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Potential antioxidant activity of *Lactobacillus fermentum* KF7 from virgin coconut oil products in Padang, West Sumatra, Indonesia

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Abstract. *Syukur S, Safrizayanti, Zulaiha S, Supriwardi E, Fahmi A, Nurfadilah KK, Purwati E. 2022. Potential antioxidant activity of* Lactobacillus fermentum *KF7 from virgin coconut oil products in Padang, West Sumatra, Indonesia. Biodiversitas 23: 1628-1634.* The isolation and characterization of five commercial Virgin Coconut Oil (VCO) products circulating in the local market of Padang city were conducted. In this study, eighteen isolates of lactic acid bacteria (LAB) were isolated and characterized microscopically, and biochemically. VCO-LAB isolates were Gram-positive bacilli and cocci, catalase-negative, and homofermentative (except VD was heterofermentative). There were three isolates of VCO-LAB (VB.3, VD and VE.4) that could produce gamma-aminobutyric acid (GABA). The highest concentration of GABA (19.5 mg/mL) was produced by isolate VB.3 after 72 hours of incubation and the addition of 7% MSG. VB.3 isolate shows the highest antioxidant activity with the addition of Monosodium glutamate (MSG), 68.13% for 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ABTS and 88.02%. In addition, GABA-producing VCO-LAB could be grown in media containing 0.2-1.0 mM hydrogen peroxide, with the lowest optical density value of 0.82 nm. The antioxidant activity of VCO-LAB was affected by the GABA produced and by secondary metabolites such as peptides and antioxidant enzymes (NADH-oxidase, superoxide dismutase, NADH peroxidase and non-heme catalase). The highest antioxidant production was obtained in isolate VB.3 or *Lactobacillus fermentum* KF7.

Keywords: Antioxidant, Gamma-aminobutyric acid, *Lactobacillus fermentum* KF7, VCO

INTRODUCTION

Virgin Coconut Oil (VCO) can be made using several procedures such as fermentation, cold press without added chemicals (Syukur et al. 2018). VCO has several advantages as a supplement, such as increased High-Density Lipoprotein (HDL), as anti-cholesterol and producing energy metabolism (Harini and Astirin 2009; Syukur et al. 2017). Several LAB has been found in VCO fermentation methods that possess antibacterial activity against S*taphylococcus aureus* and *Listeria monocytogenes* (Syukur et al. 2018; 2021). Several reports concerning Lactic Acid Bacteria as potential probiotic in buffalo milk (Dadih) (Syukur et al. 2015; 2016) and Cacao fermentation (Syukur et al. 2013), tempoyak (Hendry et al. 2021) and palm sugar (Rahmadhanti et al. 2021) makes probiotic research will be increasing. Probiotic *Lactobacillus fermentum* KF7, has antioxidant activity of gamma-aminobutyric acid (GABA) in this paper is activated by the activation of the Nrf2 (nuclear factor-E2 related factor 2) molecule which can increase gene transcription of antioxidant enzymes such as glutathione peroxidase (GPx) and superoxide dismutase (SOD) (Chen et al. 2013). GABA can inhibit lipid peroxidation, reduce the content of malondialdehyde (MDA) and increase the activity of antioxidant enzymes such as GPx, SOD and catalase (Chen et al. 2013; Di Lorenzo et al. 2016).

GABA is a non-protein amino acid resulting from glutamate decarboxylation catalyzed by the enzyme glutamate decarboxylase (GAD). GABA is also a major inhibitory neurotransmitter in the central nervous system. GABA deficiency can cause several diseases such as Huntington's, Parkinson's, Alzheimer's, schizophrenia and depression (Diana et al. 2014).

One compound that has antioxidant activity and has the potential to be developed is gamma-aminobutyric acid (GABA), GABA is formed by an irreversible -decarboxylation reaction of L-glutamic acid or its salt catalyzed by the enzyme glutamate decarboxylase (GAD; EC 4.1.1.15).

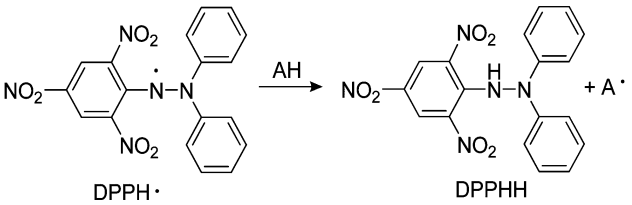

Figure 1. DPPH reaction and antioxidant compound

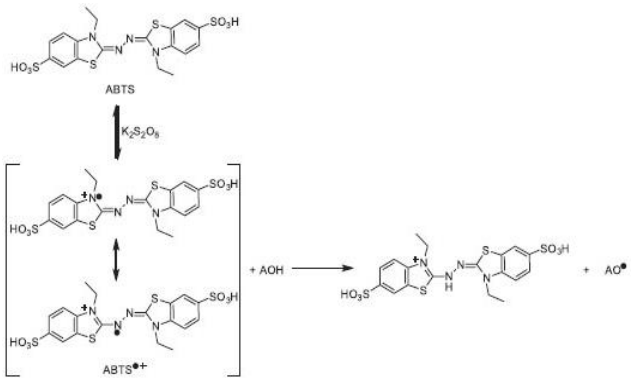

Figure 2. ABTS oxidation by potassium per-sulfate producing radical ABTS•$^{+}$ and the reaction with antioxidant compound

Several studies used Fenton's solution to form hydroxyl radicals and then tested the sample's antioxidant activity against these hydroxyl radicals Spectrophotometrically (Arasu et al. 2013; Das and Goyal 2014).

The principle of the DPPH method is based on the ability of antioxidant compounds to donate hydrogen to DPPH free radicals (Purwaningsih 2012). Compounds that are active as antioxidants will reduce DPPH free radicals to DPPHH (non-radical diphenylpicrylhydrazine compounds) as shown in (Figure 1). The antioxidant activity was indicated by a color change from purple to yellow and the absorbance was measured using a UV-Vis Spectrophotometer at a wavelength of 517 nm.

In this study, we evaluated the antioxidant activity of *L. fermentum* KF7 against DPPH. Fenton's reagent is a solution of hydrogen peroxide with iron as a catalyst to oxidize contaminants. Iron (II) is oxidized by hydrogen peroxide to iron (III), forming hydroxyl radicals and hydroxide ions. Fenton reaction and homolytic fission can be seen in the following reaction.

Fenton Reaction:

$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO\bullet + OH^-$

UV

Homolytic Reaction:

$H—O—O—H \longrightarrow 2HO\bullet$

(Das and Goyal 2014; Arasu et al. 2013).

Antioxidant activity test against free radicals ABTS produced by oxidation of potassium per-sulfate in ABTS before the addition of antioxidant compounds as shown in Figure 2. The ABTS•$^{+}$ method can be measured based on the ABTS•$^{+}$ decolorization (Han et al. 2017). ABTS•$^{+}$. The ABTS•$^{+}$ method can be measured based on the removal of ABTS•$^{+}$ color by measuring the absorbance spectrophotometrically at a wavelength of 734 nm. Percentage of antioxidant activity was measured with the equation = [(A0-A1)/A0] x 100 where A0 and A1 were the absorbance of the control (ABTS•$^{+}$ solution) and the sample, respectively (Han et al. 2017).

MATERIALS AND METHODS

Sampling

This study used 5 VCO Commercial products chosen by random sampling in local markets of Padang City, West Sumatra, Indonesia (00°44'00"-01°08''35"S, 100° 05'05"-100° 34'09"E).

Isolation and purification of LAB from 5 VCO commercial products

Isolation of LAB was carried out by the serial dilution-agar plate method. The VCO sample was diluted in MRS MERCK de-Mann Rogosa Sharpe (MRS) Broth (1:9, v/v) and incubated anaerobically at 37ºC for 24 hours. Serial dilution was carried out up to 10-8, spread on MRS Agar, and then anaerobically set at 37ºC for 48 hours. Single colonies that were round convex, shiny and yellowish-white in the color that grew separately with different diameter sizes were re-inoculated on MRS Agar by the scratch method to obtain pure isolates of VCO-LAB (LAB isolated from VCO). After purification, the isolate was stored at -80ºC in a mixture of MRS Broth and glycerol (4:6, v/v). The culture stock should be re-grown in MRS Broth (1:9, v/v) for 18-24 hours before being used for the next stage of the study (Syukur et al. 2013).

Macroscopic, microscopic and biochemical characterization of LAB-VCO

Macroscopic characterization was carried out by inoculation of LAB culture on MRS MERCK de-Mann Rogosa Sharpe (MRS) Agar to see LAB isolates shape, color, and convexity. Next, microscopic characterization was carried out through Gram staining to see the cells' shape and color. Then, the biochemical characterization was carried out utilizing a catalase test and a fermentation type test (Syukur et al. 2018).

Measurement of growth of VCO-LAB

VCO-LAB was cultivated in MRS Broth at 37ºC for 24 hours. The LAB growth was determined by calculating its optical density at a wavelength of 600 nm with an interval of 2 hours until a stationary phase was obtained (Li et al. 2010).

Analysis of VCO-LAB GABA producers by Semi-Quantitative method

VCO-LAB, which showed spot GABA in qualitative analysis, was analyzed semi-quantitatively with KK pre-staining method according to Li et al. (2009) procedure with modifications. VCO-LAB cultures were grown in MRS Broth with various MSG MERCK concentrations (1,

3, 5 and 7%) and incubation time (24, 48 and 72 hours) anaerobically at 37ºC. 2 L of the LAB culture supernatant was spotted on the chromatography paper. The separation process was carried out for 3 hours in the mobile phase of n-butanol: acetic acid:aquades (5:3:2) containing 1.2% ninhydrin. The chromatography paper was then dried at 70ºC for 80 minutes for spot color visualization. Spot GABA was cut from the paper and extracted with 5 mL of 75% alcohol (v/v):0.4% copper sulfate (w/v) (38:2, v/v) at a speed of 50 rpm and a temperature of 40ºC for 1 hour. The absorbance of the sample was measured with a UV-Vis spectrophotometer PERKIN ELMER at a wavelength of 512 nm. Before working on the sample, the absorbance measurement of the standard GABA solution was measured using the KK pre-staining method as described. A calibration curve was made to obtain the equation y = ax + b. The absorbance of the GABA extraction results in the sample could be determined by entering the absorbance value into the equation of GABA standard solution regression.

Antioxidant activity of GABA-producing VCO-LAB against DPPH

According to Lee et al. (2010), the antioxidant activity was conducted with a few modifications. A 100 L of 0.4 mM DPPH solution was mixed with 100 L of LAB culture (with or without the addition of MSG). The mixture was incubated at 37ºC in the dark for 30 minutes and then the absorbance was measured at a wavelength of 517 nm using a microplate reader. Antioxidant activity of the sample (%) = [(A0-A1)/A0] x 100, where A0 and A1 are absorbance control (DPPH solution) and sample, respectively.

Antioxidant activity of VCO-LAB, GABA producer, against ABTS•

The antioxidant activity was conducted according to Lee et al. (2010) with a few modifications. ABTS•+ was diluted in aquabides until the absorbance value was 0.70 ± 0.01 at a wavelength of 734 nm. Next, 900 L of the ABTS•+ solution was mixed with 100 L of LAB culture (with or without the addition of MSG). The mixture was incubated at room temperature in the dark for 6 minutes. The absorbance was measured at a wavelength of 734 nm and the antioxidant activity of the sample (%) = [(A0-A1)/A0] x 100 was calculated, where A0 and A1 were the absorbance of the control (ABTS•+ solution) and the sample, respectively (Arasu et al. 2013).

Antioxidant activity of VCO-LAB, GABA-producer, against hydroxyl free radical

According to Arasu et al. (2013), the antioxidant activity procedure was carried out with slight modifications. Fenton's reaction mixture consisting of 1 mL brilliant green (0.435 mM), 2 mL FeSO4.7H2O (0.5 mM), 1.5 mL H2O2 (3%, w/v) was added to 1 mL of LAB culture (with or without the addition of MSG). The mixture was incubated at room temperature for 15 minutes and the absorbance was read at a wavelength of 624 nm. The antioxidant activity of the sample is calculated in (%) = [(As-A0/A-A0)] x 100, where A0, A and As are control's absorbance (Fenton's solution without sample), blank's absorbance and absorbance of the mixture of Fenton and sample, respectively.

Resistance of VCO-LAB, GABA producer, to hydrogen peroxide

LAB cultures were grown in MRS Broth (1:9, v/v) and MRS Broth containing 0.2, 0.4, 0.6, 0.8 and 1.0 mM hydrogen peroxide. The mixture was incubated at 37ºC for 8 hours. The growth of BAL cells was calculated Spectrophotometrically at a wavelength of 600 nm (Arasu et al. 2013).

Data analysis

The data were obtained from three analysis times and shown as mean ± standard deviation (SD). Statistical significance was determined by One Way Analysis of Variance (ANOVA). The Turkey method determined the significant difference between the means results and the $p<0.05$ was expressed as the significant level.

RESULTS AND DISCUSSION

Data displayed in the average respondent's answers on organoleptic testing of 5 VCO commercial products circulating in Padang City (VA, VB, VC, VD and VE).

Based on Table 1, it can be seen that VA and VB have better color, aroma and taste characteristics than the other 3 products. The diverse characteristics and organoleptic of these 5 commercial VCO products may be due to differences in the processing methods (Anwar and Irmayanti 2020). VCO can be made by various methods such as controlled heating, fermentation (by using microbes or enzymes), centrifugation, fishing and the addition of acetic acid. Each manufacturing process shows different VCO qualities (Setiaji and Prayugo 2006). In this study, 18 colonies have been isolated and 17 VCO-LAB isolates were homofermentative and the other 1 were heterofermentative as shown in Figure 3.

Qualitative analysis of GABA-producing VCO-LAB

VCO-LAB is qualitatively analyzed for its ability to produce GABA by thin-layer chromatography method, with stationary phase and motion phase respectively being TLC plates silica gel 60 F_{254} and a mixture of *n*-butanol: acetic acid: water (5:3:2). This method is widely used because there are no expensive chemicals and special pre-treatment on samples. Some other qualitative analysis methods such as pH indicator method and enzyme-based microtiter plate assay (EBMPA) are known to require a long time of work and expensive materials, such as GABase enzymes (Li and Cao 2010).

GABA, a non-protein amino acid resulting from decarboxylation of glutamate catalyzed by the enzyme glutamate decarboxylase (GAD) can be produced by LAB because LAB has GAD enzyme activity in its cells . GAD, an intracellular enzyme of LAB, can convert GABA from its substrate in the form of glutamate or salt. This study used monosodium glutamate (MSG) as a GAD enzyme substrate in producing GABA. Generally, LAB produces

maximum GABA at the end of the stationary phase, where acidity and nutrient deficiencies will affect the metabolic activity. This acidic condition activates the enzyme GAD, thus catalyzing the formation of GABA in the cytoplasm and then secreted to a culture media (extracellular GABA (Diana et al. 2014), GABA content in VCO-LAB in this study was determined through its extracellular GABA content in VCO-LAB culture supernatant.

In this qualitative analysis, 18 isolates cultivated in MRS Broth containing 1% MSG and GABA were determined in culture supernatant using paper chromatography. 3 LAB isolates (VB.3, VD and VE.4) were selected, and the paper chromatogram can be seen in (Figure 4). The chromatogram showed that 3 selected supernatant culture isolates showed a more pronounced GABA spot than other isolated culture supernatants. Results that are not much different are also obtained by Lee et al. (2010) selected 22 LAB isolates, 6 of which showed the ability to produce GABA based on the clarity of the spot on the paper plate. Furthermore, the ability to produce GABA by VB.3, VD and VE.4 isolates is determined semi-quantitatively based on variations in incubation time (24, 48 and 72 hours) and variations in MSG concentrations (1, 3, 5 and 7%) in MRS Broth with the paper chromatography pre-staining method.

Analysis of VCO-LAB GABA producer

GABA-producing VCO-LAB is quantitatively analyzed by pre-staining methods of paper chromatography (according to Li and Cao (2010). GABA concentrations in VCO-LAB cultures are calculated by entering the absorbance value obtained in the GABA standard solution regression equation y = 0.0464x + 0.0292 (R2 = 0.9974). GABA produced by LAB VB.3, VD and VE.4 isolates with variations in incubation time and MSG concentration can be seen in Table 2. GABA concentrations continue to increase for up to 72 hours of incubation and 7% MSG. Based on the growth curve in Figure 4, the isolate is still in the static phase until the 72 hours except the VE.4 isolate which has started to enter the death phase at 2 hours). It has been mentioned earlier that GABA is produced maximum at the end of the stationary phase, this underlies the highest GABA concentration obtained at the 72 hours. In addition to incubation time, MSG concentrations also affect GABA production by LAB. This is due to an increase in substrates around the cytophilic enzyme GAD.

Based on Table 2, almost all VCO-LAB isolates showed a similar tendency in producing GABA with the highest concentrations obtained after 72 hours of incubation. However, there was a decrease in GABA concentration in VE.4 isolates after 72 hours of incubation. This fact may be due to the VE-4 isolate entering the death phase, where the cell quickly loses the ability to divide and die in a matter of hours.

The highest concentrations of GABA were produced by VB.3 isolates at 72 hours of incubation and 7% of the concentration of Mono Sodium Glutamate (MSG) in MRS Broth (19.5 mg/mL), which was significantly different from GABA concentrations produced by other isolates. This result is higher than *L. brevis* OPY-1 and *L. brevis* OPK-3 isolated from kimchi with GABA concentrations of 0.825 mg/mL and 2,023 mg/mL Also *L. brevis* BJ20 from seaweed fermentation with a GABA concentration of 2,465 mg/mL (Lee et al. 2010); *L. brevis* IFO with GABA concentration of 1,049 mg/mL and *L. brevis* CECT8183 were isolated from Spanish-made cheeses with GABA concentrations of 0.1 mg/mL. The high concentration of GABA by VB.3 is due to the high activity of GAD enzymes in their cells. The VB.3 isolate cultured in MRS Broth with 7% MSG continued for testing its activity as an antioxidant.

GABA-producing VCO-LAB antioxidant activity against ABTS•$^{+}$

The antioxidant activity of GABA-producing VCO-LAB in this method is based on its ability to donate electrons or hydrogen atoms to $ABTS^{\bullet+}$. $ABTS^{+}$ accepts these electrons or hydrogen atoms and becomes a stable. The antioxidant activity of VCO-LAB cultures containing MSG in its growth medium is significantly higher than that of cultures without MSG $P<0.05$, (Figure 5). The 3 highest antioxidant activity of VCO-LAB is indicated by VB.3+MSG (68.13%), VD+MSG (61.66%) and VD (52.89%) which is higher than *Pediococcus pentosaceus* R1 (Han et al. 2017) and LAB from kimchi with antioxidant activity against ABTS•$^{+}$ is 42.4% and above 50% respectively.

Figure 5, shows that antioxidant activity in cultures with the addition of MSG is higher than in cultures without MSG. This indicates that GABA produced in cultures affects the antioxidant activity of VCO-LAB. However, cultures without MSG also showed high antioxidant activity (52.89-43.7%). Based on this it can be said that the antioxidant activity of VCO-LAB is not only influenced by the GABA it produces, but also by secondary metabolites. Based on several tests of antioxidant activity as described, it can be said that GABA affects the antioxidant activity of VCO-LAB. The highest antioxidant activity is produced by VCO-LAB with the addition of MSG in its growth medium. The addition of MSG aims as a substrate for GAD enzymes to produce GABA. However, since the growth medium of VCO-LAB without MSG also indicates antioxidant activity, it can be concluded that the antioxidant activity of VCO-LAB is not only derived from GAB A, but also by other metabolites such as peptides (Komatsuzaki et al. 2005) and antioxidant enzymes produced by LAB such as NADH-oxidase, superoxide dismutase, NADH per-oxidase and non-heme catalase (Arasu et al. 2013).

Table 1. Characteristics of 5 commercial VCO products in Padang City

VCO Products	Characteristics		
	Colour	Flavor	Taste
VA	Very clear	Coconut Scent	Pretty good
VB	Very clear	Coconut Scent	Pretty good
VC	Clear	Slightly rancid	Pretty good
VD	Clear	Slightly rancid	Not good
VE	Clear	Slightly rancid	Not good

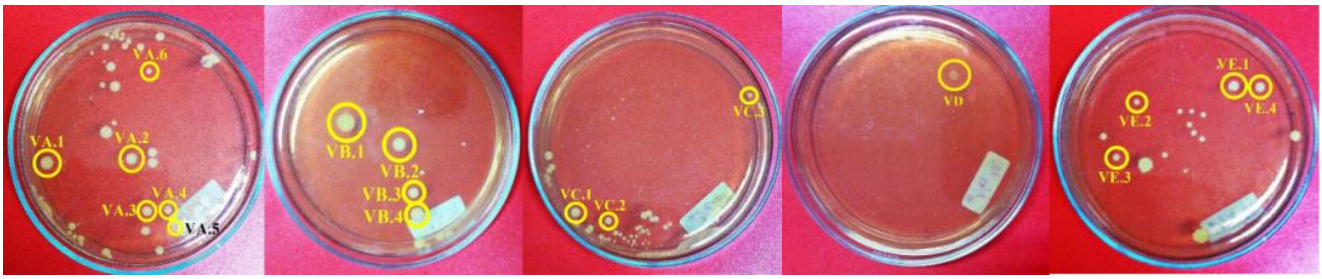

Figure 3. Eighteen (18) LAB isolates from 5 VCO Commercial Products (VCO-LAB) isolated by the serial dilution-agar plate method. A single colony that characterizes LAB was selected a VCO-LAB Panels A to E show 6 VA isolates, 4 VB isolates, 3 VC isolates, 1 VD isolate, and 4 VE isolates

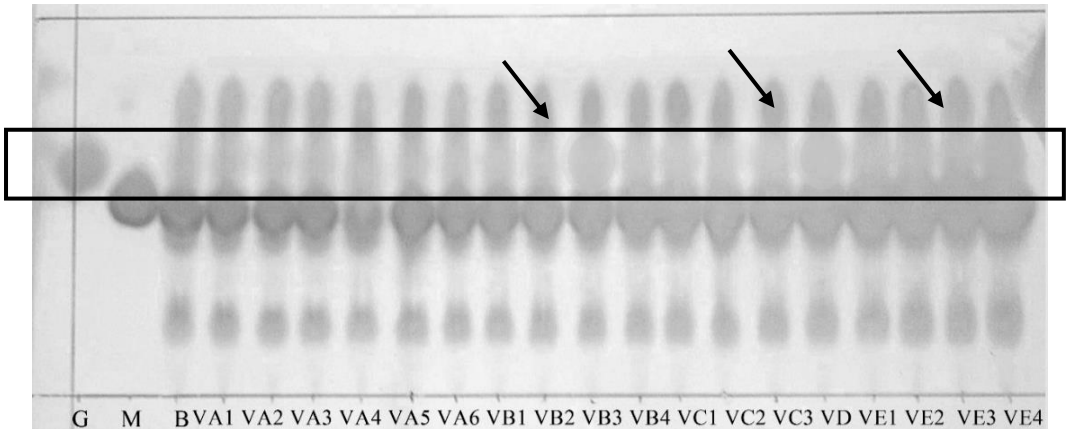

Figure 4. TLC chromatogram for qualitative analysis of VCO-LAB, producer of GABA. VCO-LAB was cultivated for 72 hours in MRS Broth containing 1% MSG and then spotted on a TLC plate. Spot 1 (G): standard GABA (1 mg/mL); spot 2 (M): standard MSG (1 mg/mL); spot 3 (B): MRS Broth + MSG; spot 4 to spot 21: 18 isolates VCO-LAB

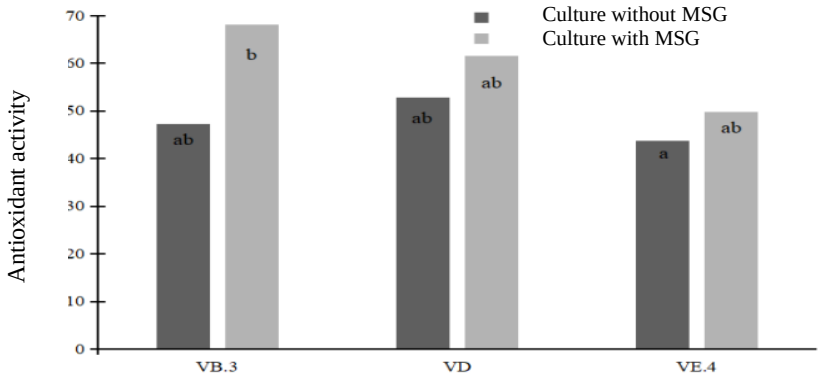

Figure 5. Antioxidant activity of VCO-LAB (with or without the addition of MSG) against ABS (%). The data is shown in the average of 3 repetitions of the measurement of antioxidant activity of VCO-LAB. Superscripts with different letters showed significantly different results ($P<0.05$)

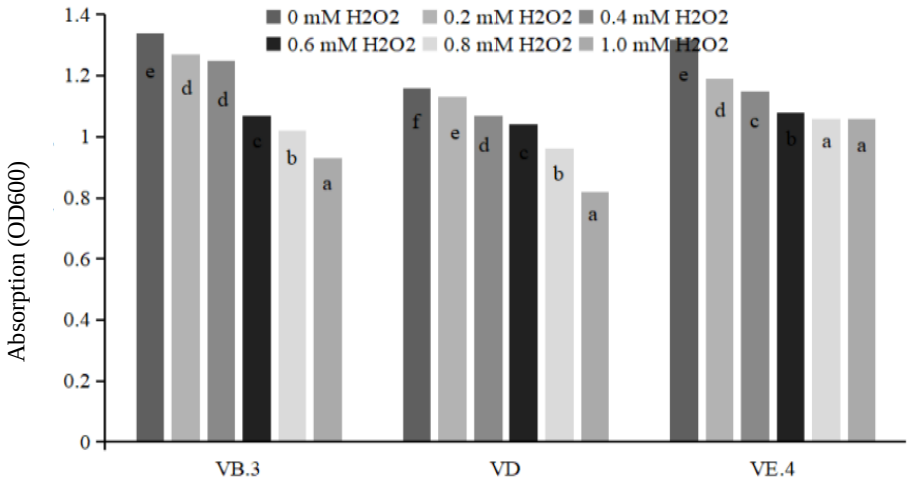

Figure 6. Resistance of o various concentrations of hydrogen peroxide. The data is shown in the average of 3 repetitions of the measurement of the resistance of VCO-LAB to H2O2. Superscripts with letters different showed significantly different results (P<0.05)

Table 2. Concentration of GABA-Producing at Variation of Incubation Time (24, 48 and 72 hours) and MSG Concentration (1, 3, 5 and 7%)

LAB isolate	GABA Concentration (mg/mL)*		
	24 hours	48 hours	72 hours
VB.3 + 1%MSG	0.45 ± 0.00^{a}	1.30 ± 2.49^{ab}	1.40 ± 1.14^{a}
VB.3 + 3%MSG	2.10 ± 1.08^{ab}	5.11 ± 1.99^{bcd}	4.41 ± 2.05^{abc}
VB.3 + 5%MSG	1.38 ± 0.66^{ab}	6.26 ± 1.18^{cd}	9.01 ± 7.25^{bc}
VB.3 +7%MSG	2.89 ± 0.87^{b}	8.34 ± 0.88^{d}	19.5 ± 2.80^{d}
VD + 1%MSG	-0.2 n± 0.25^{a}	0.17 ± 0.22^{a}	0.58 ± 0.65^{a}
VD + 3%MSG	0.66 ± 0.45^{a}	1.03 ± 0.43^{a}	1.37 ± 0.57^{a}
VD + 5%MSG	1.30 ± 0.70^{ab}	1.60 ± 0.33^{ab}	4.75 ± 0.49^{abc}
VD + 7%MSG	2.17 ± 0.33^{ab}	2.61 ± 0.66^{abc}	10.79 ± 1.47^{c}
VE.4 + 1%MSG	-0.2n ± 0.66^{a}	1.52 ± 0.90^{ab}	0.44 ± 0.45^{a}
VE.4 + 3%MSG	0.31 ± 0.23^{a}	2.09 ± 0.12^{ab}	0.58 ± 0.50^{a}
VE.4 + 5%MSG	1.03 ± 0.75^{ab}	2.74 ± 1.93^{abc}	1.66 ± 0.12^{a}
VE.4 + 7%MSG	1.75 ± 1.10^{ab}	3.60 ± 1.64^{abc}	3.39 ± 0.87^{ab}

Note: *Data are shown in mean±standard deviation of 3 repeated measurements of GABA concentrations by paper chromatography pre-staining method. Superscripts with different letters in the direction of the column showed significantly different results (P<0.05).

Resistance of VCO-LAB, GABA producer, to hydrogen peroxide

The effect of hydrogen peroxide on the growth and viability of 3 isolates of VCO-LAB producing GABA is shown in (Figure 6). All cultures of VCO-LAB showed moderate to strong resistance to various concentrations of hydrogen peroxide compared to the control (media without hydrogen peroxide). Figure 6 shows that the three isolates could survive on media containing 0.2-1.0 mM hydrogen peroxide, with the lowest OD of 0.82 after incubation for 8 hours. These results are similar to those reported by Lee et al. (2010), who reported that isolated *Lactobacillus* could survive on 1.0 mM hydrogen peroxide. Arasu et al. (2013) stated that *Lactobacillus plantarum K49* could survive on a medium containing 0.2-0.8 mM hydrogen peroxide. Hydrogen peroxide is a weak radical, but it can produce hydroxyl radicals when it reacts with transition metal ions. Organisms can produce catalase to convert hydrogen peroxide into water and O2 gas. Although VCO-LAB is catalase-negative, some types of LAB can produce NADH-peroxidase enzymes that can convert H2O2 (Komatsuzaki et al. 2005).

To conclude, VB.3 possessed an antioxidant activity against ABTS and hydroxyl radicals. The highest antioxidant activity was found by adding MSG (68.13% for ABTS and 88.02% for hydroxyl radicals). VB.3 isolate was identified as *L. fermentum* KF7.

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