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# Enhanced ceramides production by *Lactobacillus rhamnosus* IDCC 3201 and its proposed mechanism

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## Abstract

The use of probiotics has been applied for a variety of fields (e.g., immune system, mental health, and heart). In this study, the feasibility of lysates from *L. rhamnosus* IDCC 3201 for cosmetic ingredients was evaluated. More specifically, enhanced ceramides production in human epidermal keratinocytes by the lysates and its proposed mechanism were investigated through in vitro and genome analysis. In results, enhanced sphingomyelinase activity and thereby increased ceramides production by the lysates from *L. rhamnosus* IDCC 3201 was observed. Furthermore, it was found that the existence of glucosylceramidase in *L. rhamnosus* IDCC 3201 was attributed to enhanced ceramides production. Finally, it was verified that the lysates from *L. rhamnosus* IDCC 3201 was regarded as safe for its use as cosmetic materials. Thus, these findings have significant implications that might lead to the development of functional and safe cosmetic products from probiotics.

**Keywords:** Ceramides, Glucosylceramidase, Probiotics, Skin health, Sphingomyelinase

## Introduction

Recently, interest of skin health has been increased due to the escalating environmental stresses [1]. Especially, the most outer layer of the skin (stratum corneum) is thought to be a frontier of skin protection, and it is mainly composed of corneocytes and lipids [2]. These lipids mainly include several types of cholesterol, fatty acids, and ceramides [3]. Among them, ceramides, a member of sphingolipid family, play an important role in skin barrier function (e.g., skin homeostasis). Typically, ceramides are converted from glucosylceramides and sphingomyelins by glucosylceramidase (GCase) and sphingomyelinase (SMase), respectively [4]. Many clinical studies

showed that deficiency of ceramides is involved in atopic dermatitis (AD) and psoriasis directly or indirectly [5, 6]. Thus, one of the strategies for healthy skin should be to strengthen skin barrier through increased ceramides level [7]. For example, general approach is to regulate exogenous and endogenous factors involved in ceramides synthesis such as vitamin C and vitamin D [8, 9].

Currently, probiotics have received considerable attention in cosmetic industry based on long-term uses of fermented broth for skin care [10, 11]. Consistent with this notion, lysates of *Streptococcus thermophilus* S244 not only increased ceramides level of healthy elderly women but also alleviated symptoms of AD patients [12, 13]. In addition, *Lactobacillus rhamnosus* strain promoted epidermal barrier formation in human skin model [14]. However, there was no direct evidence which components of probiotics improve ceramides production in human skin and thereby skin barrier function.

In this study, enhanced ceramides production by lysates from *L. rhamnosus* IDCC 3201 and its mechanism

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were investigated through in vitro and genome analysis. For this, firstly, the lysate from a variety of bacteria that exhibited the highest SMase activity was screened. Secondly, ceramides production by the screened probiotic strain was evaluated in human epidermal keratinocytes (HEK). Then, genome and metabolome analysis of the selected strain was performed to propose the mechanism. Finally, safety of the strain was assessed to determine whether it produces toxic compounds in human skin. Thus, this study contributes to the development of functional and safe cosmetic products from probiotics.

## Materials and methods

### Bacterial strains and preparation of bacterial lysates

*L. rhamnosus* IDCC 3201 from breast-fed infant's feces [15] and *Streptococcus thermophilus* IDCC 2201 from home-made yogurt [16] were industrial strains, while the other bacterial strains were isolated from kimchi (Korean fermented food). All the strains were incubated in an in-house medium designed by Ildong Bioscience, centrifuged, and freeze-dried to obtain cell density at  $>10^{10}$  colony forming units (CFU)/g. Then, lyophilized strains were suspended in 500 mL of phosphate buffered solution (PBS) and was lysed physically by using a microfluidizer (PicoMax MN400BF; Micronox, Seongnam, Korea) to prepare bacterial lysates.

### Sphingomyelinase (SMase) activity

SMase activity was measured using a SMase assay kit (Abcam; # ab138876, Cambridge, UK). The principle of this assay kit was to quantify phosphocholine produced by the hydrolysis of sphingomyelin using colorimetric analysis at 655 nm. Briefly, 50  $\mu$ L of sphingomyelin was incubated with lysates from five bacterial strains at 37 °C for 1 h. Then, color changes were detected using a microplate reader (SpectraMax iD3; Molecular Devices, CA, USA). Then, SMase activity was calculated according to calibration curves with the absorbance values of known amounts of SMase standards.

### Cell culture

Normal human epidermal keratinocyte (HEK) cells were purchased from PromoCell (Heidelberg, Germany). Cells were grown in growth medium 2, supplemented with bovine pituitary extract, epidermal growth factor, Insulin, hydrocortisone, epinephrine, transferin and 0.06 mM of CaCl<sub>2</sub> (PromoCell). After confluence for 3–5 days at 37 °C with 5% CO<sub>2</sub>, cells were maintained by sub-culturing the detached cells with a 0.25% trypsin–EDTA solution.

### Cell viability assay

Cell viability of the HEK cells by tested bacterial lysates was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Briefly, the HEK cells were cultured in 96-well plate and treated with the lysates of *L. rhamnosus* IDCC 3201 at cell densities ranging from  $1 \times 10^7$  to  $5 \times 10^8$  CFU/mL for 24 h. Then, MTT was added to each 96-well and incubated at 37 °C for 90 min. After removal of MTT, 100  $\mu$ L of dimethyl sulfoxide (DMSO) was added to dissolve formazan. The developed color was measured at 570–630 nm using a microplate reader (SpectraMax iD3).

### Ceramides production

Ceramide contents in the suspension from the HEK cells were measured using an ELISA kit (Human Ceramides ELISA Kit; MyBioSource, CA). Briefly, The HEK cell culture suspension with SMase was treated to a 96-well plate which coated by human monoclonal antibody. The plate was incubated with biotin-labeled polyclonal antibody which is conjugated with avidin-peroxidase. Next, a chromogen solution was added into the plate for ELISA colorimetric detection at 450 nm. Finally, amounts of ceramide were calculated according to caribration curve prepared with ceramides standards.

### Real-Time PCR

Gene expression for ceramides synthesis was investigated using real-time PCR. Total RNAs were isolated from HEK cells treated with either SMase (positive control) and bacterial lysates by a Trizol method. PCR reactions were performed using CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The house-keeping gene, GAPDH was used as a constitutive control for normalization. The specific primers used for real-time PCR are listed in Table 1.

**Table 1** Sequence of the primers used in the study

Gene	Strand	Sequence(5'-3')
SMPD3	Forward	ACATCGATTCTCCCAACACCT
	Reverse	AATTCGCACAATGCAGCTGTCCTC
CERS1	Forward	ACGCTACGCTATACATGGACAC
	Reverse	AGGAGGAGACGATGAGGATGAG
CERS2	Forward	CCGATTACCTGCTGGAGTCAG
	Reverse	GGCGAAGACGATGAAGATGTTG
GAPDH	Forward	ACGGACTTCTCGGTGATAC
	Reverse	CGGTGACTGTAGCCATATTCCG

### Bacterial gene search for ceramides biosynthesis

The whole-genome sequencing of *L. rhamnosus* IDCC 3201 was performed by a PacBio RSII instrument with an Illumina platform (Macrogen, Seoul, Korea). A sequence of nucleotides was generated by single molecule real-time (SMRT) sequencing system. Contigs were constructed by pre-assembling seed reads, by generating a consensus sequence of the mapped reads, and by correcting and filtering the reads. Finally, a consensus sequence with higher quality was obtained after error-correction of the constructed contigs by Pilon (version 1.21).

To find bacterial enzymes involved in ceramides biosynthesis from probiotics, amino acid sequence of enzymes were collected: sphingomyelinase (EC 3.1.4.12); glucosylceramidase (EC 3.2.1.45); galactosylceramidase (EC 3.2.1.46), ceramides synthase (EC 2.3.1.24), and sphingolipid-4-desaturase (EC 1.14.19.17) [17] from UniProtKB database, and built hidden Markov models (HMMs), representing the conserved amino acid sequence patterns in these enzymes (Table 2). Then, genome of *L. rhamnosus* IDCC 3201 was targeted to search using the HMMsearch tool in HMMER package with the constructed profile of HMMs [18]. Finally, the candidate enzymes were verified using BLASTP tool in NCBI BLAST and HMMscan tool in HMMER package against SWISS PROT database and PFAM database, respectively.

### Metabolites analysis

Bacterial lysates were diluted in ice-cold methanol to a final concentration of 80% with a vortex for 1 min on ice. After centrifugation at 13,000g for 10 min at 4 °C, the upper layer of the supernatant was collected, concentrated to dryness in a vacuum concentrator, and stored at – 80 °C prior to derivatization and analysis by GC–MS.

The extract was derivatized with 30 µL of a solution of 20 mg/mL methoxyamine hydrochloride in pyridine (Sigma, St. Louis, MO) at 30 °C for 90 min, and 50 µL of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA; Sigma) was subsequently added at 60 °C for 30 min. A mixture of fatty acid methyl esters and fluoranthene was added to the extract as internal standards. The GC–MS analysis was conducted using a Thermo Trace 1310 GC (Waltham, MA) coupled to a Thermo ISQ LT single quadrupole mass spectrometer (Waltham, MA). A DB-5MS column with 60-m length, 0.25-mm i.d. and 0.25-µm film thickness (Agilent, Santa Clara, CA) was used for separation. For analysis, the sample was injected at 300 °C and split ratio 1:5 with 7.5 mL/min helium split flow. The metabolites were separated with 1.5 mL constant flow helium with an oven ramp of 50 °C (2 min hold) to 180 °C (8 min hold) at 5 °C/min, to 210 °C at 2.5 °C/min, and to 325 °C (10 min hold) at 5 °C/min. The mass spectra were acquired in a scan range of 35–650 m/z at an acquisition rate of 5 spectra per sec. The ionization mode was subjected to electron impact, and the temperature for the ion source was set to 270 °C. The spectra were processed by Thermo Xcalibur software using automated peak detection, and the metabolites were identified by matching the mass spectra and retention indices of the NIST Mass spectral search program (version 2.0, Gaithersburg, MD). The metabolite data were then normalized based on the intensity of the fluoranthene internal standard.

### Safety evaluation of *Lactobacillus rhamnosus* IDCC 3201

#### Genome analysis

The VFDB database was searched for virulence genes [19], and ResFider software (ver. 3.2) with the CARD database was searched for antibiotic resistance genes [20]. The search parameters were set to the identity

**Table 2** Information of ceramide biosynthesis genes

Gene	Precursor	Enzyme	PFAM domain
<i>degs</i>	Dihydroceramide	Sphingolipid 4-desaturase	PF00487 (FA_desaturase)
<i>galc</i>	Galactosylceramide	Galactosylceramidase	PF02057 (Glyco_hydro_59)
<b><i>gba</i></b>	<b>Glucosylceramide</b>	<b>Glucosylceramidase</b>	<b>PF02055 (Glyco_hydro_30)</b>
<i>gba2</i>	Glucosylceramide	Non-lysosomal glucosylceramidase	PF12215 (Glyco_hydro_116N)
<i>sph</i>	Sphingomyelin	Sphingomyelin phosphodiesterase	PF03372 (Exo_endo_phos)
<i>smpd1</i>	Sphingomyelin	Sphingomyelin phosphodiesterase	PF00149 (Metallophos)
<i>smpd2</i>	Sphingomyelin	Sphingomyelin phosphodiesterase	PF03372 (Exo_endo_phos)
<i>smpd3</i>	Sphingomyelin	Sphingomyelin phosphodiesterase	PF03372 (Exo_endo_phos)
<i>smpd4</i>	Sphingomyelin	Sphingomyelin phosphodiesterase	PF14724 (mit_SMPDase)
<i>enpp7</i>	Sphingomyelin	Sphingomyelin phosphodiesterase	PF01663 (Phosphodiester)
<i>cers1</i>	Sphingosine	Sphingoid base N-stearoyltransferase	PF03798 (TRAM_LAG1_CLN8)
<i>cers2</i>	Sphingosine	Very-long-chain ceramide synthase	PF03798 (TRAM_LAG1_CLN8)
<i>cers3</i>	Sphingosine	Very-long-chain ceramide synthase	PF03798 (TRAM_LAG1_CLN8)

of >80% and coverage of >80% for gene identification. Transposases and transferases were annotated using the protein–protein basic local search program (BLASTP) against the NCBI GenBank proteins. Prophage regions were identified using PHASTER web-based program [21].

#### In vitro analysis

*Lactobacillus rhamnosus* IDCC 3201 was evaluated for susceptibility to antimicrobials including ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline, and chloramphenicol (Sigma-Aldrich, St. Louis, MO), which are typically used to treat enterococcal infections [22]. Briefly, a single colony from plate was inoculated to MRS broth and pre-incubated for 18 h. The cultured cells and antibiotic solution were mixed in 96-well plate to obtain the cell density of  $5 \times 10^5$  CFU/mL, and the plate was then incubated at 37 °C anaerobically for 18 h. The optical density was observed using a microplate reader (BioTek, Winooski, VT). The minimal inhibitory concentrations (MICs) were determined compared to the cut-off values suggested by European Food Safety Authority (EFSA) [23].

## Results and discussion

### Screening of lactic acid bacteria with higher sphingomyelinase (SMase) activity

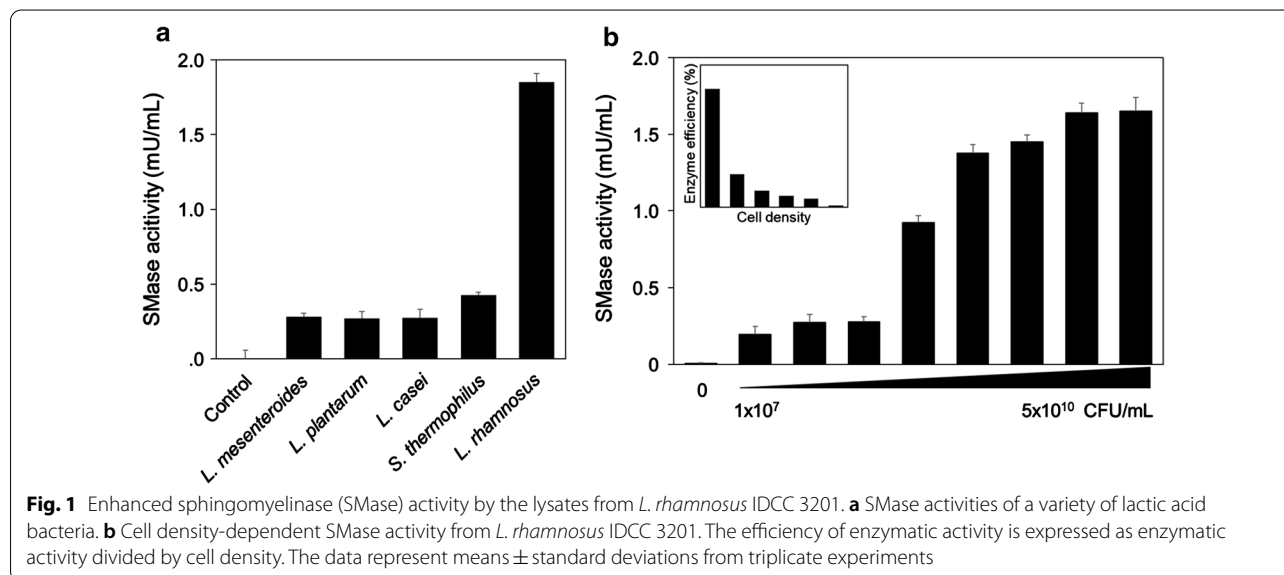
SMase is the key enzyme of sphingomyelin catabolism, converting into ceramides and phosphorylcholine [24]. Thus, decreased sphingomyelinase activity often leads to decreased ceramides level, resulting in skin troubles (e.g., atopic dermatitis) [25]. Here, various lactic acid bacteria were screened to obtain the strain(s), producing

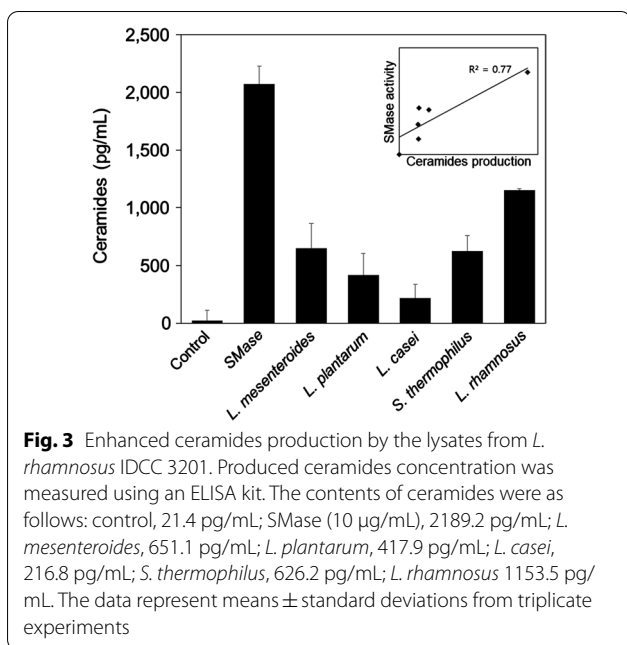
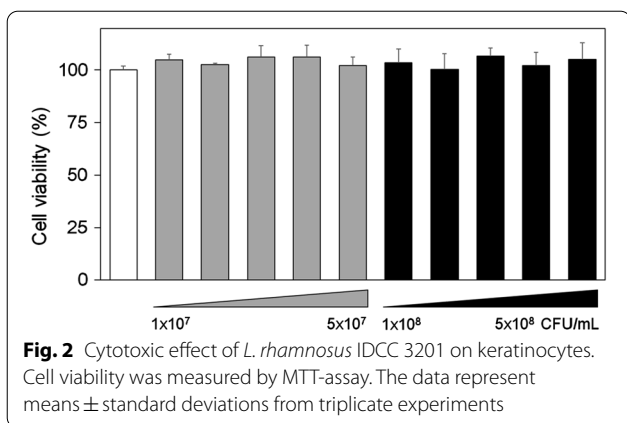
higher concentration of ceramides. For this, lysates from various lactic acid bacteria were prepared to observe whether the SMase increased. In particular, the lysates of tested strains used in this experiments were prepared from cell density at  $5 \times 10^7$  CFU/mL. As a result, *L. rhamnosus* IDCC 3201 exhibited highest SMase activity  $1.85 \pm 0.03$  mU/mL, while the other strains exhibited only at 0.28–0.43 mU/mL (Fig. 1a). The value of *L. rhamnosus* IDCC 3201 was 3.4 times higher than those of other strains, such as *L. mesenteroides*, *L. plantarum*, *L. casei*, and *S. thermophilus*. Consequently, *L. rhamnosus* IDCC 3201 strain was selected as the best performer with regard to SMase activity.

Next, optimal cell density of *L. rhamnosus* IDCC 3201 was evaluated to determine economically feasible concentration. The lysates from  $1 \times 10^7$  to  $5 \times 10^{10}$  CFU/mL were loaded onto the substrates. In results, SMase activity was observed to increase up to  $1.65 \pm 0.09$  mU/mL, as cell density increased (Fig. 1b). In contrast, in the view of efficiency of enzymatic activity (enzymatic unit/cell density), when cell density was the lowest as  $1 \times 10^7$  CFU, enzymatic efficiency of SMase was highest (Fig. 1b). Thus, cell amount of  $1 \times 10^7$  CFU/mL of *L. rhamnosus* IDCC 3201, showing the highest SMase efficiency was selected as optimal cell density for further study.

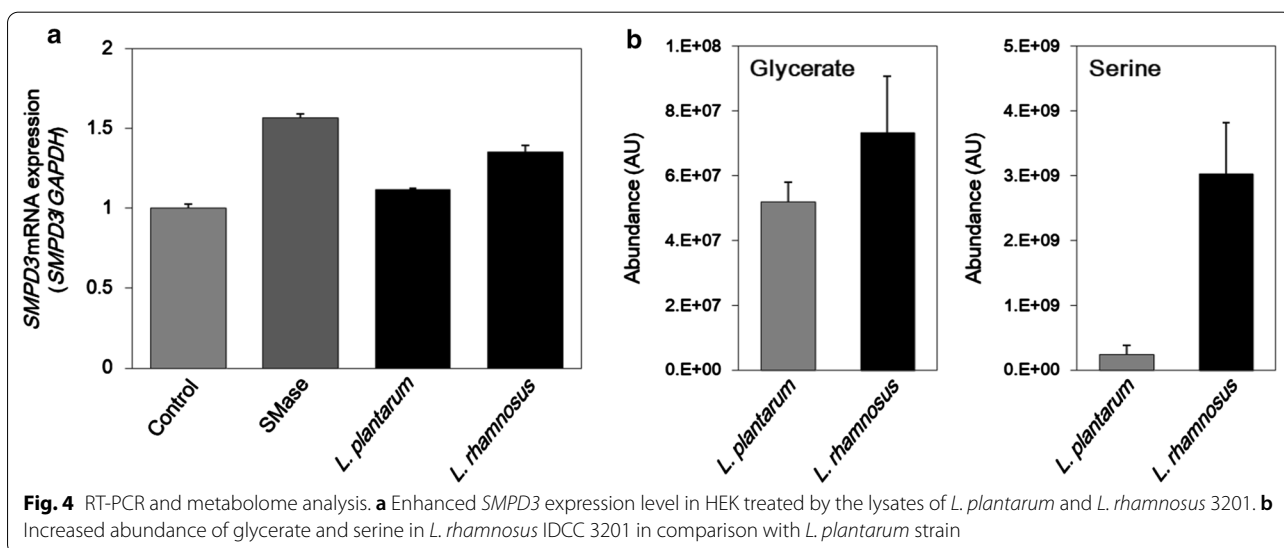
### Enhanced ceramides production by *L. rhamnosus* IDCC 3201

Ceramides, the major lipid constituents in the upper epidermal layer play a role in maintaining skin barrier integrity [26]. Thus, this layer prevents the skin from excess water loss and protects skin against various stimuli, including bacterial or microbial infection [8].





Here, ceramides production was investigated whether enhanced SMase activity by *L. rhamnosus* IDCC 3201 affects ceramides production in HEK cells. Prior to investigating the impact of lysates of *L. rhamnosus* IDCC 3201, cytotoxicity of HEK by the lysates was assessed. As a result, no significant reduction of cell proliferation was observed at cell density, ranging from  $1 \times 10^7$  to  $5 \times 10^8$  CFU/mL for 24 h (Fig. 2). Furthermore, to substantiate that increased SMase activity is responsible for enhanced ceramides production, lysates of *L. rhamnosus* IDCC 3201 and lactic acid bacteria tested in Fig. 1 were treated in HEK cells. Overall, increased ceramides production is correlated with increased SMase activity ( $R^2=0.77$ , Fig. 3) to some degree. Ceramides concentration by *L. rhamnosus* IDCC 3201 was 1.7–5.2 times higher than those by other lactic acid bacteria. More specifically,  $1153.5 \pm 334.5$  pg/mL of ceramides was measured in HEKs treated by *L. rhamnosus* IDCC 3201, while  $651.1 \pm 72.9$ ,  $417.9 \pm 55.8$ ,  $216.8 \pm 69.3$ , and  $626.2 \pm 216.5$  pg/mL of ceramides were measured in HEKs by *L. mesenteroides*, *L. plantarum*, *L. casei*, and *S. thermophilus*, respectively (Fig. 3). The observation that HEK cells treated with the lysates of *L. rhamnosus* showed higher production of ceramides, might be due to the induction of higher SMase activity (Fig. 1). To validate this notion, real-time PCR was performed to analyze the expression levels of *SMPD3*, *CERS1* and *CERS2* responsible for ceramides synthesis: *SMPD3* coding for sphingomyelin phosphodiesterase 3; *CERS1* for ceramide synthase 1; *CERS 2* for ceramide synthase 1. In results, the expression level of *SMPD3* was significantly increased in response to *L. rhamnosus* IDCC 3201, but not in *L. plantarum*. More specifically, each strain exhibited 35% and 11.4% increase of *SMPD3* expression, respectively



(Fig. 4a). Based on the results, it was concluded that comparative higher production of cermides by *L. rhamnosus* IDCC 3201 was due to the up-regulation of *SMPD3* rather than *CERS1* or *CERS2*. As a conclusion, it is expected that lysates of *L. rhamnosus* IDCC 3201 contribute to the improvement of the skin barrier through the increased level of ceramides.

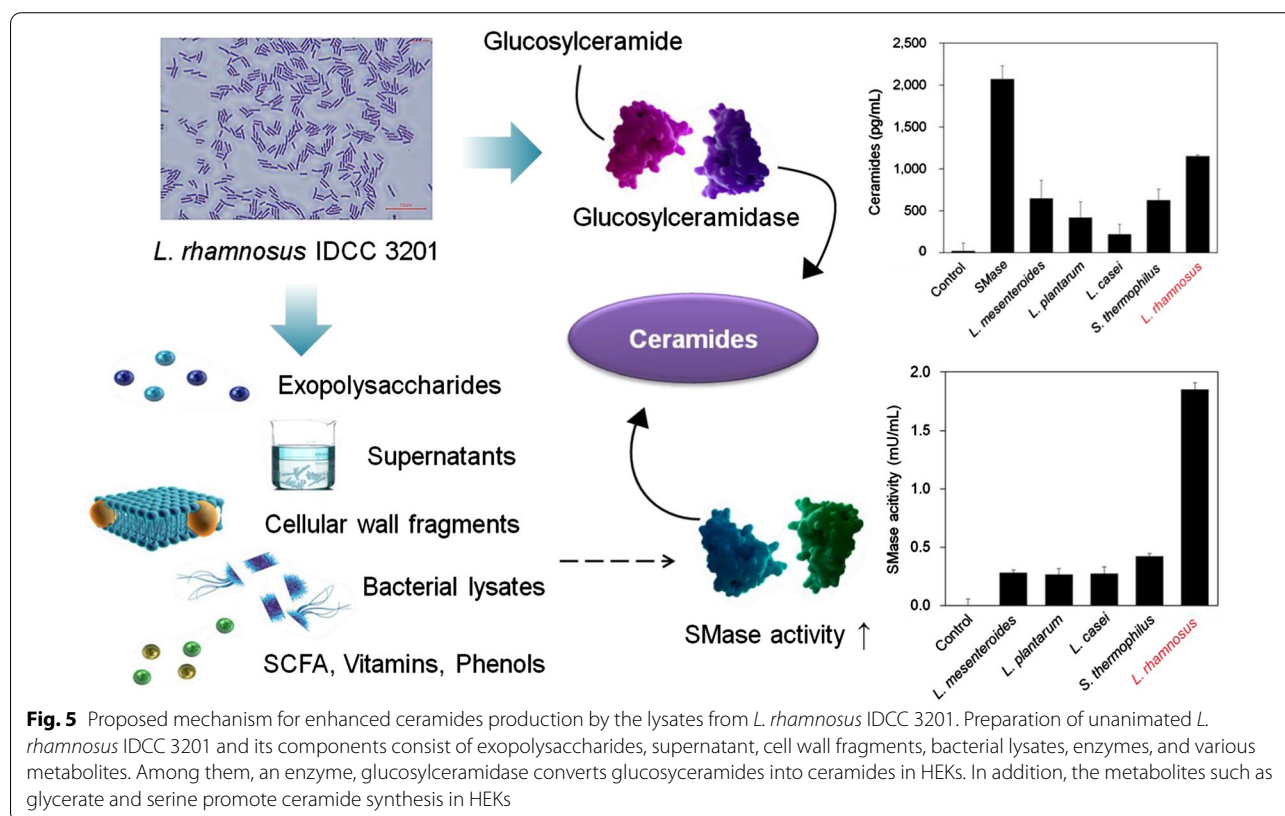
**Genome and metabolome analysis for elucidating a proposed mechanism**

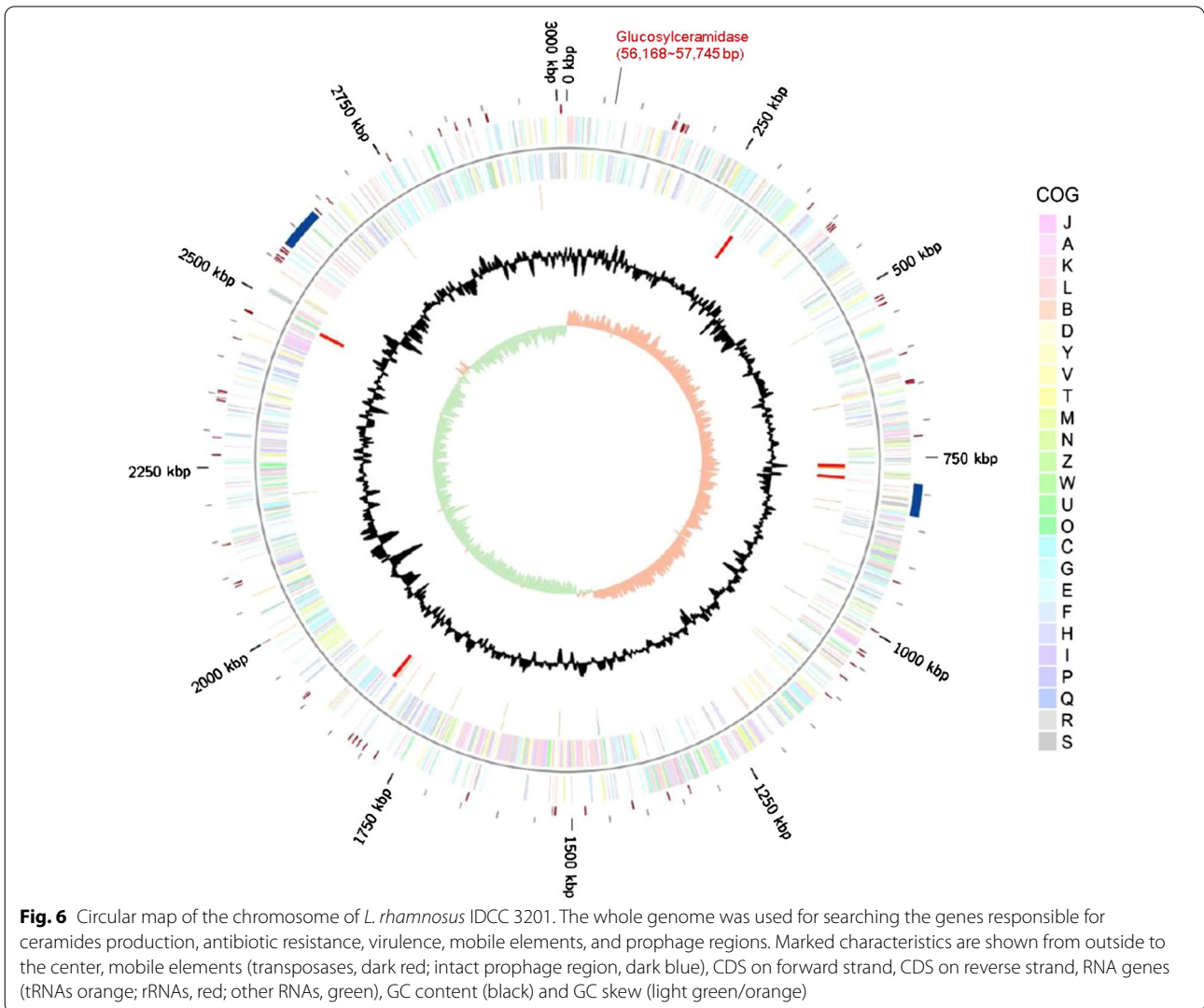
To elucidate a mechanism of enhanced ceramides production by *L. rhamnosus* IDCC 3201, information of enzymes that can synthesize ceramides in microorganisms was collected (Table 1). Among 13 genes, *L. rhamnosus* IDCC 3201 harbored *galc* and *gba*, which encodes for galactosylceramidase and glucosylceramidase, respectively. According to the gene annotation analysis using homology search, only *gba* gene was determined to be responsible for ceramides production from glucosylceramide, that is abundant in human keratinocytes [4]. Previous studies reported that the lack of glucosylceramidase encoded by the *gba* gene disturbed ceramides production and caused the failure of formation of competent skin barrier [9]. Thus, the increased ceramides in HEK cells was due to the expression of glucosylceramidase or

increased SMase activity in the lysates of *L. rhamnosus* IDCC 3201. In order to specify the possible effects of lysate components on the enhanced ceramides production, we analyzed the metabolites present in the culture supernatants of *L. rhamnosus* and *L. plantarum* using GC-TOF mass spectroscopy. As shown in Fig. 4b, we found that abundances of glycerate and serine increased in the *L. rhamnosus* culture compared to *L. plantarum*. The first step of de novo synthesis of ceramides begins with the condensation of palmitate and serine, producing 3-keto-dihydroshingosine. In this process, serine metabolism regulates ceramides and sphingolipid synthesis [27]. In addition, 3-phosphoglycerate, as one of the glycerate derivatives, is a precursor for the serine synthesis, supporting the notion that the increased glycerate and serine in the *L. rhamnosus* culture would induce the ceramides synthesis [28]. Finally, the reasons of enhanced ceramides production can be summarized by 1) indirect effect of components of the lysates (i.e., glycerate and serine) and 2) direct effect of glucosylceramidase on ceramides production (Fig. 5).

**Safety evaluation of *L. rhamnosus* IDCC 3201**

Genome analysis of *L. rhamnosus* IDCC 3201 indicated that the size of the genome was approximately 3.05 Mbps





with a GC content of 46.71% and 2,821 functional genes (Additional file 1: Tables S1 and S2) (Fig. 6). Furthermore, *L. rhamnosus* IDCC 3201 does not have any toxigenic

gene or virulence factor. According to the MIC tests, *L. rhamnosus* IDCC 3201 was susceptible to all of the antibiotics except for gentamycin and kanamycin (Table 3).

**Table 3** MICs of *L. rhamnosus* IDCC 3201 against a variety of antibiotics

	AMP	VAN	GEN	KAN	STR	ERY	CLI	TET	CHL
Cut-off value (µg/mL) <sup>a</sup>	4	n.r	16	64	32	1	4	8	4
<i>L. rhamnosus</i> IDCC 3201	1/S <sup>b</sup>	512–1024	32/R <sup>c</sup>	128/R	32/S	<0.125/S	<0.125/S	0.25/S	<0.125/S

n.r. not required

AMP ampicillin, VAN vancomycin, GEN gentamicin, KAN kanamycin, STR streptomycin, ERY erythromycin, CLI clindamycin, TET tetracycline, CHL chloramphenicol

<sup>a</sup> EFSA (European Food Safety Authority), 2018. EFSA Journal, 16(3), 5206

<sup>b</sup> S: Susceptible

<sup>c</sup> R: Resistant

These resistances should be intrinsic and not be transferable to skin microbes due to the absence of antibiotic resistance gene. Typically, many *Lactobacillus* species are shown to be tolerant to aminoglycoside antibiotics intrinsically due to the absence of cytochrome-mediated transport [29, 30]. In conclusion, *L. rhamnosus* IDCC 3201 strain is regarded as safe for its use as cosmetic materials.

#### Abbreviations

GCase: Glucosylceramidase; SMase: Sphingomyelinase; AD: Atopic dermatitis; MRS: De Man, Rogosa and Sharpe; CFU: Colony forming unit; PBS: Phosphate buffered solution; HEK: Human epidermal keratinocyte; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO: Dimethyl sulfoxide; ELISA: Enzyme-linked immunosorbent assay; MIC: Minimal inhibitory concentration; EFSA: European food safety authority; mU: Milli unit; Mbp: Mega base pair.

#### Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13765-021-00620-7>.

**Additional file 1: Table S1.** Statistical parameters of genome of *L. rhamnosus* IDCC 3201. **Table S2.** Functional genes of *L. rhamnosus* IDCC 3201.

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#### Authors' contributions

JY designed the research, supervised the project, and finalized the manuscript. MSK, ML, HO, GK, OB, and MS performed the experiments and analyzed the data. ML, MS and YHJ generated and provided analytical tools. YHJ aided in interpreting the results and provided critical revision. All authors read and approved the final manuscript.

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#### Availability of data and materials

Supplementary material is followed.

#### Declarations

#### Competing interests

The authors have no financial conflicts of interest to declare.

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