

Selection of Proteolytic Lactic Acid Bacteria and the Potential as α -Glucosidase Inhibitor Activity During Milk Fermentation

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ABSTRACT

Diabetes mellitus (DM) is a degenerative condition increasing globally, including in Indonesia, with a corresponding rise in death rates. One of the therapy methods for DM is the use of α -glucosidase inhibitor (AGI), with peptides identified as potential AGI-based food ingredients. Therefore, this study aimed to develop fermented milk products enhanced with AGI peptides by screening and identifying lactic acid bacteria (LAB) strains with proteolytic capabilities. The experiment was conducted in three main stages: (1) screening 32 LAB isolates for their proteolytic activity, (2) determining the optimal fermentation time for AGI production using selected proteolytic LAB strains, and (3) molecular identification of the selected LAB strains through 16S rRNA gene sequencing. The results showed that two isolates, SR17B and L23, identified as *Lactiplantibacillus plantarum-pentosus* SR17B and *Lactiplantibacillus plantarum-pentosus* L23, had high proteolytic activity and were capable of producing fermented milk with significant AGI activity of 35.94% and 35.15%, respectively. AGI activity progressively intensified during fermentation, peaking at 12 hours in both strains, indicating that this period of time was ideal for fermentation in order to have the largest inhibitory effect. These results suggested that selected LAB strains, particularly *L. plantarum-pentosus* SR17B and L23, could serve as functional starter cultures for development of fermented dairy products with antidiabetic potential.

Keywords: α -glucosidase inhibitor; fermentation; lactic acid bacteria; milk; proteolytic

INTRODUCTION

An increased blood sugar level is a hallmark of diabetes mellitus (DM), a chronic metabolic disease. It happens when either the body does not use the insulin produced by the pancreas efficiently or generates insufficient amount (Sapra & Bhandari, 2023). The prevalence of degenerative diseases like DM is steadily increasing worldwide, even in developing nations such as Indonesia (Ministry of Health, 2023). The IDF

diabetes atlas (Sun et al., 2022) stated that in 2021, approximately 10.5% of people between the ages of 20 and 79 had diabetes worldwide, with projection to reach 12.2% by 2045. In 2021, DM was found to affect approximately 537 million people in 20–79 age group, which was predicted to reach 643 million by 2030 and 783 million in 2045 (IDF, 2022). In Indonesia, DM prevalence increased from 10.9% in 2018 to 11.7% in 2023, with type 2 DM (T2DM) being the most common case (Ministry of Health, 2018; Ministry of Health, 2023).

Several therapeutic method for managing T2DM focus on inhibiting the breakdown of starch by α -glucosidase enzyme (Meneses et al, 2015). By slowing down the action of enzymes in the small intestine, α -glucosidase inhibitor (AGI) help reduce the absorption of glucose, preventing spikes in blood sugar levels after meals (Yin et al., 2014). Acarbose, a widely used AGI, has been applied for over two decades. However, it has been related to adverse effects including bloating, diarrhea, dizziness, vomiting, gastrointestinal distress, and hepatic problems (Dean, 2017), underscoring the necessity to investigate alternative AGIs.

According to Di Stefano et al. (2018), a variety of naturally occurring compounds such as phenolics, peptides, certain polysaccharides, and triterpenoids can slow down or inhibit the activity of α -glucosidase, an enzyme responsible for breaking down carbohydrates. Over the past decade, food-derived bioactive peptides have gained considerable attention as promising natural sources of α -glucosidase inhibitors (AGIs). The widespread availability in nature and the relative simplicity of extraction have made peptides a central focus of AGI studies. Several proteins have been identified as peptide precursors showing inhibitory activity, including whey protein hydrolysate (Konrad et al., 2014; Baba et al., 2021), douchi (Guo et al., 2023), wheat germ peptide (Liu et al., 2021), egg white protein (Yu et al., 2011), albumin (Yu et al., 2012; Sa'adah & Muhtadi, 2022), hemp (Ren et al., 2016), silkworm-cocoon protein (Zhang et al., 2016), oat globulin (Wang et al., 2018), soy (Rai et al., 2017; Ha et al., 2019; Li et al., 2023), quinoa (Vilcacundo et al., 2017), and peptides derived from *Ginkgo biloba* (Wang et al., 2023). Most inhibitory peptides are small, made up of just three to six amino acids and include residues like proline, alanine, or methionine toward the C-terminus, and threonine, serine, arginine, tyrosine, or lysine toward the N-terminus (Ibrahim, 2017).

Lu et al. (2023) stated that AGI peptides inhibited α -glucosidase by attaching to the enzyme through hydrophobic interactions, van der Waals forces, and hydrogen bonds, thereby disrupting the active site and lower glucose absorption. This activity are strengthened by hydrophobic amino acids, particularly proline and leucine (Ren et al., 2016). Milk proteins contain high amounts of these amino acids and have shown notable AGI activity in studies on whey and casein hydrolysates from cow, camel, and goat milk (Konrad et al., 2014; Baba et al., 2021; Mudgil et al., 2021; Cao et al., 2024).

Generally, a specific molecular weight is required for bioactive AGI peptides. According to Lu et al. (2023), small peptides with a molecular weight of less than 1 kDa are often more effective in inhibiting the activity of

α -glucosidase enzyme, as the size enhances attachment to enzyme active site. These small milk peptides are generated through fermentation, where lactic acid bacteria (LAB) use the proteolytic activity to break down milk proteins into smaller sizes. Fermented milk products using LAB are among the most popular dairy products with the potential to show high AGI activity.

Despite increasing results supporting the potential of food-derived peptides as natural AGI, current studies have predominantly focused on peptides extracted through enzymatic hydrolysis from various non-dairy protein sources. Although several studies explored derivatives from milk, there is limited information on how LAB contribute to the production of low-molecular-weight AGI peptides during milk fermentation. There is also a lack of comprehensive screening of LAB strains with high proteolytic activity that can be used to generate bioactive AGI peptides in functional fermented milk products. Therefore, this study aims to identify LAB isolates with strong proteolytic activity and test the potential to produce fermented milk rich in AGI peptides, contributing to the development of functional dairy products for managing DM.

METHODS

Materials

This study used pure cultures of LAB, which included five strains from the Food and Nutrition Culture Collection (FNCC) at Universitas Gadjah Mada in Yogyakarta, Indonesia. The strains included *L. plantarum* Mut 7, *L. plantarum* Mut 13, *L. plantarum* T3, *S. thermophilus* ST, and *S. thermophilus* Dad 11. There were also 28 isolates from the Research Center for Food Technology and Processing (PRTTP BRIN), namely L1, L2, L5, L6, L7, L8, L12, L13, L16, L18, L23, KM57, KM87, SR17A, SR17B, SR48, SR52, SR77, TL07, TL17, TL27, TL58, TL78, B3, CP77, GN8, and E144. Additional materials included PT Mirota SKM in Indonesia's Lactona skim milk, Merck in Germany's α -glucosidase enzyme from *S. cerevisiae*, trichloroacetic acid (TCA), MRS agar, MRS broth, p-Nitrophenyl- α -D-glucopyranoside (pNPG) from Sigma Aldrich in the United States, sodium dihydrogen phosphate (NaH_2PO_4), sodium hydrogen phosphate (Na_2HPO_4), and calcium carbonate (CaCO_3) from Merck in Germany.

Culture Preparation

A preparatory stage was conducted to ensure the availability and uniformity of the LAB isolates based on Chen et al. (2014). Pure LAB cultures were grown in MRS broth at 37 °C for 18 hours. Before usage, the

strains were cultivated twice, and the purity of the isolates was checked. Purification of the isolates was conducted by streaking on MRS agar with 1% CaCO₃, followed by incubation for 2 days. Colonies showing clear zones were further purified with the same media, with the process was repeated 3 times until uniform colonies were obtained.

Qualitative Screening of Proteolytic LAB

The qualitative analysis for proteolytic activity in LAB was conducted using block agar method as described by Aliifah et al. (2023). In wells made on MRS agar with 1% skim milk and 1% casein, bacteria isolates were cultivated and incubated for 48 hours at 37 °C. A clear zone around the well signified the presence of proteolytic activity.

Quantitative Screening of Proteolytic LAB

In addition to qualitative screening, the proteolytic activity of the bacterial isolates was also screened quantitatively. The screening was performed according to the method by Walter (1984). After centrifuging MRS broth and inoculating it with proteolytic LAB, a supernatant solution was created and cultured for a full day. A mixture of 20 µl of 500 ppm casein, 300 µl of phosphate buffer (pH 7), and 100 µl of the supernatant (sample), distilled water (blank), or 2-20 ppm tyrosine solution (standard) was vortexed. Following 60 minutes of incubation at 37 °C, 400 µl of 4% TCA was added to each treatment. The samples were centrifuged for 15 minutes at 3,500 rpm after 30 minutes of room temperature incubation. When the supernatant was diluted with phosphate buffer at pH 7, the absorbance was measured at 275 nm. Proteolytic activity was calculated based on a tyrosine standard curve and expressed in units (U), defined as the amount of enzyme required to release 1 µmol of tyrosine per minute under the assay conditions. The calculation of proteolytic activity is presented in Equation (1).

$$\text{Proteolytic activity} = \frac{(\text{tyrosine})}{M_r \text{ tyrosine}} \times \frac{V}{p \times q} \times DF \quad (1)$$

Where V is the total volume of the reaction mixture (mL), p is the volume of enzyme sample used (mL), q is the incubation time (min), DF is the dilution factor, and M_r tyrosine is the molecular weight of tyrosine (181.19 g/mol).

Fermented Milk Production

Bacteria isolates with proteolytic activity were used as starters for the fermentation of milk. Initially, bacteria were cultured in a 1% bacteria solution in 10% (w/v) skim milk and incubated at 37 °C for 24 hours.

The fermentation obtained was grown in a 5% bacteria solution in 15% (w/v) skim milk to make fermented milk starter. Another incubation was performed for 24 hours at 37 °C, followed by milk fermentation for 6, 12, 18, and 24 hours using the starter.

AGI Activity

AGI activity was tested using a method similar to Zeng et al. (2016) and Miftakhussolikhah et al. (2025). This test was conducted by seeing how an enzyme reacted with a certain substance to provide a colored outcome. All the materials used, including the α-glucosidase enzyme, acarbose, and p-Nitrophenyl-α-D-glucopyranoside, were prepared in a 0.01 M phosphate buffer saline (PBS) solution with a pH of 6.8. The reaction mixture contained 25 µl of 1% acarbose or the sample under test, 25 µl of 0.01 M PBS (pH 6.8), and 25 µl of 10 mM p-Nitrophenyl-α-D-glucopyranoside. This mixture was kept at 37 °C for 10 minutes, added with 50 µl of 1U/mL α-glucosidase enzyme, and the blend was left to incubate at 37 °C for an additional 30 minutes. Subsequently, 100 µl of 0.1 M sodium carbonate was added to stop the reaction, and color change was measured using a spectrophotometer at 405 nm. A blank solution without sample or enzyme was used as a control. The positive control included the enzyme without the sample, and the negative control used only the PBS. The percentage inhibition of α-glucosidase activity was calculated using Equation (2).

$$\text{Inhibition (\%)} = \left(1 - \frac{\text{sample absorbance} - \text{blank absorbance}}{\text{positive control absorbance} - \text{negative control absorbance}}\right) \times 100\% \quad (2)$$

Determination of Total LAB (Counting) and pH Level

The number of LAB in milk during fermentation was determined using a dilution method followed by the pour plate on MRS media (Muganga et al., 2015). Samples were diluted gradually and spread onto MRS agar that had 0.2% calcium carbonate using the pour plate method. After growing at 37 °C for 18 hours, the colonies that formed clear areas on the petri dishes were counted for each dilution. The count was expressed as CFU per milliliter, and a pH meter was used to measure pH level.

Degree of Hydrolysis

The degree of hydrolysis was determined following the procedures of Ramchandran and Shah (2008) in Miftakhussolikhah et al. (2025), which observed the amount of free amino acid in fermented milk. One milliliter of o-phthaldialdehyde (OPA) was combined with ten to fifty microliters of sample, and the mixture was agitated for five minutes. The amount of light absorbed was measured

at 340 nm using a spectrophotometer. Furthermore, the amount of peptides was found using a tryptone standard that ranged from 0 to 5 mg per milliliter. The results from the fermented milk were then compared with those from the unfermented milk (which had no fermentation time) to determine the degree of hydrolysis.

DNA Extraction

DNA extraction followed modified methods from Suhartatik et al. (2014), Matti et al. (2019), and Karyantina et al. (2020). Initially, LAB isolates were grown in MRS broth, incubated at 37 °C for 18 hours, and bacteria cell pellet was separated from the culture by centrifugation at 13,000 rpm and 4 °C. The pellet was resuspended in 50 µL of lysis solution, vortexed, and incubated for 20 minutes. After adding Proteinase K (20 µL, 20 mg/mL), the mixture was incubated for another 20 minutes, added with 40 µL of lysozyme (100 mg/mL), and incubated at 55 °C for 30 minutes. Centrifugation was used for collecting the supernatant, which was combined with phenol (1:1 v/v) and left 30 minutes. After additional centrifugation and mixing with chloroform and ethanol, the DNA genome was isolated and purified. The DNA was dried, reconstituted with TE buffer, treated with RNase, and incubated at 37 °C for 1 hour for further analysis.

Repetitive Polymerase Chain Reaction (rep-PCR) Amplification

Rep-PCR amplification was carried out using a modified Suhartatik et al. (2014) protocol. Initially, 9.5 µL of free water, 12.5 µL of MyTaq HS Red Mix, 1 µL of Primer 27F, 1 µL of Primer 1492R, and 1 µL of DNA template made up the 25 µL PCR mixture. This was followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 51.5 °C for 1 minute and 30 seconds, extension at 68 °C for 8 minutes, and final extension at 68 °C for 10 minutes comprised the PCR cycle, which started with an initial denaturation at 96 °C for 4 minutes. SYBR™ Safe was used to stain the agarose gel electrophoresis (1%) to confirm the purity of 16s rRNA. Electrophoresis was conducted for 60 minutes, and absorbance was recorded with a UV-vis spectrophotometer.

DNA Sequence and Analysis of Phylogenetic

The 16s rRNA gene sequences were analyzed against other bacteria 16s rRNA sequences in the GenBank database (NCBI) using the BLAST tool. Phylogenetic trees were constructed using the CLUSTALW program, and sequence similarities were analyzed through the neighbor-joining method with 1000x bootstrap support using MEGA-III11 software.

SPSS Analysis

Every sample was made three times, and one-way ANOVA in SPSS 27.0 software (IBM SPSS Statistics 27) was used to analyze the data. Furthermore, three replications of the data were conducted, and one-way ANOVA with Duncan's multiple comparison test was used to examine the means at a significance threshold of 0.05.

RESULTS AND DISCUSSION

Screening of Proteolytic LAB

LAB with proteolytic activity is recognized by clear zones formed around colonies. Since LAB depends on free amino acid, which are typically in short supply, proteolytic enzyme is essential for providing the cells with nitrogen molecules required for growth. The main function of this enzyme is to break down proteins into smaller molecules ingestible by bacteria cells. According to Kieliszek et al. (2021), proteolytic enzyme is made within the cells, secreted, and covalently attached to the cell wall.

In this study, 32 LAB isolates obtained from the FNCC UGM and PRTPP BRIN were evaluated for proteolytic capabilities. As shown in Table 1, 11 isolates had clear zone surrounding colonies, showing the potential for proteolysis (Aliifah et al., 2023). Skim milk agar, a popular medium for identifying bacteria capable of producing proteases, was used for the experiment (Rahmani et al., 2013). The results showed that bacteria colonies that generated protease created clear zone by breaking down casein into soluble nitrogen compounds. A clear zone is a qualitative indicator that the casein in the skim milk agar is being aggressively broken down by bacteria protease enzyme (Sabrini et al., 2021).

Previous studies reported that protease enzyme activity peaked between 24 and 48 hours. This corresponded to bacteria entering the log phase after 24 hours of development, thereby experimental incubation was performed for 48 hours (Rahmani et al., 2013). Among the 32 isolates, 11 were found to produce clear zone, namely L8, L23, KM 107, SR17A, SR17B, CP 77, Mut 7, Mut 13, T3, ST, and Dad 11.

In addition to qualitative examination, the Walter (1984) method was used to objectively assess the proteolytic activity of LAB. According to Rahmani et al. (2013), this method evaluated the activity of proteolytic enzymes in units (U), where one unit was equivalent to the quantity of enzyme required to release one micromole of tyrosine per minute. An increased protease activity signified a stronger proteolytic capability. As presented

Table 1. Screening of proteolytic LAB qualitatively (clear zone) and quantitatively (protease activity)

Isolates code	Clear zone	Protease activity (unit/mL)
L 1	-	1.22
L 2	-	0.80
L 5	-	0.82
L 6	-	1.08
L 7	-	1.29
L 8	√	0.86
L 12	-	1.25
L 13	-	0.91
L 16	-	0.83
L 18	-	1.08
L 23	√	1.94
KM 57	-	0.92
KM 87	-	0.75
KM 107	√	0.88
SR 17A	√	1.83
SR 17B	√	1,86
SR 48	-	1.13
SR 52	-	0.97
SR 77	-	0.82
TL 07	-	1.04
TL 17	-	0.94
TL 27	-	1.00
TL 58	-	0.83
TL 78	-	1.08
B 3	-	0.98
CP 77	√	0.90
GN 8	-	0.94
E 14 4	-	1.25
<i>L. plantarum</i> Mut 7	√	1.02
<i>L. plantarum</i> Mut 13	√	1.01
<i>L. plantarum</i> T3	√	1.03
<i>Streptococcus thermophilus</i> ST	√	1.08
<i>Streptococcus thermophilus</i> Dad 11	√	1.15

Description: (√) shows the presence of clear zone; (-) shows the absence of clear zone

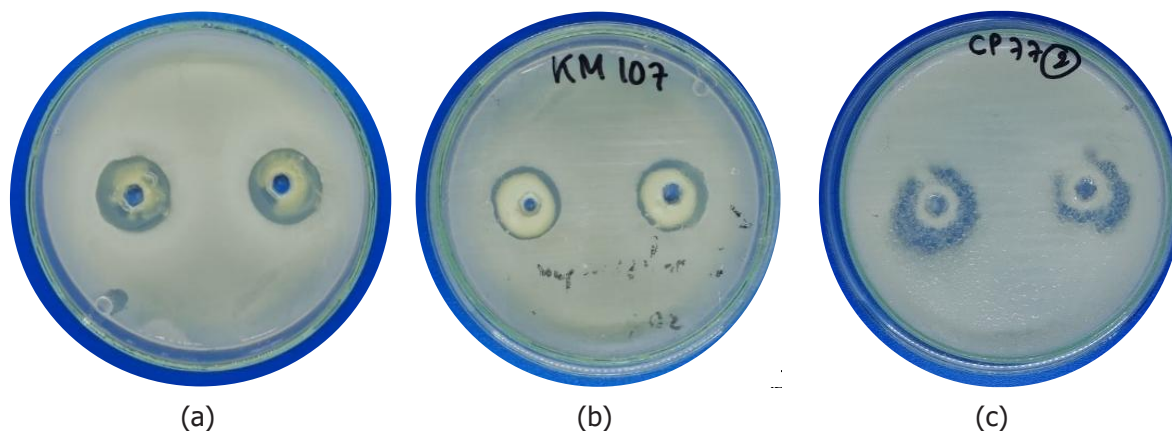


Figure 1. Clear zone in the qualitative proteolytic activity analysis of LAB using skim milk agar of *L. plantarum* Mut 7 (a); KM107 (b); CP77 (c)

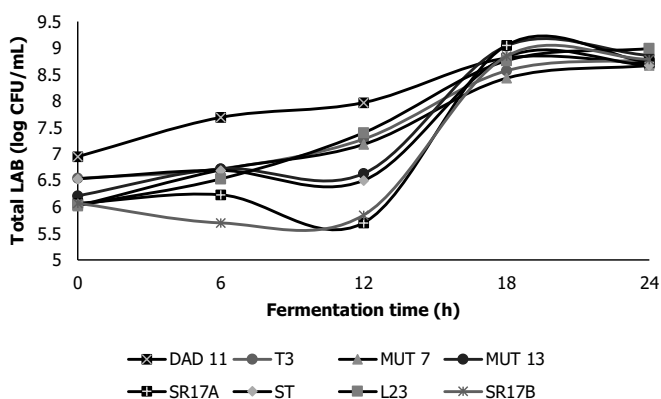


Figure 2. The quantity of LAB in fermented skim milk using specific isolates at 37 °C

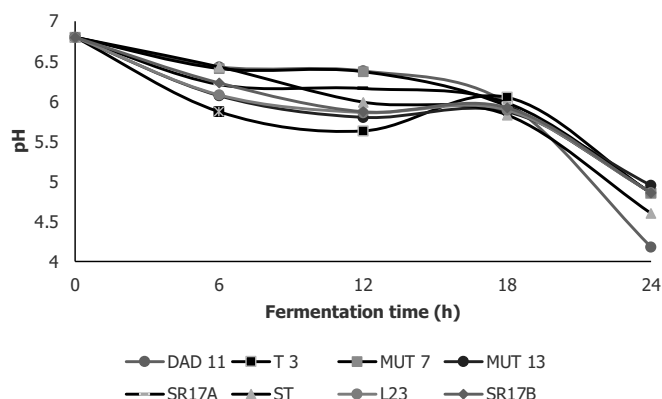


Figure 3. The pH of fermented skim milk using selected LAB isolates at a temperature of 37 °C

in Table 1, eight isolates, such as Dad 11, T3, Mut 7, Mut 13, SR17A, SR17B, ST, and L23, showed clear zone after 48 hours with a significant protease activity during incubation. In comparison, the protease activity levels in this study were higher than the values reported by Rahmani et al. (2013) and Yusmarini et al. (2010).

For other strains with high proteolytic activity but no formation of clear zones after incubation, the lack of clear zone could be attributed to insufficient incubation time. Some isolates with high protease activity (≥ 1 U/mL) would not have formed a clear zone after 48 hours due to several factors, including limited incubation time, insufficient enzyme secretion onto the agar, or the production of enzymes with low effectiveness against casein micelles in solid media. Strains have been observed to show clear zone after an extended incubation period of three or four days. The 48-hour limit indicated that only strains forming clear zones during that time were selected. Acidification can precipitate

casein and obscure zones despite proteolysis, showing method limitations. Although isolates positive in both assays were prioritized, high-activity strains without clear zone were retained for longer incubation tests.

Total Lactic Acid Bacteria and pH Value of Fermented Milk

The pH and LAB counts were monitored for 24 hours to observe bacterial growth and peptide-related changes. A total of eight isolates, including Dad 11, T3, Mut 7, Mut 13, SR17A, SR17B, ST, and L23 were selected based on proteolytic activity.

LAB increased steadily (Figure 2), while pH declined as lactic acid accumulated during fermentation (Figure 3). According to George et al. (2018), LAB are gram-positive bacteria that mostly create lactic acid during the fermentation of carbohydrates. As bacteria population increased, the amount of lactic acid produced also rose, causing a further decrease in pH of the fermented milk.

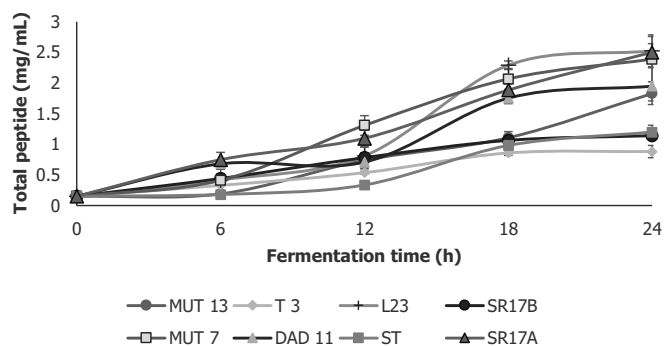


Figure 4. Total peptides of fermented skim milk using selected LAB isolates at 37 °C

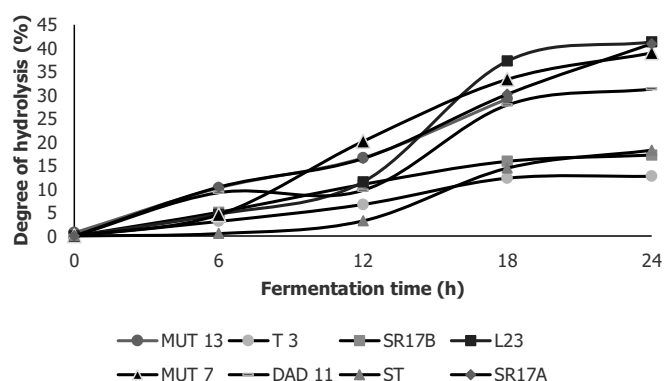


Figure 5. Degree of hydrolysis of fermented skim milk using selected LAB isolates at 37 °C

Total Peptides and Degree of Hydrolysis of Fermented Milk

The evaluation of total peptides and the degree of hydrolysis was performed on eight LAB isolates identified during the qualitative and quantitative screenings for proteolytic activity. Longer fermentation time in Figures 4 and 5 showed increased total peptides concentration and extent of hydrolysis, suggesting improved proteolytic activity in LAB. This increase is caused by LAB, which obtains growth stimulants and essential amino acids from milk proteins.

According to De Valdez et al. (1985), the fermentation process by LAB is affected by temperature and fermentation duration, which are connected to proteolytic capabilities. Certain LAB can produce essential amino acid required for growth (Savijoki et al., 2006). These bacteria require substantial levels of growth factors, including peptides and amino acids. LAB cannot grow in milk due to limited free amino acid and peptides, but has the potential to break down casein content as a source of nitrogen because of the developed sophisticated proteinase and peptidase systems.

Proteases found in the cell wall break down casein, converting into oligopeptides, which are further degraded by peptidases into smaller peptides and amino acid (Kirilov et al., 2009). Smaller peptides are generated from the hydrolysis of peptidases acting on longer oligopeptides initially produced by proteinase activity during milk fermentation. Since peptidase activity occurs intracellularly in LAB, it generally takes place after cell lysis, following fermentation (Atanasova et al., 2014). LAB proteolytic system is essential to fermented milk, enabling proliferation and the fermentation process. This proteolytic capability significantly affects the flavor and texture of products with LAB, while generating various bioactive peptides that promote health (Ramachandran and Shah, 2008)

In this study, proteolytic activity of LAB increased significantly at the beginning of milk fermentation, followed by a gradual rise towards the end. This progressive rise in the activity of the proteinase and peptidase enzymes could be explained by the acidity of the fermented milk and the self-inhibition induced by their action. Both the lactic acid bacteria population and the acidity of fermented milk increase with time, as shown in Figures 2 and 3. Since lactic acid bacteria prefer a pH range of 5.5 to 6, this increasingly acidic environment eventually lowers their activity (Sionek et al., 2024). The pH of the fermented milk generally deviates from this optimal range at the end of the fermentation process, and affects LAB's proteolytic capabilities.

AGI Activity of Fermented Milk

Table 2 shows the AGI activity of milk fermented for 24 hours with eight LAB strains. L23 and SR17B produced the highest activity at 12 hours. AGI activity rose until 12 hours for T3, L23, and SR17B, then declined at 14–16 hours, likely due to peptide degradation, nutrient loss, and low pH. Other strains reached their peak at different times, including Mut 7 and DAD 11 at 6 hours, ST at 18 hours, and Mut 13 at 24 hours. These differences highlight strain-specific behavior and the need to optimize fermentation conditions to achieve maximum AGI activity.

The results in Figures 6 and 7 showed the correlation between incubation time, degree of hydrolysis, and AGI activity in fermented milk using L23 and SR17B, with the highest AGI value. Longer fermentation time led to more hydrolysis, showing improved peptides synthesis produced by the proteolytic activity of LAB during fermentation (Konrad, 2014). However, AGI activity did not follow a linear trend with the degree of hydrolysis. After 12 hours of fermentation, AGI values for fermented milk with LAB isolates L23 and SR17B began to decline.

Hydrolysis in the formation of tripeptides and other peptides promotes AGI activity, with optimal

Table 2. AGI Activity (%) of fermented milk using several LAB isolates at different fermentation times

LAB code	0 h	6 h	12 h	18 h	24 h
<i>L. plantarum</i> Mut 7	14.20 ^a ±0.12	24.04 ^b ±2.35	15.22 ^a ±3.34	14.47 ^a ±1.96	14.01 ^a ±3.91
<i>L. plantarum</i> Mut 13	14.20 ^a ±0.12	14.74 ^a ±0.95	16.94 ^{ab} ±3.11	18.83 ^b ±1.02	27.94 ^c ±1.58
<i>Streptococcus thermophilus</i> Dad 11	14.20 ^a ±0.12	24.86 ^c ±1.65	19.74 ^{ab} ±2.07	17.50 ^{ab} ±2.10	21.39 ^{bc} ±1.28
<i>L. plantarum</i> T3	14.20 ^a ±0.12	23.48 ^b ±0.72	23.68 ^b ±0.46	22.21 ^b ±1.44	22.58 ^b ±1.10
<i>Streptococcus thermophilus</i> ST	14.20 ^a ±0.12	20.79 ^a ±2.85	21.49 ^b ±1.50	29.93 ^c ±2.15	28.55 ^c ±1.53
SR17A	14.20 ^a ±0.12	17.17 ^a ±1.15	20.42 ^{ab} ±1.30	26.62 ^b ±1.22	17.48 ^a ±0.82
SR17B	14.20 ^a ±0.12	11.98 ^a ±1.84	35.94 ^d ±1.32	18.15 ^b ±1.08	29.35 ^c ±3.26
L23	14.20 ^a ±0.12	25.61 ^b ±1.38	35.15 ^d ±0.87	25.31 ^b ±0.40	29.05 ^c ±0.44
Acarbosa (5 mg/mL)	50.56				

Values are expressed as mean ± standard deviation (SD) of triplicate experiments (n = 3). Different superscript letters in the same row indicate significant differences among fermentation times (p < 0.05).

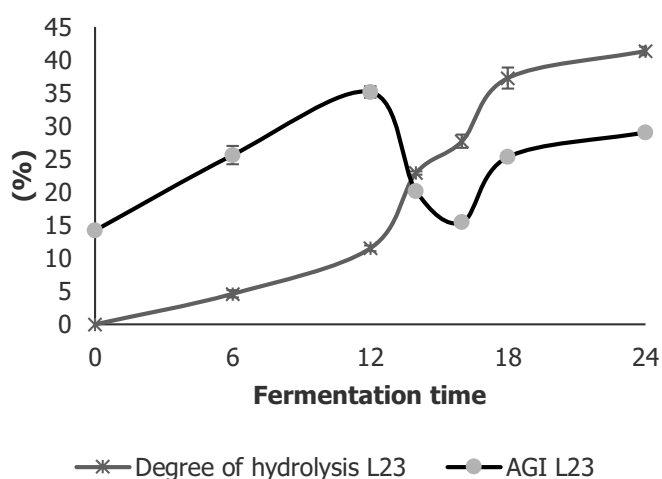


Figure 6. Degree of hydrolysis and α-glucosidase inhibitory activity of fermented skim milk using *L. plantarum-pentosus* L23 at 37 °C

fermentation times identified as 6 hours for Mut 7 and DAD 11. This was followed by 12 hours for T3, L23, and SR17B, 18 hours for ST, and 24 hours for Mut 13. After reaching maximum capacity, excessive hydrolysis caused the formation of smaller peptides less effective for AGI activity. Varying fermentation period produced 11-20 peptides with relatively strong inhibitory action based on the isolate (Ibrahim et al., 2017; Wang et al., 2018). The proteases generated by these bacteria varied according to species, media composition, and growth conditions, causing variations in relative protease activity (Hengkengbala et al., 2021).

Peptides consisting of 3 to 6 amino acids (Yu et al., 2012; Ren et al., 2016; Zhang et al., 2016; Ibrahim,

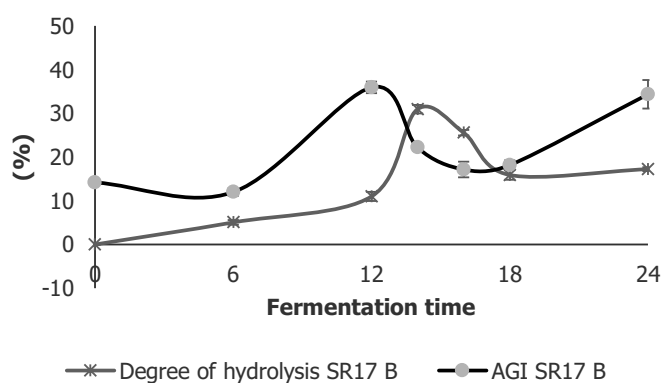


Figure 7. Degree of hydrolysis and α-glucosidase inhibitory activity of fermented skim milk using *L. plantarum-pentosus* SR17B at 37 °C

2017) or those with molecular weights between 3 to 10 kDa (Konrad et al., 2014) are essential for the inhibition of AGI. However, AGI activity levels observed in all fermented milk samples were still lower compared to acarbose activity level of 5 mg/mL (50.56%). Acarbose is a common drug used to treat T2DM due to the ability to inhibit the membrane-bound intestinal α-glucosidase hydrolase enzyme in a competitive and reversible mechanism (Ziaee et al., 2017). The mechanism of this drug includes slowing down carbohydrate digestion, which reduces glucose absorption and lowers postprandial blood glucose levels.

Molecular identification of LAB using 16S rRNA gene

Molecular identification was carried out on selected LAB isolates with proteolytic activity, such as

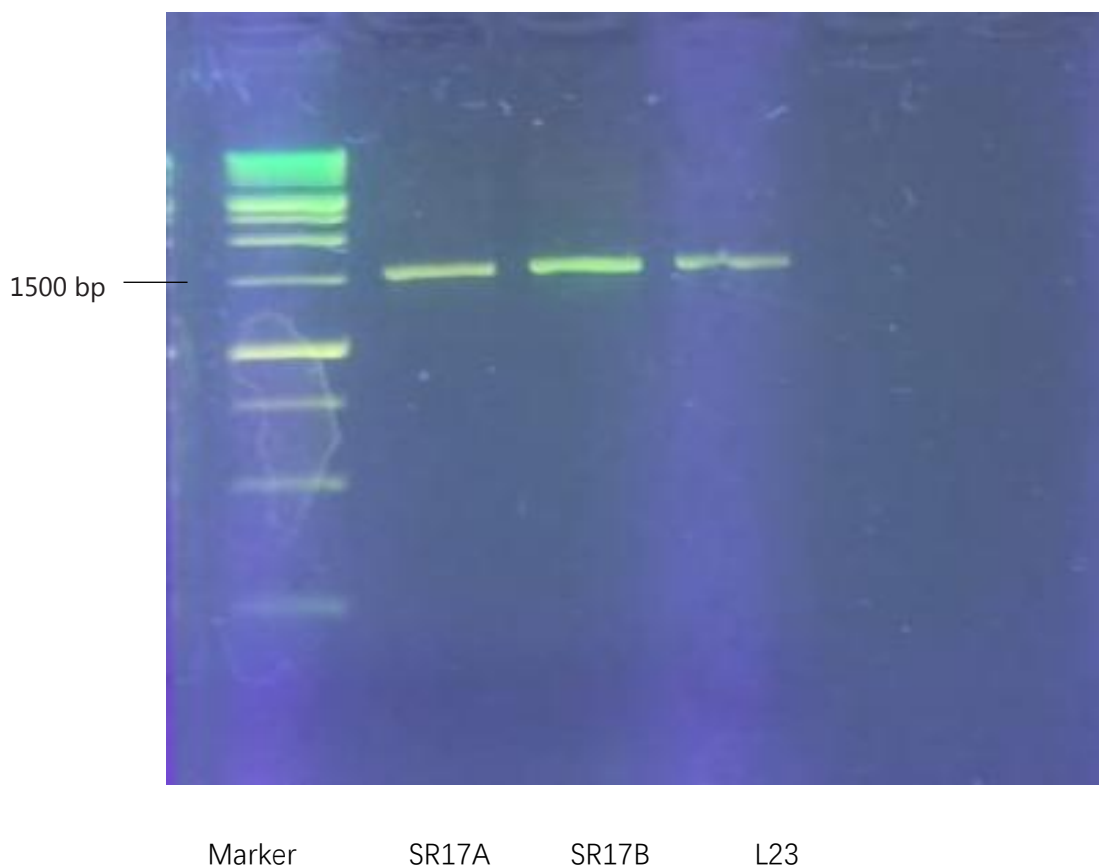


Figure 8. DNA band profile produced by repeated PCR amplification at 1500 bp in AGE

SR17A, SR17B, and L23. In Figure 8, the electrophoresis results of the 16S rRNA gene showed that the three isolates produced bands at the same region, indicating classification in the same bacteria group. Identification using 16S rRNA gene sequencing showed that the PCR products of the isolates formed a single band. This confirmed that the 16S rRNA gene was successfully amplified at approximately 1500 bp. The sequencing results of all isolates were compared with 16S rRNA gene sequences available in the GenBank database provided by NCBI (2024), with the results shown in Table 3.

The results in Table 3 showed that isolate SR17A showed 99.86% genetic similarity with *Lactiplantibacillus pentosus* strain 124-2, *Lactiplantibacillus plantarum* strain NRRL B-14768, *L. plantarum* strain JCM 1149, and *L. plantarum* strain CIP 103151. Furthermore, SR17B showed 99.93% genetic similarity with the same strains, namely *L. pentosus* strain 124-2, *L. plantarum* strain NRRL B-14768, *L. plantarum* strain JCM 1149, and *L. plantarum* strain CIP 103151. Meanwhile, isolate L23 showed 99.93% similarity with *Lactiplantibacillus plantarum* strain JCM 1149.

Based on Table 3, all isolates showed 100% query coverage, indicating that the entire tested DNA sequences

matched completely with reference sequences in the database. The identity percentage, ranging from 99.78% to 99.93%, suggested that these sequences were highly similar or nearly identical to *Lactiplantibacillus* strains listed in the NCBI database. This showed that the three isolates likely belong to the same or closely related phylogenetic species. Each isolate showed a very high degree of similarity to several *L. plantarum* and *L. pentosus* strains, suggesting minimal genetic variation. For example, SR17A and SR17B showed identical levels of similarity (99.86% to 99.93%) to the same reference strains, which indicated significant genetic resemblance. To better understand the evolutionary connections between these organisms, genes, or species based on the genetic data collected, a phylogenetic tree was created using MEGA-11 software (Figure 9).

As presented in Figure 9, phylogenetic tree showed a significant relationship between SR17A, SR17B, and L23 with several reference strains from the *Lactiplantibacillus* genus, including *Lactiplantibacillus plantarum* and *Lactiplantibacillus pentosus*. This phylogenetic tree showed the degree of genetic similarity, where the proximity of two strains or isolates indicated close evolutionary relationship.

Table 3. Results of molecular identification of LAB with 16s rRNA sequence

No	Isolates code	Reference from NCBI data base	Reference	Query cover (%)	Identities (%)
1	SR17A	<i>Lactiplantibacillus pentosus</i> strain 124-2	NR_029133.1	100	99.86
		<i>Lactiplantibacillus plantarum</i> strain NRRL B-14768	NR_042394.1	100	99.86
		<i>Lactiplantibacillus plantarum</i> strain JCM 1149	NR_117813.1	100	99.86
		<i>Lactiplantibacillus plantarum</i> strain CIP 103151	NR_104573.1	100	99.86
		<i>Lactiplantibacillus plantarum</i> strain NBRC 15891	NR_113338.1	100	99.78
2	SR17B	<i>Lactiplantibacillus pentosus</i> strain 124-2	NR_029133.1	100	99.93
		<i>Lactiplantibacillus plantarum</i> strain NRRL B-14768	NR_042394.1	100	99.93
		<i>Lactiplantibacillus plantarum</i> strain JCM 1149	NR_117813.1	100	99.93
		<i>Lactiplantibacillus plantarum</i> strain CIP 103151	NR_104573.1	100	99.93
		<i>Lactiplantibacillus plantarum</i> strain NBRC 15891	NR_113338.1	100	99.86
3	L23	<i>Lactiplantibacillus plantarum</i> strain JCM 1149	NR_115605.1	100	99.93
		<i>Lactiplantibacillus plantarum</i> strain NBRC 15891	NR_113338.1	100	99.86
		<i>Lactiplantibacillus pentosus</i> strain 124-2	NR_029133.1	100	99.86
		<i>Lactiplantibacillus plantarum</i> strain NRRL B-14768	NR_042394.1	100	99.86
		<i>Lactiplantibacillus plantarum</i> strain CIP 103151	NR_104573.1	100	99.86

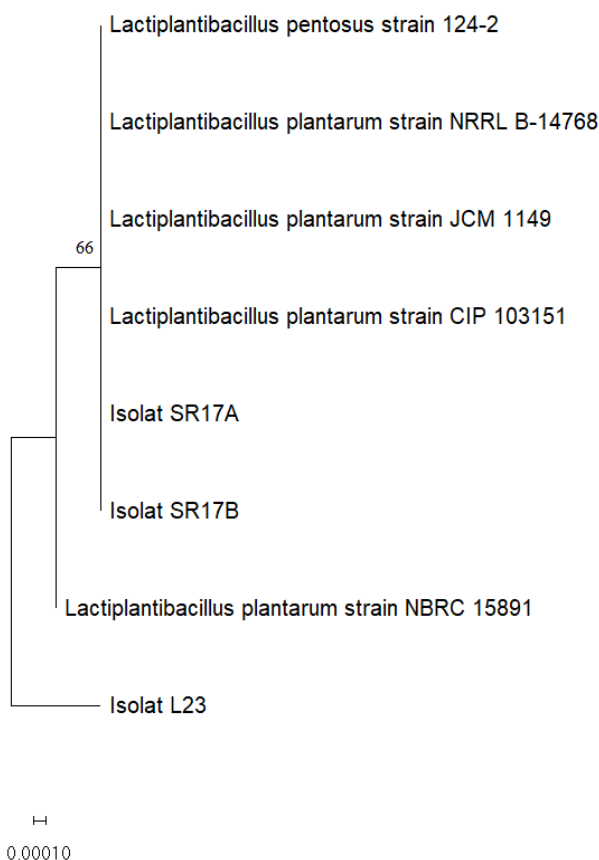


Figure 9. Neighbor-joining phylogenetic tree based on 16s rRNA- MEGA 5 of SR17A, SR17B and L23 isolates

Among all isolates, SR17A was positioned on a separate branch but closely related to a clade consisting of *L. plantarum* strain CIP 103151 and strain JCM 1149, as well as *L. pentosus* strain 124-2. This closeness indicates that SR17A is closely related in terms of the family tree, which shows sharing of similar chemical or functional traits. With a bootstrap value of 66, this relationship shows a moderate level of confidence based on phylogenetic analysis.

According to Echegaray et al. (2023), *L. plantarum* (formerly known as *Lactobacillus plantarum*) is commonly used in food fermentation. *L. plantarum* has proteolytic, probiotic properties, and various functional health benefits. Specifically, *L. plantarum* strains from food show good GI adhesion, survival, antioxidant and antimicrobial activity, and often produce bacteriocins. The probiotic traits and broad distribution offer strong health potential. *L. pentosus* converts carbohydrates to lactic acid and hydrolyzes proteins, making it important in fermented foods like kimchi, pickles, yogurt, and cheese (Behbahani et al., 2024). Among *L. plantarum* group, JCM 1149 is often found in fermented beverages, supporting gut and immune function (Horie et al., 2019). CIP 103151 also shows probiotic promise, while *L. pentosus* 124-2 from buffalo-milk fermentation (*dadih*) shows strong fermentative performance (Yuliana et al., 2023). Because of the ability to produce lactic acid, break down proteins, and have health-promoting properties, *L. plantarum* NRRL B-14768 and NBRC 15891 are widely used in the fermentation industry and are desirable constituents in food and nutraceutical compositions.

Phylogenetic analyses show that SR17A, SR17B, and L23 are closely related to well-established *Lactiplantibacillus* reference strains used in fermented-food production. The genetic similarity to well-known proteolytic and functionally active strains shows potential for application in milk fermentation and other protein-rich fermentation. This similarity also shows that isolates might share identical metabolic and technological traits advantageous for industrial fermentation.

In this study, a very high degree of genetic similarity was shown by SR17A, SR17B, and L23 to strains belonging to the *Lactiplantibacillus* genus, specifically the *L. plantarum-pentosus* species group. Therefore, SR17A, SR17B, and L23 were identified as belonging to *Lactiplantibacillus plantarum-pentosus*. As LAB, both *L. plantarum* and *L. pentosus* can produce proteases that contribute to proteolytic activity (Kuerman et al., 2024). Consequently, the three LAB isolates probably have similar abilities to break down proteins, like *L. plantarum*. The relatively high proteolytic activity shown in Table 1 supports the potential use of these isolates for producing bioactive peptides, including AGI.

CONCLUSION

In conclusion, screening of 32 LAB isolates shows that eight strains, including Dad 11, T3, Mut 7, Mut 13, SR17A, SR17B, ST, and L23, have proteolytic activity. Among these isolates, L23 and SR17B are capable of producing fermented milk with relatively high AGI activity. The results show that AGI activity in the fermented milk increases alongside fermentation time, reaching the highest level after 12 hours when using both *L. plantarum-pentosus* L23 and *L. plantarum-pentosus* SR17B. Moreover, further studies are required to explore the mechanism of AGI in fermented milk produced with these selected LAB.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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