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Anti-salmonella potential and antioxidant activity of fermented fruit-based juice by lactic acid bacteria and its biotransformation

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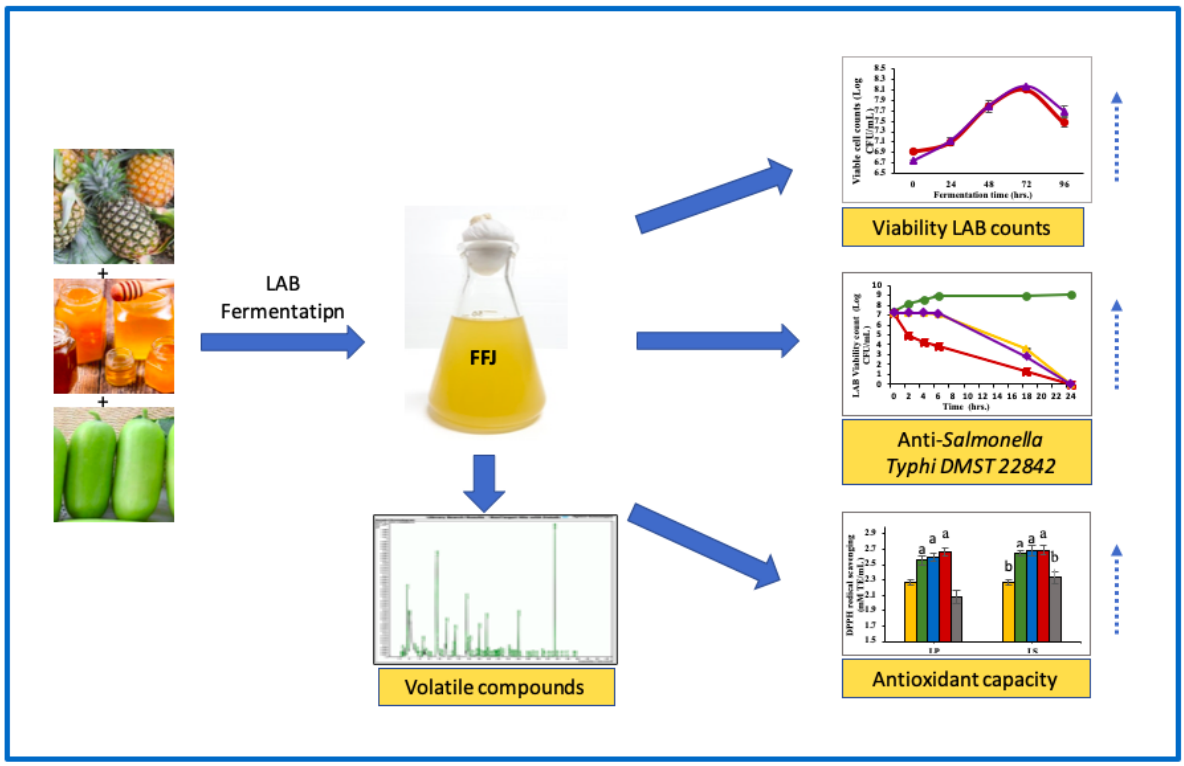
ABSTRACT

Background: Lactic acid bacteria-based fermentation clearly contributes to improving nutritional value and exhibits various health benefits. The demand for non-dairy functional beverages, such as fruit beverages, as an alternative vehicle for probiotics is increasing because of lifestyle choices or health conditions. Therefore, the objective of this study was to evaluate the anti-Salmonella potential and antioxidant activity of fermented fruit-based juice by lactic acid bacteria and its biotransformation.

Methods: In this study, to produce the fermented fruit-based juice (FFJ), the mixed fruit juice (MFJ) was fermented by *Lactobacillus plantarum* and *Lactobacillus salivarius* for 72 hrs. The potential function, anti-Salmonella by the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and antibiofilm activities of FFJ against *Salmonella* Typhi DMST 22842 was evaluated. The antioxidative capacity was determined by DPPH and FRAP assay. The active volatile compounds were identified by GC-MS.

Results: A novel functional FFJ showed excellent growth capacity with 8 log CFU/mL of probiotics *Lactobacillus plantarum* and *Lactobacillus salivarius*. MIC and MBC values in the FFJ were 500 mg/mL after 72 hrs of fermentation. After 48hrs of fermentation, biofilm formation inhibition was significant ($p < 0.05$) with 95.27% ± 2.26% inhibition; biofilm metabolic activity inhibition was also significant ($p < 0.05$) with 89.25% ± 0.18%

inhibition. The volatile compounds present in the FFJ were fruity flavors and aromas, most of have antimicrobial and antioxidant properties. These compounds comprise various classes, including alcohols, organic acid, ester, and ketone. In both LAB fermentations, the most abundant volatile alcohol was isoamyl alcohol, followed by 1-hexanol and 2,3-Butanolone; acetic acid was only present in *L. plantarum* fermentation. In addition, DPPH radical scavenging and FRAP assay showed the mixed fruit juice had dramatically increased antioxidant activity after 48 hrs of fermentation.



Conclusion: The findings of this work indicate that the obtained fermented fruit-based juice (FFJ) showed excellent growth capacity of probiotics, *Lactobacillus plantarum* and *Lactobacillus salivarius*, and produced the volatile compounds from biotransformation. This not only improved fruit flavor and aroma, but also influenced antibacterial activity against the pathogen *Salmonella Typhi* DMST 22842, as well as increased antioxidant activity. Therefore, the FFJ could be a novel functional fermented drink for vegan and non-diary consumption.

Keywords: Lactic acid bacteria, Probiotics, Biotransformation, Non-dairy functional beverage, Anti-Salmonella.

INTRODUCTION

Consumer concern and increasing knowledge of the impacts of food on health has led to the demand for food products with functional properties, as well as the consumption of functional beverages. Nowadays, drink consumers demand nutritive and healthy beverages, with pleasant flavor, low-caloric intake, and non-dairy options. The consumption of

fermented products increased worldwide along with the concomitant reduction in dairy consumption [1]. The demand for non-dairy functional beverages, such as fruit beverages, as an alternative vehicle for probiotics is increasing because of lifestyle choices or health conditions [2]. Fruits contain many bioactive compounds, including health promoting phytochemicals [3], such as antioxidants [4]. The nutritional and functional properties of fruits have been widely studied. Pineapple (*Ananas comosus* L. Merr.) and winter melon (*Benincasa hispida* Cogn) were previously evaluated as potential antioxidant food sources and they were determined to contain phytochemicals (flavonoids, phenolic acids), vitamin C, minerals, free amino acids and phytosterol [5]. Honey, a natural sweetener, and the product of *Apis mellifera* honeybees, has been a favorite foodstuff since ancient times [6]. It is often considered as the 'natural healer' due to its composition and biological properties.

In present days, lactic acid bacteria (LAB)-based fermentation has a clear contribution in improving nutritional value and enhances health benefits, including aiding digestibility of various foods, decreasing lactose intolerance, controlling potential infections, and adding further functional bioactivity [7]. It is possible that the microbial biotransformation of fruit matrices occurs because most plant-based materials contain important nutrients, including sugars for microbial growth and other phytochemicals. Accordingly, the biotransformation of biologically active compounds in fruit and vegetable juices through LAB fermentation is an active area of research. Fruit-based materials may be ideal media for cultivating beneficial bacteria, such as probiotics LAB, which concurrently enhances their nutritional functionality. Thus, various bioactive metabolites with health-promoting attributes could be obtained by biotransformation processes [8]. Moreover, due to their complex enzymes, functional benefits of lactic acid bacteria could also lead to the novel modification of flavor attributes.

Foodborne diseases remain a major public health concern worldwide and are mainly caused by the consumption of food contaminated with pathogenic organisms, including bacteria. However, foodborne pathogens can also cause various infectious illnesses in the human host, mostly associated with vomiting or diarrhea [9]. *Salmonella enterica* serovar Typhi is the most common foodborne pathogen implicated in foodborne disease [10]. *Salmonella* Typhi is a gram-negative enteropathogenic bacterium that can infect humans and cause a severe systemic infection called typhoid fever, due to its characteristic pathogenicity [11]. After entering a host, *S.* Typhi passes through the acidic stomach and duodenum, then invades the small intestine mucosa and survives in macrophages and monocytes. During this process, *S.* Typhi utilizes various strategies to overcome environmental stress through regulating gene expression, including increasing motility and biofilm formation [12]. Therefore, this study aimed to investigate *Lactobacillus salivarius* and *Lactobacillus plantarum* fermented in mixed fruit juice and their anti-Salmonella potential, antioxidant activity, as well as any volatile compound production caused by their biotransformation. The findings will inform specific function and benefits targeting humans and tropical fruits application by LAB fermentation-based biotransformation.

MATERIALS AND METHODS

***Materials*:** Pineapple (*Ananas comosus* L.Merr.) and winter melon (*Benincasa hispida* Cogn.) were purchased from the local market in southern Thailand. Longan (*Dimocarpus longan* Lour.) honey was directly purchased from beekeepers and stored at 4°C before use. Honey was used in the experiment for the first 6 months.

***Preparation of Lactic acid bacteria strains and inoculum*:** *Lactobacillus salivarius* and *Lactobacillus plantarum* were obtained in lyophilized from and were reactivated by culturing in 10 mL of de Man Rogosa and Sharpe (MRS) broth at 37˚C for 24 hrs. The resulting LAB suspensions were then used as inoculum for 100 mL of MRS broth and incubated at 37˚C for 24 hrs. The cell was harvested by centrifugation at 6000 × g at 4˚C for 10 min and washed twice with sterile water. Then optical density (OD) at 660 nm was measured and appropriate dilutions were made in sterile water to obtain an OD of 2.5 to achieve LAB cells of approximately 1×10^8 CFU/mL. The LAB suspension was used as inoculum in the fermentation process.

***Fermentation of mixed fruit juice*:** The mixed fruit juice consisted of an appropriated ratio of honey, winter melon and pineapple juices. The pH value was adjusted using food-grade Na_2CO_3. The resulting MFJ was pasteurized at 70˚C for 10 min before fermentation. The LAB suspension inoculum of approximately 1×10^8 CFU/mL was added into 1000 mL Erlenmeyer flasks containing 900 mL of sterile MFJ and incubated at 37˚C for 96 hrs. The sterile mixed fruit juice with no inoculation was treated under the same condition and was used as control. The samples were taken every 24 hrs to ensure a viable LAB count. Then the fermented fruit-based juice (FFJ) samples were centrifuged at 12000 × g at 4˚C for 10 min to obtain supernatants for physiochemical, biological activity and volatile compound analysis as described below.

***Determination of viable LAB counts*:** Viable LAB counts in the FFJ were obtained by serially diluting with 0.85% NaCl solution to 10^{-4}-10^{-6} dilution, then using the spread plate method. Each dilution consisted of 100 µL aliquots in triplicate and spread on plates containing MRS agar. The plates were incubated at 37˚C for 48 hrs and the plates containing 30-300 colonies were counted and recorded as log CFU/mL.

***Determination of total titratable acidity and reducing sugar*:** Total titratable acidity (TTA) expressed as percentage of lactic acid was determined by dissolving the sample in distilled water and titrating it. Reducing sugar (RS) content was analyzed as glucose equivalents with Dinitrosalicylic acid (DNS) reagent. The DNS method used was as described by Miller [13], with some modifications. Absorbance was measured by UV-Spectrophotometer and the curve was plotted to compare the reducing sugar content in the sample (mg/mL) against the glucose standard curve.

***Preparation of Salmonella Typhi DMST 22842 culture*:** *Salmonella* Typhi DMST 22842 were cultured in Brain Heart Infusion (BHI) broth at 37 ˚C for 24 hrs. After that, OD at 600 nm was measured and appropriate dilutions were made in BHI broth to obtain an OD_{600} of 0.1 (approximately 1×10^8

CFU/mL). The obtained bacterial culture was used for anti-Salmonella analysis.

***Determination of MIC and MBC*:** The anti-Salmonella activity was determined by finding the minimum inhibitory concentration (MIC) of the FFJ. MIC was measured using the serial broth dilution method, as outlined in the Clinical and Laboratory Standard Institute (CLSI) procedures [14]. Two-fold serial dilution of FFJ with BHI was prepared in a sterilized 96-well plate. The FFJ dilution was mixed with 100 µL bacterial cultures, resulting in approximately 1×10^8 CFU/mL and varied ranges of 15-1000 mg/mL. Gentamicin concentration of 0.01-2.5 mg/mL was used as positive control. The plates were incubated at 37˚C for 24 hrs. The MIC was defined as the lowest concentration of metabolites that inhibit the bacterial growth. To find the minimum bactericidal concentration (MBC), a 10 µL aliquot of bacterial suspensions from the wells with no bacterial growth of a previous MIC test was added into a BHI agar and incubated at 37˚C for 24 hrs. The MBC was defined as the lowest concentration that enables no growth of bacteria on agar (99.9% kill).

***Time-kill kinetics assay*:** Time-kill kinetics were performed to evaluate the killing dynamics to assess the antimicrobial effect of FFJ on *Salmonella* Typhi DMST 22842. A bacterial suspension of approximately 1×10^8 CFU/mL in BHI broth was used in assay. 5 mL of bacterial cultures were exposed to FFJ and Gentamicin at MIC value and incubated at 37˚C. A tube containing 5 mL of bacterial cultures without samples was used as a growth control. 100 µL of each sample was taken at 0, 2, 4, 6, 18 and 24 hrs and made into 10-fold serial dilutions. Then 100 µL aliquots obtained from each tube were inoculated on BHI agar plates for colony counts. The number of viable colonies was counted only from the plates containing 30-300 colonies and recorded as log CFU/mL [15].

***Biofilm biomass quantification*:** Biofilm formation assay by the method described by [16] was followed with some modifications. A 100 µL of each FFJ at MIC value was added to each well of a sterilized 96-well plate. The 100 µL gentamicin at MIC value was used as a negative control and 100 µL distilled water was used as a positive control. Then, 100 µL bacterial cultures (approximately 10^8 CFU/mL) were added into the well to obtain a final volume of 200 µL. The plate was incubated at 37˚C for 24 hrs. The supernatant was removed, and the biofilm was washed with distilled water. The plate was fixed at 60˚C for 1 hr and the biofilm was stained with 0.1% solution of crystal violet in water. After staining, samples were washed twice with distilled water. Biofilm quantification was evaluated by adding 200 µL of 30% acetic acid to obtain the biofilm formation. The absorbance was measured at 595 nm using a microplate reader.

***Biofilm metabolic activity determination*:** The metabolic activity of biofilm was determined using the 3- [4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) reduction assay of [17] with some modifications. An aliquot of 200 µL bacterial cultures (approximately 1×10^8 CFU/mL) was inoculated in a sterilized 96-well plate at 37˚C for 24 hrs. After incubation, bacterial cultures were removed, and the microplates were dried. Then, 200 µL of the FFJ were added into each well. After incubation at 37˚C for 24 hrs, wells were washed with

200 µL of PBS. Then, 200 µL of 0.25 mg/mL MTT solution was added into each well and incubated at 37°C for 3 hrs. The solution was then removed, and the insoluble purple formazan generated by the bacterial enzymatic hydrolysis of MTT was dissolved in 2% DMSO. Finally, the absorbance was measured at 570 nm by the microplate reader. Gentamicin at MIC value was included as a negative control. The FFJ's percentage of biofilm inhibition was then compared with the positive control (distilled water).

***Determination of antioxidant activity*:** The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity in FFJ was evaluated using the method previously described with some modification [18]. The 200 µL of sample solution was mixed with 1800 µL of 200 µM methanolic DPPH solution. The mixture was incubated for 30 min at room temperature in the dark. The supernatant absorbance was measured with a spectrophotometer at 517 nm. The ability of DPPH was expressed as millimolar of Trolox equivalent per milliliter of the sample.

The FRAP was performed according to the previously reported method [19] with slight modifications. The reaction mixture was prepared by mixing 100 mL of 0.3 M pH 3.6 acetate buffer, 10 mL of 40 mM 2,4,6-Tri(2-pyridyl)-1,3,5-triazine solution, prepared with 40 mM HCl and 10 mL of 20 mM $FeCl_3.6H_2O$. Then, 200 µL of the sample solution was mixed with 1800 µL of the reaction mixture and incubated at room temperature for 30 min. The absorbance was measured at 593 nm. Data was reported in millimolar of Trolox equivalent per milliliter of the sample.

***Characterization of volatile compounds*:** Volatile compounds produced by biotransformation in the FFJ were characterized by the HS-SPME/GC-MS technique following the protocol reported by [20] with slight modification. The 2 mL of FFJ was added into a 20 mL glass vial. Head space micro-extraction was placed at 40°C for 30 min. After 15 min of equilibration time, each analysis utilized a SPME fiber coated with 50/30 µm of Divinylbenzene-Carboxen-Polydimethylsiloxane (DVB/Carboxan/PDMS). Before each analysis, the fiber was conditioned by insertion into the GC-MS injector at 230°C for 2 min; volatiles were desorbed by exposing the fiber into the GC injector for 2 min at 230°C. The separation was performed on a VF-WASms capillary column (30 m × 0.25 mm × 0.25 µm) at programmed a temperature, starting from 50°C for 3 min, increasing 5°C after each minute until reaching 200°C, then maintaining this final temperature for 12 min. The transfer line temperature was 250°C. The signal acquisition mode was full scan (from 41 m/z to 500 m/z). The volatile compounds produced by LAB in FFJ were identified based on their mass spectra compared with the 90% match factor of library (NIST 14) mass by spectra. Furthermore, to obtain a more confident identification. The linear retention indices (LRIs) were calculated on the retention time with a solution of C8-C20 analysis under the same conditions applied for sample analyses.

***Statistical analysis*:** All experiments were repeated in triplicate, and data was analyzed using the SPSS 21 software. All data are expressed as the mean values ± standard deviation (SD). Analysis of variance (ANOVA) based on the control group was carried out to determine any significant differences ($p < 0.05$).

RESULTS AND DISCUSSION

***Lactic acid bacteria viability*:** Viable LAB counts changed in FFJ during fermentation at 37°C for 96 hrs with *L. salivarius* (LS) and *L. plantarum* (LP), as shown in Figure. 1. Both LAB strains showed a similar growth profile within 24 hrs and reached maximum values after 72 hrs of fermentation. The LS and LP showed maximum viable counts of 8.17 ± 0.01 log CFU/mL and 8.11 ± 0.02 log CFU/mL, respectively. An obvious decrease in the microbial population was observed with LS and LP after 72 hrs of fermentation. Since an acidic environment is created during LAB fermentation, nutrient differences potentially induced stress on the growth of LAB strains [21]. Overall, cell viability is the key factor to designing functional drink and our results indicated that the FFJ was able to sustain a viable level of LAB up to 72 hrs. This fermentation time could be used in further studies to develop functional drinks with health benefits from LAB fermentation.

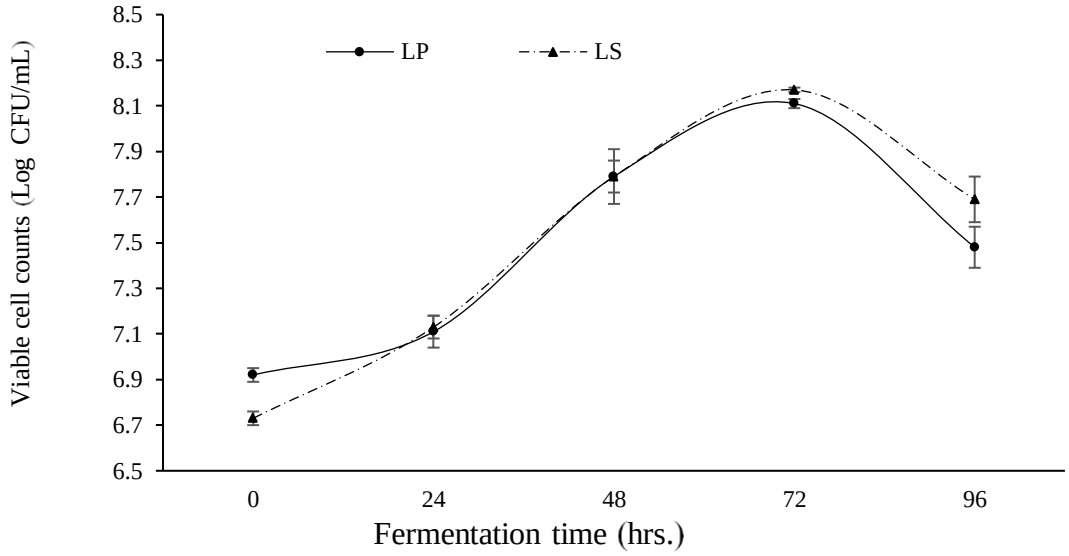


Figure 1. Viable cell counts of LAB during fermentation of fermented fruit-based juice. LP was represented *L. plantarum* and LS represented *L. salivarius*. Error bars indicate the standard deviation from three independent samples.

***Reducing sugar and total titratable acidity*:** Sugar content in the fermented fruit-based juice (FFJ) has usually been assessed in terms of reducing sugar (RS). Profile changes in RS contents in FFJ are shown in Figure. 2. During the fermentation period from beginning to optimum at 72 hrs, the values of RS decreased then stayed relatively unchanged from 72 hrs to 96 hrs in both *L. salivarius* (LS) and *L. Plantarum* (LP). The decrease of RS in FFJ with *L. salivarius* was greater than that by *L. Plantarum*, indicating that FFJ with *L. salivarius* consumed sugar at a much faster rate than *L. Plantarum*, which is concordant to the growth profiles of both LAB. The decrease in sugar concentrations during fermentation was largely due to not only bioconversion into lactic acid, but also the utilization for growth and metabolism of LAB [22]. The profile of total titratable acidity (TTA) rapidly increased throughout the fermentation period, as shown in Figure 2. For both *L. salivarius* (LS) and *L. plantarum* (LP) FFJs, TTA initially rapidly increased and reached its highest at 72 hrs. Overall, the curve showed that *L. salivarius* produced significantly

higher %TTA than *L. plantarum*. The %TTA of FFJ with LS increased from 0.33 ± 0.04% to 1.15 ± 0.07%, whereas the %TTA of FFJ with LP increased from 0.33 ± 0.04% to 1.05 ± 0.01%. Total titratable acidity increased because of acid production from sugar conversion during fermentation [23]. The greater amounts of TTA in FFJ with *L. salivarius* is potentially due to its homo-fermentative nature. This group of LAB efficiently metabolizes carbohydrates generating ATP, which is subsequently used for the biosynthesis of lactic acid. Meanwhile, *L. Plantarum*, which is facultatively hetero-fermentative, not only produces lactic acid, but also acetic acid and ethanol as main products [24].

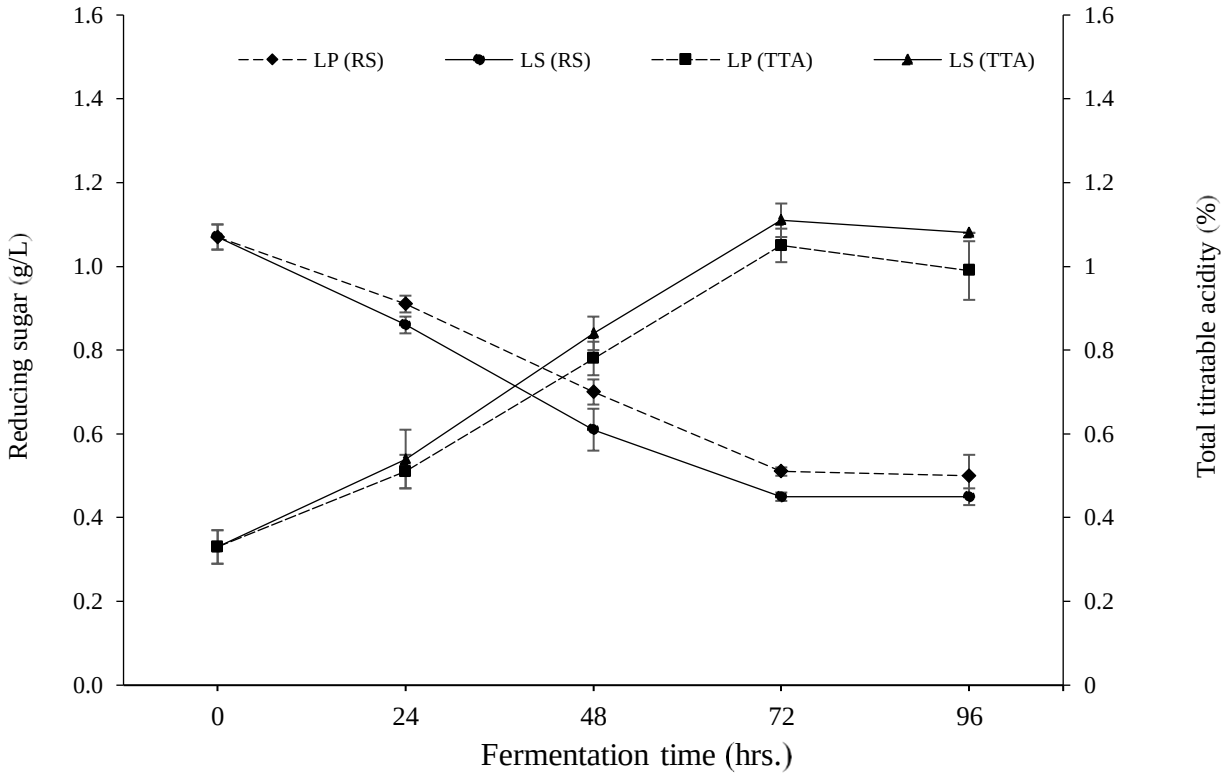


Figure 2. Changes in total titratable acidity and reducing sugar during FFJ. Error bars indicate the standard deviation from three independent samples.

***MIC and MBC*:** The anti-Salmonella activity against *Salmonella* Typhi DMST 22842 of fermented fruit-based juice (FFJ) was assessed using their minimum inhibitory concentration (MIC) and bactericidal concentration (MBC) values, as shown in Table 1. Gentamicin was used as positive control. The results showed that the non-fermented mixed fruit juice did not inhibit growth of *Salmonella* Typhi. Interestingly, the respective FFJs with *L. plantarum* and *L. salivarius* from 48to 96 hrs fermentation all inhibitedgrowth *Salmonella* Typhi with a MIC value of 500 mg/mL; the MBC values were also 500 mg/mL but only occurred at 72 hrs. Gentamicin had MIC values of 0.039 mg/mL and MBC values of 0.078 mg/mL. These anti-Salmonella effects could be due to the antimicrobial substances produced by LAB during fermentation. LAB typically produce anti-Salmonella compounds against foodborne pathogens, including bacteriocin

peptides, organic acids, free fatty acids, ammonia, diacetyl, hydrogen peroxide and enzymes [25]. Lactic acid is also known to function as a permeabilizer in Gram-negative bacteria and their outer membranes, allowing other compounds to perform synergistically with lactic acid [26].

Table 1 Minimum inhibitory concentration (MIC) and Minimum Bactericidal concentration (MBC) values of fermented fruit-based juice by *L. plantarum* (FFJ LP) and *L. salivarius* (FFJ LS) against *Salmonella* Typhi DMST 22842.

Treatments	Concentration (mg/mL)	
	MIC	MBC
Gentamicin	0.039	0.078
MFJ	-	-
FFJ LP		
24 hrs.	-	-
48 hrs.	500	-
72 hrs.	500	500
96 hrs.	500	500
FFJ LS		
24 hrs.	-	-
48 hrs.	500	-
72 hrs.	500	500
96 hrs.	500	500

- No MIC and MBC value was observed due to lack of anti-Salmonella effect.

***Biofilm biomass and biofilm methbolic activity inhibition*:** Inhibition of biofilm formation was conducted in fermented fruit-based juice, which showed at least 90% reduction (at MIC value concentration) in cell attachment of tested *Salmonella* Typhi DMST 22842 by crytal violet assay. The results showed different effects on the growth and development of a preformed biofilm, as presented in Table 2. The inhibition percentages of biofilm formation obtained for the *S.* Typhi strains was significant($p < 0.05$) reaching 90.62 ± 0.46% to 95.27 ± 2.26%. Biofilm metabolic activity inhibition was observed on the preformed biofilms. Fermented fruit-based juices were tested against 24 hr preformed biofilms using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (tetrazolium dye MTT) to stain metabolic active adhered bacteria. As presented in Table 2, the strongest metabolic activity inhibitions observed against the *Salmonella* Typhi biofilm ranged from 88.74 ± 0.27 % to 89.00 ± 0.24% . The results reveal that FFJ with *L. plantarum* and FFJ with *L. salivarius* at both 48 hrs and 72 hrs inhibit biofilm formation and biofilm methbolic activity of *Salmonella* Typhi DMST 22842.

Table 2 Biofilm formation and biofilm metabolic activity inhibition of fermented fruit-based juice (FFJ) by *L. plantarum* (LP) and *L. salivarius* (LS) at 48 hrs and 72 hrs fermentation against preformed biofilms by *Salmonella* Typhi DMST 22842.

FFJ / Control	*Salmonella* Typhi	
	Biofilm formation (%)	**Biofilm metabolic activity (%)**
Gentamicin (0.039 mg/ml)	92.98 ± 2.09^{ab}	85.19 ± 1.08^{c}
LP48	95.27 ± 2.26^{a}	89.00 ± 0.24^{ab}
LP72	93.72 ± 1.05^{ab}	88.17 ± 0.09^{b}
LS48	92.84 ± 2.19^{ab}	89.25 ± 0.18^{a}
LS72	90.62 ± 0.46^{b}	88.74 ± 0.27^{ab}

Results are expressed as means ± standard deviation (n = 3). Values in the same column with different superscript letter are significantly different ($p < 0.05$).

Time - kill kinetics curve: Time-kill kinetics profiles of the FFJs with *L. plantarum* and *L. salivarius* against *Salmonella* Typhi DMST 22842 at the MIC concentrations were evaluated. Both FFJs with *L. plantarum* and *L. salivarius* at 72 hrs fermentation inhibited the *Salmonella* growth cycle curve of *Salmonella Typhi*, as shown in Figure 3. The results showed that treatment with FFJ did not have much affect on the number of *Salmonella Typhi* viable cells over the first 2, 4 and 6 hrs, which was approximately 7.2 log CFU/mL. However, after that, the FFJs showed anti-Salmonella activity: pathogen cell viability gradually decreased from 6 to 18 hrs with cell numbers of 4.0 and 3.0 log CFU/mL for FFJs with *L. plantarum* and *L. salivarius*, respectively. The anti-*Salmonella* effect of the FFJs against *Salmonella* viable cells was significantly different ($p < 0.05$) compared to the negative control, which was approximately 8.9 log CFU/mL. The reduction continued until 24 hrs, when all cells weredestroyed; this was also observedwhen treating with the antibiotic gentamycin. *Salmonella* treated with 1x MIC of gentamicin showed rapid reduction in the number of viable cells, starting with7.2 log CFU/mL at 0 hrs to 4.9 log CFU/mL at 2 hrs, then gradually decreasing until all *Salmonella* cells were destroyed after 24 hrs of treatment. The time-kill kinetics allow anti-*Salmonella* agents to be classified as bactericidal and characterized the relationship between agent concentration and activity over time. These results illustrated the fast and sustained action of anti-*Salmonella* agents presented in the FFJs. Time-kill kinetics show that the anti-*Salmonella* agents of FFJs produced by *L. plantarum* and *L. salivarius* should be administered at 2 hr intervals following first contact with the pathogen. A study on purification and partial characterization of anti-*Salmonella* agents nameda novel bacteriocin, Paracin 1.7, which was synthesized by *Lactobacillus paracasei* HD1-7 and isolated from Chinese sauerkraut juice [27].

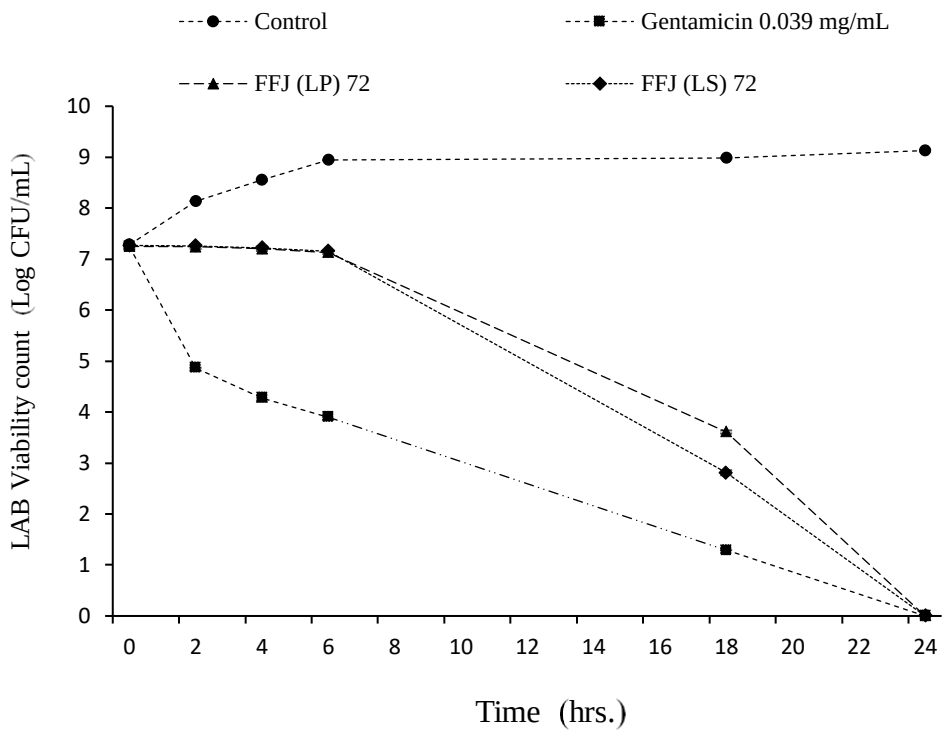


Figure 3. Time-kill kinetics curves of FFJs against *Salmonella* Typhi DMST 22842. The numbers of viable cells during treatment for 24 hrs was represented as the log reduction value of CFU counts of *Salmonella*. The error bars represent standard deviation of three replicates.

***Antioxidative capacity*:** The changes of antioxidant capacities of mixed fruit juice and fermented fruit-based juice (FFJ) with *L. salivarius* (LS) and *L. plantarum* (LP) are presented in Figure 4. The results showed that antioxidant capacity measured by DPPH and FRAP in the FFJs were higher than those in mixed fruit juice. The DPPH radical scavenging activity and ferric reducing antioxidant power showed a significant increase from 0 to 72 hrs fermentation; 72 hrshad the highest antioxidant capacity. However, it then decreased after further fermenting to 96 hrs. This correlates to the viability and growth curve of *L. salivarius* and *L. plantarum* in fermented fruit-based juice during fermentation, which increased from 0 to 72 hrs, then decreased after 96 hrs. Antioxidant activities determined by DPPH exhibited a value of 2.27 ± 0.03 mM TE/mL in fermented fruit-based juice at 0 hrs. After 24 hrs of fermentation by *L. plantarum* and *L. salivarius*, it rapidly increased to yield values of 2.55 ± 0.06 and 2.62 ± 0.05 mM TE/mL, respectively, which were significant (p < 0.05).Antioxidant activities showed little increase from 24 to 72 hrs, reaching 2.65 ± 0.05 and 2.64 ± 0.04 mM TE/mL for *L. plantarum* and *L. salivarius,* respectively. After 96 hrs of fermentation, antioxidant activity significantly (p < 0.05) lowered down to 2.08 ± 0.06 and 2.33 ± 0.05 mM TE/mL for *L. plantarum* and *L. salivarius,* respectively. An increase in DPPH radical scavenging activity from 0 to72 hrs suggests that LAB fermentation produced active compounds to improve the availability of polyphenol compounds with proton-donating properties [28], which involved the electron transfer mechanism by DPPH [29]. In this study, the phenolic compounds were evaluated in the mixed fruit juice and the 72 hr fermented fruit-based juice (FFJ). It was found that the phenolic compound significantly increased from 2.31 ± 0.01 mg GA/mL in the mixed fruit juice to 2.4 ± 0.03 mg GA/mL and 2.5

± 0.03 mg GA/mL in the *L. plantarum* and *L. salivarius* fermentation, respectively. In the same way, LAB fermentation positively affected by the FRAP assay, as shown in Figure 3B. Antioxidant capacity rapidly increased from 0 hrs with a value of 53 ± 0.35 mM TE/mL to 66.46 ± 0.26 mM TE/mL and 68.34 ± 0.80 mM TE/mL for *L. plantarum* and *L. salivarius,* respectively, after 24 hrs of fermentation. From 24 to 72 hrs, there was a significant ($p < 0.05$) increase to 71.31 ± 0.50 mM TE/mL and 72.34 ± 0.36 mM TE/mL for *L. plantarum* and *L. salivarius*, respectively. After 96 hrs fermentation, there was a significant decrease ($p < 0.05$) to 61.46 ± 0.56 mM TE/mL and 60.89 ± 0.43 mM TE/mL for *L. plantarum* and *L. salivarius*, respectively. The results indicate that FRAP assay could measure the reducing potential of an antioxidant produced during fermentation by LAB. The changes on antioxidant activity of fermented fruit-based juice were attributed to the organic acid production during fermentation, which could eventually influence antioxidant activity by changing the content and structure of phenolic compounds. LAB fermentation shows beta-glucosidase activity, which could liberate phenolic compounds after acidic and enzymatic hydrolysis of polymerized phenolic compounds during fermentation [30]. In this study, the results have shown that fermented fruit-based juice by LAB for 24 and 72 hrs improved antioxidant activity. Similar findings have been found in other studies, such as with fermented mulberry juice [31], and indicates that FFJ has overall good potential for free radical scavenging and reducing power.

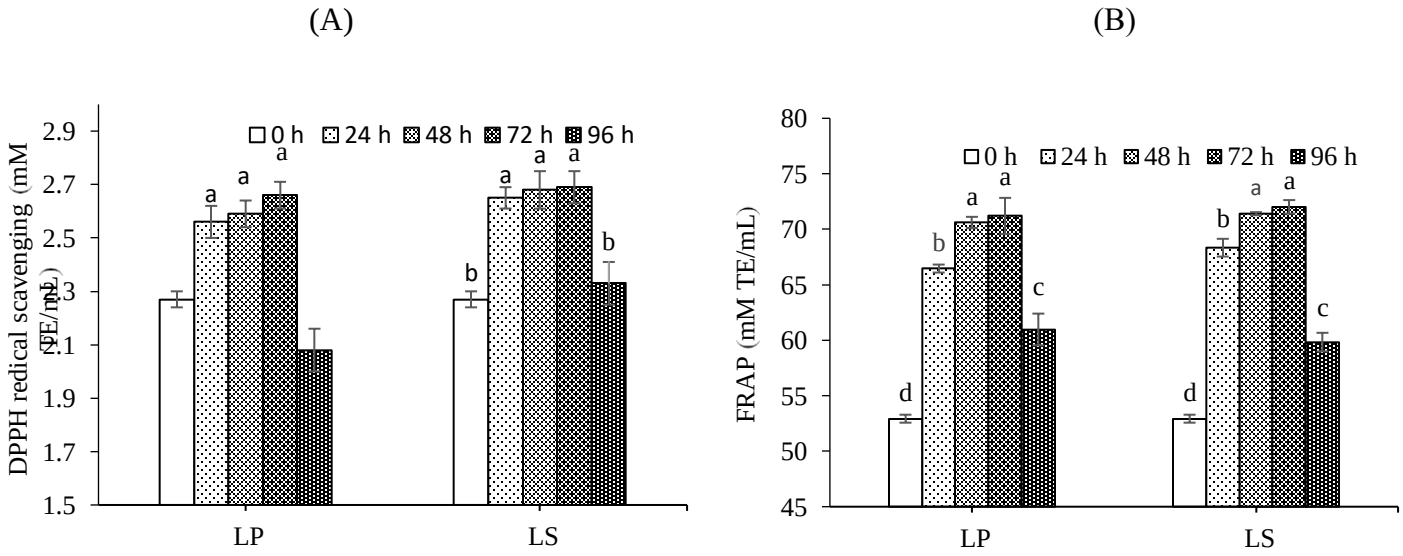


Figure 4. The antioxidant capacities of fermented fruit-based juice (FFJ) were detected and represented by (A) DPPH and (B) FRAP assay. Error bars indicate the standard deviations from two independent samples. Values in the same group column with different superscript letters are significantly different ($p < 0.05$) within a group.

***Identification of volatile compounds*:** One of the most important characteristics of food products for consumer palatability is the flavor and aroma volatile compounds [32]. Fermentation is a biological approach to produce diverse aroma compounds and strong exotic flavors due to a wide array of extracellular enzymes, especially hydrolases, produced by microorganisms [33]. Table 3 shows volatile compounds of mixed fruit juice (MFJ) and fermented fruit-based juice (FFJ) by *L. plantarum* (LP)

and *L. salivarius* (LS). 19 kinds of volatile compounds were identified in the MFJ and FFJ. The volatile profiles were identified by comparing mass spectra with the retention index database and characterized by the following classes: alcohols (7), aldehydes (2), acids (2), esters (5), ketones (1), phenol (1), and terpene (1). The most abundant volatile compounds in the FFJ were fruity aromas that have reported antimicrobial properties. Alcohols were the main dominant volatile compounds in FFJ. A major compound was isoamyl alcohol, which is a flavor aroma typically found in fruit such as pineapple [34], followed by an antimicrobial agent, benzene-ethanol [35]. Isobutyl alcohol, methionol, and benzyl alcohol were in less abundance. 1-Hexanol, a fruity flavor, and hotrienol, a fruity smelling compound, were dramatically increased in FFJ. Hexanol is derived from linoleic and linolenic acids through lipoxygenase pathway [36]. However, a 2,4-Dimethylbenzaldehydet widely used for flavor and fragrance agents was less present in FFJ compared to MFJ. The production of organic acids, such as acetic acid, was higherin FFJ fermented by *L. plantarum*, which is facultatively hetero-fermentative and produces acetic acid and ethanol as main products [24]. In contrast, *L. salivarius* is homo-fermentative and efficiently synthesizes lactic acid [22]. The presence of lactic acid in the fermented fruit-based juice (FFJ) was determined by HPLC and was present in a higher quantity than in the non-fermented mixed fruit juice (data not shown). Esters have a significant contribution to the aroma of fruits. In this study, ethyl acetate, a volatile aroma compound in pineapple, was significantly decreased in FFJ. Ethyl acetate was reported to be a potential antimicrobial agent [37]. However, another fruity odor, ethyl L-(-)-lactate was formed and detected in FFJ but not in MFJ; this also occurred for ethyl nonanoate, methyl benzoate and ethyl3-hydroxy-hexanoate. The ketone, 3-hydroxy-2-butanone, is typically used as a fruity flavor additive and was found in FFJ but not in MFJ. In addition, linalool, a monoterpene alcohol, is naturally found in many aromatic plants and known to exhibit various biological activities such as antimicrobial and antioxidant properties [38]. Interestingly, LAB fermentation significantly reduced 2,4-Bis(1,1-dimethylethyl)-phenol, (2,4-DTBP) in FFJ. This organic compound is a common toxic secondary metabolite produced by various groups of organisms; however, it was reported that 2,4-DTBP exhibits *in vitro* and *in vivo* biological activities, such as antimicrobial and antioxidant activities [39]. Several flavor and aroma volatile organic compounds appeared after MFJ was fermented by *Lactobacillus plantarum* (LP) and *Lactobacillus salivarius* (LS). Consequently, the fermented fruited-based juice possessed a strong fruity flavor and aroma, in addition to effective anti-*Salmonella* and antioxidant activity. This is similar to other fermented fruit juice research. For example, sweet lemon juice was fermented with *Lactobacillus plantarum* LS5 to produce a probiotic juice. The results showed the juice had antibacterial activity against *S. Typhimurium* and *E. coli* O157: H7. The fermented product also showed an increase of total phenolic compounds and antioxidant activity, as determined by DPPH radical scavenging and FRAP assay [40].

Table 3 Volatile compounds identified in mixed fruit juice (MFJ) and 72 hr fermented fruit-based juice (FFJ) with *L. plantarum* (LP) and *L. salivarius* (LS).

Component RT	Compound Name	% relative peak area		
		MFJ	LP	LS
	Alcohol			
6.6884	Isobutyl alcohol	-	6.0	4.7
9.4810	Isoamyl alcohol	-	99.0	78.8
13.2363	1-Hexanol	2.5	18.9	21.3
19.5323	Hotrienol	11.5	16.6	15.5
21.9279	Methionol	-	1.1	0.5
25.2658	Benzyl alcohol	-	0.7	0.7
25.9932	Benzene-ethanol	-	18.8	7.0
	Aldehyde			
2.5754	Acetaldehyde	-	0.4	0.5
24.1298	2,4-Dimethylbenzaldehyde	5.1	3.3	3.6
	Acid			
15.7147	Acetic acid	-	9.5	-
24.6451	Hexanoic acid	-	0.8	0.5
	Ester			
3.4763	Ethyl acetate	72.0	42.8	30.0
12.9393	Ethyl L -(-)- lactate	-	4.5	5.4
17.8943	Ethyl nonanoate	-	2.0	1.2
19.8459	Methyl benzoate	-	2.3	1.7
21.148	Ethyl 3-hydroxyhexanoate	-	0.6	0.7
	Ketone			
11.463	3-hydroxy-2-butanone	-	20.1	25.1
	Phenol			
33.3645	2,4-Bis(1,1-dimethylethyl)phenol	81.0	56.0	73.9
	Terpene			
18.0575	Linalool	13.5	14.3	15.5

-, Not detectable.

[a]RI (retention index) based on VF-WASms capillary column using a series of C8-C20.

[b]All compounds were identified by comparison with mass spetra and retention index database.

[c]% Relative peak area of volatile compounds is expressed as (compound peakarea/total compounds peakarea) × 100.

CONCLUSION

A novel functional fermented fruit-based juice (FFJ) made with pineapple (*Ananas comosus* L. Merr) and winter melon (*Benincasa hispida* Cogn) showed excellent growth capacity with 8 log CFU/mL of probiotics, *Lactobacillus plantarum* and *Lactobacillus salivarius*. Study showed that the FFJ exhibited increased antioxidant capacity, as well as increased anti-*Salmonella* activity against *Salmonella* Typhi DMST 22842. These effects are due to the

fermentation causing biotransformations of chemical substances in the fruit juice by LAB; this in turn formed bioactive compounds, which not only improved fruit flavor and aroma but also influenced anti-*Salmonella* and antioxidant activity. In addition to their high antioxidant and antipathogen activity, the FFJ had a reduced sugar content and is a suitable carrier for prebiotics and probiotics. Therefore, the FFJ can be served as a probiotic beverage and a functional fermented drink for vegan and non-diary consumption.

List of Abbreviations: FFJ: fermented fruit-based juice, LP: *Lactobacillus plantarum*, LS: *Lactobacillus salivarius*, MIC: minimum inhibitory concentration, MBC: minimum bactericidal concentration.

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