probiotics that create a beneficial environment in the intestinal tract. In 2001, the World Health Organization defined probiotics as "live microorganisms that, when administered in adequate amounts, confer health benefits on the host" [1]. In South Korea, according to the Ministry of Food and Drug Safety (MFDS)'s notification number 2008-12, the labeling of probiotic products as such was mandated starting in 2010 [2].

Probiotics play a role in clustering normal intestinal bacteria and eliminating harmful microorganisms that can cause disease, thus maintaining an appropriate balance of intestinal bacteria. In addition, they are known to exhibit various functions, including stabilizing the total bacterial population in the intestines, inhibiting the proliferation of harmful bacteria through the production of organic acids such as lactic acid and acetic acid, as well as proteinaceous antibacterial substances such as nisin. Probiotics are also known to alleviate conditions such as irritable bowel syndrome, lactose intolerance, and non-specific immunosuppression, as well as having various functions such as anticancer and antitumor effects [3].

These probiotics are mainly abundant in dairy products and fermented foods such as kimchi, soybean paste, yogurt, and cheese. The probiotic market in Europe, which is valued at approximately 7 trillion KRW, is the largest in the world. However, in recent years, there has been a growing trend of rapid growth in the Asian markets, including China, India, Vietnam, and Indonesia [4]. For these foods, beverages, and dietary supplements to be recognized as probiotics, they need to survive in the stomach acid and bile, reach the colon, proliferate, and colonize. They also need to demonstrate effective effects within the intestinal tract.

Additionally, there is increasing interest and utilization of fermented vinegar, as various fermented vinegar products based on a variety of ingredients such as fruits, mushrooms, and more have been proven to have antioxidant and anti-inflammatory effects [5-7]. In this study, based on the antibacterial effects of fermented fig vinegar on *Escherichia coli*, *Saccharomyces cerevisiae*, and *Staphylococcus aureus* [8], various forms of dairy products and dressing sauces were developed. These products were then analyzed for their effects on human-derived colon cells, including cell viability, antioxidant capacity, and adhesion ability of intestinal cells, similar to the human colon epithelial cells.

2. Materials and methods

2.1. Materials

The primary ingredients used for dairy product manufacturing include regular milk (original milk, Samyang Co., Seoul, Korea) and pasteurized milk (Jeju premium milk, Samyang Co., Seoul, Korea). Fig vinegar (*Ficus carica* vinegar, Newuto Local Herb Farming Association, Yeongam-gun, Jeonnam) was provided by Newuto Local Herb Farming Association. Figs were purchased from Samho Central Farm in Jeonnam, and after removing the peels, the flesh was stored at -20°C. Dried figs were prepared by drying them at 50°C for 31 hours, peeled, and stored at 4°C before use.

2.2. Drinkable yogurt production

For the production of drinkable yogurt, fig vinegar was added to regular milk and pasteurized milk at concentrations of 1%, 3%, and 5%. Additionally, 1% salt (CJ Cheiljedang, Incheon, Korea) and 1% sucrose (CJ Cheiljedang, Incheon, Korea) were added. The mixture was then fermented at 37°C for 30 hours.

2.3. Ricotta cheese production

To produce ricotta cheese, regular milk, and pasteurized milk were heated at 60°C for 10 minutes. Fig vinegar was added to each at concentrations of 1%, 3%, and 5%. Furthermore, 1% salt and 1% sugar were added. The mixture was fermented at 37°C for 30 hours. Subsequently, the ricotta cheese was then separated from the whey for 9 hours using a double layer of gauze.

2.4. Dressing sauce production

Dressing sauce was prepared by adding 5% fig vinegar to the dairy products that were used to make ricotta cheese. To each separated whey, an equal amount of fig fermentation enzyme was added, followed by the addition of 10% and 20% fig vinegar.

2.5. Measurement of probiotic bacterial colony count

For the measurement of probiotic bacterial colony counts, 5 mL of drinkable yogurt with 1%, 3%, and 5% fig vinegar, 5 g of ricotta cheese with 1%, 3%, and 5% fig vinegar, and 5 mL of dressing sauce produced by adding 10% and 20% fig vinegar to the separated whey from ricotta cheese production were collected. These samples were diluted in sterile physiological saline solution to ensure that the colony count ranged from a minimum of 30 to a maximum of 300 colonies on one plate. Each sample (100 µL) was then inoculated on Lactobacilli MRS (deMan, Rogosa, and Sharpe) agar medium (BD Difco, Fraklin Lakes, NJ, USA) and plate count agar (BD Difco, Fraklin Lakes, NJ, USA) with bromcresol purple (BCP, Sigma-Aldrich, MO, USA). The plates were cultured at 37°C using the plate count method for 48 hours. After culturing, the number of yellow colonies was measured in triplicate and the mean value was expressed as log colony forming unit (CFU) per mL.

2.6. Identification of probiotic strains

For the precise identification of isolated strains from the developed dairy products, the separated strains were cultured in Lactobacilli MRS liquid medium for 20 hours, and genomic DNA was extracted (AccuPrep, Bioneer, Daejeon, Korea). For 16S rRNA amplification, forward primer 27F (5'-aga gtt tga tcc ctc ag-3') and reverse primer 1492R (5'-ggt tac ctt gtt acg act t-3') were prepared with a final concentration of 0.4 µM each [9], using 2X EzWay Direct Master Mix (Komabiotech, Seoul, Korea) to make a total volume 20 µL. The polymerase chain reaction (PCR) cycle began with a 10-minute reaction at 95°C, followed by 30 cycles of 95°C for 30 s, 50°C for 40 s, and 72°C for 30 s. The final extension cycle was performed at 72°C for 15 min. PCR reaction products were subjected to electrophoresis on a 0.8% agarose gel at 100 V for 35 min, stained with GreenStar™ Nucleic Acid Staining Solution (Bioneer Co, Daejeon, Korea), and viewed with Kodak Gel Logic 100 image system (Easrman Kodak Co, NY, USA). Sequence analysis was outsourced to Bioneer, and sequence analysis and homology comparisons were conducted using GenBank (NIH, MD, USA).

2.7. Analysis of cell viability

In the ricotta cheese produced by adding 5% fig vinegar to regular milk, whey was separated to facilitate absorbance measurements. The impact on Caco-2 human colon cells was measured. The Caco-2 human colon cell line was purchased from the Korean Cell Line Bank. The cells in the 32nd division were used for the experiment. Cells were cultured at 37°C under 5% CO_2 and 95% O_2 (MCO-18AC, Panasonic Healthcare Co., Ltd, Japan) conditions in Minimum Essential Medium (MEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific Inc, MA, USA), 1% streptomycin/penicillin (10,000 IU/mL, Thermo Fisher Scientific Inc), 1% non-essential amino acid, 10 mM HEPES, 1 mM L-glutamate, and 1 mM sodium pyruvate. A total of 1×10^4 cells were seeded in a flat-bottom 96-well plate and incubated at 37°C in a 5% CO_2 incubator for 24 hours. After removing the medium, a new medium containing 1%, 5%, and 10% whey was added, and the cells were incubated for an additional 24 hours. The medium in each well was then removed, and 10 µL of cell counting kit-8 reagent (CCK-8, Dojindo Molecular Technologies, Inc., Kumamoto, Japan) and 100 µL of medium were added. The cells were incubated at 37°C under 5% CO_2 for 2 hours, and absorbance was measured at 450 nm using a microplate reader (Synergy™ HT Multidetection

Microplate Reader, Agilent Technologies Inc., CA, USA). Cell viability was calculated by comparing the cells with and without the addition of whey samples at different concentrations.

2.8. Antioxidant activity measurement

Caco-2 cells in the 32nd division were seeded at a concentration of 1×10^4 cells per well in a black 96-well plate. Superoxide dismutase (SOD) assay was performed according to the method provided by the superoxide dismutase assay kit-WST (WST-1,2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt, Dojindo Molecular Technologies, Inc). WST working solution, enzyme working solution, and sample solution were prepared according to the method in the previous study [10], and the reaction was carried out for 20 mins at 5% CO_2 and 37°C. The absorbance at 450 nm was measured using a microplate reader. The SOD enzyme activity of cells with addition of 1%, 5%, and 10% whey samples was compared to the antioxidant activity of arbutin at 100 μg/mL.

2.9. Analysis of the intestinal adherence of probiotic strains

Cells of the same division were used and seeded in 12-well plates at a concentration of 5×10^4/mL and incubated at 37°C, 5% CO_2 for 24 hours to form a monolayer of single cells. *Lactobacillus rhamnosus* (KCCM 42756, Korean Culture Center of Microorganisms, Seoul, Korea) strain was used as the control to analyze the relative adherence ability of the isolated strains from the developed dairy products [11]. Each strain was incubated in MRS liquid medium at 37°C for 18 h, centrifuged at 358 *g* for 4 min, 4°C. The cells were washed three times with PBS (pH 7.2) and then resuspended in serum-free MEM at a concentration of 1×10^6 CFU/mL. They were added in 0.3 portions to a 12-well plate containing Caco-2 cells forming a monolayer and incubated for 2 h at 37°C, 5% CO_2. After rinsing the monolayer twice with PBS, adherent cells were detached using 0.2% Trypsin-EDTA (ThermoFisher Scientific Inc), stained with 0.4% trypan blue (Thermo Fisher Scientific Inc), and the cell count was compared with that of the control probiotic, and the degree of adhesion was assessed.

2.10. Statistical analysis

The experimental results were analyzed using SPSS 20.0 (Statistical Package for Social Science, SPSS Inc., Chicago, IL, USA) to calculate mean values and standard deviations (SD). One-way ANOVA was conducted, followed by Duncan's multiple range test at a significance level of $P < 0.05$ to validate significant differences [12].

3. Result

3.1. Probiotic count in dairy products containing fig vinegar

The probiotic counts in the drinkable yogurt, ricotta cheese, and dressing sauce produced by mixing fig vinegar at concentrations of 1%, 3%, and 5% with regular milk or pasteurized milk were found to be over 1.0×10^7 to 1.0×10^8 CFU/mL on MRS medium and PCA medium with BCP, meeting the standards and specifications for dietary supplements [13] (**Tables 1** to **3**).

3.2. Identification of probiotic strains in dairy products containing fig vinegar

Results of identifying the strains isolated from the developed dairy products by comparing their 16S rRNA gene sequence information with sequences registered in the National Center for Biological Information (NCBI) GenBank database confirmed them as *Leuconostoc lactis* (**Table 4**).

3.3. Cell viability of human colon cell lines induced by whey from dairy products containing fig vinegar

The effect of whey from ricotta cheese made by mixing

5% FV with regular milk was evaluated based on the cell count of untreated Caco-2 cells. Whey was separated to facilitate absorbance measurements, and the cell count was compared for different concentrations. An increase in cell viability was observed when whey was added, with a significant increase of approximately 19% when treated with a 10% concentration ($P < 0.05$). This can be interpreted as whey being a non-toxic substance on the cells (**Figure 1**).

3.4. Antioxidant activity of human colon cell lines induced by whey from dairy products containing fig vinegar

The influence of whey at different concentrations on SOD enzyme activity was evaluated. When treated with 100 μg/mL arbutin as the positive control for antioxidant activity, approximately 93% of antioxidant activity was observed. When whey was treated at concentrations of 1%, 5%, and 10%, each showed

Table 1. Viable cells (log CFU/mL) count on drinkable yogurt from regular milk or pasteurized milk with the concentration of FV (mean ± standard deviation, $n = 3$)

Media	Viable cells (log CFU/mL)					
	DYRM			DYPM		
	1% FV	3% FV	5% FV	1% FV	3% FV	5% FV
PCA with BCP	7.10 ± 0.09	7.23 ± 0.09	8.31 ± 0.05	7.23 ± 0.07	7.27 ± 0.03	8.16 ± 0.03
MRS	7.78 ± 0.03	7.14 ± 0.12	8.40 ± 0.07	7.29 ± 0.04	7.39 ± 0.04	8.26 ± 0.08

Abbreviations: DYRM, drinkable yogurt from regular milk; DYPM, drinkable yogurt from pasteurized milk; FV, *Ficus carica* vinegar; PCA, plate count agar; BCP, bromcresol purple; MRS, Lactobacilli MRS agar.

Table 2. Viable cells (log CFU/mL) count on ricotta cheese from regular milk or pasteurized milk with the concentration of FV (mean ± standard deviation, $n = 3$)

Media	Viable cells (log CFU/mL)					
	RCRM			RCPM		
	1% FV	3% FV	5% FV	1% FV	3% FV	5% FV
PCA with BCP	7.31 ± 0.11	7.36 ± 0.09	7.92 ± 0.14	7.45 ± 0.07	7.61 ± 0.06	7.64 ± 0.01
MRS	7.75 ± 0.03	8.02 ± 0.05	8.38 ± 0.05	7.55 ± 0.18	7.64 ± 0.03	8.22 ± 0.01

Abbreviations: RCRM, ricotta cheese from regular milk; RCPM, ricotta cheese from pasteurized milk; See **Table 1**.

Table 3. Viable cells (log CFU/mL) count on dressing sauce from regular milk or pasteurized milk with the concentration of FV (mean ± standard deviation, $n = 3$)

Media	Viable cells (log CFU/mL)			
	Whey from RCRM with 5% FV		Whey from RCPM with 5% FV	
	10% FV	20% FV	10% FV	20% FV
PCA with BCP	7.53 ± 0.04	7.35 ± 0.03	6.82 ± 0.07	6.49 ± 0.05
MRS	7.35 ± 0.10	7.12 ± 0.09	6.61 ± 0.01	6.38 ± 0.08

Abbreviations: See **Tables 1** and **2**.

Table 4. Sequence similarity of bacteria from drinkable yogurt from regular milk with 5% FV

Identity GenBank	Accession number
Leuconostoc lactis strain CBA3625 chromosome	NZ_CP042387.1

approximately 25%, 30%, and 58% antioxidant activity, respectively (**Figure 2**).

3.5. Adhesion ability of probiotic strains in whey from dairy products containing fig vinegar

Probiotics must pass through the stomach and duodenum and adhere stably in the colon to exert their functionality. To confirm this, the Caco-2 cell line, which proliferates similarly to human colon epithelial cells, was used. *L. rhamnosus*, a commonly used commercial strain, was used as the control to confirm the relative adherence. The results showed that when the adhesion ability of *L. rhamnosus* was set to 100%, *Leuconostoc lactis* isolated from whey was found to have approximately 14% superior adhesion ability (**Figure 3**). This suggests that the isolated strains from the produced dairy products meet the basic requirements as a probiotic material.

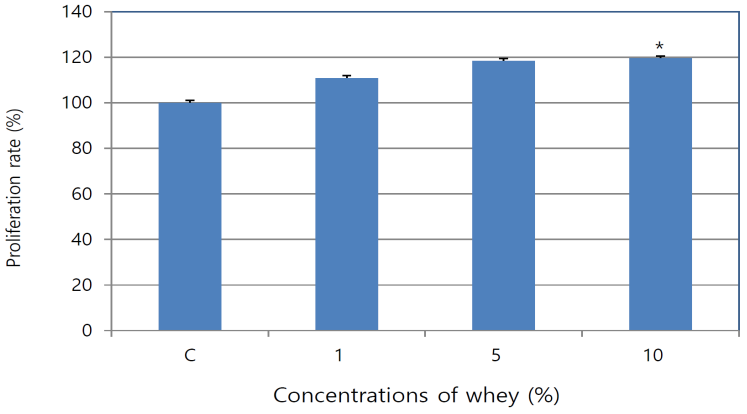

Figure 1. Percentage of proliferation rate by concentration of whey separated from ricotta cheese at Caco-2 cell line. Data are presented as mean ± standard deviation (n = 3). *P < 0.05; P-values were calculated by paired Student t-test. Abbreviation: C, control.

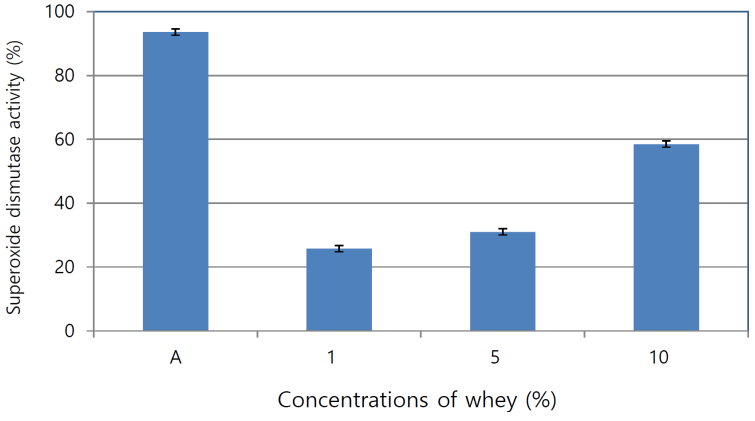

Figure 2. Superoxide dismutase activity by concentration of whey separated from ricotta cheese at Caco-2 cell line. Data are presented as mean ± standard deviation (n = 3). P-values were calculated by paired Student t-test. P = not significant. Abbreviation: A, 100 μg/mL arbutin.

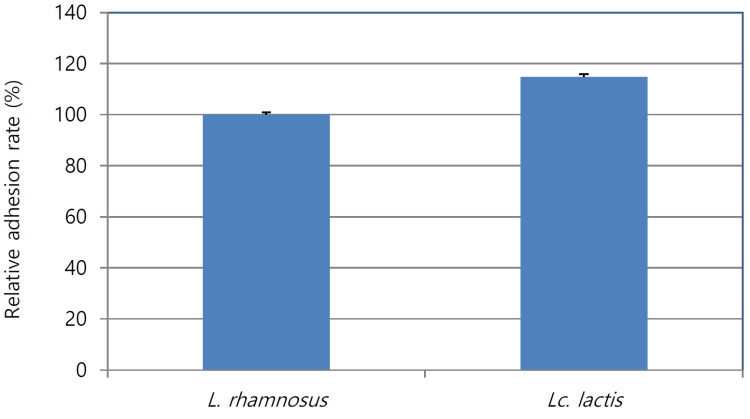

Figure 3. Adhesion ability of *Leuconostoc lactis* from whey separated from ricotta cheese at Caco-2 cell line. Data are presented as mean ± standard deviation (n = 3). P-values were calculated by paired Student t-test. P = not significant.

4. Discussion

The human gut is a complex environment inhabited by hundreds of diverse microorganisms. According to research findings, the gut microbiota of individuals suffering from gut disorders such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) differs significantly from that of healthy individuals [13,14]. Microorganisms, such as *Lactobacillus* sp., *Bifidobacterium* sp., *Bacillus subtilis*, and *Enterococcus faecium*, are known to be capable of positively altering the gut microbiota, and these live microorganisms are collectively referred to as probiotics. To be recognized as probiotics, they must survive in environments with stomach acid and bile, reach the colon, proliferate, and colonize, and their efficacy must be demonstrated in the intestinal tract. Notably, probiotics have shown remarkable effects in improving gut conditions like IBS and alleviating allergy responses, encouraging the use of probiotics in alternative medicine. Moreover, research has revealed a variety of beneficial effects such as immune system regulation, lowering blood cholesterol, anticancer properties, blood pressure regulation, and weight loss associated with probiotics [15-18]. Additionally, the term “microbiome” is a combination of the words “microbe” and “biome,” which refers to the community of microorganisms coexisting in humans, plants, animals, soil, oceans, and the atmosphere. The commercialization of the microbiome began with the sale of probiotic drinks in the food and beverage market [19,20].

The results of measuring the probiotic counts in each product in this study exceeded $1.0\times10^7 \sim 1.0\times10^8$ CFU/mL, satisfying the standards and specifications for probiotic food. The isolated probiotic strain was identified as *Leuconostoc lactis*, which is a lactic acid bacterium mainly used in the fermentation of dairy products and vegetables [21], and it is often isolated from traditional Korean fermented food, kimchi. A notable characteristic of *Leuconostoc* spp. found in kimchi is their ability to produce dextran dietary fiber [22,23]. Therefore, the dairy products produced in this study harbor probiotic strains commonly found in kimchi, which may contribute to gut health.

Furthermore, probiotics need to pass through the stomach and duodenum and adhere stably in the colon to exert their functionality. To confirm this, cell viability was observed using Caco-2 cell lines that proliferate similarly to human colon epithelial cells. When treated with a 10% concentration of whey, cell viability increased by approximately 19%, indicating that whey is not harmful to colon cells and can promote cell proliferation. In addition, when whey was treated at concentrations of 1%, 5%, and 10%, they showed approximately 25%, 30%, and 58% antioxidant activity, respectively. The analysis of the adhesion ability of probiotic strains in whey revealed that *Leuconostoc lactis* isolated from whey exhibited superior adhesion ability. Thus, it can be concluded that whey meets the basic requirements of a microbiome. Further research is required to compare the effects of fermented fig vinegar on colon cells.

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Disclosure statement

The author declares no conflict of interest.

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