Analysis of Microbiota in, and Isolation of Nisin-Producing *Lactococcus lactis* subsp. *lactis* Strains from, Indonesian Traditional Fermented Milk, Dadiah

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I. GENERAL INTRODUCTION

Dadiah is one of indigenous Minangese food that found in West-Sumatera Indonesia. This dadiah is made by fermented buffalo milk incubating on bamboo and covered with bananas leaf for two days at room temperature. Dadiah has found in West-Sumatra, Indonesia. In the past, dadiah was considered as luxurious food. It must be served when culture festival was conducted such as wedding party and important ceremonial. Unfortunately, most of the youth even from Minangese itself has started to abandoned dadiah existence. The dadiah was a symbol of Minangese identity. One of this research aims to return dadiah at deserve where it belongs.

There are four sub-provinces which still production, even the productions itself do not continuously. Several barriers like fluctuate lactating period, harvesting time and milking production are some of the reasons of un-continuously manufacture. The four sub-province areas are Batusangkar, Padangpanjang, Agam and Alahanpanjang. Every district has dadiah speciality and characteristic. Besides minor microbes, each dadiah also has different composition nutrient ingredients. It is because every district has a different environment, temperature and producer habit. The purposes of this study are: analysing microbiota community structure of dadiah, investigating identification and purification of potential Bacteriocins-producer on lactic acid bacteria

produced by strains from dadiah. There is no bacteriocin detection on dadiah recorded so far.

Usually, dadiah has enjoyed during breakfast as rice-dressing mixed with chopping chilli, dice onion and shallot, this meal locally said as Sambal (Figure. 1C). Another way to enjoyed dadiah is blending with steamed glutinous rice flake then mixed with liquid palm sugar as a toping product called as Ampiang dadiah (Surono, 2015) (Figure 1D). Nowadays, various ways have developed in order to re-socialize dadiah to society. Today, people can enjoy dadiah on traditional ice cream form. dadiah was blending with sugar and some artificial flavour to manipulate sour tasted before freeze on -20 °C. Affected with Middle East recipe, some places also use dadiah as main spicy to make mutton curry as replace yoghurt on it.

Dadiah is semi solid form product typically tofu-like consistency has face natural fermentation without pre-heating interface. Dadiah fermentation process on bamboo tube has tendency anaerobe facultative cause of covered well by banana leaf. This stage led to increasing viscosity to buffalo milk turning coagulating then semi solid. Sour tasted on dadiah caused by organic acid (lactic acid) as result of lactose fermenting, and specific flavour cause of combination between bamboo powder that found on bamboo and fermented buffalo milk volatile compound. Comparing with famous yoghurt, dadiah has thicker textures caused separation between curd and whey on milk. On dadiah, whey has absorbed

by bamboo rather than mixed back with curd. That's the reason dadiah has thicker consistency compare yoghurt.

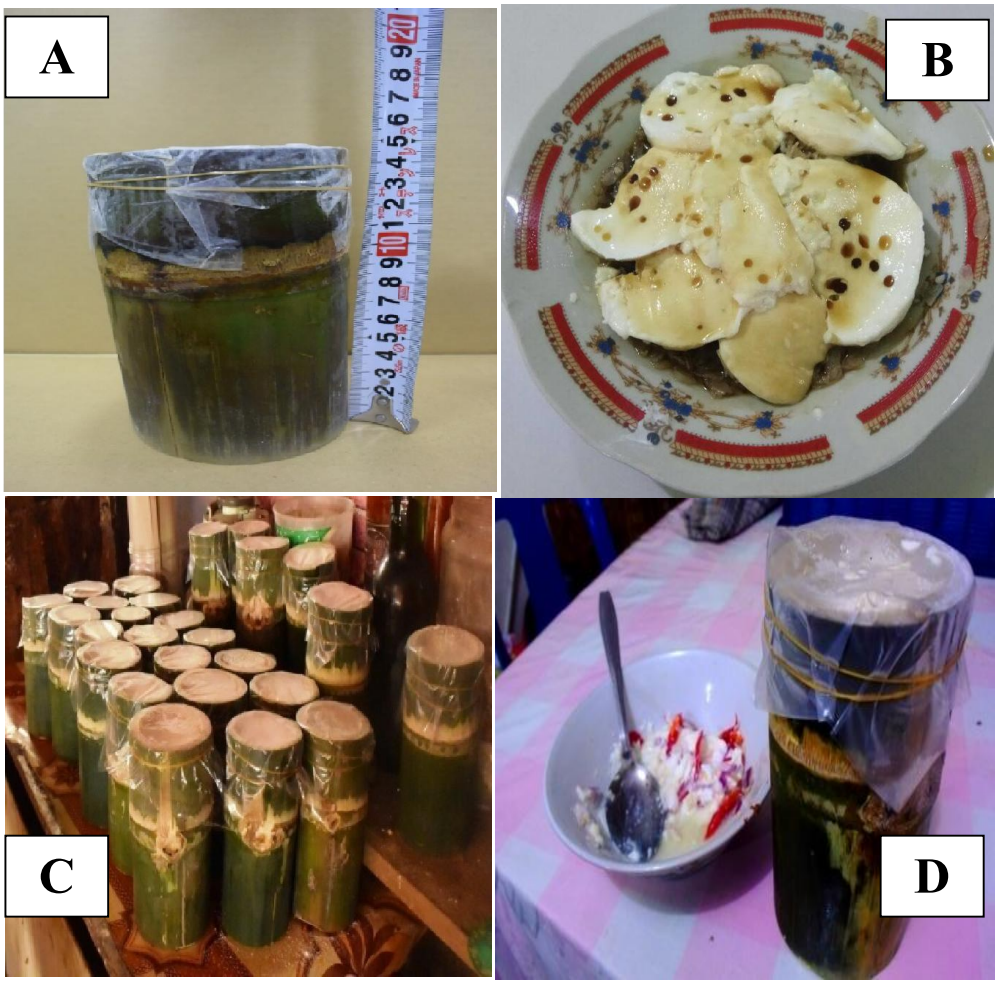


Figure 1: A. Original dadiah
B. Dadiah on display at traditional market
C. Sambal dadiah
D. Ampiangdadiah

In general, dadiah production has faced a tough challenge. Beside un-appropriate management and buffalo milk priority for calves, the buffalo type itself has using swamp buffalo rather than river buffalo. Commonly, river buffalo

is addressed as dairy buffalo. On the other hand, swamp buffalo concentrate on the power and meat commodity. Limitation milk production is one of the obstacles on dadiah manufacturing. For the same reason also lead to dadiah does not have any national standard product like yoghurt.

Like others fermented milk, dadiah contains abundant lactic acid bacteria. As the name, lactic acid bacteria identify as Gram-positive bacteria which has capability to obtain energy from carbohydrate and produce lactic acid as a result. The metabolic pathway could be as homofermentative or heterofermentative. In case of homofermentative, one single lactic acid has producedd as a result of metabolism. *Lactococcus* and *Streptococcus* are classified in this group. In order to heterofermentative, several products have generated from the metabolite process such as lactate, ethanol and carbon dioxide. Some *Lactobacillus* and *Leuconostoc* are parts of it. Some organic substance contributed on aroma and specific organoleptic properties also produced by lactic acid bacteria (Parada, Caron, Medeiros and Soccol, 2007). Some lactic acid bacteria are also known as bacteriocin producer.

Bacteriocin defines as protein or protein multi various ribosomally synthesized with bactericidal ability directed against species that are narrowly associated to the producer bacterium (Klaenhammer, 1988; O'Sullivan, Ross and Hill, 2002). Bacteriocin classifications are still faced development until right now. Although First classification has proposed by Klaenhammer (1993), some

bacteriocins still unclassified cause ambiguity information several from few-hundred bacteriocin. Generally, based on molecular weight, bacteriocin has classified on four big groups. Bacteriocin Class I or familiar with Lantibiotics has molecular weight less than 5 kDa. Lantibiotics are divided into two groups; Type A and Type B. Subclass I A consist of cationic and hydrophobic peptides has flexible structure (ex: nisin from *Lc. lactis*) than rigid globular in nature of Subclass I B (ex: mersacidin from *Bacillus subtilis*) (Jeevaratnam, Jamuna and Bawa, 2005; Chen and Hoover, 2003).

Bacteriocin Class II has known as hydrophobic heat stable peptide with molecular weight smaller than 13 kDa. Bacteriocin Class II has amphiphilic helical structures which allow them to insert membrane of target cell led to depolarization and death. Subclass II A attracts much attention due to ability on anti-*listeria*. Sakacin A from *Lactobacillus sake* is one example from Subclass II A. Subclass II B is two different component bacteriocin that working synergistically to be active. Lactococcin G produces by *Lc. lactis* has classified in this subclass. Subclass II C carry two trans-membrane segments to assist poring construction on target cell such as gassericin A. however some scientist agree to classify Subclass II C onto new other Class of bacteria cause dipeptide of bacteriocin secreted independently without involving signal peptide with no similarity sequence gene (Chen and Hoover, 2003; Parada et al., 2007; Karpiński and Szkaradkiewicz, 2013; Kaur, 2015).

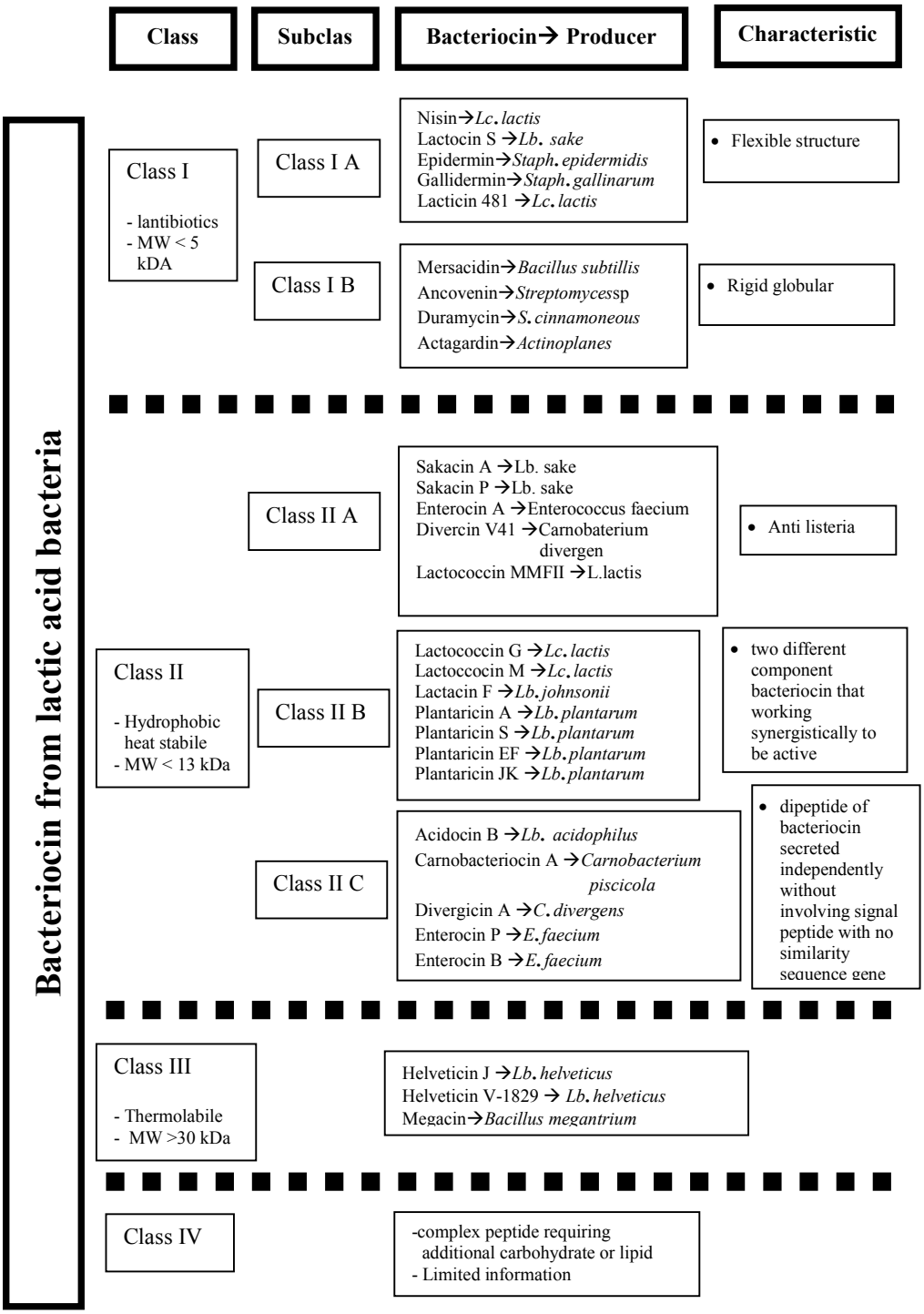


Figure 2: Bacteriocin classifications (Klaenhammer, 1993; Chen and Hoover, 2003; Jeevaratnam, Jamuna and Bawa, 2005; Karpiński and Szkaradkiewicz, 2013; Kaur, 2015).

Bacteriocin Class III has molecular weight more than 30 kDa is thermo-labile peptide. Like megacin from *Bacillus megaterium* and helveticin secreted by *Lactobacillus helveticus* is a member of this group. And the last Class IV is complex peptides requiring additional carbohydrate or lipid that has antibacterial activity. Not much information related bacteriocin Class IV so far (Chen and Hoover, 2003; Jeevaratnam, Jamuna and Bawa, 2005; Karpiński and Szkaradkiewicz, 2013; Kaur, 2015).

At a glance, bacteriocin has categorised as an antibiotic. In fact, bacteriocin is totally differenced with an antibiotic. The basic difference of bacteriocin and antibiotic is the spectrum antibacterial activity. Bacteriocin only affected on species associated with bacteriocin-producer and particularly to strain of the same species. On the other hand, antibiotic has wider antibacterial activity compare bacteriocin. Not only Gram-positive, Gram-negative also affected by antibiotic action (Zacharof and Lovitt, 2012). Furthermore, bacteriocin is ribosomally synthesized during growth phase; meanwhile antibiotic is secondary metabolites (Beasley and Saris, 2004). More detail Perez, Zendo and Sonomoto (2014) explained the differences bacteriocin and antibiotic on Table 1.

Table 1 : Differences bacteriocin and antibiotic (Perez, Zendo and Sonomoto, 2014)

Characteristics	Bacteriocin	Antibiotics
Applications	Food / Clinical	Clinical
Synthesis	Ribosomal	Secondary Metabolite
Bioactivity Spectra	Mostly Narrow	Mostly Broad
Intensity of Bioactivity	Active at Nano to Micro Molar Range	Active at Micro to Mili Molar Range
Proteolysis Enzyme of Degradability	High	Moderate to None
Thermal Stability	High	Low
Active pH Range	Wide	Narrow
Colour /Taste/Odour	No	Yes
Amenability to Bioengineering	Yes	No
Possible Mechanism of Target Cell Developing Resistance	Adaptation Through Changes in Cell Membrane Composition	Generally, Transferable Determinant That Inactivates the Active Compound
Mode of Action	Pore formation, inhibition of cell wall biosynthesis	Cell membrane or intercellular targets
Toxicity Towards Eukaryotic Cell	Relatively no	Yes

The most extensive studies currently are the production of bacteriocin by certain strains of *Lc. lactis*. Broad spectrum activity against Gram-positive bacteria is one of the reasons why scientist interested at these bacteria (Mitra et al., 2005). *Lc. lactis* is a Gram-positive bacterium usually use for milk derivate product like milk, butter and fermented milk. *Lactococcus* use an enzyme to degrade lactose in order to achieve energy on ATP. The side product to produce

ATP is a lactic acid which is useful for whey and cheese production. However, on cheese production *Lactococcus* is not the only bacteria playing a role on it. Some of the cheese productions are collaboration *Lactococcus* and other microorganisms. *Lactococcus* are coccus shape, non motil, do not form spore bacteria and occur pairing on short chain.

Lc. lactis famous as bacteriocin produce named nisin. Nisin could inhibit vary the range of the undesirable growth of Gram-positive bacteria (i.e. *Bacillus*, *Enterococcus*, *Listeria*, *Clostridium*, and *Staphylococcus*) (Noonpakdee et al., 2002). Apart from food additive use, nisin had been reported have an anti-tumour activity, edible antimicrobial film, and also food preservatives (Jeevaratnam et al., 2005; Field et al., 2008; Joo et al., 2012; Murillo-Martínez et al., 2013). The first nisin product was isolated and identified in fermented milk products. However, this type of bacteriocin actually could also be isolated from various dairy products, traditional fermented vegetables, fermented meat products, river water and human milk (Şanlibaba et al., 2009).

Based on structural and physicochemical properties, nisin had classified into Class I type A which has screw shape, flexible molecules and lead to pore formation in the cell membrane of the target organism and thereby to depolarization cytoplasmic membrane of target species (Kaur, 2015). Further, Kaur explained nisin was categorised as lantibiotics which have 2-4 kDa protein molecular weight. This structure stable at high temperature and could inhibit

Gram-positive bacteria and other lactic acid bacteria. However, it has no significant activity against Gram-negative bacteria. Most important this is safe for the animal. The Food and Drug Administration (FDA) approved nisin safety to be consumed by humans at 1988.

In the past, since nisin has commercially marketed in UK around 60 years ago, nisin has only implicating on food preservative. Safe for consuming cause inactivated by the digestive enzyme and effective against spoilage bacteria is the reason nisin became popular for extending food self-life for hot baked flour product (crumpets) and pasteurized liquid egg even in high moisture (Delves-Broughton, 1996). Nowadays, nisin has applicative not only on food preservative. Over past two decades nisin has been extending to biomedical fields. Studies have reported nisin has ability against drug-resistance-bacteria that survive against antibiotics currently like *Staphylococcus aureus, Streptococcus pneumonidae,* and *Clostridium difficile* (Shin et al., 2016). On Figure 3 has shown the development of nisin industrial, application and development in the past decades.

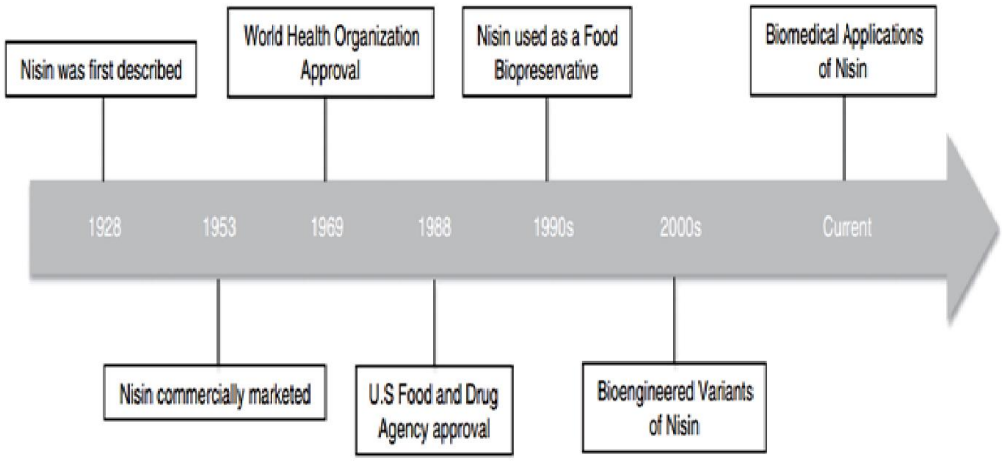


Figure 3: Timeline of nisin development (Shin et al., 2016)

Indonesia is archipelago country, located on equator make Indonesia become the richest biodiversity country in the world. Supported by comfortable weather and temperature, Indonesia is not only rich with macro-organism but also microorganism. Like two side of coin that un-separate-able, these phenomena not only giving Indonesia advantages on biodiversity but also disadvantages in the same time. One of the drawbacks is low storability of food in Indonesia.

Abundant spoilage bacteria and others microorganism led to rotten food easily in Indonesia. To manipulate this condition, most of the foods industrial compete to invent available food preservative to apply on their product in order to extend food self-life. Exploration regarding food preservative is urgent to be conducted. One of the closes potency to find is isolating from fermented food product which is developed in Indonesia.

In contrast, beside abundant spoilage bacteria, wide biodiversity also give another extra credit at Indonesia. Indonesia also known as paradise of micro biome diversity which keeps good bacteria as collection like lactic acid bacteria that support human health on probiotics track path. Some lactic acid bacteria also knew as food-preservative-producer that waiting for exploratory.

II. Analysis of Microbiota Community Structure in Traditional Fermented Milk Dadiah

ABSTRACT

Dadiah is Indonesian traditional fermented milk and is neither pasteurized nor boiled, but no food poisoning has been reported to date. Microbiota inhabiting dadiah has never been completely explored. In this study, we performed deep sequencing of 16S ribosomal RNA genes extracted from 11 dadiah samples and analyzed the dadiah microbiota at the genus level. We found that *Lactococcus*, *Lactobacillus*, and *Leuconostoc* were predominant among the dadiah microbiota. Unexpectedly, *Klebsiella* and *Chryseobacterium* considered potential pathogens were also found in some of the dadiah samples. There was little difference in the microbiota among samples taken from the same bamboo tube. In contrast, there were differences between the dadiah microbiota from different bamboo tubes, even those collected from the same sampling area. Furthermore, the composition of the dadiah microbiota showed large differences between sampling areas. We believe that our findings will lead to further improvement in the preparation of dadiah.

1. INTRODUCTION

Among fourth area producer dadiah produce from Alahanpanjang has its own characteristic. Not like other dadiah generally that pouring raw fresh buffalo milk on bamboo on once pouring, dadiah from Alahanpanjang has several processes pouring to bamboo. At least four up to seven times pouring process on four up to seven days length period led to four up to seven layers created on dadiah from Alahanpanjang. The numerous layers evoke assumption about microbe biodiversity on each layer.

Besides microbe biodiversity on dadiah Alahanpanjang, the microbe diversity also predicts on difference dadiah area. This hypothesis based on differences of location, local producer practice and even different height of bamboo use are factor of microbe diversity on dadiah. Although *Lactococcus* and *Lactobacillus* are the dominant bacteria on dadiah (Surono, 2003), the community structure on dadiah has not explored yet until right now.

2. MATERIALS AND METHODS

A. Dadiah samples, purification of bacterial genome DNA the samples and amplification of 16S ribosomal RNA gene from bacterial genome DNA

Eleven samples were obtained from four local areas which are Batusangkar (A1, A2), Alahanpanjang (B1, B2, B3, B4, B5), Padang Panjang (C1, C2), and Agam (D1, D2) in West-Sumatra, Indonesia (Fig. 4). Because

dadiah is rarely manufactured as a large-scale dairy product, we obtained the samples from traditional markets in each area. All the samples were transported by air to Okayama University, Japan, were kept below freezing temperature during transit, and were then stored at –20 °C in the laboratory until they were required for DNA extraction.

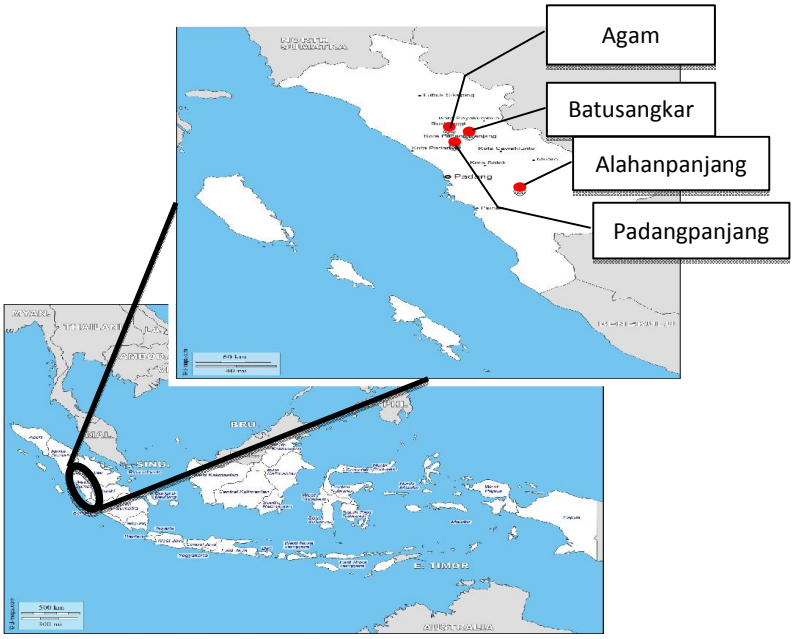


Figure 4: Sample area taken West Sumatera Province of Indonesia.

Preparing 16S rRNA gene amplicons. Bacteria and bacterial-DNA were prepared using a modification of our previous method (Morita et al., 2007). DNA was purified by treatment with ribonuclease A (Wako), followed by precipitation with 20% PEG6000 (NacalaiTesque) in 2.5 M NaCl. The pelleted DNA was rinsed with 75% ethanol, and dissolved in TE buffer. The V3-V4 hypervariable region of the bacterial 16S rRNA gene was polymerase chain reaction (PCR)-

amplified using the barcode tag universal primer sets 341F–805R (341F 5'-CCTACGGGNGGCWGCAG-3' and 805R 5'-GACTACHVGGGTATCTAATCC-3') (Klindworth et al., 2013). The PCR amplification was performed using the following program: 95°C for 3 min, 25 cycles (95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s), 72 °C for 30 s, then hold at 4°C. Agilent 2100 Bioanalyzer (Agilent Technologies) was used to determine the size (approximately 550 base pairs) of the PCR fragments. The PCR products were purified using Agencourt AMPure XP Beads (Beckman Coulter). Nextera XT Index Kit (Illumina) was used for labelling the amplicons with different dual barcodes.

B. Deep sequencing and data analysis.

Deep paired-end sequencing was performed using the Illumina MiSeq platform. The obtained raw sequence data comprising 2,028,824 reads were initially processed using Mothur v1.38.1 (Schloss et al., 2009) for barcode splitting. The resulting demultiplexed sequences were clustered into bins called Operational Taxonomic Units (OTUs) based on 97% sequence similarity, using the Ribosomal Database Project classifier (Wang et al., 2007). Only genera containing over 1% of total OTUs were used for subsequent analysis. The taxonomy of each OTU was assigned using the SILVA rRNA database (Quast et al., 2013). SPSS program v23.0 (Field, 2009) was used for principal components analysis (PCA). The sequence data sets supporting the results of this article are

available in the NCBI Sequence Read Archive under project accession number PRJNA379875.

3. RESULTS

To investigate the bacterial composition in dadiah, we first collected 11 dadiah samples from six bamboo tubes, obtained from four local areas in West-Sumatra, Indonesia (Fig. 5A). Samples from Batusangkar and Alahanpanjang were obtained from different sites inside the same bamboo tube. On the other hand, each sample from Padang Panjang and Agam was obtained from different bamboo tubes. The V3-V4 region of the 16S rRNA gene was amplified from the 11 samples and subjected to deep sequencing. We obtained 39,677 high-quality filtered reads per sample. The read was clustered into 1,054 phylotypes (OTU's: at 97% sequence identity), and their representative sequences were used in the taxonomic analysis. Thus, the OTU was assigned to 154 genera, 16 of which showed over 1% of the total OTU's and were used for subsequent analysis.

The bacterial composition of the 11 samples was determined for each taxonomic level according to the read counts of the 16 genera in each sample. As we expecting, the microbiota of all the samples was highly abundant in *Lactococcus* and *Lactobacillus* (Fig. 5B). This core microbiota (*Lactococcus* and *Lactobacillus*) accounted for more than 40% of the total reads of the 16 genera in all the samples except D1. In sample D1, *Leuconostoc* was the most predominant genus. These results indicate that indigenous lactic acid bacteria (*Lactococcus*,

Lactobacillus, and *Leuconostoc*), which are derived from buffalo milk, bamboo tubes and/or banana leaves, may be involved in the fermentation of dadiah without starter culture.

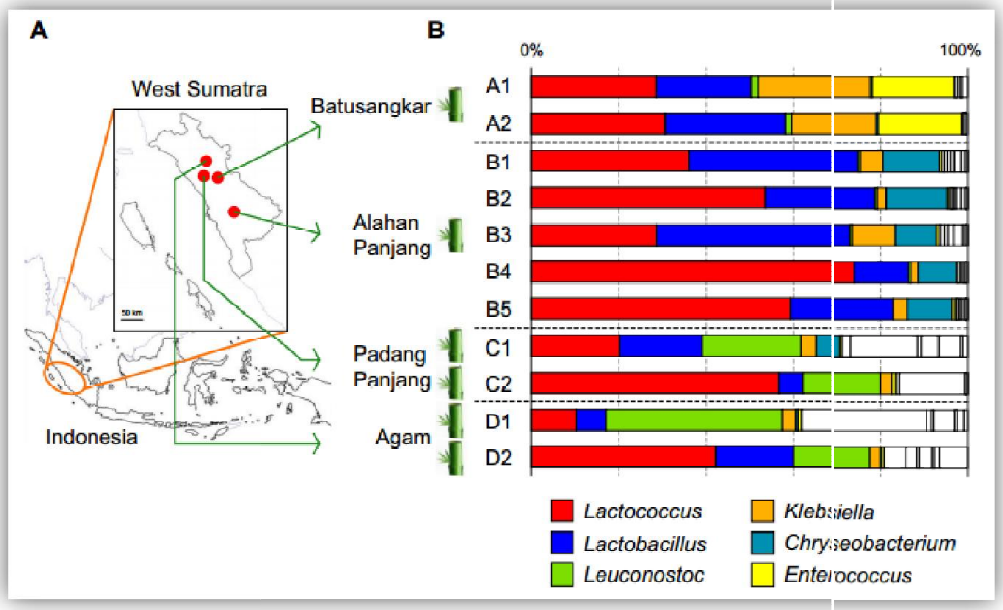


Figure 5: A. Sample area taken in West Sumatera Indonesia.
B. Microbiome distribution genes on various dadiah producer areas.

There were some subdominant genera enriched in dadiah. Interestingly, Gram-negative bacteria also detected. *Klebsiella* and *Chryseobacterium*, which are considered as potential pathogens, were also found in dadiah. Genus composition in dadiah varied substantially between the four sampling areas (Fig. 5B). The greatest number of *Klebsiella* had detected on a sample from Batusangkar (Sample A1-A2), meanwhile, greatest number of *Chryseobacterium* had detected on a sample from Alahanpanjang (Sample from B1-B5). The dadiah

microbiota from Padangpanjang (Sample C1-C2) and Agam (Sample D1-D2) were abundant in *Leuconostoc*.

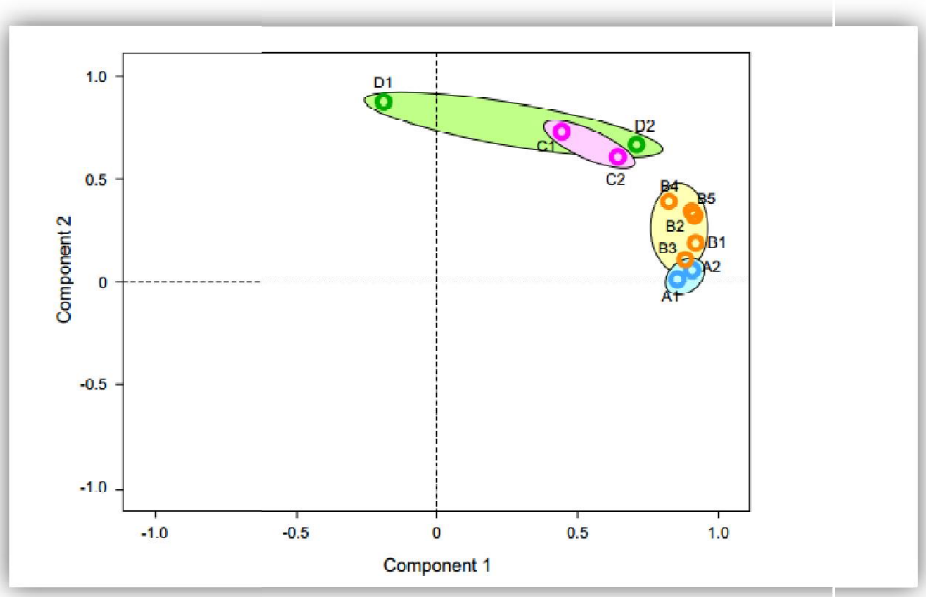


Figure 6: Microbiota community closeness variety on each area has described on PCA chart.

The diversity of the dadiah microbiota from the four sampling areas was analyzed by PCA (Fig. 6). There was little difference among the samples (A1–A2 and B1–B5) obtained from the same bamboo tube, showing that the predominant microbiota in dadiah remains relatively constant in a single bamboo tube. In contrast, there were large differences between the samples (C1–C2 and D1–D2) obtained from different bamboo tubes, even those collected from the same sampling area (Fig.5, Fig. 6). Interestingly, the microbiota of D2 more closely resembled that of C2 than D1 (Fig. 6). These results indicate that the composition of dadiah microbiota is different in every bamboo tube. Because dadiah is still a

homemade product made by traditional methods in Indonesia, different microbiota may be generated in every bamboo tube and every local area.

4. DISCUSSION

The result has confirmed that dadiah has dominated by lactic acid bacteria as main microbiota community structure on dadiah. Lactic acid bacteria like *lactococcus* and *lactobacillus* are belonging on the top of list. *Lactococcus, lactobacillus* and *leuconostoc* also documented as predominant fermented milk from various fermented milk like dahi in Bangladesh (Rashid, Ueda and Miyamoto, 2009), kefir in Ireland (Marsh, O'Sullivan, Hill, Ross and Cotter, 2013) datshi in Bhutan (Shangpliang, Sharma, Rai and Tamang, 2017), and koumiss in Mongolia (Yao et al., 2017). *Lacotococcuc* and *lactobacillus* domination also noted by previous Indonesian traditional food scientist (Surono, 2003; Mustopa and Fatimah, 2014; Nuraida, 2015). Unfortunately, so far scientist only interest on lactic acid bacteria potential as probiotic for human health led to microbiota community structure unnoticeable yet.

However, lactic acid bacteria source of dadiah is still unknown surely until right now whether it is from bamboo or it is come from buffalo milk itself as an indigenous microbe. Recorded by Li et al., (2016) lactic acid bacteria was predominant on raw buffalo milk within on 24 hours and keep surviving after pasteurize storability up 7 days then replace by *paenibacillus* on 21 days keeping. This is the evidence that lactic acid bacteria one of the indigenous bacteria consisted of raw buffalo milk. On the other side lactic acid bacteria also found as

native microbe contains on bamboo like reported by Sonar and Halami (2014) and Romi, Ahmed and Jeyaram (2015) whom succeed identify native lactic acid bacteria in bamboo shoot product.

Anyway, where ever lactic acid bacteria come from whether come from bamboo or even come from native raw milk, they are already accomplished simplify lactose on buffalo raw milk on to monosaccharide that easier to absorb by the human body, especially for the person who get lactose intolerance. Lactose intolerance is a condition where deficiency of lactase on the human body that would lead to abdominal pain, diarrhoea when detecting lactose existence inside the body (Deng, Misselwitz, Dai and Fox, 2015). Lactic acid bacteria helped intolerance patient in order to enjoyed milk along with benefit followed it.

Klebsiella belongs to the *Enterobacteriaceae* family and is used as an indicator of fecal contamination (Martin et al., 2016), whereas *Chryseobacterium* has been found in soil and water environments (Vishnu et al., 2014). *Enterococcus*, which is also an indicator of fecal contamination (Staley et al., 2014) was also found in dadiah, especially in samples A1–A2 (Fig. 5B). Because dadiah is neither pasteurized nor boiled, it is possible that these potential pathogens could contaminate dadiah during the production process, which is not ideal, although no food poisoning has been reported to date (Surono, 2015). Lactic acid and bacteriocin which are produced by the core microbiota (*Lactococcus* and *Lactobacillus*) may contribute to the suppression of potential pathogens in dadiah.

This is not the first time potential pathogen bacterial existing on dairy product has reported. *Coliform* contamination also had reported by Jayarao and Henning (2001). The main source contamination was come from bulk tank milk rather than from feces itself. Milk contamination by *enterococci* and *coliform* had been reported before by Kagkli, Vancanneyt, Vandamme, Hill and Cogan (2007).

Studies have reported that nisin has capability to prevent *Streptococcus pneumonia* which has knowing as antibiotic-resistance (Shin et al., 2016; Kim et al., 2016). Like *Klebsiella pneumonia, Streptococcus pneumonia* also known as *pneumonococcus* has known as major causes of pneumonia disease on late 19's (Kenneth and Ray, 2004). Dharmawan et al. (2005) describe the adhesion properties ability of probiotic consist on dadiah has showing impact to minimize effect of pathogen bacteria at human digestive track. In addition, Collado et al. (2007) clarify adhesion properties ability make pathogen bacteria entering human body just passing through without having chance to effect body's health. However, further research is needed to clarify whether bioactive compound on dadiah has same effect on inhibiting *Klebsiella* symptom at pneumonia respiratory track in human like nisin does to *Steptococcus penumonia*, and the reason no food borne illness has not occurred as a result of dadiah consumption.

5. CONCLUSIONS

In summary, this study revealed the microbiota present in dadiah by deep sequencing. The predominant genera, *Lactococcus*, *Lactobacillus*, and *Leuconostoc*, were evenly distributed among the dadiah microbiota. Potential

pathogens such as *Klebsiella* were also found in dadiah. We believe that our findings provide a basis for further improvements in the preparation of dadiah.

The microbiota consist on dadiah tube has unique each other whether it came from same producer, same procession time and same raw milk material. The difference predicts cause of difference height of bamboo tube was use on making process.

III. Isolation Nisin-Producing *Lactococcus lactis* subsp. *lactis* Isolated from Dadiah

ABSTRACT

Dadiah contains abundant lactic acid bacteria, which can produce bacteriocin that could be applied as a food preservative. One strain on dadiah (D4) has been indicated as Bacteriocin-producer. 16S-rRNA sequence and API 50 CHL confirmed 100% identically *Lc. lactis* subsp. *lactis*. Characteristics studies have revealed that the bacteriocin is heat stable even at an autoclaving temperature (121 °C for 15 minutes) and still active over seven days depository on temperatures 4 °C and 25 °C even there is decrease activity along storage. The bacteriocin was active in the wide range of pH (2.0 - 11.0). Protease tested shown the bacteriocin could be inactivated by proteinase-K. SDS-PAGE discovered the bacteriocin had molecular weight around 3.5 kDa. The anti-bacterial broad spectra showed wide spectrum on inhibiting strains foodborne pathogens and food spoilage bacteria. Gene encoding was amplified by Polymerase Chain Reaction (PCR) with Nisin gene-specific primer definite the strain as nisin Z producer. *Lc. lactis* subsp. *lactis* D4 has draft genome GC content 34.83% which is approximately 2.5 Mb in size and encode 2,378 ORF's.

1. INTRODUCTION

As mention on the previous chapter that dadiah has dominated by lactic acid bacteria which are need on human health support. *Lactococcus* is one of abundant lactic acid bacteria found on dadiah. Delves-Broughton (1996) stated *Lactococcus lactis* as one of *Lactococcus* strain has produce bacteriocin as active antimicrobial compound named nisin.

For year nisin has been found and developed on wide aspect application like an anti tumor (Joo et al., 2012), edible antimicrobial film (Murillo-Martinez et al., 2013) and the most application on food preservative (Field et al., 2008). Nisin could inhibit a wide range the growth of several undesirable Gram-positive bacteria in the genera *Bacillus, Enterococcus, Listeria, Clostridium* and *Staphylococcus* (Noonpakde et al., 2003).

Islam et al. (2012) explained the mechanism of Type I A bacteriocin like nisin work on inhibit wide range of Gram-positive bacteria. Furthermore, principles of the mechanism are by inhibiting peptidoglycan biosynthesis and pore formation on Gram-positive cell wall. These explanations are the reason why nisin only working on Gram-positive bacteria which has peptidoglycan component on the cell wall.

2. Material and Methode

A. Bacterial strains and growth media

Colonies were isolated from dadiah sample from three sub-districts (Batusangkar, Padangpanjang and Kayu-Aro) after previously dadiah was dilute with sterile physiological saline. Colonies were cultivated on BCP (nissui, Japan) and MRS (oxoid, Hampshire, UK) agar then incubate on 25 ℃, 30 ℃, and 37 ℃ for two days. Next step was screening bacteriocin existence. *Lc. lactis* IFO 12007 (nisin A Producer), NBRC 10933 (non-nisin producer) and JCM 7638 (nisin Z producer) was used as a control. Besides control strains, nisin A (Nisui, Japan) also used on this research. All control was inoculated on MRS broth and incubated at optimum temperature. Optimum temperature decided by comparing inhibitory zone that created by incubated Cell-Free-Supernatant (CFS) culture media. The biggest inhibitory zone was settled as optimum temperature.

Listeria monocytogenes VTU 206 also used as an indicator strain to pointing bacteriocin existence. *Listeria monocytogenes* VTU 206 was cultivated on TYLG broth then inoculated on Nutrient Agar (Eiken Chemical, Tokyo, Japan). The culturestock was maintained at -80℃ on skim milk. All strains were cultivated twice in the appropriate broth overnight to get optimum growth before application.

Potential cultures which assume produce antibacterial activity will be evaluated by some method. The methods are; Gram staining microscopy, API 50

CHL, and 16S rRNA sequencing. 16S rRNA gene was amplified using universal primer F27 (5'-AGA GTT TGA TCM TGG CTC AG-3'; positions 8 to 27f and 1525r (5'-AAG GAG GTG WTC CAR CC-3') (Arakawa et al.; 2015) was used. The conditions consisted of an initial denaturation at 95 ℃ for 5 min, continued by a -35 series at 95℃ for one minute, 55 ℃ for 2 minutes, and then 72 ℃ for 3 minutes, final extension step consisting of 72 ℃ for 10 min.

The PCR products were electrophoresed on 1% agarose gel (Merck, Germany) in 1×TBE buffer stained with ethidium bromide. These products were visualized with the Gene Genius Bio-imaging system (Isik et al., 2014). Sequences were matched to those in the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) and also compared to the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp).

B. Antimicrobial activity assay

The antimicrobial activity could be identified using 50 µl cell-free-culture-supernatant of each colony that isolated from dadiah. This colony was added into solidified media that have been inoculated with an indicator strain. As much 50 µl of indicator strain was inoculated on 20 ml appropriate media, then pour into sterile petri-dish for this antimicrobial activity assay. Indicator strain media that have been added to colonies were incubated at 37 ℃ for overnight. Target strains were examined by clear-zone that has created around the well.

Wide clear-zone creation and the explicit clear-zone boundary will use as a potential strain produced bacteriocin indicator.

C. Genome DNA purification for sequencing

To sequencing targeting strain, firstly the target strain DNA has to purify through the pellet taken from centrifuging 200 ml MRS media that cultivated overnight process. The purified process began with dissolving the pellet in 10 ml 1x TE then added 1 ml lysozyme continued with incubated on water bath 37 ℃ for 1 hour to demolish cell wall. To optimize the cell lysis, 50 µl achromopeptidase was added followed with 37 ℃ incubating for another 30 minute. As the result the protein will separate and removed by added 1 ml proteinase-K treatment in the presence of 1.5 ml 10% SDS incubating at 55 ℃ for 1 hour. To denature and precipitate the protein, 15 ml phenol/chloroform/isoamyl alcohol (25:24:1) was added. The precipitated material was removed by centrifuge at 12,000 rpm, 30 minutes, 4 ℃. The upper layer consists DNA was taken then repeat the denture and precipitate process at least twice to remove excess protein out of DNA layer. DNA precipitation was conducted by added 1/10 vol. NaOAc (sodium acetate, pH = 5.2) and 1 vol. isopropanol. After the solution mixed well, the solution was centrifuged 12,000 rpm, 30 minutes, 4 ℃. The supernatant was discarded, left precipitate material to wash twice with -20 ℃ 75% ethanol. The precipitated DNA was air dried around 20 minutes then diluted with 600 µl 1x TE and incubated at 4 ℃ overnight.

To remove RNA consist on a solution, 1 µl RNAse A was implicated then incubated at 37 °C for 1 hour. In order get pure DNA, 300 µl 1.6 M NaCl and 300 µl 26% PEG (polyethylene glycol) were mixed with solution. The mixture was incubated at 4 °C for 30 minutes. Precipitating pure DNA achieved by implicating 500 µl cold 75% ethanol continued with a maximum centrifuge for 15 minutes at 4 °C. After discarding the supernatant, the pellet was air dried then diluted on 1x TE buffer and keep on 4 °C. Spectrophotometer and electrophoresis with 0.7 % agarose in 1x TAE buffer were used for examining DNA quality and quantity. Before entering sequencing process, the DNA could be stored at -20 °C.

Sequencing process for target strain DNA was paired-end sequenced using Miseq Technology Sequencer platform (Illumina®, San Diego, CA, USA) with the whole genome shotgun strategy. Genomic libraries containing 600-1000 bp insert were constructed and sequenced. The sequence reads were assembled using CLC Genomics Workbench version 9.0.1.

D. Effect of enzymes, pH and heat treatment

Potential targeting strain will be evaluated by the effect of a various enzyme, pH and heat adjust treatment to explore kind of bacteriocin contain on dadiah. The effect of numerous enzymes on bacteriocin activity was determined as described by (Arakawa et al., 2015) with slight modification. CFS target strains were treated with 50 U/mg of the certain enzyme solution. They are; Proteinase-K (pH 7.5; NicalaiTesque), α-chymotrypsin (pH 7.8; NicalaiTesque), Trypsin (pH 8;

Wako), Pepsin (pH 2; Sigma), catalase (pH 7; Sigma). All solution incubated for 2 hours at 37 °C then continued with enzyme inactivation by heated at 95 °C for 10 minutes. The untreated solution was used as a control. Post reaction, the pH solution will neutralize before sterile filtration to get CFS.

Evaluation of pH sensitivity of active substance was estimated by arranging pH of cell-free-culture-supernatant between 2 to 11 by using 0.5-3 N NaOH and 0.5-3N HCl. Incubated solution at room temperature for 1 hour then injected CFS onto media indicator strain gel. CFS strain NBRC 100933^{T} solution was used as a control.

Evaluation to heat stability observation was assessed using neutralized CFS was incubated in water bath at 65 °C for 60, 45, 30, 15 minutes and no heated as a control. CFS also heated on the high temperature at 95 °C, 110 °C and 121 °C for 15 min. Storage time figure was evaluated using CFS treated at 4 °C and 25 °C for seven days. After processed in several ways (enzyme, pH, heat and stockpiling), CFS was observed in agar well assay against indicator strain. The treated cell-free-culture-supernatant will compare with control by clear-zone was created.

E. Antibacterial wide spectrum assay

Antibacterial broad spectrum method well determined by agar diffusion assay. Indicator strains related to food spoilage and pathogenic bacteria were given in Table 4. Five strains tested were grown in MRS medium at the optimum

temperature for 24 hours to get cell-free supernatant (CFS). After that, the cell was removed by centrifugation (3,000 rpm for 20 minutes at room temperature). The CFS was arranged onto neutral pH by using 3 M NaOH then sterilized with micro-filter (0.2 μm pore size, Sartorius®, Hannover, Germany).

Indicator strain was inoculated 2% (v/v) on appropriate media then incubated at suitable temperature (30 °C and 37 °C). Inoculation was arranged twice to get log phase growth of bacteria indicator then injected on proper agar media (MRS Agar and Nutrient Agar). Solidified-indicator-strain-media-agar was punched as 6 mm to create a hole/well on agar media. Each well was filled with 50 μl CFS then agar incubated at their suitable temperature (37 °C). The diameter of the inhibitory area (in mm) created after 24 hours incubation. This zone was measured by callipers then recorded.

F. Confirmation Nisin-Producing Strain

PCR method was used to confirm nisin production on strains. Nisin specific primer was used on it. Forward and reverse were as followed: Primer 1: primer 5'-CCG GAA TTC ATA AGG AGG CAC TCA AAA TG-3'and Primer 2: primer 5'-CGG GGT ACC TAC TAT CCT TTG ATT TGG TT-3'.The condition consisted of 30 cycles of 90 °C for 1 min, 55 min for 1 min and 72 °C for 2 min. The primers were deliberate from nisin A structural genes, which were complementary to region 17 bp upstream and two bp downstream of the coding region (Mulders et al., 1991; Dodd et al., 1996).

G. SDS-PAGE and *in-situ* activity assay

Total soluble complete cell proteins were evaluated using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). This method also used to estimate molecular mass of bioactive peptides. The gel was divided into two vertical parts after SDS-PAGE to determine the apparent molecular weight of bacteriocin. One staining (containing marker and sample) has set with Coomassie Brilliant Blue R-250 (Eztain Aqua; Atto Corporation, Tokyo, Japan) and the other (containing sample) for running in situ assay. In the in-situ assay, 50 µl of indicator strain was inoculated on 20 ml MRS agar on Petri dish that has been laying down SDS-PAGE gel result. Agar media were incubated at 37 °C overnight to see clear-zone creation. Wide scale clear-zone use to determine the antibacterial activity (modified Batdorj, 2005).

3. RESULTS

A. Isolation and identification of bacteriocin-producing strain

404 colonies were isolated from dadiah that taken from three areas in West-Sumatera Indonesia. All of the colonies were tested against indicator strains (*Listeria monocytogenes* VTU 206). Six colonies (F8, F4, D5, D4, C5 and C1) indicated bacteriocin-producer by creating broad zone inhibition on strain-indicator-media was selected. All six colonies bacteriocin–production was detected on dadiah taken from Batusangkar district only. This study is the first to achieve bacteriocin production on dadiah.

The selected colonies were identified based on morphological and carbohydrate fermentation characteristics. Microscopic analyze of colonies figure out that all six colonies have cocci shape in short chains and indicate Gram-positive bacteria. API 50 CHL carbohydrate fermentation profile discovered selected colonies have the ability to ferment arabinose, ribose, xylose, galactose, glucose, fructose, mannose, mannitol, acetyl glucosamine, amygdaline, arbutin, esculine, cellobiose, maltose, lactose, saccharose, trehalose, amidon, gentiobiose, and potassium gluconate. Based on carbohydrate fermentation ability, selected colonies were classified as *Lc .lactis* subsp. *lactis*.

One representing target colonies and other three strains control will get into the further test. The other three strains are IFO 12007 (Nisin A producer), JCM 7638 (nisin Z producer), and NBRC 100933^{T} (as non-nisin producer). All four colonies will continue growing at three different temperatures, which are 25 °C, 30 °C, and 37 °C for 24 hours to determine bacteriocin optimum production. Bacteriocin optimum production tested shown 30 °C and 25 °C for 24 hours shown optimum antibacterial substance better than incubating on 37 ^{0}C. In Indonesia, dadiah was produced on Indonesia's room temperature (around 28 °C). This condition explains the reason why incubation temperature of 25 °C and 30 °C does not show the significant difference range of inhibition area.

B. Identification of the isolated strain and its draft genome sequence

PCR sequence amplified around 1,400 nucleotides (Fig. 7) of the 16S rRNA bacterium and subjected to 16S RNA sequence analysis. This sequencing is

used to determine the phylogenetic position of target colonies. Based on BLAST database entire target colonies was 100 % identically *Lc. lactis* subsp. *lactis*. Carbohydrate fermentation pattern on API 50 CHL also confirm the same strain *Lc. lactis* subsp. *lactis* found on dadiah.

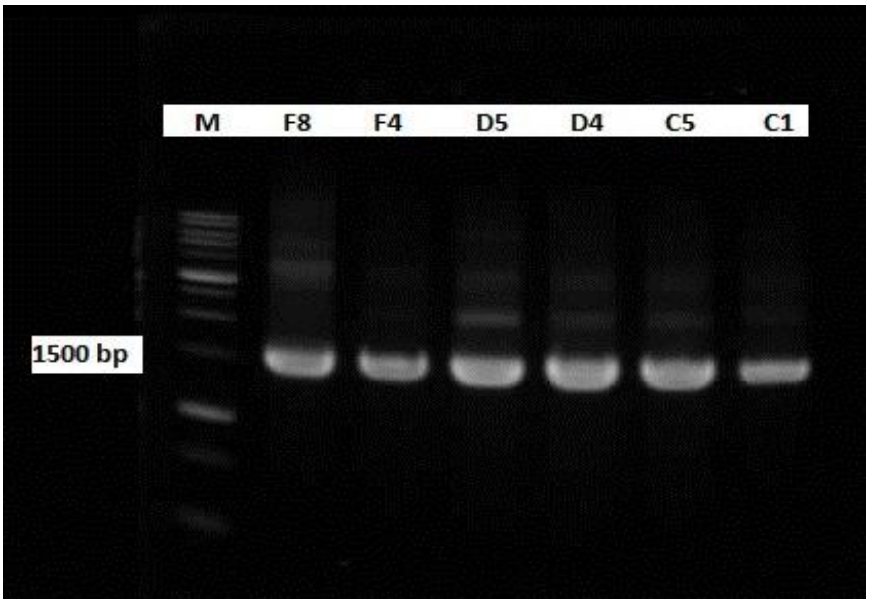


Figure 7 : DNA sequence of approximately 1.400 bp for 16S rRNA gene using Primer 27F and 1525R

Regarding to observe relationship among all six selected isolates, the selected isolates were examined by fingerprinted at strain level using repetitive sequence based (rep) – PCR analysis using three combinations mixing primer (Odamaki, 2011) along with all the control strains. All six isolates were showed to be identical genetically by the rep-PCR finger print analysis (Fig. 8). For entering next tested, strain D4 was selected randomly to represent all six isolates.

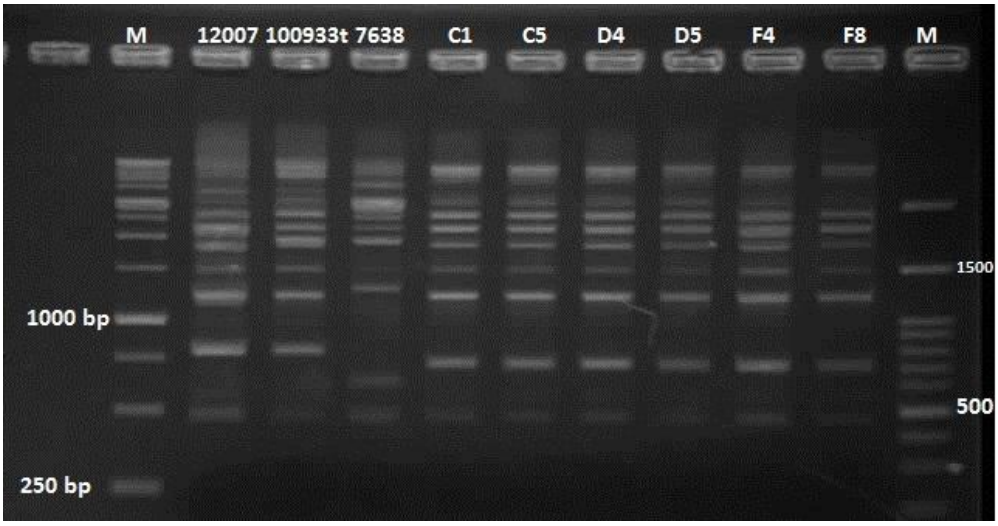


Figure 8: Six isolate colonies fingerprinting rep-PCR along with three control strains

The draft genome of *Lc. lactis* strain D4 disclosed having low GC content that belongs to 34.8 % with total protein gene encode 2,378 ORF's with estimated genome length 2,502,845 bp consist of 25 contigs. The *Lc*. *lactis* strain D4 shares 1,940 ORFs with *Lc. lactis* subsp. *cremoris* and 1,780 ORFs with *Lc. lactis* subsp. *lactis*. The statistically template mapping *Lc. lactis* strain D4 was shown on Table 2 and Table 3.

Table 2 : Nucleotide distribution (G+C content = 34.83 %).

No	Nucleotide	Number	Frequency
1.	Adenine (A)	813,386	32.49
2.	Cytosine (C)	439,017	17.53
3.	Guanine (G)	433,145	17.30
4.	Thymine (T)	817,374	32.65
5.	Any Nucleotide (N)	73	0.00

Table 3: Genome features of the draft genome sequence of strain D4 and eight complete genomes sequence of *Lc. lactis* subsp. *lactis* various sources.

Strain	Genome Length (Mb)	G+C Content (%)	ORF	Source
D4	**2.5**	**34.8**	**2,378**	**Dadiah**
IL1403	**2.4**	**35.3**	**2,406**	**Cheese Starter**
KF 147	2.6	34.9	2,595	Sprout of Soybean
CV 56	2.5	35.0	2,533	Vagina of Healthy Woman
IO-1	2.4	35.1	2,343	Water in the Drain pit of Kitchen Sink
NCDO 2118	2.6	34.9	2,545	Frozen Peas
KLDS 4.0325	2.6	35.4	2,648	Homemade Koumiss in Xinjiang, China
S0	2.5	35.2	2,482	Cow Milk
A12	2.7	35.5	2,685	Wheat Sourdough

In this study, we comparing *Lc. lactis* strain D4 with other strains *Lc. lactis* especially with strain IL1403 complete genome isolated from cheese starter (Bolotin et al, 2001) as similarity dairy derivate product that was shown in Figure 9. On the green box of Figure 9 shown ORF length absence draft genome *Lc. lactis* strain D4 to strain IL1403 indicated both of strains were un-similarity each other.

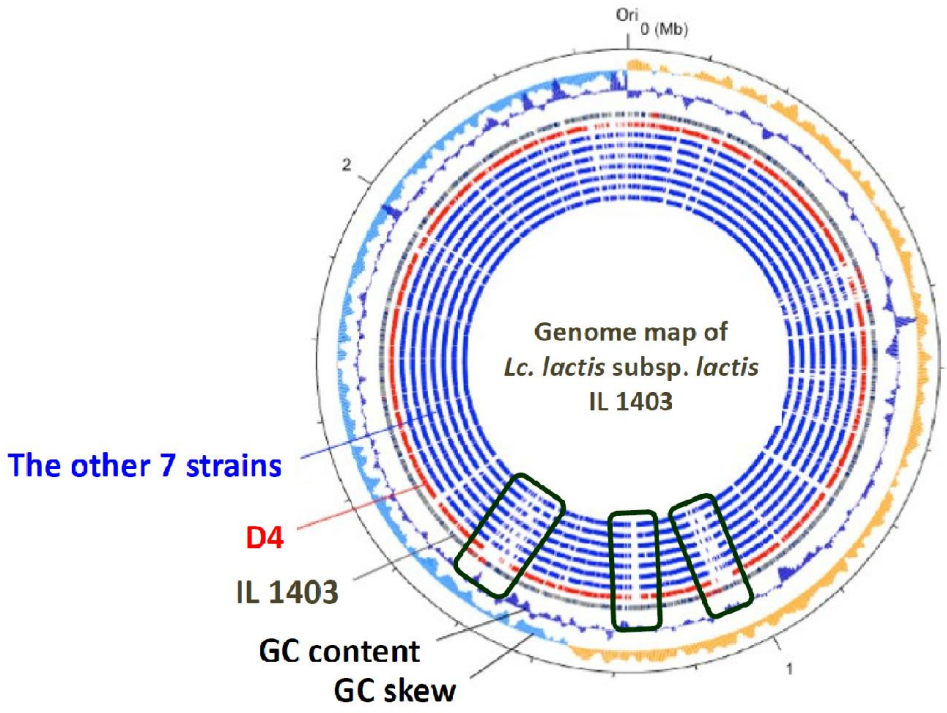


Figure 9: Mapping of *Lc. lactis* subsp. *lactis* strain D4 draft genome sequence to *Lc. lactis* subsp. *lactis* strain IL1403 complete genome. On the outside circle is genome size in base-pair. D4 strain mapping shown in red. The green box showed the difference between strain IL1403 and other strains.

The phenomenon explained in detail on Figure 10 that shown ORF encode which has responsibility for amino acid and carbohydrate transport and metabolism perspective. From the graph revealed ORF *Lc. lactis* strain D4 has involving carbohydrate transport and metabolism genes greater than amino acid transport and metabolism. In contrary, *Lc. lactis* strain IL1403 has amino acid dominance activity than on carbohydrate genes functional.

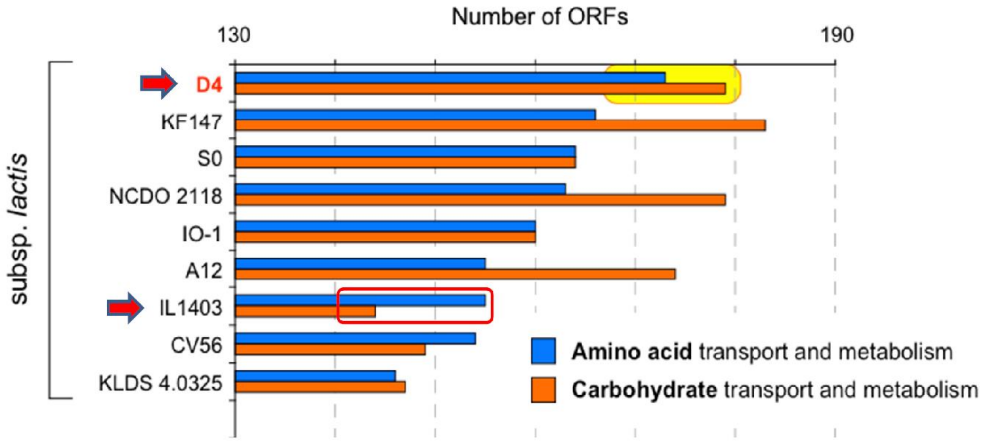


Figure 10: Number of gene encoded function (amino acid and carbohydrate metabolism) by each genomic sequence.

C. Effect of pH to antibacterial bacteriocin

Effect of various pH on bacteriocin produced by D4 was shown in Figure 11. As seen on the graph there were slightly similarity trends antibacterial activity between IFO 12007 as nisin A producer and JCM 7638 as nisin Z producer compared to NBRC 100933^{T} as non-nisin producer. As a non-bacteriocin producer, NBRC 100933^{T} has antibacterial activity at lower pH 2–4 then completely gone when turning into pH 5. Strain IFO 12007 as nisin A producer

shown decreasing antibacterial activity steadily among pH range of 2 to 9 from 20 mm to 15 mm. after that, inactive directly on pH bigger than 10. Strain JCM 7638 as nisin Z producer has decreased gradually antibacterial activity from around 25 mm to around ten between pH 2–11. The further information explains that strain D4 showed the same trend that given by JCM 7638. Furthermore, Figure 11 reviled bacteriocin on CFS D4 as target strain still active even on alkaline condition (pH 11). For NBRC 100933^{T} who recorded antimicrobial activity because the acid condition that makes indicator strain inconvenience growth just like control (pH adjustment blank media) shown the same trend.

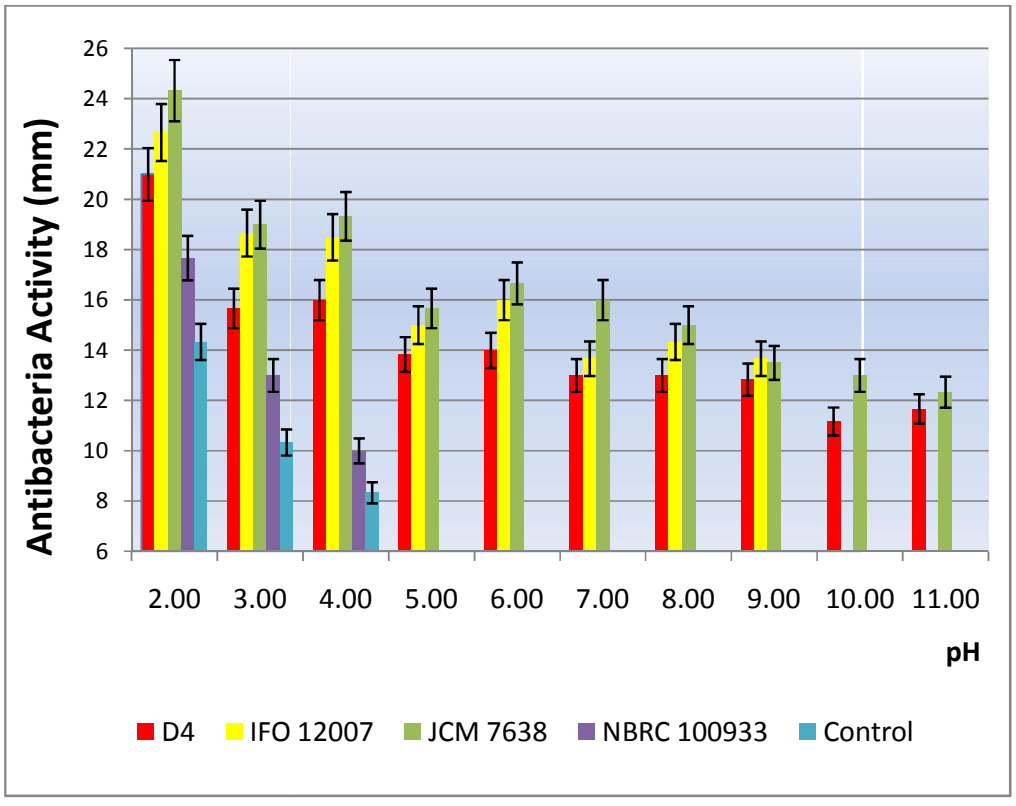


Figure 11: Effect of pH to antibacterial bacteriocin.

D. Effect of heat treatment on antibacterial activity

Impact heating and storage treatment on antibacterial activity on CFS D4 as target strain were evaluated by comparing with control strains (IFO 12007 and JCM 7638). The comparison was divided on two conditions, which are; pH unadjusted CFS and Neutralized CFS. As shown in Figure 12, the highest antimicrobial activity was showed on pH-unadjusted control and heat treatment at 65 °C for 30-minute. Meanwhile, the lowest antimicrobial activity founded neutral pH at seven days storage treatment.

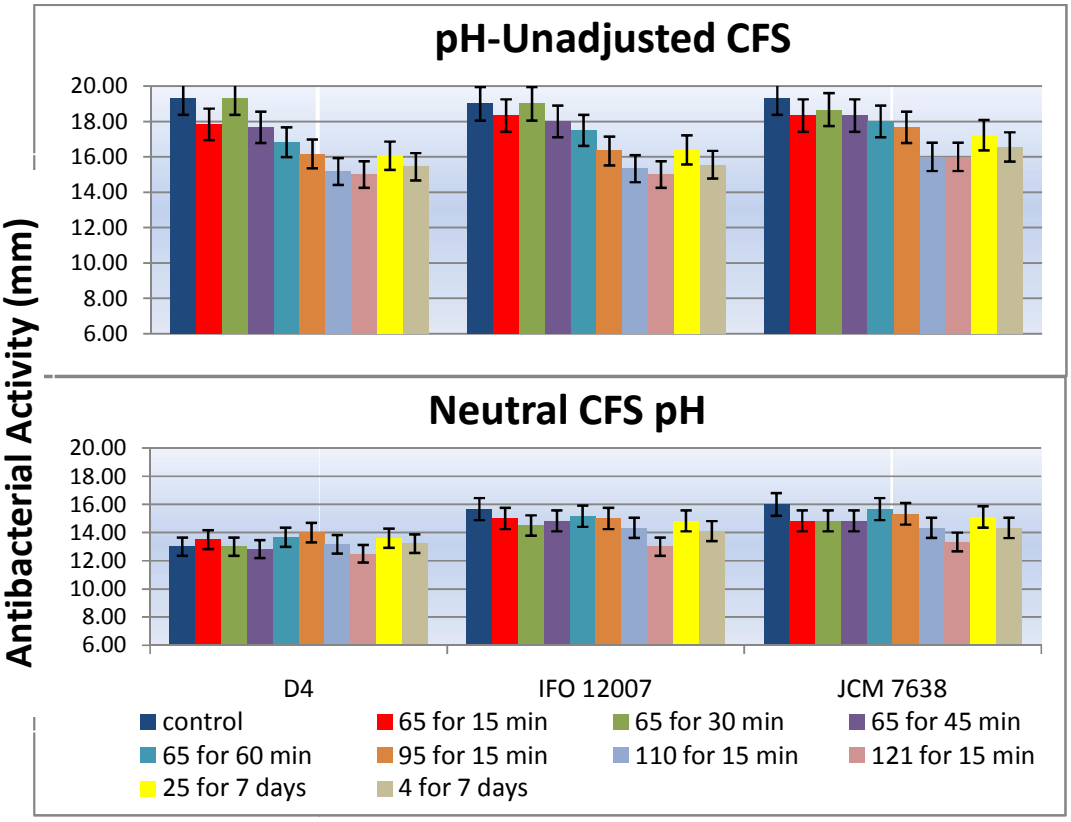


Figure 12: Effect of various temperatures to bacteriocin antibacterial activity.

For neutralized CFS, bacteriocin contains it relatively stable until days seven and even facing autoclaves temperature. Lactic acid in existence is the only reason for it. Heat stability gave advantage on bacteriocin, especially when it is used as a food preservative. In food processing field, the procedure of heating up is used to minimized spoilage bacteria especially from Gram-negative bacteria (Gálvez et al., 2007).

E. Effect of enzyme treatment

The bar chart in Figure 13 gives information about the effect of several digestive enzymes in some strains. On this study, the digestive enzymes were represented by trypsin, α–chymotrypsin, proteinase–K, catalase, and pepsin. From all enzymes tested, antibacterial activity only inactivated by Proteinase–K. Meanwhile, NBRC 100933^{T} as the non-bacteriocin producer does not show any antibacterial activity that corresponds to none inhibitory zone created. Another enzyme that has an effect on antibacterial activity showed on α – Chymotrypsin. It is proved by smaller inhibitory creation zone compare on untreated CFS.

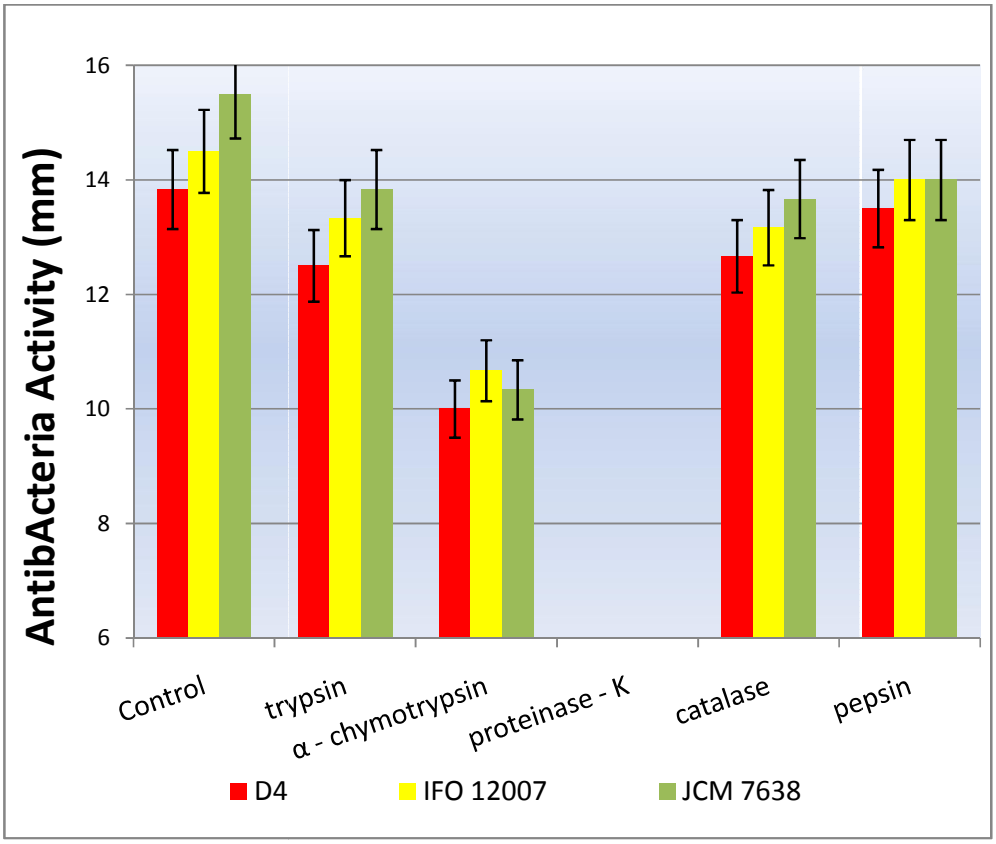


Figure 13: Effect of various enzymes to bacteriocin antibacterial activity.

F. Antibacterial spectrum assay

The inhibitory spectrum assay produced by *Lc. lactic* strain D4 was showing Table 4. Some 30 strains pathogen bacteria and food spoilage bacteria were used to analyze their spectrum. The species are *E. coli*, *Listeria*, *Staphylococcus*, *Lactobacillus*, *Enterococcus* and many others. The bacterium was not effective against Gram-negative bacteria like *E. coli*. These result show, bacteriocin production have broad spectrum bacterial growth inhibitory.

Antibacterial spectrum assay indicates that bacteriocin producer on dadiah has a wide range activity to inhibit spoilage and pathogen bacteria such as *Listeria, Staphylococcus, Lactobacillus, and Enterococcus*. Unfortunately, it only can inhibit most of Gram-positive bacteria but failed to inhibit negative bacteria like *E. coli*.

Table 4: Antimicrobial spectrum assay cell-free supernatant (CFS) of tested strains

Species	Strain	Clear Zone (mm ± SD) [A]			
		D4	IFO 12007	NBRC 100933^T	JCM 7638
Escherichia coli	JCM 1649^T	-	-	-	-
Listeria ivanovii subsp. *ivanovii*	JCM 7681^T	22.00 ± 0.00	20.00 ± 0.00	-	20.00 ± 1.00
Staphylococcus aureus subsp. *aureus*	JCM 20624^T	11.00 ± 0.00	-	-	9.00 ± 0.50
Bacillus cereus	JCM 2152^T	16.00 ± 1.00	8.33 ± 0.33	-	10.00 ± 0.00
Enterococcus faecalis	JCM 5803^T	8.33 ± 1.15	7.67 ± 0.33	-	8.83 ± 0.08
Enterococcus faecium	JCM 5804^T	9.33 ± 0.58	8.83 ± 0.08	-	9.83 ± 0.08
Lactococcus lactis subsp. *cremoris*	NBRC 100676^T	29.67 ± 0.58	22.33 ± 0.33	-	25.67 ± 0.33
Lactococcus lactis subsp. *hordniae*	NBRC 100931^T	-	23.00 ± 1.00	-	-
Leuconostoc mesenteroides subsp. *cremoris*	NBRC 107766^T	-	12.67 ± 0.33	-	-
Leuconostoc mesenteroides subsp. *dextranicum*	NBRC 100495^T	19.00 ± 0.00	21.00 ± 1.00	-	26.67 ± 0.33
Leuconostoc mesenteroides subsp. *mesenteroides*	NBRC 100496^T	-	14.00 ± 0.00	-	-
Leuconostoc lactis	JCM 6123^T	17.33 ± 0.58	16.00 ± 1.00	-	21.67 ± 0.33

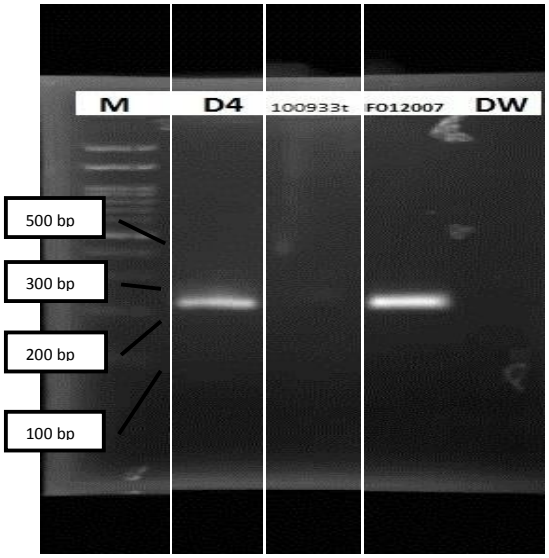
Pediococcus acidilactici	JCM 8797^T	10.00 ± 0.00	13.00 ± 0.00	-	12.33 ± 2.33
Pediococcus pentosaceus	JCM 5890^T	17.67 ± 0.58	14.67 ± 0.33	-	15.67 ± 0.33
Streptococcus thermophilus	JCM 17834^T	11.00 ± 0.00	9.67 ± 0.33	-	10.00 ± 1.00
Weissella confuse	JCM 1093^T	14.67 ± 0.58	13.33 ± 2.33	-	17.33 ± 0.33
Weissella viridescens	JCM 1174^T	13.33 ± 0.58	12.67 ± 0.33	-	15.33 ± 0.33
Lactobacillus helveticus	JCM 1120^T	17.33 ± 0.58	20.67 ± 0.33	-	19.67 ± 0.33
Lactobacillus delbrueckii subsp. *lactis*	JCM 1248^T	29.00 ± 0.00	25.33 ± 2.33	-	28.00 ± 1.00
Lactobacillus delbrueckii subsp. *delbrueckii*	ATCC 9649^T	18.67 ± 0.58	19.33 ± 4.33	-	19.33 ± 0.33
Lactobacillus reuteri	JCM 1112^T	9.33 ± 0.58	12.33 ± 0.33	-	11.33 ± 0.33
Lactobacillus fermentum	JCM 1173^T	13.67 ± 0.58	14.00 ± 1.00	-	14.67 ± 0.33
Lactobacillus rhamnosus	JCM 1136^T	21.00 ± 0.00	20.33 ± 0.33	-	21.67 ± 1.33
Lactobacillus casei	NBRC 15883^T	23.00 ± 0.00	21.33 ± 0.33	-	23.67 ± 0.33
Lactobacillus casei	JCM 11302	-	-	-	-
Lactobacillus paracasei subsp. *paracasei*	NBRC 15889^T	-	-	-	-
Lactobacillus salivarius	JCM 1231^T	12.67 ± 0.58	11.67 ± 1.33	-	14.67 ± 0.67
Lactobacillus brevis	NBRC 107147^T	16.33 ± 1.53	16.67 ± 2.33	-	12.00 ± 1.00
Lactobacillus plantarum subsp. *plantarum*	JCM 1149^T	17.33 ± 0.58	15.50 ± 0.75	-	15.33 ± 0.33
Lactobacillus sakei subsp. *sakei*	JCM 1157^T	28.67 ± 0.58	28.33 ± 0.33	-	28.33 ± 1.33

[A] Mean diameter (mm ± SD) of clear zone formed on agar well diffusion assay,
" - " no inhibitory zone,
JCM = Japan Collection of Microorganism
NBRC = NITE Biological Resource Center

G. Nisin production confirmation

PCR analysis using the published sequence of the nisin structural gene was used to prove bacteriocin production (Dodd et al., 1990). The result was shown in Figure 14. A 227 bp fragment was amplified from genomic DNA of *Lc. lactis* strain D4. This DNA was identical to *Lc. lactis* IFO 12007 as nisin A producer, except transfusion C position to A position at 148 bp. Same location also with 27^{th} amino acid residue as asparagines (AAT) instead histidine (CAT) (Taniguchi et al., 2010). This PCR analysis proved that *Lc. lactis* D4 were Nisin Z producer instead of Nisin A producer that was shown on *Lc. lactis* IFO 12007 showed the difference of amino acid sequence gives nisin Z greater solubility for application on food preservation (Gálvez et al., 2007).

A



B

	AAG	GAG	GCA	CTC	AAA	1 ATG Met	AGT Ser	ACA Thr	AAA Lys	GAT Asp	TTT Phe	AAC Asn	TTG Leu	GAT Asp	TTG Leu	**30**
31	GTA Val	TCT Ser	GTT Val	TCG Ser	AAG Lys	AAA Lys	GAT Asp	TCA Ser	GGT Gly	GCA Ala	TCA Ser	CCA Pro	CGC Arg	ATT Ile	ACA Thr	**75**
76	AGT Ser	ATT Ile	TCG Ser	CTA Leu	TGT Cys	ACA Thr	CCC Pro	GGT Gly	TGT Cys	AAA Lys	ACA Thr	GGA Gly	GCT Ala	CTG Leu	ATG Met	**120**
121	GGT Gly	TGT Cys	AAC Asn	ATG Met	AAA Lys	ACA Thr	GCA Ala	ACT Thr	TGT Thr	C AAT Asn	TGT Cys	AGT Ser	ATT Ile	CAC His	GTA Val	**165**
166	AGC Ser	AAA Lys	TAA End	CCA Pro	AAT Asn	CAA Arg	AGG Arg	ATA Ile	GTA Val	GGT Gly						

Figure 14: A. Agarose gel electrophoresis of PCR product within nisin-specific gene primer, with marker 100–3000 bp.

B. The nucleotide sequence of nisin Z isolated from D4 by using nisin-specific primer. At this sequence has shown exchanging histidine (CAT) on Nisin A to Asparagines (AAT) as nisin Z specific.

H. Estimation of peptide molecular weight

Molecular weight estimation of the peptide was conducted using in-situ antibacterial activity assay after SDS-PAGE has run. The band detection performed by Coomassie Brilliant Blue Staining (CBB Staining). Beside CBB staining, in situ assay also performed by put the gel down in the bottom of Petridis then pour indicator strain agar to observe clear zone was created (Fig. 15). Figure 15 shows SDS-PAGE 18% that laying down on the lower part of strain indicator agar media from the picture, inhibitory zone estimation was located around 3.5 kDa. Nisin was classified as lantiboitic Class I type A that has 2–4 kDa (Kaur, 2015). Furthermore, (Mitra et al., 2005) also detected nisin existence on milk fermented that has peptide molecule weight 3.5 kDa.

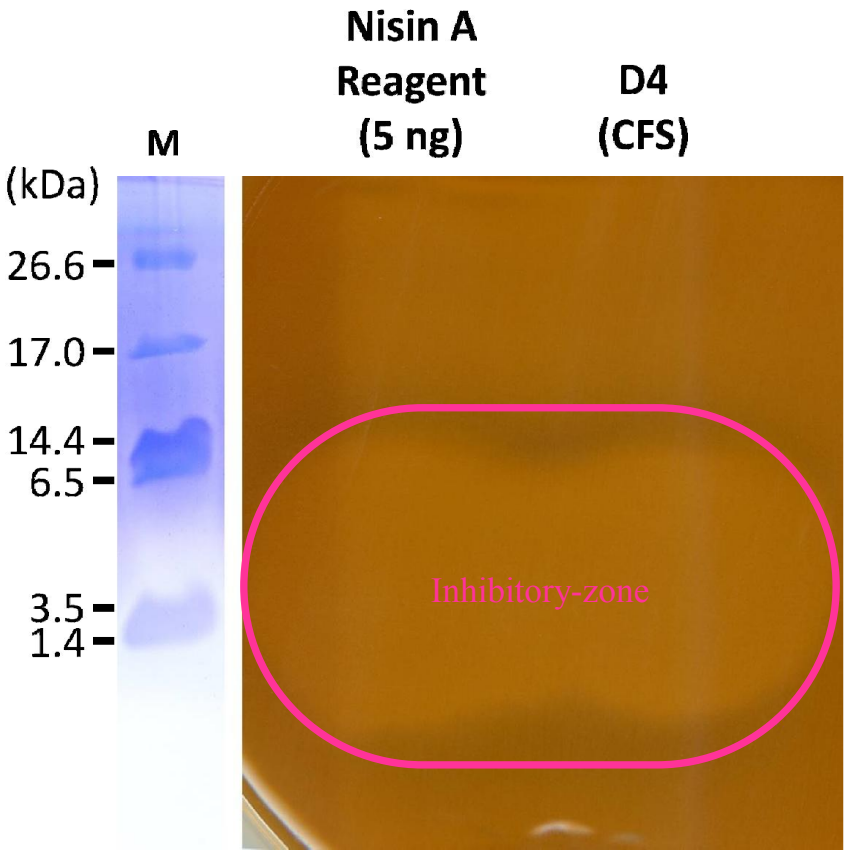


Figure 15: SDS-PAGE In situ assay analysis purification of nisin and commercial nisin. Inhibitory zone cell-free-supernatant appears around molecular weight 3.5 kDa of *Lc. lactis* D4 and commercial nisin. *Listeria monocytogenes* VTU 206 was used as indicator strain

4. DISCUSSION

One strain indicated bacteriocin-producer (strain D4) has identified as *Lc. lactis* subsp. *lactis* through 16S rRNA and API 50-CHL method. *Lc. lactis* subsp. *Lactis* also detected by Surono (2003), Mustopa and Fatimah (2014) and Nuraida (2015). *Lc. lactis* widely has used on dairy industrialized product almost entirely come from milk fermentation, plant-origin *Lc. lactis* strain isolated shown low-

quality performance. However, nowadays *Lc. lactis* also isolated from plant niche origin cause give several benefits like flavour-forming that hardly ever found on dairy basis (Rademaker et al, 2007).

At the present time, *Lc. lactis* can be isolated from various environmental, could be from plant or dairy based source. Usually *Lc. lactis* was isolated on industrial purposes of fermented dairy product like sour cream, soft and hard cheese and lactic casein. Strong evidence of the differences *Lc. lactis* isolated from plant and dairy basis was exposure by Siezen et al. (2008). According to Siezen et al (2008), *Lc. lactis* isolated from the plant has a wider range of carbohydrate substrates compare *Lc. lactis* dairy basis that has greater amino acid auxotrophies. These theory revealed an assumption the *Lc. lactis* strain D4 isolated from dadiah was from the plant instead of dairy raw material.

Moreover, van Hycklama Vlieg et al. (2006) supported by Siezen (2011) explained arabinose phenotypes is one of specific character on *Lc lactis* isolated from plant. The API 50 CHL confirmed arabinose existence in *Lc. lactis* strain D4 dadiah basis. These observable facts emphasize *Lc. lactis* strain D4 originates from plant (bamboo or banana leaf).

The CFS sample strain D4 was observing the effect to antibacterial of bacteriocin with respectively pH, heat and storage also digestive enzyme treatments. On the effect of various pH to antibacterial activity shown active compound produced by strain D4 has wide range pH condition. The antibacterial

active compound is still given away activity even at acid and alkaline condition.The Same pattern also claimed by Noonpakdee et al. (2002) who found nisin on Thai fermented sausage that has stability even in alkaline condition and Şanlibaba et al. (2009) which isolate nisin from Turkish raw dairy.

pH very related with nisin activity. The activity of the compound is dependent upon the pH of the solution. Documented, nisin solution has the greatest activity at pH 3-4 and the lowest activity noted on pH 11 (Davidson, Juneja and Branen, 2002). Furthermore, explained pH above that level the activity getting reduced and storage at neutral pH at room temperature decreasing activity occur happened.

In order to heat and storage treatment, Antibacterial *Lc. lactis* strain D4 shown highest activity on pH-unadjusted control and heat treatment at 65 °C for 30-minute. On the other hands, the lowest antimicrobial activity founded neutral pH at seven days storage treatment. This trend proofed that bacteriocin on CFS working synergically with lactic acid. Bacteriocin and lactic acid worked optimally at the beginning, then the activity sagging until days seventh.

Regarding effect of enzyme treatment, α–chymotrypsin, proteinase–K are two protease enzyme affected on antibacterial activity. Noonpakdee et al. (2002) and Şanlibaba et al. (2009) disclose the same pattern that the nisin are they isolated from Thai fermented sausage and Turkish fermented milk respectively have only affected by Proteinase-K and α – Chymotrypsin.

Based on Table 4 has seen nisin D4 had ability to inhibited 24 from 30 indicator spoilage and pathogen bacterias. It is only Gram-negative bacteria and some *lactococcus* and *lactobacillus* have nisin-resistance features. As known, nisin has wide broad spectrum in order to bacterial inhibiting Gram-positive bacteria growth (Hansen, 1994; Field et al., 2008; Molloy et al., 2013; Katharopoulos, Touloupi and Touraki, 2016). Nisin only effects on Gram-positive bacteria but not effects on Gram-negative because of differences of cell wall structure on both of type bacteria. Gram-positive cell walls composed of peptidoglycan. On the other hand, the outer membrane of Gram-negative bacteria enamelled with lipopolysaccharide that disturbing nisin binding on cell wall bacteria (Davidson, Juneja and Branen, 2002).

Mierau and Kleerebezem (2005) and Islam et al. (2012) describe nisin mechanism on inhibiting Gram-positive bacteria by binding Lipid II as precursor polymer biosynthesis peptidoglycan. Continued by (Draper, Cotter, Hill and Ross, 2015), peptidoglycan as a polymer of reiterate N-acetylmuramic (MurNAc) and GlcNAc motif utilize lipid II as a substrate in order to cross-linking (transpeptidation) peptidoglycan cell wall composition. In addition, by nisin existence has binding lipid II to interfere peptidoglycan biosynthesis.

Davidson, Juneja and Branen (2002) explained that nisin during storage has variable stability, especially at neutral pH. Additionally, in Swiss cheese, nisin has degradation rapidly after 8 days and after 20 days very little amount of nisin was detected. Nisin degradation also reported in meat slurries and pasteurized

process cheese spread at elevated temperature storage (Delves and Broughton, 1996).

On Table 4 also shown some Gram-positive bacteria like *Lactococci, Lactobacilli* and *Leuconostoc* have nisin resistance. The nisin-resistance-substance called nisinase. Anti-nisin has detected first time since 1967 in extract of *bacilli* and endospores organism. Further investigation nisinase also isolated from some *Bacillus* sp., *Lactobacilli, Streptococci, Clostridium, Lactococcus, Enterococcus,* and *Staphylococcus* (Davidson, Juneja, Branen, 2002; Draper et al., 2015). Beside nisinase metabolite as a reason for nisin resistance, nisin-producer-strains also had reported have immunity proteins to protect cell from nisin (Mantovani and Russell, 2001).

Regarding nisin-producing strain confirmation, PCR analysis revealed *Lc. lactis* subsp. *lactis* D4 is nisin Z producer. Figure 11 shown 227 bp fragment was amplified. Since there is the difference at amino acid residue on position 27th which is histidine (CAT) transformed into aspargine (AAT) indicating D4 is nisin Z producer. Nisin consists of 34 amino acids sequence. The first nisin type discovered is nisin A on 1928, right three years after the first bacteriocin (colicin) has exposed (Davidson, Juneja and Branen, 2002). Since then, various nisin had been explored. One of nisin A variant is nisin Z that first isolated from *Lc. lactis* NIZO-22186 from a dairy product. The difference is just one amino acid in the final active peptide His27Asn, C-to-A transversion right on position 148

(Noonpakdee et al., 2002; Piper, Hill, Cotter and Ross, 2011). Beside PCR analysis, SDS-PAGE in-situ assay proved the size of nisinZ produced by *Lc. lactis* strain D4 less than 4 kDa. The Same size also shown by Piper et al. (2011) and Zendo et al. (2008) exposed nisin molecular weigh on 3.3 kDa.

Other nisinA variants are nisin Q and nisin F. First-time nisin Q produce by *Lc. lactis* strain 61-14 was isolated from the river in Japan that has two additional variations on two amino acid residue at spot Ala15Val and Met21Leu (Zendo et al, 2003). Meanwhile, variant nisin F was fabricated by *Lc.lactis* strain F10 isolated from Catfish in South Africa has different on two amino acid residue at position His27Asn and Ile30Val (De Kwaadsteniet, Doeschate, and Dicks, 2008).

5. CONCLUSIONS

One strain in dadiah has been indicated as Bacteriocin-producer. 16S-rRNA sequence and API 50-CHL confirmed 100% identically *Lc. lactis* subsp. *lactis*. Characteristics studies have revealed that the bacteriocin is heat stable even at an autoclaving temperature (121 °C for 15 minutes) and still active over seven days depository on temperatures 4 °C and 25 °C also was active in the wide range of pH (2.0-11.0). Bacteriocin could be completely inactivated by proteinase-K. SDS-PAGE discovered the bacteriocin had molecular weight around 3.5 kDa. The anti-bacterial wide spectrum showed wide spectrum on inhibiting strains

foodborne pathogens and food spoilage bacteria. Gene encoding was amplified by Polymerase Chain Reaction (PCR) with Nisin gene-specific primer definite as nisin Z producer.

IV. SUMMARY

Dadiah is Indonesian traditional fermented milk (yoghurt-like product) that is consumed in West Sumatra, Indonesia. Milk is safe to consume after pasteurization, but dadiah is produced by pouring fresh, raw, unheated (unpasteurized) buffalo milk into bamboo tubes covered with banana leaves. The bamboo tube is incubated at room temperature (28–32 ℃) for two days, allowing it to ferment spontaneously. The most common lactic acid bacteria genera in raw dairy milk are *Lactococcus*, *Lactobacillus*, *Leuconostoc*, and *Enterococcus*, with *Lactobacillus*, *Lactococcus*, and *Leuconostoc* also being dominant in dadiah.

In this study, deep sequencing of 16S ribosomal RNA genes extracted from 11 dadiah samples and analyzed the dadiah microbiota at the genus level had performed. Expectedly that *Lactococcus*, *Lactobacillus*, and *Leuconostoc* were predominant among the dadiah microbiota. Interestingly, *Klebsiella* and *Chryseobacterium*, potential pathogens, were also found in some of the dadiah samples. There were differences between the dadiah microbiota from different bamboo tubes, even those collected from the same sampling area, and large differences between sampling areas (different buffalo milk sources). These fluctuate microbe composition has led to a variation of the bioactive compound on dadiah. It is necessary to develop beneficial bacteria isolated from dadiah as a starter culture in order to similarity valuable bioactive compound on dadiah.

Six colonies were isolated from dadiah in this study and indicated as bacteriocin-producing with agar-well diffusion assays of culture supernatant against indicator strains, *Listeria monocytogenes* VTU 206. All six colonies were identified as *Lc. lactis* subsp. *lactis* using the API 50 CHL test and 16S rRNA gene sequencing. The genetic characteristic through rep-PCR indicated selected colonies belong on one strain only that represent by Strain D4. The antibacterial activity culture supernatant was comparatively stable between pH 2.0–11.0 and resistant to heating (121 °C, 15 min) and storage (25 °C, 7 days). In addition, the activity was resistant to some enzymatic treatments such as catalase, pepsin and trypsin. However, it decreased and completely disappeared by digestion with α-chymotrypsin and Proteinase-K, respectively.

The culture supernatant also inhibited the growth of 24 strains among 30 tested strains of food borne pathogens, food spoilage bacteria and lactic acid bacteria. The stain D4 genome was paired-end sequenced using Illumina's MiSeq platform. The total length of the draft genome was 2.5 Mbp. The genome had a G+C content of 34.6% and contained 2,378 predicted protein-coding genes (ORF's). The strain D4 was shown to have the nisin Z structural genes by PCR and genome sequencing. This is the first report showing the isolation of a nisin-producer from dadiah.

Lc. lactis strain D4 has proofed producing nisinZ as an antibacterial activity that isolated on dadiah. This strain has an opportunity in order to use as

starter culture on dadiah manufacturing. By spread *Lc. lactis* D4 strain among dadiah producer (no longer only found in Batusangkar area), it will increase bargaining position on dadiah as well as generalizing antibacterial composition on dadiah produce in Indonesia, especially in West-Sumatera.

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It doesn’t matter how many times you fell down, what the matter is how many time you rise and keep struggling. Keep moving forward, even you have to crawling.

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