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Metagenomic analysis and biodiversity of Lactic Acid Bacteria (LAB) on masin (fermented sauce) from Sumbawa, West Nusa Tenggara, Indonesia

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Abstract. *Manguntungi B, Saputri DS, Mustopa AZ, Ekawati N, Afgani CA, Sari AP, Triratna L, Sukmarini L, Fatimah, Kusmiran A, Yanti S, Irawan S, Prasetyo MD, Fidien KA. 2020. Metagenomic analysis and biodiversity of Lactic Acid Bacteria (LAB) on masin (fermented sauce) from Sumbawa, West Nusa Tenggara, Indonesia. Biodiversitas 21: 3287-3293.* Masin is a spontaneously fermented sauce from Sumbawa, West Nusa Tenggara Indonesia that is made of shrimp paste, chili, turmeric flower, and herbs mixed with some spices. This study aims to isolate Lactic Acid Bacteria (LAB) and analyze the metagenomic and biodiversity of its bacteria. Genome isolation for metagenomic analysis was using ZymoBIOMICS™ DNA Miniprep Kit. Sequencing analysis to identify LAB strain form masin was using 16S rRNA universal primer. Metagenomic analysis showed that relative abundance bacteria in masin for order taxon was Lactobacillales, the family taxon was Enteroccocaceae, and the genus was Tetragenoccocus. Six different groups were obtained from the phylogenetic tree analysis of 40 isolates found in masin. The representatives of each group taken were isolates number 2, 17, 11, 34, 28, and 5. Based on the results of sequencing analysis, the 6 isolates found in masin are *Staphylococcus pisciferm*entans strain CIP103958 (Isolate Code 2), *S. pisciferm*entans strain BULST54 (Isolate Code 17), *S. pisciferm*entans strain SK03 (Isolate Code 11), *S. pisciferm*entans strain ATCC 51136 (Isolate Code 34), *S. pisciferm*entans strain PCM 2409 (Isolate Code 28) and *S. pisciferm*entans *strain* PU-87 (Isolate Code 5). Through this research, it has been described a whole diversity of bacteria contained in masin, one of them is Lactic acid bacteria. The six LAB isolates that have been identified can be developed as starter candidates for masin production.

Keywords: Biodiversity, LAB, masin, metagenomics

INTRODUCTION

Masin is a fermented product by using shrimp as the main ingredient and is widely consumed in Sumbawa. Bacteria that ferment masin were unidentified. The process was spontaneous without a starter. Masin producers prefer to rely on the quality of their production on natural microorganisms. In some fermented products, commercial starters often against the possibility in obtaining a unique product, change the sensory quality, and no longer distinguishable for production technology and geographical origin (Speranza et al. 2017). Spontaneous fermentation could develop potential pathogens (Manguntungi et al. 2020) and toxic compounds that lead to the production of undesired metabolites (Lavefve et al. 2019).

One type of beneficial bacteria that is often found in food fermentation products is LAB. Bacteria isolated from *peda* (Indonesian fermented fish product) consist of *Lactobacillus plantarum*, *Lactobacillus curvatus*, *Lactobacillus murinus*, *Streptococcus thermophilus* (Putra et al. 2018). *L. plantarum* and *Lactobacillus pentosus* isolated from pekasam, Malaysian fermented fish (Muryany et al. 2017). *Staphylococcus*, *Micrococcus* and *Bacillus* were found in punti shidal and phasa shidal, fermented fish products in India (Majumdar et al. 2016). *Staphylococcus pisciferm*entans was found in Cincaluk, fermented shrimp from Riau Indonesia, while *L.*

plantarum, Staphylococcus xylosus, Saccharomyces cerevisiae were found in low salt traditional Chinese fermented fish, suanyu (Azam et al. 2017). However, the research on lactic acid bacteria in masin is still limited.

LAB can improve the quality of the products. The main bioactive compounds produced by LAB during fermentation are vitamins, γ-aminobutyric acid, bioactive peptides, bacteriocins, enzymes, conjugated linoleic acid, and exopolysaccharide. These compounds are the main biopreservative and have functional properties such as antioxidant and anti-diabetic (Speranza et al. 2017; Muryany et al. 2017; Linares et al. 2017; Mokoena et al. 2016).

LAB also has the ability to inhibit pathogen growth by disrupting the outer membrane of bacteria that causes lysis. LAB produces bacteriocins such as nisin, lactococcin, and lactacin by *Lactobacillus lactis*, pediocin by *L. plantarum*, and garvieacin by *Lactobacillus garvieae*. These antimicrobial peptides at a low concentration 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 cell. 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 (Generally Recognized as Safe) 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).

This study aimed to analyze the metagenome and biodiversity of LAB on masin. Through this metagenomic analysis, both potential and pathogenic bacterial diversity would be found in masin. Therefore, masin producers would be able to develop better products.

MATERIALS AND METHODS

Study area

Sumbawa is one of the districts in West Nusa Tenggara (NTB). Sumbawa is famous for one of its local fermented products, which is masin. The study began from June to August 2019. The research was conducted at the Laboratory of Applied Genetic Engineering and Protein Design, Biotechnology Research Center, Indonesian Institute of Sciences, Bogor, West Java, Indonesia.

Procedures

Sample preparation

Masin samples were obtained from Empang Sub-district, Sumbawa District, West Nusa Tenggara, Indonesia. Masin is stored at 4˚C to avoid spoilage.

Genome isolation for metagenomic analysis

Genome isolation was using ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, USA) 250 mg of masin was diluted in 1 mL ddH_2O and centrifuged at 1000 ×g for 10 minutes. Supernatant was transferred into Lysis tube and centrifuged at 6000 rpm for 10 minutes. 750 µL Lysis solution was added to pellet and mixed for 20 minutes. Solution then was centrifuged at 10000 ×g for 1 minute. 400 µL supernatant was filtered and centrifuged at 8000 ×g for 1 minute. 1200 µL DNA binding buffer was mixed with the filtrate. The mixture was transferred to IICR column and centrifuged at 10000 ×g for 1 minute. DNA wash buffer was added 3 times (400, 700, and 200 µL) followed by 1-minute centrifugation at 10000 ×g for each addition. The IICR column transferred to a clean microtube, 45 µL of DNase/RNase Free Water was added, incubated for 1 minute and centrifuged at 10,000 ×g for 1 minute to elute the DNA. The result was visualized with gel electrophoresis on 1% (w/v) agarose gel by using 1xTAE buffer. The gel was stained in a solution of Ethidium bromide and viewed using UV transilluminator.

Sequencing analysis of metagenomic

The next-generation sequencing analysis was performed at Novogene CO., Ltd Japan using Illumina (MiSeq) platform (paired-end reads). Paired-end reads were merged using FLASH (Magoč and Salzberg 2011). Primers used in this sequencing were V3-V4 region for bacteria (341F: 5'-CCT AYG GGR BGC ASC AG-3'; 806R: 5'-GGA CTA CNN GGG TAT CTA AT-3'). Quality filtering on the raw tags was performed under specific filtering conditions to obtain the high-quality clean tags (Bokulich et al. 2013) according to the Qiime (V1.7.0) (Caporaso et al. 2010) quality controlled process. In order to detect chimera sequences using UCHIME algorithm (Edgar et al. 2011). Sequence analysis was performed by Uparse software (Edgar RC 2013) and the similarity of OTU was aligned with references from SILVA database (Quast et al. 2013).

LAB isolation

Isolation was carried out by suspending 100 mg of masin in 5 mL of MRS Broth with various concentrations of lactose and glucose. There are ten various compositions and concentrations (MRS control, MRS + glucose 1%, MRS + glucose 2%, MRS + lactose 1%, MRS + lactose 2%, M17 control, M17 + glucose 1%, M17 + glucose 2%, M17 + lactose 1%, and M17 + lactose 2%). The isolates then incubated overnight at 37°C. After incubation, 100 µL cultures were serially diluted in 900 µL of NaCl 0.85%. Serial dilution was conducted until dilution 10^{-7}. A total of 100 µL from 10^{-5}, 10^{-6} and 10^{-7} dilutions were taken and spread on MRS Agar with the treatment of adding lactose and glucose. The culture was incubated for 24 hours at 37°C. A single colony that grows then subcultured on MRS Broth for DNA isolation.

DNA isolation

1.5 mL of liquid culture was taken in microtube and centrifuged at 6000 ×g for 10 minutes at 4°C. 1.5 mL of liquid culture was added to pellet and re-centrifuged at 6000 ×g for 10 minutes at 4°C. Supernatant was discarded and pellet was added with 500 µL TE buffer pH 8 and 40 µL Lysozyme (60 mg/mL), then incubated 37°C for 60 minutes. After incubation, 200 µL 10% SDS, 100 µL 5 M NaCl, 80 µL 10% CTAB were added to microtube and then incubated at 60°C for 30 minutes (invert microtube every

10 minutes). 700 µL of chloroform 1: 1 was added and centrifuged at 13000 ×g for 10 minutes at 4°C. The supernatant was transferred to new microtube and added 0.6 × v isopropanol, then incubated for 2 hours at-20°C. After incubation, microtube was centrifuged at 13000 ×g for 10 minutes, 4°C. The supernatant was discharged. Pellets were added with 1 mL of ethanol 70% and centrifuged at 13000 ×g for 10 minutes, 4°C. The supernatant was discarded and the pellet is dried overnight. The pellets were added 30 µL ddH_2O and 5 µL RNAse (1 mg/mL) and then incubated 37°C for 60 minutes. After that, the results of DNA isolation were stored at a low temperature to avoid DNA degradation (Lahiri and Schnabel 1993) and analyzed with gel electrophoresis on 2% (w/v) agarose gel by using 1 × TAE buffer. Then the gel was stained in ethidium bromide solution and using UV transilluminator to analyze it.

RAPD bacteria isolates from masin

Random Amplified Polymorphism DNA is a PCR-based discrimination method in which short arbitrary primers anneal to multiple random target sequences, resulting in patterns of diagnostic value (Mohania et al. 2008). RAPD used to identify the level of genetic diversity in LAB. Each reaction mixture (final volume 15 µL) was contained 3 µL ddH2O, 7.5 µL 5 × My Taq (Bioline), 1.5 µL primers and 3 µL DNA Templates (LAB Genome). The primers used are GTG5. The PCR method performed using the GTG5 primer (5'-GTGGTGGTGGTGGTG-3 ') was previously (Gevers et al. 2001). The cycling program consisted of 1 cycle of 95°C for 7 minutes; 30 cycles of 95°C for 1 minute, 55°C for 1 minute, and 65°C for 8 minutes; and finally 1 cycle of 65°C for 16 minutes. PCR products were analyzed by gel electrophoresis on 2% (w/v) agarose gel using 1 × TAE buffer. The gel was stained in a solution of Ethidium bromide and viewed using UV transilluminator.

16S rRNA PCR analysis and sequencing analysis

PCR16S rRNA is a step to identify LAB strains found in masin using a 16S rRNA universal primer. The primers sequences used are 8F (5'-AGAGTTTGATCATGGCTCAG-3') and 15R (5'-AAGGAGGTGATCCAACCGCA-3'). Positions 1541 to 1522 bp are used to amplify the overall fragment length of 16S rRNA bacteria (Kazuko et al. 1992). The reaction mixture of PCR includes ddH_2O 38.5 µL, 5 × MyTaq Green 7.5 µL, 1 µL of each primer, DNA templates (LAB genome) 2 µL so that the total is 50 µL. The PCR conditions were 96°C for 5 minutes; 35 cycles consisting of 96°C for 1 minute, 55°C for 30 seconds, and 72°C for 1 minute; and 72°C for 7 minutes. The PCR products were subjected to electrophoresis gel on 1% agarose gel, followed by ethidium bromide staining. Sequencing results were analyzed using the Basic Local Alignment Search Tool (BLAST) in the NCBI and MegaX Software.

RESULTS AND DISCUSSION

Results

Molecular analysis is one of the methods to identify bacteria in masin. These bacteria are potential for further development into commercial starters. Through this research, metagenomic mapping of bacteria contained on masin was compiled. Figure 1 and Figure 2 are results of analysis metagenomic data from bacteria contained on masin. The results obtained all the bacteria found in the masin. Most of the bacteria belong to the bacilli class. In the taxon order, bacteria are dominated by the order Lactobacillales, whereas in the taxon family is dominated by Enteroccocaceae.

Based on that metagenomic analysis, some of the bacteria may be lactic acid bacteria. The selection of LAB was done by growing the bacteria in various modifications of growth media special for LAB as shown in Table 1. LAB has different media compositions according to their respective types. Some LABs display colony forms and the production of secondary metabolites which vary according to the type of medium (Hajar and Hamid 2013). Based on the results obtained in Table 1., after incubated for 2-4 days most LAB colonies were found on MRS + Lactose 2% media.

DNA was isolated to identify the types of LAB contained on masin. Bacterial DNA isolation was done by taking 4 isolates in each type of media, so there were 40 isolates obtained. Figure 3 shows the result of RAPD analysis conducted on 40 isolates.

Figure 4 shows the phylogeny of 40 LAB isolates. It appears that there are 6 large groups of LAB types with the highest genetic relation. DNA structure of one isolate for each group was analyzed to determine the LAB type.

From the phylogeny tree of 40 LAB isolates found on masin, there were 6 different groups. These groups were obtained based on the similarity of each isolate. The representatives of each group taken were isolates numbers 2, 17, 11, 34, 28, and 5 each of which was then sequenced to determine the specific isolate types. The results of the sequence analysis of the 6 isolates are figured out in Table 2.

Based on the results of sequencing analysis, the 6 isolates found in masin are *S. pisciferm*entans strain CIP103958 (Isolate Code 2), *S. pisciferm*entans strain BULST54 (Isolate Code 17), *S. pisciferm*entans strain SK03 (Isolate Code 11), *S. pisciferm*entans strain ATCC 51136 (Isolate Code 34), *S. pisciferm*entans strain PCM 2409 (Isolate Code 28) and *S. pisciferm*entans *strain* PU-87 (Isolate Code 5). The results of DNA analysis carried out on LAB isolated from masin showed the same species in all isolate groups, which is *S. pisciferm*entans. However, these species have different strains. The amount of these species ranges from 0.099%-0.10% of all bacteria found in the masin (Figure 2). These results indicate that all of bacteria mapped through previous metagenomic analysis, some of them are Lactic acid bacteria of the type *S. pisciferm*entans.

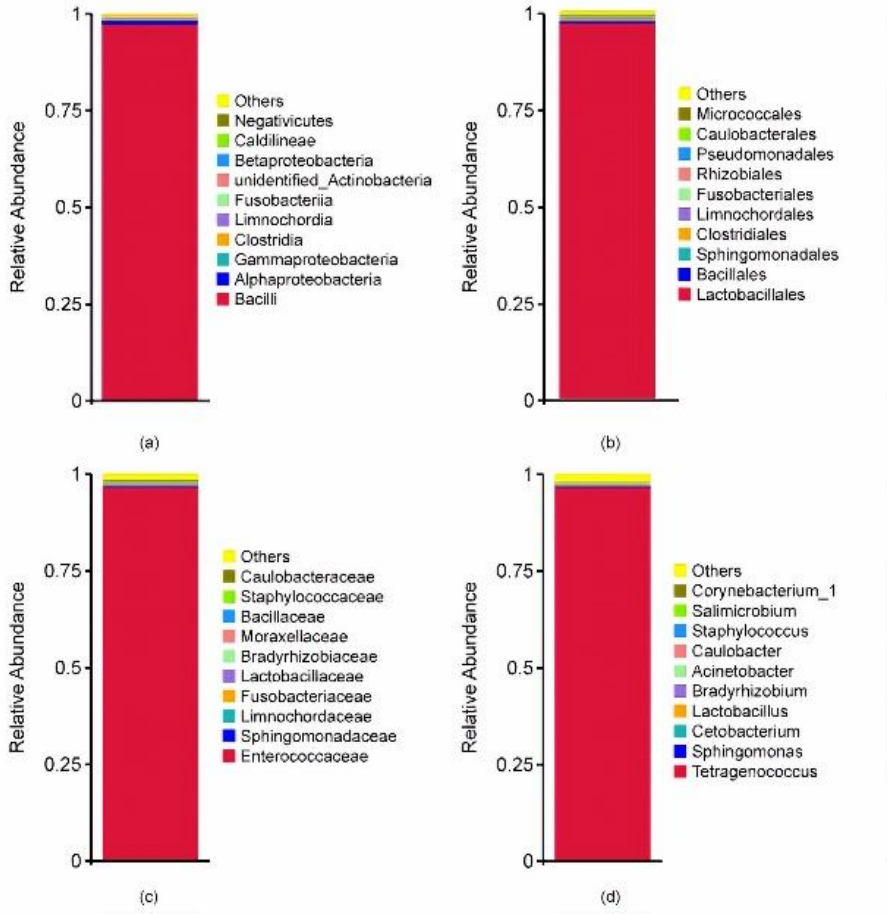

Figure 1. Relative abundance of bacteria in masin. The average bacterial abundance at each taxon level found in masin products is based on metagenomic analysis. A. Relative abundance of classes in bacteria isolated from masin; B. Metagenomic of the order in bacteria isolated from masin; C. Metagenomic of family in bacteria isolated from masin; D. Metagenomic of genus in bacteria isolated from masin.

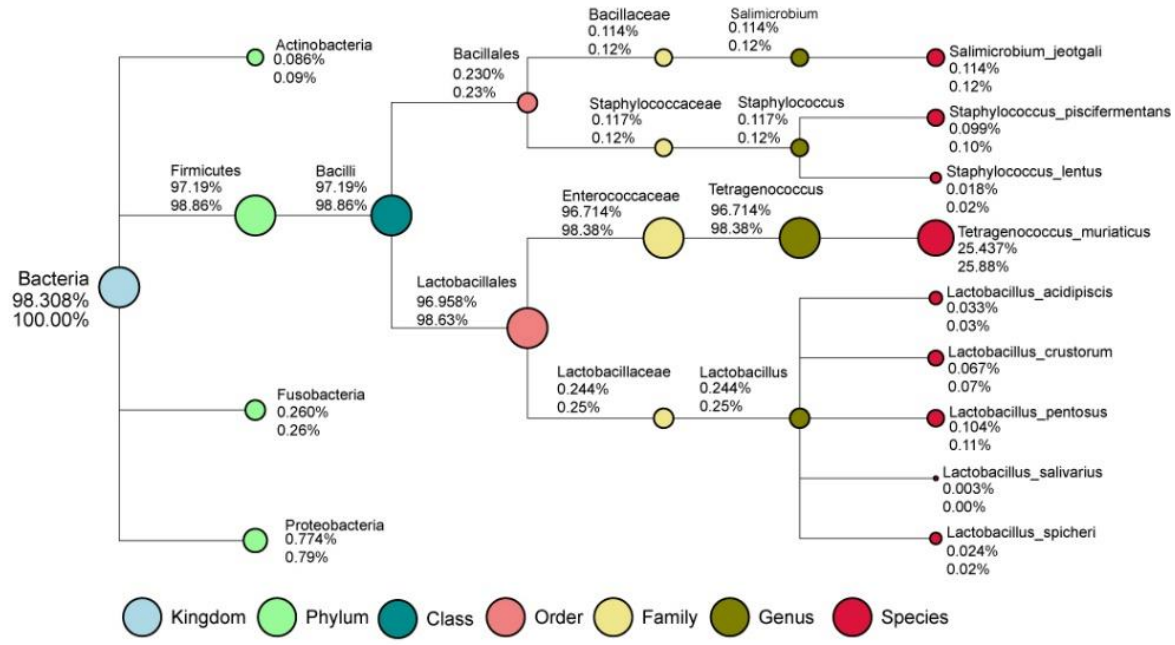

Figure 2. Phylogenetic of bacteria in masin. Phylogenetic of bacteria found in masin based on metagenomic analysis. The larger circle/sign indicates the greater number of bacteria at each taxon level which is also indicated by a percentage

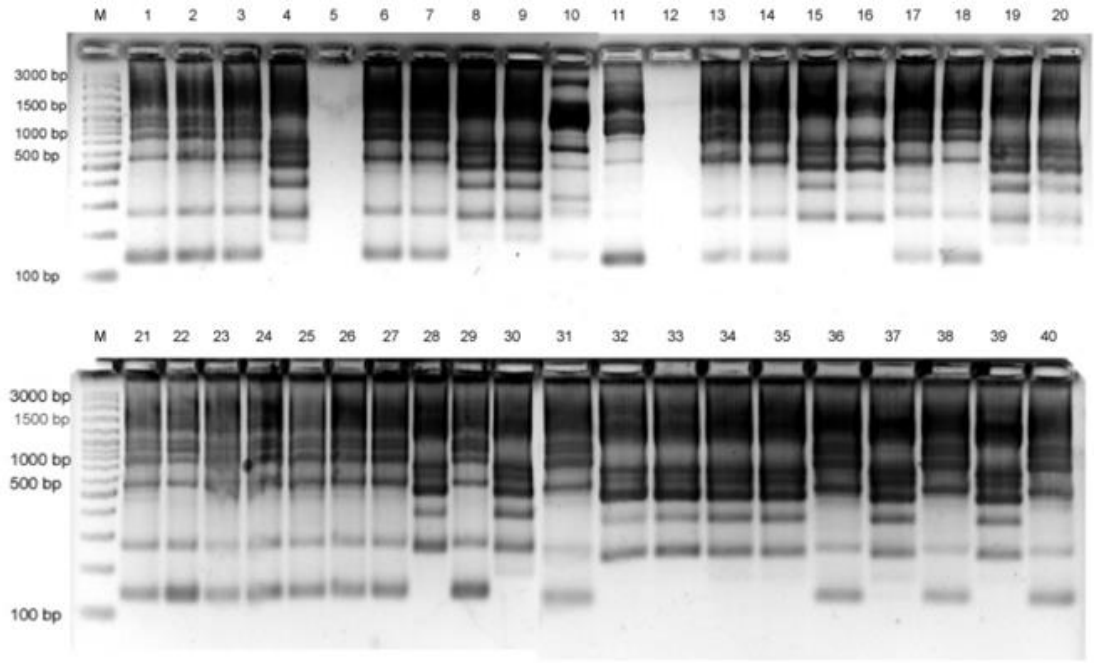

Figure 3. Visualization of PCR-RAPD band pattern of bacteria isolate code 1-40

Discussion

Bacilli class are the most dominant bacteria in masin (97.19%-98.86%). At the level of taxonomic order, *Lactobacillales* is the most dominant (96,958%-98.63%). This order is most represented by Lactic acid bacteria and has the ability to produce lactic acid through carbohydrate fermentation (Subagiyo et al. 2015; Mekadim et al. 2018). Then at the Genera level, *Tetragenoccocus* became the most dominant (98.38%). Meanwhile, at the species level, *Tetragenoccocus muricatus* is the most dominant species (25%). In a study that isolated Lactic acid bacteria from the penaeid shrimp intestine, it was found that one of the bacteria was *T. muricatus* which grew well under medium conditions with the addition of 7% NaCl, but produced the highest lactic acid at 15% NaCl addition (Reuter 1985). During the production of a fermented product, microorganisms transform raw material into a product that has better quality, generally by extending the shelf life of the raw materials and increasing the nutritional value of the product by improving the production of organoleptic attributes. Metagenomic approach has enabled exploration of microbial compositions in a range of traditional fermented foods while bypassing the need for cultivation, allowing the identification of a vast array of microorganisms never previously isolated in culture (Zhang et al. 2016).

Table 1. Population of LAB in several media

Media treatment	Isolate code	CFU/g (10^8)
MRS Control	1-4	15.85
MRS + Glucose 1%	5-8	18.90
MRS + Glucose 2%	9-12	16.57
MRS + Lactose 1%	13-16	17.00
MRS + Lactose 2%	17-20	20.65
M17 Control	21-24	4.50
M17 + Glucose 1%	25-28	9.85
M17 + Glucose 2%	29-32	13. 65
M17 + Lactose 1%	33-36	8.50
M17 + Lactose 2%	37-40	12.65

Table 2. The result of sequencing analysis of 6 LAB isolates

Isolates code	Species	% identity	Accession number
2	*Staphylococcus pisciferm*entans strain CIP103958	99.93 %	NR_116436.1
17	*Staphylococcus pisciferm*entans strain BULST54	97.71 %	MK774756.1
11	*Staphylococcus pisciferm*entans strain SK03	99.53%	NR_036981.1
34	*Staphylococcus pisciferm*entans strain ATCC 51136	99.65 %	NR_112035.1
28	*Staphylococcus pisciferm*entans strain PCM 2409	100 %	MF678955.1
5	*Staphylococcus pisciferm*entans *strain PU-87*	99.86 %	Y15753.1

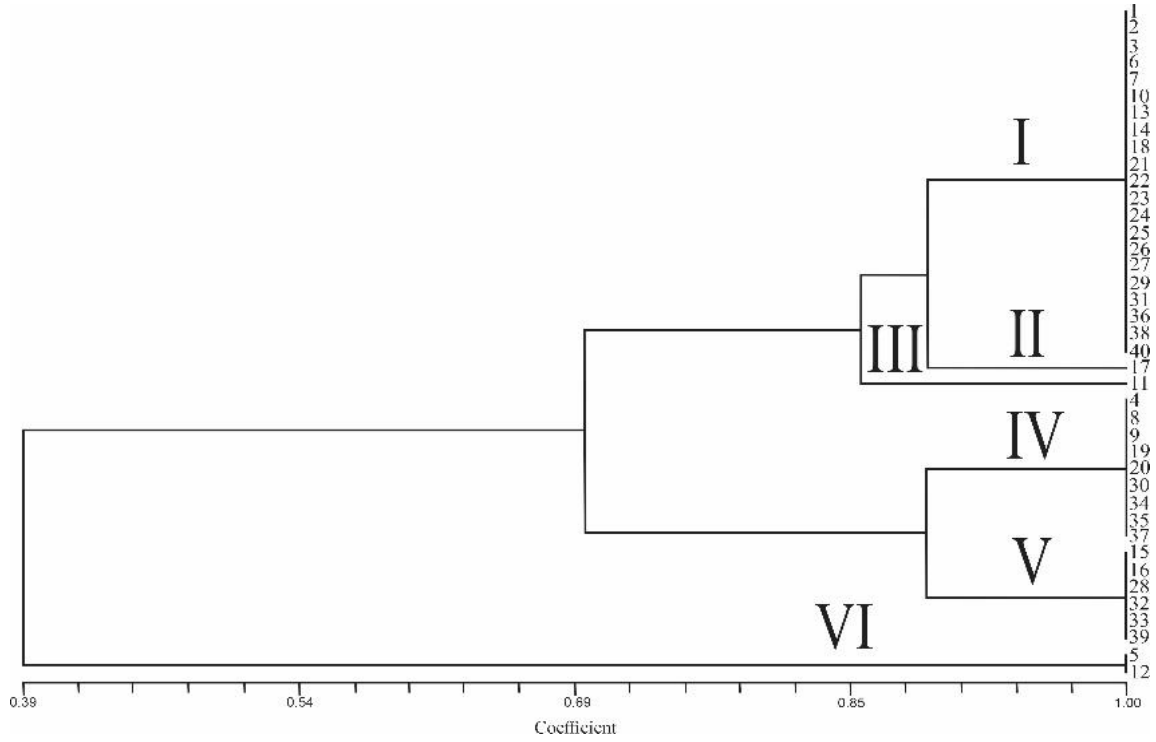

Figure 4. Phylogenetic tree of LAB found on masin

However, several LAB also isolated from fermented fish products. Yucha, traditional fermented food made from cooked rice and fresh fish. At the genus level, yucha contains an abundant amount of *Lactobacillus* (*Firmicutes* phylum) (Zhang et al. 2016). Alkaliphilic LAB belonging to the genera *Marini lactobacillus* and *Jeotgalibaca* have been detected in fermented skate, also known as hongeo, a traditional fermented fish product in South Korea. The difference of bacterial structure in different hongeo samples might be associated with the different fermentation environments in different fish processing plants, such as temperature, humidity, and other processing steps (Zhao and Eun 2020). LAB *Tetragenococus halophilus* were found in fermented fish sauce from Vietnam (Nga et al. 2017). *Gammaproteobacteria*, *Bacilli*, *Psychrobacter* and *Lactobacillus* isolated from Norwegian fermented fish (rakfisk), made of mild salting salmonid freshwater fish (Bjerke et al. 2019).

The results of DNA analysis carried out on LAB isolated from masin showed the presence of similar species in all isolate groups, which is *S. piscifermentans*. This species is also found in typical Malaysian shrimp fermentation products (cincaluk) and in fermented fish products and soy sauce from Thailand (Tanasupawat et al 1992).

Staphylococcus piscifermentans strain is Generally Recognized as Safe (GRAS) organism that commonly found in fermented foods including fermented fish, soy sauce, sausages, and traditional salted meat. *S. piscifermentans* is a non-pathogenic Gram-positive that is used as part of starter cultures for fish fermentation combined with *S. canosus* and *S. condimentii*. In fermented fish product in India, *S. piscifermentans* has shown the capability to prevent the growth of undesirable bacteria, decrease pH, hydrogen peroxide, develop flavor and red color (Gupta et al. 2018; Ouobaa et al. 2019).

Food such as dairies and vegetables is the right ecosystem for LAB. Hence, it is common to find bioactive molecules in fermented products. LAB proteolytic system has the capability to produce molecules from proteins present in food matrices. These bioactive compounds are effective for anti-hypertensive, anti-thrombotic, cholesterol-lowering, metal-chelating, antimicrobial, antioxidant, immune-modulating and to treat reproductive behavior (Pessione and Cirrincione 2016).

LAB strains produce substances such as reuterin, reutericyclin, diacetyl, fatty acids, and hydrogen peroxide, propionate, phenyl-lactate, hydroxyphenyl-lactate and 3-hydroxy fatty acids that play a role as antimicrobial and antifungal for foods biopreservation (Castellano et al. 2017). Lacticin from *Lactococci*, macedovicin from *Streptococcus macedonicus*, reuterin from *Lactobacillus reuteri*, sakacin from *Lactobacillus sake*, curvacin A, curvaticin and lactocin from *Lactobacillus curvatus*, pediocin from *Pediococcus acidilactici*, plantaricins from *Lactobacillus plantarum* are bacteriocins that have proved to control of spoilage and pathogenic bacteria in food (Bintsis 2018).

With the identification of bacteria types in masin products through metagenomics, the development of fermented products is expected to be better. Specifically through the use of LAB as a starter for the production of masin is expected to improve the quality of the product itself.

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