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Article

Development and Evaluation of Bioconverted Milk with Anti-Microbial Effect Against Oral Pathogens and α-Glucosidase Inhibitory Activity

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Abstract: To decrease oral pathogens and increase the number of beneficial bacteria, probiotics and bioactive compounds made via microbial bioconversion are recently used. In addition, the interest regarding probiotics-mediated bioconversion with popular medicinal plants is increasing. These compounds produced during the process exhibit synergistic effect and enhance a variety of biological activities including pathogen inhibition in host. Therefore, we developed bioconverted milk with high inhibitory activity against oral pathogens and α-glucosidase. *Lactiplantibacillus plantarum* SMFM2016-RK was chosen as the probiotic due to its beneficial characteristics such as high acid and bile tolerance, antioxidant activity, and α-glucosidase inhibition. Based on the minimal bactericidal concentration against three oral pathogens, the following appropriate concentrations of *Artemisia herba-alba* extract were added to milk: 5 mg/mL of *A. herba-alba* ethanol extract and 25 mg/mL of *A. herba-alba* hot-water extract. Four bioconverted milks (BM), BM1, BM2, BM3, and BM4, were produced by combining *L. plantarum* SMFM2016-RK alone, *L. plantarum* SMFM2016-RK and ethanol extract, *L. plantarum* SMFM2016-RK and hot-water extract, and *L. plantarum* SMFM2016-RK with both extracts. As a result of antimicrobial activity, BM3 inhibited the growth of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* the most, and BM4 suppressed the growth of *Fusobacterium nucleatum* the most. In addition, bioconverted milk containing *A. herba-alba* (BM2, BM3, and BM4) inhibited α-glucosidase more effectively than BM1. In conclusion, *A. herba-alba*-added milk bioconverted by *L. plantarum* SMFM2016-RK displayed the growth inhibitory effect on oral pathogens and the α-glucosidase inhibitory activity, thus it necessitates to evaluate the effects on the alleviation of oral diseases and glycemic control through future animal experiments.

Keywords: bioconverted milk; probiotics; *Artemisia herba-alba;* oral pathogens; α-glucosidase

1. Introduction

Oral disease is a major global health concern that affects a large portion of the world's population. According to a report by the World Health Organization (WHO), almost half of the world's population suffer from oral diseases [1]. Furthermore, oral infections and associated inflammatory responses can have a negative impact on blood glucose levels, and previous study reported that more than 90% of diabetic patients experienced oral complications [2,3]. The α-glucosidase enzyme affects blood glucose levels in company with oral infections. It plays a major role in raising blood sugar, breaks down complex carbohydrates into simple sugars and then absorbs them into the bloodstream. Therefore, it is known that inhibiting the α-glucosidase enzyme helps to prevent glucose levels from rising rapidly by allowing glucose to be gradually absorbed into the bloodstream [4]. Thus, many researchers have made an effort to identify potent α-glucosidase inhibitor [5–8]. The treatment and

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prevention of periodontitis involve various medications and therapeutic approaches. Some of the commonly used medications include antibiotics, antiseptics, and carbamide peroxide topical [9]. However, these treatments have certain limitations such as antibiotic resistance [9]. Moreover, antibiotic resistance genes are often exchanged among bacteria within these biofilms, further complicating treatment [9]. Due to the increase in antibiotic resistance, the focus of periodontitis treatment is on restoring the balance between the oral microbiota and the host's periodontal tissue [9]. For this reason, probiotics markets targeting oral health is experiencing significant growth and development. The market size for probiotics specifically for oral health was valued at USD 2.5 billion in 2022 [10]. This growth is driven by research into specific probiotic strains, such as *Lactobacillus* and *Bifidobacterium* species, which have shown potential benefits in reducing the risk of dental caries, gum disease, and bad breath [11]. Furthermore, various new quality-related components such as phenolic acids, flavones and their glycosides, alkaloids, and terpenoids have been identified due to changes in chemical composition during microbial bioconversion with plants in recent years [12]. Microbial bioconversion showed a synergistic effect compared to simple application of lactic acid bacteria (LAB), such as increasing the fermentation ability due to soybean protein and enhancing the inflammatory bowel disease-alleviating effect by boosting the bioactivity of anthocyanin [13,14].

Artemisia herba-alba, commonly known as desert or white wormwood, has been recognized for its potential medicinal properties [15]. Studies on this plant have identified various beneficial compounds such as herbalbin, *cis*-chryanthenyl acetate, flavonoids (hispidulin and cirsilineol), monoterpenes, and sesquiterpene [15]. It is used in folk medicine for treating a range of diseases [15]. Because of a very low level of toxicity, the aerial portions of *A. herba-alba* are appropriate for various uses [15]. This plant has shown promise in pharmacological and toxicological properties, but further studies are needed to integrate it more effectively into the healthcare system [15]. Therefore, this study aimed to select LAB as a probiotic strain and develop bioconversion products with *A. herba-alba* by the selected LAB that alleviate oral disease by inhibiting the growth of oral pathogenic bacteria and enhance glycemic control by impeding the α-glucosidase activity.

2. Materials and Methods

2.1. Selection of Probiotic Candidate Strain

2.1.1. Preparation of Lactic Acid Bacteria Isolates

Seventy-four LAB isolates obtained from 106 kimchi samples [16] were screened to identify a candidate probiotic strain. The isolates were cultured in 10 mL Lactobacilli MRS broth (Becton, Dickinson and Company, Franklin, NJ, USA) and incubated at 35°C for 24 h. The cultures were centrifuged at 1,912×g (S750–4B swing rotor, Combi 514R, Hanil Science Inc., Gyeonggido, Korea) and 4°C for 15 min and washed twice with 10 mL phosphate buffered saline (PBS; pH 7.4; 0.2 g KCl, 0.2 g KH_2PO_4, 8.0 g NaCl, and 1.5 g $Na_2HPO_4{\cdot}7H_2O$ in 1 L distilled water).

2.1.2. Hemolytic Analysis

Hemolytic activity was analyzed by streaking fresh cultures on Columbia agar (BioMerieux, Marcy l'Etoile, Lyon, France), containing 5% sheep blood (w/v), and incubating them at 35°C for 48 h. Blood agar plates were examined for signs of α-hemolysis (green-hued zones around colonies), β-hemolysis (clear zones around colonies), and γ-hemolysis (no zone around colonies).

2.1.3. Analysis of β-Glucosidase and β-Glucuronidase Activities

β-glucosidase and β-glucuronidase enzymatic activities were evaluated using the API ZYM test kit (BioMerieux) according to the manufacturer's instructions. The LAB isolates were diluted until their OD_{500} reached 1.048; 65 μL of the aliquots was then inoculated into the API ZYM test kit wells and incubated at 35°C for 4 h. The results were graded from 0 (no activity) to 5 (>40 nanomoles) by comparing color intensity. Results with grades >2 were considered positive.

2.1.4. Analysis of Acid and Bile Salt Tolerance

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For acid tolerance, 500 μL of washed bacterial pellets were inoculated in 500 μL Lactobacilli MRS broth adjusted to pH 2.5 and then incubated at 35°C. Bacterial cells were counted after 0 and 3 h of exposure to acidic conditions. Aliquots of 100 μL were serially diluted and spread onto Lactobacilli MRS agar plates (Becton, Dickinson and Company). The bacterial colony-forming units were counted after 24 h of incubation at 35°C. The results of acid tolerance were compared to those of *Lacticaseibacillus rhamnosus* GG (LGG, ATCC53103). To evaluate bile salt tolerance, 500 μL culture was inoculated in 500 μL Lactobacilli MRS broth, containing 3% oxgall (Becton, Dickinson and Company), in 96-well microplates (SPL Life Sciences, Pocheon-si, Korea) and incubated at 35°C. After 24 h of incubation, 100 μL aliquots were serially diluted and plated on Lactobacilli MRS agar. The bacterial cells were counted after 24 h of incubation at 35°C. These results were compared to those of LGG.

2.1.5. Determination of ABTS-Scavenging Activity

2.6. mM potassium persulfate and 7.4 mM 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Sigma-Aldrich, St. Louis, MO, USA) solutions were mixed in a 1:1 ratio. The mixture was placed in the dark for 20 h until it became blue-green owing to radical formation. The mixture was diluted until its OD_{734nm} reached 0.7±0.02. Approximately 500 μL of the isolates (9 Log CFU/mL) was added to the solution (500 μL) and incubated in the dark at 37°C for 30 min. The absorbance of the samples was measured at 734 nm using a microplate spectrophotometer (BioTeck Instruments Inc., Winooski, VT, USA). The ABTS-scavenging activity of the isolates was calculated as follows; ABTS radical scavenging (%) = {(OD_{734nm} of control-OD734nm of sample)/OD_{734nm} of control}×100.

2.2. Preparation of Artemisia herba-alba Extracts

Hot-water and ethanol were used to extract the hydrophilic and hydrophobic compounds from the dried *A. herba-alba* leaves (Bedel food, Incheon, Korea), respectively. The hydrophobic compounds were extracted from 100 g dried *A. herba-alba* in 1 L of 95% ethanol (Samchun, Gyeonggido, Korea) at 60°C for 24 h. The hydrophilic compounds were extracted from 100 g dried *A. herba-alba* in 1 L of distilled water at 90°C for 1 h. The extracts were filtered with Advantec NO. 1 filter paper (Advantec Toyo Kaisha Ltd., Taito-ku, Tokyo, Japan), and the filtrates were concentrated through a rotary evaporation (Laborota 4001 WB, Viertrieb, Germany) under vacuum at 80°C. The concentrates were frozen at -80°C for 24 h, lyophilized at -80°C for 3 days using a freeze dryer (FDB-5503, Operon Co Ltd., Gyeonggi-do, Korea), and the powder was collected.

2.2.1. Minimum Bactericidal Concentration of Artemisia herba-alba Extracts against Periodontal Pathogens

To select the optimal concentrations of the two *A. herba-alba* extracts, the minimum bactericidal concentration (MBC) was analyzed. The standard broth dilution method (CLSI M07-A8) was used to determine the antimicrobial efficacy of *A. herba-alba* extracts by evaluating the growth of the following periodontal microbial pathogens: A*ggregatibacter actinomycetemcomitans* ATCC43718, *Fusobacterium nucleatum* ATCC10953, and *Porphyromonas gingivalis* ATCC33277. The strains were cultured in 10 mL Wilkins Chalgren Anaerobe Broth (Oxoid, Basingstoke, Hampshire, UK) and incubated at 35°C for 48 h in a 90% N_2, 5% CO_2, and 5% H_2 atmosphere. The cells were collected through centrifugation at 1,912×g and 4°C for 15 min and washed twice with 10 mL PBS. The two types of *A. herba-alba* extracts were diluted in Brain Heart Infusion broth (Becton, Dickinson and Company) to concentrations of 102.4–0.4 mg/mL and dispensed into a 96-well microplate. Periodontal bacterial cultures were inoculated into all wells, and the plates were incubated as described above. The culture was streaked on Columbia agar containing 5% sheep blood (w/v) and incubated as described above. The MBC was determined based on colony formation.

2.2.2. Growth of Probiotic Candidate Strains in the Presence of Artemisia herba-alba Extracts

To determine whether *A. herba-alba* extracts inhibit the growth of LAB, the growing ability of the isolates when cocultured with *A. herba-alba* was evaluated. The LAB isolates, including *Lactobacillus*

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curvatus SMFM2016-NK, which was previously identified as an effective anti-periodontitis isolate [17], were diluted to 3.0±0.5 Log CFU/mL using Buffered Peptone Water (Becton, Dickinson and Company). The diluted aliquots were inoculated into four different liquid media—Lactobacilli MRS broth, Lactobacilli MRS broth containing 25 mg/mL *A. herba-alba* ethanol extract, Lactobacilli MRS broth containing 5 mg/mL *A. herba-alba* hot-water extract, and Lactobacilli MRS broth containing 25 mg/mL *A. herba-alba* ethanol extract and 5 mg/mL *A. herba-alba* hot-water extract. The samples were incubated at 37°C for 20 h, and they were plated on Lactobacilli MRS agar. The bacterial cells were enumerated after a 24 h incubation at 35°C. The viable cell counts were used to calculate the growth rate using the following formula;

$$\text{Growth rate}=\{LN(D_2) - LN(D_1)\}/(t_2-t_1)$$

where t_1 denotes 0 h; t_2 denotes 20 h; D_1 represents the number of viable cells at 0 h; D_2 represents the number of viable cells at 20 h.

The mean generation time (Td) was calculated as Td = LN(2)/growth rate [18].

2.2.3. Antimicrobial Effects of Artemisia herba-alba Cocultured Broths against Periodontal Pathogens

Coculture broths containing *A. herba-alba* extracts and LAB were analyzed for their antimicrobial effects against periodontal pathogens. Three types of broth (10 mL) were prepared—Lactobacilli MRS broth, Lactobacilli MRS broth containing 5 mg/mL *A. herba-alba* ethanol extract, and Lactobacilli MRS broth containing 25 mg/mL *A. herba-alba* hot-water extract. These broths, along with the probiotic candidate strains, were cultured at 35°C for 24 h. *A. actinomycetemcomitans* ATCC43718 and *F. nucleatum* ATCC10953 were inoculated into 10 mL Wilkins Chalgren Anaerobe Broth (Oxoid) and incubated at 35°C for 48 h in a 90% N_2, 5% CO_2, and 5% H_2 atmosphere. The cell pellets of periodontal bacteria were harvested through centrifugation at 1,912×g at 4°C for 15 min and washed twice with 10 mL PBS. One hundred microliters of the aliquots were plated on Columbia agar (BioMerieux) and dried for 30 min; then, 10 µL bioconversion broth was spot-inoculated on Columbia agar and incubated at 35°C for 48 h in a 90% N_2, 5% CO_2, and 5% H_2 atmosphere. The inhibition zone from the edge of the spot to the clear zone was measured.

2.2.4. α-Glucosidase Inhibitory Activity of Artemisia herba-alba Cocultured Broths

To analyze the α-glucosidase inhibitory activity, pre-reaction mixtures comprising 50 µL coculture broth samples, 50 µL of 200 mM phosphate buffer (pH 6.5; Sigma-Aldrich), and 50 µL of 0.75 units/mL α-glucosidase (Sigma-Aldrich) were prepared. The reaction mixture was incubated at 37°C for 10 min. Subsequently, the reaction was initiated by adding 100 µL p-nitrophenyl α-glucopyranoside (Sigma-Aldrich) diluted 10 times in dimethyl sulfoxide (Duksan Pure Chemicals Co., Ltd., Gyeonggi-do, Korea), followed by incubation at 37°C for 10 min. The reaction was terminated by adding 750 µL of 0.1 M sodium carbonate (Duksan Pure Chemicals Co., Ltd.). p-nitrophenyl release was assessed by measuring the absorbance at 405 nm (BioTeck Instruments Inc.). A control sample was prepared by replacing the coculture sample with sterile distilled water. Percentage of α-glucosidase inhibition was calculated using the following formula:

α-glucosidase inhibitory activity (%)= {1-(OD_{405nm} of sample/OD_{405nm} of control)}×100.

2.3. Preparation of Bioconverted Milk

Four bioconverted milk (BM) samples, including milk with *L. plantarum* SMFM2016-RK (BM1), BM1 and 5 mg/mL *A. herba-alba* ethanol extract (BM2), BM1 and 25 mg/mL *A. herba-alba* hot-water extract (BM3), and BM1, 5 mg/mL *A. herba-alba* ethanol extract, and 25 mg/mL *A. herba-alba* hot-water extract (BM4), were prepared as follows. Approximately 100 mL of 10% skim milk (Becton, Dickinson and Company) containing 0.5% yeast extract (Becton, Dickinson and Company) was pasteurized at 100°C for 10 min. The pasteurized milk was cooled to 40°C. Then, 5 mg/mL *A. herba-alba* ethanol extract, 25 mg/mL *A. herba-alba* hot-water extract, and both 5 mg/mL *A. herba-alba* ethanol extract and 25 mg/mL *A. herba-alba* hot-water extract were added in the milk to prepare BM2, BM3, and BM4,

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respectively. The milk was stirred for 1 min. Then, 5 mL of the L. plantarum SMFM2016-RK inoculum was inoculated, and the milk was stirred again for 1 min. The inoculated milk samples were incubated at 42°C; when the pH of the BM fell below 5.0, the samples were stored at 4°C for 24 h. The BMs were stored at -80°C for 24 h, and whole frozen BMs were lyophilized at -80°C for 3 days. The lyophilized BM samples were dissolved in sterile distilled water at a concentration of 1 g/10 mL for further use.

2.3.1. Antimicrobial Effects of Bioconverted Milk against Periodontal Pathogens

The antimicrobial effects of four types of BM samples against periodontal pathogens were analyzed using the paper disc diffusion inhibition method. *A. actinomycetemcomitans* ATCC43718, *F. nucleatum* ATCC10953, and *P. gingivalis* ATCC33277 were prepared as described in 2.2.1. Approximately 100 μL of the aliquots was plated on Columbia agar and air-dried for 30 min. Sterilized paper discs were placed at different areas on the surface of the plate. Then, 10 μL BMs were spot-inoculated on the paper disc and dried for 10 min. The dried plates were incubated at 35°C for 48 h in a 90% N_2, 5% CO_2, and 5% H_2 atmosphere. The inhibition zone, from the edge of the disc to the clear zone, was measured.

2.3.2. Analysis of the α-Glucosidase Inhibitory Activity of Bioconverted Milk

The α-glucosidase inhibitory activity of the four types of BM samples was analyzed as described in 2.2.4.

2.4. Whole-Genome Analysis of Novel Probiotics

2.4.1. DNA Extraction and Library Preparation

DNA of the selected LAB was extracted with the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Library was constructed with 5 μg of the extracted DNA sample using the SMRTbell™ Template Prep Kit 1.0 (PN 100-259-100) (Pacific Biosciences, Menlo Park, CA, USA) in accordance with the manufacturer's instructions. Using the BluePippin size selection technique (Sage Science, Beverly, MA, USA), fragments of the SMRTbell template less than 20 kb were eliminated to create large-insert libraries. For quality control, the generated library was confirmed using an Agilent 2100 Bioanalyzer (Santa Clara, CA, USA).

2.4.2. De Novo Sequencing

The DNA/Polymerase Binding Kit P6 (Pacific Biosciences) was used to bind DNA polymerase to the complex after the SMRTbell libraries were annealed to sequencing primers. The PacBio RS II sequencing platform (Pacific Biosciences) was used to sequence the polymerase-SMRT bell adaptor complex after it was inserted into SMRT cells. *De novo* assembly was used to create long contigs, and then gene annotation and prediction were carried out to examine their genetic characteristics.

2.4.3. Comparison with Other Lactic Acid Bacteria

The sequence of the final probiotic isolate, identified as *L. plantarum*, was compared with those of seven other *L. plantarum* strains (E1, MF1298, SRCM103473, SRCM103472, K259, NCU116, and CNEI-KCA4) that were shown to have high similarity through BLAST analysis by NCBI GenBank database. Chromosomal characteristics, such as isolated source and location, chromosomal genome size, and the number of tRNAs and rRNAs, of *L. plantarum* were compared among the strains. The sequence data of seven *L. plantarum* strains were downloaded from the NCBI to calculate the ANI (%) among the chromosomal DNA of the *L. plantarum* strains, using the unweighted pair group method with arithmetic mean tree, created using the CLC program (Insilicogen, Yongin, Korea)

3. Results and Discussion

3.1. Determination of A. herba-alba Extract Concentration

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A. herba-alba ethanol extract exhibited the highest antimicrobial effect against the oral pathogen *P. gingivalis*, with an MBC of 1.4 mg/mL. Its MBCs against *F. nucleatum* and *A. actinomycetemcomitans* were 4.3 and 5.3 mg/mL, respectively (Table 1). *A. herba-alba* hot-water extract exhibited the highest antimicrobial effect against *F. nucleatum*, with an MBC of 5.9 mg/mL. Its MBCs against *P. gingivalis* and *A. actinomycetemcomitans* were 26.5 and 10.7 mg/mL, respectively. There was difference between ethanol extract and hot-water extract as a result of the MBC test of *A. herba-alba* extracts. It could be attributed to whether concentration process was conducted during extract preparation or not. The *A. herba-alba* ethanol extract was concentrated to remove the solvent after extraction and then lyophilized, whereas the hot-water extract was directly lyophilized. Another possible cause may be difference between compositions of antimicrobial substances contained in two extracts. The bioactive components extracted from plants generally varies based on the extraction solvent and method. The extraction yields also vary; therefore, desirable antimicrobial activities appear at different concentrations [19]. Accordingly, 5 mg/mL *A. herba-alba* ethanol extract and 25 mg/mL *A. herba-alba* hot-water extract efficiently were used to prepare bioconversion products with the chosen probiotic candidate strain.

Table 1. Minimum bactericidal concentrations (mg/mL; mean ± standard deviation) of *Artemisia herba-alba* extracts against periodontal pathogens.

***Artermisia herba-alba* extracts**	***F. nucleatum* ATCC43718**	***A. actinomycetemcomitans* ATCC10953**	***P. gingivalis* ATCC33277**	**Total average**
Ethanol extracts	4.3±1.7^{C}	5.3±1.7^{C}	1.4±0.4^{C}	4.0±2.1
Hot-water extracts	5.9±1.3^{C}	10.7±3.3^{B}	26.5±2.0^{A}	13.6±9.1

A-C; different letters indicate a significant difference.

3.2. Selection of Probiotic Candidate Strain

α-Hemolysis and β-hemolysis are major virulence indicators of pathogenic bacteria, while LAB isolates with γ-hemolytic activity are considered safe for consumption, as they exhibit low virulence [20]. Hemolytic analysis is essential to determine whether a strain can be safely used by humans and animals as probiotics [21]. Among the 74 LAB isolates examined in this study, 35 isolates exhibited γ-hemolysis, 36 isolates exhibited α-hemolysis, and 3 isolates exhibited β-hemolysis (data not shown). The 35 isolates showing γ-hemolysis were selected for further analyses of β-glucuronidase and β-glucosidase activities. Thirteen strains were negative to produce β-glucosidase and β-glucuronidase (data not shown) and were selected as candidate probiotic strains. When the toxic substances enter the body, β-glucoside and glucuronic acid molecules are transferred from the liver to the colon as glucuronic acid conjugates after being detoxified [22]. However, in the colon, β-glucuronidase disrupted this connection, producing amines, poisons, or mutations that could act as carcinogens [22]. β-Glucosidase hydrolyzes glycosides, and the undigested glycosides are transported to the colon, where bacterial β-glucosidase further hydrolyzes them [23]. Aglycones formed during this conversion are often toxic and carcinogenic [23]. Therefore, both are considered as harmful enzymes [24]. The 13 isolates were assessed for their tolerance to acid and bile salt. The acid and bile salt tolerance of the 13 isolates was 56.6–102.2% and 87.5–110.5%, respectively (Figure 1). The acid tolerance of most isolates, except that of *Lactobacillus pentosus* SMFM2016-NK7 (66.0%), *Leuconostoc citreum* SMFM2016-YK (57.3%), and *Lactobacillus curvatus* SMFM2016-NK2 (68.6%), was higher than that of LGG (92.6%) (Figure 1A). In the bile tolerance analysis, *L. pentosus* SMFM2016-NK7 showed the highest tolerance of 110.5%, which was significantly higher (p<0.05) than that of LGG (107.1%) (Figure 1B). Additionally, all isolates, except *L. curvatus* SMFM2016-NK2 (87.5%), did not show a significant difference when compared to LGG. Therefore, the *L. pentosus* SMFM2016-NK7, *L. citreum* SMFM2016-YK, and *L. curvatus* SMFM2016-NK2 isolates, which showed significantly lower (p<0.05) tolerance than LGG, were excluded.

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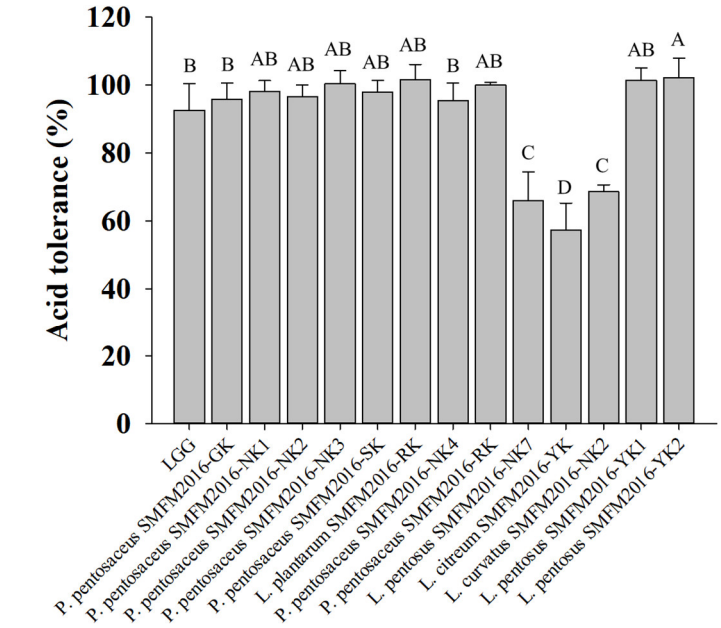
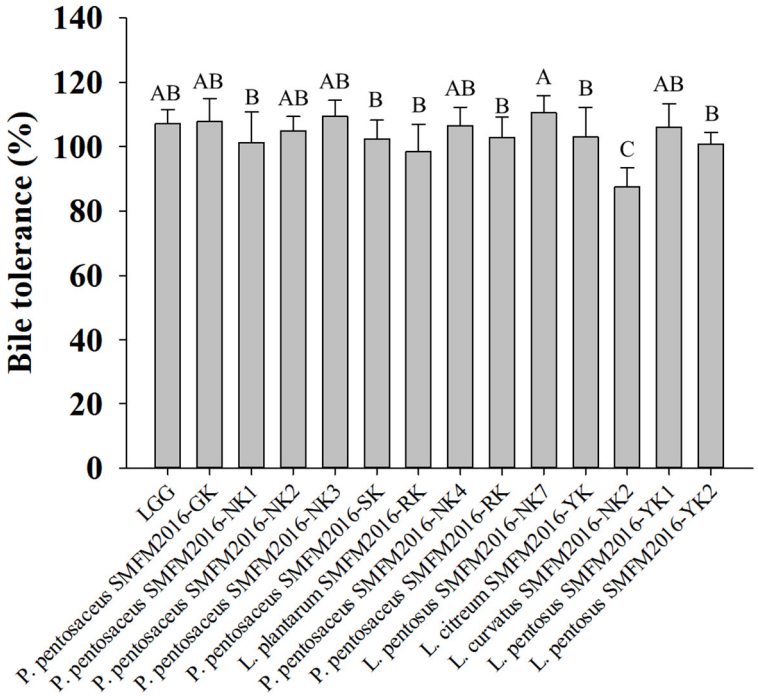


(A)



(B)

Figure 1. Acid tolerance (A) and bile salt tolerance (B) of the lactic acid bacteria isolates. A-D; different letters indicate a significant difference.

The remaining ten isolates were analyzed for ABTS radical-scavenging activity; their activities were >40% (Figure 2). Among the ten isolates, *L. plantarum* SMFM2016-RK showed the highest activity (78.4%), with no significant difference in 0.2 mM ascorbic acid (79.8%). *L. pentosus* SMFM2016-YK1 (42.7%) and *L. pentosus* SMFM2016-YK2 (50.8%) exhibited significantly lower ($p<0.05$) activities than the other bacteria. Therefore, these two strains were excluded from the list of probiotic candidate strains in this study, and the remaining eight isolates were subjected to further analysis.

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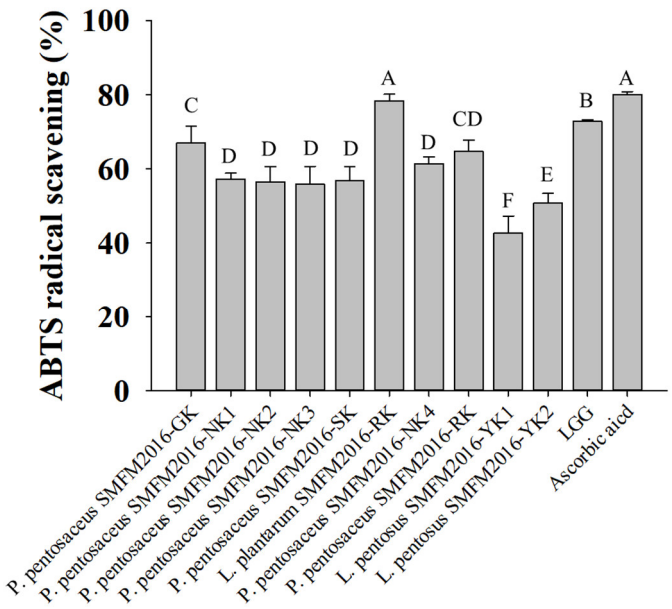

Figure 2. 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid; ABTS) scavenging activity of the lactic acid bacteria isolates. A-F; different letters indicate a significant difference.

Among the nine LAB isolates, including *L. curvatus* SMFM2016-NK, the growth rate averaged 0.051 and the mean generation time was 13.5 h (Tables 2 and 3). None of the isolates showed significantly altered growth rates upon addition of the two types of *A. herba-alba* extracts. However, the growth rate of LGG in *A. herba-alba* ethanol extract was significantly lower and its generation time in *A. herba-alba* ethanol extracts were significantly longer (p<0.05) than that in the MRS broth or *A. herba-alba* hot-water extract. This indicated that *A. herba-alba* ethanol extract suppressed the growth of LGG.

Table 2. Specific growth rate of lactic acid bacteria in MRS broth containing different *Artemisia herba-alba* extracts (mean ± standard deviation).

Lactic acid bacteria strain	MRS broth	MRS broth+ *A. herba-alba* ethanol extract (5 mg/mL)	MRS broth+ *A. herba-alba* hot-water extract (25 mg/mL)
Pediococcus pentosaceus SMFM2016-GK	0.054±0.002^{Aa*}	0.052±0.002^{Aab}	0.052±0.002^{Aab}
P. pentosaceus SMFM2016-NK1	0.052±0.002^{Aab}	0.051±0.003^{Aab}	0.050±0.001^{Aab}
P. pentosaceus SMFM2016-NK2	0.052±0.001^{Aab}	0.052±0.004^{Aab}	0.052±0.003^{Aab}
P. pentosaceus SMFM2016-NK3	0.053±0.002^{Aab}	0.051±0.002^{Aab}	0.051±0.002^{Aab}
P. pentosaceus SMFM2016-SK	0.053±0.001^{Aa}	0.053±0.004^{Aab}	0.052±0.002^{Aab}
Lactiplantibacillus plantarum SMFM2016-RK	0.049±0.001^{Ab}	0.048±0.002^{Ab}	0.049±0.002^{Ab}
P. pentosaceus SMFM2016-NK4	0.053±0.002^{Aa}	0.052±0.002^{Aab}	0.052±0.002^{Aab}
P. pentosaceus SMFM2016-RK	0.053±0.002^{Aab}	0.050±0.002^{Aab}	0.050±0.002^{Aab}

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Lactobacillus curvatus SMFM2016-NK	0.050±0.002^{Ab}	0.050±0.001^{Ab}	0.049±0.002^{Ab}
Lactobacillus rhamnosus GG	0.042±0.004^{Ac}	0.028±0.006^{Bc}	0.44±0.004^{Ac}

*Means with the same row with different superscript capital letters and same column with different superscript small letters are significantly different.

Table 3. Generation time of lactic acid bacteria in MRS broth containing different *Artemisia herba-alba* extracts (h; mean ± standard deviation).

Lactic acid bacteria strain	MRS broth	MRS broth+ *A. herba-alba* ethanol extract (5 mg/mL)	MRS broth+ *A. herba-alba* hot-water extract (25 mg/mL)
Pediococcus pentosaceus SMFM2016-GK	12.9±0.6^{Ac}	13.5±0.4^{Ab}	13.3±0.5^{Ab}
P. pentosaceus SMFM2016-NK1	13.3±0.4^{Aab}	13.6±0.8^{Ab}	13.9±0.2^{Ab}
P. pentosaceus SMFM2016-NK2	13.2±0.3^{Aab}	13.3±0.9^{Ab}	13.3±0.7^{Ab}
P. pentosaceus SMFM2016-NK3	13.2±0.4^{Aab}	13.5±0.6^{Ab}	13.7±0.6^{Ab}
P. pentosaceus SMFM2016-SK	13.1±0.4^{Aab}	13.2±0.9^{Ab}	13.3±0.5^{Ab}
Lactiplantibacillus plantarum SMFM2016-RK	14.3±0.3^{Ab}	14.5±0.6^{Ab}	14.2±0.5^{Ab}
P. pentosaceus SMFM2016-NK4	13.0±0.5^{Aab}	13.4±0.4^{Ab}	13.2±0.6^{Ab}
P. pentosaceus SMFM2016-RK	13.1±0.5^{Aab}	13.9±0.5^{Ab}	13.8±0.7^{Ab}
Lactobacillus curvatus SMFM2016-NK	13.8±0.5^{Aab}	3.9±0.4^{Ab}	14.3±0.5^{Ab}
Lactobacillus rhamnosus GG	16.7±1.6^{Ba}	25.6±6.3^{Aa}	15.7±1.4^{Ba}

*Means with the same row with different superscript capital letters and same column with different superscript small letters are significantly different.

The antimicrobial analysis of the ethanol or hot-water extract against the nine isolates revealed that the extracts bioconverted by *L. plantarum* SMFM2016-RK or *L. curvatus* SMFM2016-NK exhibited higher inhibitory effect *on A. actinomycetemcomitans* ATCC43718 and *F. nucleatum* ATCC10953 than those bioconverted by other isolates (Table 4). *L. plantarum* SMFM2016-RK and *L. curvatus* SMFM2016-NK were more effective on inhibiting the growth of two oral pathogens in a coculture broth with *A. herba-alba* extracts when compared to the isolates grown in MRS broth. Among the cultures, the culture broth of *L. plantarum* SMFM2016-RK with *A. herba-alba* ethanol extract showed the highest inhibitory effect against *A. actinomycetemcomitans* ATCC43718, with an inhibition zone of 2.1 mm (p<0.05). The culture broth of *L. curvatus* SMFM2016-NK with *A. herba-alba* ethanol extract exhibited the highest inhibition size 4.0 mm against *F. nucleatum* ATCC10953 (p<0.05). Although it was difficult to evaluate the antimicrobial effect of LAB and *A. herba-alba* extracts against *P. gingivalis*, their effects on the inhibition of *P. gingivalis* biofilm were observed (data not shown). Probiotic-mediated bioconversion releases bioactive metabolites, such as antimicrobial peptides, immunopeptides, and bioactive polyphenols, that are effective on maintaining oral health [25–27]. Therefore, *A. herba-alba* coculture broths containing *L. plantarum* SMFM2016-RK or *L. curvatus* SMFM2016-NK could be considered for a possible alleviator of periodontal disease, as they inhibit the growth of oral pathogens. Hence, *L. plantarum* SMFM2016-RK and *L. curvatus* SMFM2016-NK were selected for analyzing α-glucosidase activity. α-glucosidase inhibition disrupts carbohydrate digestion and absorption, thereby alleviating hyperglycemia, and reduces the angiotensin-converting

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enzyme inhibitory potential [28,29]. Accordingly, the isolate with a high α-glucosidase activity could be effective on alleviating the systemic disease caused by periodontitis. The α-glucosidase inhibitory effects vary from 0 to 85.23% for the different strains of LAB [29,30]. *L. plantarum* SMFM2016-RK cultured in MRS broth displayed the highest α-glucosidase inhibitory activity of 85.2±0.3% (Table 5). The three coculture broths containing *L. curvatus* SMFM2016-NK showed significantly lower activities than the other coculture broths (p<0.05). Eventually, *L. plantarum* SMFM2016-RK was chosen as the probiotic candidate strain to execute the bioconversion of *A. herba-alba*. Both types of *A. herba-alba* extracts were effective on different functionalities; therefore, both extracts were used for developing BM.

Table 4. Sizes of inhibition zones produced by bioconverted broth with lactic acid bacteria (mm; mean ± standard deviation) against *Aggregatibacter actinomycetemcomitans* ATCC43718 and *Fusobacterium nucleatum* ATCC10953.

Lactic acid bacteria strain	MRS broth		MRS broth + *A. herba-alba* ethanol extract (5 mg/mL)		MRS broth + *A. herba-alba* hot-water extract (25 mg/mL)	
	Aggregatibacter actinomycetemcomitans ATCC43718	*Fusobacterium nucleatum* ATCC10953	*A. actinomycetemcomitans* ATCC43718	*F. nucleatum* ATCC10953	*A. actinomycetemcomitans* ATCC43718	*F. nucleatum* ATCC10953
Pediococcus pentosaceus SMFM2016-GK	1.0±0.0^{Ab}	1.5±0.6^{ABbc}	0.5±0.6^{Bd}	1.8±0.3^{Ac}	1.1±0.3^{Ab}	0.7±0.6^{Bc}
P. pentosaceus SMFM2016-NK1	1.0±0.0^{Ab}	1.3±0.5^{Abc}	1.0±0.0^{Ac}	1.4±0.5^{Ac}	1.1±0.3^{Ab}	0.3±0.5^{Bc}
P. pentosaceus SMFM2016-NK2	1.3±0.3^{Ab}	1.4±0.5^{ABbc}	1.0±0.0^{Ac}	1.8±0.3^{Ac}	1.1±0.3^{Ab}	0.7±0.6^{Bc}
P. pentosaceus SMFM2016-NK3	1.0±0.0^{Ab}	1.5±0.9^{Abc}	1.0±0.0^{Ac}	1.2±1.0^{Ac}	1.1±0.3^{Ab}	0.0±0.0^{Bc}
P. pentosaceus SMFM2016-SK	1.0±0.0^{Ab}	2.0±0.5^{Ab}	1.0±0.0^{Ac}	1.6±0.5^{Ac}	0.8±0.8^{Ab}	0.3±0.5^{Bc}
Lactiplantibacillus plantarum SMFM2016-RK	1.4±0.5^{Ca}	3.1±0.3^{Aa}	2.1±0.3^{Aa}	2.8±0.9^{Ab}	2.0±0.0^{Ba}	3.1±0.3^{Aa}
P. pentosaceus	1.0±0.4^{Ab}	0.9±0.6^{Ac}	1.0±0.0^{Ac}	1.1±0.3^{Ac}	0.9±0.3^{Ab}	0.8±0.3^{Ac}

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SMFM2016-NK4						
P. pentosaceus SMFM2016-RK	1.0±0.0^{Ab}	0.8±0.5^{Ac}	1.1±0.3^{Abc}	1.0±0.0^{Ac}	1.0±0.0^{Ab}	1.0±0.0^{Abc}
Lactobacillus curvatus SMFM2016-NK	1.0±0.0^{Bb}	3.1±1.0^{Ba}	1.9±0.3^{Aab}	4.0±0.9^{Aa}	1.6±0.5^{Aa}	2.6±0.9^{Ba}
Lactobacillus rhamnosus GG	1.0±0.0^{Bb}	1.5±0.6^{Abc*}	1.5±0.6^{Ab}	2.1±0.9^{Abc}	1.8±0.5^{Aa}	1.8±0.5^{Ab}

*Means with the same row with different superscript capital letters and same column with different superscript small letters are significantly different.

Table 5. α-Glucosidase inhibitory activities (%; mean ± standard deviation) of bioconverted broths with lactic acid bacteria.

Lactic acid bacteria strain	MRS broth	MRS broth+ *A. herba-alba* ethanol extract (5 mg/mL)	MRS broth+ *A. herba-alba* hot-water extract (25 mg/mL)
Positive control (*Lactobacillus rhamnosus* GG)	83.2±1.7^{Aa*}	60.0±5.5^{Ba}	26.2±0.9^{Cb}
Lactiplantibacillus plantarum SMFM2016-RK	85.2±0.3^{Aa}	60.8±5.0^{Ba}	35.0±0.2^{Ca}
Lactobacillus curvatus SMFM2016-NK	0.0±0.0^{Bb}	2.7±1.2^{Ab}	5.9±2.6^{Ac}

*Means with the same row with different superscript capital letters and same column with different superscript small letters are significantly different.

3.3. Efficacy Evaluation of Bioconverted Milk

When the BM reached an optimum pH of 4.5–5.0, it was considered fermented. The pH of milk containing *A. herba-alba* extracts and *L. plantarum* SMFM2016-RK (BM2, BM3, and BM4) was slightly lower than that of milk without addition of *A. herba-alba* extracts (BM1) at 0 h (Table 6). BM3 and BM4 reached the optimum pH of 4.62±0.30 and 4.58±0.25, respectively, after 6 h of fermentation, and the LAB cell count was 9.2±0.1 Log CFU/mL. Collectively, *A. herba-alba* hot-water extract exhibited a synergistic effect that improved the growing ability of *L. plantarum* SMFM2016-RK. BM1 reached a pH of 4.42±0.57 after 24 h, and the LAB cell count was 8.8±0.2 Log CFU/mL. BM2 reached a pH of 4.58±0.50 only after a long fermentation time of 37 h, and the LAB cell count was 8.8±0.3 Log CFU/mL. Therefore, different fermentation conditions were applied to produce each type of BM. The prepared BM samples were analyzed for their antimicrobial effects against periodontal pathogens.

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Table 6. pH and lactic acid bacteria cell count (mean ± standard deviation) of the bioconverted milk with *Lactiplantibacillus plantarum* SMFM2016-RK and *Artemisia herba-alba* extracts during fermentation at 35°C.

Sample	0 h		6 h		24 h		37 h	
	pH	cell counts (Log CFU/mL)	pH	cell counts (Log CFU/mL)	pH	cell counts (Log CFU/mL)	pH	cell counts (Log CFU/mL)
BM1	6.57±0.08	8.3±0.0	-	-	4.42±0.57	8.8±0.2	-	-
BM2	6.39±0.07	8.3±0.0	-	-	-	-	4.58±0.50	8.8±0.3
BM3	6.20±0.05	8.3±0.2	4.62±0.30	9.2±0.1	-	-	-	-
BM4	6.11±0.05	8.2±0.1	4.58±0.25	9.2±0.1	-	-	-	-

BM1: bioconverted milk with *L. plantarum* SMFM2016-RK, BM2: BM1 and 5 mg/mL *A. herba-alba* ethanol extract, BM3: BM1 and 25 mg/mL *A. herba-alba* hot-water extract, BM4: BM1, 5 mg/mL *A. herba-alba* ethanol extract, and 25 mg/mL *A. herba-alba* hot-water extract.

The inhibition zones of *A. actinomycetemcomitans* ATCC43718, *F. nucleatum* ATCC10953, and *P. gingivalis* ATCC33277 were 2.6–3.6, 1.6–2.9, and 1.6–1.9 mm, respectively (Table 7). The average inhibition zones for periodontal pathogens when using BM1, BM2, BM3, and BM4 were 2.0±0.8, 2.4±0.8, 2.5±1.1, and 2.6±1.1 mm, respectively. Bioconversion products with *A. herba-alba* extracts (BM2, BM3, and BM4) exhibited marginally higher antimicrobial effects than those without the extract (BM1). Among these products, BM3 showed the highest antimicrobial activity against *A. actinomycetemcomitans*, with an inhibition zone of 3.6±0.8 mm (p<0.05). BM4 showed the highest inhibition zone of 2.5±0.6 mm for *F. nucleatum* ATCC10953, which was significantly higher than that achieved with BM1 (1.6±0.5 mm; p<0.0.5). BM1 and BM3 showed the same inhibition zone of 1.9±0.3 mm for *P. gingivalis* ATCC33277.

Table 7. Sizes (mm; mean ± standard deviation) of inhibition zones produced by bioconverted milk with *Lactiplantibacillus plantarum* SMFM2016-RK and *Artemisia herba-alba* extracts against periodontal pathogens.

Sample	Periodontal pathogen			**Total average**
	Aggregatibacter actinomycetemcomitans ATCC43718	*Fusobacterium nucleatum* ATCC10953	*Porphyromonas gingivalis* ATCC33277	
10% skim milk	0.0±0.0^{D}	0.0±0.0^{D}	0.0±0.0^{D}	**0.0±0.0**
BM1	2.6±1.1^{B}	1.6±0.5^{C}	1.9±0.3^{BC}	**2.0±0.8**
BM2	3.0±0.8^{AB}	2.5±0.6^{BC}	1.8±0.5^{BC}	**2.4±0.8**
BM3	3.6±0.8^{A}	1.9±1.0^{BC}	1.9±0.3^{BC}	**2.5±1.1**
BM4	3.3±1.5^{AB}	2.9±0.6^{AB}	1.6±0.5^{C}	**2.6±1.1**

BM1: bioconverted milk with *L. plantarum* SMFM2016-RK, BM2: BM1 and 5 mg/mL *A. herba-alba* ethanol extract, BM3: BM1 and 25 mg/mL *A. herba-alba* hot-water extract, BM4: BM1, 5 mg/mL *A. herba-alba* ethanol extract, and 25 mg/mL *A. herba-alba* hot-water extract; A-C; different letters indicate a significant difference.

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Bioconversion products containing *A. herba-alba* extracts (BM2, BM3, and BM4) exhibited significantly higher (p<0.05) α-glucosidase inhibitory activity than BM1 (Figure 3). Notably, BM3 displayed a significantly higher activity (12.4±0.2%) than the other samples (p<0.05).

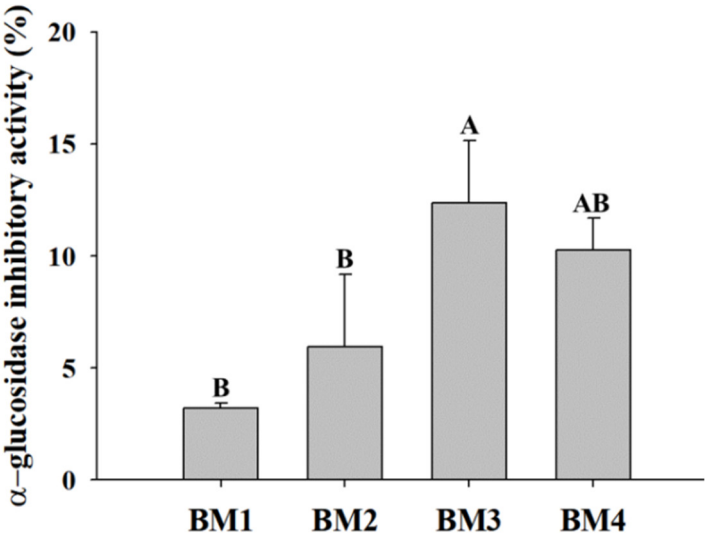

Figure 3. Comparison of α-glucosidase inhibitory activity on treatment with bioconverted milk. A-B; different letters indicate a significant difference.

3.4. Whole-Genome Analysis of Novel Probiotics

L. plantarum SMFM2016-RK was sequenced using *de novo* assembly, and the whole genome was obtained. Three contigs—Contig1, Contig2, and Contig3—were assembled, and their lengths were 3,320,843 bp, 60,102 bp, and 39,997 bp, respectively. Using BLAST, the best match for L. plantarum SMFM2016-RK was identified as *Lactiplantibacillus plantarum*, with 99.96% identification rate. For gene function analysis, gene annotation and gene prediction were performed using the information provided by the gene ontology database. Contig1 (chromosome) was predicted to contain 3,135 coding sequences (CDS). The 67 tRNA and 16 rRNA genes and their locations were predicted from the CDS. The structural and functional properties of Contig1 were described using DNA plots (Figure 4). The predicted functional genes were divided into three primary categories—biological process, molecular function, and cellular component—according to their characteristics. Two-thousand-nine-hundred-and-twelve transcripts were included under biological process; 2,280 transcripts under molecular function; and 1,378 transcripts under cellular component (Table 8). In the biological process, metabolic process had the highest number (1,089) of transcripts. This included transcripts corresponding to various chemical reactions and pathways of organisms and the processes such as protein synthesis and degradation.

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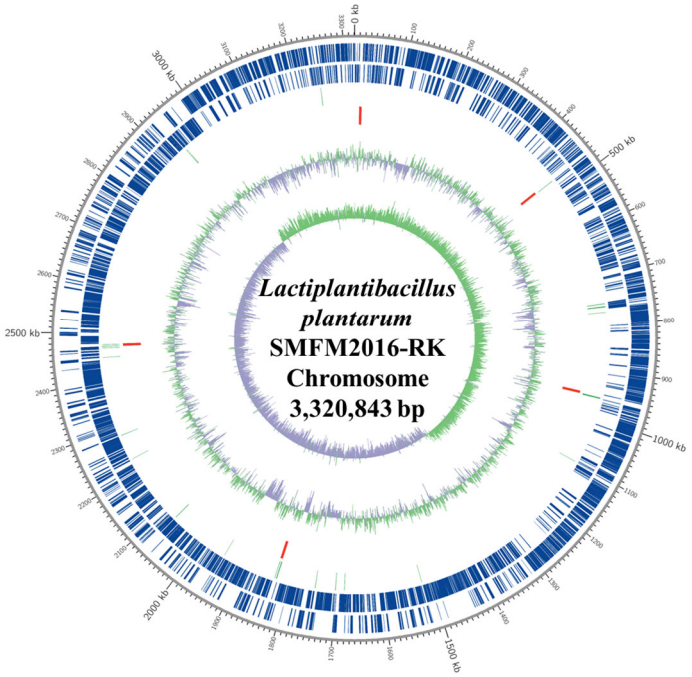

Figure 4. Chromosomal genome properties of *Lactiplantibacillus plantarum* SMFM2016-RK. Outer scale; base pairs, the first (the outer-most) and second ring; forward and reverse open reading frame (ORF), the third and fourth ring; forward and reverse ORF by gene annotation, the fifth and sixth ring; rRNA and tRNA genes, the seventh and eighth ring; positive and negative GC content values, the inner-most circle; GC skew.

Table 8. Gene ontology by gene prediction of *Lactiplantibacillus plantarum* SMFM2016-RK chromosome.

Category	Gene ontology	Number of transcripts
Cellular component	Cell part	263
	Cell	263
	Protein-containing complex	120
	Organelle	61
	Extracellular region	5
	Membrane	397
	Membrane part	249
	Extracellular region part	4
	Organelle part	16
Molecular function	Catalytic activity	1,087
	Binding	805
	Molecular carrier activity	2
	Transport activity	219
	Antioxidant activity	8
	Transcription regulator activity	105
	Molecular function regulator	1
	Structural molecule activity	51
	Molecular transducer activity	2

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Biological process	Localization	317
	Response to stimulus	104
	Metabolic process	1,089
	Cellular process	845
	Biological regulation	223
	Regulation of biological process	217
	Cellular component organization or biogenesis	56
	Negative regulation of biological process	9
	Multi-organism process	9
	Signaling	22
	Developmental process	9
	Immune system process	1
	Biological adhesion	8
	Detoxification	2
	Carbon utilization	1

Gene annotation analysis using the Evolutionary genealogy of genes: Non-supervised Orthologous Groups (EggNOG) database revealed that *L. plantarum* SMFM2016-RK had a high ability for carbohydrate transportation and metabolism (9.25%) and amino acid transportation and metabolism (6.57%; Table 9). *L. plantarum* SMFM2016-RK had some CDS related to proteolysis and amino acid metabolism (Table 10). The genes known as oligopeptide ABC transportation system (*oppF, oppD, dtpT, pepF1*, and *pepO*) and the serine-related metabolism (*glyA, dsdA*, and *sdhA*) were mostly identified. In addition, the arginosuccinate metabolism operon clustered with aspartate aminotransferase (*asp*), lyase (*argH*) and synthase (*argG*), which can be used for energy production and NADH regeneration, was also observed. These genes might influence various proteins and amino acids present in BM3, thus improving the growth of *L. plantarum* SMFM2016-RK and reducing the fermentation time. *L. plantarum* SMFM2016-RK was also shown to possess *lpdC* (gallate decarboxylase) and *padC* (phenolic acid decarboxylase), which encode phenolic acids-metabolizing enzymes (Table 11). In relation to this, several research reported that phenolic acids were the main bioactive ingredients of *A. herba-alba* [31,32]. The phenolic compounds produced by LAB from precursors improve efficacy in the body or enable the production of bioactive metabolites through changes in gut microbiome [33,34]. According to Sun and Miao (2020), phenolic compounds can lower the glycemic index by altering the digestibility of food, and flavonoids and proanthocyanidins are effective in inhibiting α-glucosidase activity [35]. Likewise, in this study, it can be assumed that the inhibition of α-glucosidase by BM3 is due to the action of these phenolic metabolites produced during bioconversion of milk and *A. herba-alba* extracts by LAB.

Table 9. Distribution of predicted open reading frames (ORFs) over functional class in *Lactiplantibacillus plantarum* SMFM2016-RK.

Description	Number of ORFs	Ratio (%)
Translation, ribosomal structure, and biogenesis	150	4.7847
Transcription	258	8.2297
Replication, recombination, and repair	196	6.2520
Cell cycle control, cell division, chromosome partitioning	26	0.8293
Defense mechanisms	66	2.1053
Signal transduction mechanisms	70	2.2329
Cell wall/membrane/envelope biogenesis	179	5.7097
Cell motility	4	0.1276
Intracellular trafficking, secretion, and vesicular transport	25	0.7974

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Posttranslational modification, protein turnover, chaperones	69	2.2010
Energy production and conversion	111	3.5407
Carbohydrate transport and metabolism	290	9.2504
Amino acid transport and metabolism	206	6.5710
Nucleotide transport and metabolism	86	2.7432
Coenzyme transport and metabolism	63	2.0096
Lipid transport and metabolism	62	1.9777
Inorganic ion transport and metabolism	125	3.9872
Secondary metabolites biosynthesis, transport, and catabolism	20	0.6380
General function prediction only	349	11.1324
Function unknown	780	24.8804
Total	**3,135**	**100**

Table 10. Coding DNA sequences (DNA) related to proteolysis and amino acid metabolism identified in the gene annotation of *Lactiplantibacillus plantarum* SMFM2016-RK.

Start	End	Product	Gene	Identity	e-value	Bit score
1849164	1850129	Oligopeptide transport ATP-binding protein OppF	*oppF*	77.636	0.0	518
1850136	1851215	Oligopeptide transport ATP-binding protein OppD	*oppD*	79.883	0.0	570
2470957	2472429	Di-/tripeptide transporter	*dtpT*	66.189	0.0	648
962965	964776	Oligoendopeptidase F, plasmid	*pepF1*	57.333	0.0	728
3234367	3236283	Neutral endopeptidase	*pepO*	59.528	0.0	791
504486	505796	D-serine dehydratase	*dsdA*	57.619	9.40e-175	501
2530776	2531678	L-serine dehydratase, alpha chain	*sdhA*	72.069	5.73e-138	397
820964	822202	Serine hydroxymethyltransferase	*glyA*	70.270	0.0	607
1173945	1175741	Aspartate--tRNA ligase	*aspS*	74.617	0.0	917
2291674	2292909	Argininosuccinate synthase	*argG*	73.350	0.0	634
2290271	2291674	Argininosuccinate lyase	*argH*	71.024	0.0	699

Table 11. Coding DNA sequences (DNA) related to phenolic acid decarboxylase identified in the gene annotation of *Lactiplantibacillus plantarum* SMFM2016-RK.

Start	End	Product	Gene	Identity	e-value	Bit score
335672	337144	Gallate decarboxylase	*lpdC*	82.857	0	841
3008485	3009021	Phenolic acid decarboxylase PadC	*padC*	87.64	3.70E-116	332

The microbial genome similarity and the molecular phylogenetic relations were determined by analyzing the phylogenetic tree and average nucleotide identity (ANI) (%). The ANI of the seven *L. plantarum* strains in the NCBI GenBank database were compared to that of *L. plantarum* SMFM2016-RK (Figure 5A). The seven *L. plantarum* strains were isolated from various foods, and differences in genome size, GC content, and the numbers of tRNA and rRNA were observed. The ANI (%) between *L. plantarum* strains was 99.07–99.84%, and *L. plantarum* SMFM2016-RK was closest to the *L. plantarum* E1 strain, according to the unweighted pair group method with arithmetic mean tree by ANI (Figure 5A and 5B). Since ANI ≥96.5% is common in genomes within the same species, it was necessary to confirm whether *L. plantarum* SMFM2016-RK was a new strain [36]. Accordingly, whole genome annotation was performed with *L. plantarum* E1, which showed the highest similarity, using CLC workbench. Differences were observed between the two strains in various regions of the whole genome (Figure 5C). Therefore, *L. plantarum* SMFM2016-RK was confirmed to be a new strain.

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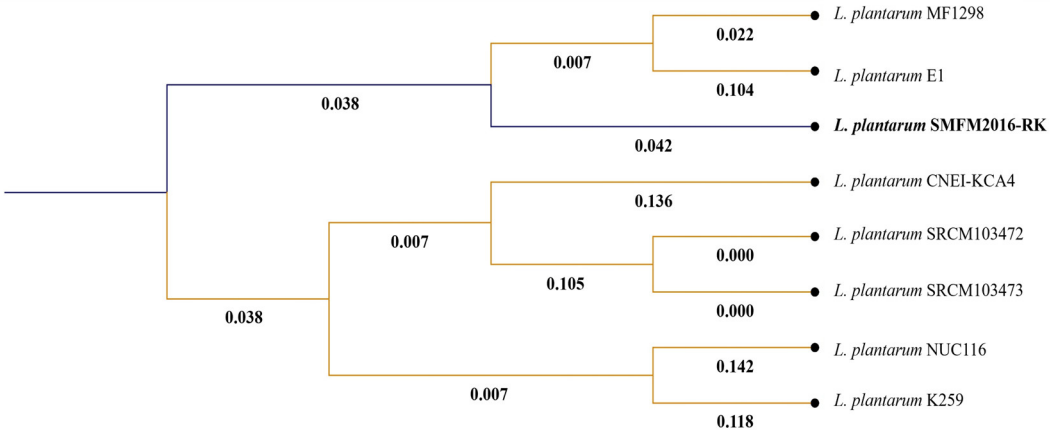
(A)

		1	2	3	4	5	6	7	8
***L. plantarum* SMFM2016-RK**	1		99.84	99.81	99.28	99.18	99.28	99.07	99.22
***L. plantarum* E1**	2	97.23		99.87	99.30	99.20	99.30	99.15	99.28
***L. plantarum* MF1298**	3	96.64	99.15		99.34	99.18	99.34	99.13	99.27
***L. plantarum* SRCM103473**	4	90.34	91.91	91.54		99.23	100.00	99.15	99.27
***L. plantarum* K259**	5	89.38	91.31	90.94	90.37		99.23	99.18	99.24
***L. plantarum* SRCM103472**	6	90.34	91.91	91.54	99.97	90.37		99.15	99.27
***L. plantarum* NCU116**	7	90.14	90.60	90.10	88.30	89.29	88.30		99.10
***L. plantarum* CNEI-KCA4**	8	88.10	89.35	89.08	89.40	87.41	89.40	88.03	

(B)



(C)

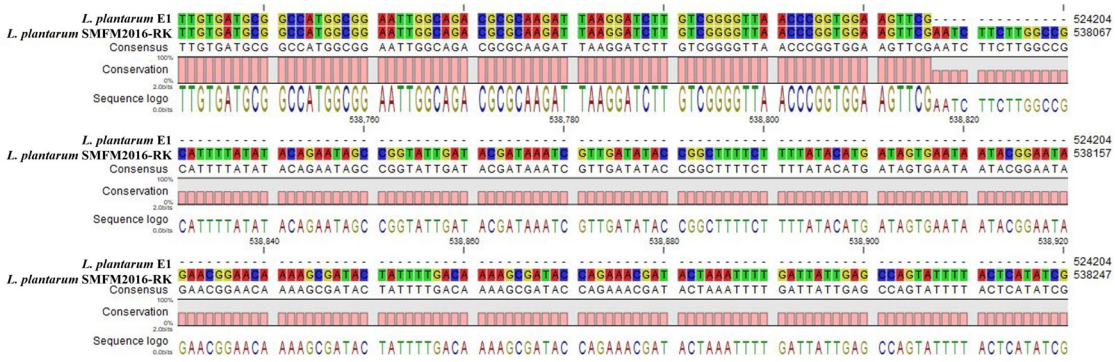


Figure 5. Average nucleotide identity (ANI) (A), unweighted pair group method with arithmetic mean (UPGMA) tree by average nucleotide identity (ANI) (B), and comparison of genome annotation with *Lactiplantibacillus plantarum* E1 (region 538,067-538,247) (C) of *L. plantarum* SMFM2016-RK.

5. Conclusions

This study identified *L. plantarum* SMFM2016-RK, which might be safe and inhibited the growth of oral pathogens and the activity of α-glucosidase. Whole-genome sequence analysis indicated that *L. plantarum* SMFM2016-RK is a new strain that could be used as a novel probiotic. The *A. herba-alba* extract added to skim milk was bioconverted by the newly characterized probiotic, and four BM also showed the effects on impeding the growth of oral pathogenic bacteria and the activity of α-glucosidase. Taken together, probiotic-mediated bioconversion of *A. herba-alba* extract might be effective on oral disease and glycemic control. However, since the efficacy of the BM developed in this study was examined *in vitro*, it is necessary to apply it to animals in the future study to evaluate the efficacy *in vivo*.

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Conflicts of Interest: The authors declare no conflicts of interest.

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