

Article

# Cosmetics Pattern Map Considering their Effects on Skin Microbiota-Principal Component Analysis of the Effects on Short Chain Fatty Acid Production by Skin Microbiota *Staphylococcus epidermidis*

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**Abstract:** Cosmetics have various characters, but there have been no studies which classified the properties of cosmetics based on their effects on skin-domesticated bacteria. In this study, we established an anaerobic culture and short-chain fatty acid measurement system using *Staphylococcus epidermidis*, a representative bacterium of indigenous skin bacteria. Furthermore, this system was used to extract and classify the characteristics of 9 cosmetics. Cosmetics containing ferments of rice and soy were clustered in the vicinity, although one cosmetic containing soy ferment was clustered distant from other similar cosmetics. Cosmetics from South Asia and those containing natural plant extracts were clustered in the vicinity. These results show that this method can be utilized as a cosmetics pattern map, and will provide a method to prevent growth of opportunistic bacteria.

**Keywords:** cosmetics; skin microbiota; *Staphylococcus epidermidis*; lactic acid; PCA

## 1. Introduction

Cosmetics are applied to the skin for the purpose of enhancement of beauty. Before the establishment of modern chemical industry, there were few concepts of make-up and skin care, and most cosmetics used plant-derived ingredients or minerals. In Japan, make-up products such as white powder, safflower, and eyebrow ink, and plant-derived skin care products such as loofah water and flower dew [1,2] began to become popular among the general public during the Edo period (AD 1600-1868). The establishment of the chemical industry in the 18th and early 19th centuries led to the use of glycerin as a moisturizing ingredient and the spread of cosmetics made with chemical products. For this reason, the cosmetics industry established in the 20th century, combined with the petrochemical industry established in the early 20th century, almost exclusively used petroleum-derived chemical products. However, such chemical products have not been applied to human skin in the past, and due to the problem of petroleum-derived trace constituents, they have been causing various health problems since the 1970s. For example, skin diseases such as melanosis [3] and chemical sensitivity have been reported. As a result, from the 1980s, many cosmetics using naturally derived ingredients began to appear on the market. Furthermore, taking advantage of traditional Japanese fermentation technology, many cosmetics containing fermented products were developed in Japan [4,5]. However, there have been no studies to date that have revealed the effects of cosmetics containing such natural or fermentation-derived ingredients on skin bacteria, let alone cosmetics made with chemical products. There are cosmetics from various cultural backgrounds in the world, and they have completely different characteristics. However, with the development of logistics,

cosmetics from all over the world are now available, and there have been no studies comparing the effects of these cosmetics to skin microbiota.

Properties of cosmetics vary dependent on the cultures of countries. For example, in Japan, many cosmetics are based on fermented products because of the country's long tradition of fermented foods. On the other hand, in South Asia, cosmetics based on medicinal herbs are considered common due to the Ayurvedic tradition of using medicinal herbs. In Europe, where the cosmetics industry first emerged, many cosmetics are chemical-based, and many are dairy- and plant-based due to the traditions of dairy farming and Unani medicine. However, there are no studies that report such regional characteristics because there is no system to identify the properties of cosmetics.

Skin is said to be "the largest organ of the human body " [6] since it covers our entire body, occupying a total area of about 1.6 square meters (for an adult) and weighing about 15-16% of our body weight. Therefore, it is believed that maintaining healthy skin leads to the maintenance of the overall health of the body. Human skin is inhabited by about 1,000 species of bacteria, which are referred to as dermal commensal bacteria. The skin commensal flora is said to be the second largest flora in the human body after the intestinal flora [7], and it is classified into three major categories according to their functions for the host. They are classified into three categories according to their function on the host: beneficial bacteria, opportunistic bacteria that change their role depending on the balance between beneficial and harmful bacteria on the skin, and harmful bacteria that cause skin problems. Among these, *Staphylococcus epidermidis*, which is the most ubiquitous bacterium on human skin and is known as the representative of good bacteria, degrades sweat and sebum on the skin and produces glycerin and fatty acids. The glycerin produced by these indigenous skin bacteria strengthens the skin barrier function [8] and the fatty acids keep the pH level of the epidermis acidic and inhibit the growth of bad bacteria [9]. Other short-chain fatty acids derived from indigenous skin bacteria have also been reported to have various effects on the skin. For example, Lactic acid has been reported to have a peeling effect on the skin and a softening effect on the stratum corneum [10]. In addition, it has recently been reported that lactate produced by indigenous skin bacteria [11] and *Staphylococcus aureus* act on the immune response of the host through the production of IL-10 and control skin pathogens [12]. Succinic acid inhibits the growth of *Propionibacterium acnes*, a causative agent of acne [13], and propionic acid inhibits the growth of *Staphylococcus aureus*, a representative of hazardous bacteria [14]. On the other hand, acetic acid and formic acid are known to be highly irritating to the skin [15]. Therefore, insight into the effects of cosmetics on short-chain fatty acids produced by *S. epidermidis* will have an impact on skin health.

In this study, we attempted to establish a cosmetics pattern map that can extract and compare the characteristics of cosmetics based on the short-chain fatty acids produced by *S. epidermidis*.

## 2. Materials and Methods

### 2.1. Materials

The bacteria used were *Staphylococcus epidermidis* (NBRC100911) purchased from the National Institute of Biotechnology and Life Science (NBRC).

The nine cosmetics used in this study and the main ingredients used in the cosmetics are listed below.

- (1) Cosmetic A containing Soy Milk Fermentation Liquid (Made in Japan) (Soy.fermentA)  
Soy milk fermentation liquid
- (2) Cosmetics containing Soy Milk Fermentation Liquid (Made in Japan) (Soy.fermentB)  
Soy milk fermentation liquid
- (3) Antibacterial cosmetics (Made in Japan) (Antimicrobial)  
Antimicrobial ingredient
- (4) cosmetics containing plant extracts (Made in South Asia)(SouthAsia)  
Plant oil
- (5) cosmetics claiming natural ingredients (Made in Japan) (Natural)  
Soy milk fermentation liquid, vegetable oil

- (6) cosmetics containing loofah water (Made in Japan) (Loofar)  
Loofah extract
- (7) cosmetics containing plant extracts (Made in Japan) (Plant)  
Plant extract
- (8) Lotion containing rice ferment (Made in Japan) (Rice.fermentA)  
Rice fermentation liquid, vegetable oil
- (9) cosmetics containing rice ferment (Made in Japan) (Rice.fermentB)  
Rice fermentation liquid

## 2.2. Reagents

The following culture media and reagents were used to culture the bacteria.

Bacterial pre-culture: 702 medium (High polypeptone [Wako Pure Chemical Industries, Ltd.], yeast extract [Wako Pure Chemical Industries, Ltd.], magnesium sulfate heptahydrate  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  [Nakalai Tesque Co.]). After preparation of the medium, pH was adjusted to 7.0. Co-culture of bacteria and cosmetics: GAM medium (Modified GAM Bouillon [Nissui Pharmaceutical Co.]).

## 2.3. Nitrogen replacement in the culture vessel

A 10 mL headspace vial (vial, 45 mm × 22 mm, Agilent technologies Inc., USA) was used as the culture vessel. Nitrogen was supplied from the connecting tube of a nitrogen gas cylinder to the vials using silicone tubes (9 × 12 mm), 1000-5000  $\mu\text{L}$  Pipet Tip. GAM medium was added to 5 mL vials. 5  $\mu\text{L}$  of resazurin diluent (1 mg/mL), a redox indicator, was added at the time of GAM medium preparation, and the color of the medium changed from reddish purple to yellow derived from the medium as a discrimination of anaerobic condition. The tip of the nitrogen inflow device was inserted into vials. The entire area was covered with aluminum foil, and holes were drilled for oxygen exit. An anaerobic environment was created by flowing nitrogen gas and bubbling for 5 minutes. After gas displacement, a crimp cap (septum type: molded PTFE/butyl, Agilent technologies Inc., USA) was immediately set on the vial and immediately sealed using a hand crimper (G.L. Science Corporation). The culture medium was then sterilized by autoclaving.

## 2.4. Co-culture of *S. epidermidis* and cosmetics

*S. epidermidis* was inoculated into 5 mL of 702 medium and pre-cultured at 37°C for 24 hours. In the main culture, the pre-culture solution was added to the GAM medium prepared in the method to achieve an initial bacterial concentration of  $1.0 \times 10^7$  cells/5 mL. A micro syringe (Ito Manufacturing Co., Ltd.) was used for the addition. 0.25 mL (5% of the total volume of 5 mL) of the cosmetic product to be studied was added and incubated at 37°C for 24 and 48 h. A total of 6 samples per cosmetic product were prepared: vehicle-added control group (0, 24, 48 h) and cosmetic product-added group (0, 24, 48 h).

## 2.5. Extraction of exogenous metabolites (removal of foreign substances)

After co-culturing as described above, the culture medium was centrifuged (24,000 rpm, 5 min) and 2.0 mL of the supernatant was collected as exogenous metabolites. Further centrifugation (14000 rpm, 1 min) was performed to ensure removal of bacteria, and 1.5 mL of the supernatant was removed of remnants using a 0.45  $\mu\text{m}$  membrane filter (Shimadzu GLC Corporation). 1.0 mL was added to a vial for HPLC, covered with a lid, and set in an autosampler for HPLC (L-2200, Hitachi, Ltd. Hitachi, Ltd. Randomization was performed using the random function in Microsoft Excel for the analysis, and all analyses of each cosmetic were performed on the same day.

## 2.6. Short-chain fatty acid (BTB method) analysis by high-performance liquid chromatography (HPLC)

Analysis by HPLC was performed using an RSpak KC-811 column (particle size 6.0  $\mu\text{m}$ , inner diameter 8 mm, length 300 mm, G.L. Science Corporation), column oven: 40°C (FS-420, Pro Devices

Corporation), flow rate: 0.5 mL/min (A), 0.6 mL/min (D), buffer A: 3 mM HClO<sub>4</sub>, buffer D: 0.1 mM BTB and 15 mM Na<sub>2</sub>HPO<sub>4</sub>, analysis time: 80 min.

### 2.7. Peak identification

Peak identification was based on the retention time in the chromatogram. The obtained area value data for short-chain fatty acids were converted to an Excel file and used for subsequent statistical analysis.

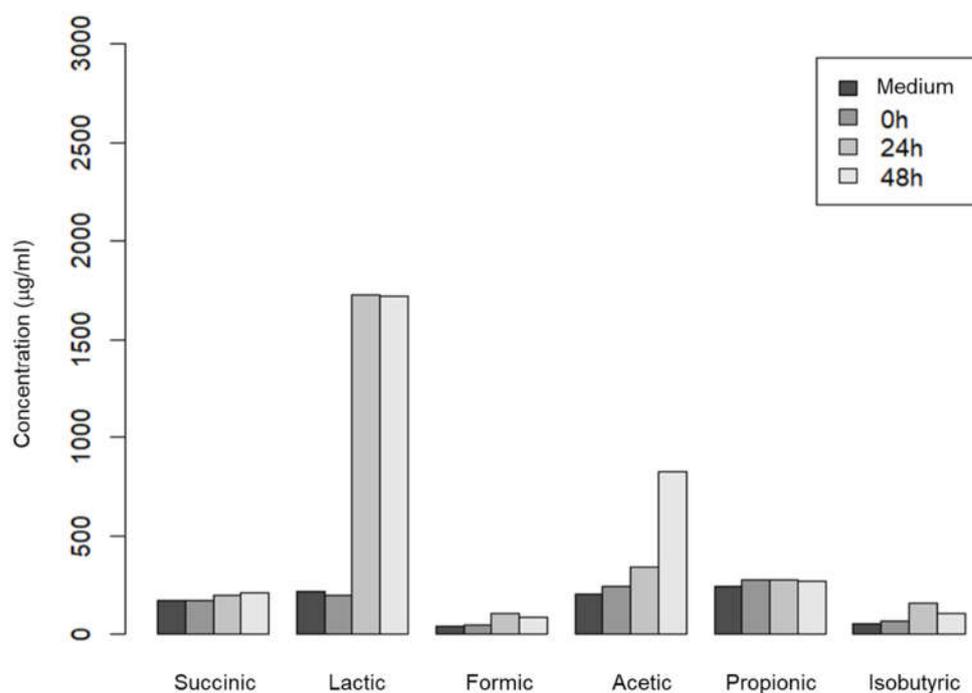
### 2.8. Statistical Analysis

SIMCA-P+ ver. 13.0 (Umetrics, Umeå, Sweden) was used for principal component analysis.

## 3. Results

1. Establishment of an anaerobic culture and short-chain fatty acid production measurement system for *S. epidermidis*.

First, to investigate whether metabolites of *S. epidermidis*, an indigenous skin bacterium, can be quantitatively detected in an anaerobic environment that mimics skin pores, media with or without *S. epidermidis* cultured anaerobically for 0, 24, and 48 hours were subjected to HPLC for analysis. As a result, six peaks were detected and identified as six short-chain fatty acids (succinic acid, lactic acid, formic acid, acetic acid, propionic acid, and isobutyric acid). This suggested that *S. epidermidis* metabolites could be quantitatively detected under these culture conditions. The concentrations ( $\mu\text{g/mL}$ ) of the short-chain fatty acids were calculated from the detected peak area values, calibration curves for each short-chain fatty acid. Using these values, we compared the amount of short-chain fatty acid production by incubation time, and found that lactic acid and acetic acid increased in the 24- and 48-hour incubation samples (Fig. 1). From this result, we considered that we could construct a system to simulate the metabolism of indigenous skin bacteria in skin pores.



**Figure 1.** Short-chain fatty acid concentration of the culture of *S. epidermidis*. *S. epidermidis* was cultured in nitrogen-purged GAM medium anaerobically. Concentrations of cultures incubated with *S. epidermidis* for 0, 24 and 48 h were analyzed using HPLC.

### 3.1. Change in short-chain fatty acid production by *S. epidermidis* upon addition of cosmetics

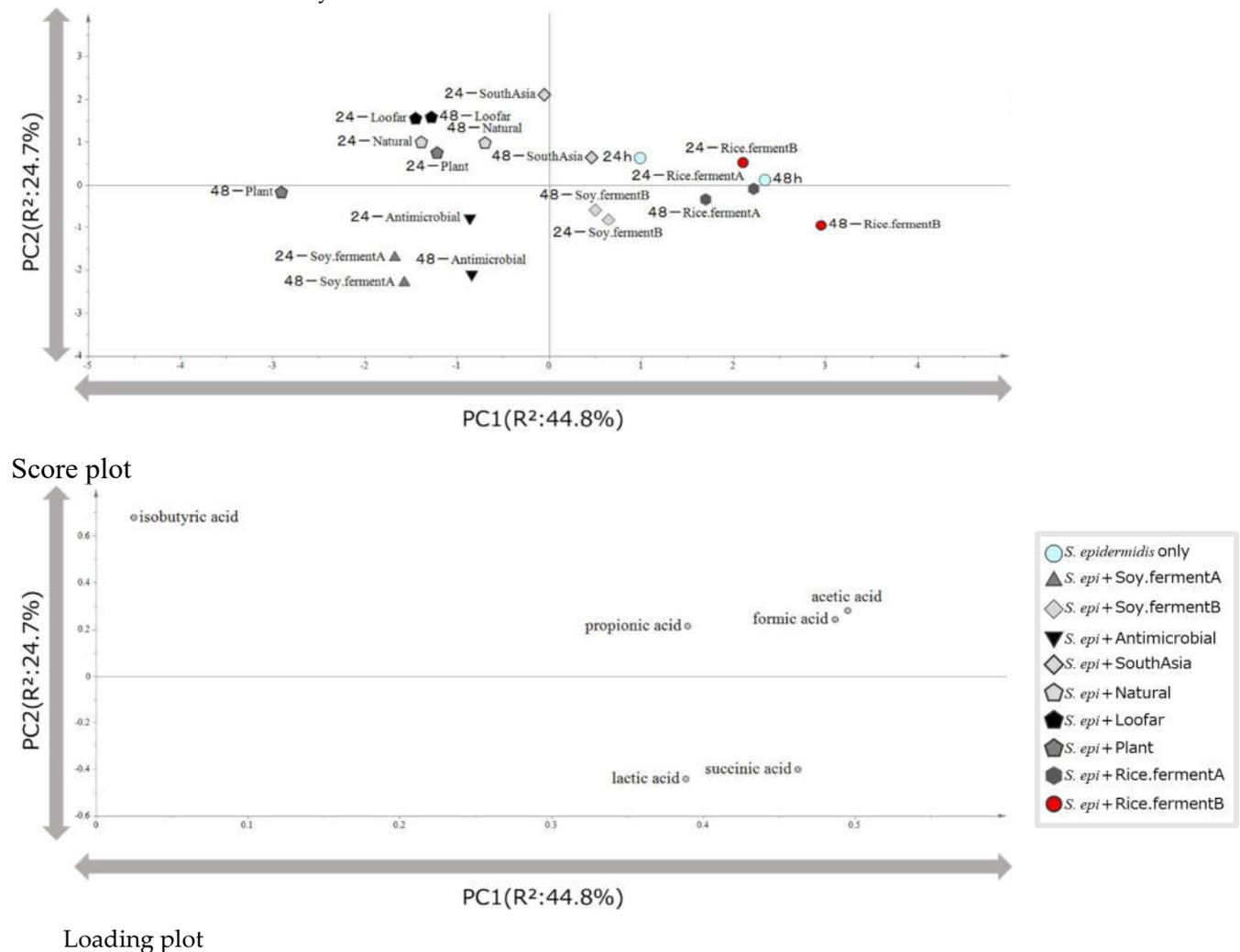
Since we were able to construct a system for analyzing metabolites of *S. epidermidis* as described above, we decided to examine whether the metabolites changed when actual cosmetics were added to the culture. *S. epidermidis* alone cultured for 0, 24, and 48 hours, and *S. epidermidis* added with various cosmetics for 0, 24, 48 hours, were subjected to HPLC and analyzed. The results showed that there were various patterns depending on the cosmetics (Table 1). The results showed that there were various patterns depending on the cosmetics, which may allow us to distinguish and clarify the effects of cosmetics on indigenous skin bacteria.

**Table 1.** Short-chain fatty acid concentrations ( $\mu\text{g/ml}$ ) of *S. epidermidis* cultured with various cosmetics.

	antimicrobial	Soy.fermentA	Loofar	Natural	Plant	Soy.fermentB	SouthAsia	Rice.fermentA	Rice.fermentB
0h									
succinic	154	190	150	169	180	165	173	173	167
lactic	169	217	166	182	199	182	367	245	217
formic	39	49	43	34	35	42	44	52	28
acetic	238	225	242	242	235	242	236	312	239
propionic	264	299	293	280	292	284	293	315	289
isobutyric	110	70	149	130	97	88	136	112	67
24h									
succinic	164	200	180	179	201	191	193	166	189
lactic	1523	1690	1458	1468	1801	1716	1549	1939	2000
formic	107	93	100	107	80	87	104	88	94
acetic	357	341	363	362	376	379	379	371	417
propionic	282	300	301	287	280	277	272	289	278
isobutyric	167	151	302	232	191	199	335	146	211
48h									
succinic	175	199	176	184	191	196	181	207	183
lactic	1937	2123	1808	1691	2300	2153	1821	2683	2556
formic	100	95	104	107	103	88	100	119	83
acetic	408	359	374	397	394	417	391	579	511
propionic	276	282	257	294	301	265	297	321	283
isobutyric	184	156	202	213	185	161	184	147	228
plus cosmetics									
0h									
succinic	143	179	144	153	169	179	157	169	167
lactic	147	237	177	193	190	158	183	236	233
formic	50	52	32	46	38	42	56	44	33
acetic	218	230	226	231	229	227	233	260	283
propionic	306	293	279	294	280	276	292	303	294
isobutyric	109	49	166	103	79	69	117	98	94
24h									
succinic	165	174	158	166	188	184	159	181	161
lactic	1386	1924	1311	1772	1970	1838	1542	2566	2139
formic	84	79	88	85	93	70	93	84	72
acetic	331	281	354	309	339	330	372	397	383
propionic	280	252	257	256	288	274	304	349	278
isobutyric	200	125	241	280	157	188	255	186	183
48h									
succinic	155	177	166	157	182	177	162	190	172
lactic	1430	2299	1717	2174	2151	2433	1965	2854	2678
formic	61	73	70	91	87	70	94	94	67
acetic	290	281	358	332	342	347	426	359	389
propionic	280	247	266	266	294	246	269	282	261
isobutyric	165	129	313	263	179	127	179	260	172

### 3.2. Classification of cosmetics based on principal component analysis of the amount of short-chain fatty acids produced by indigenous skin bacteria

In order to gain a comprehensive insight into the pattern of short-chain fatty acid production by each cosmetic product obtained above, a principal component analysis was conducted using the values as explanatory variables. As a result, an axis separating lactic acid and other short-chain fatty acids as the first principal component was obtained, and an axis separating succinic acid, formic acid and isobutyric acid as the second principal component (Figure 2). The contribution of the first and second principal components (PC1 and PC2) was 44.8% and 24.7%, respectively. In the score plot, the samples were divided into two groups. First group was the groups of rice-fermented cosmetics and soy-fermented cosmetics and the second group was South Asian cosmetics, plant extract cosmetics and natural cosmetics. One cosmetic containing soy ferment was clustered in the vicinity of antimicrobial. This finding suggested that it would be possible to classify cosmetics according to their characteristics and to identify the substances that contribute to this classification.



**Figure 2.** Results of principal component analysis of short-chain fatty acids in cosmetics and cultured *S. epidermidis*.

Principal component analysis was performed using concentrations of six short-chain fatty acids obtained from HPLC analysis of cosmetics and cultured *S. epidermidis* cultures. (A) Score plot (B) Loading plot.

## 4. Discussion

Until now, there have been no studies classifying the properties of cosmetics based on their effects on indigenous skin bacteria, but in this study, for the first time, we succeeded in establishing

a system for classifying the properties of cosmetics based on their effects on *S. epidermidis*, one of the indigenous skin bacteria.

Among the nine cosmetics investigated in this study, rice- and soy-fermented cosmetics were plotted in the close proximity on the score plot; the fact that these cosmetics were located near succinic acid and formic acid on the loading plot suggests that these cosmetics have similar effects on *S. epidermidis*. The fact that these cosmetics were located near formic acid, acetic acid in the loading plot suggests that they are highly effective in promoting these acids production towards *S. epidermidis*. In contrast, South Asian cosmetics, loofar cosmetic, natural cosmetic, and plant-based cosmetic formed clusters at positions near isobutyric acid. These results suggest that these cosmetics may have similar properties and may enhance production of isobutyric acid. Since South Asian cosmetics contain abundant plant-derived ingredients, it was considered that cosmetics with high plant ingredients would be located in the vicinity. There were two bean-fermented cosmetics, but each was located in a different cluster, suggesting that the same bean-fermented cosmetics may have very different properties.

Cosmetics plotted onto the loading plot space generates many insights into the characteristics of the cosmetics. As described above, lactic acid has beneficial effects on the physical properties and immunity of the skin. Succinic acid has been reported to inhibit the growth of *Propionibacterium acnes*, the causative agent of acne, without causing significant irritation to the skin [16]. On the other hand, acetic acid and formic acid are known to be highly irritating to skin [13]. Based on these reports, we hypothesized that cosmetics showing a positive correlation with lactic acid and succinic acid in the principal component analysis would be highly effective towards *S. epidermidis*, while cosmetics showing a positive correlation with acetic acid and formic acid would be less effective.

Safety of cosmetics is evaluated by several indices, such as skin stimulation, allergic potential, light toxicity, toxicity, and eye stimulation [17]. However, there has been no study to elucidate the effects of cosmetics on skin microbiota. Indeed, some patients resort skin inflammation upon local administration of cosmetics, but the cause was not known. These symptoms might be explained by the disorder of skin microbiota in the future, considering these researches.

Several ingredients might influence the growth and short chain fatty acid production by *S. epidermidis*. For example, oligosaccharides and amino acids might enhance the growth and short chain fatty acid production. Antibiotics contained in cosmetics might hinder the growth of *S. epidermidis*.

It is now not known if ingredients in cosmetics such as emollients, lubricants, emulsifiers, fatty acids, alcohols, gelling agents, thickeners and antibiotics might affect growth or metabolism of skin microbiota. This study will shed light on the effect of these constituents towards skin microbiota, and will enable design of a more functional cosmetics that function on skin microbiota.

Recently, many cosmetics that appeal improvement of skin microbiota are commercially available. However, there is no indices that objectively evaluate the competence of the cosmetics on skin microbiota. This confused situation gives rise to survival of low quality cosmetics, and is unbeneficial for consumers. Therefore, this research will be an effective method to evaluate those cosmetics in terms of their function on skin microbiota, which is beneficial for consumers.

The problem of multidrug resistance bacteria is one of the strongest threat to the human beings now. MRSA is generated from *Staphylococcus aureus*, one of skin microbiota. It is known that *S. aureus* is sensitive to organic acids including lactic acid, acetic acid and propionic acid [18]. Indeed, *S. epidermidis* is known to prevent colonization of *S. aureus* through delta-lysin, esp and antimicrobial peptide [19-22], and skin microbiota abundant in *S. epidermidis* or *Corynebacterium acnes* is proposed to be healthy [23]. Imbalance in skin microbiota generates nonpathogenic but unfavorable status of the skin, such as dry skin and sensitivity [24]. Moreover, *S. epidermidis* act on TLRs and enhances production of antimicrobial peptides, which also leads to prevention of opportunistic pathogenic bacteria [25]. *S. epidermidis* also prevents adhesion and biofilm formation by *S. aureus* [26]. It is proposed that the lipoteichoic acid of *S. epidermidis* can prevent skin inflammation mediated by TLR2 and TLR3 [27]. Therefore, regulation of lactic acid by *S. epidermidis* will be a potent approach to the regulation of *S. aureus*, which leads to prevention of the rise of MRSA.

So far, *in vitro* on mono- or co-cultures of skin bacteria to measure the effects of cosmetics by growth measurement using qPCR has been described [28]. Studies using 3D skin models have been

applied to elucidate the interaction between skin microbiota and cosmetics [29]. These are considered *ex vivo* models, and the knowledge obtained in this study can be in the future utilized in these *ex vivo* models. However, this is the first study to extract information on cosmetics by using data obtained from *in vitro* culture of skin microbiota.

Cosmetic ingredients used that are either functional ingredients, such as preservatives, oils and emulsifiers, or active ingredients, impact the skin microbiota and require caution. Indeed, conventional skincare or hygiene products such as soap, gel and cream contain preservatives and natural and synthetic chemicals that impact microbiota even if these effects are not fully investigated in detail until now [30, 31].

This research has several limitations. For example, it did not adopt 3D-structured skin model [29]. However, when focusing onto the metabolism of skin microbiota, this research will provide some information on the properties of cosmetics. Secondly, this research used only *S. epidermidis*. Skin microbiota contain other bacteria, such as *Propionibacterium acnes*, *Corynebacterium acnes* and *S. aureus*. Therefore, in the future, these bacteria should also be taken into account. The composition of media is artificial. Media with which bacteria grow in the actual skin pores are triglycerides and amino acids secreted from the sebaceous glands. However, since the growth using these components requires enormous time, media containing glucose was used to facilitate the analysis. Therefore, knowledge obtained through this study should take these into account.

Skin pH varies dependent on the parts of the skin. For example, pHs of the skin of Axillary vault, and antecubital crease tend to be high. In contrast, pH of the skin of glabella and alar crease tend to be low [32, 33]. Therefore, administration of cosmetics and decrease of pH through *S. epidermidis* should be considered in terms of the control of pH.

Recently, fungi which proliferate in skin pores, such as *Malassezia furfur* [34], cause pancreatic oncogenesis and is a serious threat to the human health. Since fungi generally cannot grow in an acidic environment, increase of short-chain fatty acids by *S. epidermidis* might be favorable to prevent proliferation of these fungi, which is a significant target of future research.

Some cosmetics contain detergents. Since detergents interfere with skin bacteria, these might have impacted the results obtained in this research. This should be considered in the future research.

It was not clear from this study which components of these cosmetics stimulated the production of these acids. However, plant extracts and fermented products contain plant and microbial components and microbial metabolites, these components may have stimulated the production of the short-chain fatty acids by *S. epidermidis*.

Atopic dermatitis is one of the major skin diseases in the developed countries. It is caused by skin barrier dysfunction such as 1) deficiency in ceramide, cholesterol and free fatty acids, 2) deficiency in differentiation of keratinocyte proteins (filaggrin, loricin and involucrin), 3) sensitivity to immune stimulus, 4) decrease in antimicrobial peptides and 5) decrease in the diversity of skin microbiota [35]. Indeed, it is reported that *S. epidermidis* is less and *S. aureus* is abundant in atopic dermatitis patients. Both *S. epidermidis* and *S. aureus* share the characteristics of biofilm formation, but *S. aureus* is more frequent in atopic dermatitis patients [36]. Therefore, usage of specific cosmetics might cause dysfunction of skin microbiota and lead to atopic dermatitis.

Cosmetics market is rapidly growing. The global cosmetics market size was estimated to be USD 375.30 billion in 2022. It is expected to grow at a compound annual growth rate (CAGR) of 5.1% from 2021 to 2030 [37]. Therefore, people in the world will more and more use cosmetics. However, since legal regulations of cosmetics vary greatly depending on the countries, some subjective criteria for evaluation of the influence of cosmetics on the skin microbiota is needed. The method proposed in this study will be one such criteria.

It is reported that with increasing age, at the phylum level, *Proteobacteria* increased and *Actinobacteria* decreased, and at the genus level, *Corynebacterium* increased and *Cutibacterium* decreased in Caucasian women [38]. Therefore, it is hypothesized that alteration of skin microbiota is related to aging, and manipulation of skin microbiota might lead to anti-aging.

So far, the effects of cosmetics on skin microbiota have been analyzed. For example, the effects of cosmetics on the growth, virulence, cytotoxicity on HaCat keratinocytes, biofilm formation of *S.*

epidermidis and Cutibacterium acnes have been reported [39-41]. However, there has been no study on the short-chain fatty acids of *S. epidermidis* and this report is the first study to report it.

In summary, a cosmetics-pattern map using *S. epidermidis* was established in this study. This novel method can be used to pattern cosmetics and infer their effects on skin microbiota.

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