not easy to escape these preservative-containing cosmetics. Therefore, the application of these cosmetics on the skin may influence the composition of the skin microbiota. In the present study, preservatives potently inhibited pathogens and skin-resident microbiota. Among those preservatives, phenoxyethanol exhibited the most potent effect against *S. aureus* and *P. aeruginosae* and skin-resident bacteria. However, *E. coli* was inhibited by propyl paraben most potently. The commercial preservative-contain-

ing cosmetics also inhibited the growth of pathogens. Furthermore, the application of phenoxyethanol-containing cosmetics (PEC) to the skin caused the disturbance of skin microbiota, such as the increase of Proteobacteria phylum and *4P004125_s* and *Propionibacterium acnes* species and the decrease of *Propionibacterium humerusii*. Based on these findings, preservative-containing cosmetics may disturb the microbiota and cause superinfection.

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