ANTIDIABETIC, ANTIOXIDANTS AND ANTIBACTERIAL ACTIVITIES OF LACTIC ACID BACTERIA (LAB) FROM MASIN (FERMENTED SAUCE FROM SUMBAWA, WEST NUSA TENGGARA, INDONESIA)

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Abstract

The study aimed to determine the effectiveness of metabolites from Lactic Acid Bacteria (LAB) derived Masin (fermented sauce from Sumbawa) as antioxidant, antidiabetic, and antibacterial compounds. The LAB isolates were isolated from various strains of *Staphylococcus pisciferm*entan which consisted of *Staphylococcus pisciferm*entans strain CIP103958 (code: 2), strain BULST54 (code: 17), strain SK03 (code: 11), strain ATCC 51136 (code: 34), strain PCM 2409 (code: 28) and strain PU-87 (code: 5). The Metabolites of LAB were analyzed by the bioprospecting test to indicate antidiabetic, antioxidant and antibacterial activities. The isolate (Code: 5) at 500 ug/ml showed the most effective antioxidant activity up to 71%. The isolate (code: 28), at 300 ug/ml revealed to have the most antidiabetic activity up to 43 %. The isolate (code: 2) showed moderate antibacterial activity with the inhibition zone of 5.59 mm. The results of the antidiabetic, antioxidant and antibacterial activity showed that the secondary metabolites produced by LAB from the Masin have broad activities as an antidiabetic, antioxidant and antibacterial.

Keywords: Antidiabetic, Antioxidant, Antibacterial, Lactic Acid Bacteria (LAB), Masin

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Introduction

Masin is a spontaneously fermented sauce from Sumbawa, West Nusa Tenggara Indonesia made from shrimp paste, chili, turmeric powder, and herbs mixed with some spices. Spontaneous fermentation could develop potential pathogens (Manguntungi *et al*., 2020) and toxic compounds leading to novel metabolites production (Lavefve *et al*., 2019). Commercial starters for masin have not been developed yet. This study aims to examine bioprospective activities of antioxidant, antidiabetic and antibacterial compounds in LAB isolated from masin.

Lactic acid bacteria are dominant microorganisms in many fermented fisheries products. During fermentation, LAB produce several bioactive compounds such as vitamins, gamma-amino butyric acid, bioactive peptides, bacteriocins, enzymes, conjugated linoleic acid, and exopolysaccharide that have a functional properties such as antioxidant, antidiabetic and antibacterial effect (Linares *et al*. (2017); Muryany *et al*. (2017); Speranza *et al*. (2017); Mokoena *et al*. (2016).

Pomace grape, mulberry juice and quinoa flour dough, fermented by *Lactobacillus plantarum*, camel milk fermented by *Lactobacillus lactis* showed a high antioxidant effect. Antioxidative enzymes of LAB such as superoxide dismutase and glutathione peroxidase isolated from fermented food play an important role in scavenging free radicals (Cai *et al*. (2019); Kwaw *et al.* (2018); Campanella *et al*. (2017); Rizzello *et al*. (2017); Soleymanzadeh *et al*. (2016). In vivo study of *L. casei* and *L. rhamnosus* showed a decrease in

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plasma glucose level that improves insulin imbalances. *Lactobacillus brevis* produces gamma-aminobutyric acid and high α-glucosidase inhibitory activities to treat diabetes (Azam *et al*. (2017); Evivie *et al*. (2017); Son *et al*. (2017).

LAB also showed antibacterial acitivity. *L. plantarum* and *L. Casei* isolated from fermented food had shown strong antimicrobial activity for inhibiting *E.coli, S. typhimurium* and *S. aureus* (Darsanaki *et al*. (2012); Lelise *et al*. (2014); Inglin *et al*. (2015) ; Ren *et al*. (2018). The antibacterial activity of these lactic acid bacteria may be due to various antibacterial compounds such as bacteriocins, organic acids by decreased pH levels, or hydrogen peroxide (Luo *et al*., 2011). Lactic acid bacteria produce bacteriocins such as nisin, lactococcin and lactacin by *L. lactis*, pediocin by *L. plantarum*, and garvieacin by *L. garvieae*. These antibacterial proteins or peptides at a concentration as low as picomolar to nanomolar exhibit the ability to permeabilize the cytoplasmic membrane of the receptor bacteria, resulting in a leakage of ions and small molecules into the cells. Bacteriocin-producing cultures have been applied to inhibit a wide range of Gram-positive genera, including staphylococci, streptococci, *Listeria* spp., bacilli, and enterococci in various fermented foods. LAB bacteriocins are GRAS in food because it can be digested by proteases and have no or little influence on the gut microbiota (Mokoena *et al*. (2016); Woraprayote *et al*. (2016); Silva *et al*. (2016). The study aimed to determine the effectiveness of metabolites from Lactic Acid Bacteria (LAB) derived masin (fermented sauce from Sumbawa) as antioxidant, antidiabetic, and antibacterial compounds.

Materials and Methods

1. Isolates Preparation

Preparation of tested isolates begins with the rejuvenation process and production of LAB metabolites from Masin. All of the LAB isolates were obtained from previous study (Manguntungi *et al.,* 2020*)* (Table 1).

Table1. LAB from Masin

No.	Isolates Code	Species
1	2	*Staphylococcus pisciferm*entans strain CIP103958
2	17	*Staphylococcus pisciferm*entans strain BULST54
3	11	*Staphylococcus pisciferm*entans strain SK03
4	34	*Staphylococcus pisciferm*entans strain ATCC 51136
5	28	*Staphylococcus pisciferm*entans strain PCM 2409
6	5	*Staphylococcus pisciferm*entans strain PU-87

The Isolates were grown on selective media, MRS Agar (de man, Rogosa, Sharpe) (HiMedia, India) containing sodium azide and incubated at 37° C in a shaker incubator for 3 x 24 hours to maximize metabolite production. The bacterial culture was then extracted using 96% ethanol and centrifuged at 10000 rpm for 10 minutes to separate the crude components of the extract. Afterwards, the LAB metabolites were separated from its crude extract through the process of solvent evaporation (ethanol) using a Rotary Evaporator for 6 hours. The metabolite produced is then stored in a freezer at 4° C.

2. Antioxidant Activity

The DPPH radical scavenging activity was assayed with some modifications (Zahratunnisa *et al*., 2017). Briefly, 180 µl of DPPH solution (0.2 mM DPPH in methanol) was mixed with 20 µl of sample with various concentrations. In triplicate, the mixture was incubated at room temperature for 30 minutes. Then, the absorbance of the mixture was measured at 540 nm. 50 ppm Vitamin C was used as the positive control (Hazimah *et al*., 2013). The DPPH radical scavenging activity was calculated by the following formula:

$$\text{scavenging activity (\%)} = \frac{Ac - AS}{Ac} \; x100\%$$

Where, A_c and A_s define the absorbance of control and sample, respectively.

3. Antidiabetic Activity

Antidiabetic activity was measured with the inhibition of alpha-glucosidase test (Yuniarto & Selifiana, 2018). The reaction mixture consisting 30 µl of samples at various concentrations was premixed with 36µl

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phosphate buffer pH 6.8 and 17 µl of 5 mM p-nitrophenyl- α-D-glucopyranoside. After preincubating at 39°C for 5 minutes, 17 µl alpha-glucosidase (0.045 units/mL) was added and incubated at 39°C for 15 minutes. The reaction was terminated by adding 100 µl Na_2CO_3 200 mM. Inhibition of alpha-glucosidase was determined at 400 nm using microplate reader by measuring the quantity of p-nitrophenol released from p-NPG. 100 ppm acarbose was used as positive control of α-glucosidase inhibitor. The concentration of the extract required to inhibit 50% of α-glucosidase activity under the assay conditions was defined as the IC_{50} value.

4. Antibacterial Activity

The antibacterial activity test conducted by well diffusion method. The specimens of tested pathogenic bacteria were *S. epidermidis*, *S. aureus*, *B. pumilus*, *B. subtilus*, *EPEC*, *S. typhymirium*, *L. monocytogenes*, *E. zakazaki*, *DPT proteus* and *E. coli.* These Bacteria were collecteted from Indonesian Institute of Sciences (LIPI). All bacteria specimens, thereafter, were cultured on 10 mL of Nutrient Broth media (Oxoid, U.S) and incubated for 24 hours at 37ºC. The positive control used is 20 µL ampicillin antibiotic on paper disc with concentration 0.5 µg/µL (MP Biomedicals, USA). A total of 3 mL of bacterial culture test and 20 ml of Nutrient Agar (Oxoid, U.S) were poured into a sterile petri dish. The plates were allowed to dry and the wells (6 mm in diameter) were made using micro tip. A total of 50 µL of LAB secondary metabolite extract was put into the well (Purwijantiningsih, 2014). After incubation at 37°C for 20 hours, the diameter (mm) of the inhibition zone around the wells were measured with criteria 0 is no inhibition zone; 5-10 is moderate inhibition zone; 11-15 is strong inhibition zone: 16-18 is very strong inhibition zone (Messi *et al*., 2000). Inhibition zone indicates that the sample has antibacterial activity.

5. Statistical Analysis

The test was carried out using a Completely Randomized Design (CRD) in three replications. The data was test by Analysis of variance (ANOVA), and SPSS 20.0. Data signify the means plus or minus the standard error of mean (means ± S.E.M.) of three samples and are representative of three independent experiment.

Results

1. Antioxidant Activity

Table 2 shows the results of data analysis on the antioxidant activity of LAB isolates, where the control treatments were significantly different from all treatments on various types of isolates and concentrations.

Table 2. Antioxidant activity of LAB in Masin

Concentration (µg/ml)	Isolate Code/Antioxidant Activity (%)					
	2	17	11	34	28	5
100 µg/ml	7 ± 0,005^{a}	12 ± 0,012^{a}	8 ± 0,007^{a}	42 ± 0,014^{a}	40 ± 0,008^{a}	38 ± 0,014^{a}
200 µg/ml	38 ± 0,007^{a}	13 ± 0,004^{a}	8 ± 0,008^{a}	42 ± 0,005^{a}	43 ± 0,005^{a}	42 ± 0,003^{a}
300 µg/ml	49 ± 0,011^{b}	45 ± 0,021^{bc}	21 ± 0,003^{b}	59 ± 0,002^{b}	62 ± 0,002^{b}	50 ± 0,002^{b}
400 µg/ml	49 ± 0,011^{b}	40 ± 0,005^{b}	25 ± 0,004^{bc}	60 ± 0,001^{bc}	63 ± 0,004^{b}	49 ± 0,002^{b}
500 µg/ml	52 ± 0,006^{b}	55 ± 0,011^{c}	31 ± 0,001^{c}	64 ± 0,005^{c}	64 ± 0,001^{b}	71 ± 0,006^{c}
Control +	81 ± 0,003^{d}	81 ± 0,003^{d}	81 ± 0,003^{d}	81 ± 0,003^{e}	81 ± 0,003^{d}	81 ± 0,003^{d}

Note: The data obtained is a representative of mean ± S.E.M. Numbers followed by the same letters in the same column show no significant difference in the one-way ANOVA test, α = 0, 05.

In each isolate, the most effective concentration as an antioxidant was 500 µg/ml. Among six isolates, secondary metabolite powder from isolate (code: 5) was the most effective antioxidant with 71% scavenging activity.

2. Antidiabetic Activity

The result of antidiabetic activity in Table 3 shows that all treatments were significantly different from controls.

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Table 3. Antidiabetic Activity of LAB in Masin

Concentration (µg/ml)	Isolate Code/Anti-diabetic Activity (%)Sampel					
	2	17	11	34	28	5
100 µg/ml	8 ± 0,002^{a}	11 ± 0,003^{a}	14 ± 0,003^{a}	7 ± 0,001^{a}	1 ± 0,004^{a}	0 ± 0,003^{a}
200 µg/ml	11 ± 0,002^{a}	13 ± 0,004^{a}	14 ± 0,002^{a}	12 ± 0,000^{ab}	0 ± 0,003^{a}	0 ± 0,003^{a}
300 µg/ml	13 ± 0,002^{a}	13 ± 0,004^{a}	15 ± 0,003^{a}	14 ± 0,001^{ab}	0 ± 0,003^{a}	0 ± 0,003^{a}
400 µg/ml	10 ± 0,002^{a}	17 ± 0,003^{ab}	17 ± 0,002^{a}	16 ± 0,003^{ab}	6 ± 0,003^{a}	0 ± 0,001^{a}
500 µg/ml	16 ± 0,002^{a}	15 ± 0,005^{a}	13 ± 0,001^{a}	17 ± 0,002^{b}	2 ± 0,002^{A}	0 ± 0,000^{a}
Control +	75 ± 0,037^{c}	75 ± 0,037^{c}	75 ± 0,037^{b}	75 ± 0,037^{d}	75 ± 0,037^{c}	75 ± 0,037^{c}

Note: The data obtained is a representative of mean ± S.E.M. Numbers followed by the same letters in the same column show no significant difference in the one-way ANOVA test, α = 0. 05.

Among isolates code 2, 11 and 5, the highest anti-diabetic ability was found at a concentration of 500 µg/ml. For the isolates (code: 17) and (code: 34), the highest antidiabetic ability was found at a concentration of 400 µg/ml and decreased at a concentration of 500 µg/ml. Whereas in the isolate (code: 28) was the highest antidiabetic ability with a concentration of 300 µg/ml and decreased at concentrations of 400 µg/ml and 500 µg/ml.

3. Antibacterial Activity

Antibacterial activity results are shown in the Table 4. The isolate (Code: 2) has a moderate antibacterial activity against pathogen *B. pumilus* with the inhibition zone length of 5.59 mm.

Table 4. Antibacterial activity of LAB in masin

Pathogens	Control (+)	Isolate Code/Antimicrobial Activity (mm)					
		2	17	11	34	28	5
S. epidermidis	13,46 ± 1,110^{bc}	1,58 ± 0,160^{b}	2,01 ± 0,145^{b}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
S. aureus	15,35 ± 0,873^{bcd}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
B. pumilus	17,38 ± 1,663^{de}	5,59 ± 0,337^{c}	3,37 ± 0,909^{c}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
B. subtilis	17,80 ± 0,457^{de}	1,81 ± 0,821^{b}	1,26 ± 0,080^{b}	1,52 ± 0,375^{b}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
EPEC	2,55 ± 0,179^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
S. typhimirium	17,95 ± 3,356^{de}	1,27 ± 0,168^{b}	1,49 ± 0,316^{b}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
L. monocytogenes	15,35 ± 1,006^{bcd}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
E. zakazaki	12,95 ± 1,762^{b}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
DPT Proteus	17,07 ± 0,204^{cd}	1,93 ± 0,452^{b}	1,43 ± 0,410^{b}	0 ± 0^{a}	0 ± 0^{a}		0 ± 0^{a}
E. coli	20,97 ± 0,429^{e}	1,30 ± 0,280^{b}	1,46 ± 0,262^{b}	0 ± 0^{a}	1,47 ± 0,133^{b}	1,40 ± 0,080^{b}	0 ± 0^{a}

Note: The data obtained is a representative of mean ± S.E.M. Numbers followed by the same letters in the same column show no significant difference in the one-way ANOVA test, α = 0.05.
0: no inhibition zone; 5-10: moderate inhibition zone; 11-15: strong inhibition zone; 16-18: very strong inhibition zone (Messi *et al*., 2000)

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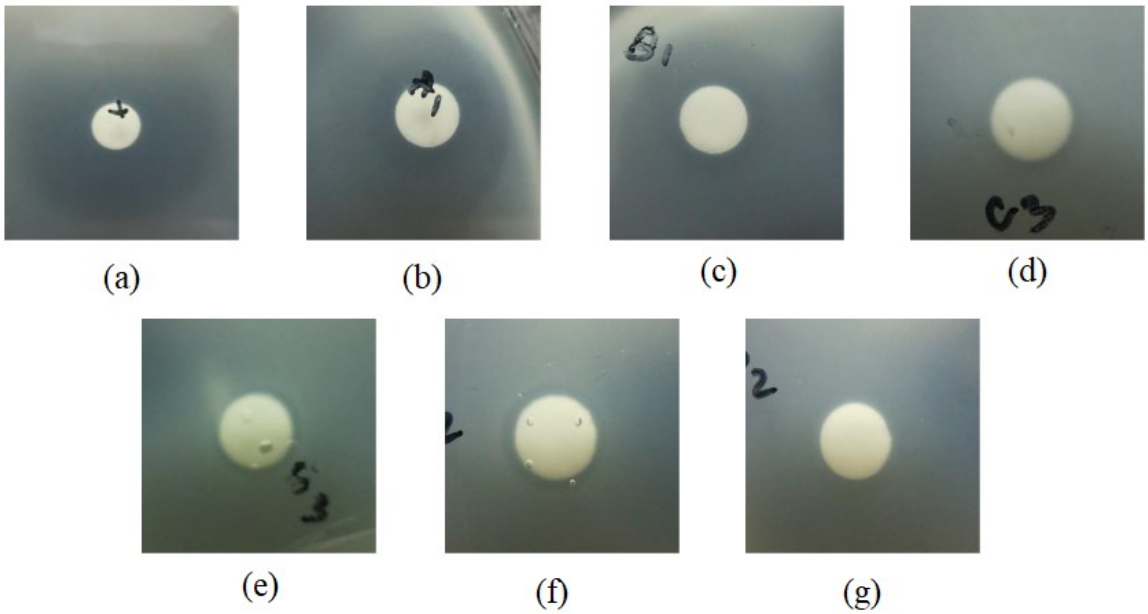

Figure 1. LAB antibacterial activity of masin (a) Control + AMP; (b) Isolate (code: 2)against *B. pumilus*; (c) Isolate (code: 17) against *B. pumilus*; (d) Isolate (code: 11) against *B. subtilis*; (e) Isolate (code: 34) against *E. coli*; (f) Isolate (code: 28) against *E. coli*; (g) Isolate (code: 5) against *E. Zakazaki*.

Isolate (code: 17) had an antibacterial activity against pathogen *B. pumilus* with the inhibition zone length of 3.37 mm. Isolates code 11, 34, and 28 only have an activity on one type of pathogen *B. subtilis* and *E. coli*, respectively. While isolate (code: 5) had no antibacterial activity in inhibiting the growth of pathogens. Isolate (code : 2) has the best antibacterial activity against *B. pumilus* bacteria.

Discussion

Masin is made by spontaneous fermentation resulting in a very high diversity of bacteria, such as lactic acid bacteria (Manguntungi *et al*., 2020). LAB play an important role as probiotics that can inhibit the growth of pathogenic bacteria (Manguntungi *et al*., 2020). LAB are able to produce various compounds that can inhibit the growth of pathogenic bacteria such as lactic acid, carbon dioxide, diacetil, and bacteriocin (Mbolaji and Wuraola, 2011). LAB are also able to produce hydrogen peroxide (H_2O_2) compounds (Manguntungi *et* al., 2020). Lactic acid compounds are organic acids from BAL fermentation (Ammor *et al*., 2006). Lactic acid has an antibacterial ability because it is able to disrupt the structure of cell membranes, inhibit active transport, reduce intracellular pH and inhibit various metabolic functions. The activity of LAB as antibacterial against pathogens has been widely reported. Cho *et al*. (2015) reported antimicrobial activity of LAB isolated from korean traditional fermented food against *Staphylococcus aureus* and *Salmonella enterica.* Antagonist test of *Staphylococcus piscifermentans* using dics diffusion method showed growth inhibitory activity of *S. tiphymirium* and *E. coli* (Hajar and Hamid, 2013). In this study, six LAB strains have been isolated from Masin. Five out of six showed antimicrobial activity against several pathogens. Isolate (Code: 5) was the most effective LAB from masin that inhibited *B. pumilus* bacteria. Antibacterial activity of LAB can be caused by various antibacterial compounds such as organic acids with low pH, hydrogen peroxide, or the presence of bacteriocin (Luo *et al*., 2011).

Assay of antioxidant activity in LAB is measured by DPPH free radical scavenging ability. DPPH free radical scavenging is widely used to determine the antioxidant activity of LAB because of its easiness, speed, sensitivity and productivity compared to other methods (Milardovic *et al*., 2006). The principle of the assay is based on the reduction of ethanolic DPPH solution in the presence of a hydrogen donating antioxidant, leading to the formation of non radical form DPPH-H. The antioxidant is able to reduce the stable radical DPPH from purple to yellow colored diphenyl picrylhydrazine (Zhang *et al*., 2011).

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Antioxidant activity of *Lactobacillus plantarum* strains isolated from traditional Chinese fermented foods was reported by Li *et al*. (2012). In this study, LAB isolate *Staphylococcus piscifermentans* (code: 5) was the most effective producer of antioxidant in which at a concentration of 500 µg/ml showed 71% scavenging activity. The antioxidant activity of LAB is determined by bacterial strains and their proteolytic enzymes activity (Papademas *et al*., 2015). Proteolytic enzymes is well established to hydrolyze the food protein to be smaller peptides of biological activity (El Salam and El Shibiny, 2013). The development of 4–20 kDa peptides was found to correlate well with high antioxidant capacity. Virtanen *et al*. (2007) described antioxidant activity of selected lactic acid bacteria had a high propotion of peptides representing a molecular mass of 4–20 kDa.

Diabetic is a global epidemic with obesity, high calorie diets and physical activity as some of the main causes of type 2 diabetic in people who have a genetic predisposition (Everard and Cani, 2013). The ability of LAB bacteria as antidiabetic has been investigated (Honda *et al*., 2012). LAB is known to produces exopolysaccharides which can inhibit the activity of the enzyme a glucosidase (Ramchandran and Shah, 2009). LAB species that have been reported as antidiabetic potential are *L. Plantarum*, *L. acidophilus* (Muganga *et al*, 2015) *L. sakei* (Bajpai *et al*., 2016) and *L. brevis* (Son *et al*., 2017). In line with the results in this study, where six LAB isolates had antidiabetic activity. LAB strains have been reported to produce gamma aminobutyric acid (GABA) and polyunsaturated fatty acid (PUFA) that both have antidiabetic activity (Linares *et al*, 2017). Diabetic can be treated by inhibiting α-glucosidase, postponing digestion and absorption of carbohydrates. Exopolysaccharides produced by Lactobacillus strains has the α-glucosidase inhibitory activity (Son *et al*., 2017). In vivo analysis showed that antidiabetic activity of LAB depends on the bacterial strain and whether the bacteria are viable when it arrives in the intestine (Evivie *at al*., 2017).

The results obtained from the bioprocessing activities tests above show that the secondary metabolites produced by LAB have a fairly good ability as antioxidant and antidiabetic. This is very useful for consumers of Masin because antioxidants have the ability to bind free radical compounds which are toxic in the body. Likewise anti-diabetic, is expected to control sugar levels in Masin consumers.

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