Molecular Identification of Lactic Acid Bacteria from Broiler Chicken Meat

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Abstract

Lactic acid bacteria are bacteria used to ferment food. Molecular identification of isolates of lactic acid bacteria has a high degree of accuracy compared to morphological identification. This study aimed to molecularly identify isolates of lactic acid bacteria from broiler chickens. Molecular identification is carried out in 3 stages, namely: rRNA gene amplification stage, squencing and phylogeny tree. PCR electrophoresis results obtained amplicone results measuring about 1,500 bp. The results of BLAST analysis showed that the gen 16S rRNA sequence of BR 17 lactic acid bacteria isolate had a 97-98% similarity with the genome sequence of the Lactobacillus fermentum strain. Development of phylogenetic tree isolates of single clusters of lactic acid bacteria BR 17 with strains of Lactobacillus fermentum CAU2036, Lactobacillus fermentum JCM 7772, and Lactobacillus fermentum 10-18. The identification results of lactic acid bacteria isolate BR 17 showed Lactobacillus fermentum BR 17.

Keywords: *broiler chicken, identification, lactic acid bacteria, molecular*

1. INTRODUCTION

Lactic acid bacteria are a group of Gram positive bacteria, do not form spores, almost all strains are not able to produce the enzyme catalase, most are facultative anaerobic and able to ferment lactose with lactic acid end products (Krockel, 2013). Lactic acid bacteria are beneficial microorganisms used as starter cultures for functional food processing (Aritonang et al., 2017) These lactic acid bacteria are not toxic to the host and have the ability to kill pathogenic bacteria. Many studies have reported that lactic acid can have significant antimicrobial effects (Gao et al., 2019) Researchers have developed Lactic Acid Bacteria that can be used for food fermentation (Mathur et al., 2020) Most of the use of lactic acid bacteria for milk fermentation (Widyastuti, and Febrisiantosa, 2014)(Montet et al., 2014)(Soro-Yao et al., 2014)(Tsuda et al., 2012)and lactic acid bacteria as starter on the manufacture of fermented sausages (Franciosa et al., 2018)

Lactic acid bacteria are found naturally in foodstuffs including chicken meat (Sakaridis et al., 2014) (Arief et al., 2015) succeeded in isolating LAB from beef as a probiotic. Lactic acid bacteria BR 12, BR 17, BR 11, BR 10, and BR 7 obtained from broiler chicken meat resulted in the growth of lactic acid bacteria with continuous growth with the highest lactic acid levels and the lowest pH of all Lactobacillus genes (Mudawaroch et al., 2020). BR 17 isolate is more efficient in using sucrose when compared to BR 12. BR 17 isolate requires 0.246 g of sucrose in every 100 ml of medium to produce an optimal bacterial growth rate, while BR 12 is 0.34 g/100ml (Mudawaroch et al., 2019) *Lactobacillus fermentum* can be used as an starter in meat fermentation (Ruiz-Moyano et al., 2011)

Traditionally, bacteria were commonly identified through morphological characteristics, metabolites and other phenotypes, and this method was considered inefficient and no longer accurate because it relied on phenotypic expression. There are many differences in results between morphological identification and molecular identification (Ahmadi et al., 2015)**.** This study aims to molecularly identify BR 17 isolates from broiler chicken meat.

2. MATERIAL AND METHODS

BR 17 isolate was obtained from the isolation of lactic acid bacteria from broiler chicken meat (Mudawaroch et al., 2019) (Mudawaroch et al., 2020).

2.1 Research Method

Molecular identification consists of 3 stages, namely: rRNA gene amplification stage, squencing and phylogeny tree.

2.1.1 Amplifcation of 16S rRNA genes.

Lactic Acid Bacteria Isolate is harvested by fish method again in MRS Agar media until a pure isolate is obtained.

A. Isolation of DNA isolates.

Bacterial culture was grown into 5 ml of sterile MRS medium and incubated at 37ºC for 12 hours. Cells were harvested by centrifugation of bacterial culture as much as 2 ml at a speed of 13,000 rpm for 5 minutes. Cell deposits are taken and the supernatant is discarded. The bacterial culture precipitate was resuspended using a 750µL lysis buffer, then homogenized (vortex). Then 20µL (10mg/mL) of proteinase K was added and incubated at 55°C for 30 minutes. Stock lysozyme (100mg/mL) was added as much as 40µL, incubated at 55°C for 30 min and centrifuge at 13,000 rpm for 5 min. The supernatant is transferred into a new 2 ml microtube and 750µL of phenol is added. The sample was vigorously jogged, for 15 minutes, centrifuge returned to 13,000 rpm for 10 minutes. The formed top layer, carefully taken and transferred into a 2 ml microtube. Cold chloroform is added in a ratio of 1:1 (v / v) and gently cornered for 10 minutes, then centrifuge at a speed of 13,000 rpm for 10 minutes. Take the top layer carefully, transfer it into a new 1.5 ml microtube. 1:1 (v/v) cold absolute ethanol is added and stored at -80 C °for2 hours or -20°C overnight. Next, the sample was centrifuge at 13,000 rpm for 10 min. The supernatant was removed and the precipitate was washed using 0.5 ml of 70% ethanol and centrifuge again at 13,000 rpm for 10 minutes. The pellets are formed, dried and 50µL TE is added. The DNA extract is then stored at 4°C overnight, then transferred to -20ºC.

B. PCR

The PCR procedure follows the GoTaq Green mastermix PCR Kit procedure. The material mixture for uk PCR consists of 25 µl master mix 2xGoTaq Green readymix PCR, forward and reverse primers of 2 µl each, The primer used is forward 5'-AGAGTTTGA(C/T)(A/C)TGGCTCA-'3, Reverse 5'-CA(G/T)AAAGGAGGTGATCC-'3, DNA template 2 µl, and water free-DNase 19 µl. The PCR program in the thermal cycle used consists of an initial denaturation of 95 ºC for 3 minutes as much as 1 cycle; followed by 30 cycles which include denaturation temperature 94 ºC for 30 seconds; annealing temperature 55 ºC, elongation 72 ºC for 2 min; and 1 cycle covering final elongation of temperature 72 ºC for 5 min.

The PCR product was then analyzed for the size of 16S DNA obtained with 1% agarose electrophoresis gel (Harisha, 2007). Agarose gel (Bioron) as much as 2% [1 g in 100 ml TAE stok 1x (20 ml TAE and 980 ml aquades)]. The stock composition of 1000 ml TAE electrophoresis buffer (Fermentation) consists of 40 mM Tris, 20 mM acetic acid and 1 mM EDTA. Agarose and TAE are dissolved until homogeneous by microwave and printed in trays and fitted with combs to form a spool. After cooling, the gel is placed in an electrophoresis device (BioCRAFT BE 520) and given TAE 1x until submerged. Samples and DNA ladders (1 kb DNA ladder Gene Rule-Fermentas) of 5 µl each were inserted in the well. Electrophoresis is performed at 100 volts for 40 minutes. Upon completion, the gel is taken and soaked in ethidium bromide (25 µl EtBr in 500 ml TAE 1x) for 20 minutes. The results of electrophoresis are observed with UV-transilluminators.

2.1.2 Sequencing.

Theg sequence was performed by 1stBASE Sequencing in Malaysia, using ABI PRISMTM 3730-XL 1406-022 instrument. A sample of 100 µl PCR product in a parafilm-coated PCR tube. The result of combining forward and reverse readings to obtain the DNA sequence sampel.

2.1.3 Tree phylogeny.

The amplified sequence data was then compared with the sequence data obtained from the Gene Bank (http://www.ncbi.nlm.nih.gov). The gene sequences obtained from the data base were aligned and gene phylogeny construction was carried out using the MEGA 6 program with the Maximum likehood (ML) algorithm.

3. RESULTS AND DISCUSSION

Molecularly identified is necessary to determine exactly the genus and species as well as proximity to other isolates. Molecular identification is used because of its high level of accuracy and can be used to identify isolates down to strain level (Taufiq et al., 2017). Characterization of lactic acid bacteria is based on 16S rRNA sequence analysis to determine genus and strain (Julendra et al., 2017).

Isolate lactic acid bacteria from broiler chicken meat broiler chickens obtained 21 isolates. From 21 isolates were selected by looking at the lowest pH value until 13 isolates were obtained. Of the 13 isolates selected growth acceleration, the highest lactic acid levels. Based on the use of the least energy source, namely isolate BR 17. BR 17 isolate Pure BR 17 isolate followed by DNA isolation of lactic acid bacteria isolate until DNA is obtained. Pure BR 17 isolates are followed by DNA solation of lactic acid bacterial isolates to obtain DNA. DNA isolation includes cell breakdown, protein and RNA removal and DNA purification (Agrawal & Prakash, 2013).

Amplification with *Polymerase Chain Reaction* (PCR) is carried out after DNA isolation of the genome*. Polymerase Chain Reaction* (PCR) is a system for DNA replication that allows DNA to selectively "target" DNA sequences several million times in just a few hours (Agrawal & Prakash, 2013). PCR consists of denaturation at 90 - 94ºC to open DNA strands, annealing at 50 - 65°C for primary hybridization of "anneal" (via hydrogen bonding) to its complement and elongation (elongation) at 72ºC is the appropriate temperature for polymerase against template. This cycle is repeated 30 – 40 times.

The results of DNA amplification by PCR, observation of the presence of DNA was carried out at the end of the reaction using agarose gel after an electrophoresis process (Pranawaty et al., 2012) PCR product detection is performed electrophoresis with 2% agarose gel and observed under UV lamp. The results of genomic DNA amplification are shown in Fig.1. The results of PCR electrophoresis obtained amplicom measuring about 1.500 bp of DNA. The PCR results are then sequenced to find out the nucleotide sequence.

DNA sequencing is the process of determining the sequence of nucleotide bases, namely adenine, thymine, guanosine, and cytosine in the DNA sequence, this DNA sequence is important for the gene information of a living thing. Sequencing was conducted by 1st BASE Sequencing in Malaysia, using ABI PRISMTM 3730-XL 1406-022 instrument. 16S rDNA sequencing is then followed by checking the similarity of nucleotides p a da GeneBank which is one of the molecular detection methods that is quite ideal to determine the kinship between bacteria because the 16S rDNA sequence is a gene found in all microbes and is indispensable in maintaining life.

The sequencing results were used for analysis of the Basic Local Alligment Search Tool (BLAST) program. BLAST is an algorithm for comparing primary biological sequence information, such as amino acid sequences or DNA sequences and RNA sequences. BLAST serves to compare the order of queries with libraries or databases, and identify database sequences that resemble the order of queries. The results of sequence homology analysis with the data base in NCBI are presented in Table 1.

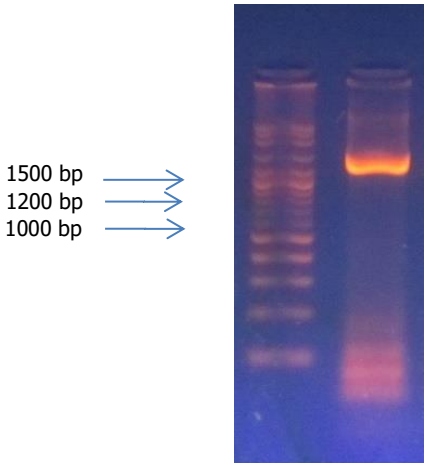

Figure 1. DNA electrophoresis

Table 1. Results of sequence homology analysis with data base in NCBI

No	Description	IDENT	Accession
1	*Lactobacillus fermentum* strain CAU2036 16S ribosomal RNA gene, partial sequence	9.8%	MF42-4967.1
2	*Lactobacillus fermentum* strain 10-18 16S ribosomal RNA gene, partial sequence	98%	KY458550.1
3	*Lactobacillus fermentum* strain FTDC 8312, complete genome	97%	MF992210.1
4	*Lactobacillus fermentum* strain SNUV175, complete genome	97%	LC311073.1
5	*Lactobacillus fermentum* strain BCS9 16S ribosomal RNA gene, partial sequence	97%	KM257692.1

The results of BLAST analysis showed that the 16S rRNA gene sequence of the BR 17 lactic acid bacteria isolate had 97 to 98% similarity to the genome sequence of the Lactobacillus fermentum strain. Within one species there is only a difference of around 3% in the 16S rDNA sequence or it can be said to have a sequence homology of ≥97%. Sequence homology with a value of ≥97% is equivalent to a hybridization similarity of 70%, the minimum value used to state that two bacteria belong to one species (Madigan & Martinko., 2012)

To better see the proximity of BR 17 isolates to several partial sequences and genomes of *Lactobacillus fermentum*, phylogenetic analysis is necessary (Fig.2). Phylogenetic analysis can provide a further picture of the closeness between bacteria. The phylogeny tree is described according to a scale with the length of the branches in the same unit as kinship (Taufiq et al., 2017). The scale length of the tree is 0.0 2, with the branch length in units equal to the evolutionary distance used to infer the phylogenetic tree. Trees are drawn with scales, with branch length measured in the number of substitutions per location (Tilahun et al., 2018) Based on the phylogeny tree, the isolates of lactic acid bacteria BR 17 are clustered with Lactobacillus fermentum strain CAU2036, Lactobacillus fermentum *strain* JCM 7772 and *Lactobacillus fermentum* strain 10-18.

The results of molecular identification of lactic acid bacteria isolate BR 7 from broiler chicken meat are *Lactobacillus fermentum* species. These results are the same as those reported(Lengkey et al., 2009) who get *Lactobacillus fermentum* bacteria from broiler chicken meat (Han et al., 2017). identified isolates of lactic acid bacteria from fermented sausages, as well as *Lactobacillus fermentum* bacteria. Lactobacillus fermentum bacteria as a starter in the manufacture of fermented sauceis (Chen et al., 2016(Chen et al., 2016)(Domínguez et al., 2016).

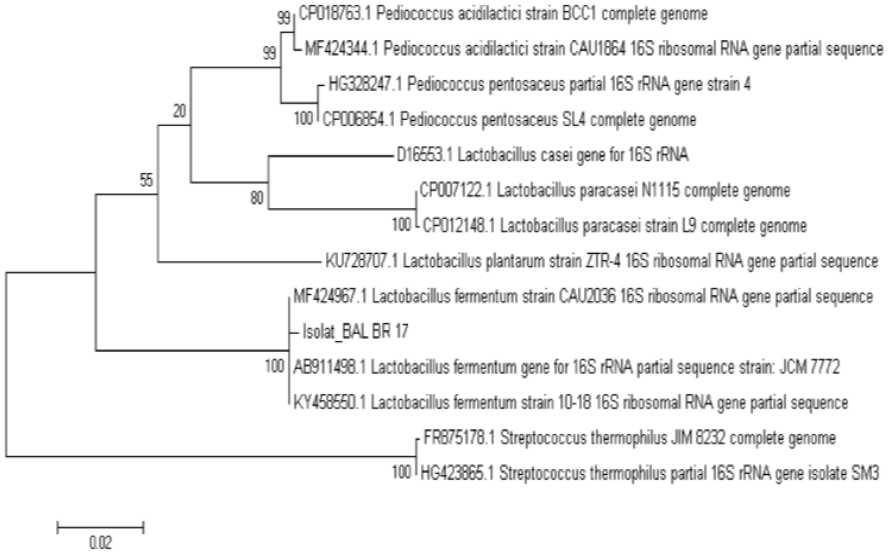

Figure 2. Phylogenetic tree of lactic acid bacteria isolate BR 17

4. CONCLUSIONS

The results of electrophoresis PCR isolate of lactic acid bacteria BR 17 obtained an amplicom measuring about 1.500 bp. sequence homology with data base in NCBI isolate of lactic acid bacteria BR 17 amounting to 97-98% is a strain of *Lactobacillus fermentum*. The phylogeny *tree Lactobacillus fermentum* BR 17 has a close relationship with the sequence in the NCBI database.

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