Antimicrobial potential of *Pediococcus acidilactici* ~~PB22~~ from Bekasam, fermentation of sepat rawa fish (*Tricopodustrichopterus)* from Banyuasin, Sumatera Selatan, Indonesia

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Abstract. This study aimed to determine the antimicrobial potential of lactic acid bacteria isolated from bekasam. Bekasam is a result of Sepat Rawa fermentation from Banyuasin District, South Sumatra, Indonesia. The results showed that the morphological and biochemical properties of lactic acid bacteria were Gram positive and cocci, negative catalase and included in homofermentative groups. The biggest antimicrobial activity was shown by Bekasam isolate to *Escherichia coli* O157: H7 (21.26 mm), followed by *Staphylococcus aureus* ATCC25923 (18.23 mm) and *Listeria monocytogenes* CFSAN004330 (5.10 mm), while diameter barriers for crude bacteriosin supernantant isolates lactic acid bacteria to *Escherichia coli* O157: H7, *Staphylococcus aureus* ATCC25923 were 14.99 mm, 17.69 mm, and *Listeria monocytogenes* CFSAN004330 had no antimicrobial activity at neutral pH. The results of molecular identification with 16S rRNA showed that lactic acid bacteria isolated from Bekasam (Bk Isolate) was *Pediococcus acidilactici* strain PB22 that has antimicrobial potential against pathogenic bacteria.

Keywords: bekasam, fermented fish, lactic acid bacteria, Sepat Rawa, antibacterial activity ~~16S rRNA~~

Running title: Antimicrobial potential of *Pediococcus acidilactici*

INTRODUCTION

Traditional fermented natural food is very diverse in Indonesia because the territory of Indonesia is very wide and has distinctive food characteristics for each region one of them is curd. Curd is a natural fermentation of buffalo milk from West Sumatra that is beneficial for health (Surono, 2003) and contains several types of lactic acid bacteria (Venema and Surono, 2019). *Lactococcus lactis ssp. Lactis, Lactobacillus plantarum ssp. Plantarum, L. lactis ssp. cremoris, Pediococcus pentosaceus* and *Lactobacillus pentosus* are some types of bacteria that are naturally found in curd (Wirawati *et al*. (2019). Tempoyak, a natural fermentation product from Durian, is also a natural fermentation product (Juliyarsi *et al*. (2018) and tempoyak also contains lactic acid bacteria that have the potential as probiotics (Hartini et al., 2019 and Ahmad et al. 2018). In addition, there are also naturally occurring fish fermented products called Budu originating from West Sumatra. Budu contains Bacillus and Micrococcus (*Bacillus sphaericus, Bacillus polymyxa, Bacillus cereus, Bacillus pantothenticus and Micrococcus lactis*) (Yusra et al. 2014), and *lactic acid bacteria* that have the potential as antimicrobial Bacillus cereus strain HVR22 (Yusra et al. (2013). All natural fermentation product contain lactic acid bacteria that are very beneficial for health.

Bekasam is a traditional food originating from several regions in Indonesia such as Java, South Sumatra and South Kalimantan. Bekasam is the result of spontaneous fermentation of fish. According to Desniar *et al*. (2013), Bekasam is used as a processed fish product by fermentation that tastes sour. Fish that can be used as exam is the type of freshwater fish. The raw material in the form of cork fish, betam, siam and swamp spikes with the addition of salt about 15-20%, and added 15% sangria rice, then fermented for about one week to produce a distinctive aroma and taste.

There are several previous studies about the content of lactic acid bacteria in Bekasam. Wikandari *et al* (2012), found lactic acid bacteria that have proetolytic activity namely *L. plantarum* B765, *L. plantarum* T2565, *L. plantarum* N2352, *L. plantarum* B1465, *L. pentosus* B2555, and *Pediococcus pentosaceus* B1666. Desniar *et al*. (2013), in their study revealed the presence of antimicrobial activity of lactic acid bacteria isolates to *Staphylococcus aureus*, that was caused by the ability of organic acids as antibacterial compounds. Then Afriani *et al*. (2015) isolated lactic acid bacteria of Bekasam from Jambi, that also had proteolytic activity, namely *Lactobacillus pentosus* BS15, *Lactobacillus plantarum* 1 BS22 and *Lactobacillus plantarum* 1 BL12. Melia, et *al*. (2018), tested the antibacterial activity of lactic acid bacteria Bekasan against *Staphylococcus aureus* ATCC 25923, *Escherechia coli* ATCC 25922 and *Salmonella sp*. However, this research

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has not been observed many. This study will evaluate ~~see~~ the potential of lactic acid bacteria from the fermented Sepat Rawa fish that is a typical type of fish used in the Banyuasin region, which has antimicrobial activity and is a potential source bacteriocin, which can later be used as probiotic and biopreservative.

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MATERIALS AND METHODS

Bekasam Processing

The process of making Bekasam is very simple. The ingredients consist of fish, rice, and salt. The fish was cleaned, then added salt and rice sufficiently, put in a bottle and closed tightly. The bottle was stored at room temperature for 3 days (Figure 1).

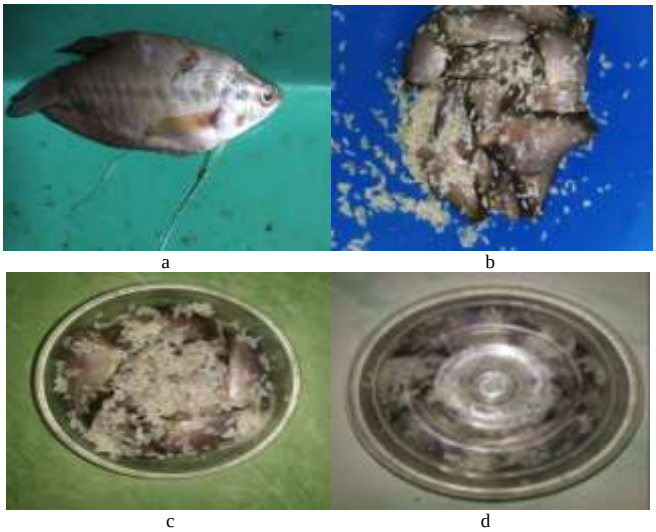

Figure 1. Bekasam Processing (a). Clean sepat fish scales, (b). Give salt and rice, (c). Store in jar bottles (d). Bekasam is fermented for 3 days at room temperature.

Sampling

The material of this study was 4 Bekasam samples originating from Sepat Rawa fermented fish obtained from 4 producers from Banyuasin, South Sumatra, Indonesia (Figure 2), namely Pulau Harapan Village (Producer 1), Mainan Village (Producer 2), Sei Rengit Village (Producer 3) and Santan Sari Village (Producer 4).

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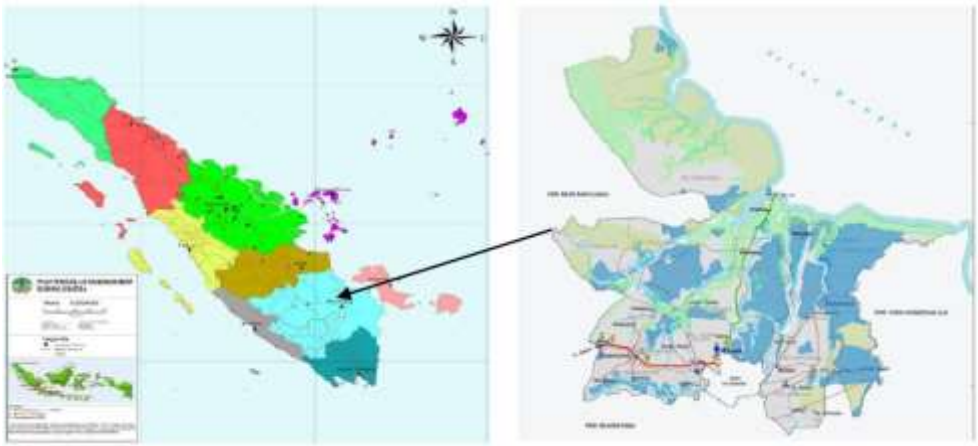

Figure 2. Study area in Banyuasi, South Sumatera, Indonesia (1,3° - 4°S, 103° - 105° E.)

Isolation and Identification of Lactic Acid Bacteria

The isolate of lactic acid bacteria from Bekasam was cultured in broth De Man Rogosa Sharpe (MRS) media (Merck, Germany) and planted in MRS agar media (Merck, Germany) that was incubated at 37^{o}C in an anaerobic jar for 48 hours. Furthermore, morphological properties (shape and color) were observed, and biochemical properties (Gram staining, catalase test and fermentation type) Phikunthong and Yunchalard (2010).

Antimicrobial Activity Test

Modification of the Yang *et al.*, (2012) method was used for antimicrobial activity test against pathogens. The method used was well diffusion assay. In short, cell free supernatants were obtained from lactic acid bacteria grown in MRS Broth, for 24 hours at 37 °C, anaerobic conditions and centrifuged at 10,000 rpm, 5 minutes at 4°C. A 50 µl supernatant was inserted into a well (6 mm) that was perforated with a cock borer. Previously Nutrient Agar (Merck, Germany) has been grown by pathogenic bacteria. As a control it was compared to antibiotics (penicillin 10µg, kanamycin 30µg, ampicillin 10µg). The clear zone formed can be read after 24 hours.

Antimicrobial Test of Crude Bacteriocin Supernantant

One ml of culture was incubated for 24 hours in 9 ml MRS broth for 24 hours at 37 °C. Then centrifuged at 14,000 rpm for 5 minutes. The supernatant was filtered with a 0.22 µl membrane filter. Cell-free supernatant was regulated with pH 6.5 with 1 N NaOH to eliminate the effect of barriers because of the presence of organic acids (Yang et al., 2012). Pathogenic bacteria were grown aerobically at 37^{o}C for 24 hours. Then the pathogenic bacterial culture was 0.2% into 20 ml of MHA at 50 ^{o}C. After agar became solid, well was with a size of 6 mm using the corek borer. Then the supernatant was taken as much as 50 µl and inserted into each well and allowed to stand for 15-20 minutes, then incubated for 24 hours at 37^{o}C in aerobic conditions. The inhibitory zone was measured by using the caliper. If the well is found, the inhibition zone can be said to be BAL isolate that contains bacteriocin compounds.

DNA Genomes Isolation of Lactic Acid Bacteria and 16S rRNA

Single colony lactic acid bacterial isolates from MRS Broth were piped as much as 1000 µl and included in the new eppendorf. Centrifuged as 14000 rpm for 2 minutes. Then the supernatant is removed and the pellet is taken. Added with 480µl 50mM EDTA. Then, 120µl of Lysozyme were added. Next, Incubation in 37ºC water bath for 60 minutes. Centrifuge for 2 minutes 14000 rpm, then remove the supernatant and pellet is taken. Added with 600 µl nuclei lysis solution. Incubated 80ºC for 5 minutes, then let it stand at room temperature. Added 3µl of RNase Solution, incubated in waterbath 37ºC for 60 minutes. Added with 200 µl of the protein precipitation solution then vortex. 600 µl of isopropanol was added. Centrifuged for 2 minutes 14000 rpm, then pellets are taken and the supernatant is removed. Added 600 µl of ethanol 70% and then homogenized. Centrifuged for 2 minutes 14000 rpm, then pellets were taken and the supernatant was removed. Pellet DNA rehydration by adding 10 - 100 µl of Rehydration solution for 30 minutes at 65ºC. Primer R (16S-1492R, Tm 47°C, 5'-GTT TAC CTT GTT ACT ACT-3') and F (16S-27F, Tm 54.3 °C, 5'-AGA GTT TGA TCC TGG CTC AG-3'), prepared (concentration of 10 pM). Take 90µl dH2O + 10µl (Primary R and F). (Primary R and F in TE buffer (concentration 100 µM). Cocktail PCR in1 eppendorf (Master Mix 12.5 µl, Primary F 1 µl, Primary R 1 µl, Template DNA 1 µl, ddH2O 9.5 µl), with PCR denaturation 95°C 45 seconds, annex 56°C 45 seconds, Extention 72°C 1 minutes 40 seconds, final extention72°C 10 minutes. Electrophoresis of 10 µl samples into the well agar, inserted 4 µl of the DNA ladder. Set to 100 V for 45 minutes. The gel placed in a container plus TBE until submerged. The gel, then seen under the UV lamp. The 16S rRNA gene sequences of the isolate were submitted to the NCBI for a BLAST search. The MEGA version 6.0 (http://www.megasoftware.net) was used to create phylogenetic trees using the neighbor joining (NJ) method.

RESULTS AND DISCUSSION

Morphological and biochemical characteristics of lactic acid bacteria isolate

Fifty-six isolates of lactic acid bacteria were isolated from bekasam, morphologically round (cocci), rod shape, and cream colored. The testing of biochemical properties showed that the results of Gram positive and negative catalog as well as isolated of lactic acid bacteria were homofermentative as indicated by the absence of gas bubbles in the Durham tube. Mumtianah *et.al* (2014) showed that lactic acid bacteria isolates from Bekasam were gram-positive, non-motile, negative catalase, positively acid-forming and had proteolytic activity.

Desniar *et al.* (2013), also stated that in general the LAB isolates from the bekasam were homofermentative. Chandra *et al.* (2007) and Irianto (2007) states that bacterial isolates from Bekasam consists of Gram positive cocci and Gram positive Bacilli.

Antimicrobial Activity of Lactic Acid Bacteria Isolate

Of the fifty-six isolates of lactic acid bacteria, there were 4 isolates that had antimicrobial activity, but only Bk isolates had antimicrobial activity against the three tested pathogenic bacteria, *Escherichia coli* O157: H7, *Staphylococcus aureus*

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ATCC25923 and *Listeria monocytogenes* CFSAN004330. *Antimicrobial activity* of Bk isolates from bekasam on pathogenic bacteria, *Escherichia coli* O157: H7, *Staphylococcus aureus* ATCC25923 and *Listeria monocytogenes* CFSAN004330 can be seen in Table 1. Antibiotics used positive controls (Penicillin 10µg, Ampicilin 10µg and Kanamycin 30µg).

Table 1. Antimicrobial activity Bk Isolate and antibiotic test

Inhibitory source	**Clear zone (mm)**		
	E. coli O157: H7	***S. aureus* ATCC 25923**	***L. monocytogenes* CFSAN004330**
Isolate LAB	21.26 ± 0.03	18.23 ± 0.01	5.10 ± 0.01
Penicillin10µg	2.70 ± 0.03	-	-
Ampicillin10µg	14.19 ± 0.05	21.26 ± 0.02	-
Kanamycin 30µg	16.21 ± 0.09	13.18 ±0.05	10.15 ±0.08

The value is expressed as the mean ± standard deviation; n=3

In Table 1, the largest antimicrobial activity was shown by Bk isolate against *E. coli* O157: H7, with a clear zone diameter of 21.26 mm followed by *S. aureus* ATCC 25923 18.23 mm and *L. monocytogenes* CFSAN004330 5.10 mm. LAB isolate, had greater antimicrobial activity against *E. coli* O157: H7 than the three antibiotics used, namely penicillin 10 µg, kanamycin 30 µg, ampicillin 10 µg. However, as a whole in Figure 3, it can be seen that Bk isolate from the stain has antimicrobial activity against all pathogenic bacteria, compared with penicillin which does not inhibit growth of *S. aureus* ATCC 25923 and *L. monocytogenes* CFSAN004330 and kanamycin had no antimicrobial activity to *L. monocytogenes* CFSAN004330. Desniar (2012) states that the antimicrobial test against the BALs of tilapia extracts has the ability to inhibit five types of pathogenic bacteria: *E. coli*, *S. typimurium* ATCC 14028, *Bacillus aureus*, *S. aureus* and *L. monocytogenes*. The results showed that inhibited zones on pathogenic bacteria had high antimicrobial activity. Pan *et al* (2009) states that the diameter of the inhibited zone against 0-3 mm pathogenic bacteria showed low antimicrobial activity that is > 3-6 mm medium antimicrobial activity and > 6 mm had high antimicrobial activity.

The same result is also shown by Saithong *et al.* (2010), using *L. reuteri* IFRDP P17 in Plaa-som, a typical Thai fish fermentation product, capable of suppressing growth, but in contrast with Desniar *et al.* (2013), which states that isolate LAB from exteriors from Indralaya, Ogan Komiring Ilir (South Sumatera) and Indramayu (West Java), the largest activity of lactic acid bacteria antimicrobials was against *S. aureus*. Lactic acid bacteria werethe dominant bacteria found in fermented fish products (Wikandari *et al.*, 2011, Olympia *et al.*, 1992 and Ostergaard *et al.*, *1*998). Mumtianah *et al.* (2014), states that lactic acid bacteria are bacteria that can produce organic acids, lactic acid and acetic acid that also play a role in inhibiting the growth of bacterial rot. Melia, *et al.* (2017) states that adding lactic acid bacteria is able to inhibit *L. monocytogenes*. It can also inhibit *S. aureus* ATCC 25923 (Melia, *et al.*, 2018). The main role of lactic acid bacteria is to ferment carbohydrates that produce organic acids, which can lead to a decrease in pH. The low pH and presence of organic acids, the main is lactic acid, is a major factor in the process of preservation in fermented fish products. Generally, pH between 4.5 - 5.0 can inhibit pathogenic bacteria and decomposers (Owen and Mendoza, 1985). Organic acid produced by Lactic Acid Bacteria has antibacterial activity (Theron and Ludes, 2011).

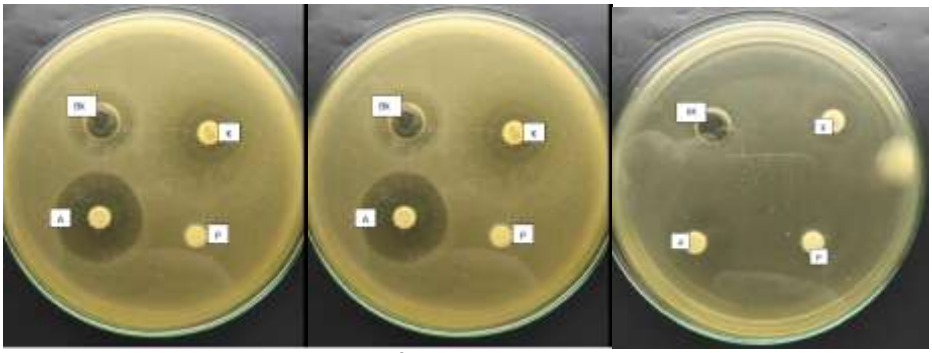

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Figure 3. Antimicrobial activity of Bk isolate to *E. coli* O157: H7 (A), *S. aureus* ATCC 25923 (B) and *L. monocytogenes* CFSAN004330 (C). (Description: Bk = Isolate LAB Bekasam, A = Ampicilin, K = Kanamycin and P = penicillin)

Antimicrobial Activity of Crude Bacteriocin Supernantant

The measurement of antimicrobial activity of isolates Bk crude bacteriocin supernatant was obtained after neutralizing pH in the supernatant of lactic acid bacteria, so that the antimicrobial activity of organic acid was not present. According to Palludan-Muller *et al.* (2002), components of organic acids, especially lactic acid is the main components of the antimicrobial compounds of lactic acid bacteria. The results of the study can be seen in Tables 2 and 3 that showed antimicrobial activity after pH of the lactic acid bacterial supernatant was neutralized to the *E. coli* O157: H7 wasie

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14.99 mm and *S. aureus* ATCC25923 that was 17.69 mm, but defention activities were not shown to *L. mMonocytogenes* CFSAN004330.

The result was higher than Melia, et *al.* (2018) study. In their study, the crude bacteriosin LAB isolates activity from Bekasam was against *S. aureus* ATCC 25923 (13.1 mm) and *E. coli* ATCC 25922 (12.7 mm) (Figure 4a and Figure 4b). Whereas in the Desniar *et al.*(2013), LAB isolates from Bekasam did not have antimicrobial activity after supernatant pH was neutralized so that it was thought, antimicrobial activity originated from organic acids produced by lactic acid bacteria. Furthermore Desniar *et al.* (2016) isolated *L. plantarum NS* (9) from Bekasam Tilapia Atin that produced antibacterial activity from organic acids. The highest antibacterial activity against E. *coli*, *B. cereus* and *L. monocytogenes* at the end of the exponential growth phase (12-15 hour incubation) while *S. aureus* and *S. tTyphimurium* ATCC 14028 on the 21st and 24th incubation hours.

Furthermore, Fall et al. (2018), revealed that the antimicrobial activity of supernatant cell free culture from *Lactobacillus plantarum* and *L. brevis* isolated from fermented fish meat (guedj) in Senegal was able to inhibit *E. coli* and *L. monocytogenes*. Srionnual et al. (2007), found that Weissellicin 110, a class II bacteriocin produced by Weissellicin 110 isolated from Pla-som was able to inhibit gram-positive bacteria, but did not have antimicrobial activity against *Listeria monocytogenes.* Nurhikmayani et al. (2010), crude bacteriocin from lactic acid bacteria was isolated from Chao, against *S. aureus* FNCC0047 and *E. coli* FNCC0049.

According to Islam *et al.* (2012), there are several mechanisms to inhibit the destruction of target cells by bacteriocins. Basically inhibiting the formation of lipids II (cell wall precursors) can be done by following mechanism L a. Inhibition of cell wall biosynthesis, b. Stabilize the formation of membrane target pores. Added by Bahar, and Ren (2013), and Song and Zheng (2015)that when the peptide attaches the target cell membrane, the positive end of the peptide will bind the fatty acids in the fospolipid layer on the target bacterial membrane. This stage involves binding the peptide with a membrane like a monomer, so that separation occurs that leads to the formation of pores, ultimately causing death in the cell.

Table 2. Antimicrobial Activity of Bk Isolate Crude Bacteriocin Supernatant

Pathogenic bacteria	Diameter clear zone (mm)
E. cColi O157: H7	14.99 ± 0.03
S. aureus ATCC 25923	17.69 ± 0.01
L. mMonocytogenes CFSAN004330	-

The value is expressed as the mean ± standard deviation; n=3

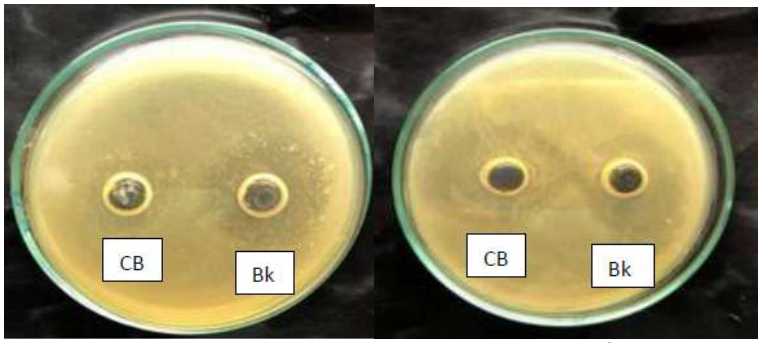

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Figure 4. Antimicrobial activity of crude bacteriocin after neutral pH (CB) and before neutral pH (Bk) ~~to~~ against *E. cColi* O157: H7 (4a), ~~and Figure 4b. Antimicrobial activity of crude bacteriocin after neutral pH (CB) and before neutral pH (Bk) to~~ against *S. aureus* ATCC 25923 (4b).

Results of 16S rRNA gene amplification by PCR

At Figure 5, it can be seen that the amplification of the area of the 16S rRNA gene isolates lactic acid bacteria from Bekasam. It can be seen by the appearance of PCR fragment of size 1542 bp using R Primer– (16S-1492R, Tm 47 °-C, 5'-GTT TAC CTT GTT ACT ACT-3´) and F (16S-27F, Tm 54.3 °-C, 5-'-AGA GTT TGA TGCC CTC AG-3´). Phylogenetic trees based on 16S rRNA gene sequence analysis can be seen in Figure 6. Sequencing results of Bk isolates compared to Gene Bank data using the BLAST program on the NCBI website (http://www.ncbi.nlm.nih.gov) showed a similarity rate of 99% with PB22 strain *Pediococcus acidilactici*, so it can be concluded that the lactic acid bacteria isolate from Bekasam (Bk Isolate) is *Pediococcus acidilactici* strain PB22. This isolate lactic acid bacteria is a new strain found in Bekasam or other fermented fish.

Rinto *et al.* (2015), isolated *Lactobacillus acidophilus* and *Lactobacillus delbruckii sp* acid bacteria. Delbruckii using API 50CH and API 50CHL, as statin producers. Afriani *et al.* (2015), isolated lactic acid bacteria from Bekasam from Jambi, which also has proteolytic activity, namely *Lactobacillus pentosus* BS15, *Lactobacillus plantarum* 1 BS22 and

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(9) (PDF) Characterization of lactic acid bacteria isolated from an Indonesian fermented fish (bekasam) and their antimicrobial activity against pathogenic bacteria. Available from: https://www.researchgate.net/publication/2338718_Characterization_of_lactic_acid_bacia_isolated_from_an_Indonesian_fermented_h_bekasam_and_their_antimicrobial_activit

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Lactobacillus plantarum 1 BL12. —Nurhikmayani et al. (2019), found *Lactobacillus plantarum* and *Pediococcus pentosaceus* from Chao, fermented fish from South Sulawesi using 16S rRNA.

In addition there are also several sources of lactic acid bacteria from Thai Plaa-som fermented fish products such as *Pediococcus pentosaceus, Lactobacillus alimentarius / farciminis, Weisella confusa, L. plantarum* and *Lactococcus garviae* from Plaa-som, fermented fish products from local producers in Songkhla province, Southern Thailand (Paludan-Mu¨ller et al., 2002), *Lactococcus garvieae, Streptococcus bovis, Weissella cibaria, Pediococcus pentosaceus, Lactobacillus plantarum,* and *Lactobacillus fermentum* (Kopermsub and Yunchalard, 2010), *Lb. plantarum* and *Pediococcus pentosaceous* (Nicomarat et al., 2018).

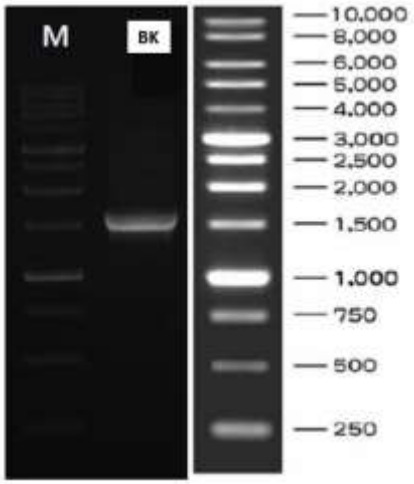

Figure 5. PCR electrophoresis product of lactic acid bacteria isolates from Bekasam (Bk) from Banyuasin, South Sumatra, (M = 1 kB DNA Ladder).

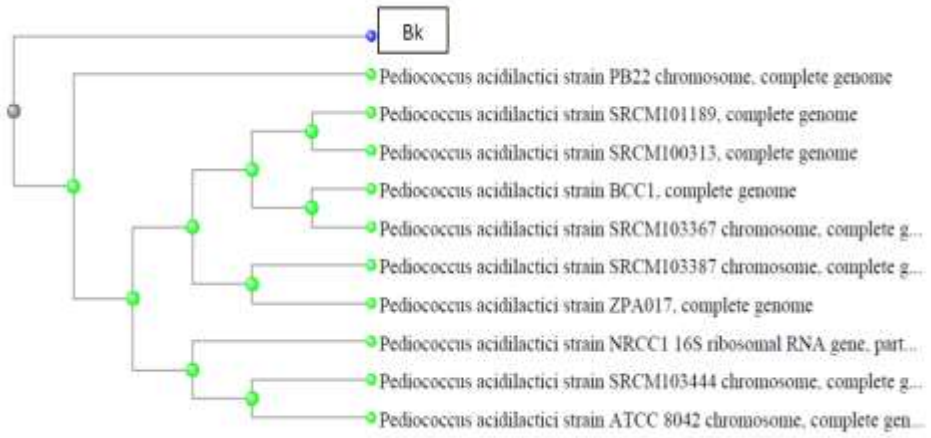

Figure 6. Phylogenetic isolate of lactic acid from Bekasam (Bk)

In Summary, the results of molecular identification with 16S rRNA showed the potential lactic acid bacteria (Bk isolate) as an antimicrobial isolation from Bekasam from South Sumatra Banyuasin was *Pediococcus acidilactici* strain PB22. *Pediococcus acidilactici* strain PB22, is the new strain found in Bekasam. Furthermore, *Pediococcus acidilactici* strain ~~PB22~~ can later be used as probiotics and biopreservatives.

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ACKNOWLEDGEMENTS

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Antimicrobial potential of *Pediococcus acidilactici* ~~PB22~~ from Bekasam, fermentation of sepat rawa fish (*Tricopodustrichopterus)* from Banyuasin, Sumatera Selatan, Indonesia

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Abstract. This study aimed to determine the antimicrobial potential of lactic acid bacteria isolated from bekasam. Bekasam is a result of Sepat Rawa fermentation from Banyuasin District, South Sumatra, Indonesia. The results showed that the morphological and biochemical properties of lactic acid bacteria were Gram positive and cocci, negative catalase and included in homofermentative groups. The biggest antimicrobial activity was shown by Bekasam isolate to *Escherichia coli* O157: H7 (21.26 mm), followed by *Staphylococcus aureus* ATCC25923 (18.23 mm) and *Listeria monocytogenes* CFSAN004330 (5.10 mm), while diameter barriers for crude bacteriosin supernantant isolates lactic acid bacteria to *Escherichia coli* O157: H7, *Staphylococcus aureus* ATCC25923 were 14.99 mm, 17.69 mm, and *Listeria monocytogenes* CFSAN004330 had no antimicrobial activity at neutral pH. The results of molecular identification with 16S rRNA showed that lactic acid bacteria isolated from Bekasam (Bk Isolate) was *Pediococcus acidilactici* strain PB22 that has antimicrobial potential against pathogenic bacteria.

Keywords: bekasam, fermented fish, lactic acid bacteria, Sepat Rawa, antibacterial activity ~~16S rRNA~~

Running title: Antimicrobial potential of *Pediococcus acidilactici*

INTRODUCTION

Traditional fermented natural food is very diverse in Indonesia because the territory of Indonesia is very wide and has distinctive food characteristics for each region one of them is curd. Curd is a natural fermentation of buffalo milk from West Sumatra that is beneficial for health (Surono, 2003) and contains several types of lactic acid bacteria (Venema and Surono, 2019). *Lactococcus lactis ssp. Lactis, Lactobacillus plantarum ssp. Plantarum, L. lactis ssp. cremoris, Pediococcus pentosaceus* and *Lactobacillus pentosus* are some types of bacteria that are naturally found in curd (Wirawati *et al*. (2019). Tempoyak, a natural fermentation product from Durian, is also a natural fermentation product (Juliyarsi *et al*. (2018) and tempoyak also contains lactic acid bacteria that have the potential as probiotics (Hartini et al., 2019 and Ahmad et al. 2018). In addition, there are also naturally occurring fish fermented products called Budu originating from West Sumatra. Budu contains Bacillus and Micrococcus (*Bacillus sphaericus, Bacillus polymyxa, Bacillus cereus, Bacillus pantothenticus and Micrococcus lactis*) (Yusra et al. 2014), and *lactic acid bacteria* that have the potential as antimicrobial Bacillus cereus strain HVR22 (Yusra et al. (2013). All natural fermentation product contain lactic acid bacteria that are very beneficial for health.

Bekasam is a traditional food originating from several regions in Indonesia such as Java, South Sumatra and South Kalimantan. Bekasam is the result of spontaneous fermentation of fish. According to Desniar *et al*. (2013), Bekasam is used as a processed fish product by fermentation that tastes sour. Fish that can be used as exam is the type of freshwater fish. The raw material in the form of cork fish, betam, siam and swamp spikes with the addition of salt about 15-20%, and added 15% sangria rice, then fermented for about one week to produce a distinctive aroma and taste.

There are several previous studies about the content of lactic acid bacteria in Bekasam. Wikandari *et al* (2012), found lactic acid bacteria that have proetolytic activity namely *L. plantarum* B765, *L. plantarum* T2565, *L. plantarum* N2352, *L. plantarum* B1465, *L. pentosus* B2555, and *Pediococcus pentosaceus* B1666. Desniar *et al*. (2013), in their study revealed the presence of antimicrobial activity of lactic acid bacteria isolates to *Staphylococcus aureus*, that was caused by the ability of organic acids as antibacterial compounds. Then Afriani *et al*. (2015) isolated lactic acid bacteria of Bekasam from Jambi, that also had proteolytic activity, namely *Lactobacillus pentosus* BS15, *Lactobacillus plantarum* 1 BS22 and *Lactobacillus plantarum* 1 BL12. Melia, et *al*. (2018), tested the antibacterial activity of lactic acid bacteria Bekasan against *Staphylococcus aureus* ATCC 25923, *Escherechia coli* ATCC 25922 and *Salmonella sp*. However, this research

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has not been observed many. This study will evaluate ~~see~~ the potential of lactic acid bacteria from the fermented Sepat Rawa fish that is a typical type of fish used in the Banyuasin region, which has antimicrobial activity and is a potential source bacteriocin, which can later be used as probiotic and biopreservative.

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MATERIALS AND METHODS

Bekasam Processing

The process of making Bekasam is very simple. The ingredients consist of fish, rice, and salt. The fish was cleaned, then added salt and rice sufficiently, put in a bottle and closed tightly. The bottle was stored at room temperature for 3 days (Figure 1).

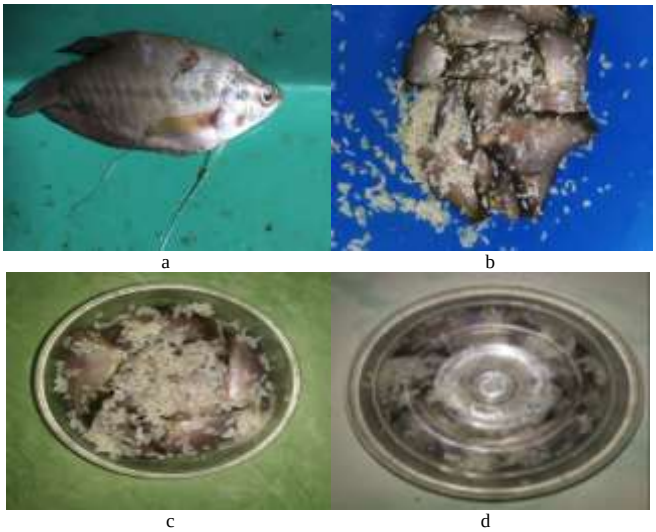

Figure 1. Bekasam Processing (a). Clean sepat fish scales, (b). Give salt and rice, (c). Store in jar bottles (d). Bekasam is fermented for 3 days at room temperature.

Sampling

The material of this study was 4 Bekasam samples originating from Sepat Rawa fermented fish obtained from 4 producers from Banyuasin, South Sumatra, Indonesia (Figure 2), namely Pulau Harapan Village (Producer 1), Mainan Village (Producer 2), Sei Rengit Village (Producer 3) and Santan Sari Village (Producer 4).

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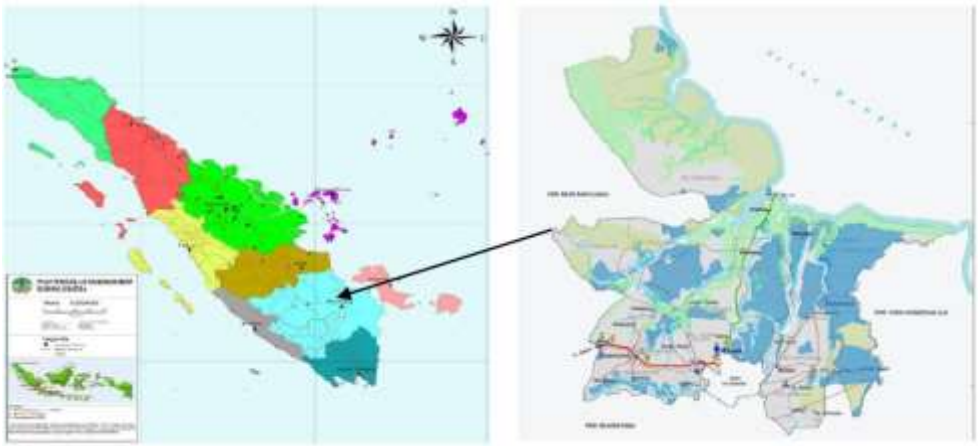

Figure 2. Study area in Banyuasi, South Sumatera, Indonesia (1,3° - 4°S, 103° - 105° E.)

Isolation and Identification of Lactic Acid Bacteria

The isolate of lactic acid bacteria from Bekasam was cultured in broth De Man Rogosa Sharpe (MRS) media (Merck, Germany) and planted in MRS agar media (Merck, Germany) that was incubated at 37^{o}C in an anaerobic jar for 48 hours. Furthermore, morphological properties (shape and color) were observed, and biochemical properties (Gram staining, catalase test and fermentation type) Phikunthong and Yunchalard (2010).

Antimicrobial Activity Test

Modification of the Yang *et al.*, (2012) method was used for antimicrobial activity test against pathogens. The method used was well diffusion assay. In short, cell free supernatants were obtained from lactic acid bacteria grown in MRS Broth, for 24 hours at 37 °C, anaerobic conditions and centrifuged at 10,000 rpm, 5 minutes at 4°C. A 50 µl supernatant was inserted into a well (6 mm) that was perforated with a cock borer. Previously Nutrient Agar (Merck, Germany) has been grown by pathogenic bacteria. As a control it was compared to antibiotics (penicillin 10µg, kanamycin 30µg, ampicillin 10µg). The clear zone formed can be read after 24 hours.

Antimicrobial Test of Crude Bacteriocin Supernantant

One ml of culture was incubated for 24 hours in 9 ml MRS broth for 24 hours at 37 °C. Then centrifuged at 14,000 rpm for 5 minutes. The supernatant was filtered with a 0.22 µl membrane filter. Cell-free supernatant was regulated with pH 6.5 with 1 N NaOH to eliminate the effect of barriers because of the presence of organic acids (Yang et al., 2012). Pathogenic bacteria were grown aerobically at 37^{o}C for 24 hours. Then the pathogenic bacterial culture was 0.2% into 20 ml of MHA at 50 ^{o}C. After agar became solid, well was with a size of 6 mm using the corek borer. Then the supernatant was taken as much as 50 µl and inserted into each well and allowed to stand for 15-20 minutes, then incubated for 24 hours at 37^{o}C in aerobic conditions. The inhibitory zone was measured by using the caliper. If the well is found, the inhibition zone can be said to be BAL isolate that contains bacteriocin compounds.

DNA Genomes Isolation of Lactic Acid Bacteria and 16S rRNA

Single colony lactic acid bacterial isolates from MRS Broth were piped as much as 1000 µl and included in the new eppendorf. Centrifuged as 14000 rpm for 2 minutes. Then the supernatant is removed and the pellet is taken. Added with 480µl 50mM EDTA. Then, 120µl of Lysozyme were added. Next, Incubation in 37ºC water bath for 60 minutes. Centrifuge for 2 minutes 14000 rpm, then remove the supernatant and pellet is taken. Added with 600 µl nuclei lysis solution. Incubated 80ºC for 5 minutes, then let it stand at room temperature. Added 3µl of RNase Solution, incubated in waterbath 37ºC for 60 minutes. Added with 200 µl of the protein precipitation solution then vortex. 600 µl of isopropanol was added. Centrifuged for 2 minutes 14000 rpm, then pellets are taken and the supernatant is removed. Added 600 µl of ethanol 70% and then homogenized. Centrifuged for 2 minutes 14000 rpm, then pellets were taken and the supernatant was removed. Pellet DNA rehydration by adding 10 - 100 µl of Rehydration solution for 30 minutes at 65ºC. Primer R (16S-1492R, Tm 47°C, 5'-GTT TAC CTT GTT ACT ACT-3') and F (16S-27F, Tm 54.3 °C, 5'-AGA GTT TGA TCC TGG CTC AG-3'), prepared (concentration of 10 pM). Take 90µl dH2O + 10µl (Primary R and F). (Primary R and F in TE buffer (concentration 100 µM). Cocktail PCR in1 eppendorf (Master Mix 12.5 µl, Primary F 1 µl, Primary R 1 µl, Template DNA 1 µl, ddH2O 9.5 µl), with PCR denaturation 95°C 45 seconds, annex 56°C 45 seconds, Extention 72°C 1 minutes 40 seconds, final extention72°C 10 minutes. Electrophoresis of 10 µl samples into the well agar, inserted 4 µl of the DNA ladder. Set to 100 V for 45 minutes. The gel placed in a container plus TBE until submerged. The gel, then seen under the UV lamp. The 16S rRNA gene sequences of the isolate were submitted to the NCBI for a BLAST search. The MEGA version 6.0 (http://www.megasoftware.net) was used to create phylogenetic trees using the neighbor joining (NJ) method.

RESULTS AND DISCUSSION

Morphological and biochemical characteristics of lactic acid bacteria isolate

Fifty-six isolates of lactic acid bacteria were isolated from bekasam, morphologically round (cocci), rod shape, and cream colored. The testing of biochemical properties showed that the results of Gram positive and negative catalog as well as isolated of lactic acid bacteria were homofermentative as indicated by the absence of gas bubbles in the Durham tube. Mumtianah *et.al* (2014) showed that lactic acid bacteria isolates from Bekasam were gram-positive, non-motile, negative catalase, positively acid-forming and had proteolytic activity.

Desniar *et al.* (2013), also stated that in general the LAB isolates from the bekasam were homofermentative. Chandra *et al.* (2007) and Irianto (2007) states that bacterial isolates from Bekasam consists of Gram positive cocci and Gram positive Bacilli.

Antimicrobial Activity of Lactic Acid Bacteria Isolate

Of the fifty-six isolates of lactic acid bacteria, there were 4 isolates that had antimicrobial activity, but only Bk isolates had antimicrobial activity against the three tested pathogenic bacteria, *Escherichia coli* O157: H7, *Staphylococcus aureus*

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ATCC25923 and *Listeria monocytogenes* CFSAN004330. *Antimicrobial activity* of Bk isolates from bekasam on pathogenic bacteria, *Escherichia coli* O157: H7, *Staphylococcus aureus* ATCC25923 and *Listeria monocytogenes* CFSAN004330 can be seen in Table 1. Antibiotics used positive controls (Penicillin 10µg, Ampicilin 10µg and Kanamycin 30µg).

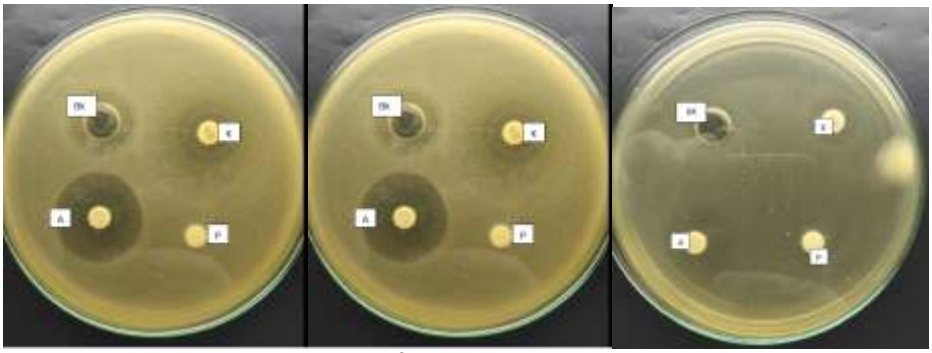
Table 1. Antimicrobial activity Bk Isolate and antibiotic test

Inhibitory source	**Clear zone (mm)**		
	E. coli O157: H7	***S. aureus* ATCC 25923**	***L. monocytogenes* CFSAN004330**
Isolate LAB	21.26 ± 0.03	18.23 ± 0.01	5.10 ± 0.01
Penicillin10µg	2.70 ± 0.03	-	-
Ampicillin10µg	14.19 ± 0.05	21.26 ± 0.02	-
Kanamycin 30µg	16.21 ± 0.09	13.18 ±0.05	10.15 ±0.08

The value is expressed as the mean ± standard deviation; n=3

In Table 1, the largest antimicrobial activity was shown by Bk isolate against *E. coli* O157: H7, with a clear zone diameter of 21.26 mm followed by *S. aureus* ATCC 25923 18.23 mm and *L. monocytogenes* CFSAN004330 5.10 mm. LAB isolate, had greater antimicrobial activity against *E. coli* O157: H7 than the three antibiotics used, namely penicillin 10 µg, kanamycin 30 µg, ampicillin 10 µg. However, as a whole in Figure 3, it can be seen that Bk isolate from the stain has antimicrobial activity against all pathogenic bacteria, compared with penicillin which does not inhibit growth of *S. aureus* ATCC 25923 and *L. monocytogenes* CFSAN004330 and kanamycin had no antimicrobial activity to *L. monocytogenes* CFSAN004330. Desniar (2012) states that the antimicrobial test against the BALs of tilapia extracts has the ability to inhibit five types of pathogenic bacteria: *E. coli*, *S. typimurium* ATCC 14028, *Bacillus aureus*, *S. aureus* and *L. monocytogenes*. The results showed that inhibited zones on pathogenic bacteria had high antimicrobial activity. Pan *et al* (2009) states that the diameter of the inhibited zone against 0-3 mm pathogenic bacteria showed low antimicrobial activity that is > 3-6 mm medium antimicrobial activity and > 6 mm had high antimicrobial activity.

The same result is also shown by Saithong *et al.* (2010), using *L. reuteri* IFRDP P17 in Plaa-som, a typical Thai fish fermentation product, capable of suppressing growth, but in contrast with Desniar *et al.* (2013), which states that isolate LAB from exteriors from Indralaya, Ogan Komiring Ilir (South Sumatera) and Indramayu (West Java), the largest activity of lactic acid bacteria antimicrobials was against *S. aureus*. Lactic acid bacteria werethe dominant bacteria found in fermented fish products (Wikandari *et al.*, 2011, Olympia *et al.*, 1992 and Ostergaard *et al.*, *1*998). Mumtianah *et al.* (2014), states that lactic acid bacteria are bacteria that can produce organic acids, lactic acid and acetic acid that also play a role in inhibiting the growth of bacterial rot. Melia, *et al.* (2017) states that adding lactic acid bacteria is able to inhibit *L. monocytogenes*. It can also inhibit *S. aureus* ATCC 25923 (Melia, *et al.*, 2018). The main role of lactic acid bacteria is to ferment carbohydrates that produce organic acids, which can lead to a decrease in pH. The low pH and presence of organic acids, the main is lactic acid, is a major factor in the process of preservation in fermented fish products. Generally, pH between 4.5 - 5.0 can inhibit pathogenic bacteria and decomposers (Owen and Mendoza, 1985). Organic acid produced by Lactic Acid Bacteria has antibacterial activity (Theron and Ludes, 2011).



a b c

Figure 3. Antimicrobial activity of Bk isolate to *E. coli* O157: H7 (A), *S. aureus* ATCC 25923 (B) and *L. monocytogenes* CFSAN004330 (C). (Description: Bk = Isolate LAB Bekasam, A = Ampicilin, K = Kanamycin and P = penicillin)

Antimicrobial Activity of Crude Bacteriocin Supernantant

The measurement of antimicrobial activity of isolates Bk crude bacteriocin supernatant was obtained after neutralizing pH in the supernatant of lactic acid bacteria, so that the antimicrobial activity of organic acid was not present. According to Palludan-Muller *et al.* (2002), components of organic acids, especially lactic acid is the main components of the antimicrobial compounds of lactic acid bacteria. The results of the study can be seen in Tables 2 and 3 that showed antimicrobial activity after pH of the lactic acid bacterial supernatant was neutralized to the *E. coli* O157: H7 wasie

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14.99 mm and *S. aureus* ATCC25923 that was 17.69 mm, but defention activities were not shown to *L. mMonocytogenes* CFSAN004330.

The result was higher than Melia, et *al.* (2018) study. In their study, the crude bacteriosin LAB isolates activity from Bekasam was against *S. aureus* ATCC 25923 (13.1 mm) and *E. coli* ATCC 25922 (12.7 mm) (Figure 4a and Figure 4b). Whereas in the Desniar *et al.*(2013), LAB isolates from Bekasam did not have antimicrobial activity after supernatant pH was neutralized so that it was thought, antimicrobial activity originated from organic acids produced by lactic acid bacteria. Furthermore Desniar *et al.* (2016) isolated *L. plantarum NS* (9) from Bekasam Tilapia Atin that produced antibacterial activity from organic acids. The highest antibacterial activity against E. *coli*, *B. cereus* and *L. monocytogenes* at the end of the exponential growth phase (12-15 hour incubation) while *S. aureus* and *S. tTyphimurium* ATCC 14028 on the 21st and 24th incubation hours.

Furthermore, Fall et al. (2018), revealed that the antimicrobial activity of supernatant cell free culture from *Lactobacillus plantarum* and *L. brevis* isolated from fermented fish meat (guedj) in Senegal was able to inhibit *E. coli* and *L. monocytogenes*. Srionnual et al. (2007), found that Weissellicin 110, a class II bacteriocin produced by Weissellicin 110 isolated from Pla-som was able to inhibit gram-positive bacteria, but did not have antimicrobial activity against *Listeria monocytogenes.* Nurhikmayani et al. (2010), crude bacteriocin from lactic acid bacteria was isolated from Chao, against *S. aureus* FNCC0047 and *E. coli* FNCC0049.

According to Islam *et al.* (2012), there are several mechanisms to inhibit the destruction of target cells by bacteriocins. Basically inhibiting the formation of lipids II (cell wall precursors) can be done by following mechanism L a. Inhibition of cell wall biosynthesis, b. Stabilize the formation of membrane target pores. Added by Bahar, and Ren (2013), and Song and Zheng (2015)that when the peptide attaches the target cell membrane, the positive end of the peptide will bind the fatty acids in the fospolipid layer on the target bacterial membrane. This stage involves binding the peptide with a membrane like a monomer, so that separation occurs that leads to the formation of pores, ultimately causing death in the cell.

Table 2. Antimicrobial Activity of Bk Isolate Crude Bacteriocin Supernatant

Pathogenic bacteria	Diameter clear zone (mm)
E. cColi O157: H7	14.99 ± 0.03
S. aureus ATCC 25923	17.69 ± 0.01
L. mMonocytogenes CFSAN004330	-

The value is expressed as the mean ± standard deviation; n=3

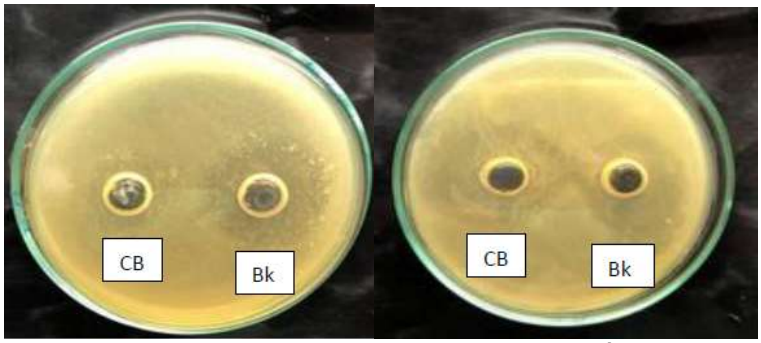

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Figure 4. Antimicrobial activity of crude bacteriocin after neutral pH (CB) and before neutral pH (Bk) ~~to~~ against *E. cColi* O157: H7 (4a), ~~and Figure 4b. Antimicrobial activity of crude bacteriocin after neutral pH (CB) and before neutral pH (Bk) to~~ against *S. aureus* ATCC 25923 (4b).

Results of 16S rRNA gene amplification by PCR

At Figure 5, it can be seen that the amplification of the area of the 16S rRNA gene isolates lactic acid bacteria from Bekasam. It can be seen by the appearance of PCR fragment of size 1542 bp using R Primer– (16S-1492R, Tm 47 °-C, 5'-GTT TAC CTT GTT ACT ACT-3´) and F (16S-27F, Tm 54.3 °-C, 5-'-AGA GTT TGA TGCC CTC AG-3´). Phylogenetic trees based on 16S rRNA gene sequence analysis can be seen in Figure 6. Sequencing results of Bk isolates compared to Gene Bank data using the BLAST program on the NCBI website (http://www.ncbi.nlm.nih.gov) showed a similarity rate of 99% with PB22 strain *Pediococcus acidilactici*, so it can be concluded that the lactic acid bacteria isolate from Bekasam (Bk Isolate) is *Pediococcus acidilactici* strain PB22. This isolate lactic acid bacteria is a new strain found in Bekasam or other fermented fish.

Rinto *et al.* (2015), isolated *Lactobacillus acidophilus* and *Lactobacillus delbruckii sp* acid bacteria. Delbruckii using API 50CH and API 50CHL, as statin producers. Afriani *et al.* (2015), isolated lactic acid bacteria from Bekasam from Jambi, which also has proteolytic activity, namely *Lactobacillus pentosus* BS15, *Lactobacillus plantarum* 1 BS22 and

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Comment [u18]: 99% is not enough to say they are the same strain.

Comment [u19]: Did you consider Desnia et al. 2013? They cited: Yahya et al. (1997 reported that LAB isolated during bekasam fermentation from Nile tilapia (Oreochromis mossambicus) were Leuconostoc mesenteroides which was isolated from sample of 1 – 7 days fermentation. Lactobacillus acidophilus from day 5 to 7 L. plantarum and L. fermentans on the first day, L. buchneri, L. reuteri on the third day, Pediococcus pentacaseus, L. bifermentans, L. tolerans, Pediococcus acidilactici..." Then, P. acidilactici is not new in bekasa

(9) (PDF) Characterization of lactic acid bacteria isolated from an Indonesian fermented fish (bekasam) and their antimicrobial activity against pathogenic bacteria. Available from: https://www.researchgate.net/publication/2338718_Characterization_of_lactic_acid_bacia_isolated_from_an_Indonesian_fermented_h_bekasam_and_their_antimicrobial_activit

Comment [u20R19]:

Comment [u21]: Reference?

Comment [u22]: From bekasam?

Comment [u23]: Is it complete the idea?

Lactobacillus plantarum 1 BL12. —Nurhikmayani et al. (2019), found *Lactobacillus plantarum* and *Pediococcus pentosaceus* from Chao, fermented fish from South Sulawesi using 16S rRNA.

In addition there are also several sources of lactic acid bacteria from Thai Plaa-som fermented fish products such as *Pediococcus pentosaceus, Lactobacillus alimentarius / farciminis, Weisella confusa, L. plantarum* and *Lactococcus garviae* from Plaa-som, fermented fish products from local producers in Songkhla province, Southern Thailand (Paludan-Mu¨ller et al., 2002), *Lactococcus garvieae, Streptococcus bovis, Weissella cibaria, Pediococcus pentosaceus, Lactobacillus plantarum,* and *Lactobacillus fermentum* (Kopermsub and Yunchalard, 2010), *Lb. plantarum* and *Pediococcus pentosaceous* (Nicomarat et al., 2018).

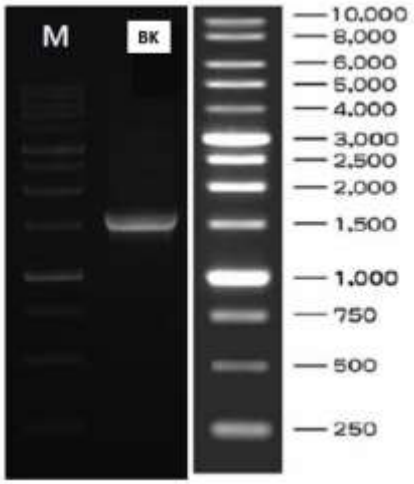

Figure 5. PCR electrophoresis product of lactic acid bacteria isolates from Bekasam (Bk) from Banyuasin, South Sumatra, (M = 1 kB DNA Ladder).

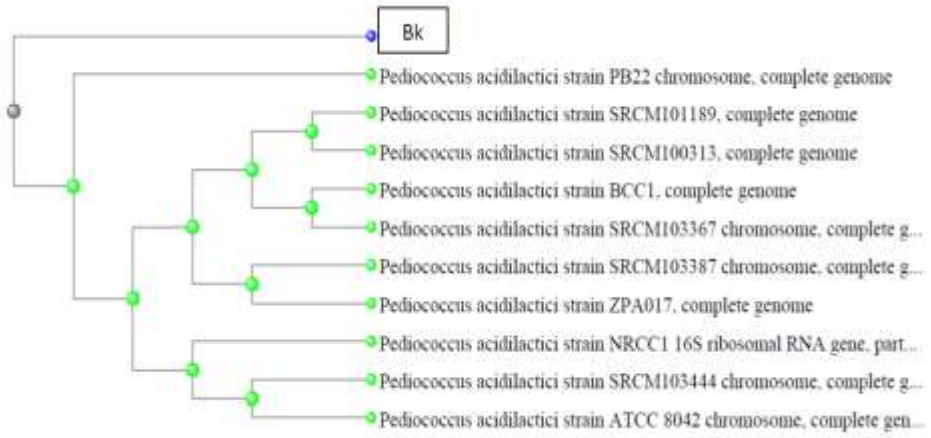

Figure 6. Phylogenetic isolate of lactic acid from Bekasam (Bk)

In Summary, the results of molecular identification with 16S rRNA showed the potential lactic acid bacteria (Bk isolate) as an antimicrobial isolation from Bekasam from South Sumatra Banyuasin was *Pediococcus acidilactici* strain PB22. *Pediococcus acidilactici* strain PB22, is the new strain found in Bekasam. Furthermore, *Pediococcus acidilactici* strain ~~PB22~~ can later be used as probiotics and biopreservatives.

Comment [u24]: Figure 5 is not relevant

Comment [u25]: Consider the corresponding bootstrap values (100 replications) on each branch.

Comment [u26]: Suggestion: Phylogenet tree of 16S rRNA sequences Pediococc pentosaceus BCNU 9070 among the Pediococcus strains using Neighbor-joining method. Bk is the isolate describ in this paper, other sequences were obtained from GenBank.
------Consider the corresponding bootstr values (100 replications) on each branch

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ACKNOWLEDGEMENTS

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Reviewer Comments Antimicrobial Potential of Pediococcus acidilactici PB22 From Bekasam, Fermentation of Sepat Rawa Fish (Tricopodustrichopterus) from Banyuasin, Sumatera Selatan, Indonesia

For author

This paper is about the isolation of lactic acid bacteria (LAB) from bekasam from Indonesia and determination of the antimicrobial properties of the LAB and their bacteriocins. However, this study is very similar to Melia et al., 2018. I cannot see any new result from this study. In addition, there is no clear inhibition zone observed after neutralizing the organic acid (Figure 4). The isolated strains are not bacteriocin producers as considered by the author. The conclusion that *Pediococcus acidilactici* strain PB22 can later be used as probiotics and biopreservatives is not accepted at this stage because the author does not study any probiotic characteristics for this isolate.

Major comments:

1. This study is very similar to previous study (same source, same pathogens tested, etc.) and it is not adding any valuable information to the knowledge in this field.
2. The inhibition zone related to bacteriocin in Figure 4 could not be considered as a "clear" zone.
3. In the abstract, the author doesn't mention the challenge or the importance for doing this work.
4. The paper is not well written, it needs revision

2.2. Minor comments:

1. Line 15, bacteriocin (not bacteriosin)
2. Line 32, Bacillus in italic
3. Line 70, the isolation of the LAB from the sample should be mentioned not only the bacterial culture
4. *4. Line 70, De Man, Rogosa and Sharpe (MRS) Broth*
5. Line 84, to pH 6.5
6. Line 91-92 needs revision
7. Line 90-99, please mention the kit used for DNA extraction if available

Lactic acid bacteria isolates were cultured in MRS broth at 37°C for 24 h. Isolation of genomic DNA was carried out using Promega DNA Kit,

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