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# Antioxidant Production Of Lactic Acid Bacteria Isolated From Indonesian Traditional Fermented Buffalo Milk (*Dadih*)

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***Abstract:*** *One of antioxidant sources is foods that contain peptides from enzymatic hydrolysis and/or fermentation by lactic acid bacteria (LAB). The availability of antioxidants in fermented food, particularly milk, is associated with the presence of LAB. Dadih is traditionally made by keeping the fresh raw buffalo milk in a bamboo tube capped with banana leaf and allowing it to ferment spontaneously at room temperature for two days. This research aimed to obtain bacteria from dadih with the highest antioxidants. Isolate Lac 3 and Lac 13 displayed the highest antioxidant activity, with inhibition capacity of 64.2% and 63.79% respectively against DPPH radical. The result showed that 6 best isolates (Lac 3, Lac 13, DS 6, DS 10, DS 12, and DS 14) were not significantly different. Lac 3 was found to be identical with Lactococcus lactic subsp lactic, while Lac 13 was identical with Lactobacillus plantarum with the percentage of similarity of 98% and 96% respectively. Lactococcus lactic subsp lactic displayed the highest antioxidant activity at the early stationary phase and at the lowest pH value of culture.*

***Keywords:*** *antioxidant activity, dadih, lactic acid bacteria, milk*

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## I. Introduction

*Dadih* is fermented milk popular among people of West Sumatra, Indonesia. It is traditionally made by keeping fresh raw buffalo milk in a bamboo tube capped with banana leaf. The milk is left to ferment spontaneously at room temperature for two days [1]. The traditional production of *dadih* does not use any starter and relies completely on indigenous bacteria to do the fermentation. Therefore, the bacteria involved in the process are unknown (spontaneous fermentation). Previous studies reported that *L. plantarum* dominated the bacteria in *dadih* from Solok, West Sumatra [2] while *Lactobacillus sp.*, *Lactococcus sp.*, and *Leuconostoc sp.* were predominant in the one from Bukit Tinggi, West Sumatra [3].

Lactic acid bacteria (LAB) can be a source of natural antioxidants. Some compounds from LAB activities were reported to possess antioxidative activity, such as peroxiredoxin peptide [4], Dpr protein (ferritin-like iron binding protein) [5], lactic acid group [6], and exopolysaccharides [7]. Foods fermented by LAB contain peptide derivatives from enzymatic hydrolysis and fermentation [8, 9, 10]. Coda *et al.* [11] suggested that protein from either enzymatic hydrolysis or fermentation have antioxidative activity. Milk protein is rich in active peptides [12]. Fermentation of milk by LAB produces peptides and amino acids with various functional benefits, e.g. as angiostensin converting enzyme (ACE) inhibitor [13], immune modulator [14], and as antioxidant [15].

A study of the search for natural antioxidants can start with a screening on some *dadih* isolates with the highest antioxidant activity. Laboratory of Applied Genetic Engineering and Protein Design, Biotechnology Research Center, Indonesian Institute of Sciences (LIPI) have isolated and analyzed the diversity of 29 LAB isolates from *dadih*. The results from RAPD (Random Amplified Polymorphic DNA) test suggested 20 isolates with different LAB species. The antioxidant activity of those 20 isolates was unknown. Therefore, screening process would be done to choose the best one out of them.

This research aimed to obtain the LAB isolate from *dadih* with good antioxidative potential, to analyze the time of optimum growth and the optimum pH of LAB isolate culture in producing antioxidant.

## II. Material and methods

### 2.1 Materials

Twenty isolates of dadih from Solok, Bukit Tinggi, and Padang, West Sumatra, Indonesia, 2,2-Diphenyl-1-picrylhydracyl (DPPH), M17 broth media (Oxoid and Himedia), Cetyl trimethylammonium bromide (CTAB) 10%, Sodium Dodecyl Sulfate (SDS) (Sigma), RNase, lysozyme (Sigma), agarose, TBE 1x, loading dye, EtBr (Etidium bromida), ddH2O, dNTPs, buffer QG, buffer PE, nuclease free water, Kappa Polymerase, Primer Forward (5'AGAGTTTGATCCTGCCTCAG 3'), Primer Reverse (5' AAGGAGGTGATCCAGCC 3'), and buffer PCR.

### 2.2 Methods

2.2.1 Screening of LAB

*2.2.1.1 Preparation of bacteria culture [16]*

Isolate (10 µL) was transferred into a test tube containing 0.5% M17 glucose medium (5 mL) and incubated for at least 18 h at 30oC. The incubated culture was then centrifuged using centrifugation tube (FalconR) at 6,000 x g speed at 4oC for 10 min. The supernatant was collected and measured for its antioxidant activity. The measurement was performed only for the supernatant since the highest antioxidant activity belongs to this part [17].

*2.2.1.2 Measurement of antioxidant activity [18]*.

The antioxidant activity was assessed with 2,2-Diphenyl-1-picrylhydracyl (DPPH) method using microplate 96 well. Ascorbic acid 1 mM was used as a positive control. The final volume of each reaction was 200 µL, consisted of 180 µL DPPH in methanol solution and 20 µL sample. The absorbance was measured with microplate reader, Thermo Scientific$^{R}$. Antioxidant activity was determined as percentage of inhibition with the following formula:

$$\%inhibition = \frac{1 - absorbance\ of\ sample}{absorbance\ of\ blank} x100\%$$

Equation (1). Persen Inhibition

*2.2.1.1 Optimization of Bacterial Growth Time [19]*.

The best isolate obtained from antioxidant activity test (1% from total volume of the media) was added into the M17 glucose 0.5% 250 mL . The culture was incubated at 30oC for 48 h. Every 2 h, some bacteria culture was transferred into a 1.5 mL sterile microcup and measured for its absorbance (at 600 nm), pH, and antioxidant activity. The growth of the isolate, pH of the culture, and antioxidant activity during 48 h incubation were plotted in one graph.

2.2.2 Identification of Potential LAB

2.2.2.1 *Genomic DNA Isolation [20]*

Bacteria culture was centrifuged at 6,000 rpm for 5 min. Pellet was collected and separated from the supernatant by using EppendorfR tube, added with buffer TE pH 8 (500 µL). As much as 40 µL of lysozyme (60 mg/mL) was added into the sample and resuspensed. Sample was incubated at 37oC for 75 min, then was added with SDS 10% (200 µL), NaCl 5 M (100 µL), and CTAB 10% (80 µL). Sample was incubated at 68oC for 30 min and shaken up and down every 10 min. Absolute chloroform (700 µL) was added into the sample followed by centrifugation at 13,000 rpm, at 4oC for 10 min. The clear layer on the top (450 µL) was transferred into a new EppendorfR tube. The solution was added with isopropanol 100% (0.6 times of the total volume) at 4oC and incubated at -20oC for 2 h. The solution was centrifuged again at 13,000 rpm at 4oC for 10 min. The obtained pellet was collected and dried overnight at room temperature. The pellet was added with H2O containing RNAse with concentration of 0.1 mg/mL (30 µL), then incubated at 37oC for 30 min. Sample was then subjected to electrophoresis.

2.2.2.2 *Characterization of Genomic DNA with Agarose Gel Electrophoresis [21]*.

Agarose gel 0.8% was prepared by dissolving 0.4 g of agarose in 50 mL of TBE and heating it in oven. The gel was poured into a mould and left to harden. Isolated genomic DNA (5 µL) was mixed with loading dye (2 µL). The electrophoresis was carried out at 100 V for 30 min. The gel obtained in the electrophoresis was stained with EtBr for 15 min. The bands of DNA were visualized by using UV-ray.

2.2.2.3 *Amplification of 16S rRNA Gene [22]*.

Amplification was carried out by using PCR technique. Kappa Taq buffer (10 µL), dd$H_2O$ (33 µL), $MgCl_2$ (3.5 µL), dNTPs (1.5 µL), primer forward with final concentration of 10 µM (0.25 µL), primer reverse with final concentration of 10 µM (0.25 µL), Kappa Taq Polymerase (0.5 µL), and template (1 µL) were mixed in a microtube and homogenized with capsulefuge. *S*ample was then subjected to PCR with 30 cycles. The PCR program used was predenaturation at 94$^{o}$C for 5 min, denaturation at 94$^{o}$C for

1 min, annealing at 45°C for 1 min, extension at 72°C for 3 min, and final extension at 72°C for 3 min. The result was used for agarose gel electrophoresis with gel concentration of 1.5%. The resulting bands were then visualized with UV Transilluminator.

2.2.2.4 *Purification of 16S rRNA from Gel (Qiagen gel purification kit).*

Electrophoresis gel with specific band resulted from PCR was cut and weighed. Gel was dissolved in buffer QG (3x volume) at 45-50oC for 10 min. Sample was pipetted and put into column to be centrifuged at 13,000 rpm for 1 min, then the flow through liquid was removed. Buffer PE (750 µL) was added and re-centrifuged for 1 min at 13,000 rpm. The bin, where the flow through liquid was collected, was replaced with the new sterile tube containing 30 µL ddH2O . Sample was incubated at room temperature for 2 min, then centrifuged at 13,000 rpm for 2 min to get pure DNA.

2.2.2.5 *Sequencing and Analysis of Base Order (1st Base).*

Sequencing of bacterial DNA was conducted in company of 1st Base. The result (in the ab1 format) was processed with BioEdit program, stored in the fasta format, and was identified using BLASTN tools in NCBI cites.

## III. Results

### 3.1 Antioxidant Activity of Isolates

The results revealed 3 isolates with the highest antioxidant activity, isolate Lac 3, Lac 13, and DS 6. All isolates displayed antioxidative activity, each at different level (Fig. 1).

### 3.2 Identification of Potential LAB

The obtained sequence of 16S rRNA was analyzed using BLAST program. The observation showed that isolate Lac 3 had a 98% similarity to *Lactococcus lactis* subsp *lactis* while isolate Lac 13 had a 96% similarity to *L. plantarum*. The result of the observation and accesion number are presented in Table 1. Amplification result of 16S rRNA gene from Lac 3 and Lac 13 genomes is presented in Fig. 2.

### 3.3 Growth Curve, pH Decrease, and Antioxidant Activity of Isolate Lac 3

Isolate Lac 3 was selected for its highest antioxidant activity among others. Fig. 3 illustrated 3 curves of isolate Lac 3; growth curve, curve of pH decrease, and curve of antioxidant activity.

## IV. Discussion

### 4.1 Screening of LAB

Isolate Lac 3 displayed the highest antioxidant activity with 64.25% inhibition, followed by Lac 13 and DS 6 with 63.79% and 63.32% inhibition respectively (Fig. 1). Meanwhile Lac 5 showed the lowest activity (30.81% inhibition). The antioxidant activity of five isolates (Lac 3, Lac 14, DS 6, DS 10, DS 12, and DS 14) was not significantly different to each other, but was to the other isolates. Ascorbic acid as control was found to have inhibition value of 92.66%.

The antioxidants observed in supernatant could originate from protein breakdown in the medium or from bacterial secretion. The supernatant of the isolate contains extracellular compounds secreted by bacteria [23, 24]. This supernatant shows the highest antioxidant activity of the LAB [17]. Additionally, Wu *et al*. [24] suggested that sample from bacterial extracellular secretion has the highest antioxidant activity compared to cell sample.

Peptides with biological activities can be synthesized through enzymatic hydrolysis or fermentation [8, 9, 10]. Fermentation by LAB produces peptides of 4-20 kDa which play a role in radical scavenging activity [25]. Some LAB such as *L. plantarum* produce antibacterial protein called plantaricin. Plantaricin IIb, a class of plantaricin, is of ≤ 10 kDa and is heat resistant [26]. Sogandi *et al.* [19] purified plantaricin from *L. plantarum* U10, obtaining protein of 4.5-9.8 kDa. Marzani *et al.* [27] stated that plantaricin A (Pln A), a type of plantaricin, can increase the antioxidant activity in human keratinocyte NCTC 2544 cells. Its antioxidative activity is presumably due to its molecular size as antioxidative proteins are all of 4-20 kDa. Such compounds or cellular products make LAB a potential source for antioxidant development.

LAB also has peptide compartment which could directly act as antioxidant, such as peroxiredoxin [4]. Besides, there is also ferritin-like iron binding protein (Dpr) which serves as new antioxidative protein in LAB [5].

The antioxidants produced by *L. plantarum* have been identified as L-3-(4-hydroxyphenyl) lactic acid (HPLA) and L-indol-3-lactic acid [6] while that from *Lactococcus lactis* have been known to be its exopolysaccharide [7].

### 4.2 Identification of Isolate Lac 3 and Lac 13

For further identification, isolate Lac 3 and Lac 13 were selected for their highest antioxidant activity among the six isolates which were statistically indifferent (Fig. 1). Genome isolation was carried out for Lac 3 and Lac 13, following the procedure of Romero and Goni [20].

Results from the amplification of 16S rRNA gene from Lac 3 and Lac 13 genomes are presented in Fig. 2. The two bands resulted from the amplification were of ±1500 bp in size (Fig. 2). According to Patel [28], the size of 16S rRNA gene is 1500 bp and it contains a lot of genetic information. The nucleotide sequence of the 16S rRNA band was determined through sequencing with BLAST tools in NCBI. Isolate Lac 3 was revealed to be identical with *Lactococcus lactis* subsp *lactis*, while isolate Lac 13 was identical with *L.plantarum*.

*Lactococcus lactis* and *L. plantarum* belong to LAB. This group of bacteria is widely used as probiotic which is beneficial for the ecosystem of intestinal flora [29]. LAB has been known to produce antimicrobial compounds such as hydrogen peroxide and bacteriocins which could kill or inhibit the growth of pathogenic bacteria [30].

*L. plantarum* displays antioxidant activity either in the culture, extracellular, and intracellular level [17]. Fermentation by *L. plantarum* has showed potential to improve the functionality of antioxidative enzymes and to reduce the oxidative stress induced by hyperlipidemia [29]. Meanwhile, the exopolysaccharides produced by *Lactococcus lactis* could serve as antioxidant [7]. *Lactococcus lactis* subsp. *lactis* grow well with the presence of glucose, acetate, vitamin, and 8 amino acids in the media [31].

### 4.3 Growth Curve, pH Decrease, and Antioxidant Activity of Isolate Lac 3

The growth curve of isolate Lac 3 (Fig. 3) showed that the log phase started after 2 h of incubation. The exponential phase took place after 10 h until 26 h of incubation. After 26 h, stationary phase started and was still observed until 48 h. The death phase was yet to be seen in this study due to the short incubation time. The medium's pH decreased from 7.2 at the start of the incubation to 5.8. Significant pH decrease occurred during exponential phase after being nearly constant at around 5.8 during stationary phase. The antioxidant activity also increased rapidly in the exponential phase. The highest and the constant antioxidant activity were displayed in the early stationary phase, but tended to decrease after 40 h.

Isolate Lac 3 was used in the analysis of growth curve, since isolate Lac 3 and Lac 13 were not statistically different based on the previous statistical test. Isolate Lac 3 showed similarity with *Lactococcus lactis.* The result showed that antioxidant activity increased during exponential phase and tended to be high in the beginning of stationary phase (Fig. 3). The increase of antioxidant activity was followed by the decrease of culture pH due to production of lactic acid by the bacteria [32]. It gives an indication that the antioxidant present in the culture is in the form of acid from bacterial activities.

*Lactococcus lactis* grow in the medium that contains at least glucose, acetic acid, vitamin, and 8 amino acids [31]. The addition of 19 amino acids could increase the growth rate of *Lactococcus lactis.* The bacteria grow well at 30-37$^{o}$C. Incubation temperature and pH of the medium could influence the metabolites produced while the observed fermentation process (growth curve) remained similar. Incubation in maltose at pH of 5.0 and temperature of 37$^{o}$C reduced the fermentation products and cell mass compared to when pH was 6.5 and incubation temperature was 30$^{o}$C. In addition to a decrease in cell mass, incubation at 37$^{o}$C would result in the increase of lactic acid, but the decrease of other substances such as formic acid, acetic acid, and ethanol [32]. Another study by Ăkerberg *et al.* [33] reported that the formation of lactic acid by *L. lactis 19435* occurred at incubation temperature of 33.5$^{o}$C. This indicated that incubation temperature is influential to the formation of bacterial metabolites.

The antioxidant activity of LAB comes from the lactic acid they produced, although other substances might as well contribute to it. Production of lactic acid by this group of bacteria is affected by the incubation temperature, nutrient availability, pH of medium, and other factors. The completeness of nutrition, particularly the carbon source, is influential to the production of antioxidant by LAB [34]. The presence of antioxidant in the culture medium was proven to promote the growth of the bacteria [35].

## V. Figures and Tables

**Table 1**. Equalization result of 16S rRNA isolate gene sequence in NCBI

| Isolate | Sequence size (pb) | Species | Identity (%) | Accesion number |
|---|---|---|---|---|
| **Lac 3** | 1166 | *Lactococcus lactis subsp.lactis* strain NM141-1-16S ribosomal RNA gen, partial sequence | 98 | HM218559.1 |
| **Lac 13** | 1197 | *Lactobacillus plantarum* strain L601(LBF2)F02 16S ribosomal RNA gen, partial sequence | 96 | KM269713.1 |

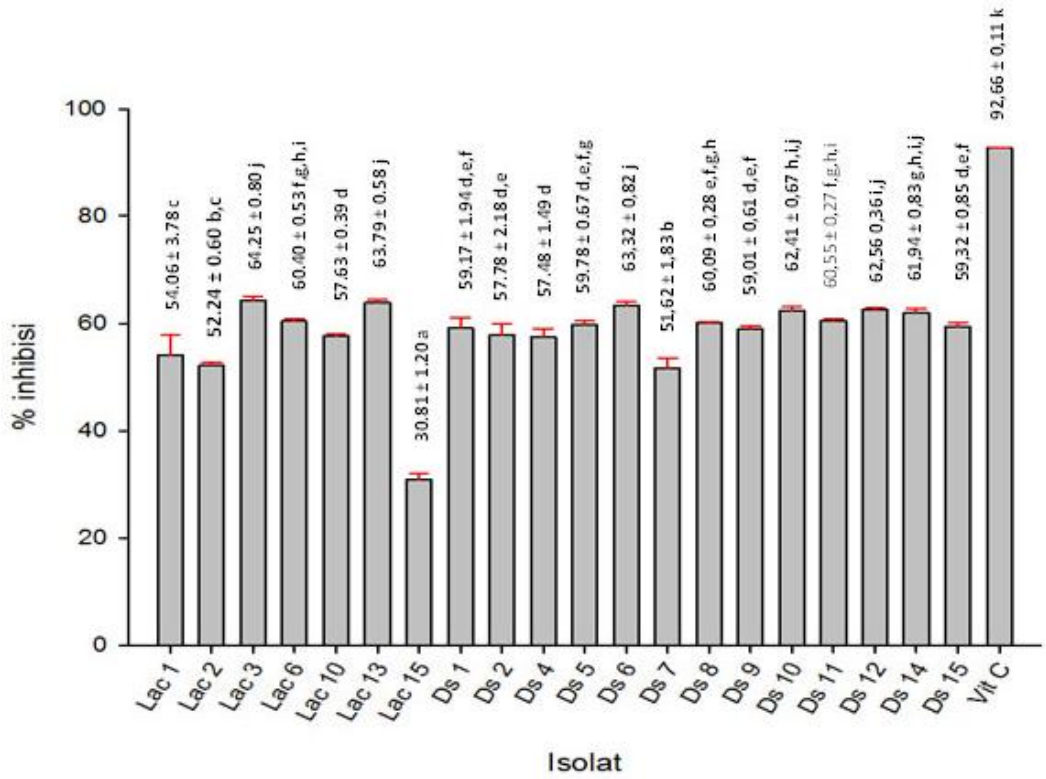


**Fig 1**. Antioxidant activity of isolates

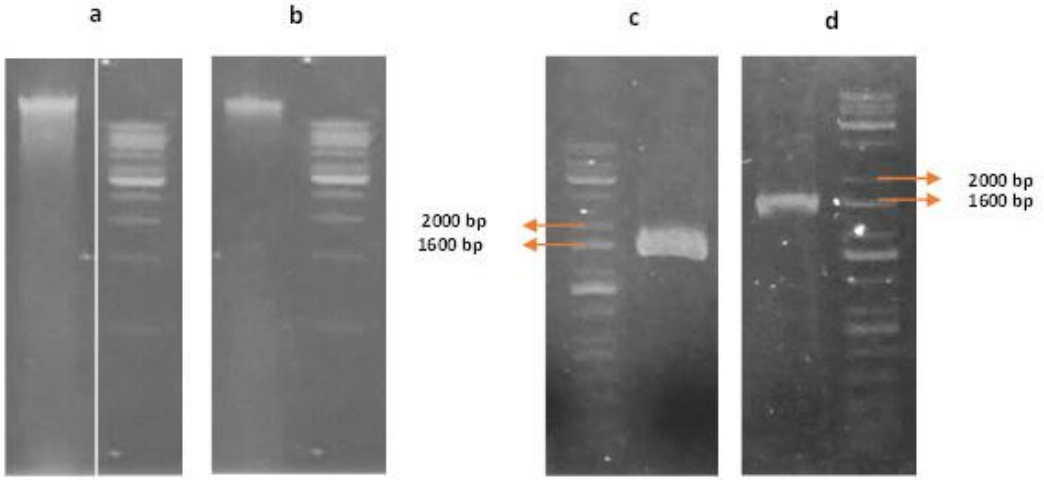


**Fig 2.** Genomic DNA isolation of isolate Lac 3 (a), Lac 13 (b) and amplication result of 16S rRNA for Lac 3 (c) and Lac 13 (d) with size of ± 1500 bp

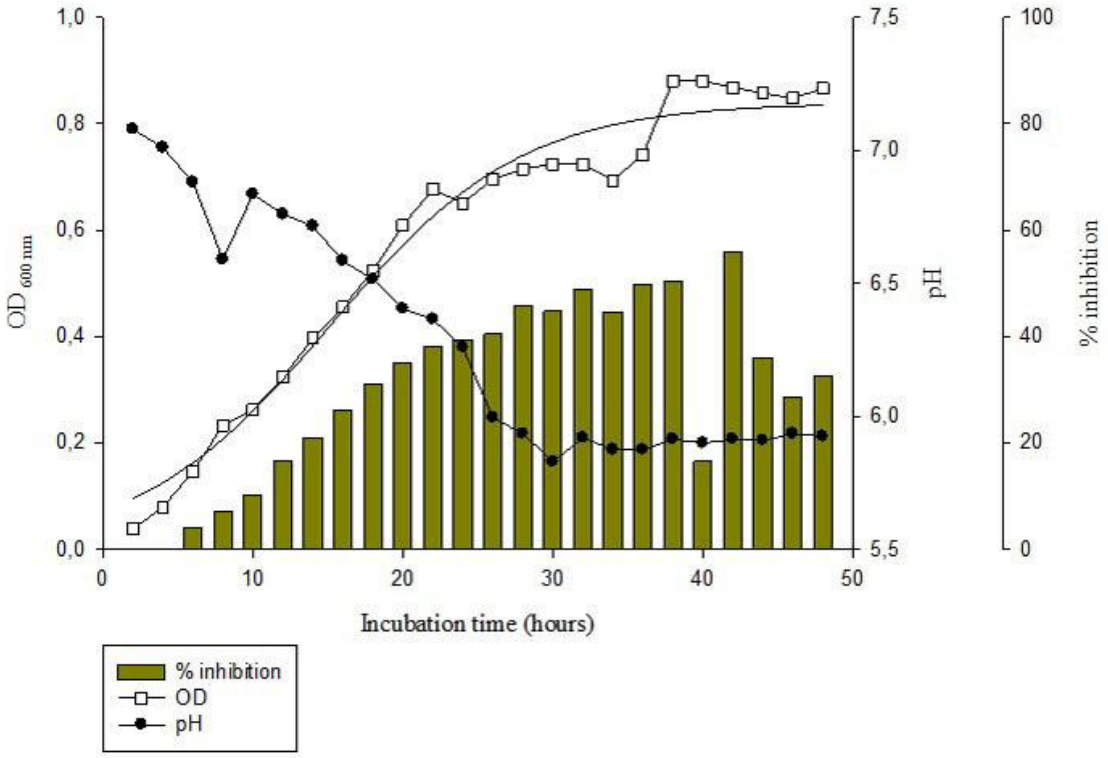


**Fig 3.** Growth curve, pH change, and antioxidant activity of isolate Lac 3

## VI. Conclusion

*Dadih* could be utilized as a good source of natural antioxidant, which was available as the product of the lactic acid bacteria involved during the making of *dadih*. Lac 3 and Lac 13 were two lactic acid bacteria isolates from *dadih* with the highest antioxidant activity. Lac 3 was found to be identical with *Lactococcus lactic* subsp *lactic*, while Lac 13 was identical with *L. plantarum* with the percentage of similarity of 98% and 96% respectively. *Lactococcus lactic* subsp *lactic* displayed the highest antioxidant activity in the early stationary phase and at the lowest pH value of culture.

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