# Novel probiotic lactic acid bacteria isolated from fermented foods : Kimchi and Sauerkraut in the treatment of skin aging

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## Introduction:

The importance of research on skin barrier enhancement related to skin immunity through changes in the external environment of the COVID-19, and probiotics has proven skin immunity from viruses through outside – inside theories related to skin immunity barriers through various papers. Therefore, the demand for the development of probiotics materials is increasing. Dr. Jean Bousquet, honorary professor of Pulmonary Medicine at Montpellier University, France, and his team study of the correlation between the number of COVID-19 deaths and regional dietary differences found that fewer deaths were found in countries based on fermented cabbage.(*For foreign countries, Germany, which eats sauerkraut made from pickled cabbage, has fewer deaths than other countries (France, Italy). There are a lot of reports that eating fermented cabbage reduces the chances of COVID-19 penetrating the human body. Consuming high levels of fermented cabbage brings down the number of ACE2, making it more difficult for the virus to enter the body. High in antioxidants, fermented cabbage is also good for boosting immunity. Especially fermented cabbage has high in antioxidants, it also good for boosting immunity [1]. The Kimchi Starters developed in this study is a lactic acid bacteria (LAB) that alone leads to more than 80% of kimchi fermentation in a harsh environment where mixed fermentation is carried out by more than 200 kinds of early microorganisms derived from raw materials. Sauerkraut, also called German kimchi, means sour cabbage, which is a food that marinates various cabbages with salt and then matured by lactobacillus. Through this study, lactic acid bacteria (LAB) that can lead microbial succession can be secured during the fermentation process and can be used as high functional anti-aging materials that can cope with skin aging. Plant origin lactic acid bacteria (PLAB) are lactobacillus isolated from fermented foods of plant raw materials, such as pickles and kimchi, and are more diverse and more adaptable to the external environment than Animal origin lactic acid bacteria (ALAB). In particular, plant origin lactic acid bacteria (PLAB) separated from kimchi are known to be highly productive of various physiological active substances because they grow in a harsh environment. Accordingly, the development of cosmetics materials using lactobacillus culture products separated from kimchi and killed lactic acid bacteria is actively carried out. Kimchi is a food fermented by lactobacillus, and the lactobacillus separated from kimchi is Leuconostoc mesenteroides, Leuconostoc dextranicum, Lactobacillus brevis, Lactobacillus plantarum, Pediococcus pentosacues. In this study explain to the development of lactobacillus materials that are safe for skin that can comprehensively provide functions such as anti-oxidative, anti-inflammatory, moisturizing, wrinkle improvement, whitening, etc. Fermented foods have been widely used for the human health. Ethnic and cultural diversity contributed to production of various types of typical lactic acid-fermented foods such as kimchi, sauerkraut, cheese, yogurt etc. A number of studies reported that metabolites and cell wall components of lactic acid bacteria (LAB) have been associated with beneficial health effects [2,3,4,5]. The effects of LAB could be used for healthy skin as well as healthy gut with prebiotics, probiotics and postbiotics, etc. This study aimed to find novel probiotic lactic acid bacteria (LAB) from fermented foods – Kimchi and Sauerkraut in the treatment of skin aging.

## Materials & Methods:

1. Isolation of Microorganisms : Lactic acid bacterial strains were isolated from commercial Kimchi were collected from different regions in Korea. To prepare Sauerkraut, fresh ingredients including cabbage, red cabbage, brussels sprouts and salt were purchased from a grocery store in Jeju, Korea, 2020. Three types of Sauerkraut were made in the laboratory. The Kmchi and Sauerkraut samples diluted with 0.85% NaCl solution were spread on MRS agar (Difco, Detroit, MI, USA). Selected isolates were identified first by using an genomic DNA extraction kit (iNtRon Biotechnology, Korea). Screened strains were identified by 16S rRNA gene sequencing which was amplified by PCR. PCR was performed using the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGCTACCTTGTTACGACTT-3′). PCR conditions were 35 cycles at 95°C for 1 min, 45°C for 1 min, and 72°C for 1.5 min. PCR mplified products were confirmed by performing agarose gel electrophoresis, and nucleotide sequences were determined at Macrogen Co. in Seoul, Korea. The BLAST program was used to identify homologous 16S rRNA gene sequences in the database.
2. Antimicrobial Activity : The pathogenic bacteria used as indicators were Propionibacterium acnes, Staphylococcus aureus Staphylococcus. epidermidis and Staphylococcus. capatis. S.aureus S. epidermidis and S. capitis were cultured for 24 h. P. acnes was cultured for 48h. The concentration was adjusted to 107 CFU/ml, plated to MRS agar, and dried. 10x concentrated cell-free supernatants was loading 10μl the plate smeared with pathogenic bacteria. This was incubated at 37°C for 48 h and checked for the appearance of a clear inhibition zone. Each assay was performed in triplicate.
3. Lyophilization Medium : After the antibacterial experiment, the medium of the selected three strains (WiKim 125, 126 and 127) were obtained by freeze-drying for 48 h (Eyela FUD-1100, Tokyo Rikakikai Co., Tokyo, Japan), and then the experiment was performed.
4. ABTS Radical Scavenging Activity : ABTS radical scavenging activity was evaluated according to the method of Gorinstein et al [6]. A mixture (1:1) of 7.0 mM ABTS and 2.45 mM potassium persulphate was prepared and stored in dark conditions for 16 h to get the green–blue free radical ABTS. Then the solution was diluted with ethanol until the absorbance was 0.7±0.02 at 734 nm. 0.5 ml of lyophilization medium samples (WiKim 125, 126 and 127) were mixed with 3.0ml of ABTS working solution. After 10 min of reaction, the absorbance was taken at 734 nm. The ABTS radical scavenging activity was calculated as the following equation; * ABTS radical scavenging effect (%) =(1-As/Ac)×100 where As is the absorbance in the presence of sample, and Ac is the absorbance of control reaction.
5. NO Scavenging Activity : Different concentrations of 1ml of lyophilization medium samples were incubated with 2ml of 10 mM sodium nitroferricyanide (III) dihydrate at 25℃ for 150 min. 0.5ml of the reaction mixture was then treated with 1ml of 1% sulfanilamide (dissolved in 30% acetic acid) for 10 min and further incubated with 2ml of 0.1% N-(naphthyl)ethylenediamine dihydrochloride (dissolved in 60% acetic acid) at 25℃ for 30 min. The absorbance was measured at 540 ㎚ using a spectrophotometer. The NO radical scavenging activity was calculated as the following equation;
* NO radical scavenging effect (%) =(1-As/Ac)×100 where As is the absorbance in the presence of sample, and Ac is the absorbance of control reaction.
6. Collagenase Inhibitory Assay : Collagenase inhibitory activity was determined by the method of Dongwook K. el al (2014) with slight modification [7]. For the reaction, 4 mM CaCl2 was added to 0.1. M Tris-HCl buffer (pH 7.5) and 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-Arg (0.3 mg/ml). Next, 0.1 ml of lyophilization medium samples were mixed with 0.25 mL. 0.15 ml of collagenase (0.2 mg/ml) was added and incubated for 20 minutes at room temperature. 0.5 ml of 6% citric acid was added to stop the reaction then 2.5 ml ethylacetate was added. The absorbance was measured at 320㎚ using a spectrophotometer. Collagenase inhibition activity was determined using the following equation.
* Collagenase inhibition activity (%) =(1-As/Ac)×100 where As is the absorbance in the presence of sample, and Ac is the absorbance of control reaction.
7. Tyrosinase Inhibitory Activity : Tyrosinase inhibitory activity was determined according to the method of Chang et al. (2005) with a slight modification [8]. 2.3 ml of 10 mM substrate (L-DOPA dissolved in 50 mM phosphate buffer, pH 6.8) was mixed with 0.2 ml of the tested samples. 0.5 ml of tyrosinase (110 Unit/ml) was added to initiate the reaction. at 25 ºC for 10 min. The increase in absorbance at 475 nm due to the formation of dopachrome was monitored with a spectrophotometer. The percentage inhibition of tyrosinase activity was expressed as follows.
* Tyrosinase inhibition activity (%) =(1-As/Ac)×100 where As is the absorbance in the presence of sample, and Ac is the absorbance of control reaction.
8. Hyaluronidase Inhibitory Activity : In the ase hyaluronidase inhibition experiment, as the samples are treated, the substrates sodium hyaluronic acid (HA) and ρ-dimethylaminobenzaldehyde react and affect the activity of hyaluronidase to change the color of the reactant. Briefly, 0.1 ml hyaluronidase solution (10mg/ml) dissolved in 0.1 M acetate buffer (pH 3.5) and 0.1 ml of samples were mixed and incubated for 20 min at 37 °C. Then, HA (6 mg/ml) dissolved in 0.25 ml of 0.1 M acetate buffer (pH 3.5) was added as substrate. Next, 0.1 ml of 0.4 N potassium tetraborate and 0.1 ml of 0.4 N sodium hydroxide solution was added to the reacted mixture, boiled in a water bath. After cooling, 2.5 ml DMAB solution, was added to the cooled mixture and incubated for 20 min at 37 °C. The absorbance was measured at 540㎚ using a spectrophotometer.
* Hyaluronidase inhibition activity (%) =(1-As/Ac)×100 where As is the absorbance in the presence of sample, and Ac is the absorbance of control reaction.
9. Cell Culture
Hs68 human skin fibroblasts and Human epidermal keratinocyte (HaCaT) cells obtained from the ATCC(American Type Culture Collection, USA). B16F10 murine melanoma cells and RAW 264.7 cells were supplied by the Korean Cell Line Bank. All cells were maintained in DMEM supplemented with 10% Fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37˚C in a 5% CO2 incubator.
9-1. Melanin contents : Melanin content in B16F10 at 4 × 104 cells/well were seeded in 6-well plates and incubated for 24 h. After that, the cells were treated with new medium containing 200 nM α-MSH and lyophilization medium samples at various concentrations and incubated for 72 h. Cell pellets were dissolved with 1N NaOH, and kept in a 80 °C for 1 h. The melanin content was measured at 405 nm using an ELISA ((Molecular Device, USA) reader.
9-2. Analysis of Inhibition of NO Production.
RAW 264.7 cells at 5 ×104 cells/well were seeded in 96-well plates and incubated for 24 h at 37°C in a humidified atmosphere 5% CO2. The cells were incubated in a serum-free medium containing the sample and 1 μg/ml LPS. After incubation for an additional 24 h, the NO content was evaluated by using the Griess assay. The 100 ml supernatant was obtained, Griess was added 100 ml and the absorbance at 540 nm was recorded. The inhibition of NO production was calculated from equation. NO production inhibition(%)= (sample O.D/control O.D) × 100
9-3. Real-time PCR for MMP-1
Hs68 human skin fibroblasts cells at 5 ×105 cells/well were seeded in 60mm plates and incubated for 24 h at 37°C in a humidified atmosphere 5% CO2. The Hs68 cells were pretreatment with lyophilization medium samples for 45 min prior to the induction with TNF-α (20 ng/mL) for 24 h. Then the cell supernatant was discarded and washed with PBS. Total RNA was extracted from cell lysates using TRIzol reagent (Ambion, U.S.A.), and cDNA was prepared using a Revertra ACE-α- (Toyobo, Japan), according to the manufacturer's instructions. Quantification of mRNA by qRT-PCR was carried out using Taqman RT-PCR Master Mix. The cDNA was amplified by using the following primers are MMP-1 (Hs00899658_m1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs02786624_g1).
9-4. Real-time PCR for HAS-2
HaCaT cells at 6 ×105 cells/well were seeded in 60mm plates and incubated for 24 h at 37°C in a humidified atmosphere 5% CO2. Cells were treatment with lyophilization medium samples for 24 h. Then the cell supernatant was discarded and washed with PBS. Total RNA was extracted from cell lysates using TRIzol reagent (Ambion, U.S.A.), and cDNA was prepared using a Revertra ACE-α- (Toyobo, Japan), according to the manufacturer's instructions. Quantification of mRNA by qRT-PCR was carried out using Taqman RT-PCR Master Mix. The cDNA was amplified by using the following primers are HAS-2 (Hs00193435_m1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs02786624_g1).
9-5. Real-time PCR for iNOS
RAW 264.7 cells at 5 ×105 cells/well were seeded in 6 well plate and incubated for 24 h at 37°C in a humidified atmosphere 5% CO2. Cells were treatment with lyophilization medium samples for 24 h. Then the cell supernatant was discarded and washed with PBS. Total RNA was extracted from cell lysates using TRIzol reagent (Ambion, U.S.A.), and cDNA was prepared using a Revertra ACE-α- (Toyobo, Japan), according to the manufacturer's instructions. Quantification of mRNA by qRT-PCR was carried out using Taqman RT-PCR Master Mix. The cDNA was amplified by using the following primers are iNOS (Mm00440502_m1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Mm99999915-g1).

## Results & Discussion:

### 1. Development of Novel Probiotic Lactic Acid Bacteria


**Figure 1.** Novel Probiotics isolated from Kimchi & Sauerkraut.

### 2. Strains isolated from Kimchi and Sauerkraut

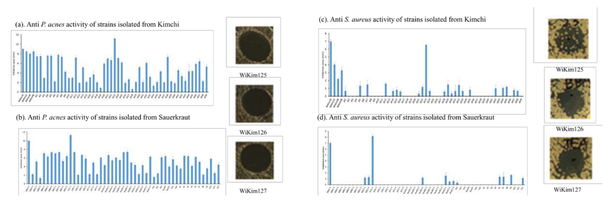


**Figure 2.** Anti *Propionibacterium acnes* and *Staphylococcus aureus* activity of 53 strains isolated from Kimchi and 51 Strains isolated from Sauerkraut.

### 3. Anti *S. epidermidis* and *S. capitis* activity

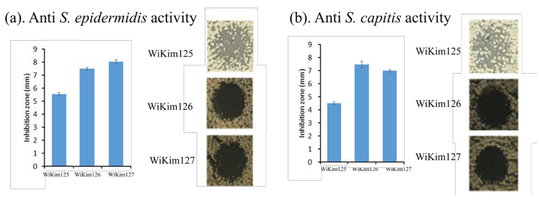


**Figure 3.** Anti *S. epidermidis* and *S. capitis* activity of Wikim 125, 126 and 127

### 4. The Result of *in vitro* screening

Table 1. *in vitro* activity of WiKim 125, 126, 127 and Novel Probiotics

| Strains | ABTS radical scavenging activity (%) | NO radical scavenging activity (%) | Collagenase inhibitory activity (%) | Tyrosinase inhibitory activity (%) | Hyaluronidase Inhibitory activity (%) |
|---|---|---|---|---|---|
| Wikim125 (1mg/ml) | 35.06 ± 0.29 | 40.61 ± 3.69 | 22.16 ± 2.03 | 39.23 ± 1.64 | 43.76 ± 4.47 |
| Wikim126 (1mg/ml) | 50.69 ± 1.25 | 24.44 ± 3.34 | 2.59 ± 1.52 | 6.67 ± 2.38 | 41.14 ± 4.61 |
| Wikim127 (1mg/ml) | 40.03 ± 0.33 | 35.15 ± 2.78 | 14.39 ± 1.01 | 34.95 ± 1.49 | 31.95 ± 2.73 |
| Novel Probiotics (1mg/ml) | 55.64 ± 0.75 | 69.33 ± 1.49 | 31.43 ± 1.96 | 33.47 ± 1.90 | 48.11 ± 3.61 |

### 5. The Result of MMP-1 expression

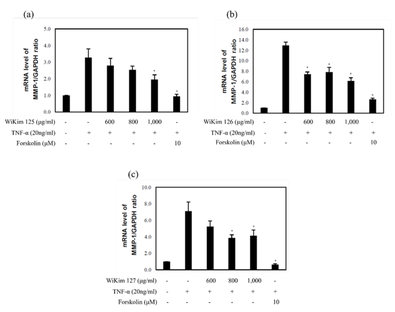


**Figure 4.** Inhibition effects of MMP1 mRNA expression of induced TNF-α in Hs68 cells. Forskolin was positive control. *p<0.05 compared with the control.

### 6. The Result of melanin content

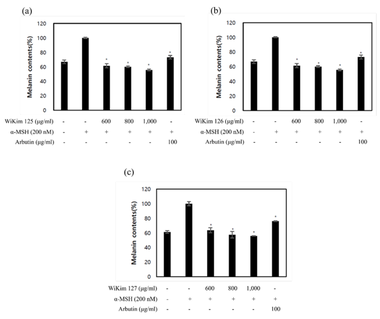


**Figure 5.** Inhibition effects of melanin contents in B16F10 cells. Arbutin was positive control. *p<0.05 compared with the control.

### 7. The Result of HAS-2

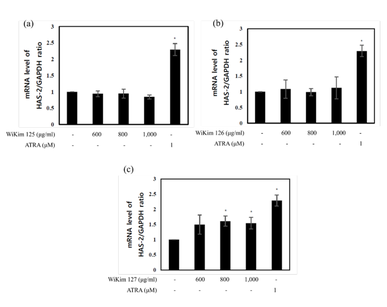


**Figure 6.** Effects of HAS-2 expression in B16F10 cells. ATRA was positive control. *p<0.05 compared with the control.

### 8. The Result of NO production

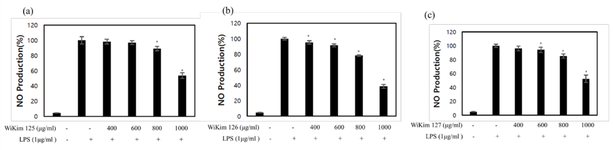


**Figure 7.** Inhibition effects of NO production induced LPS in Raw 264.7 cells.

### 9. The Result of iNOS mRNA

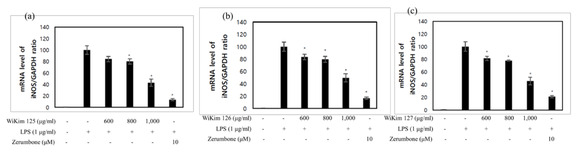


**Figure 8.** Inhibition effects of iNOS mRNA expression induced LPS in Raw 264.7 cells.

## Conclusions:

The skin is the largest organ of the body, which protects our body from the harmful environment and helps to regulate our body temperature. The skin is composed of three layers, epidermis, dermis, and the hypodermis. The outermost layer of the skin, the epidermis, is a multi-layered epithelial tissue, and the barrier function of the skin is mainly provided by the stratum corneum. The stratum corneum serves as a selective permeability barrier against infectious or toxic substances [9]. The stratum corneum is composed of keratinocytes called corneocytes, that are attached to each other by corneodesmosomes and lamellar lipids. The structural organization of this barrier is also referred as a 'brick and mortar' structure with the keratin microfibrils, filaggrin and cornified envelopes forming the bricks and the lipids forming the mortar to seal together the cornified envelopes [10]. Also tight-junctions formed by desmosomes and proteins like loricrin or filaggrin contribute to the protective skin barrier [11]. Although the barrier is self-maintaining and self-renewing, skin barrier dysfunction commonly occurs in inflammatory skin disorders such as atopic dermatitis and psoriasis [11]. Disruption of the skin barrier causes the collapse of moisture balance in the epidermis, allowing the penetration of allergens or chemicals into the deeper layers of the skin, delaying remission or aggravating the diseases [12,13]. Probiotics are live microorganisms used for various health benefits on the host. Traditionally, most probiotics are developed as foods or dietary supplements, targeting benefits on the intestine [14]. However, recent studies have shown that probiotics can have favorable on heart, liver, lung, and even on mental health [15] as well. There is also escalating interest in the topical application of probiotics for skin health. Bacterial strains that are adherent to the human skin have been studied to identify probiotics [16]. Currently, consumers demand multi-functional cosmetics that go beyond fragmentary functions to provide complex anti-aging effects like antioxidant, anti-inflammatory, moisturizing, wrinkle improvement, whitening improvement, etc. As a result, it is necessary to provide multi-functional cosmetics that meet consumer needs through the development of lactic acid bacteria materials that are safe for the skin. This study completed the invention by discovering that the culture of Lactobacillus Plantarum Wikim 125 (BP1910798), Lactobacillus Plantarum Wikim 126 (BP1910799), Lactobacillus Plantarum Wikim 127 (BP1910800), separated from kimchi, and its killed lactic acid bacteria, are skin-friendly, safe, and naturally derived materials that can improve skin function, promoting skin aging prevention and improvement through anti-oxidation, anti-inflammatory, moisturizing, skin tone improvement, elasticity and wrinkles improvement. The 3 LABs isolated from Kimchi, and Sauerkraut in this study may be used as probiotics, with isolate Wikim 125, 126, 127 (Lactobacillus spp.) as the most promising novel probiotics for anti-aging applications based on its excellent cell activity. Through this study, a cosmetic composition for anti-aging or improvement can be provided that contains one or more types of killed lactic acid bacteria and one or more types of lactic acid bacteria selected from the group composed of Lactobacillus Plantarum Wikim 125 (BP1910798), Lactobacillus Plantarum Wikim 126 (BP1910799), Lactobacillus Plantarum Wikim 127 (BP1910800). In this study, natural-derived lactic acid bacteria, which is skin-friendly, safe, and improves skin function, and culture using these lactic acid bacteria can be used to promote skin aging prevention and improvement through antioxidant, inflammatory, moisturizing, skin tone, elasticity and wrinkles.

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