APPLICATION OF *Lacticaseibacillus casei* JCM 1134^T FOR THE PRODUCTION AND PARTIAL ANALYSIS OF BIOACTIVE PEPTIDES ENCODED IN CARABAO MILK

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ABSTRACT

This study was conducted to generate and characterize bioactive peptides encrypted in carabao milk through lactic acid bacteria (LAB) proteolysis. Thirteen LAB strains from the genus *Lactococcus, Lactiplantibacillus, Latilactobacillus, Pediococcus, Leuconostoc, Weissella, Lacticaseibacillus, Lactobacillus*, and *Limosilactobacillus* were screened for proteolytic activity using litmus milk test (LMT) and pH measurement. Lacticaseibacillus casei JCM 1134^T was selected to ferment (3% v/v) carabao milk at 37°C for 5 days. Milk protein hydrolysate from Lacticaseibacillus casei JCM 1134^T-fermented carabao milk was purified and separated using hydrophobic interaction chromatography (HIC), ion exchange chromatography (IEC), ultrafiltration (UF), reversed-phase high performance liquid chromatography (RP-HPLC), and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fractions were tested for antimicrobial (microbial inhibition), antioxidant (DPPH free radical scavenging), and antihypertensive (ACE inhibition) activities. Some fractions only had antimicrobial activity against *Bacillus subtilis* JCM 1465^T, antioxidant activity decreased as the purification process progressed, and high antihypertensive activity was observed in hydrolysates and retentates from whey and casein proteins. However, when subjected to electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS), all of the peaks were impure and inactive. These peaks underwent another RP-HPLC run but cannot be detected anymore. Further optimization of purification methods to generate bioactive peptides is highly recommended.

Keywords: bioactive peptides, carabao milk, fermentation, lactic acid bacteria, proteolysis

INTRODUCTION

Consumer preference and intake are shifting toward functional foods due to the high prevalence of diet-related chronic diseases such as cancer, diabetes, hypertension, and heart diseases. Aside from the basic role of food in providing energy, functional foods exhibit health-promoting properties including antimicrobial, antioxidant, and antihypertensive activities. Biologically active or "bioactive" peptides derived from milk fermented with lactic

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acid bacteria (LAB) are considered to be examples of functional foods (Donkor *et al*., 2007). In this study, carabao milk was fermented with LAB to generate potential bioactive peptides. The importance of using carabao milk was mainly due to these reasons: 1) it is produced by carabaos which are domestic swamp-type water buffaloes native to the Philippines, 2) limited research has been conducted on the biomolecular characterization of its potential bioactive peptides, 3) possible additional monetary and nutritional value benefitting Filipino dairy farmers and consumers, respectively, and, 4) addresses the "product development and processing" section in the Livestock Research and Development Agenda 2022-2028 in the Updated Harmonized National Research and Development Agenda in Agriculture, Aquatic and Natural Resources (HNRDA-AANR) published by the Department of Science and Technology-Philippine Council for Agriculture, Aquatic and Natural Resources Research and Development (DOST-PCAARRD, 2021).

Strains of LAB were used to ferment carabao milk because they are generally recognized as safe (GRAS) microorganisms as evident in manufacturing commercial dairy products such as fermented milk, yogurt, and cheese. But most importantly, they were reported to possess an efficient proteolytic system capable of releasing bioactive peptides encrypted in the native milk protein sequence. These microorganisms have cell wall bound proteinases that initiate the hydrolysis of extracellular milk proteins into oligopeptides, tripeptides, and dipeptides. Hydrolyzed peptides are subsequently taken up into the microbial cell through specific peptide transporters and undergo further degradation into shorter peptides and free amino acids (Savijoki *et al.*, 2006). Carabao milk is primarily comprised of two major milk proteins namely casein and whey proteins. Casein proteins consist of α_{s1}-casein (α_{s1}-CN), α_{s2}-casein (α_{s2}-CN), β-casein (β-CN), and κ-casein (κ-CN) while whey proteins consist of α-lactalbumin (α-LA) and β-lactoglobulin (β-LG). These milk proteins differ in amino acid sequence, hydropathy index, and degrees in glycosylation and phosphorylation (Pessione, 2012). Hydrolytic cleavage of these milk proteins can produce antimicrobial (microbial inhibition), antioxidant (free radical scavenging), and antihypertensive (ACE inhibition) peptides that can potentially modulate physiological systems in the human body (Thakur *et al*., 2012).

Every LAB strain has varying proteolytic activity resulting to varying decrypted bioactive peptides. Nowadays, bioactive peptides can be purified and separated using different approaches such as hydrophobic interaction chromatography (HIC), ion exchange chromatography (IEC), ultrafiltration (UF), reversed-phase high performance liquid chromatography (RP-HPLC), and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Methods such as electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS) and amino acid sequencing can characterize the bioactive peptides and determine which major milk proteins they were hydrolyzed from. In addition, structural analysis using various software can potentially elucidate the structure-activity relationship of the bioactive peptides. The overall aim of this study was to generate and characterize bioactive peptides encrypted in carabao milk through LAB proteolysis.

MATERIALS AND METHODS

Propagation of lactic acid bacteria strains

Table 1 showed the thirteen LAB Strains that were used in this study along with their

accession number, incubation temperature, and isolation source. Activation was performed by inoculating 1% (v/v) or 50 µL of each LAB strain from its previous glycerol stock in to 5 mL of de Man, Rogosa, and Sharpe (MRS) broth then incubated at 30°C or 37°C for 24 hours. The inoculated MRS broth was subcultured to another MRS broth with the same incubation condition to prepare a working culture. A 15% (v/v) glycerol stock of each LAB strain was prepared by inoculating 900 µL of working culture into 900 µL of 30% (v/v) glycerol stock. The working culture can be stored at 4°C and used for approximately one month. The glycerol stock can be stored indefinitely in a biomedical freezer at -80°C. Each vial of glycerol stock can be used for activation for three times.

Table 1. Lactic acid bacteria strains that were used in the study.

STRAIN	CODE	ACCESSION NUMBER	INCUBATION TEMPERATURE	ISOLATION SOURCE
Lactococcus lactis subsp. *lactis*	A1	ATCC 19435	30°C	dairy starter
Lactococcus raffinolactis	A2	JCM 5706^T	30°C	raw milk
Lactiplantibacillus plantarum subsp. *plantarum*	A3	JCM 1149^T	30°C	pickled cabbage
Latilactobacillus curvatus	A4	JCM 1096^T	30°C	milk
Pediococcus acidilactici	A5	JCM 8797^T	30°C	barley
Leuconostoc mesenteroides subsp. *mesenteroides*	A6	JCM 6124^T	30°C	fermenting olives
Weissella minor	A7	JCM 1168^T	30°C	milking machine slime
Lacticaseibacillus casei	B1	JCM 1134^T	37°C	cheese
Lactobacillus acidophilus	B2	JCM 1132^T	37°C	human feces
Lactobacillus delbrueckii subsp. *bulgaricus*	B3	JCM 1002^T	37°C	Bulgarian yogurt
Lactobacillus helveticus	B4	JCM 1120^T	37°C	Emmental (Swiss) cheese
Limosilactobacillus fermentum	B5	JCM 1173^T	37°C	fermented beets
Weissella viridescens	B6	JCM 1174^T	37°C	cured meat products

Litmus milk test (LMT)

This method was used to determine the proteolytic activity of each LAB strain in liquid milk media. In a test tube, 1% (v/v) of each working culture was inoculated into 5 mL of litmus milk (10% v/v skimmed milk carabao milk + 0.0005% litmus + 0.0005% NA_2SO_3). Depending on the LAB strain, the tightly sealed test tube was incubated at 30°C or 37°C for 7 days. The metabolic reactions happening in the test tube was recorded for 7 consecutive days. The following can be observed: lactose fermentation (pink color), litmus reduction (white color), milk protein coagulation (curd formation), peptonization (medium clarification), and no fermentation (purple color). These results may appear together in a variety of combinations. Curd formation and medium clarification can be considered as indicators of proteolytic activity (Aryal, 2022). The pH value of each test tube was also measured after 7 days using a pH meter (HM-41X, DOA-TKK Corporation, Tokyo, Japan). It also served as a basis for selecting the LAB strain to be used in the subsequent analyses.

Preparation of fermented carabao milk

High temperature short time (HTST) pasteurized carabao milk was purchased from Philippine Carabao Center (PCC) at the University of the Philippines Los Baños (UPLB). The carabao milk was freeze dried using a fabricated freeze dryer (Gecar Machine Solutions, Inc., Quezon City, Philippines) serviced at the Institute of Food Science and Technology (IFST), College of Agriculture and Food Science (CAFS), UPLB. Freeze drying employs a low temperature dehydration process that generally preserved the native structure of proteins. This preservation method was also used for easy storage and transportation to Kyushu University, Japan. Before every experiment, the freeze dried carabao milk was reconstituted (18% w/v) with Milli-Q water and centrifuged at 12,000 rpm and 4°C for 15 minutes to skim milk fat. Whole carabao milk usually contains 18% (w/v) total solids. Milk fat was removed from the top layer and skimmed milk was passed through one layer of 110 nm qualitative filter paper. The working culture of *Lacticaseibacillus casei* JCM 1134^T was inoculated (3% v/v) into the skimmed carabao milk and incubated at 37°C for 5 days with an AnaeroPack™. The inoculation rate and incubation period were determined during the preliminary experiments.

Table 2. Indicator strains that were used in the study.

STRAIN	ACCESSION NUMBER	INCUBATION TEMPERATURE	MEDIUM
Enterococcus faecalis	JCM 5803^T	37°C	MRS broth
Listeria innocua	ATCC 33090	37°C	TSB-YE
Escherichia coli	JM 109	37°C	TSB-YE
Bacillus coagulans	JCM 2257^T	37°C	TSB-YE
Bacillus subtilis	JCM 1465^T	37°C	TSB-YE
Pseudomonas putida	ATCC 12633	37°C	LBB

Spot-on-lawn assay (SOLA) for antimicrobial activity

To prepare the LAA-MRS agar plate, 1% (v/v) or 100 µL of each indicator strain was inoculated into 10 mL of melted Lactobacilli agar (LAA). The indicator strains used in the study were presented in Table 2. A prepared sterilized MRS agar plate (1.2% w/v) was overlayed with the inoculated LAA and hardened inside a clean bench. The LAA-MRS agar plate was continuously dried inside a 55°C incubator in an inverted position. In the plate, 10 µL of sample was spotted. The spotted plate in an inverted position was incubated for 37°C for 24 hours. The presence of clear zones is indicative of antimicrobial activity (Ennahar *et al*., 2001).

DPPH antioxidant assay (DPPHAA) for antioxidant activity

The principle of this method was to determine if a sample can reduce or scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals. It can be visualized through the color change of the DPPH working solution from purple to colorless. Using the DPPHAA kit, the antioxidant capacity of a sample is expressed as the Trolox equivalent antioxidant capacity (TEAC). However, in this study, TEAC cannot be expressed yet in numerical form since the protein concentration of the samples were still unknown (Shimamura *et al*., 2014; Dojindo Laboratories, 2020a). In the meantime, the inhibition ratio of the samples was measured and calculated from the following equation:

$$\text{Inhibition ratio of sample (\%)} = [(A\,CS - A\,S) / A\,CS] \times 100$$

where: A CS: Blank 1 – Blank 2
A S: Absorbance of samples – Blank 2
Plot the inhibition ratio (y) against the sample concentration (x) and draw a regression line ($y = ax + b$).

ACE inhibition activity assay (ACEIAA) for antihypertensive activity

The colorimetric detection system in the ACE Kit-WST determines the amount of 3-hydroxybutyric acid (3HB) generated from 3-hydroxybutyryl-Gly-Gly-Gly by inhibiting angiotensin converting enzyme (ACE). It can be visualized through the color change of the indicator working solution from yellow to colorless (Dojindo Laboratories, 2020b). ACE inhibition of colored samples can be calculated from the following equation:

$$\text{ACE inhibition (\%)} = [(A\,\text{blank 1} - A\,\text{sample} - A\,\text{sample blank})/(A\,\text{blank 1} - A\,\text{blank 2})] \times 100$$

Hydrophobic interaction chromatography (HIC)

Collection of culture supernatant: The working culture of *Lacticaseibacillus casei* JCM 1134^T was inoculated (3% v/v) into 500 mL of skimmed carabao milk and incubated at 37°C for 5 days in a tightly sealed reagent bottle. After incubation, the fermented carabao milk (FCM) was shaken well and equally dispensed into two 250 mL ultracentrifuge bottles. The bottles were centrifuged at 8,500 *x g* and 4°C for 25 minutes using an ultracentrifuge. The culture supernatant was collected in a 2 L Erlenmeyer flask.

Purification of milk hydrolysate: The culture supernatant was mixed with 30 g of activated-washed Amberlite® XAD16N resin (Supelco, Inc., Pennsylvania, USA). The resin was activated with 100 mL of 50% (v/v) isopropanol for 24 hours and washed with 100 mL of deionized water for five times until the odor disappears. The mixture was shaken for 3

hours in a low temperature for the resin to fully adsorb the culture supernatant. The principle of this method is to separate proteins according to differences in their surface hydrophobicity. It maintains the biological activity of the proteins due to the use of matrices that operate under less denaturing conditions. After shaking, the mixture was poured into a column (30 cm, ø 2.5 cm) and eluted to collect the through fraction. A 100 mL of deionized water was poured into the column and rotationally stirred for 10 minutes. The mixture was eluted, and the first wash 1 fraction was obtained. This step was done for one more time. A 100 mL of 40% (v/v) ethanol was poured into the column and rotationally stirred for 15 minutes. The mixture was eluted, and the first wash 2 fraction was collected. This step was performed for four more times. A 100 mL of 70% (v/v) isopropanol + 0.1% (v/v) trifluoroacetic acid (TFA) was poured into the column and rotationally stirred for 20 minutes. This step was done two times. Every eluted fraction, elution 1 and 2, was collected in reagent bottles. Lastly, a 100 mL of 100% isopropanol was poured into the column and rotationally stirred for 25 minutes. The mixture was eluted, and the wash 3 fraction was obtained. For subsequent use, a 100 mL of 50% (v/v) isopropanol was poured into the column. Its crevices and openings were sealed with parafilm and stored at 4°C (Iwatani *et al*., 2007). Every eluted fraction collected was filtered-sterilized using a 0.20 µm cellulose acetate membrane filter then concentrated using a SpeedVac concentrator with no heat at 05.1 pressure for 40 to 50 minutes. The antimicrobial and antioxidant activity of the culture supernatant and eluted fractions were tested using SOLA and DPPHAA, respectively.

Ion exchange chromatography (IEC)

Purification of HIC elution 1: 100 mL of HIC elution 1 was subjected to a rotary vacuum evaporator at 90 mbar and 40°C for approximately 30 to 40 minutes. The evaporated sample was diluted 5-fold with 50 mM phosphate budder (PB) (30 mL evaporated HIC elution 1 + 120 mL 50 mM PB) and pumped into a column half-filled with degassed-equilibrated SP Sepharose® Fast Flow resin (Cytiva, Massachusetts, USA). It is important that the resin should not be exposed to air at all times. The principle of this method involved the separation of ionizable proteins based on their total charge and affinity to the ion exchanger. The sample passed through the column and eluted to collect the through fraction. A 100 mL of 50 mM PB was pumped into the column and eluted to obtain the wash fraction. 30 mL of 50 mM PB containing 0.5 M NaCl was pumped into the column and eluted to collect the 0.5 M NaCl elution. Lastly, 30 mL of 50 mM PB containing 1.0 M NaCl was pumped into the column and eluted to obtain the 1.0 M NaCl elution. For subsequent use, 100 mL of 50 mM PB was pumped into the column. Its crevices and opening were wrapped with parafilm and stored at 4°C. Every eluted fraction was filtered-sterilized using a 0.20 µm cellulose acetate membrane filter. The antimicrobial and antioxidant activity of the eluted fractions were tested using SOLA and DPPHAA, respectively.

Ultrafiltration (UF)

Fractionation of milk hydrolysate: Ultrafiltration is a pressure-driven membrane method used for protein concentration, purification, or fractionation (protein-protein separation). The FCM was centrifuged at 5,000 x g and 4°C for 10 minutes to separate whey and casein hydrolysates.

For whey hydrolysate: The culture supernatant or whey hydrolysate (W) was obtained and filtered-sterilized using a 0.20 µm cellulose acetate membrane filter. The

W was ultrafiltered using a 1,000 molecular weight cut-off (MWCO) centrifugal device (Macrosep®, Pall Corporation, New York, USA) and centrifuged at 5,000 *x g* and 4°C for 10 minutes. After ultrafiltration, the whey retentate (WR) and whey permeate (WP) were collected.

For casein hydrolysate: The pellet was reconstituted with Milli-Q water back to its initial volume to collect the casein hydrolysate 1 (C1). The C1 was centrifuged at 5,000 *x g* and 4°C for 10 minutes to remove LAB cell pellets and obtain the casein hydrolysate 2 (C2). The C2 was ultrafiltered using a 1,000 or 3,000 MWCO centrifugal filter and centrifuged at 5,000 *x g* and 4°C for 10 minutes. After ultrafiltration, the casein retentate (CR) and casein permeate (CP) were collected. The antimicrobial, antioxidant, and antihypertensive activity of every fraction were tested using SOLA, DPPHAA, and ACEIAA, respectively.

Reversed-phase high performance liquid chromatography (RP-HPLC)

Preparation of samples: HIC eluted fractions (HIC elution 1, HIC elution 2, or HIC wash 3) should be subjected to a rotary vacuum evaporator at 90 mbar and 40°C for approximately 30 to 40 minutes prior to injection. When evaporating HIC wash 3, 30 mL of Milli-Q water was added then evaporated. IEC eluted fractions, WR, WP, CR, and CP can be directly injected into the HPLC machine.

Purification of samples: The principle of this method is to separate proteins on the basis of their hydrophobicity. It mainly depends on the hydrophobic binding of the solute from the mobile phase to the immobilized hydrophobic ligands in the stationary phase. The samples were analyzed using an Atlantis™ T3 column (5 µm, 4.6 mm x 150 mm) (Waters, Wexford, Ireland) in a LC-2000Plus HPLC system (JASCO, Tokyo, Japan). This HPLC system was comprised of a binary inert pump, UV/VIS detector, dynamic mixer, 3-line degasser, and manual injection block. The data acquisition and data processing of the HPLC system was controlled by the HY-PCR software. The elution program consisted of a gradient of Solvent A and Solvent B at a flow rate of 1 mL/min with varying gradient concentrations and run times (see Results and Discussion). Solvent A contains Milli-Q water and TFA in a ration of 99.9:0.1 (v/v) while Solvent B contains acetonitrile and TFA in a ration of 99.9:0.1 (v/). The injection volume was 3 mL. The column was washed (Solvent A 0.100 mL/min and Solvent B 0.900 mL/min) and equilibrated (depending on the initial gradient concentration) prior to every run. Peaks were detected at 220 nm, collected in low protein binding micro tubes, and concentrated using a SpeedVac concentrator with no heat at 05.1 pressure for 40 to 50 minutes. The antimicrobial and antioxidant activity of the RP-HPLC peaks were tested using SOLA and DPPHAA, respectively.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Separation of proteins: The principle of this method is to obtain high resolution separation of proteins based on their molecular weight. In addition, this method was performed to visualize the hydrolysis of milk proteins in carabao milk fermented by LAB. The gel cassette was inserted in the electrophoresis tank (AE-6500, ATTO Corporation, Tokyo, Japan). Anode buffer (Tris) and cathode buffer (Tris/Tricine/SDS) were poured in the outer and inner chamber of the electrophoresis tank. To prepare a sample, 50 µL of 2x SDS buffer and 50 µL of sample was mixed together and heated at 95°C for 5 minutes. 70 µL of the prepared samples and markers (SeeBlue™ Plus2 Pre-stained Protein Standard, 3-198 kDa; FastGene BlueStar Prestained Protein Marker, 10-180 kDa) were each pipetted into the

wells of the gel cassette. 300 volts with 30 mA (myPowerII AE-8135, ATTO Corporation, Tokyo, Japan) per gel cassette were applied to the gel electrophoresis setup for 1 hour initiating protein separation. After running, the gel cassette was retrieved and the stacking gel was removed. The resolving gel was soaked in a fixing solution for 20 minutes then washed with deionized water for 5 times. The resolving gel was submerged in deionized water and shaken for 10 minutes. The deionized water was poured out and the resolving gel was soaked in Coomassie Brilliant Blue G-250 for 30 minutes. After staining, the resolving gel was destained with a destaining solution every 10 minutes for 3 times. The destaining solution was removed and the stained gel was photographed.

Electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS)

The RP-HPLC peaks were subjected to ESI-TOF MS using a JMS-T100LC AccuTOF™ LC-TOF MS (JEOL, Tokyo, Japan) and interphased with an Agilent 1100 Series HPLC system (Agilent Technologies, California, USA) to determine their molecular weights. Impure RP-HPLC peaks were subjected to another run in the HPLC machine while pure RP-HPLC peaks were further analyzed. The conditions of ESI-MS were as follows: ionization mode, positive; desolvation temperature, 260°C; needle voltage, 2000 V; orifice voltage, 75 V; and, ring lens voltage, 10 V. The total ion chromatogram, mass chromatogram, and mass spectrum were recorded using a JEOL MassCenter program (JEOL, Tokyo, Japan) (Zendo *et al*., 2008). Solvent A contains Milli-Q water and TFA in a ration of 99.95:0.05 (v/v) while Solvent B contains acetonitrile and TFA in a ration of 99.95:0.05 (v/v). The injection volume and flow rate were 10 μL and 0.200 mL/min, respectively.

RESULTS AND DISCUSSION

Proteolytic activity of lactic acid bacteria strains in carabao milk using LMT

30°C LAB strains: The LMT results of 30°C LAB strains inoculated to litmus carabao milk were presented in Figure 1. After 1 day of incubation, lactose fermentation and litmus reduction were already observed in A1 and A2. Although, as the incubation period progressed, only A1 exhibited curd formation and medium clarification. Casein proteins, in particular, can be hydrolyzed into shorter peptides and amino acids. As a result, the large protein aggregates responsible for the turbidity of milk are solubilized, causing the milk to clarify. The peptides may also have different interactions with water molecules, altering the light scattering properties of milk and resulting in increased transparency. After 7 days of incubation, A1 also had the lowest pH value among the 30°C LAB strains (Table 3). The growth of LAB is closely tied to the fermentation process wherein lactose in milk is metabolized to lactic acid. The accumulation of lactic acid leads to a decrease in pH resulting to an acidic environment that may favor LAB growth and inhibit competing spoilage microorganisms. The proteolytic activity of LAB in milk can be greatly influenced by pH. A1 was reported to display higher proteolytic activity in low pH values typically around ≤ pH 4.6. The lower pH can regulate the expression and activation of proteolytic enzymes and promote hydrolysis of milk proteins. The pH of milk can influence the solubility and conformation of proteins which can affect the accessibility of proteolytic enzymes to their target substrates.

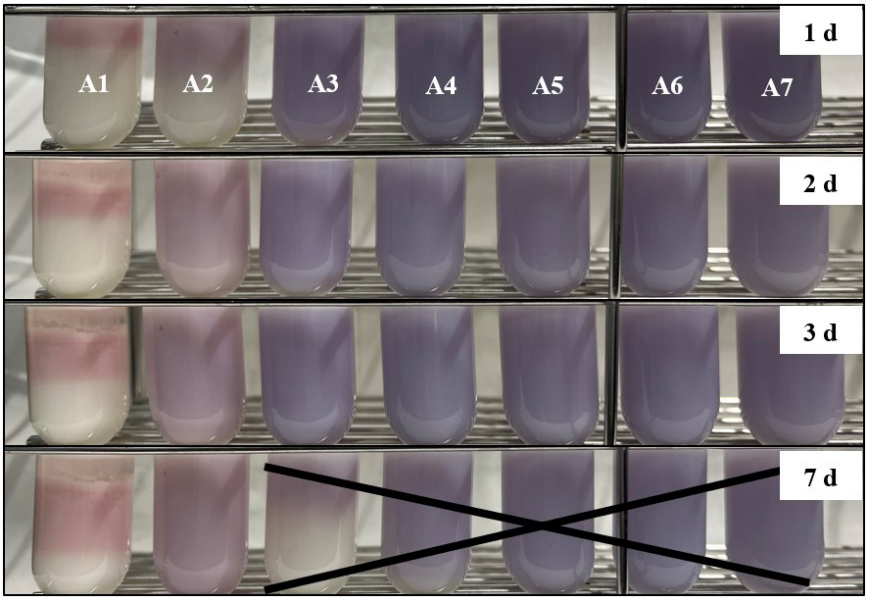

Figure 1. Litmus milk test (LMT) results of 30°C LAB strains inoculated to litmus carabao milk.

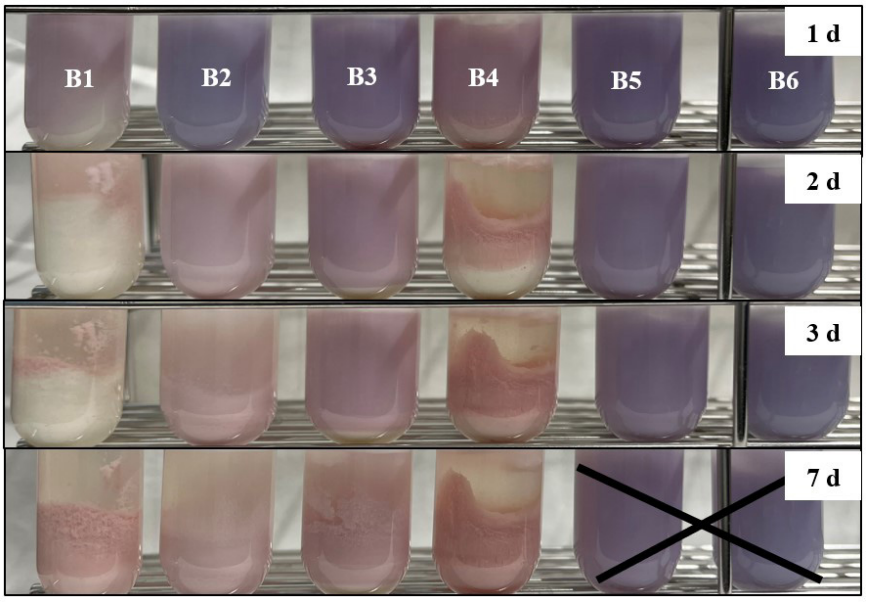

Figure 2. Litmus milk test (LMT) results for 37°C LAB strains inoculated to litmus carabao milk.

37°C LAB strains. The LMT results of 37°C LAB strains inoculated to litmus carabao milk were presented in Figure 2. B1 and B4 exhibited lactose fermentation after 1 day of incubation followed by B2 and B3 after 2 days of incubation. At the end of the 7-day incubation period, milk protein coagulation and peptonization were observed in B1, B2, B3, and B4. However, B1 and B4 exhibited an earlier and more pronounced formed curd and clarified medium. Among the 37°C LAB strains, B1 and B4 also had the lowest pH values.

pH values of fermented carabao milk

The pH values of LAB-fermented carabao milk (FCM) incubated for 7 days at 30°C or 37°C were shown in Table 3. Among the 30°C LAB strains, only A1 and A2 exhibited proteolytic activity in LMT (Figure 1) and pH values less than pH 4.6. On the other hand, among the 37°C LAB strains, B1, B2, B3, and B4 attained pH values less than pH 4.6 which were evident in their LMT results (Figure 2). Taking into consideration the LMT results and pH values, *Lacticaseibacillus casei* JCM 1134^T (B1) was selected to ferment carabao milk to generate potential bioactive peptides in the next study.

Table 3. pH values of LAB-fermented carabao milk (FCM) incubated for 7 days at 30°C or 37°C.

SAMPLE	CODE	pH
Lactococcus lactis subsp. *lactis*-FCM	A1-FCM	4.08
Lactococcus raffinolactis-FCM	A2-FCM	4.48
Lactiplantibacillus plantarum subsp. *plantarum*-FCM	A3-FCM	6.07
Latilactobacillus curvatus-FCM	A4-FCM	6.32
Pediococcus acidilactici-FCM	A5-FCM	6.61
Leuconostoc mesenteroides subsp. *mesenteroides*-FCM	A6-FCM	5.61
Weissella minor-FCM	A7-FCM	6.51
Lacticaseibacillus casei-FCM	B1-FCM	3.55
Lactobacillus acidophilus-FCM	B2-FCM	3.71
Lactobacillus delbrueckii subsp. *bulgaricus*-FCM	B3-FCM	4.05
Lactobacillus helveticus-FCM	B4-FCM	3.28
Limosilactobacillus fermentum-FCM	B5-FCM	5.00
Weissella viridescens-FCM	B6-FCM	6.75
Untreated CM	CM	6.76

Biological activity of HIC and IEC eluted fractions

HIC eluted fractions. Among the six indicator strains used for determining antimicrobial activity, culture supernatant, through, elution 1, elution 2, and wash 3 of B1-FCM exhibited weak antimicrobial activity against *Bacillus subtilis* JCM 1465^T as shown in Figure 3. Culture supernatant and through may contain the same components since through was only eluted after shaking the culture supernatant and Amberlite® XAD16N resin

together. These fractions may be comprised of metabolites such as organic acids, hydrogen peroxide, and soluble proteins or peptides secreted by LAB during fermentation in carabao milk. Wash 1 did not have antimicrobial activity since only deionized water was applied as the mobile phase. Wash 2 also did not exhibit antimicrobial activity even though 40% (v/v) ethanol was employed as the mobile phase. Ethanol modulates the hydrophobic interactions between the stationary phase and the sample to achieve separation. It promotes the binding of hydrophobic proteins to the hydrophobic stationary phase; this allows for selective retention of the target proteins based on their hydrophobicity. It is utilized in the elution step to disrupt the hydrophobic interactions and release the bound proteins from the stationary phase. Elution 1 and elution 2 had slight antimicrobial activity as observed in the faint clearing around their spots. 70% (v/v) isopropanol + 0.1% (v/v) TFA was used as the mobile phase to elute these fractions. Isopropanol is occasionally used as an alternative to ethanol due to its similar properties. It modulates hydrophobic interactions, binds hydrophobic proteins, elutes bound proteins, and aids in the solvation of proteins. TFA is commonly used as an additive to enhance the separation and elution of hydrophobic proteins. It acts as an ion-pairing agent by forming ion pairs with positively charged amino acid residues on proteins and peptides which reduces their net positive charge. This modification of the surface charge improves the interaction between the stationary phase and hydrophobic proteins. TFA is an acidic compound which can adjust the pH of the system to an appropriate range for the separation of hydrophobic proteins. It can also suppress unwanted interactions such as non-specific adsorption or aggregation which allows for better resolution and purity of the eluted fractions. Wash 3 also had slight antimicrobial activity even though 100% isopropanol was used as the mobile phase. In theory, a high concentration of isopropanol in the mobile phase increases the hydrophobicity of the system leading to stronger retention of hydrophobic

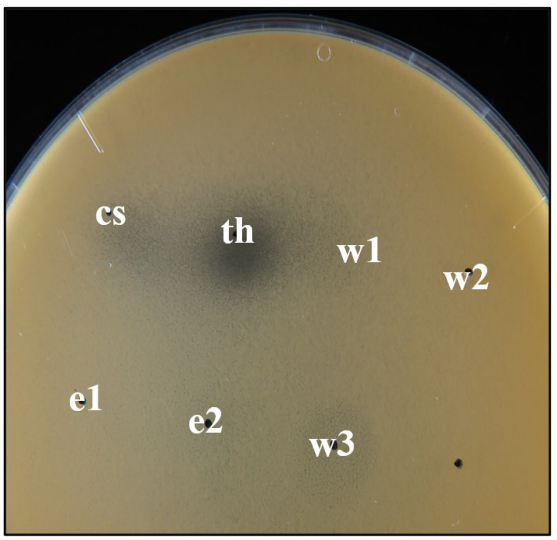

Figure 3. Spot-on-lawn assay (SOLA) results of hydrophobic interaction chromatography (HIC) eluted fractions from *Lacticaseibacillus casei* JCM 1134^{T}-fermented carabao milk (B1-FCM) against *Bacillus subtilis* JCM 1465^{T}. **cs** culture supernatant, **th** through, **w1** wash 1, **w2** wash 2, **e1** elution 1, **e2** elution 2, and **w3** wash 3.

proteins. No or low antimicrobial activity of HIC eluted fractions that used ethanol and isopropanol as mobile phases may suggest that the potential peptides active against *Bacillus subtilis* JCM 1465^T in the culture supernatant and through fractions are hydrophilic in nature.

Numerous studies have documented the antimicrobial activity displayed by different strains of *Lacticaseibacillus casei*. Using the agar well diffusion assay (AWDA), *Lacticaseibacillus casei* FBL6 had a broad antimicrobial spectrum. It exhibited antimicrobial activity against Gram-negative bacteria and Gram-positive bacteria such as *Listeria monocytogenes*, *Bacillus cereus*, *Escherichia coli* O157:H7 and O1:K1:H7, *Salmonella enteritidis*, and *Listeria ivanovii*. The antimicrobial activity is mainly mediated by the production of some inhibitory substances such as bacteriocins and lactic acid (Kim *et al*., 2022). Using AWDA, the extracted bacteriocin derived from *Lacticaseibacillus casei* VITCM05 exhibited a wide-ranging antibacterial effect, targeting various Gram-positive bacteria including *Listeria monocytogenes* and *Staphylococcus aureus*, as well as Gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhii*. The antibacterial activity displayed by the extracted bacteriocin was found to be effective across a broad spectrum (Siddique and Vaithilingam, 2023). *Lacticaseibacillus casei*-01 was reported to produce antimicrobial substances (organic acids, fatty acids, and hydrogen peroxide) and reduce the counts of *Staphylococcus aureus* TISTR118, *Escherichia coli* TISTR780, *Micrococcus luteus* TISTR884, *Bacillus cereus* TISTR687, and *Enterococcus faecalis* TISTR1482 (Chaikham *et al*., 2013; Parsaeimehr *et al*., 2017). Lin *et al.* (2015) observed that *Lacticaseibacillus casei*-01 inhibited the growth and biofilm formation of *Streptococcus mutans*, mainly through acid production. They also suggest that this strain could produce bacteriocins with a molecular weight lower than 3000 Da (Pimentel *et al*., 2021). *Lacticaseibacillus casei* T1 exhibited broad bacteriostatic spectrum against *Helicobacter pylori*, *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, *Levilactobacillus brevis* (Yu *et al*., 2023). Whey-milk beverages fermented with *Lacticaseibacillus casei*-01 produced AMPs derived from α_{s1}-CN and β-CN (Rosa *et al*., 2023). Caseicin (A, B, and C) are other bacteriocins produced by *Lacticaseibacillus casei*.

Table 4 presented the inhibition ratio (%) of HIC eluted fractions from B1-FCM using DPPHAA. The principle of this assay is based on the ability of the antioxidant in the sample to scavenge free radicals. The DPPH molecule is a stable free radical with a deep purple color in its oxidized form. It accepts an electron or hydrogen atom from an antioxidant resulting in the reduction of the DPPH free radical to a stable and colorless DPPH-H molecule. The reduction of DPPH is accompanied by a change in color from purple to yellow which can be measure spectrophotometrically. The inhibition ratio increased from culture supernatant to through then decreased in wash 1 and increased again in wash 2. As the purification progressed after wash 2, the inhibition ratio of the succeeding eluted fractions continuously declined. This downward trend might suggest that the potential peptides exhibiting antioxidant activity are hydrophilic in nature.

Several studies have reported the antioxidant activity exhibited by different strains of *Lacticaseibacillus casei*. The activated cell of *Lacticaseibacillus casei* FBL6 exhibited 60% DPPH free radical scavenging activity (Kim *et al*., 2022). The addition of *Lacticaseibacillus casei*-01 in food products increased antioxidant activity (Pimentel *et al*., 2021). *Lacticaseibacillus casei* T1 could effectively improve the low expression of glutathione peroxidase (GSH-PX) and superoxide dismutase (SOD) induced by *Helicobacter*

pylori infection. Thus, the outcomes confirmed that *Lacticaseibacillus casei* T1 can alleviate oxidative stress (Yu *et al*., 2023). During fermentation, starter and probiotic cultures can synthesize cell-surface proteinases capable of hydrolyzing milk proteins, releasing different peptides into the medium (Kocak *et al*., 2020; Rosa *et al*., 2023). The higher antioxidant activity of probiotic products may be related to some of these bioactive peptides exhibiting free radical scavenging and inhibiting enzymatic and non-enzymatic lipid peroxidation (Korhonen and Pihlanto, 2006). Labneh, a Middle Eastern cheese made from strained yogurt, fermented by untreated and photobiostimulated *Lacticaseibacillus casei* NRRL B-1992 (same strain as *Lacticaseibacillus casei* JCM 1134^T) exhibited DPPH scavenging activity (Elshaghabee *et al*., 2022). *Lacticaseibacillus casei* can produce glutathione-like peptides, dipeptides, tripeptides, and hydrophobic peptides that possess the ability to scavenge free radicals and reduce oxidative stress in the body. These peptides contain amino acid residues such as histidine, tyrosine, tryptophan, cysteine, methionine, phenylalanine, leucine, and isoleucine.

Table 4. Inhibition ratio (%) of hydrophobic interaction chromatography (HIC) eluted fractions from *Lacticaseibacillus casei* JCM 1134^T-fermented carabao milk (B1-FCM) using DPPH antioxidant assay (DPPHAA).

SAMPLE	HIC ELUTED FRACTION	INHIBITION RATIO (%)
Lacticaseibacillus casei JCM 1134^T-FCM (B1-FCM)	Culture Supernatant	50.492
	Through	87.213
	Wash 1	69.508
	Wash 2	71.803
	Elution 1	41.639
	Elution 2	9.508
	Wash 3	0.000

Untreated Carabao Milk: 1.544%

Figure 4 presented the chromatograms of HIC eluted fractions (elution 1, elution 2, and wash 3) using the following gradient programs: **A** HIC elution 1, 30-70% Solvent B for 45 minutes, **B** HIC elution 2, 30-60% Solvent B for 30 minutes, and **C** HIC wash 3, 30-60% B for 30 minutes. The gradient concentration and run time were narrowed down as peaks were no longer observed beyond the 60% mark of Solvent B. The principle of RP-HPLC is based on the differential partitioning of analytes between a nonpolar stationary phase and a polar mobile phase. In this case, Atlantis™ T3 column containing C18 (octadecylsilane) was used as the stationary phase to retain hydrophobic analytes ranging from nonpolar

or moderately polar compounds. Simultaneously, it allows the quick elution of polar compounds. The detected peaks did not exhibit any antimicrobial or antioxidant activities. Based on this, it might be proposed that the potential peptides demonstrating antimicrobial and antioxidant activities possess a hydrophilic character and were lost during subsequent purification stages.

IEC eluted fractions. All of the IEC eluted fractions (through, wash, 0.5 M NaCl, and 1.0 M NaCl) were not active against *Bacillus subtilis* JCM 1465^{T} using SOLA. Antioxidant activity was also not detected using DPPHAA. Since HIC elution 1 was used for IEC, these results might be expected since HIC elution 1 demonstrated weak antimicrobial activity

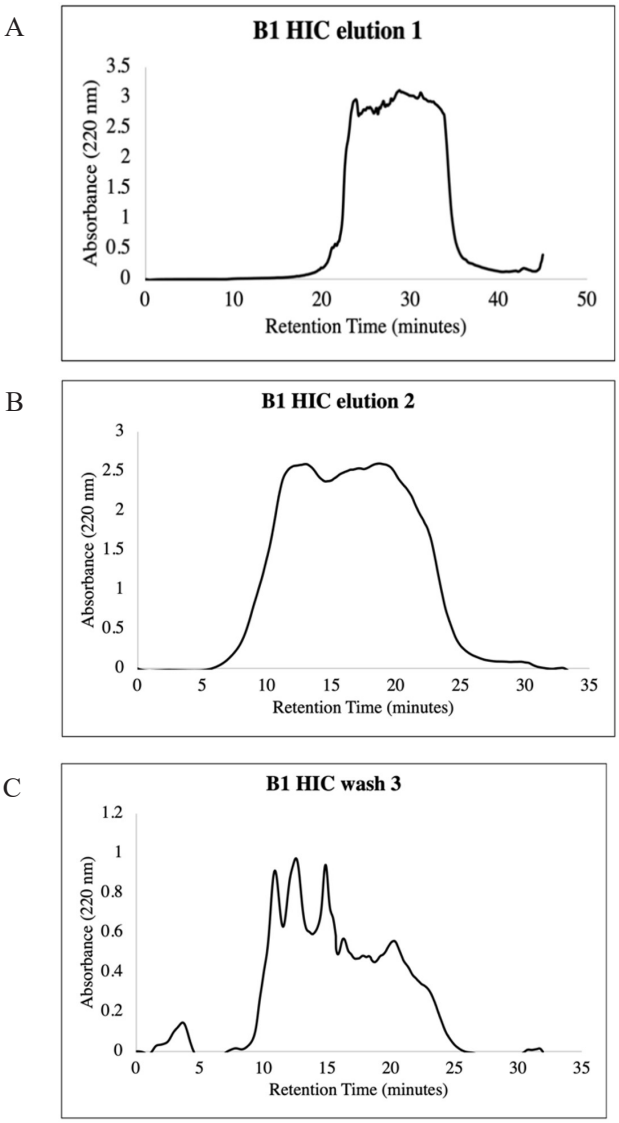

Figure 4. Chromatograms of HIC eluted fractions using the following gradient programs: **A** HIC elution 1, 30-70% Solvent B for 45 minutes, **B** HIC elution 2, 30-60% Solvent B for 30 minutes, and **C** HIC wash 3, 30-60% Solvent B for 30 minutes.

against the same indicator strain. Meanwhile, potential antioxidant proteins or peptide could have been probably lost as the purification process proceeded, similar to what occurred in HIC (Table 4). From these results, further purification by RP-HPLC of IEC eluted fractions was not performed. These observations could also indicate that these potential bioactive peptides possess a negative surface charge rather than a positive charge, rendering this purification method unsuitable.

Biological activity of milk protein hydrolysates from ultrafiltration

Table 5 showed the spot-on-lawn assay (SOLA), DPPH antioxidant assay (DPPHAA), and ACE inhibition activity assay (ACEIAA) results of milk protein hydrolysates from *Lacticaseibacillus casei* JCM 1134^T-fermented carabao milk (B1-FCM). Whey hydrolysate (W), casein hydrolysate (C1), whey retentate (WR), casein retentate (CR), whey retentate peak 3 (WRp3), and casein retentate peak 3 (CRp3) demonstrated antimicrobial activity against *Bacillus subtilis* JCM 1465^T. W and WR might contain potential AMPs with similar size and molecular weight since WR was obtained from ultrafiltered W. Ultrafiltration is a pressure-driven filtration process using a semi-permeable membrane. It operates on the principle of size and molecular weight exclusion; allowing smaller molecules to pass through the membrane (permeate) while retaining larger molecules (retentate). A centrifugal device with a 1000 MWCO was used for ultrafiltration. Hence, it might be suggested that these potential AMPs in W and WR have a molecular weight of ≥1000 Da. WRp3 and CRp3 were initially isolated from WR and CR through RP-HPLC (Figure 5), respectively. However, they were no longer detectable following subsequent purification using another round of RP-HPLC.

The antimicrobial activity of these hydrolysates can be attributed to several mechanisms. AMPs disrupt the integrity of microbial cell membranes leading to cell lysis. They can also inhibit microbial growth by interfering with essential cellular processes such as DNA replication, protein synthesis, and enzyme activity. AMPs can act individually or synergistically with other bioactive compounds such as organic acids and bacteriocins in inhibiting their target microorganism. *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli* were inhibited by whey fractions fermented with *Lacticaseibacillus casei* NRRL B-1922 (same strain as *Lacticaseibacillus casei* JCM 1134^T) (Mohamed *et al*., 2020). AMPs derived from α_{s1}-CN and β-CN were identified in whey-milk beverages fermented by *Lacticaseibacillus casei*-01 (Rosa *et al*., 2023). Both *Lacticaseibacillus casei* PRA205 and *Lacticaseibacillus casei* 2006 hydrolyzed α_{s1}-CN and β-CN producing AMPs (Solieri *et al*., 2022).

Only W (35.59%) and WR (34.21%) exhibited antioxidant activity through scavenging DPPH free radicals. It could be implied that these hydrolysates were comprised of potential antioxidant peptides with similar size and molecular weight. However, RP-HPLC peaks of WR did not exhibit antioxidant activity anymore. Several studies have reported that antioxidant peptides were decrypted from β-CN by *Lacticaseibacillus casei*-01 (Taha *et al*., 2017; Abdel-Hamid *et al*., 2019; Ali *et al*., 2019). Skim milk fermented by *Lacticaseibacillus casei* NRRL B-1922 (same strain as *Lacticaseibacillus casei* JCM 1134^T) displayed antioxidant capacity. Moreover, red laser irradiation on *Lacticaseibacillus casei* NRRL B-1922 before skim milk fermentation increased its antioxidant capacity (Mohamed *et al*., 2020). Water-soluble extracts of retentates and permeates (3 kDa and 10 kDa) from *Lacticaseibacillus casei* NK9-fermented camel milk expressed antioxidant activity in terms

of ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) activity, hydroxyl free radical scavenging activity, superoxide radical scavenging activity (SRSA) (Dharmisthaben *et al*., 2022). *Lacticaseibacillus casei*-01 showed high titratable acidity, low pH value, and the presence of antihypertensive peptides (α_{s1}-CN, α_{s2}-CN, and β-CN) in fermented whey-milk beverages during storage (Rosa *et al*., 2023). *Lacticaseibacillus casei* PRA205 and *Lacticaseibacillus casei* 2006 both produced antihypertensive peptides fragmented from α_{s1}-CN and β-CN (Solieri *et al*., 2022).

Table 5. Spot-on-lawn assay (SOLA), DPPH antioxidant assay (DPPHAA), and ACE inhibition activity assay (ACEIAA) results of milk protein hydrolysates from *Lacticaseibacillus casei* JCM 1134^T-fermented carabao milk (B1-FCM).

SAMPLE	SOLA[1]	DPPHAA[2] (%)	ACEIAA[2] (%)
Whey hydrolysate (W)	+	35.59%	82.91%
Whey retentate (WR)	+	34.21%	85.57%
Whey retentate peak 1 (WRp1)	-	-	nt
WRp2	-	-	nt
WRp3	+	-	nt
Casein hydrolysate 1 (C1)	+	nt[3]	nt
Casein hydrolysate 2 (C2)	-	-	88.51%
Casein retentate (CR)	+	-	88.61%
Casein retentate peak 1 (CRp1)	-	-	nt
CRp2	-	-	nt
CRp3	+	-	nt

[1]Active (+) or inactive (-) against *Bacillus subtilis* JCM 1465^T.
[2]Inhibition ratio.
[3]Not tested.

Figure 5 illustrated the chromatograms of WR and CR from *Lacticaseibacillus casei* JCM 1134^T-fermented carabao milk (B1-FCM) using the gradient program: 30-60% Solvent B for 30 minutes. These hydrolysates were subjected to RP-HPLC to isolate and purify their potential bioactive peptides. Only WRp3 and CRp3 inhibited *Bacillus subtilis* JCM

1465^{T}. All RP-HPLC peaks were subjected to ESI-TOF MS to determine their purity (Figure 6). The results showed that both WRp3 and CRp3 do not contain a singular peptide but may contain several peptides with similar hydrophobic characteristics. Hence, another run of RP-HPLC was employed to further purify these peaks. However, these peaks were not detected anymore using a modified and specific gradient program (WRp3: 50-55% Solvent B for 45 minutes; CRp3: 40-45% Solvent B for 45 minutes). Inhibitory effect in terms of antimicrobial and antioxidant activities were completely lost when further purified. These results suggested that other metabolites may be involved in expressing antimicrobial and antioxidant activities. It can also be speculated that synergism of other metabolites and potential bioactive peptides can take place.

A gradient program with a wider range of gradient concentration and longer run time (0-80% Solvent B for 60 minutes) was applied as presented in Figure 7. The potential antimicrobial and antioxidant peptides observed in the preceding findings might have remained unrecoverable due to their hydrophilic nature. Thus, the previous purification methods (HIC, IEC, and RP-HPLC) were unsuccessful in purifying milk-derived bioactive peptides.

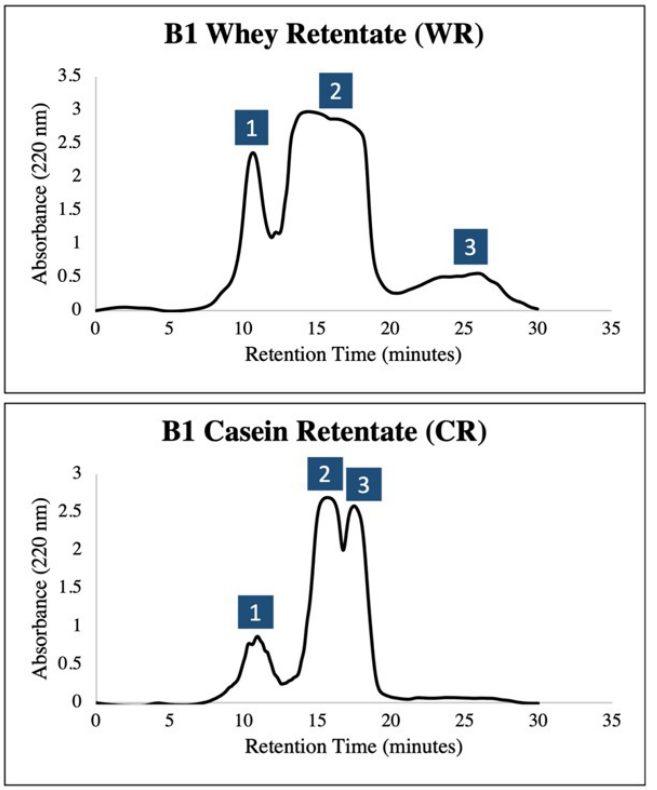

Figure 5. Chromatograms of whey and casein retentates from *Lacticaseibacillus casei* JCM 1134^{T}-fermented carabao milk (B1-FCM) using the gradient program: 30-60% Solvent B for 30 minutes.

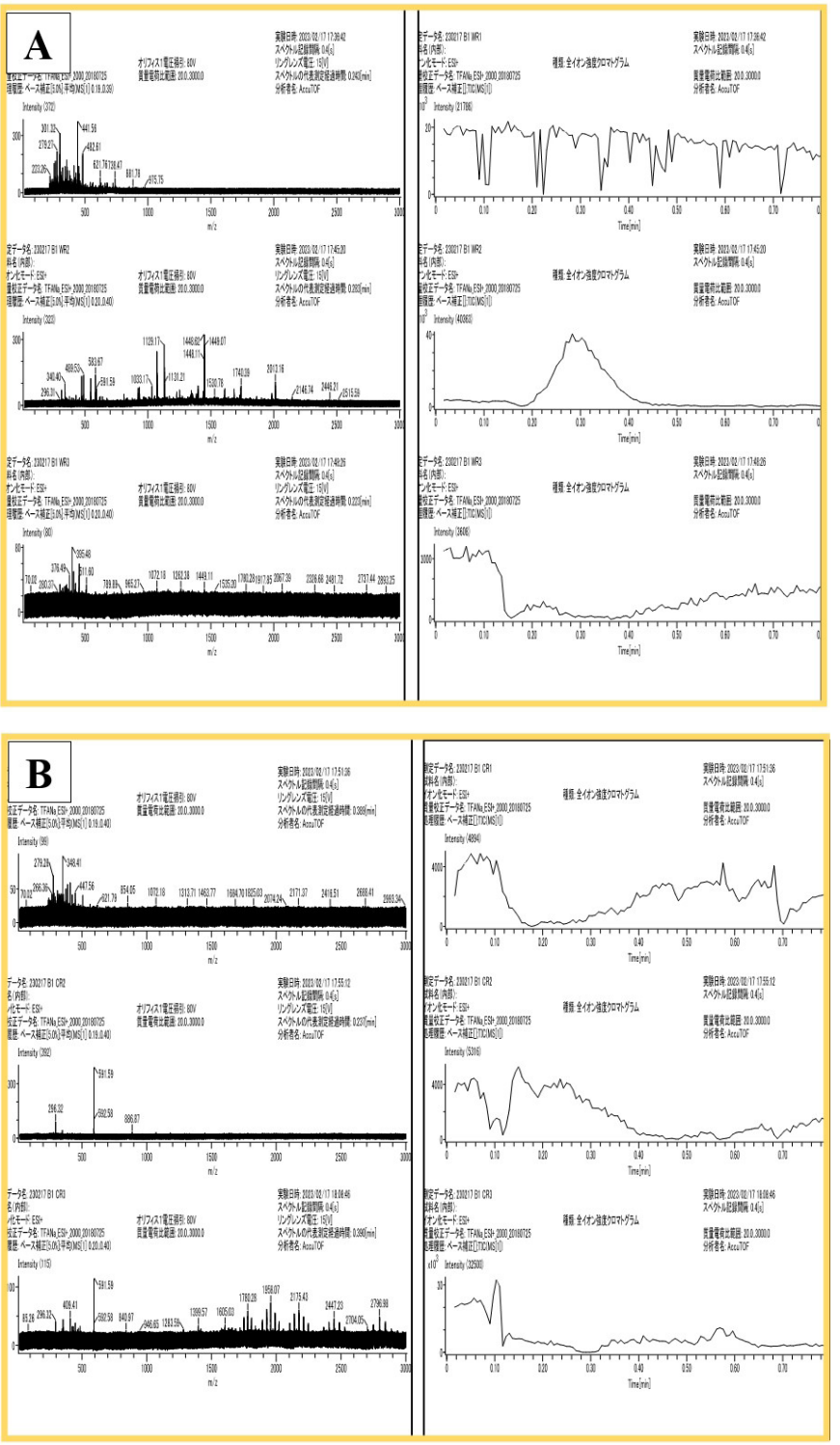

Figure 6. Total ion chromatograms (TIC) and mass spectra (MS) of RP-HPLC peaks from **A** whey retentate and **B** casein retentate both from *Lacticaseibacillus casei* JCM 1134^{T}-fermented carabao milk (B1-FCM).

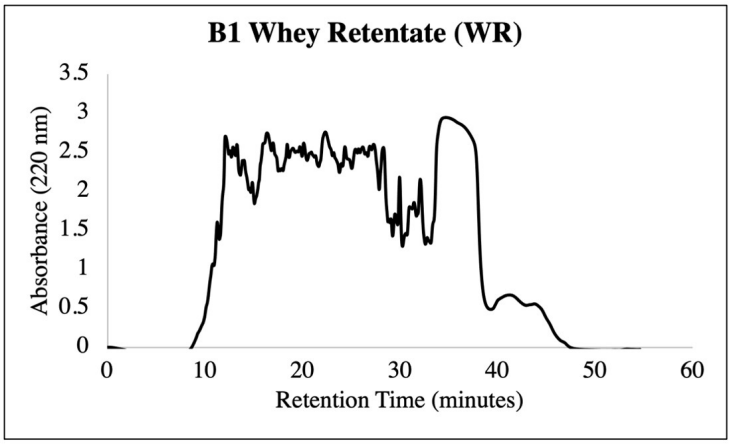

Figure 7. Chromatogram of whey retentate from *Lacticaseibacillus casei* JCM 1134^{T}-fermented carabao milk (B1-FCM) using the gradient program: 0-80% Solvent B for 60 minutes.

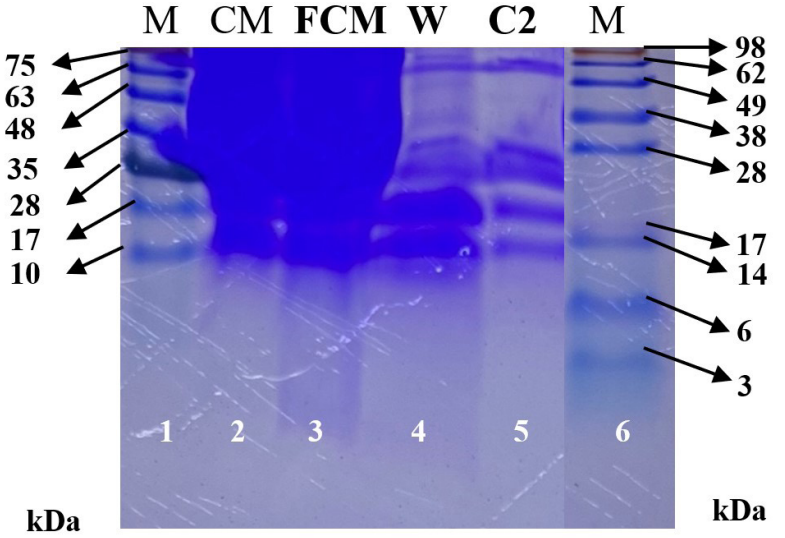

Figure 8. Electrophoretograms of carabao milk, fermented carabao milk, milk protein hydrolysates, and protein standards. **From left to right: Lane 1** FastGene BlueStar Prestained Protein Marker (10-180 kDa) (marker, M), **Lane 2** carabao milk (CM), **Lane 3** *Lacticaseibacillus casei* JCM 1134^{T}-fermented carabao milk (FCM), **Lane 4** B1 whey hydrolysate (W), **Lane 5** B1 casein hydrolysate 2 (C2), and **Lane 6** SeeBlue^{TM} Plus2 Pre-stained Protein Standard (3-198 kDa) (marker, M).

The electrophoretograms of carabao milk, fermented carabao milk, milk protein hydrolysates, and protein standards were shown in Figure 8. The migration distance of bands from CM (Lane 2) and B1-FCM (Lane 3) were closely spaced. The bands also appeared

intense which might suggest that they contain a high protein concentration. The lowest molecular weight observed for these samples was approximately 10 kDa. In addition, smear-like patterns were observed in B1-FCM which are indicative of protein degradation. On the contrary, the band separation of W and C2 was evident, albeit somewhat indistinct. These results suggested that whey and casein proteins in carabao milk were hydrolyzed by the LAB strains. W (Lane 4) and C2 (Lane 5) exhibited five similar bands with approximate molecular weights of 62-63 kDa, 28-35 kDa, 25-28 kDa, 17-18 kDa, and 10-14 kDa. Smearing was present in W and the two bands at the bottom were intense compared to C2.

Figure 9 presented the electrophoretograms of different milk protein hydrolysates. W (Lane 2), WR (Lane 3), and WP (Lane 4) had similar bands even though ultrafiltration with a 1000 MWCO was used. The same observation was visualized in C2 (Lane 5), CR (Lane 6), and CP (Lane 7). Some bands were undetected in casein hydrolysates compared to whey hydrolysates suggesting that they differ in hydrolyzed proteins or peptides.

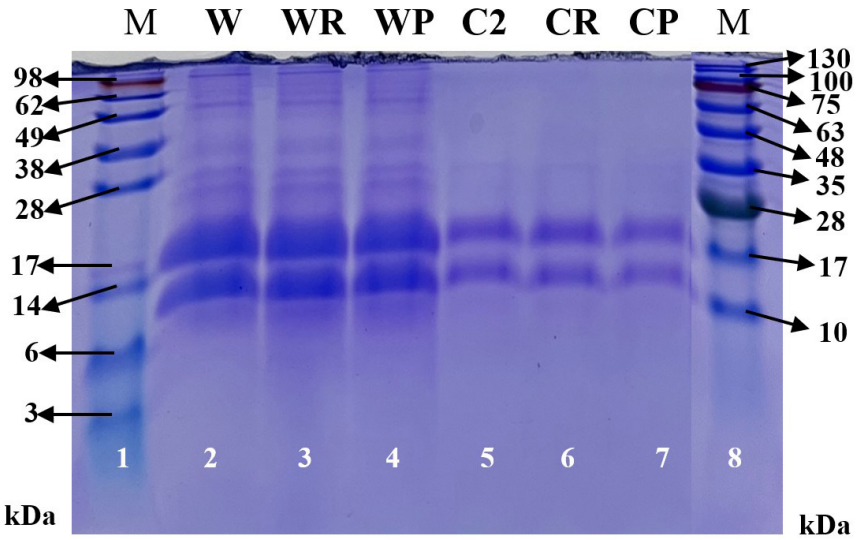

Figure 9. Electrophoretograms of milk protein hydrolysates and protein standards. **From left to right: Lane 1** SeeBlue™ Plus2 Pre-stained Protein Standard (3-198 kDa) (marker, M), **Lane 2** B1 whey hydrolysate (W), **Lane 3** B1 whey retentate (WR), **Lane 4** B1 whey permeate (WP), **Lane 5** B1 casein hydrolysate 2 (C2), **Lane 6** B1 casein retentate (CR), **Lane 7** casein permeate (CP), and **Lane 8** FastGene BlueStar Prestained Protein Marker (10-180 kDa) (marker, M).

CONCLUSION

This research aimed to generate and characterize bioactive peptides encrypted in carabao milk through lactic acid bacteria (LAB) proteolysis. The first study was conducted to screen and select LAB strains with high proteolytic activity. A total of thirteen LAB strains from the genus *Lactococcus*, *Lactiplantibacillus*, *Latilactobacillus*, *Pediococcus*, *Leuconostoc*, *Weissella*, *Lacticaseibacillus*, *Lactobacillus*, and *Limosilactobacillus* was screened due to diversity representation and isolation source. Skim milk agar plate (SMAP),

litmus milk test (LMT), and pH measurement were used to assess proteolytic activity. LAB strains with high proteolytic activity were selected to ferment carabao milk. The second study was performed to generate and purify milk-derived bioactive peptides from milk protein hydrolysates. Various purification and separation techniques were applied including hydrophobic interaction chromatography (HIC), ion exchange chromatography (IEC), ultrafiltration (UF), reversed-phase high performance liquid chromatography (RP-HPLC), and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fractions were tested for biological activity such as antimicrobial (microbial inhibition), antioxidant (DPPH free radical scavenging), and antihypertensive (ACE inhibition) activities. Purity of the fractions was determined by electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS).

The first study yielded inconclusive findings with regard to SMAP, leading to the inclusion of results from LMT and pH measurement for consideration. *Lacticaseibacillus casei* JCM 1134^T (B1) was selected to ferment (3% v/v) carabao milk at 37°C for 5 days. In the second study, milk protein hydrolysates from B1-fermented carabao milk were purified and separated. Some fractions only had weak antimicrobial activity against *Bacillus subtilis* JCM 1465^T, antioxidant activity declined as the purification process continued, and high antihypertensive activity was observed in hydrolysates and retentates from whey and casein proteins. These fractions were subjected to RP-HPLC to isolate potential antimicrobial and antioxidant peptides. Nevertheless, despite employing different gradient programs, the peaks were often inactive, impure, or undetected. Hence, it is highly recommended to further optimize the purification methods to isolate bioactive peptides. Once completely purified, the amino acid sequence and protein concentration of the bioactive peptides should be performed using Edman degradation via an automated protein sequencer and Pierce bicinchoninic acid (BCA) protein assay, respectively. The amino acid sequence can determine the novelty or identity of the bioactive peptides. It is also important in visualizing the structure of the bioactive peptides which can explain its mode of biological activity.

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