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Abstract

To prevent degenerative joint diseases in dogs, the development of fermented whey peptides that enhance calcium absorption is essential. This study examined the physiological traits of lactic acid bacteria (LAB) derived from canine feces. The functional characteristics of whey peptides produced through fermentation with these bacterial strains were also investigated. To assess this, three strains—*Lactobacillus acidophilus* D5, D6, and *Lactiplantibacillus plantarum* D7—were chosen, and their respective whey protein hydrolysis levels were evaluated after fermentation. Among these strains, *L. plantarum* D7 exhibited the highest hydrolysis rate. Antioxidant and anti-inflammatory activities, along with calcium solubility, were evaluated. Fraction 2 displayed significant antioxidant activity and effectively inhibited pro-inflammatory cytokines. Additionally, 17 amino acids, including leucine, were identified. These findings suggest that *L. plantarum* D7 has the potential to be used as a functional food ingredient to promote canine health.

Keywords whey protein, bioactive peptide, calcium solubilization, companion dog, lactic acid bacteria

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INTRODUCTION

The phenomenon of "pet humanization," where companion animals are treated as family members or close companions, has gained significant attention in urban households (Lee et al., 2022). In 2020, pet-related expenditures in the United States reached $10.36 billion, with approximately 67% of households owning a pet during the same year (Wangyi et al., 2022). In South Korea, an estimated 6.04 million households, representing 11.6 million individuals, owned companion animals, with an average monthly expenditure of 110,000 KRW ($97) on food and veterinary care (Baritugo et al., 2023).

Advancements in vaccinations, dietary practices, and environmental conditions have extended the lifespan of companion dogs, leading to a rise in age-related conditions such as degenerative joint arthritis, a common chronic joint disease in older dogs (Hoffman et al., 2022). As a result, interest in functional foods designed to support joint health in dogs has grown, reflecting the broader trend of pet humanization, where pet health is given similar priority to human health (Hobbs and Shanoyan, 2018).

Milk proteins are widely recognized as functional ingredients due to their diverse health benefits, such as promoting muscle development, reducing inflammation, and exhibiting free radical scavenging activity (Nongonierma and FitzGerald, 2015). Key examples include casein-derived CPP (Casein phosphopeptide) and

whey protein hydrolysate, both known for their ability to enhance calcium absorption and support musculoskeletal health. Whey proteins, which contain bioactive components including lactoferrin, β-lactoglobulin, and α-lactalbumin, are particularly notable for promoting bone formation and improving metabolic processes (Xu, 2009; Jo et al., 2020). Hydrolysis of whey proteins through enzymatic methods or fermentation with LAB produces peptides with enhanced functional properties (Leila et al., 2023). While enzymatic hydrolysis is effective, its high production costs present challenges (Němečková et al., 2009). Alternatively, LAB fermentation offers a more cost-effective approach, with additional advantages such as better palatability and improved sensory qualities (Fiorentino et al., 2008).

This study aims to isolate LAB from canine feces and utilize them to produce specific bioactive peptides from whey proteins. By exploring their biochemical properties, this research aimed to develop functional peptides tailored to the unique health needs of dogs, contributing to advancements in companion animal nutrition.

Materials and Methods

Isolation of LAB from dog feces

LAB was isolated from dog feces collected from a Border Collie (2 years, Sex: Male, Nutritional status: Normal). Fecal samples (10 mL) were mixed with 40 mL of PBS (pH 7.4) and thoroughly vortexed for homogenization. A 1 mL portion of the homogenized sample was serially diluted using sterile 0.85% saline, and 100 μL from each dilution was plated onto De Man-Rogosa-Sharpe (MRS) agar (BD Difco Co., USA) supplemented with 0.02% sodium azide. The inoculated plates were then incubated at 37℃ for 48 hours. LAB colonies that appeared on the plates were selected and transferred into MRS broth to prepare stocks. All cultures were preserved at -72℃ in MRS broth with 20% glycerol for long-term storage.

Screening and identification of LAB

The selected strains were evaluated based on their colony shape, microscopic morphology, and Gram staining results. Additional analysis was performed through 16S-rRNA gene sequencing, and the obtained sequences were aligned and compared to those registered in the GenBank database (Macrogen, Korea).

Tolerance to artificial gastric and bile juice

The ability of selected LAB strains to withstand acidic and bile conditions was assessed following the method of

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Kobayashi et al. (1974). For acid tolerance, LAB cultures were incubated in MRS broth (BD Difco, USA) at 37°C for 2 hours after adjusting the pH to 1.0, 1.5, and 2.5 using 1.0 N HCl (Sigma-Aldrich, USA). The number of surviving colonies was determined by plating on BCP agar (BD Difco, USA) and incubating at 37°C for 48 hours. For bile tolerance assessment, LAB strains were transferred to MRS broth supplemented with 0.3% oxgall (BD Difco, USA) and incubated at 37°C for 24 and 48 hours. Viability was measured using the same plating method, and colony-forming units (CFUs) were enumerated to determine the survival rate.

Proteolytic activity

Proteolytic activity was assessed following the method described by Riffel and Brandelli (2006), with minor modifications with minor modifications. The selected LAB strains were screened for proteolytic activity using an agar-well diffusion assay on skim milk agar containing 2.0% (w/v) agar (BD Difco, Franklin Lakes, USA). After centrifugation at 5,000×g for 10 minutes using a Microfuge 20R (Beckman Coulter, Indianapolis, USA), 100 µL of the supernatant was transferred into 9 mm-diameter wells on the skim milk agar plates. Proteolytic activity was determined based on the formation of clear zones around the wells, indicating enzymatic hydrolysis of milk proteins. The diameters of these clear zones were measured every 24 hours for up to 72 hours of incubation at 37°C in a Heratherm incubator (Thermo Fisher Scientific, Waltham, USA) to evaluate protease activity.

***β*-Galactosidase activity assay**

The *β*-galactosidase activity was determined following the method outlined by Jalili et al. (2009), with slight modifications. Specifically, 1 mL of bacterial culture was mixed with 50 mL of 0.1 M phosphate buffer (Sigma-Aldrich, St. Louis, USA) containing 0.001 M magnesium sulfate ($MgSO_4$; Sigma-Aldrich, St. Louis, USA) and 0.05 M *β*-mercaptoethanol (Sigma-Aldrich, St. Louis, USA). To this solution, two drops of chloroform (J.T. Baker, Phillipsburg, USA) and one drop of 1% (w/v) sodium dodecyl sulfate (SDS; Sigma-Aldrich, St. Louis, USA) were added to 1 mL of the diluted sample. The mixture was vortexed for 10 seconds using a Vortex-Genie 2 (Scientific Industries, Bohemia, USA) and incubated in a water bath (Thermo Fisher Scientific, Waltham, USA) at 28°C for 5 minutes. Next, 0.2 mL of a 4 mg/mL solution of o-nitrophenyl-*β*-D-galactopyranoside (ONPG; Sigma-Aldrich, St. Louis, USA) was added, followed by vortexing for 10 seconds. The enzymatic reaction catalyzed by *β*-galactosidase was stopped after 10 minutes by adding 0.5 mL of 1 M sodium carbonate (Na_2CO_3; Sigma-Aldrich, St. Louis, USA). The mixture was then centrifuged at 15,000×g for 15 minutes using a Sorvall Legend X1R

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centrifuge (Thermo Fisher Scientific, Waltham, USA), and the optical density was measured at 420 nm using a Multiskan EX355 Microplate reader (Thermo Fisher Scientific, Waltham, USA). Enzyme activity was calculated using the following formula:

$$\text{Activity of } \beta - \text{galactosidase (unit/mL)} = A420/tv \times 100$$

where t, v, and A_{420} denote reaction time (min), sample volume (mL), and absorbance (420 nm), respectively.

Preparation of whey protein concentrate (WPC) solution

WPC (80% protein) was sourced from Hilmar™, USA, with its chemical composition provided by the manufacturer (Table 1). To prepare the solution, WPC (Hilmar™, USA) was mixed with distilled water to reach a final concentration of 7% (w/v). To enhance the proliferation of LAB, glucose (3% w/v; Sigma Aldrich Co., USA) was subsequently incorporated. The pH of the mixture was then regulated to the optimal level for LAB activity using 1 N NaOH (Sigma Aldrich Co., USA). The solution was then sterilized by heating at 65°C for 30 minutes. After heat treatment, the whey protein solution was stored at 4°C and used within 1 week.

Assay for the degree of hydrolysis of whey protein concentrate

The degree of hydrolysis (DH) of the WPC solution in fermented samples was assessed every 4 hours for six intervals using the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method (D. Spellman et al., 2003). The TNBS reagent was prepared by dissolving 0.1% (w/v) TNBS (Sigma-Aldrich, St. Louis, USA) in distilled water. All samples and standard solutions were made with 1% (w/v) sodium dodecyl sulfate (SDS; Sigma-Aldrich, St. Louis, USA). To prepare the samples, 2 mL of the solution was diluted in distilled water at a 10% (v/v) concentration in a test tube, followed by the addition of 4 mL of 0.72 N trichloroacetic acid (TCA; Samchun Chemical, Seoul, Korea). The samples were incubated at 25°C for 20 minutes. A 0.2 mL aliquot of each sample was then filtered using a sterile syringe filter (0.45 µm PVDF; SIGL, Mainz, Germany) and transferred into a test tube containing 2 mL of 0.2125 M sodium phosphate buffer (pH 8.2; Sigma-Aldrich, St. Louis, USA). Subsequently, 2 mL of TNBS reagent was added, and the mixture was incubated in the dark at 50°C for 60 minutes. The reaction was terminated by adding 4 mL of 0.1 N hydrochloric acid (HCl; Sigma-Aldrich, St. Louis, USA) to each tube, and the samples were allowed to cool to room temperature for 30 minutes.

Absorbance was measured at 420 nm using a Multiskan EX355 microplate reader (Thermo Fisher Scientific,

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Waltham, USA) . The degree of hydrolysis (DH) was calculated as follows (Lee et al, 2024):

$$DH\ (\%) = \frac{hs - h0}{htot - h0} \times 100$$

where hs is the A - N content of the WPC hydrolysate, h_{tot} is the A - N content after acid hydrolysis (6 N HCl, 110℃, 24h) of the sample, and $h0$ is the A - N content of sample its self. All values are calculated using L-leucine as the standard substance.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted to analyze the peptide profile of the fermented whey protein concentrate. Using 12% acrylamide gels (Bio-Rad, Hercules, USA) and the Mini-Protean Tetra Electrophoresis System (Bio-Rad, Hercules, USA), the molecular weights of the peptides were estimated according to the method described by Laemmli (1970). Protein bands were visualized using Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, USA) staining, with molar mass standards provided by Precision Plus Protein Dual Xtra Standards (Bio-Rad, Hercules, USA).

Fast protein liquid chromatography (FPLC)

Peptide fractions from fermented whey protein concentrate (WPC) were obtained through gel filtration using a preparative chromatography system (Waters Co., USA). The setup included a Waters W600 Preparative Liquid Chromatography system, a Waters Preparative Pump, a Waters W717 Autosampler, and a Waters Dual λ Absorbance Detector (W2487). For the initial purification, fermented WPC was dissolved in 50 mM sodium phosphate buffer (pH 7.0) supplemented with 0.15 M NaCl (Sigma Aldrich Co., USA). The resulting solution was filtered using a 0.45 μm PVDF syringe filter (SIGL Inc., Germany) to remove particulates. The sample was then applied to a Hiprep 16/60 Sephacryl S-100 HR column (GE Healthcare Life Sciences, USA) and eluted at a constant flow rate of 1 mL/min. Absorbance was monitored at 280 nm. Collected fractions were stored at –20℃ for further analysis (Table 2).

ABTS radical scavenging activity

ABTS radical scavenging activity was assessed by first preparing a 7 mM ABTS solution in ethanol (Samchun Chemical, Seoul, Korea) using ABTS powder (Sigma-Aldrich, St. Louis, USA). To generate the $ABTS^{\bullet+}$ radical

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solution, the ABTS stock was mixed with 2.45 mM potassium persulfate (Sigma-Aldrich, St. Louis, USA) in a 1:1 (v/v) ratio and incubated at ambient temperature in the dark for 12–16 hours. The resulting $ABTS^{\bullet+}$ solution was diluted with ethanol to achieve an absorbance of 0.70 ± 0.02 at 734 nm, measured using a Multiskan EX355 microplate reader (Thermo Fisher Scientific, Waltham, USA). For the assay, 190 µL of the diluted $ABTS^{\bullet+}$ solution was added to 10 µL of each sample fraction in a 96-well plate (SPL Life Sciences, Pocheon, Korea). After 3 minutes of reaction time, absorbance at 734 nm was measured, and antioxidant activity was expressed as Trolox equivalent antioxidant capacity (TEAC) in mM/L.

DPPH radical scavenging activity

The DPPH free radical scavenging activity was determined using the method described by Wei et al. (2014) with slight modifications. For the experiment, a DPPH solution (0.2 mM) was prepared by dissolving 2,2-Diphenyl-1-picrylhydrazyl along with 2,4,6-tripyridyl-s-triazine (Sigma Aldrich Co., USA) in ethanol. This solution was then mixed with fermented WPC samples in equal volumes (1:1, v/v), followed by incubation in the dark at room temperature for 30 minutes. After the reaction, absorbance was recorded at 570 nm, and the antioxidant capacity was quantified as ascorbic acid equivalents (mM), using ascorbic acid (Sigma Aldrich Co., USA) as the standard.

Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power (FRAP) assay was performed according to the method described by Benzie et al. (1996). The FRAP solution for the experiment was prepared as follows: A 300 mM acetate buffer was made by mixing Sodium acetate trihydrate (Sigma Aldrich Co., USA) with Acetic acid (J.T Baker Co., USA). A 10 mM TPTZ solution was created by dissolving 2,4,6-tri(2-pyridyl)-1,3,5-triazine (Sigma Aldrich Co., USA) into 40 mM HCl (DAEJUNG Co., Korea). Additionally, a 20 mM Iron(III) chloride hexahydrate solution was prepared by dissolving Iron(III) chloride hexahydrate 97% A.C.S reagent (Sigma Aldrich Co., USA) in ethanol (Samchun Chemical Co., Korea). These three solutions were mixed in a 10:1:1 (v/v) ratio. For the assay, 1.5 mL of the FRAP solution, preheated to 37℃, was combined with 50 µL of fermented WPC fraction samples or standard solutions. The mixture was vortexed and allowed to react at room temperature in a dark room. Absorbance was measured at 593 nm, and the results were expressed as Ascorbic acid (Sigma Aldrich Co., USA) equivalent capacity in mM. The protein concentration of all fermented WPC fraction samples was adjusted to 200 µg/mL.

Calcium solubilization capacity

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In the experiment calcium solubilization capacity was measured with slight modifications to the methods reported by Yamaguchi et al. (2024) and Yin et al. (2020), focusing on the degree of precipitation following calcium phosphate formation. A 10 mM calcium chloride solution (Sigma Aldrich Co., USA) and a 20 mM sodium phosphate buffer were first prepared. Subsequently, each fermented WPC fraction sample (0.5 mL) was mixed with an equal volume (0.5 mL) of the calcium chloride solution, followed by the addition of 1.0 mL of 20 mM sodium phosphate buffer to the resulting mixture. The solution was incubated at 37℃ for 2 hours and subsequently centrifuged at 2,000×g for 30 minutes at 25℃ to remove the precipitated insoluble calcium phosphate. Calcium levels were quantified using a calcium colorimetric assay kit (Bioassay Systems Co., USA), analyzing both the complete reaction mixture and the supernatant obtained after centrifugation. For the colorimetric measurement, 10 μL of each sample (i.e., unseparated mixture and centrifuged supernatant) was transferred into a 96-well microplate, followed by the addition of 90 μL of chromogenic reagent and 60 μL of calcium assay buffer. The mixture was allowed to react in the dark at room temperature for 5 minutes, after which absorbance was measured at 570 nm. Calcium concentration was determined using the first equation described below, and the calcium solubilization capacity was calculated using the second. All fermented whey protein concentrate fractions were adjusted to a uniform protein concentration of 2.0 mg/mL prior to testing.

$$\text{Calcium concemtration (mg/mL)} = \frac{Sa}{Sv}$$

· Sa = Sample amount form standard curve

· Sv = Sample volume

$$\text{Calcium solubility (\%)} = \frac{\text{Calcium concentration in supernatant}}{\text{Calcium concentration in whole solution}} \times 100$$

Cell culture

RAW 264.7 cells were maintained in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS at 37℃ in a 5% CO_2 atmosphere. The medium was replaced every two days. To assess cell protection, RAW 264.7 cells were seeded into 96-well plates at a density of 1×10^5 cells/mL (100 μL per well) for nitric oxide production inhibition and cell viability assays.

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Cell viability assay

Cell viability was assessed using the MTT assay, following the protocols outlined by Moon et al. (2019) and Y. Peng et al. (2019). RAW 264.7 cells were seeded into 96-well plates and allowed to adhere for 24 hours. After the culture medium was removed, cells were treated with various concentrations of the fraction samples diluted in serum-free medium and incubated for an additional 24 hours. Following this treatment, MTT solution (5 mg/mL) was added to each well, and the cells were incubated for 3 hours at 37°C in a 5% CO_2 atmosphere. After incubation, the supernatant was carefully discarded, and the formazan crystals, produced by metabolically active cells, were solubilized using DMSO. Absorbance was recorded at 540 nm with a microplate reader (Bio-Tek Instruments, Winooski, VT, USA) to evaluate cell viability. The viability percentage was determined by comparing the absorbance values of the treated groups to that of the untreated control group, which was considered to have 100% viability.

Nitric oxide (NO) assay

Nitric oxide (NO) levels were determined as nitrite using the Griess reaction, based on the method outlined by Fiorentino et al. (2008). RAW 264.7 cells were incubated in serum-free medium with different concentrations of fraction samples for 2–3 hours after removing the existing medium. Subsequently, LPS (100 ng/mL) was added in an equal volume of serum-free medium, and the cells were incubated for 20 hours to induce stimulation. Following this, 0.1 mL of the reaction mixture was collected and placed into a 96-well microplate, where Griess reagent was added. The mixture was allowed to react in the dark at room temperature for 15 minutes, and absorbance was measured at 540 nm. NO scavenging activity was calculated as a percentage, comparing the absorbance at 540 nm of the samples to that of the blank experiment.

Cytokine production assay

RAW 264.7 cells were seeded into 96-well plates and cultured for 24 hours. Once the medium was removed, the cells were exposed to different concentrations of fraction samples prepared in serum-free medium and incubated for an additional 2–3 hours. Subsequently, lipopolysaccharide (LPS, 100 ng/mL) was added using the same volume of serum-free medium to stimulate the cells for 20 hours. After incubation, the supernatants were collected, and cytokine levels (IL-1α, IL-6, and TNF-α) were quantified using a mouse ELISA kit (Komabiotech, Korea) based on the enzyme-linked immunosorbent assay (ELISA) method. Absorbance was measured at 450 nm.

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Profiling of amino acids

The amino acid composition of the purified fraction was analyzed following the procedure outlined by Alaiz et al. (1992). Fractions that demonstrated strong bioactivity were further examined via high-performance liquid chromatography (HPLC), employing the method reported by Liu et al. (1995). Amino acid separation was carried out on an AccQ-Tag™ column (3.9 mm × 150 mm; WAT052885, Waters Co., USA) with a two-eluent system. Sample derivatization was performed using the Waters AccQ-Tag·Fluor reagent kit. Samples were hydrolyzed in 6 N hydrochloric acid (HCl) 5 mL in sealed test tubes at 110℃ for 24 hours. The hydrolysate was dried in an oven at 75℃, reconstituted in 1 mL of triple-distilled water, and filtered through a 0.22 μm sterile syringe membrane filter (SIGL Inc., Germany). For derivatization, 10 μL of the filtrate was mixed with 70 μL of AccQ·Flour borate buffer and 20 μL of AccQ·Fluor reagent (3 mg/mL in acetonitrile), vortexed for 5 seconds, and incubated at 55℃ for 10 minutes in a heating block. The column temperature was maintained at 37℃ during the analysis, with a flow rate of 1 mL/min. Fluorescence detection was conducted at an excitation wavelength of 250 nm and an emission wavelength of 395 nm. An automatic amino acid analyzer (Waters Co., USA) with a Waters 717 autosampler was used for the analysis. The mobile phases consisted of (A) AccQ-Tag eluent A (prepared by mixing 100 mL of AccQ-Tag A concentrate [WAT052890, Waters Co., USA] with 900 mL of triple-distilled water and adjusting to pH 5.02 using phosphoric acid) and (B) 60% (v/v) acetonitrile (HPLC grade, Fisher Scientific, USA) in triple-distilled water. Calibration was performed using amino acid standards (Sigma-Aldrich Co., USA), with sample amounts ranging from 0.02 to 0.08 μg (20–1,000 pmol). The gradient program used for analysis is presented in Table 3, and additional parameters are detailed in Table 4. Before starting the gradient, the column was equilibrated with 100% mobile phase A for 10 minutes. After completing the analysis, the column was washed with triple-distilled water for 30 minutes and conditioned with mobile phase B for 15–20 minutes.

Peptide identification by mass spectrometry

The fraction sample was examined using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with a Vanquish UHPLC system (Thermo Fisher Scientific Inc., USA) connected to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific Inc., USA) at Proteinworks Co., Korea. Detailed analytical parameters are outlined in Table 5.

Results

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Isolation of LAB from dog feces

LAB was isolated from dog feces collected from various dogs. Colonies were observed on MRS agar plates after being subcultured three times at 37℃ for 18 to 24 hours. The samples were then stored at -72℃ in a mixture of MRS broth and 20% glycerol.

Screening and Identification of LAB

The morphological characteristics of the isolated LAB strains were examined microscopically. Various microorganisms were observed, and all strains were confirmed to be Gram-positive. Seven strains were identified through 16S-rRNA sequencing. Based on the 16S-rRNA sequence analysis, the lactic acid-producing strains were classified as four strains of *Lactobacillus acidophilus*, one strain of *Enterococcus faecalis*, one strain of *Lactiplantibacillus plantarum*, and one strain of *Lacticaseibacillus paracasei*. The 16S-rRNA gene sequences are detailed in Table 6. After excluding *Enterococcus faecalis* D4, identified as a potentially harmful intestinal bacterium, the remaining isolated strains were named as follows: *Lactobacillus acidophilus* D2, D3, D5, and D6; *Lactiplantibacillus plantarum* D7; and *Lacticaseibacillus paracasei* D8.

Tolerance to artificial gastric and bile juice

The ability of LAB strains to survive in artificial gastric juice was evaluated. The selected LAB strains were incubated in the artificial gastric environment for 2 hours, after which viable cell counts were determined before and after treatment using BCP agar. In dogs, the gastric transit time is approximately 120 minutes, with gastric pH ranging from 0.9 to 2.5 and an average of 1.5 (Jennifer, 1986). To replicate these conditions, the pH was set to 1.0, 1.5, and 2.5, and an incubation time of 120 minutes at 37℃ was used to simulate transit time. The LAB strains showed excellent tolerance under these simulated gastric conditions (Table 7). These results are consistent with the findings of Mechai et al. (2014), who indicated that probiotic strain tolerance during gastrointestinal passage varies depending on the strain. In addition to gastric tolerance, resistance to intestinal bile salts is a critical criterion in selecting probiotics. A simulated digestive model was employed for *in vitro* assessment, in which LAB strains were treated with artificial gastric fluid for 2 hours at 37℃. After treatment, viable cells were enumerated to assess their resistance to artificial bile components. The findings revealed that the LAB strains exhibited high survivability under bile salt conditions (Tables 8 and 9).

Proteolytic Activity

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The proteolytic activity of the bacterial strains was assessed by their ability to produce clear zones on skim milk agar, following the method described by Pailin et al. (2001). Using the agar well diffusion technique, it was observed that all seven screened LAB strains demonstrated the capacity to hydrolyze milk proteins. Clear zones were detected around the wells for six of the strains, confirming their proteolytic activity. Among these, *Lactiplantibacillus plantarum* D7 exhibited the highest level of proteolytic activity, with a clear zone diameter exceeding 26 mm on skim milk agar (Table 10). These findings identify *L. plantarum* D7 as the most efficient strain for proteolytic activity among the strains tested.

β-Galactosidase Activity

β-Galactosidase is an essential enzyme responsible for lactose hydrolysis, contributing significantly to improved lactose digestion and the conversion of lactose into short-chain fatty acids, which offer various health benefits to the host (Naidu et al., 1999; Bras et al., 2010). The activity of *β*-galactosidase was found to be strongly influenced by enzyme production within the cells, which varied depending on the type of carbohydrate and the availability of nitrogen sources in the medium. Among the tested strains, *Lactobacillus acidophilus* D3, D5, D6, and *Lactiplantibacillus plantarum* D7 demonstrated the highest levels of *β*-galactosidase activity, as presented in Fig. 1. These findings emphasize the potential of these strains for applications requiring effective lactose hydrolysis.

Degree of Hydrolysis of WPC Solution

Lactic acid bacteria (LAB) possess the ability to hydrolyze proteins through complex peptidase activity, converting proteins into amino acids and nitrogen, making them a valuable source of nutrients for the body. Protein fermentation was assessed by analyzing soluble protein content and the capacity of primary amino groups for hydrolysis. LAB strains were inoculated into the protein solution, and hydrolysis levels were monitored every 4 hours (Fig. 2). The proteolytic activity of LAB led to an increase in peptide formation and a rise in total free amino group concentration. Within the first 0–4 hours, all three LAB strains exhibited a sharp increase in hydrolytic activity. After this period, hydrolysis progressed more slowly, reaching a plateau around 20 hours, with negligible changes observed beyond that point. These results indicate that LAB strains achieved the highest rate of protein proteolysis after 20 hours and that the extent of proteolysis was time-dependent. This demonstrates that the LAB strains exhibit proteolytic activity in whey protein concentrate (WPC) solutions, supporting their role in protein hydrolysis (Fig. 2).

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SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE analysis of whey protein hydrolyzed by *Lactiplantibacillus plantarum* D7 is shown in Fig. 3. Protein bands with molecular weights ranging from 13 to 250 kDa were identified. Major whey protein components, such as lactoferrin, bovine serum albumin, immunoglobulins, β-lactoglobulin, and α-lactalbumin, appeared as bands within the 13 to 100 kDa range. During the hydrolysis process, higher molecular weight proteins were broken down. After 20 hours of hydrolysis, significant degradation of lactoferrin, bovine serum albumin, and immunoglobulins was observed, demonstrating the efficient proteolytic activity of *L. plantarum* D7 on whey protein components.

Fast protein liquid chromatography (FPLC)

Whey protein hydrolysis was analyzed using SDS-PAGE and subsequently subjected to preparative-scale fractionation for further analysis. The fermentation time was determined based on the degree of hydrolysis and SDS-PAGE results. The fermented WPC was fractionated using a HiPrep 16/60 Sephacryl S-100 HR column connected to a preparative liquid chromatography system. Separation was achieved based on molecular size, yielding three distinct fractions (Fig. 4). These fractions were compared to the original fermented WPC through SDS-PAGE analysis (data not shown).

ABTS radical scavenging activity

The Trolox equivalent antioxidant capacity assay evaluates a compound's ability to scavenge or neutralize radicals, using Trolox as the antioxidant reference standard (Leung et al., 2009). The ABTS assay results are presented in Fig. 5. All fractions, except for F3, demonstrated greater ABTS radical scavenging activity compared to non-hydrolyzed whey protein. Among the fractions, fraction 2 exhibited the highest activity.

DPPH radical scavenging activity

DPPH, a stable free radical, is commonly used to evaluate the free radical scavenging activity of antioxidants (Lin and Chang, 2000). The antioxidant activity of each fraction was assessed by determining its DPPH scavenging capacity. DPPH radical scavenging values were quantified by referencing a standard curve generated using ascorbic acid (Vitamin C), and statistical analysis was subsequently performed. The results, shown in Fig. 5, indicate that fractions 1 and 2 exhibited DPPH radical scavenging activity comparable to that of CPP..

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Ferric reducing antioxidant power (FRAP)

The FRAP assay measures the ability of antioxidants to convert ferric ions (Fe^{3+}) into ferrous ions (Fe^{2+}), producing a blue-colored Fe^{2+}–TPTZ complex that exhibits maximum absorbance at 593 nm. This reaction reflects the electron-donating ability of antioxidants, which plays a crucial role in disrupting free radical chain reactions (Lin and Chang, 2000). The decrease in absorbance is directly associated with the antioxidant content (Benzie et al., 1996). As shown in Fig. 5, fraction 2 demonstrated ferric-reducing antioxidant power comparable to CPP, underscoring its strong antioxidant potential based on its electron-donating capability and ferric ion reduction ability.

Calcium solubilization capacity

Casein phosphopeptide (CPP) is rich in phosphoserine, which interacts with calcium to enhance its solubility. In this study, calcium solubility was assessed to determine whether the individual fractions exhibited calcium-solubilizing properties similar to those of CPP. The results revealed that CPP achieved a high calcium solubility of 87.15%. The phosphoserine in CPP plays a key role in dissolving calcium and improving its solubility. Although all hydrolysate fractions showed lower calcium solubilization capacity compared to CPP, they demonstrated greater capacity than unhydrolyzed whey protein. Among the fractions, fraction 2 exhibited approximately 80% calcium solubilization capacity, which was slightly lower than CPP but still significant (Fig. 6). These findings suggest that fraction 2 may have potential calcium-solubilizing capabilities.

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Cell viability (MTT assay)

Cytotoxicity tests using the MTT assay are commonly employed in in vitro toxicology studies. The experimental findings demonstrated that higher concentrations enhanced cell viability. Furthermore, all fractions maintained cell viability above 80% (Fig. 7). These results confirmed that the macrophage fractions were non-cytotoxic.

Nitric oxide (NO) assay

Nitric Oxide (NO) plays a crucial role in blood coagulation, blood pressure regulation, and immune responses

in cancer cells. However, it is oxidized into Reactive Oxygen Species (ROS), leading to the formation of active NO. These reactive species can further generate oxidants, which may result in cytotoxicity. NO production increases in cells exposed to inflammatory mediators due to tissue damage and inflammatory diseases. The results of this study indicated that NO production was suppressed as the concentration of the fractions increased (Fig. 8). Additionally, MTT assay results confirmed that the inhibition of NO production was not due to cytotoxic effects.

Cytokine production assay

Maintaining immune homeostasis requires either direct or indirect communication between immune cells. Cytokines play a critical role in regulating immune responses by promoting the proliferation, differentiation, and functional modulation of various immune cells. Many diseases are closely linked to inflammatory processes, during which immune cells release proinflammatory cytokines that drive inflammation (Barland et al., 2004). In the present study, the expression levels of IL-1α, IL-6, and TNF-α were quantified. ELISA-based cytokine analysis revealed that the production of these three cytokines was significantly reduced in all sample-treated groups compared to the LPS-stimulated positive control. These findings indicate that the hydrolysate fractions exhibit anti-inflammatory properties. Furthermore, the notably decreased secretion of TNF-α by macrophages treated with the fractions may contribute to the suppression of other proinflammatory cytokines, as TNF-α plays a central role in triggering downstream inflammatory cascades (Fig. 9). This suggests a potential mechanism by which the samples may alleviate inflammatory responses.

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Profiling of amino acids (AccQ-Tag system)

Following these findings, fraction 2 was chosen for further analysis. Using the AccQ-Tag system to determine the amino acid composition of the selected fraction, a total of 17 distinct amino acids were identified, along with several unidentified amino acid components (Fig. 10, Table 11). Threonine, leucine, phenylalanine, and valine are amino acids that stimulate muscle protein synthesis.

Peptide identification by mass spectrometry

Peptide separation and sequence identification of fraction 2 was performed using LC-MS and LC-MS/MS analysis. Mass spectrometry revealed the presence of numerous peptides, though only two were completely sequenced, indicating that further purification of fraction 2 might be necessary. Two peptides were identified in this fraction (Table 12), with molecular weights of 902.457 Da and 804.446 Da, respectively. The corresponding molecular masses and MS/MS fragmentation patterns are presented in Figs. 11 and 12.

Discussion

The canine gut microbiome has a unique composition and physiological characteristics that are different from humans and other animals, and previous studies suggest that canine-derived probiotics may be effective in improving gut health and immune function in dogs (Kumar et al., 2017; Lin et al., 2023; Liu et al., 2024). It is anticipated that the biochemical metabolites produced by strains of canine origin may confer greater benefits to dogs, given their host-specific adaptation and compatibility. In this study, the functional characteristics of whey-derived peptides were examined using lactic acid bacteria isolated from canine feces as fermentation agents.

In the calcium solubilization test, fraction 2 demonstrated a solubilization level of 91.8% relative to CPP.
The superior calcium solubilization capacity of casein phosphopeptides (CPP) is attributed to their peptide sequences, which are rich in serine (Ser) and glutamic acid (Glu) residues. These residues confer a multi-negative charge, enabling stable chelation with calcium ions (Guo liu et al., 2022). LC/MS sequence analysis of the fermented whey peptide fraction 2, produced using *Lactiplantibacillus plantarum* D7, identified two peptide sequences: RELEELN (Arg-Glu-Leu-Glu-Glu-Leu-Asn) and VQTSTAV (Val-Gln-Val-Thr-Ser-Thr-Ala-Val). Notably, RELEELN exhibits a high degree of similarity to the previously reported CPP sequence REELEELN, characterized by multiple glutamic acid (E) residues that impart a negative charge. Furthermore, REELEELN exhibits 71.4% sequence identity (5/7 residues) at the N-terminal region with the β-casein-derived mineral-binding peptide RELEELNVPGEIVESLSSSEESITR, suggesting conserved structural homology that may underpin shared calcium-chelating functionalities (Skrzypczak et al., 2019). Although this sequence does not directly correspond to the phosphorylation sites of conventional CPP, its structural resemblance suggests

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potential functionality related to calcium ion binding.

Amino acid analysis of fraction 2, a whey peptide fraction fermented with *Lactoplantibacillus plantarum* D7, revealed a composition enriched with leucine, valine, isoleucine, serine, threonine, and aromatic amino acids such as phenylalanine and tyrosine. Notably, the branched-chain amino acids (BCAAs) leucine, valine, and isoleucine are known to regulate calcium absorption, suggesting that fraction 2 may exhibit a high calcium absorption rate (Mohammad et al., 2022). This hypothesis is further supported by previous studies indicating that peptides with sequences similar to casein phosphopeptides (CPP) can enhance calcium absorption (Yong et al., 2017; Liu et al., 2021). In addition, fraction 2 was found to contain arginine and lysine that are absent in conventional CPP but are critical for osteoblast formation highlighting its potential as an alternative calcium absorption enhancer. Among the amino acids in fraction 2, threonine was the most abundant. Threonine is not only essential for muscle function and bone formation (Ziquan et al., 2022) but also plays a crucial role in mucosal immunity and immune modulation. The high content of serine, an amino acid critical for phosphorylation and calcium binding in CPP, further indicates a strong potential for mineral-binding capacity. Furthermore, the presence of aromatic amino acids, which are known for their antioxidant properties, contributes to the overall bioactivity of fraction 2.

The antioxidant activity test revealed that whey peptides fermented with *Lactiplantibacillus plantarum* D7 exhibited significant antioxidant activity. These findings are consistent with previous studies that have demonstrated the antioxidant effects of whey fermented with *L. spp* strains (Skrzypczak et al., 2020; Li and Chen, 2024). This antioxidant effect can be attributed to the presence of aromatic amino acids, such as phenylalanine and tyrosine, which are known for their radical-scavenging properties. Specifically, these aromatic amino acids can donate hydrogen atoms to electron-deficient radicals through a process known as resonance stabilization, thereby enhancing the radical-scavenging capacity of the peptides (Ruijia Hu et al., 2020).

The anti-inflammatory activity of fraction 2 was also confirmed in this study. Fraction 2 was found to suppress

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the secretion of pro-inflammatory cytokines and their associated mediators, highlighting its potential role as an anti-inflammatory peptide. While the REELEELN sequence shares structural homology with immunomodulatory peptides, direct evidence for its anti-inflammatory activity remains limited in current literature. In contrast, VQTSTAV is recognized as a bioactive peptide with notable antioxidant, antimicrobial, and immunomodulatory properties (López-Expósito et al., 2006). This sequence analysis indicates that the peptides identified in fraction 2 may serve as alternatives to conventional CPP, offering not only enhanced antioxidant and antimicrobial activities but also immunomodulatory capacity.

The fermented peptides demonstrated antioxidant, calcium-solubilizing, and anti-inflammatory properties through *in vitro* assays, highlighting their potential as functional ingredients. However, as these findings are based solely on *in vitro* evaluations, they may not fully reflect the complex physiological conditions of living organisms. To address this limitation, *in vivo* validation particularly in companion animals such as dogs is essential. Previous studies have shown that casein phosphopeptide (CPP) administration in beagle dogs can enhance immune function by increasing serum cytokine and immunoglobulin levels, without inducing toxicity (Wang et al., 2022). These results suggest that similar bioactive peptides may confer physiological benefits in canines. Despite this potential, *in vivo* studies remain scarce. To bridge this research gap, we plan to conduct well-designed *in vivo* trials to evaluate the health effects of whey derived peptides in dogs. Our future investigations will focus specifically on their roles in alleviating joint inflammation and supporting muscle health both of which are critical for canine well-being. These studies will not only confirm the *in vivo* bioactivity of the peptides but also provide insights into their bioavailability, metabolic pathways, and applicability in functional food development. Ultimately, this research aims to support the creation of targeted dietary solutions to promote canine health. The findings of this study serve as a foundational step toward the development of functional food ingredients specifically designed to support the well-being of companion animals.

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Tables

Table 1. The chemical composition of whey protein concentrates

Sample	Protein(%)	Moisture(%)	Fat(%)
Hilmar^{TM} 8000 Whey Protein Concentrate	81.88	4.30	6.70

Table 2. Fast protein liquid chromatography (FPLC) conditions

Instrument	Condition
Column	Hiprep 16/60 Sephacryl S-100 HR column (GE Healthcare Life Sciences)
Mobile phase	0.05 M Sodium phosphate buffer (0.15 M NaCl, pH 7.0)
Detector (Detection)	Waters Dual λ Absorbance Detector W2487 (280mm)
Flow rate	1 mL/min
Injection volume	1 mL

Table 3. The amino acid analysis conditions (HPLC)

Time (min)	% A	% B	Curve
0.00	100	0	*
0.5	98	2	6
15.0	93	7	6
19.0	90	10	6
32.0	67	33	6
33.0	67	33	6
34	0	100	6
37	0	100	6
38	100	0	6

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Table 4. The amino acid analysis conditions (HPLC)

Instrument	Condition
Column	Waters AccQ-Tag™ (Waters Co., USA)
Column temperature	37 ℃
Mobile phase	A:Waters AccQ-Tag eluent A B: 60% (v/v) acetonitrile in water
Detector (Detection)	Waters 474 scanning fluorescence detector
Flow rate	1 mL/min
Injection volume	5 μL

Table 5. Chromatographic conditions for LC-MS

Instrument	LC Condition	
Analysis instrument	Vanquish UHPLC (Thermo Fisher Scientific Inc., USA)	
Column	AdvanceBio Peptide Mapping 120Å, 2.1 x 100 mm, 2.7 μm	
Injection volume	10 μL	
Flow rate	100 μL/min	
Column temperature	30°C	
Mobile phase/Time (min)	Solvent composition	
	A(%)	B(%)
0	98	2
2.0	98	2
25.0	80	20
30.0	50	50
31.0	2	98
36.0	2	98
37.0	98	2
45.0	98	2
Instrument	**Q Exactive Plus (Thermo Fisher Scientific Inc., USA)**	
Polarity	Positive	

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Flow rate	200 μL/min	
Ionization mode	ESI, positive mode	
Scan type: Full MS	Resolution	70,000
	AGC target	3e6
	Maximum IT	100 ms
	Scan range	200 to 1500 m/z
Scan type: dd-MS2	Resolution	17,500
	AGC target	1e5
	Maximum IT	100 ms
	Isolation window	1.6 m/z
	(N)CE / stepped nce	16

A: H_2O/FA = 100/0.1 (v/v), B; Acetonitrile/FA = 100/0.1 (v/v)

Table 6. Identification of strains isolated from dog feces with the 16s-rRNA sequence

Strain	**16s-rRNA sequence**	
	species	**% ID**
D2	*Lactobacillus acidophilus* strain L2-16 16S ribosomal RNA gene, partial sequence	99
D3	*Lactobacillus acidophilus* strain L2-16 16S ribosomal RNA gene, partial sequence	99
D4	*Enterococcus faecalis* strain BSR2 16S ribosomal RNA gene, partial sequence	99
D5	*Lactobacillus acidophilus* strain L2-16 16S ribosomal RNA gene, partial sequence	97
D6	*Lactobacillus acidophilus* strain L2-16 16S ribosomal RNA gene, partial sequence	99
D7	*Lactobacillus plantarum* strain KLB 415 16S ribosomal RNA gene, partial sequence	96
D8	*Lactobacillus paracasei* strain CAU5144 16S ribosomal RNA gene, partial sequence	99

Table 7. Survival of lactic acid bacteria D strains in artificial gastric acid

Strains	**Viable cell Counts (CFU/mL)**				
	0 h	**2 h**			
		pH 7.0 (control)	**pH 1.5**	**pH 2.0**	**pH 3.0**

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LA-5	1.3×10^9	1.6×10^8	2.2×10^5	8.3×10^7	2.5×10^7
D2	4.5×10^9	7.6×10^7	3.8×10^5	5.3×10^7	1.5×10^8
D3	3.8×10^9	5.0×10^7	2.5×10^6	1.2×10^8	1.2×10^8
D5	4.1×10^9	4.6×10^8	2.9×10^6	4.8×10^8	7.4×10^8
D6	3.1×10^9	1.3×10^7	2.8×10^5	2.9×10^7	7.2×10^7
D7	3.9×10^9	3.1×10^8	2.4×10^6	5.3×10^8	6.5×10^8
D8	1.7×10^9	1.2×10^7	2.9×10^5	1.2×10^7	2.2×10^7

*All value were mean ± SD of triplicates

*All samples incubated at 37 ℃ for 24h in broth containing 0.3% Oxgall

*LA-5: *Commercial strain Lactobacillus acidophilus*

D2, D3, D5, D6: Lactobacillus acidophilus*

D7: Lactiplantibacillus plantarum*

D8: Lacticaseibacillus paracasei*

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Table 8. Bile acid tolerance of lactic acid bacteria D strains for 24h

Strains	**Counting(CFU/mL)**			
	24h			
	pH 7.0 (control)	**pH 3.0**	**pH 2.0**	**pH 1.5**
LA-5	1.3×10^9	7.1×10^6	3.1×10^5	7.2×10^3
D2	1.2×10^9	2.6×10^6	1.2×10^5	3.1×10^4
D3	2.2×10^9	3.4×10^6	3.1×10^5	2.0×10^4
D5	2.1×10^9	9.8×10^6	3.3×10^5	6.4×10^4
D6	2.1×10^9	6.8×10^6	3.2×10^5	3.2×10^4
D7	2.1×10^9	2.7×10^7	3.3×10^6	6.5×10^5
D8	2.4×10^9	2.9×10^6	3.9×10^5	1.2×10^4

*All value were mean ± SD of triplicates

*All samples incubated at 37 ℃ for 24h in broth containing 0.3% Oxgall

*LA-5: *Commercial strain Lactobacillus acidophilus*

D2, D3, D5, D6: Lactobacillus acidophilus*

D7: Lactiplantibacillus plantarum*

D8: Lacticaseibacillus paracasei*

Table 9. Bile acid tolerance of lactic acid bacteria D strains for 48h

Strains	Counting(CFU/mL)			
	48h			
	pH 7.0 (control)	pH 3.0	pH 2.0	pH 1.5
LA-5	7.5×10^7	2.7×10^6	1.6×10^4	4.0×10^3
D2	2.2×10^8	3.4×10^6	3.1×10^6	2.0×10^3
D3	4.3×10^7	1.3×10^6	3.2×10^5	N.D
D5	1.1×10^8	9.8×10^6	3.3×10^6	6.4×10^3
D6	1.1×10^8	6.8×10^6	3.2×10^5	1.2×10^3
D7	5.1×10^7	2.7×10^7	5.3×10^6	6.5×10^3
D8	2.4×10^7	2.9×10^6	3.9×10^5	N.D

*All value were mean ± SD of triplicates

*All samples incubated at 37 ℃ for 48h in broth containing 0.3% Oxgall

* N.D: Not detected < 10^2

*LA-5: *Commercial strain Lactobacillus acidophilus*

D2, D3, D5, D6: Lactobacillus acidophilus*

D7: Lactiplantibacillus plantarum*

D8: Lacticaseibacillus paracasei*

ACCEPTED

Table 10. Measurement of proteolytic activity of probiotic lactic acid bacteria isolated from dog fe ces at 24, 48, and 72 hours

Sample	Diameter of clear zone (mm)		
	24h	48h	72h
LA-5	11±0.12	13±0.16	23±0.21
D2	13±0.12	14±0.24	20±0.11
D3	12±0.02	14±0.22	21±0.16
D5	14±0.11	18±0.17	25±0.09
D6	13±0.01	15±0.12	23±0.02
D7	15±0.13	19±0.18	26±0.21
D8	12±0.10	14±0.20	22±0.22

*All value were mean ± SD of triplicates

*Diameter of clear zone: mm

*All samples incubated at 37 ℃

*LA-5: *Commercial strain Lactobacillus acidophilus*

**D2, D3, D5, D6: Lactobacillus acidophilus*

**D7: Lactiplantibacillus plantarum*

**D8: Lacticaseibacillus paracasei*

ACCEPTED

Table 11. Amino acids concentration in fraction 2

AAs	RT[1)]	Area (%)[2)]	Amino acid content(pmol/uL)[3)]
	Complex Peptides		
Asp	12.105	861775	329.840
Ser	13.333	2682535	811.706
Glu	14.265	1670472	592.867
Gly	15.072	3434452	967.440
His	16.018	1039059	265.638
Arg	20.050	583283	155.154
Thr	20.515	5474927	1097.881
Ala	22.058	1815415	460.810
Pro	25.065	1460623	666.224
Cys + Tyr	28.731	2096539	542.564
Val	30.169	4389837	510.061
Met	32.498	2166372	287.712
Lys	33.109	573279	129.081
Ile	33.558	6241195	560.661
Leu	34.103	11848250	945.565
Phe	34.401	8157521	537.084

ACCEPTED

* [1]: Retention time, [2]: Amount of total amino acids (%), [3]: Amino acid content(ρmol/μL) in fraction 2

Table 12. Characterization of fraction 2 identified by LC-MS

Peak No.	RT	m/z	Charge	Calc. mass[M+H]	Sequence
1	21.46	451.733	2	902.457	RELEELN
2	22.75	402.727	2	804.446	VQVTSTAV

Figures

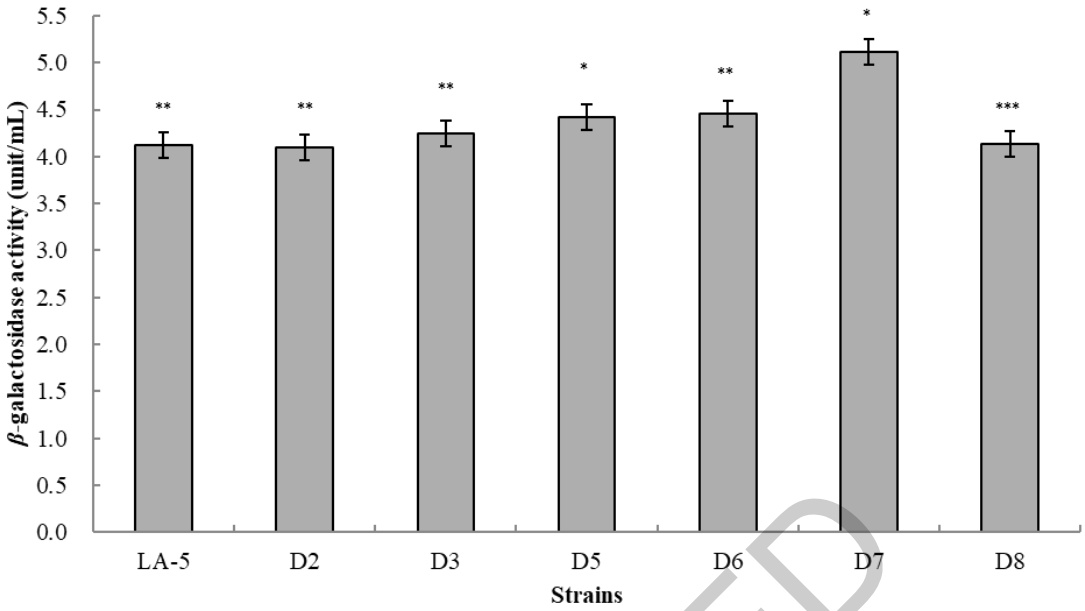

Fig. 1. β-galactosidase activity of probiotic lactic acid bacteria isolated from dog feces

*LA-5: Commercial strain *Lactobacillus acidophilus*

*D2, D3, D5, D6: *Lactobacillus acidophilus*

*D7: *Lactiplantibacillus plantarum*

*D8: *Lacticaseibacillus paracasei*

*Statistical difference : *p<0.05, **p<0.01, ***p<0.001

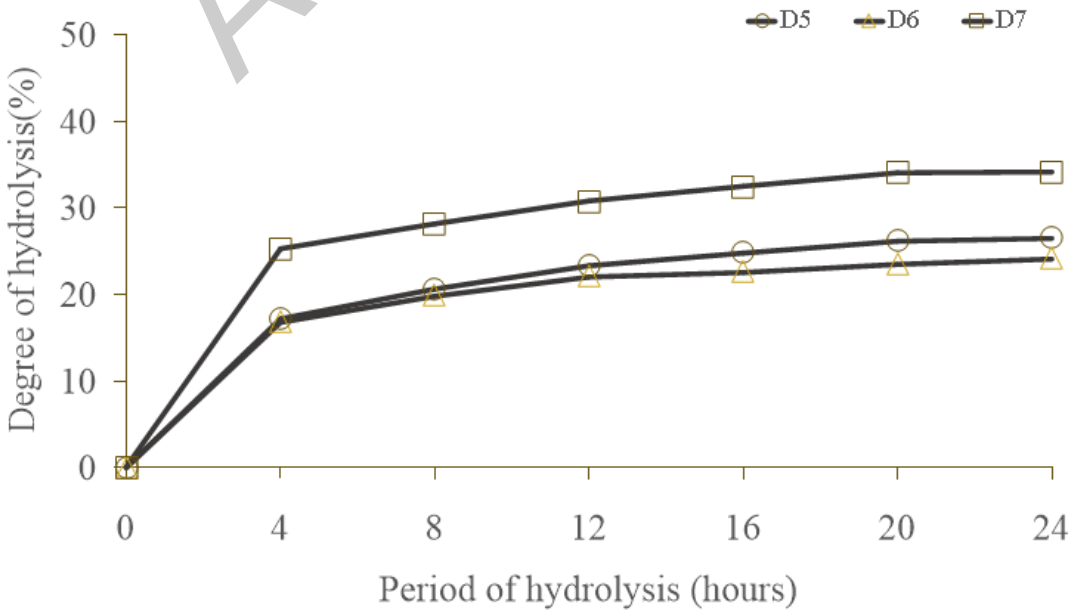

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Fig. 2. Degree of hydrolysis (DH%) of fermented whey protein concentrate with D strains

*Reactions were carried out in a water bath (37 ℃, 150 rpm)

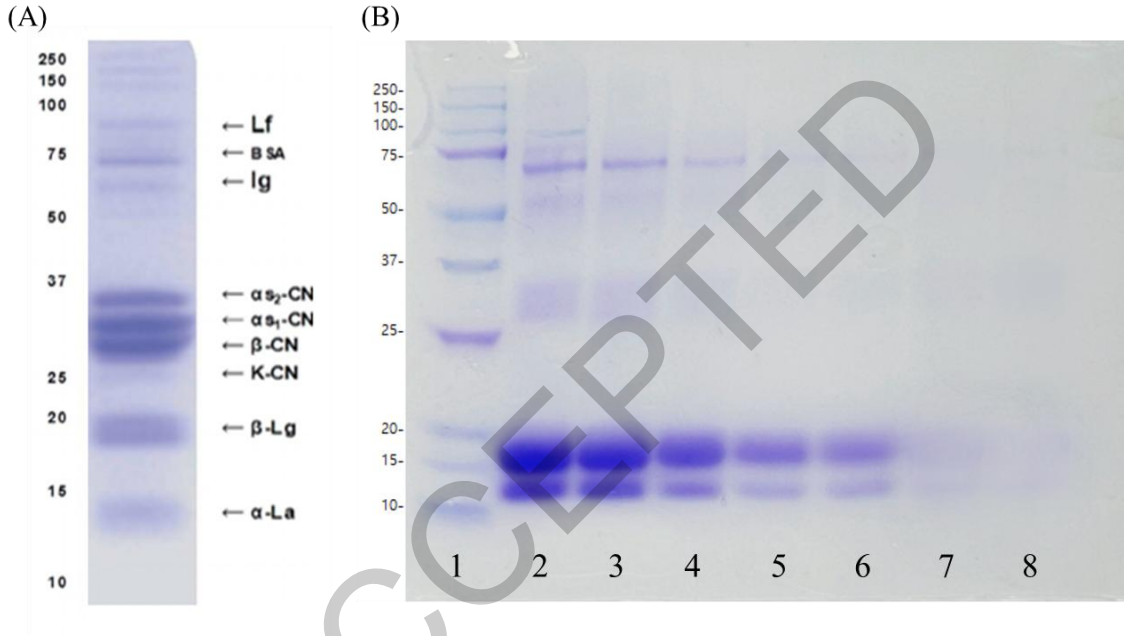
*All value were mean ± SD of triplicates

*All LAB were applied at a concentration of 1% (v/v)

*D5, D6: *Lactobacillus acidophilus*

*D7: *Lactiplantibacillus plantarum*

Fig. 3. SDS-PAGE patterns of fermented whey protein concentrate with D7 for each time



*(A): Raw bovine milk

*(B) lane 1: Precision plus protein dual xtra standards (Bio-rad, U.S.A); lane 2, 3, 4, 5, 6, 7, 8: hydrolysates obtained after 0, 4, 8, 12, 16, 20, 24 hours of hydrolysis

*D7: *Lactiplantibacillus plantarum*

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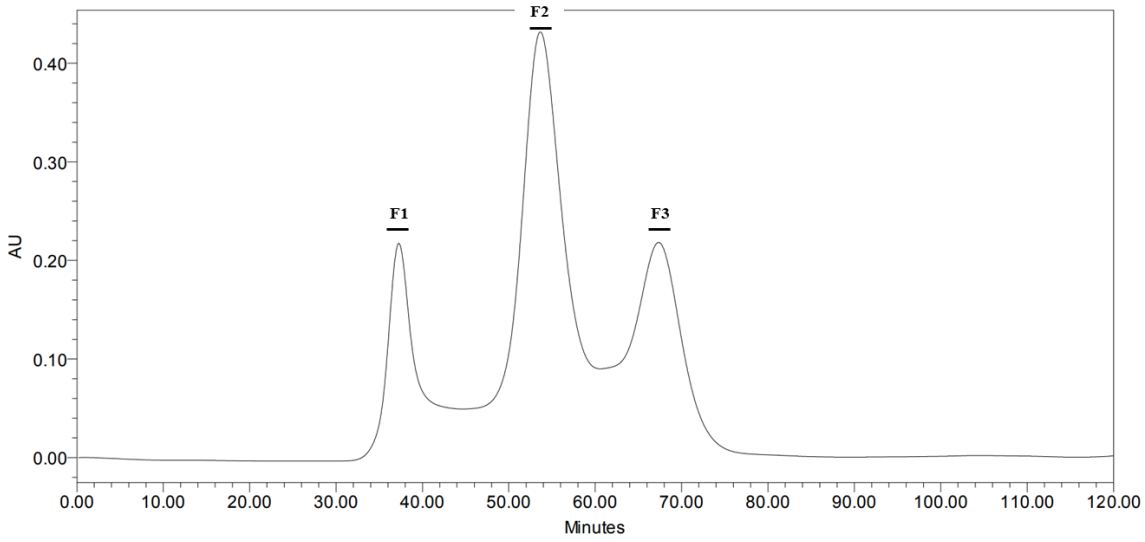

Fig. 4. Separation offermented whey protein concentrate using D7 (20h) in FPLC system on Hiprep 16/60 Sephacryl S-100R column

*F#: Fraction number in FPLC system

*D7: *Lactiplantibacillus plantarum*

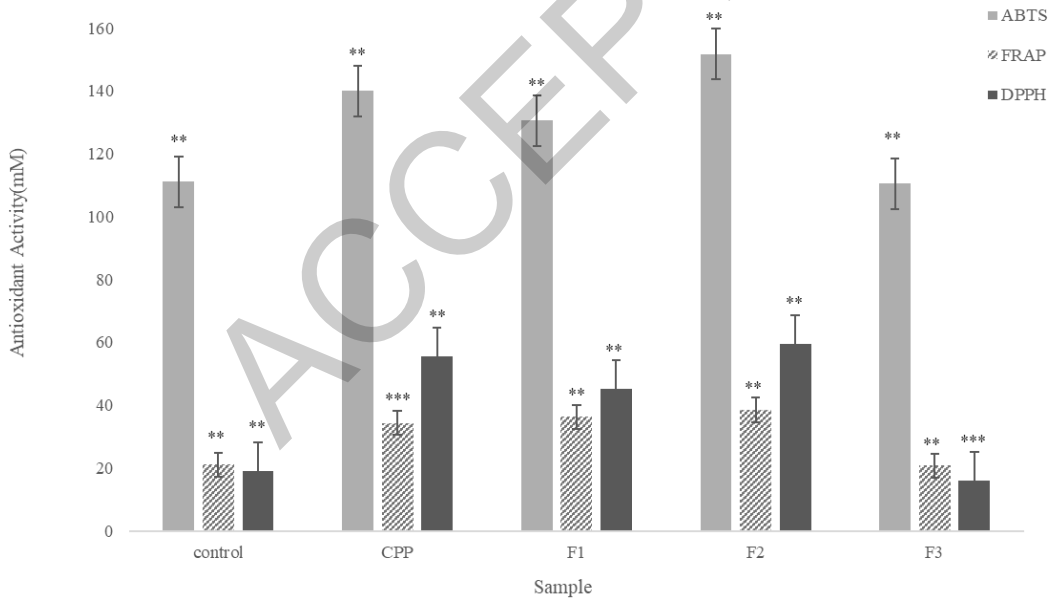

Fig. 5. Antioxidant activities of fermented whey protein concentrate fractions

*Control : WPC solution after pasteurization

*CPP: Casein phosphopeptide

*F#: Fraction number in FPLC system

*All samples protein concentration were 200 μg/mL

*Statistical difference: *p<0.05, **<0.01, ***p<0.001

ACCEPTED

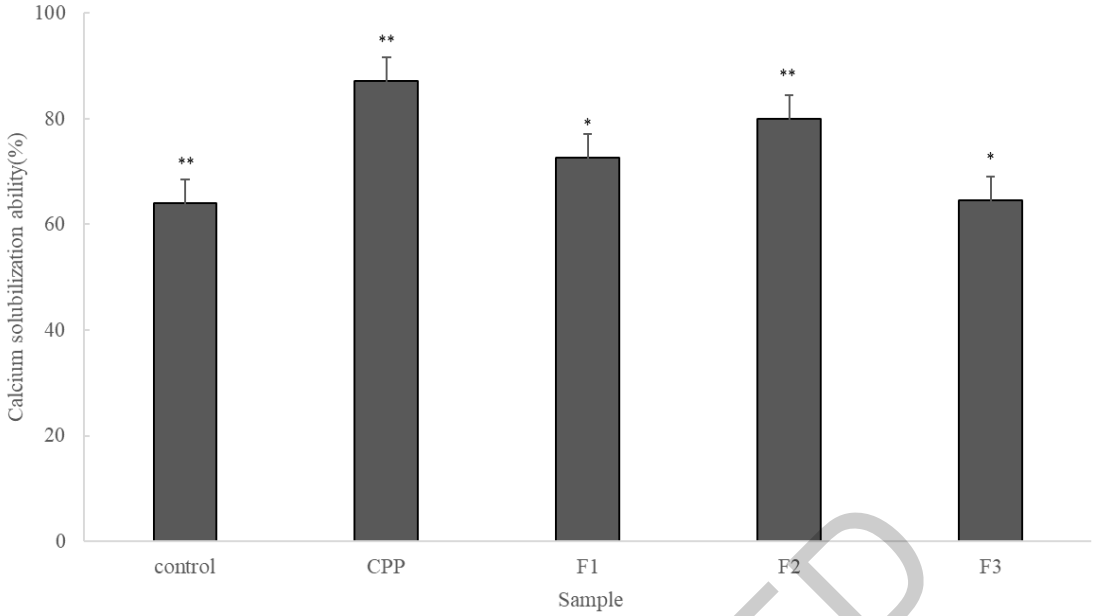

Fig. 6. Calcium solubilization capacity of fermented whey protein concentrate fractions

*Control : WPC solution after pasteurization

*CPP : casein phosphopeptide

*F# : fraction number in FPLC system

*All samples protein concentration were 2.0mg/mL

*Statistical difference *p<0.05; **p<0.01

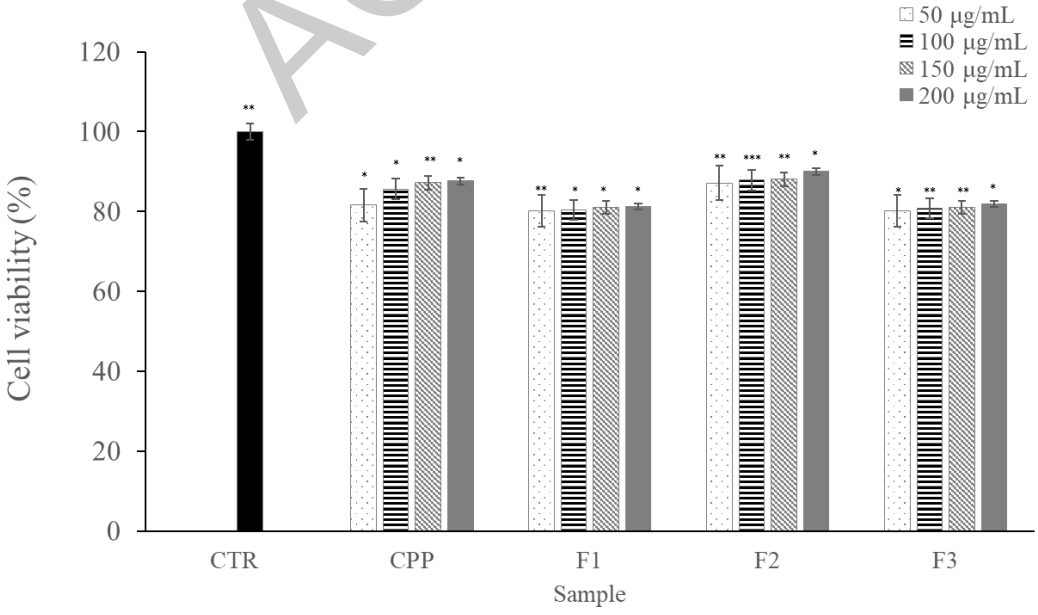

Fig. 7. Effect of fermented whey protein concentrate fractions on cell viability in RAW 264.7 cells

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*CPP: Casein phosphopeptide

*F#: Fraction number in FPLC system

*200, 150, 100, 50 µg/mL: Samples protein concentration

*All values were mean ± SD of triplicates.

*Statistical difference: *p<0.05, **p<0.01, ***p<0.001

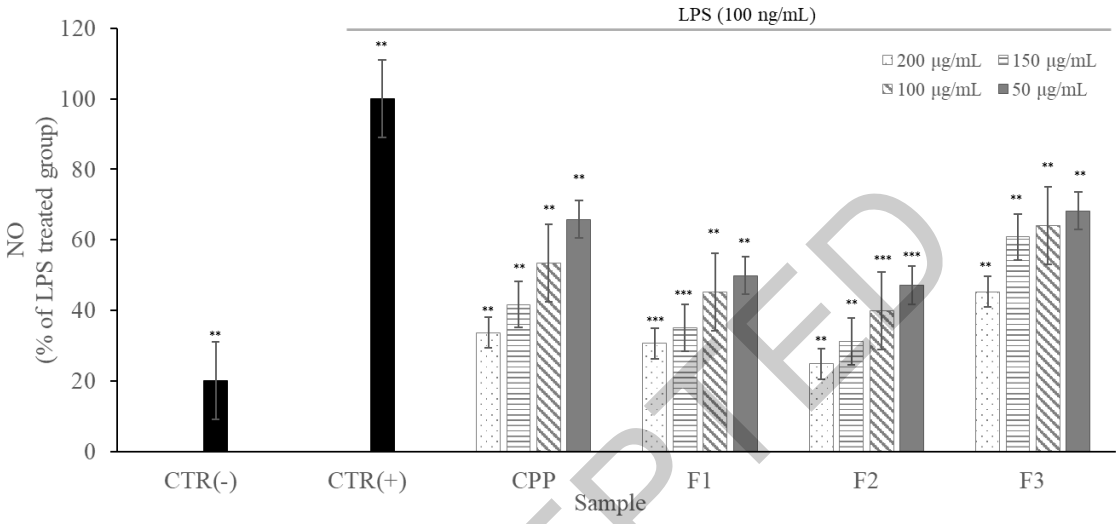

Fig. 8. Effect of fermented whey protein concentrate fractions on nitric oxide in LPS activated RAW 264.7 cells

*CPP: Casein phosphopeptide

*F#: Fraction number in FPLC system

*200, 150, 100, 50 µg/mL: Samples protein concentration

*Statistical difference: *p<0.05, **p<0.01, ***p<0.001

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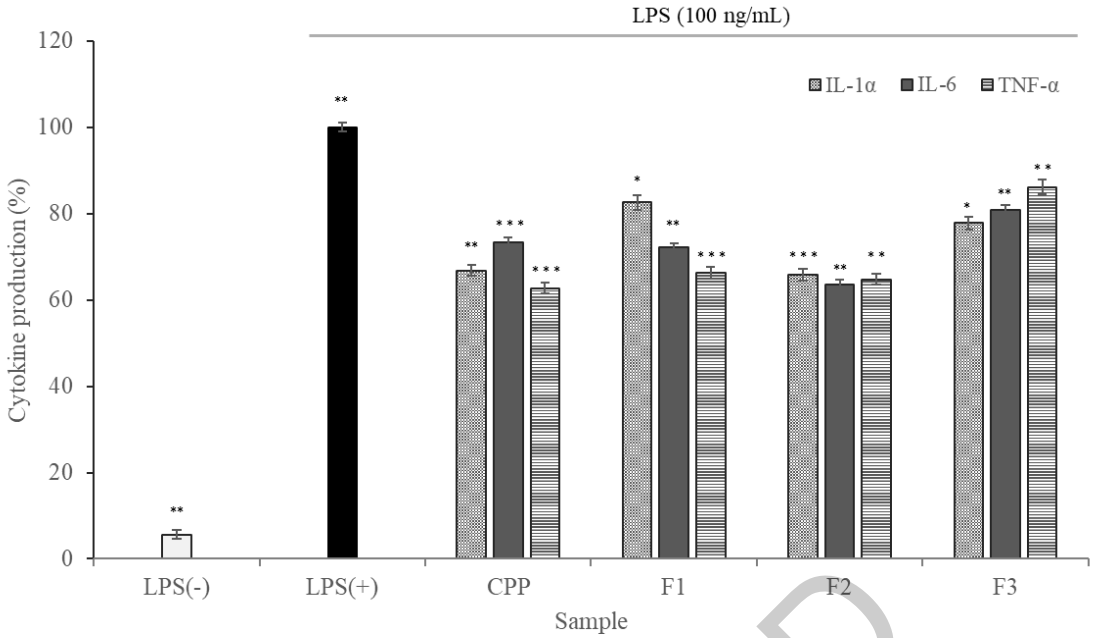


61

62 **Fig. 9. Comparison of IL-1α, IL-6 and TNF-α production of plant in LPS-unstimulated RAW264.7 cells**

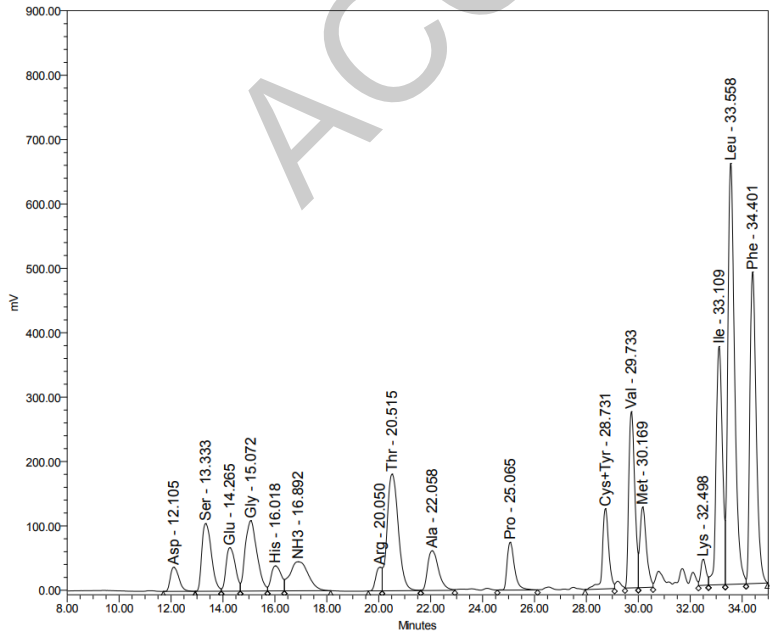
63 *CPP: Casein phosphopeptide

64 *F#: Fraction number in FPLC system

65 *200 μg/mL: Samples of protein concentration

66 *Statistical difference: *p<0.05, **p<0.01, ***p<0.001

67



68

69 **Fig. 10. HPLC chromatogram of amino acids of the fraction 2**

ACCEPTED

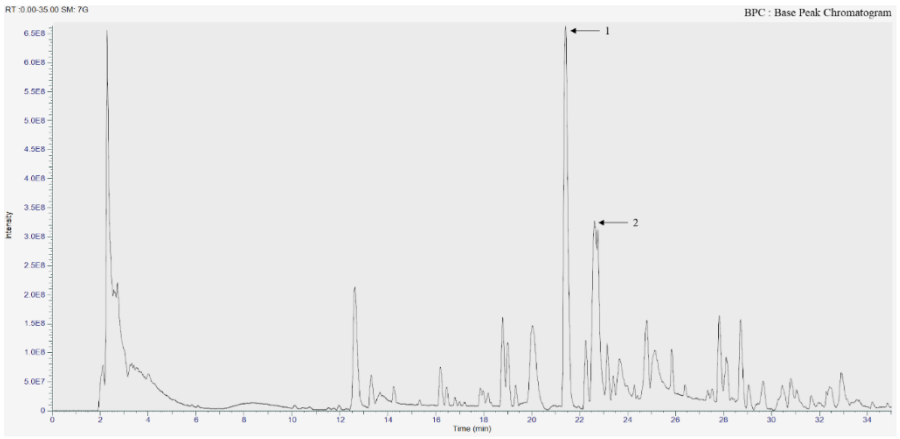


Fig. 11. LC-MS base peak chromatogram of fraction 2

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(1)

(2)

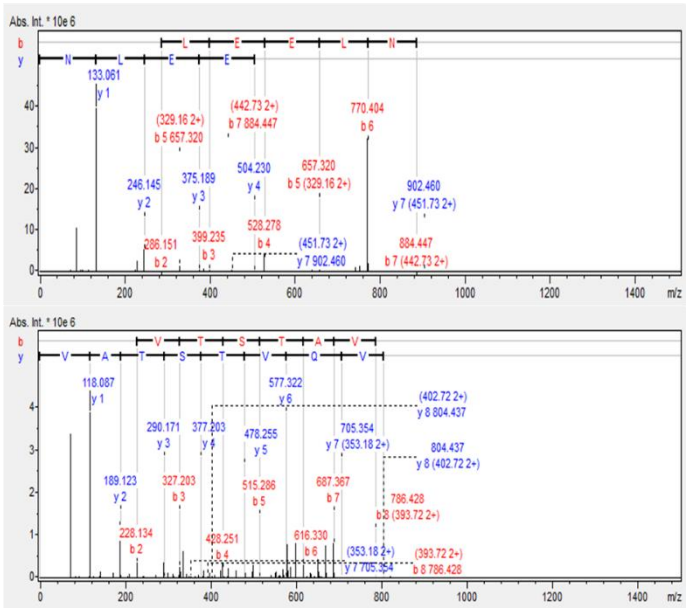

Fig. 12. Mass spectrometric de novo sequencing

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