



α -glucosidase Inhibitory Activity of Probiotic Isolate LBSU₉ Isolated from Traditional Food “Trites”: a Preliminary Study

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ABSTRACT

As the prevalence of type 2 diabetes mellitus continues to increase over the years, research into antidiabetic drugs needs to continue. Probiotics are non-pathogenic microorganisms that have potential as α -glucosidase inhibitors. This study aimed to characterize and determine the α -glucosidase inhibitor activity of LBSU₉ isolate, a probiotic isolated from "trites," a traditional food of North Sumatra. The results showed that LBSU₉ had bacillus morphology, Gram positive, negative catalase test, TSIA (Triple Sugar Iron Agar) A/A test and non-hemolysis. LBSU₉ had good tolerance to simulated gastric acid and bile salts, with growth percentages of 66.54% and 64.74%. LBSU₉ also has potent antimicrobial activity against *S.aureus* and *E.coli*, with 20 mm and 17 mm inhibition zones, respectively. The alpha-glucosidase inhibitor activity of LBSU₉ was 98.4% greater than that of acarbose, which was 97%. Based on the results of this study, it is concluded that LBSU₉ isolate has the potential to be a complementary therapy to prevent or treat type 2 diabetes mellitus.

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1. INTRODUCTION

Type 2 diabetes mellitus (T₂DM) is recognized as a critical public health concern that has a significant impact on human life and healthcare costs. Rapid economic development and urbanization have increased the prevalence of diabetes in many parts of the world [1]. In the past, Type 2 Diabetes Mellitus (T₂DM) was previously known as non-insulin dependent diabetes or adult-onset diabetes with insulin resistance that could escalate to total resistance. Over the past decade, impaired β -cell function has been discovered as a major issue in T₂DM. About 90-95% of diabetics have type 2 diabetes [2].

Acarbose, an alpha-glucosidase inhibitor (AGIs), is a common oral hypoglycemic medication. AGIs primarily focus on inhibiting α -glucosidase in the small intestine, an enzyme responsible for converting complex carbs that cannot be absorbed into easily absorbable monosaccharides. Acarbose functions as a competitive and reversible inhibitor of glucosidase, an enzyme found in the small intestine's brush border. By doing so, it hinders the digestion of starch and sucrose, thereby slowing the absorption of glucose and fructose in the upper part of the small bowel. This reduces blood glucose levels, particularly after eating [3]. Although these inhibitors reduce glucose absorption, they are not widely used due to adverse gastrointestinal side effects. As a result, research efforts continue to identify new inhibitors with increased efficacy and minimum adverse effects [4].

One of the potential source of α -glucosidase inhibitors is Lactic Acid Bacteria. Several species of Lactic Acid Bacteria possess this inhibitor activity. Lactic acid bacteria (LAB) are classified into many genera and have long been utilized as probiotics. LAB can be found in a variety of environments, including the gastrointestinal (GI) tract, oral cavities, vaginal tracts of people and animals, fermented foods, silages, and composts. They provide numerous health benefits to the host, including increased immunological function, better digestion, control of inflammatory bowel illnesses, relief of constipation, and strengthening the mucosal barrier [5]. Research on the use of probiotics as an alternative therapy in lowering blood glucose levels is still in its early stages, but some studies have shown potential especially in α -glucosidase inhibition. *Lactobacillus casei* 2W and *Lactobacillus rhamnosus* Z7 are reported to have α -glucosidase inhibitor activity [6]. *Lactobacillus pentosus* isolated from the Kersen plant (*Muntingia calabura*) was reported to have activity as an α -glucosidase inhibitor in vitro although 1.27 times lower than acarbose [7]. Traditional fermented food have been identified as potential sources of probiotics due to their high nutritional value and the presence of lactic acid bacteria (LAB). These LAB, which are integral to many fermented foods, have been shown to have probiotic characteristics, suggesting that these foods could be beneficial for gut health [8].

In North Sumatra, there is one traditional food that has potential as a source of probiotics, called "trites". Trites are made from grass derived from the rumen of buffalo. Based on a report by [9], the rumen of livestock is one of the sources of probiotics, apart from milk and even feces. In his research, it was stated that bacteria isolated from the rumen, milk, and feces of livestock showed antimicrobial activity against pathogenic bacteria such as *Bacillus* spp, *E.coli*, *Staphylococcus aureus*, *Salmonella* sp, and *Listeria* spp. 16SrRNA gene sequencing results showed that the bacteria were identified as *Enterococcus lactis*, *Pediococcus pentosaceus*, and *Lactococcus lactis*. This statement is also reinforced by [10], who reported in his research that probiotics identified as *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus fermentum*, and *Lactobacillus johnsonii* can be isolated from cow rumen fluid. These probiotics are reported to have antimicrobial activity against pathogenic bacteria such as *Pseudomonas aeruginosa* (ATCC9027) and *Staphylococcus aureus* (ATCC 6538).

LBSU9 is one of the bacterial isolates that we isolated from trites. In this study, we characterized LBSU9 probiotics based on antimicrobial activity, tolerance to simulated gastric acid and tolerance to bile salts. We determined the safety test based on hemolytic activity. However, research on the potential of LBSU9 as α -glucosidase inhibitors has not been reported.

2. RESEARCH METHOD

2.1. Isolation of LBSU9 from Trites

One gram of trites sample was cultured into 9 mL of sterile MRS broth and incubated for 18-24 hours under anaerobic conditions. After the incubation process, serial dilution was carried out up to a dilution of 10^{-7} . A total of 0.1 mL of the 10^{-7} th dilution was then inoculated onto the surface of MRS agar media supplemented with 1% CaCO_3 to distinguish acid-producing bacteria from other bacteria characterized by a clear zone around the bacteria. Bacterial isolate were then randomly selected and continued with purification by re-streaking into new MRS media. The isolate were stored in 40% glycerol stock media at -20°C [11].

2.2. Characterization of LBSU9

The purified colonies were then characterized, including Gram staining, TSIA (Triple Sugar Iron Agar) test, hemolysis, and catalase test. Morphological and culture examination used the Gram staining method described by Hans Christian Gram (1884). This test was used to check for catalase enzyme production. For this test, a clean microscopic slide was taken. A drop of 3% H_2O_2 is taken on the microscopic slide aseptically. One ose of bacterial culture was taken, mixed with 3% H_2O_2 solution on the slide, and observed for bubble production [12]. The TSIA test is performed by inoculating the TSIA medium by piercing through the center of the medium to the bottom of the tube and then scraping the agar surface obliquely. Incubate the tube at $35^\circ\text{--}37^\circ\text{C}$ in open air for 18-24 hours. After incubation, check for discoloration of the slanted and bottom portions, appearance, and cracks in the media [13].

2.3. Characterization Test and Safety Evaluation of Probiotics

The probiotic characteristics of LBSU₉ isolates were tested based on tolerance to acidic pH, simulation of gastric juice and bile salt, and hemolytic test. Tolerance to acidic pH and gastric juice was carried out to simulate the ability of isolates to survive in gastric conditions. In contrast, tolerance to bile salt was carried out to simulate conditions in the duodenum. To evaluate the capacity for tolerance to acidic conditions, 0.1 mL of LBSU₉ cultured for 24 hours was inoculated into MRS broth, which had been adjusted to pH 3 using 1 M HCl. OD values of LBSU₉ growth at MRS pH 3 and pH 6.5 were measured at a wavelength of 600 nm after incubation at 37°C for 4 hours with the formula [5]:

$$\text{Percentage of growth} = \frac{\text{growth in MRS broth pH3}}{\text{growth in MRS broth pH 6.5}} \times 100\% \quad (1)$$

The range of tolerance to bile salt was assessed by assessing the growth ratio of MRS broth containing 0.3% bile salt to MRS broth without bile salt. Simulated gastric fluid was prepared by dissolving 3 mg/ml pepsin in physiological NaCl and then set at pH 2.5. This solution was inoculated for 4 hours with 1% mL of LAB culture (v/v) incubated overnight [14].

The hemolytic assay was determined using Columbia agar containing 5% (w/v) sheep blood, and Petri dishes were incubated at 37°C for 48 hours. After incubation, the hemolytic activity of the isolate was evaluated and classified based on the lysis of red blood cells around the colony. A green zone around the colony included α -hemolysis, and a clear zone around the colony included β -hemolysis. If there was no zone around the colony, it included γ -hemolysis. Only γ -hemolysis is considered safe [15].

2.4. Antibacterial activity of LBSU₉

LBSU₉ and indicator bacteria, as many as 4-5 colonies, were dissolved into 5 mL of sterile Nutrient Broth and compared with 0.5 McFarland solution, equivalent to 1.5×10^8 CFU/mL. Indicator microorganisms *Staphylococcus aureus* and *Escherichia coli* were utilized. The indicator microorganisms were transferred using a sterile cotton swab onto the nutrient agar surface. Sterile discs were dipped into the LBSU₉ isolate culture attached to the nutrient agar surface and incubated for 24 hours at 37°C. The zone of inhibition was measured using a caliper [16].

2.5. Inhibitory α -glucosidase activity of LBSU₉

Isolates of LBSU₉ were cultivated for 24 hours at 37°C in MRS broth medium. 15 minutes of centrifugation at 3500 rpm were required to separate the metabolite extract from the bacteria. Metabolites contained in the supernatant were tested for α -glucosidase enzyme inhibition activity. An enzyme solution of 1 unit/ml was prepared by dissolving 2,695 mg α glucosidase in 50 mL phosphate buffer pH 7. The reaction mixture containing 2 μ l supernatant, 48 μ l phosphate buffer (100 mM, pH 7), and 25 μ l α -glucosidase enzyme (0.25 unit/mL) was incubated at 37°C for 5 minutes. After incubation, 25 μ l of p-nitrophenyl α D glucopyranoside substrate (20mM) was added to the mixture. Incubation was continued for 15 minutes at 37°C, and then 100 μ l of 200mM Na₂CO₃ was added to stop the reaction. The absorbance was measured using a microplate reader at λ 415nm. Acarbose, 1% solution, was prepared as a comparison (positive control) by dissolving Glucose tablets in phosphate buffer pH 7 (1:100). The complete reaction design for 1 sample with a total volume of 200 μ l can be seen in the following table:

Table 1. Enzymatic reaction design of α -glucosidase inhibitor

	mixture			
	A ₀ (μ L)	A _i (μ L)	A ₁₀ (μ L)	A _n (μ L)
Supernatant	-	-	2	2
MRS media	2	2	-	-
Buffer	48	48	48	48
Enzyme	-	25	-	25
Incubate at 37°C for 5 minutes				
substrate	25	25	25	25
Incubate at 37°C for 15 minutes				

	mixture			
	A _o (μL)	A _i (μL)	A _{io} (μL)	A _{ii} (μL)
Na ₂ CO ₃	100	100	100	100

The inhibitory activity of isolate and acarbose against α-glucosidase enzyme was calculated by the following formula : [17]

$$\text{Percentage of inhibition} = \frac{(A1 \text{ absorbance} - A0) - (AI \text{ absorbance} - AI0)}{(A1 \text{ absorbance} - A0)} \times 100\% \tag{2}$$

3. RESULTS AND DISCUSSIONS

3.1. Characterization of LBSU₉

Before further testing, isolate LBSU₉ was first characterized morphologically and biochemically. Tests include determining Gram staining and morphology, catalase test, TSIA test, Gas production, and hemolysis test. Characterization needs to be done to ascertain whether or not the LBSU₉ isolate is classified as lactic acid bacteria. The characterization results of isolate LBSU₉ are shown in table 2 below:

Table. 2. Characterization of LBSU₉ isolate

isolate	characterization					
	Gram staining	morphology	catalase	TSIA test	Gas production	Haemolysis
LBSU ₉	positive	rod	negative	A/A	-	γ

From Table. 2, it is known that isolate LBSU₉ is Gram positive and catalase negative. The identification of LAB is typically confirmed through a combination of morphological, physiological, and biochemical tests, including the catalase test and Gram staining. Lactic acid bacteria (LAB) are Gram-positive, catalase-negative, and non-spore forming organisms, with a variety of shapes including cocci and rod-shaped cells [18]. Gram staining results of LBSU₉ are shown in Figure 1 below.

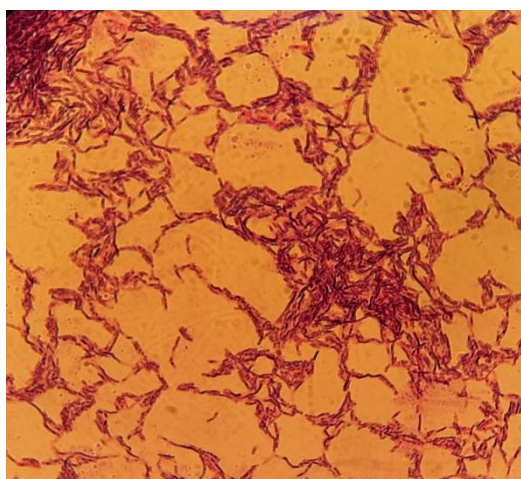


Figure 1. Gram staining results of isolate LBSU₉.

Figure. 1 was obtained from observations using a light microscope at 400x magnification. From Figure. 1, it can be seen that the LBSU₉ bacterial colony has a bacillus or rod-shaped morphology. The dark purple color observed from the results of Gram staining indicates that isolate LBSU₉ is Gram positive. The violet or purple color of Gram-positive bacteria is due to their ability to retain the crystal violet-iodine complex after treatment with alcohol.

The TSIA (Triple Sugar Iron Agar) test showed that the LBSU₉ isolate showed A/A results, which means that the bacterial isolate is able to ferment glucose, lactose and sucrose. TSIA media

contains phenol red which is an indicator and ferrous sulfate which is an indicator for hydrogen sulfide. The change in the media to a yellow color is because the bacteria produce acid so that it lowers the pH and the media becomes yellow [19]. TSIA test results are shown in Figure 2 below.



Figure 2. TSIA test result of isolate LBSU9

3.2. Characterization Test and Safety Evaluation of Probiotics

Hemolysis test results show that isolate LBSU9 is hemolysis γ . Bacteria are frequently classified into several families and groups. This classification is based in part on the bacteria's physical appearance, as well as some chemical and biological properties. The organism does not cause hemolysis, the agar under and around the colony remains unaffected, and it is referred to as non-hemolytic or gamma hemolytic (γ -hemolysis) [20]. The correlation between hemolytic activity of bacteria and pathogenicity is well-established. Previous study found that these properties are characteristic of pathogenic strains, with a wide variation in their activity against different human blood group cells [21]. This is further supported by [22], who reported that hemolytic activity is pronounced in dermatophytes and may play a role as a virulence factor. The hemolysis test result are shown in Figure 3.

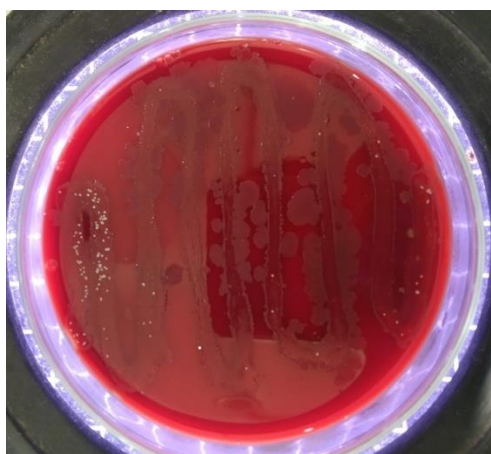


Figure 3. Hemolysis test result of LBSU9

Numerous studies have demonstrated that probiotics do not induce hemolysis. A prior investigation documented the combination of three prospective probiotics—*Bacillus amyloliquefaciens* (L9, isolated from the blue swimming crab), *Lysinibacillus fusiformis* (A2, isolated from a microalga), and *Enterococcus hirae* (LAB3, isolated from the Asian seabass)—which exhibited in vitro antagonistic activity towards *Aeromonas hydrophila*. Additionally, streaking the probiotic mixture onto sheep blood agar (5%), which contained hemolytic agents, did not yield any discernible effects. However, *A. hydrophila* exhibited α -hemolysis [23]. Similarly, [24] and [25] reported that *Lactobacillus gasseri* MA-2 and various yeast strains, respectively, also showed no hemolytic activity. These findings collectively support the safety of probiotics in this regard. Simulated gastric fluid was made by adjusting the pH of MRS broth to 3 and added with 3% pepsin. The growth of LBSU9 isolate in simulated gastric acid solution and MRS media pH 6.5 is shown in table 3.

Table. 3. Growth of LBSU9 isolate in MRS broth media (pH =6.5) compared to growth in simulated gastric juices

Isolate code	Growth on MRS broth pH 6.5	Growth on simulated gastric fluid	% growth
LBSU9	1.81	1.20	66.54%

Based on the Table. 3, it can be seen that isolate LBSU9 has a good tolerance to simulated stomach acid. The ability of bacteria to grow on acidic media is influenced by a variety of factors. In marine environments, acidification has been shown to decrease microbial community diversity and bacterial metabolic activity [26]. Research has shown that certain strains of probiotics, such as *Lactobacillus pentosus*, have inherent resistance to stomach acid, which is associated with the over-production of specific proteins [27]. Similarly, *Lactobacillus* spp. strains isolated from the human stomach have demonstrated good resistance to low pH, making them potential candidates for probiotic use [28]. However, the impact of exposure to stressful conditions, such as acid, on the functional properties of probiotics is not fully understood. Further research is needed to explore the relationship between stress exposure and probiotic properties [29]. Another probiotic criterion is resistance to bile salts. LBSU9 isolate resistance to bile salt solution is shown in the following table.

Table 4. Growth of LBSU9 isolate in MRS broth media (pH =6.5) compared to growth in bile salt 0.3%

Isolate code	Growth on MRS broth pH 6.5	Growth on bile salt 3%	% growth
LBSU9	1.9	1.23	64.74%

Based on the Table. 4 it can be seen that isolate LBSU9 has a good tolerance to simulated stomach acid. Research has shown that certain probiotic strains, such as *Pediococcus acidilactici* Ro1 and Ro2, are resistant to bile salt and acid conditions, making them potential probiotic candidates [30]. Similarly, *Lactobacillus* spp strains isolated from locally fermented food products have been found to be tolerant to bile salt, indicating their potential as probiotics [31]. The resistance of these probiotics to bile salt is attributed to specific mechanisms, such as efflux of bile salts or protons, modification of sugar metabolism, and prevention of protein misfolding [32].

3.3. Antibacterial activity of LBSU9

The antimicrobial activity of the isolates was tested against *E.coli* ATCC 25922 and *S.aureus* ATCC 25923 by disc diffusion method. Amoxycillin was used as positive control. The antimicrobial activity of isolate LBSU9 is shown in Figure 4.

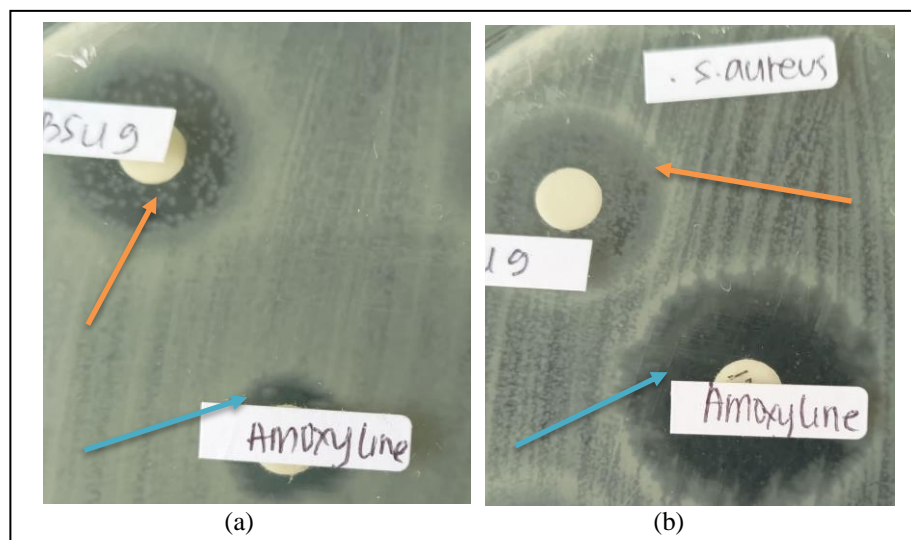


Figure 4. Antimicrobial activity of LBSU9 againsts *E.coli* ATCC 25922 (a) and *S.aureus* ATCC 25923 (b). the brown arrow indicates the antibacterial activity of LBSU9 while the blue arrow indicates the antibacterial activity of amoxycillin

Figure 4 above shows the ability of LBSU9 isolate to inhibit the growth of *E.coli* and *S. aureus* bacteria. The zone of inhibition of LBSU9 isolate against these pathogenic bacteria is indicated by a clear zone around the disc. Figure. 2 shows that the inhibition zone of LBSU9 isolates against *E. coli* bacteria is greater than the zone of inhibition of amoxycillin. Diameter of the inhibition zone of LBSU9 and amoxycillin are shown in the following table.

Table 5. Diameter of the inhibition zone of LBSU9 and amoxycillin

Isolate / positive control	Inhibition zone (mm)	
	<i>E.coli</i>	<i>S.aureus</i>
LBSU9	17	20
Amoxycillin	11	22

From the Table. 5, it can be seen that LBSU9 can inhibit the growth of *E.coli* and *S.aureus* bacteria, which can even show better antimicrobial activity than Amoxycillin in inhibiting the growth of *E.coli*. Research has consistently shown the antimicrobial activity of probiotics against *E. coli* and *S. aureus*. From previous study, [33] found that probiotics isolated from yoghurts and their metabolites exhibited significant antibacterial effects, with the highest activity against *S. aureus* which is 10.3 mm. In another study, [34] highlighted the potential of certain lactic acid bacteria, classified as probiotics, to produce antimicrobials active against *S. aureus* and other skin pathogens, suggesting their use in the development of living antimicrobials for skin disorders. Table. 5 demonstrates that the growth inhibition against *S. aureus* is more potent than the inhibition against *E. coli*. This is attributed to variations in their cellular wall structure and composition. The increased thickness of the peptidoglycan layer in Gram-positive bacteria renders it more susceptible to antimicrobial chemicals, whereas the outer membrane of Gram-negative bacteria functions as a protective barrier, rendering them more resilient [35]. Efflux pumps in Gram-negative bacteria can also contribute to antimicrobial resistance [36].

3.4. Inhibitory α -glucosidase activity of LBSU9

The value A_0 , A_t , A_{10} , and A_{11} were determined by measuring the absorbance of the solution based on Table 1 using a microplate reader at $\lambda = 415$ nm. The α -glucosidase inhibitor activity was determined by the formula (2):

Table 6. Inhibitory α -glucosidase activity of LBSU9

sample	A _o	A _i	A _{io}	A _{ii}	% inhibition
LBSU9	o	4.714	o	0.074	98.4%
acarbose	o	4.324	o	0.1	97%

From the table 6, it is known that the activity of alpha glucosidase inhibitor LBSU9 is higher than the activity of acarbose. A range of studies have explored the potential of probiotics to inhibit α -glucosidase activity, a key enzyme in carbohydrate digestion. In previous research it was reported that certain lactic acid bacteria strains, including *Lactocaseibacillus rhamnosus* and *Lactobacillus*, exhibited α -glucosidase inhibitory activities [37]. These findings are supported by the work of [38] who demonstrated that specific probiotics, such as *Lactobacillus paracasei* and Bifidobacterium animals, can stimulate glucagon-like peptide-1 (GLP-1) secretion, which can help regulate blood glucose levels. Furthermore, [39] identified specific bioactive components in probiotics, such as secreted proteins and metabolites, that can enhance intestinal barrier function and display antioxidant and anti-inflammatory potential, respectively. A range of bioactive compounds produced by probiotics have been found to inhibit the enzyme α -glucosidase. These include antioxidants and anti-inflammatory agents such as those produced by *Lactobacillus plantarum* KU15149 [40]. Postbiotics, such as bacteriocins and organic acids, have also been identified as having inhibitory effects on alpha glucosidase [41]. These findings suggest that a variety of bioactive compounds produced by probiotics can play a role in inhibiting α -glucosidase. The limitation of this study is that the test is still on an in vitro scale and the secondary metabolites that act as alpha glucosidase inhibitors are not yet known. For further research, it is hoped that researchers can conduct in vivo tests using type 2 diabetes mellitus models and conduct whole genomic sequencing analysis to predict secondary metabolites that act as alpha glucosidase inhibitors.

4. CONCLUSION

From this study, Trites, a traditional food typical of North Sumatra, contains probiotics that can potentially produce bioactive compounds that inhibit the α -glucosidase enzyme. The probiotic isolate coded LBSU9 has an inhibitor activity of 98.4%, even more significant than the activity of acarbose, which is 97%. Based on the result, it is concluded that LBSU9 has the potential as a complementary therapy to prevent or treat type 2 diabetes mellitus. The limitation in this study is that the LBSU9 isolate has not been identified to the molecular level. So that in future studies, it is necessary to carry out molecular identification of LBSU9 isolates and in vivo tests using experimental rats. In addition further research is needed to determine the genomic characteristics of this LBSU9 isolate through whole genomic sequencing before in vivo testing.

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