Changes in folate characteristics and its identification in broccoli (*Brassica oleracea Italica*) extract fermented by Lactic Acid Bacteria Mixed Culture (LAB)

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Abstract. Broccoli (*Brassica oleracea Italica*) was fermented by cultures of lactic acid bacteria (LAB) as a potential source of natural folic acid. This study aimed to evalte characteristic changes and to identify folate compounds from broccoli extract, fermented by mixed LAB cultures (*L. bulgaricus, S. thermophulus, L.acidophilus, Bd. bifidum*). The formulation of broccoli extract was fermented with variation of LAB starter culture with concentrations of 10 and 20%(v/v), and the change of characteristic of folic acid compound during fermentation (0 to 48 hours) with an interval of 8 hours was evaluated. The results showed that the fermentation of broccoli extract with different concentration of LAB culture had an effect on the concentration of folic acid produced, as well as the change of concentration of folic acid during the fermentation time interval. The optimum condition was obtained based on the highest folic acid concentration of 6.74%, at culture concentration of 20% during 24 hour fermentation with the value of folic acid concentration of 72.11 µg/mL, pH value of 4.29, total sugars of 34.61%, total acids of 0, 97%, dissolved protein of 14.64 mg/mL and total LAB of log 13.02 + 0.05 cfu / ml.

1 Introduction

Vegetables from the *Cruciferous* family such as broccoli (*Brassica oleracea Italica*) are foods that are often consumed and can good for health. The benefits provided from broccoli secondary plants are linked to compounds that have bioactivity; Glucosinolates and phenolic acids [1]. Broccoli contains less fat. It is low in calories and rich in vitamins, inorganic substances, and fiber [2]. Broccoli is also rich in β-carotene, ascorbic acid, selenium, quercetin, and glutathione, potentially as antioxidants. In addition, it contains high enough folic acid (folate).
Humans cannot synthesize folate, hence, it is a need for human to intake folate to prevent the deficiency of this vitamin [3]. The recommended daily intake (RDI) of folate for adults is 200-400 µg [4, 5]. For pregnant women is recommended to be 400-600 µg. Although folate is always present in every food consumed by humans, folate deficiency is still common, even in developing countries [6]. Milk and fermented milk products are folate-derived foods. Folate has a very unstable nature. Therefore, to synthesize folate, culture of lactic acid bacteria in fermented milk products combined with broccoli vegetables can be used to increase folate content.

Lactic acid bacteria (LAB) include *lactobacilli* and *bifidobacteria* that are dominant in the gut and able to produce lactic acid through saccharide fermentation. LAB also acts as a live bacterial activator that can promote the growth of beneficial bacteria in the body, prevent various diseases, and regulate physiological activity by improving digestive function, inhibits cholesterol absorption, controlling immunity, enhances absorption, improves nutrient utilization and others [7,8] . It is well known that it is not only yogurt starter culture and *Lactobacillus lactis* which has the ability to produce folate, but other lactic acid bacteria (LAB) also possess the ability to produce folate. *L. acidophilus* is reported to be able to increase the concentration of folate in fermented milk [9]. It has also been observed that *L. plantarum* is capable of producing folate, even at low folate levels, when it is grown on a folate-free medium with a certain chemical composition [10]. In addition, recent research has shown that some probiotic microorganisms have the ability to synthesize folic acid such as *bifidobacteria* and propionibacteria. In this study we examined changes in the characteristics of folate and its identification in fermented broccoli beverages with the addition of lactic acid bacteria enriched with FOS as soluble fiber that is beneficial for the health of the body.

2 Experimental

2.1 Materials

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The ingredients used in this study were broccoli vegetable concentrate, fructooligosaccharide (FOS), skim milk, mixed cultures LAB powder (*L. bulgaricus, S. thermophulus, L. acidophilus, Bifidum*), supplied by Hansel. Co. Ltd. MRSA, MRSB, 3-aminophenol, Hydrochloric (4M), Sodium Nitrite, Sulfamic Acid were supplied by Sigma-Aldrich (St. Louis, MO). Ethanol, Folin-Ciocalteau reagent, HCl, KH_2PO_4, potassium ferric cyanide, trichloroacetic acid (TCA), NaOH, Buffer Phosphate were obtained from E-Merck.

2.2 Fermentation process

Cleaned broccoli vegetables were blanched for 5 minutes at 80ºC, followed by blending process of vegetable and water ratio of 1: 4, so that broccoli vegetable porridge was obtained. The next step of filtration was done with 80 mesh filters to obtain filtrate/broccoli vegetable extract. The broccoli extract is enriched by the addition of skim milk and FOS as a source of prebiotic oligosaccharides at 9% (w/v) for each. The result of the broccoli extract formulation was sterilized at 121 °C for 15 minutes. The sterilized formulated broccoli extract was rapidly cooled to a temperature of 45 °C, and then inoculated with 10% and 20% (v/v) of LAB starter mix culture (containing about 10^9 cfu/mL) obtained from 1% (w/v) mixed LAB powder culture (*L Bulgaricus, S. thermophulus, L. acidophilus, B. bifidum*), inoculated against MRSB-based media and incubated at 37 °C for 24 hours. The obtained subculture was then inoculated with variations of 10% and 20% (v/v) on the sterile formula of 15% (w/v) of skim milk solution and 15% (w/v) of FOS, with incubation temperature of 37 °C for 0–48 hours, with an interval of 8 hours.

2.3 Cell number of LAB, pH, total sugar and Total acid (TA)

LAB fermented broccoli extract was analyzed for bacterial growth, determined by cell number of LAB calculation from several series dilution with 0,1 mL sample to 10 ml of sterile water, then using plating method with MRSA, which was done as much as 2 replicates (duplo), incubated at temperature 37 °C for 48 hours. The number of colonies grown in the medium was calculated by multiplying the average number of colonies by dilution factor [11]. Similarly, the pH value measurements of the samples were measured by digital pH meter and total sugar measurements were made by the phenol sulfate method [12] performed at 0–48 hours. The total acid was determined by titration of each sample with 0.1 M NaOH. The result was expressed as a percentage of lactic acid, determined according to standard procedure [13].

2.4 Analysis of folic acid

Folic acid analysis was performed using spectrophotometric method based on the reaction of diazotizing p-aminobenzoylglutamat acid produced after the reduction reaction of folic acid and 3-aminophenol to form a complex of yellow-orange. About 1 mL of standard folic acid or sample was added into 1 ml of 4 M HCl , 1 mL of 1% (w/v) sodium nitrite, 1 mL of 1% (w/v) sulfamic acid, and 1 mL of 1% (w/v) 3-aminophenol. After mixing, it formed a yellow-orange complex solution. Furthermore, the absorbance was measured using UV–VIS spectrophotometer at a wavelength of 460 nm [14].

2.5 Folic acid identification by LC-MS

After fermentation process, fermented brocoli extract were then filtered by microfiltration. Identification of folic acid and glutamate acid compounds in brocoli fermentation at the optimum condition. The obtanied permeate or purified brocoli extracts and folic acid standard. Oligomer analysis was performed by LC-MS using Mariner Biospectrometry integrated with Q-tof mass spectrometer (MS) through ESI (electrospray ionisation) system where the scan mode performed in the range of 100-1200 sqm at 140°C. C18 column Supelco (RP 18, 250 x 2 mm with a particle size of 5 μ) was used for the LC (Hitachi L 6200). Solvent was a mixture of water containing 0.3% acetic acid (A) and methanol containing 0.3% acetic acid (B) at a ratio of 80% methanol and 20% water with a flow rate of 1 mL/min. The injection volume 20 μL was used in this analysis [15].

3 Results and Discussion

3.1 Material Characterization

Fresh broccoli (*Brassica oleracea L.*), blanching broccoli at 80 °C for 5 minutes, and blend broccoli at ratio of broccoli and water (1:4) as broccoli pulp, and broccoli extract filtered through 100 mesh sieve showed a difference in chemical compositions, as tabulated in Table 1. This difference was showed at filtrate 100 mesh (broccoli extract) containing total solids 1.54% higher compared with total solids 0.79% and in broccoli pulp. This difference also seen at total polyphenol in broccoli pulp (0.4740%) and broccoli extract (0.1836%) were lower compared with fresh broccoli (0.0179%). but different from the content of folic acid in fresh broccoli, folic acid is lower than the blanching treatment.

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Table 1. Composition of broccoli as substrates in LAB fermentation

Type of broccoli	Composition		
	Total solids (%)	Total polyphenol (%)	Folic acid (µg/mL)
Fresh broccoli	25.51	0.0179	8.76
Blanched broccoli*	0.5129	0.2704	32.11
Broccoli pulp**	0.79	0.4740	30.53
Broccoli extract***	1.54	0.1836	37.72

**result of blanching 80 °C for 5 minutes*
***blending at a mixture of 1 part of broccoli and 4 parts of water*
****filtered via 100 mesh*

Blanching process decreased possibility components contained in broccoli, besides pulverizing and filtration. Broccoli extract showed that polyphenol was trapped in other components so that not significant difference in pulp and extract. Soluble component effect on total solids, although this matter was affected by solubility properties of polyphenol in water. It had been known that both components were eased to solve in water [16].

3.2 Cell number of LAB

Characteristics of fermentation of broccoli extract with mixed LAB culture to total LAB count continued to increase depending on the amount of LAB culture concentration added to broccoli extract medium. The main factor influencing the increase of LAB population was the ability of lactic acid bacteria as starter culture that serves as a trigger of a metabolic process, in this case in particular is fermentation. The nature of the LAB is its ability to ferment sugars to acid and other factors that were suspected to cause an increase in LAB population was the condition of the media environment that supports optimum LAB growth. The LAB population for the 10% concentration of culture reached a maximum of 11.26 log cfu/ml at 48 hours fermentation time and for a culture concentration of 20% reached a maximum of 13.68 log cfu/mL at 40 fermentation hours, as shown in Figure 1.

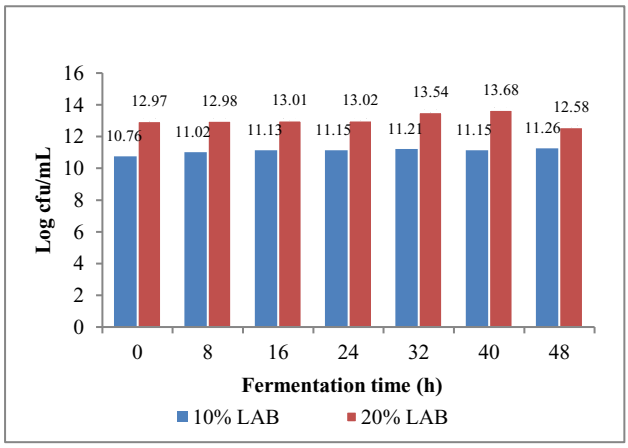


Figure 1. Changes in cell number of LAB (Log cfu/mL) from broccoli extract (*Brassica oleracea Italica*) fermented by mixed culture of Lactic acid bacteria (LAB) for 0-48 hours

3.3 pH and Total acid (%)

The concentration of free hydrogen ions is reflected by the pH value [17]. Changes in pH and total acid during fermentation of broccoli extract inoculated by mixtures of lactic acid bacteria and *bifidobacteria* were presented in Figure 2.

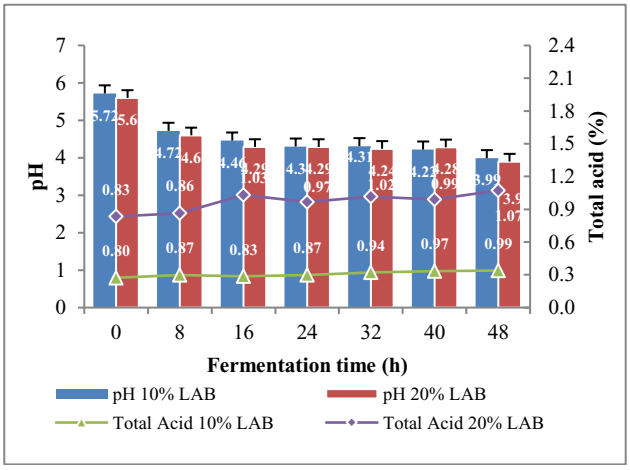


Figure 2. Relation of total acid and pH to characteristic changes of broccoli extract (*Brassica oleracea Italica*) is fermented by mixed culture of Lactic acid bacteria (LAB)

In general, broccoli extract during the fermentation time of 0 to 48 hours decreased the pH and increased the total acid. The concentration of lactic acid bacteria influenced the pH change. Low pH values and higher acid production were obtained from both 10% of LAB starch culture concentrations and 20% of fermented broccoli extracts.

The decrease of pH value after 48 hours incubation in broccoli extract was significantly different from each concentration of starter culture LAB added. In this study, the culture concentrations of 10% and 20% had a pH value of 5.72 and 5.60 at the beginning of fermentation (0 hours) and at the end of fermentation (48 hours) ranged from 3.98 and 3.90, while the total acid ranged from 0.83 and 0.80 up to 1.07% and 0.99% at the end of fermentation (48 hours). The decrease in pH occurred along with the production of various organic acids during the fermentation of broccoli extract by LAB. The total acid (TA) value after fermentation by LAB increased significantly depending on the concentration of LAB culture added to the broccoli extract medium. This was due

to the measurement of TA that the measured acid component consists of dissociated and un-dissociated acids, whereas for pH only measures the dissociated acid component in the form of H^+ ions [17]. Similarly, the pattern of TA value change in treatment was consistent with the change in pH values (Figure 3). Similarly, the pattern of TA value changes in starter culture was consistent with the pattern of pH value changes in starter culture. Similar to pH values, the pattern of TA values change in general was consistent with the pattern of TA value changes in starter control.

3.4 Total Sugar and Reducing sugar

The change in total sugar is in line with the changes that occur in reducing sugars. The pattern of changes in total sugar and reducing sugars occurred in both culture concentration of 10% and 20% of LAB culture during fermentation 0 to 48 hours. Changes in starter culture concentration and fermentation time can be seen in Figure 3.

Changes in total sugar decrease occur at 20% concentration, this is because the greater the total content of sugar will accelerate bacterial activity in converting to acid [18]. In other words, it indicates that a single carbon source derived from prebiotics FOS (DP 2-8) in broccoli beverages is utilized by BAL for its growth, or an existing carbon source can be metabolized in β(2-1) fructans with a DP Higher than 2 (two). The total sugar increased during the fermentation process (0-48 h) at the culture concentration of 10% to 20%, the increase of total sugar and reducing sugar showed that too large BAL concentration resulted in inefficient culture in metabolizing FOS compounds in the time range until 48 hours at concentrations of 10% and 32 hours at a concentration of 20% of LAB cultures, this may be influenced by physical and chemical factors from the tested sample.

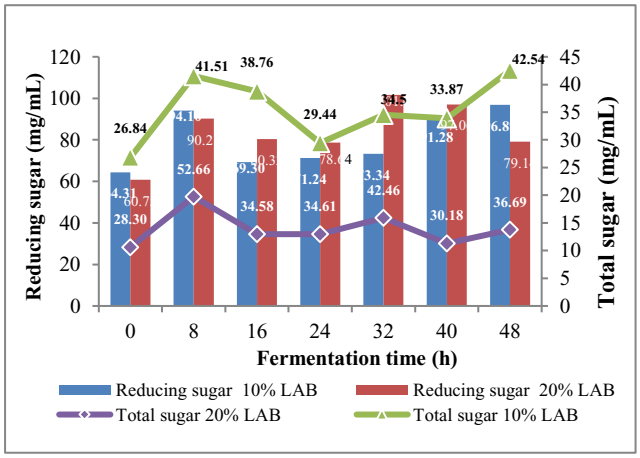


Figure 3. Relationship of total sugar and sugar reduction to the characteristic changes of broccoli extract (*Brassica oleracea italica*) fermented by mixed cultures Lactic acid bacteria (LAB)

3.5 Folic acid and Dissolved protein

The tendency of yield on the rate of fermentation to folic acid, the data showed during the fermentation time of broccoli extract gave the result of folic acid fluctuated, the data obtained was significantly different during the fermentation time.

Folic acid at optimum condition was obtained at initial fermentation (32 hours) at 10% LAB culture concentration while at optimum 20% culture concentration at 24 hours fermentation time, the concentration of folic acid in each starter culture was 71.20 and 72.11 µg / mL. Folic acid is a compound that is very sensitive to light, oxygen and temperature, therefore fermentation by using lactic acid bacteria culture can improve the recovery of natural folic acid because it is done under anaerobic conditions to minimize interaction with light and oxygen. In addition, microbial activity in the culture of lactic acid bacteria is expected to help the stability of natural folic acid during fermentation. The fermentation time will increase the chemical composition change from the fermentation of broccoli extract. Changes in dissolved protein levels during the fermentation time occur because of the role of the enzyme produced by the BAL during the fermentation process takes place. Protease is an enzyme capable of hydrolyzing peptide bonds in proteins. Lactic acid bacteria have the ability to produce proteolytic enzymes around cell walls, cytoplasmic membranes, or in cells [19]. Increased levels of dissolved protein in fermented broccoli extract may also occur due to increased growth and proliferation of BAL cells (*L. acidophilus, Bd. bifidum, L. bulgaricus and S. thermophilus*). This is due to the protein in the extract of broccoli undergoing hydrolysis process by proteolic enzyme produced by BAL during fermentation. Rahman [20] states that lactic acid-forming bacteria in fermentation produce proteolic enzymes that help protein degradation.

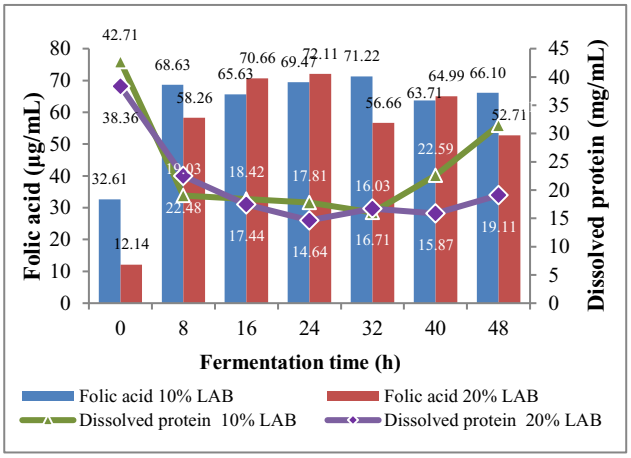


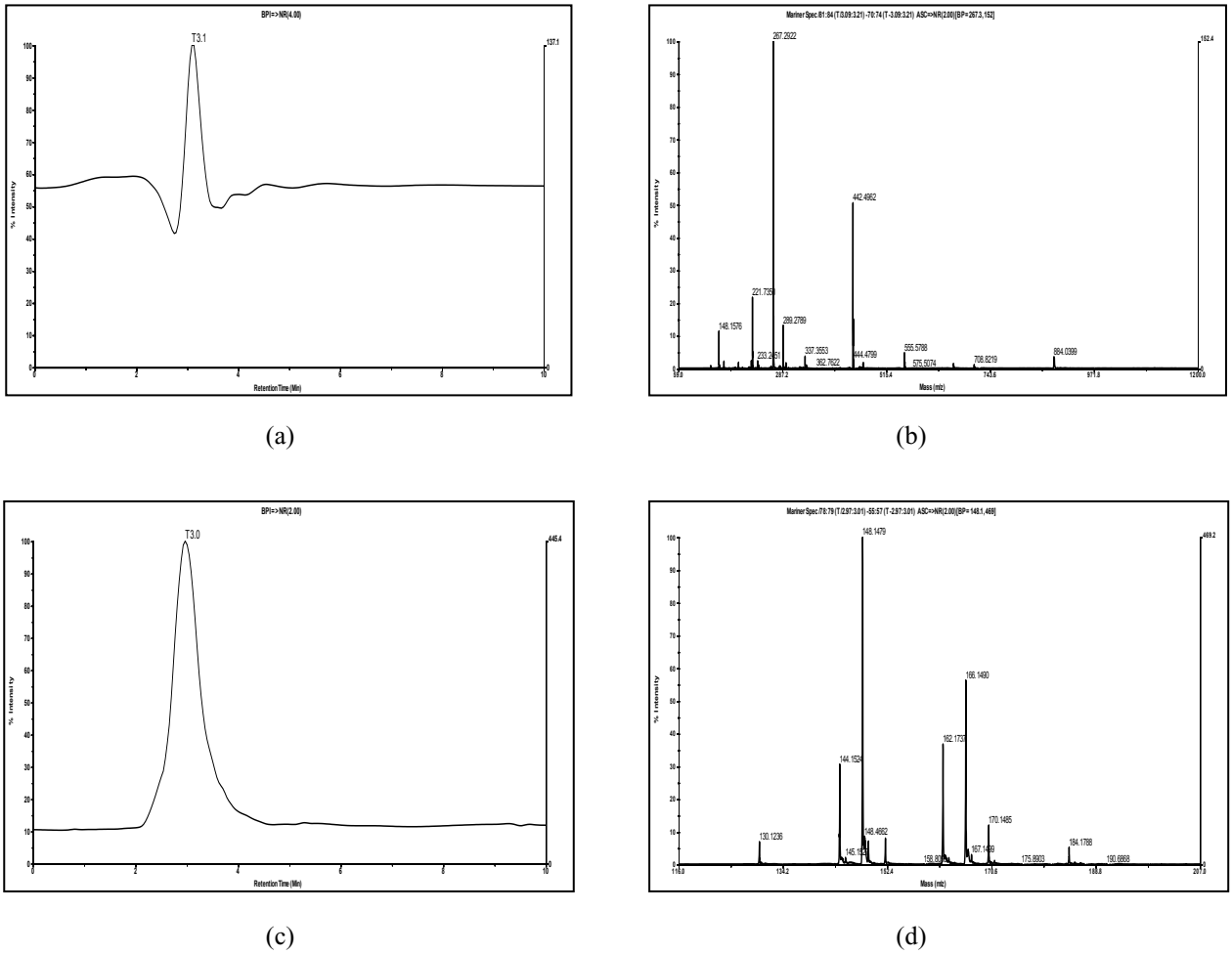
Figure 4. The relation of soluble protein and folic acid to changes in the characteristics of broccoli extract (*Brassica oleracea italica*) fermented by mixed cultures Lactic acid bacteria (LAB).

Folic acid at optimum condition was obtained at initial fermentation (32 hours) at 10% LAB culture concentration while at optimum 20% culture concentration at 24 hours fermentation time, the concentration of folic acid in each

starter culture was 71.20 and 72.11 μg / mL. Folic acid is a compound that is very sensitive to light, oxygen and temperature, therefore fermentation by using lactic acid bacteria culture can improve the recovery of natural folic acid because it is done under anaerobic conditions to minimize interaction with light and oxygen. In addition, microbial activity in the culture of lactic acid bacteria is expected to help the stability of natural folic acid during fermentation. The fermentation time will increase the chemical composition change from the fermentation of broccoli extract. Changes in dissolved protein levels during the fermentation time occur because of the role of the enzyme produced by the BAL during the fermentation process takes place. Protease is an enzyme capable of hydrolyzing peptide bonds in proteins. Lactic acid bacteria have the ability to produce proteolytic enzymes around cell walls, cytoplasmic membranes, or in cells [19]. Increased levels of dissolved protein in fermented broccoli extract may also occur due to increased growth and proliferation of BAL cells (*L. acidophilus, Bd. bifidum, L. bulgaricus and S. thermophilus*). This is due to the protein in the extract of broccoli undergoing hydrolysis process by proteolic enzyme produced by BAL during fermentation. Rahman [20] states that lactic acid-forming bacteria in fermentation produce proteolic enzymes that help protein degradation.

3.6 Identification of folic acid with LCMS

Broccoli extract contains high enough folic acid and can be seen the changes during the fermentation process with the addition of lactic acid bacteria. Folic acid is a heterocyclic compound with a conjugated pentoric acid structure with one or more L-glutamates linked via an amino acid-carbonyl group. Folic acid has one L-glutamic residue with the name "pteroilglutamic acid". Folic acid can be reduced to H2 folate or tetrahydrofolate (H4 folate) which is an active form of coenzyme vitamin [21].



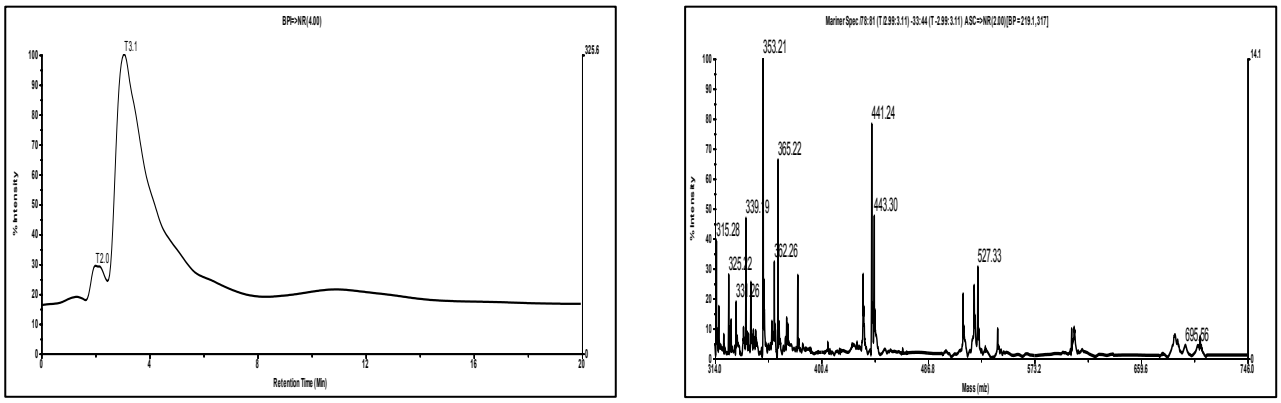
(a) (b)

(c) (d)

Figure 5. Standard solution of glutamate (c) and mass spectra from T 3.0 from chromatogram folic acid standard (b), Standard solution of glutamate (c) and mass spectra from T 3.0 from chromatogram glutam e standard (d)

Figures 5a and 5b show that the standard of folic acid is dominated by compounds with BM 442,5, 443,16, 443,51 and 444,48 Da, respectively. With the successive intensities of 50.55, 8.35, 15 and 2.44% in other words folic acid is possible on the BM. In Figs. 5c and 5d show that the standard of glutamic acid is dominated by compounds with BM 148.15, 148.47 and 149.14 Da respectively. With successive intensities of 100, 8.51 and 7% in other words glutamic acid is possible on the BM. The results of mass spectra of broccoli extract fermentation by mixed LAB cultures were obtained, at a culture concentration of 20% during 24-hour fermentation

(Fig. 6) than the standard mass spectra of folic acid and glutamate (Figs. 5a and 5c), identified eight compounds at T 3.7 where 2 (two) compounds are identical as folic acid with molecular weight 441.23, and 443.29, with the relative intensity respectively of 3.48% and 2.11%.



(a) (b)

Figure 6. Chromatogram of fermented broccoli (a) and mass spectra of T3.07 from chromatogram of fermented broccoli and optimum process conditions based on highest folic acid in a culture concentration of 20% during fermentation time of 24 h (b)

While the identical compound as glutamic acid is obtained 5 (five) compounds with molecular weight of 145.08, 159.046, 163.09, 175.10, and 191.08, with relative intensity of 0.86%, 12.15%, 1.58%, 2.95%, and 28% of 43 compounds respectively. The presence of folic acid in the fermentation of broccoli extract is presented in Table 2.

Table 2. Identification of folic acid and degraded folic acid compounds in fermented extract broccoli using lactic acid bacteria (LAB) mix culture by LC-MS

No.	Centroid Mass	Relative Intensity (%)	Area	Represented Compounds
1.	145.156904	0.86	26.85	2-aminopentanedioateglutamate $C_5H_7NO_4^{-2}$
2.	159.060508	12.15	265.59	Lithium L-glutamate C5H8NNaO4
3.	163.167017	1.58	30.97	2-amino-4-hydroxypteridine
4.	175.183459	2.95	59.96	L-Glutamic acid 5-ethyl ester C7H13NO4
5.	191.118699	28	653.26	L-Glutamic acid disodium salt C5H7NNa2O4
6.	192.073319	1.2	24.69	Pterin-carboxylic acid C7H5N5O3
7.	441.397255	3.48	101.33	Isofolic Acid C19H19N7O6
8.	443.417706	2.11	40.96	(6S)-5,6,7,8-tetrahydrofolate dianion C19H21N7O6-2

4 Conclusion

Characteristics changes of the bioactive component of fermented broccoli extract mixed of starter cultures of LAB (*L. bulgaricus, S. thermophilus, L. acidophilus, Bd. bifidum*) with different concentrations of LAB cultures and different time of fermentation affect to folic acid, dissolved protein, total sugar, total acids, and total BAL. The optimum conditions obtained by the highest concentration folic acid at a concentration of 20% during the fermentation time of 24 h with a value of folic acid concentration of 72.11 μg/mL, pH value of 4.29, total sugars of 34.61%, total acids of 0, 97%, dissolved protein of 14.64 mg/mL and total LAB of log 13.02 + 0.05 cfu / ml. Identification of glutamate and folic acid through LCMS showed identified eight compounds at T 3.7. Where 2 (two) compounds are identical as folic acid and as glutamic acid is obtained 5 (five) compounds.

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