Journal of Veterinary and Animal Sciences
DOI: https://doi.org/10.24843/JVAS.2021.v04.i02.p03
pISSN 2550-1283; eISSN:2622-0571
August 2021 Vol. 4 No.2: 47-57

Molecular Analysis of Lactic Acid Bacteria SR4 Strain Isolated from Gastric's Juice of Bali Cattle

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Abstract. Lactic acid bacteria SR4 strain is known to have potential as a probiotic source according to the previous study. The isolate derived from the gastric's juice of bali cattle has characteristics such as its resistance to growth at low pH, namely pH 2, 3, and 4, its tolerance to bile concentration of 0.6 mM, and its inhibition against Gram-positive and negative bacteria. On the other hand, a complete molecular analysis of the isolate has never been carried out. Based on these considerations, the molecular analysis of the LAB SR4 strain becomes interesting to present. The study was initiated by cultivating LAB SR4 isolate on an MRS broth medium. The isolate was further confirmed molecularly through analysis of the 16S rRNA gene which was initiated by isolating genomic DNA followed by PCR reaction using universal primers 27F and 1492R. The PCR product of the 16S rRNA gene was sequenced and further analyzed using the MEGA 5.2 software. The data were then analyzed descriptively and presented in the form of tables or figures. The results showed that the LAB strain SR4 was identified as a *Pediococcus pentosaceus*. This strain potentially to develops as a source of bacteriocin and it can be applied as a bio preservative, improvement of the meat taste, inhibition of inflammation, and also as antagonism of cancer.

Key words: Lactic acid bacteria SR4 strain, molecular analysis, probiotic

I. INTRODUCTION

Lactic acid bacteria (LAB) are known as bacteria that are very beneficial for health, have been used widely, and are safe to use with minimal side effects. These bacteria are known as GRAS (generally recognized as safe). The bacteria have antagonistic effects against bacterial pathogens[1], immunomodulating[2], anti-mutagenic[3], antioxidant and anti-imflammatory[4], and anti-carcinogenic[5].

Recently, the number of LAB studies has shown increasing specifically for their antagonizes pathogens through secreting bacteriocins[6]. For example, Pediocin K 23-2 with 5 kDa molecular weight produced by *P. pentosaceus* K23-2 from Kimchi was found to inhibit various Gram-positive pathogens[7]. Moreover, both pentocin L and pentocin S with 27 and 25 kDa molecular weight, respectively were also known able to inhibit an extensive spectrum of pathogens, especially *Clostridium sporogenes* ATCC 11,259[8].

As a normal flora, LAB is very easy to isolate from the digestive tract of animals or humans. It is estimated not less than 500 species out of 10^{12} bacteria per gram of gastrointestinal contents of animals or humans are lactic acid bacteria[9]. Generally, The LAB composition of the normal flora in the gastrointestinal tract is site-specific. Several factors such as physical (bowel movements), chemical (changes in pH) and food (diet) consider making a significant contribution to changes in the normal flora of the digestive tract[9]. Their easy to adapt to a less favorable environment and have a high digestibility of fibrous foods of bali cattle[10, 11] are predicted to affect the genetics of bacteria in their body.

Furthermore, the LAB SR4 strain isolated from gastric' juice of bali cattle is known to have potential as a probiotic source according to the previous study. The isolate has characteristics such as its resistance to growth at low pH, namely pH 2, 3, and 4, its tolerance to bile concentration of 0.6 mM, and its inhibition against Gram-positive and negative bacteria[12]. On the other hand, a complete molecular analysis of the LAB SR4 strain has never been carried out. Based on these considerations, the molecular analysis of the SR4 strain becomes interesting to present.

II. MATERIALS AND METHODS

Cultivation od isolate

LAB SR4 isolate was taken from 30% glycerol stock stored at -20^{o}C storage temperature. The isolate was then thawed at 4°C for 15 minutes before being planted at room temperature in sterile MRS broth media. The culture was being incubated at 37°C for 24 hours, and it was ready to be used for further tests[12].

DNA extraction

The genomic DNA of the LAB SR4 isolate was isolated by centrifugation of bacteria at 7500 rpm for 5 minutes. The supernatant was discarded, proceeded with 180 µl of ATL buffer, and 20 µl of proteinase K solution was added to the cell pellet. The dilution was mixed by vortex for 5 seconds, added with 200 µl of AL buffer and then vortexed for 15 seconds, followed by incubating it in a water bath at 56^{o}C for 10 minutes, added with 200 µl of absolute 96-100% ethanol, and then vortex for 15 seconds.

The next step was the preparation of a 2 ml tube containing a filter tube (QIAamp Mini spin column), and the lysate was put into the filter, centrifuged 6000 g (8000 rpm) for 1 minute, followed by DNA Washing stage. As much as 500 µl of washing buffer (AW1 buffer) was added and let stand for 5 minutes, centrifuge at 8000 rpm for 1 minute, discard the remaining liquid collected and replace with a new 2 ml tube, added with 500 µl of washing buffer (buffer AW2) and let stand for 5 minutes, centrifuge at full speed (14,000 rpm for 3 minutes), and then proceed to the DNA eluting stage. This stage begins by placing the QIAamp Mini spin column into a new sterile efendorf before being added with 100 µl of elution buffer (AE buffer). and allowing it to stand at room temperature for 5 minutes. The DNA was then centrifuged at 8000 rpm for 1 minute and the tube has been contained pure DNA. To avoid repeated freezing and thawing of DNA, pure DNA is stored at 4°C before being used[13].

PCR amplification of the 16S rRNA gene

The universal primer B27F (5'-AGAGTTTGATCCTGGCTCAG-3') and U1492R (5'-GGTTACCTTGTTACGACTT-3') were used to amplify the 16S rRNA gene. A total of 36 µl reaction volume containing 2 µl DNA template, 25 µl My Taq HS Red Mix, 7 µl distilled water, and 1 µl (20 pmol/µl) of primer 27F and U1492R was used in this method. The PCR amplification was performed by an initial DNA denaturation at 94°C for 5 min and followed by 30 cycles of denaturation at 94°C for 1 min. This procedure used annealing at 45°C for 45 sec and an extension at 72°C for 1 min. The amplification process was completed by a

final extension at 72°C for 5 min. Furthermore, 5 µl of PCR product was analyzed by electrophoresis in 1% agarose[13, 14].

Sequencing and Phylogenetic Analysis

ABI Prism 3130 and 3130xl Genetic Analyzer was used for sequencing of the 16S rRNA gene through the Institute for sequencing service providers at PT Genetika Science, Jakarta. The sequencing process used similar primers with the previous PCR reactions. The sequence results were edited and corrected with the MEGA 5.2 version software (https://www.megasoftware.net/) [13]. The edited gene sequences were compared automatically using the BLAST program against the sequences of bacteria available in databanks (www.ncbi.nlm.nih. gov). The neighbor-joining algorithm method was then used to construct the phylogenetic tree[13, 14].

III. RESULTS AND DISCUSSIONS

Identification and Molecular Analysis of BAL SR4 isolate.

The cultivation of LAB isolates SR4 taken from 30% glycerol stock showed characteristics as LAB isolates, namely growing as single colonies on MRS broth media. The result of the Gram stain shows the shape of the cocci as in Figure 1

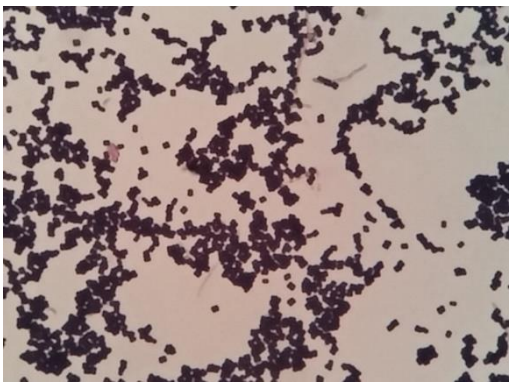

Figure 1. Gram staining of LAB SR4 isolate

pISSN 2550-1283; eISSN:2622-0571
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The bacteria that grew were then isolated for their DNA and continued with the molecular identification stage of bacterial strains by PCR method using universal primers, namely: B27F 5'-AGAGTTTGATCCTGGCTCAG-'3 and U1492R 5'-GGTTACCTTGTTACGACTT-'3[13, 14]. The PCR results were then electrophoresed and showed results as shown in Figure 2

Figure 2 shows that the 16S rRNA gene of SR4 isolate was successfully amplified with a PCR product length of 1587 bp. The PCR products were then sequenced to determine the composition of their constituent nucleotides. The nucleotides of SR4 isolate were blasted in the NCBI program and showed similarities to the nucleotide arrangement of *Pediococcus pentosacueus* (MT000144) as shown in Table 1.

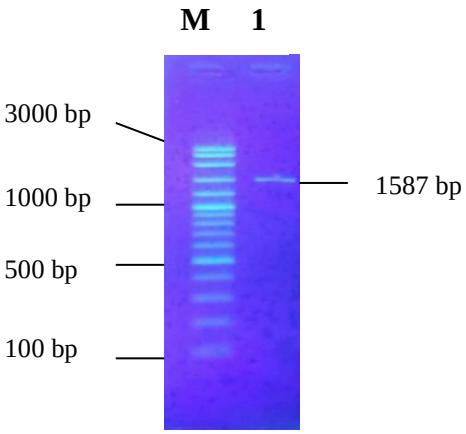

Figure 2. Electrophoresis results of 16S rRNA gene detection with B27(F) and U 1492(R) primers on 1% agarose. M: markers; 1: Isolate SR4.

Table 1. Blast result of the 16S rRNA gene of LAB SR4 strain (query) against *Pediococcus pentosacueus* (MT000144) (subjct).

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Query  1    TGCAGTCGAACGAACTTCCGTTAATTGATTATGACGTACTTGTACTGATTGAGATTTTAA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  18   TGCAGTCGAACGAACTTCCGTTAATTGATTATGACGTACTTGTACTGATTGAGATTTTAA  77

Query  61   CACGAAGTGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCAGAAGTAGGGGAT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  78   CACGAAGTGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCAGAAGTAGGGGAT  137

Query  121  AACACCTGGAAACAGATGCTAATACCGTATAACAGAGAAAACCGCATGGTTTTCTTTTAA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  138  AACACCTGGAAACAGATGCTAATACCGTATAACAGAGAAAACCGCATGGTTTTCTTTTAA  197

Query  181  AAGATGGCTCTGCTATCACTTCTGGATGGACCCGCGGCGTATTAGCTAGTTGGTGAGGCA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  198  AAGATGGCTCTGCTATCACTTCTGGATGGACCCGCGGCGTATTAGCTAGTTGGTGAGGCA  257

Query  241  AAGGCTCACCAAGGCAGTGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGAC  300
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                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  258   AAGGCTCACCAAGGCAGTGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGAC  317

Query  301   TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCA  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  318   TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCA  377

Query  361   AGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGT  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  378   AGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGT  437

Query  421   TAAAGAAGAACGTGGGTAAGAGTAACTGTTTACCCAGTGACGGTATTTAACCAGAAAGCC  480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  438   TAAAGAAGAACGTGGGTAAGAGTAACTGTTTACCCAGTGACGGTATTTAACCAGAAAGCC  497

Query  481   ACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTT  540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  498   ACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTT  557

Query  541   ATTGGGCGTAAAGCGAGCGCAGGCGGTCTTTTAAGTCTAATGTGAAAGCCTTCGGCTCAA  600
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  558   ATTGGGCGTAAAGCGAGCGCAGGCGGTCTTTTAAGTCTAATGTGAAAGCCTTCGGCTCAA  617

Query  601   CCGAAGAAGTGCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGACAGTGGAACTCCAT  660
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  618   CCGAAGAAGTGCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGACAGTGGAACTCCAT  677

Query  661   GTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGG  720
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  678   GTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGG  737

Query  721   TCTGCAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTA  780
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  738   TCTGCAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTA  797

Query  781   GTCCATGCCGTAAACGATGATTACTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAG  840
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  798   GTCCATGCCGTAAACGATGATTACTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAG  857

Query  841   CTAACGCATTAAGTAATCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAAGAATT  900
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  858   CTAACGCATTAAGTAATCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAAGAATT  917

Query  901   GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCT  960
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  918   GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCT  977

Query  961   TACCAGGTCTTGACATCTTCTGACAGTCTAAGAGATTAGAGGTTCCCTTCGGGGACAGAA  1020
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  978   TACCAGGTCTTGACATCTTCTGACAGTCTAAGAGATTAGAGGTTCCCTTCGGGGACAGAA  1037

Query  1021  TGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA  1080
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1038  TGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA  1097

Query  1081  ACGAGCGCAACCCTTATTACTAGTTGCCAGCATTAAGTTGGGCACTCTAGTGAGACTGCC  1140
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1098  ACGAGCGCAACCCTTATTACTAGTTGCCAGCATTAAGTTGGGCACTCTAGTGAGACTGCC  1157

Query  1141  GGTGACAAACCGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGACCTGGG  1200
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1158  GGTGACAAACCGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGACCTGGG  1217

Query  1201  CTACACACGTGCTACAATGGATGGTACAACGAGTCGCGAGACCGCGAGGTTAAGCTAATC  1260
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1218  CTACACACGTGCTACAATGGATGGTACAACGAGTCGCGAGACCGCGAGGTTAAGCTAATC  1277

Query  1261  TCTTAAAACCATTCTCAGTTCGGACTGTAGGCTGCAACTCGCCTACACGAAGTCGGAATC  1320
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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1278  TCTTAAAACCATTCTCAGTTCGGACTGTAGGCTGCAACTCGCCTACACGAAGTCGGAATC  1337

Query  1321  GCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGC  1380
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1338  GCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGC  1397

Query  1381  CCGTCACACCATGAGAGTTTGTAACACCCAAAGCCGGTGGGGTAACC  1427
            |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1398  CCGTCACACCATGAGAGTTTGTAACACCCAAAGCCGGTGGGGTAACC  1444
```

Table 1 shows, identically of LAB SR4 nucleotide sequences against *Pediococcus pentosaceus* (MT000144) of 1427 nucleotide compared. Moreover, the LAB SR4 nucleotide sequences also alignment with other LAB nucleotide sequences available in Genbank such as *Tetragenococcus halophilus* (AB681196), *Vagococcus salmoninarum* (X54272), *Lactobacillus rhamnosus* (D16552), *Pediococcus siamensis* (AB258357), *Leuconostoc lactis* (AJ970316), *Lactococcus lactis* (AB100796), *Streptococcus salivarius* (AB608747), *Bifidobacterium biavatii* (AB559506), and *Pediococcus pentosaceus* (MT000144). Comparison analysis of the constituent nucleotide bases of the 16S rRNA gene between LAB SR4 strains against other bacterial strains are presented in Table 2.

Table 2. Comparison analysis of the constituent nucleotide bases of the 16S rRNA gene between LAB SR4 strains against other bacterial strains.

	T(U)	C	A	G	Total
Tetragenococcus halophilus (AB681196)	20.8	23.5	25.7	30.1	1390
Strain SR4	**22.0**	**22.2**	**27.0**	**28.8**	**1410**
Vagococcus salmoninarum (X54272)	22.0	23.2	25.2	29.6	1347
Lactobacillus rhamnosus (D16552)	22.1	22.3	25.9	29.7	1399
Pediococcus siamensis (AB258357)	21.9	22.7	26.1	29.2	1413
Leuconostoc lactis (AJ970316)	21.6	22.0	26.9	29.5	1385
Lactococcus lactis (AB100796)	21.9	21.3	27.1	29.7	1382
Streptococcus salivarius (AB608747)	21.9	22.3	25.7	30.1	1383
Bifidobacterium biavatii (AB559506)	20.8	23.6	21.3	34.4	1368
Pediococcus pentosaceus (MT000144)	22.1	22.1	26.8	29.0	1398

The genetic distances analysis of LAB SR4 strain against several strains available in Genbank based on the 16S rRNA gene sequences as a proceed analysis of the data in Table 2 shown in Table 3.

Table 3 shows the genetic distances among bacteria. It is shown, LAB SR4 strain was identical with *Pediococcus pentosaceus* (MT 000144). Further analysis to see the

group of bacteria in the form of phylogenetic trees is presented in Figure 2.

Table 3. The genetic distances analysis of LAB SR4 strain against several strains available in Genbank based on the 16S rRNA gene sequences.

	Tetragenococcus halophilus (AB681196)	Strain SR4	Vagococcus salmoninarum (X54272)	Lactobacillus rhamnosus (D16552)	Pediococcus siamensis (AB258357)	Leuconostoc lactis (AJ970316)	Lactococcus lactis (AB100796)	Streptococcus salivarius (AB608747)	Bifidobacterium biavatii (AB559506)	Pediococcus pentosaceus (MT000144)
Tetragenococcus halophilus (AB681196)										
Strain SR4	0.152									
Vagococcus salmoninarum (X54272)	0.108	0.127								
Lactobacillus rhamnosus (D16552)	0.134	0.083	0.119							
Pediococcus siamensis (AB258357)	0.155	0.070	0.128	0.092						
Leuconostoc lactis (AJ970316)	0.165	0.173	0.176	0.154	0.182					
Lactococcus lactis (AB100796)	0.172	0.157	0.143	0.157	0.170	0.195				
Streptococcus salivarius (AB608747)	0.156	0.160	0.135	0.161	0.181	0.188	0.109			
Bifidobacterium biavatii (AB559506)	0.302	0.278	0.287	0.265	0.283	0.318	0.305	0.296		
Pediococcus pentosaceus (MT000144)	0.153	0.000	0.127	0.083	0.071	0.172	0.155	0.160	0.277	

pISSN 2550-1283; eISSN:2622-0571
August 2021 Vol. 4 No.2: 47-57

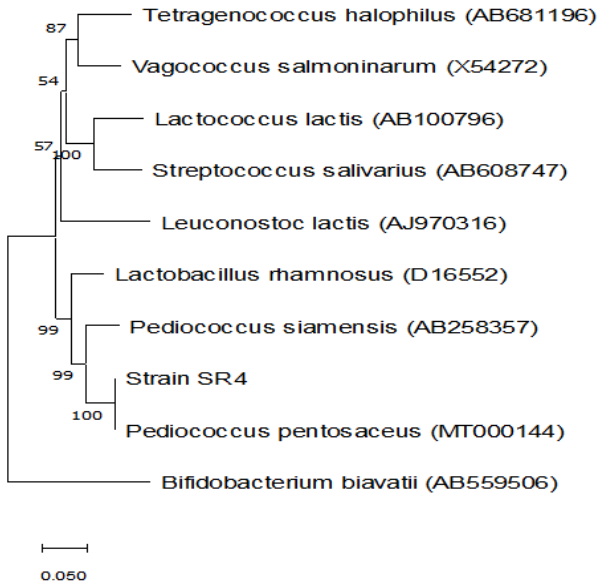

Figure 2. Phylogram of the 16S rRNA sequences based on the Neighbor-Joining algorithm[15]. The numbers in the phylogram branch indicate the bootstrap value (%) with 1000 replicates. The scale indicates a 5 per 100 nucleotide substitution in the 16S rRNA gene sequence.

Figure 2 shows the grouping of SR4 isolates into the same clade as *Pediococcus pentosaceus* (MT000144) with a bootstrap value of 100%. The results of this analysis indicate that the SR4 strain is a *Pediococcus pentosaceus* strain. This is supported by the theory of Janda and Abbott (2007)[16] confirming the rules for microbial identification based on the 16S rRNA sequencing including (i) 16S rRNA genes that must be sequenced at least 500-525 bp from ideally 1300-1500 bp (doubtful position < 1%), and (ii) criteria for identification of similarity of species, ie the same sequence is minimum >99%, from ideally >99.5%. This strain is different from the result of the previous study that found LAB SR9 strain as *Lactococcus lactis spp lactis* 1[17], LAB SR2 strain as *Lactococcus lactis*[12], and LAB 9A strain as S*treptococcus bovis*[18].

Pediococcus pentosaceus, one type of LAB, has played an increasingly pivotal role in LAB applications in recent years. Isolated from fermented food, aquatic products, raw animal, plant products, and feces, many strains of *P. pentosaceus* were finally proven to have links to the human gastrointestinal tract (GIT)[19]. To date, there is increasing experimental evidence indicating that *P. pentosaceus* may be usable as a bio preservative for foods,

plants, or animals or as an emerging possible probiotic candidate[20]. Several studies about the application of *Pediococcus pentosaceus* have been conducted such as to improve the taste of the meat flavor[21], inhibition of inflammation[22], and also as antagonism of cancer specifically their efficacy on Caco-2 and MCF-7 cell line[23]. It is known, anti-cancer activity of *Pediococcus sp* induced apoptosis in the cancerous cells through increasing the BAX protein expression and decreasing the Bcl-2 protein expression[24].

IV. CONCLUSION

Molecular analysis of lactic acid bacteria SR4 strain isolated from Gastric Juice of bali cattle identified the strain as *Pediococcus pentosaceus*. This strain potentially develops as a source of bacteriocin and applicate it as a bio preservative, improvement of the meat taste, inhibition of inflammation, and also as antagonism of cancer.

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