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Lactic Acid Bacteria Producing Sorbic Acid and Benzoic Acid Compounds from Fermented Durian Flesh (Tempoyak) and Their Antibacterial Activities Against Foodborne Pathogenic Bacteria

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Abstract

Background and Objective: Antibacterial compounds produced by lactic acid bacteria are believed to replace functions of chemical preservatives. The objectives of this study were to identify lactic acid bacteria, which produced antibacterial compounds, from fermented durian flesh (tempoyak) and to assess antibacterial activities of the isolates.

Material and Methods: Two bacterial identification techniques were used, including API 50 CHL kit with supplementary medium and matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF/MS).

Results and Conclusion: Four various lactic acid bacteria strains of *Lactobacillus buchneri*, *Lactobacillus plantarum*, *Lactobacillus brevis* I and *Lactobacillus acidophilus* I were identified using API 50 CHL Kit and five various others of *Lactobacillus paracasei* DSM 2649, *Lactobacillus buchneri* DSM 20057T, *Lactobacillus parabuchneri* DSM 57069, *Lactobacillus paracasei* DSM 20020 and *Lactobacillus farcimini* CIP 103136T using MALDI-TOF/MS. Cell-free supernatant extracted from *Lactobacillus plantarum*, *Lactobacillus buchneri*, *Lactobacillus brevis* I and *Lactobacillus acidophilus* I included strong inhibitory effects against *Vibrio cholera* O1 (Inaba type), *Vibrio cholera* O139 (Bengal type), *Vibrio parahaemolyticus* ATCC 17802, *Escherichia coli* ATCC 11795, *Escherichia coli* O157, *Salmonella typhimurium* ATCC 14028 and a total of 23 serotypes of *Salmonella* spp. associated with outbreaks of food poisoning from raw chicken, egg shell and water samples. Only *Lactobacillus buchneri* DSM 20057T was identified by MALDI-TOF/MS as a strain producing sorbic and benzoic acids. This strain can potentially be used as food preservative to decrease growth of foodborne pathogenic bacteria.

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1. Introduction

Food contamination with pathogenic bacteria has become a global concern since it can result in severe hazards to human health [1,2]. The particular interests are due to a global outbreaks associated to *Vibrio* (*V.*) *cholera* and *Escherichia coli* O157, causing tremendously high numbers of fatalities worldwide. From 1998 to 2008, more than 13,000 cases of food poisoning outbreaks were documented in the United States [3]. In 2018, the foodborne diseases active surveillance network (FoodNet) identified 25,606 cases of infections; from which, 5893 were hospitalizations and/or dead [4]. Food poisoning incidences in schools of Malaysia have increased by 57% from 30 incidences in 2015 to 45 incidences in 2016 [5,6]. Despite stringent rules of the ministry of health to govern hygienic practices of local food handlers, food poisoning cases are still high. The major causative factor behind food poisoning outbreaks in

Malaysia is the hot and humid weather that promotes the growth of pathogenic bacteria. Food poisoning becomes more common as drug resistance increases as a result of incorrect use of antibiotics in treatments. Recently, use of farming practices, natural antibiotics, nano-antibiotics, lactic acid bacteria (LAB), bacteriocins, cyclopeptides, bacteriophages, synthetic biology and predatory bacteria have been studied to replace traditional antibiotics [7]. Fermented durian flesh or tempoyak is one of the valuable food heritage products in Malaysia and Indonesia, which is usually served as a condiment with local herbs [8,9]. Malaysians typically produce tempoyak by fermenting durian pulps in closed jars for up to several days (4-7 days). After this time, fermentation process produces an acidic and strongly flavored semi-solid durian flesh with a pH range of 3.96-4.08 [9,10]. It has previously been verified by [11] that tempoyak includes a variable diversity of LAB microflora depending on the quality of the durian flesh. Recently, LAB strains have been reported to suppress growth of pathogenic bacteria [12,13]. These bacteria include probiotic properties with health benefits [14-16]. However, no studies are available on public safety of tempoyak consumption, indicating that tempoyak is safe.

Lactobacillus spp. are the predominant LAB microflora in tempoyak. However, types of LAB microflora are affected by the places, where the products are prepared [17]. Various LAB strains such as *Lactobacillus* (*L*). *plantarum*, *L. brevis*, *L. mali*, *L. fermentum* have been isolated from tempoyak in Malaysia [11-14,18-20]. In contrast, tempoyak from Indonesia has been reported to contain LAB strains such as *L. plantarum*, *L. casei*, *L. corynebacterium* and *L. casei* [21,22]. Although LAB metabolites are able to inhibit the growth of pathogenic bacteria, they are categorised as generally recognised as safe [23]. Furthermore, LAB have been verified to help trigger immune reactions, prevent contaminations of enteropathogenic bacteria and treat diarrhoea [24]. Mostly, supernatants of LAB such as *L. casei* and *L. rahmnosus* include several antimicrobial components in forms of organic acids, hydrogen peroxide, aroma components, fatty acids and low-molecular weight compounds that can destroy pathogenic bacteria [25,26].

Therefore, it is critical to identify novel LAB strains from Malaysian tempoyak to prevent outbreaks of pathogenic bacteria. Furthermore, use of LAB in foods has become popular, compared to toxic chemical preservatives. Thus, purposes of this study were to identify LAB from tempoyak that produced antibacterial compounds and to assess antibacterial activities of these LAB.

2. Materials and Methods

2.1. Sampling of fermented durian flesh (tempoyak)

A total of eight samples of fermented durian flesh (approximately 100 g each) were purchased from local sellers in food markets of Kuala Terengganu and Marang, Terengganu, Malaysia. These samples were labelled as T001, T002, T004, T006, T007, T008, T009 and T010 and transferred to the Food Microbiology Laboratory, Faculty of Fisheries and Food Science at University Malaysia Terengganu, Terengganu, Malaysia, using cold chain. After arrival, samples were stored at 4 ℃ until use.

2.2. Isolation of Lactic Acid Bacteria

Isolation of LAB was carried out based on a method by Muhialdin et al. [18] with some modifications. Approximately, 25 g of the fermented durian flesh were diluted with 0.1% sterile peptone water (Oxoid, UK) at a ratio of 1:10. Samples were homogenised to achieve six serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}). Then, 100-µl aliquot of each sample was spread onto four selective media of de Man, Rogosa and Sharpe agar (MRSA), MRSA with 0.8% calcium carbonate, tomato juice agar (TJA) and M17 agar. All media were purchased from Oxoid, UK. Various types of selective media (MRSA, MRSA and $CaCO_3$, TJA and M17 agar) were used to isolate LAB species from tempoyak samples. All plates were incubated anaerobically at 30 ℃ with 5% of carbon dioxide (CO_2) using anaerobic incubator for 48-72 h. Presumptive LAB colonies were Gram-positive rod or cocci, which were typically identified based on morphological characteristics as colony-forming flat, rough grey to whitish-like bacteria with irregular edges and diameters of 1-3 mm.

2.3. Identification of LAB isolates using biochemical tests and matrix-assisted laser desorption ionisation-time of flight mass spectrometry

Briefly, LAB isolates were identified using two various techniques of 1) API 50 CHL Kit with supplementary media, and 2) matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF/MS). The presumptive isolates of LAB that were Gram-positive rod or cocci and included negative results for catalase and oxidase tests were preserved in a semi-solid agar of MRS covered with 20% glycerol. In biochemical tests using API 50 CHL Kit with supplementary media (BioMerieux, Montalieu-Vercieu, France), color changes for 50-wells carbohydrate fermentation strips of the isolates were assessed after incubation at 35 °C for 48 h. Then, API profiles were analysed using API profile index number and API Web System (BioMerieux, Montalieu-Vercieu, France). The second identification was carried out using MALDI-TOF/MS. The protocol was adapted from Callaway et al. [27] with some modifications. Eight isolates of LAB were identified using direct transfer and smears of biological materials on target plates by the addition of 1 µl of α-cyano-4-hydroxycinnamic acid matrix (HCCA) (Sigma-Aldrich, USA). The matrix was dissolved with a standard solvent system containing acetonitrile, water and trifluoroacetic acid at a ratio of 20:19:1. Then, compounds were identified using MALDI-TOF/MS (Bruker, Germany). Mass spectra of the compounds were achieved using MALDI Biotyper Software V.3.0 with at least 10^4 CFU/ml.

2.4. Preparation of the cell-Free supernatant of LAB Strains

Four strains from the identified LAB *L. plantarum* (V1), *L. buchneri* (V8), *L. brevis* I (V11) and *L. acidophilus* I (V14)] using API technique were harvested aerobically at 30 ℃ for 24 h in MRS broth until they reached a density of nearly 10^7 CFU ml^{-1}. Then, Cell-free supernatant (CFS) from each LAB strain was prepared by centrifugation at 10,000 rpm for 5 min. Supernatants were collected for their antibacterial activities according to Perez et al. [28].

2.5. Antibacterial screening of the cell-Free supernatant of Lactic Acid Bacteria strains using well diffusion method based on Perez et al. [28]

Antibacterial activity of the CFS for each LAB strain was assessed against 23 types of *Salmonella* spp. as well as other associated pure cultures and ATCC bacteria using well-diffusion method and method described by Perez et al. [28]. A total of 23 serotypes of *Salmonella* spp. From the outbreaks of food poisoning were collected from the Laboratory of Food Safety and Quality Division, Terengganu and Kelantan Departments of Health, Ministry of Health, Malaysia. Other bacterial strains such as *S. typhimurium* ATCC 14028, *V. cholera* non O1-0139 (Bengal type), *V. cholera* O1 (Inaba type), *V. parahaemolyticus* ATCC 17802, *E. coli* ATCC 11795 and *E. coli* O157 cultures were provided by the Microbiological Unit, Food Safety and Quality Division, Terengganu, Ministry of Health, Malaysia. All strains of the pathogenic bacteria were involved in foodborne diarrhoeal cases in Malaysia or other countries. In total, 23 strains of *Salmonella* spp., *S. typhimurium* ATCC 14028 and pathogenic strains were diluted using 0.1% peptone water to achieve 0.5 McFarland turbidity standard. Targeted pathogenic bacteria were freshly cultured onto Muller-Hilton agar (Merck, Germany). Then, 6-mm diameter size wells were prepared in plates and a total of 80 µl of CFS from each LAB strain were transferred into each well. All plates were aerobically incubated at 37 ℃ for 18-24 h. Then, zones of inhibition were measured and reported. The MRS broth with no bacteria was used as negative and ampicillin (500 µg ml^{-1}) was used as positive controls.

2.6. Identification of bioactive compounds from semi-purified cell-free supernatant of *L. buchneri* DSM 20057T using high-performance liquid chromatography

Semi-purification of CFS from *L. buchneri* DSM 20057T was carried out following the method described by Brown et al. [29] using series of methanol fractions at concentrations of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% into a Sep-Pak C18 Solid Phase Extraction Cartridge Column (Agilent Technologies, USA) to separate their bioactive compounds before identifying the compounds using high-performance liquid chromatography (HPLC). Then, methanol fractions at 70 and 80% were pooled and 10-µl filtrates were injected into HPLC using ZORBAX C18 Column (Agilent Technologies, USA) with dimensions of 150 mm (length) × 4.1 mm (width) × 5 µm (internal diameter). This was allowed to flow at a rate of 1.0 ml min^{-1}, column temperature of 40 ℃ and detection of wavelength at 227 nm. The mobile phase was prepared using a mixture of 1900 ml of 0.03 M phosphate buffer (3.8 g of K_2HPO_4 and 5 g of KH_2PO_4 in 2 l of distilled water, pH 6.5) and 100 ml of 95% methanol. Then, samples from C18 Solid Phase Extraction Column were concentrated and loaded onto a C18 Column (TSK gel Tosho Bioscience, USA). Benzoic and sorbic acids (40 mg/kg each) were prepared as positive controls and 4-aceramidophenol as internal standard. Moreover, MRS broth was used as negative control. Presence of benzoic and sorbic acids was verified by not more than 5% retention time differences between the sample and standard solution peaks and spectral index capabilities of a photo-diode array detector (FQC laboratory method, E04-002, 2013). For the assessment of sorbic acid and benzoic acid concentrations, the Eq. 1was used [29]:

$$\text{Compound concentration (mg/kg)} = \frac{\text{HPLC reading (mg/L)} \times \text{final volume (ml)}}{\text{Weight (g)}} \quad \text{Eq. 1}$$

3. Results and Discussion

The LAB count of the samples varied based on the type of media, ranging 10^4–10^6 CFU/g (Table 1). All LAB isolated from tempoyak were identified as Gram-positive rods and were negative for catalase and oxidase assays (Table 1). In this study, morphology of the presumptive *Lactobacillus* spp. on MRSA was less common reported and differentiated from the regular *Lactobacillus* strains. Strains were irregular or included rough marginal shapes.

Out of eight isolated LAB using API 50 CHL Kit and MALDI-TOF/MS, four isolates (V3, V7, V8, V9 and V13) included similar identification results of *L. buchneri* (Table 2). However, four LAB isolates (V1, V11, V9 and V14) showed contradictory results of species identification, comparing the two methods (Table 2 and Figure 1(a,b)).

After 24-48 h of incubation, LAB isolates demonstrated color changes with various sugar compositions. The *L. plantarum* and *L. brevis* showed positive reactions with at least 24 sugar types (Table 3). Moreover, *L. buchneri* and *L. acidophilus* showed positive reactions with a fewer sugar types of 12 and 15, respectively (Table 3). All LAB isolates produced six sugar types such as galactose, D-glucose, D-fructose, D-mannose, maltose and saccharose. Eight sugar types, including N-acetyl glucosamine, amygdaline, salicine, cellobiose, lactose, trehalose, β-gentibiose and D-turanose, were produced by all LAB isolates, except *L. buchneri*. The *L. plantarum* produced rhamnose, D-raffinose, β-methyl-D-mannoside and gluconate, compared to other LAB isolates.

Table 1. Bacterial colony counts and basic characteristics of lactic acid bacteria in various culture media

Sample ID	Media	Basic Confirmation Tests			LAB count (CFU g^{-1})
		Gram's Staining	Catalase	Oxidase	
T001	MRS Agar	+	-	-	2.0 X 10^6
	MRS + 0.8 % $CaCO_3$	N/A	N/A	N/A	NG
	TJA	N/A	N/A	N/A	NG
	M17	N/A	N/A	N/A	NG
T002	MRS Agar	+	-	-	4.4 X 10^6
	MRS + 0.8 % $CaCO_3$	N/A	N/A	N/A	NG
	TJA	N/A	N/A	N/A	NG
	M17	N/A	N/A	N/A	NG
T004	MRS Agar	+	-	-	3.0 X 10^6
	MRS + 0.8 % $CaCO_3$	N/A	N/A	N/A	NG
	TJA	+	-	-	4.2 X 10^6
	M17	+	-	-	3.7 X 10^6
T006	MRS Agar	+	-	-	3.0 X 10^5
	MRS + 0.8 % $CaCO_3$	+	-	-	2.5 X 10^5
	TJA	+	-	-	2.0 X 10^5
	M17	N/A	N/A	N/A	NG
T007	MRS Agar	+	-	-	5.7 X 10^6
	MRS + 0.8 % $CaCO_3$	+	-	-	2.0 X 10^6
	TJA	+	-	-	5.4 X 10^6
	M17	N/A	N/A	N/A	NG
T008	MRS Agar	+	-	-	3.4 X 10^6
	MRS + 0.8 % $CaCO_3$	+	-	-	4.0 X 10^6
	TJA	+	-	-	4.4 X 10^6
	M17	+	-	-	1.0 X 10^6
T009	MRS Agar	+	-	-	5.6 X 10^5
	MRS + 0.8 % $CaCO_3$	+	-	-	4.0 X 10^4
	TJA	+	-	-	8.1 X 10^5
	M17	N/A	N/A	N/A	NG
T010	MRS Agar	+	-	-	1.9 X 10^5
	MRS + 0.8 % $CaCO_3$	+	-	-	1.4 X 10^5
	TJA	+	-	-	1.7 X 10^5
	M17	N/A	N/A	N/A	NG

*NG: No growth, N/A: Not applied, Lactic Acid Bacteria: LAB

Table 2. Comparisons of API 50 CHL Kit and MALDI-TOF/MS identification methods for lactic acid bacterial isolates

Sample ID	API 50 CHL		MALDI-TOF/MS		
	Proportion of similarity (%)	Identification of genus	NCBI Identifier	Score value	Identification of genus
V1	99.9	*L. plantarum*	47714	2.396	*L. paracasei** DSM 2649
V3	96.4	*L. buchneri*	1581	1.701	*L. buchneri* DSM 20057T
V7	99.6	*L. buchneri*	1581	2.08	*L. buchneri* DSM 20057T
V8	99.6	*L. buchneri*	1581	2.08	*L. buchneri** DSM 20057T
V9	99.6	*L. buchneri*	152331	1.74	*L. parabuchneri* DSM 57069
V11	99.6	*L. brevis* I	47714	2.443	*L. paracasei* DSM 20020 DSM
V13	99.7	*L. buchneri*	1581	1.89	*L. buchneri* DSM 20057T
V14	95.5	*L. acidophilus* I	1612	2.053	*L. farciminis* CIP 103136T

* An example of MALDI-TOF mass spectra of *L. paracasei*, V1 (Figure 1a) and *L. buchneri*, V8 (Figure 1b), *L.* = *lactobacillus*

Insulin was detected in *L. brevis*, while 5-ceto-gluconate was detected in *L. buchneri*. Only *L. acidophilus* was not able to produce L-arabinose, ribose and melizitose. Furthermore, *L. plantarum* and *L. brevis* could produce mannitol, sorbitol and arbutine. Three separate sugar types of melibiose, D-tagalose and D-xylose were reported in three combinations of LAB strains, including *L. plantarum* and *L. buchneri*, *L. brevis* and *L. acidophilus* and *L. buchneri* and *L. brevis*, respectively.

In general, *L. plantarum* (V1) showed strong inhibition against 17 strains of *Salmonella* spp. (inhibition zone diameters of 16-20 mm) (Table 4). Moreover, *L. plantarum* demonstrated intermediate inhibition against seven strains of *Salmonella* spp. (inhibition zone diameters of 11-14 mm) (Table 4).

The *L. buchneri* (V8) included strong inhibition against 13 strains of *Salmonella* spp. (inhibition zone diameter of 15-21 mm) (Table 4).

Table 3. Biochemical characteristics of *Lactobacillus* spp. isolated from tempoyak after 24-48 h of incubation using API 50 CHL Kit

Sugar Fermentation	*L. plantarum* (V1)	*L. buchneri* (V3, V7, V8, V9, V13)	*L. brevis* (V11)	*L. acidophilus* (V14)
Control	-	-	-	-
Glycerol	-	-	-	-
Erythritol	-	-	-	-
D-Arabinose	-	-	-	-
L-Arabinose	+	+	+	-
Ribose	+	+	+	-
D-Xylose	-	+	+	-
L-Xylose	-	-	-	-
Adonitol	-	-	-	-
B-Methyl Xylose	-	-	-	-
Galactose	+	+	+	+
D-Glucose	+	+	+	+
D-Fructose	+	+	+	+
D-Manose	+	+	+	+
L-Sorbose	-	-	-	-
Rhamnose	+	-	-	-
Dulcitol	-	-	-	-
Inositol	-	-	-	-
Mannitol	+	-	+	-
Sorbitol	+	-	+	-
β-Methyl-D-mannoside	+	-	-	-
β-Methyl-D-glucoside	-	-	-	-
N-acetyl glucosamine	+	-	+	+
Amygdaline	+	-	+	+
Arbutine	+	-	+	-
Esculine	-	-	-	-
Salicine	+	-	+	+
Cellobiose	+	-	+	+
Maltose	+	+	+	+
Lactose	+	-	+	+
Melibiose	+	+	-	-
Saccharose	+	+	+	+
Trehalose	+	-	+	+
Insuline	-	-	+	-
Melizitose	+	+	+	-
D-Raffinose	+	-	-	-
Amidon	-	-	-	-
Glycogene	-	-	-	-
Xylitol	-	-	-	-
β-Gentibiose	+	-	+	+
D-Turanose	+	-	+	+
D-Lyxose	-	-	-	-
D-Tagalose	-	-	+	+
D-Fucose	-	-	-	-
L-Fucose	-	-	-	-
D-Arabitol	-	-	-	-
Gluconate	+	-	-	-
2-ceto-gluconate	-	-	-	-
5-ceto-gluconate	-	+	-	-

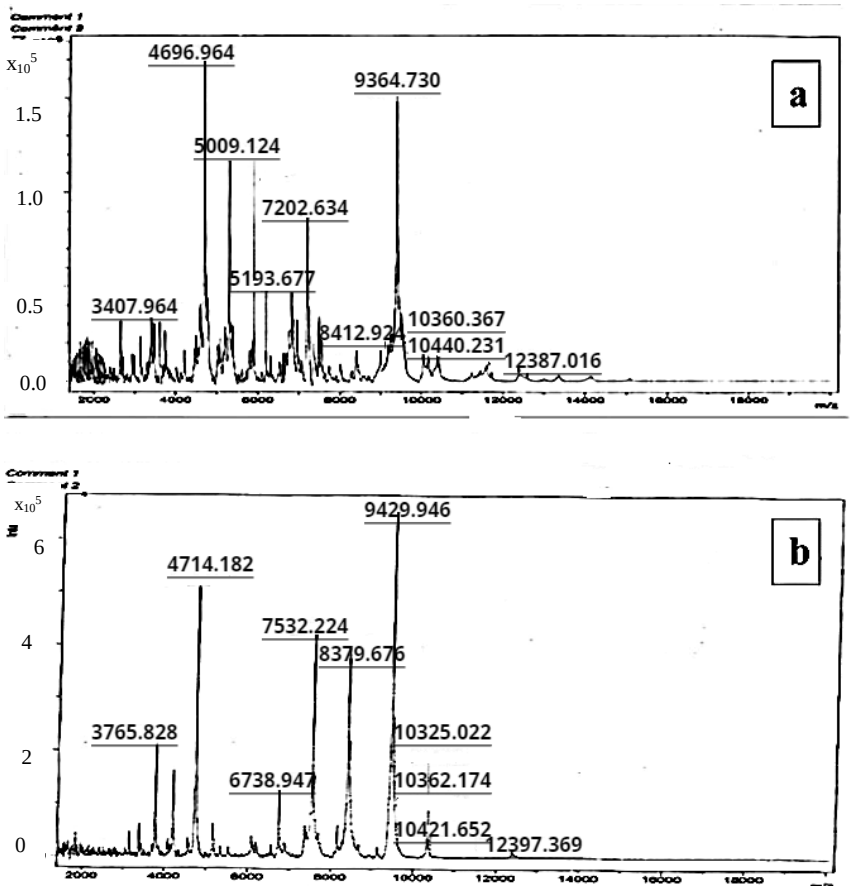

Figure 1. MALDI-TOF mass spectra of *L. paracasei* V1 (1a) and *L. buchneri* V8 (1b), *L= lactobacillus*

Table 4. Antibacterial activities of V1, V8, V11 and V14 LAB strains against *Salmonella* spp. and *Salmonella typhimurium* ATCC strain

Salmonella spp. Test Strain	Diameter of Inhibition (mm)			
	L. plantarum (V1)	*L. buchneri* (V8)	*L. brevis* (V11)	*L. acidophilus* 1 (V14)
Salmonella typhimurium (ATCC 1408)	18±0	18±0.3	20±0	20±0
S. typhimurium (Raw chicken)	18±0.3	17±0.3	20±0.3	19±0.3
S. enteriditis (Well water)	18±0.3	14±0.3	18±0	20±0.3
S. paratyphi B (Well water)	18±0.3	11±0.3	13±0.3	14±0
S. chester (Pipe water)	14±0.3	12±0.3	13±0.3	14±0.3
S. sarajane (Well water)	13±0.3	11±0.3	15±0.3	15±0
S. richmond (Pipe water)	14±0.3	13±0.3	15±0.3	18±0.3
S. chingola (GFS water)	13±0.3	9±0.3	12±0.3	15±0.3
S. paratyphi A (Tube well water)	16±0.3	12±0.3	15±0.3	15±0.3
S. louga (Pipe water)	11±0.3	12±0.3	15±0.3	16±0.3
S. mountpleasant (Well water)	14±0.3	10±0.3	14±0	14±0.3
S. sandiago (Well water)	18±0.3	16±0.3	16±0.3	21±0.3
S. winslow (Pipe water)	17±0.3	16±0.3	17±0.3	17±0.3
S. borbeck (Tube well water)	15±0.3	13±0.3	15±0	18±0
S. rhydyfellin (Coconut milk sieve)	17±0.3	17±0.3	16±0.3	18±0.3
S. weltevreden (GFS pipe water)	20±0.3	21±0.3	22±0.3	22±0.3
S. bareilly (Well water)	20±0.3	20±0.3	20±0.3	20±0.3
S. panama (Well water)	21±0	17±0.3	18±0.3	21±0.3
S. farsta (Chicken dish)	20±0	19±0.3	19±0.3	19±0.3
S. corvalis (Raw chicken)	20±0.3	17±0.3	20±0.3	20±0.3
S. albany (Well water)	19±0.3	14±0.3	17±0.3	18±0.3
S. adamstua (Stream water)	18±0.3	17±0.3	18±0.3	18±0
S. tsevie (Well water)	14±0.3	15±0.3	16±0.3	18±0.3
S. livingston (Egg shell)	20±0	20±0.3	22±0.3	22±0

The strength of antibacterial activity (S: Strong, I: Intermediate, W: Weak). Diameter of Inhibition (mm) of *Lb. plantarum* ($9 \leq W$; $10 \leq I \leq 14$; $S \geq 15$)

A total of ten strains of *Salmonella* spp. showed intermediate inhibition by *L. buchneri* (inhibition zone diameter of 11-14 mm) (Table 4). Only *Salmonella (S.) chingola* was weakly inhibited by *L. buchneri*. A total of 11 strains of *Salmonella* spp. were strongly inhibited by *L. brevis* I (V11). Furthermore, *L. brevis* demonstrated intermediate inhibition against four strains of *Salmonella* spp. with diameter of inhibition zones of 12-14 mm (Table 4). In contrast, *L. acidophilus* 1 (V14) strongly inhibited 21 strains of *Salmonella* spp. and three strains included intermediate responses (Table 4). All LAB isolates responded strongly to all strains of *Vibrio* spp. and *E. coli* O157 (Table 5), except *E. coli* ATCC 11795, which intermediately responded to *L. plantarum* (V1) and *L. buchneri* (V8) (Table 5).

Two major compounds were identified in semi purification of CFS from *L. buchneri* DSM 20057T. Based on the chromato-grams of HPLC, these compounds included benzoic and sorbic acids (Table 6) with the presence of three predominant peaks at retention times of 8.502, 10.957 and 17.102 min, respectively. Sorbic acid included the highest yield (6.5 mg kg^{-1}), compared to that the benzoic acid did (3.4 mg kg^{-1}).

Antibacterial activity of the semi-purified CFS from *L. buchneri* DSM 20057T using various methanol fractions (10–100%) demonstrated that the 70-100% methanol fractions included activities against *S. typhimurium* ATCC 14028, *E.coli* ATCC 11795, *E. coli* O157, *S. aureus* ATCC 25923 and *V. cholerae* O139 (Figure 2). Inhibition zone diameters of all these foodborne pathogens ranged 8–12 mm. Results were similar to those from the control (water-treated sample) and 10% methanol (inhibition zone diameter of 10-15 mm). No inhibitions were shown by the pathogens for 20, 30, 40, 50 and 60% methanol fractions.

In this study, MRS agar was the only media capable of supporting growth of all samples because it contained high carbon, nitrogen, yeast extract and mineral contents [30]. The MRS agar was designed to facilitate growth of LAB, which included species of the following genera of *Lactobacillus*, *Streptococcus*, *Pediococcus* and *Leuconostoc* [30]. According to Yang et al. [31], the optimal growth conditions for three species of LAB (*L. curvatus*, *Enterococcus faecium* and *L. paracasei*) were described using MRS media at pH range of 6.2-8.5 and optimal temperature of 37 ℃. At pH values lower than 4.5, researchers have discovered that the LAB growth becomes slower and is sometimes suppressed because the enzymatic reactions are controlled by pH [32,33]. The five LAB samples were demonstrated to include relatively good to excellent identifications with similarity proportions of 96.4-99.7 % using API 50 CHL Kit. These results are similar to those of MALDI-TOF/MS, which produced a score value range of 1.70–2.08, indicating possible and secure genera and species identification. In supporting these results, Leisner et al. [11] identified five dominant strains of LAB in fermented durian pulps with *L. plantarum* as the major bacteria. Four other strains included *L. brevis*, *L. fermentum*, *L. mali* and *L. mesenteroides*. These differences between the biochemical and molecular identifications were particularly because the fundamental approaches of the two techniques principally varied. Technically, the API 50 CHL Kit function was based on comparisons of fermentation patterns for 50 various types of carbohydrates with registered bacteria in APIweb Database. Advantages of this method included easy and rapid performances and results depended solely on visualization of their phenotypic properties. In fact, identification depended on registered bacteria in APIweb Database while biochemical profiles of LAB were partially listed in the database and therefore certain LAB might improperly be identified [34].

In this study, the two assays (API 50 CHL Kit and MALDI-TOF/MS) have shown various results, especially in identification of V1, V11 and V14 LAB strains. Several researchers have reported that specificity of the API 50 CHL Kit is unclear due to various phenotypic patterns and non-reactivity shown by the identified LAB strains, leading to misidentification or misinterpretation of the results [35-38].

Table 5. Antibacterial activities of V1, V8, V11 and V14 LAB strains against *Vibrio* spp. and *Escherichia coli* strains

Test Strains	Diameter of Inhibition (mm)				
	L. plantarum (V1)	*L. buchneri* (V8)	*L. brevis* (V11)	*L. acidophilus* 1 (V14)	Ampicillin (positive control)
Vibrio cholera NON 01-0139 (Benggal type)	30±0.3	23±0.3	28±0.3	26±0.3	25±0.3
V. cholera 01 (Inaba type)	22±0.3	18±0.3	22±0.3	22±0.3	24±0.3
V. parahaemolyticus ATCC 17802	18±0.3	16±0.3	22±0.3	22±0.3	24±0.3
Escherichia coli ATCC 11795	18±0.3	12±0.3	20±0.3	18±0.3	25±0.3
E. coli 0157	20±0.3	16±0.3	20±0.3	20±0.3	25±0.3

The strength of antibacterial activity (S: Strong, I: Intermediate, W: Weak). Diameter of Inhibition (mm) of *Lb. plantarum* ($9 \leq W$; $10 \leq I \leq 14$; $S \geq 15$).

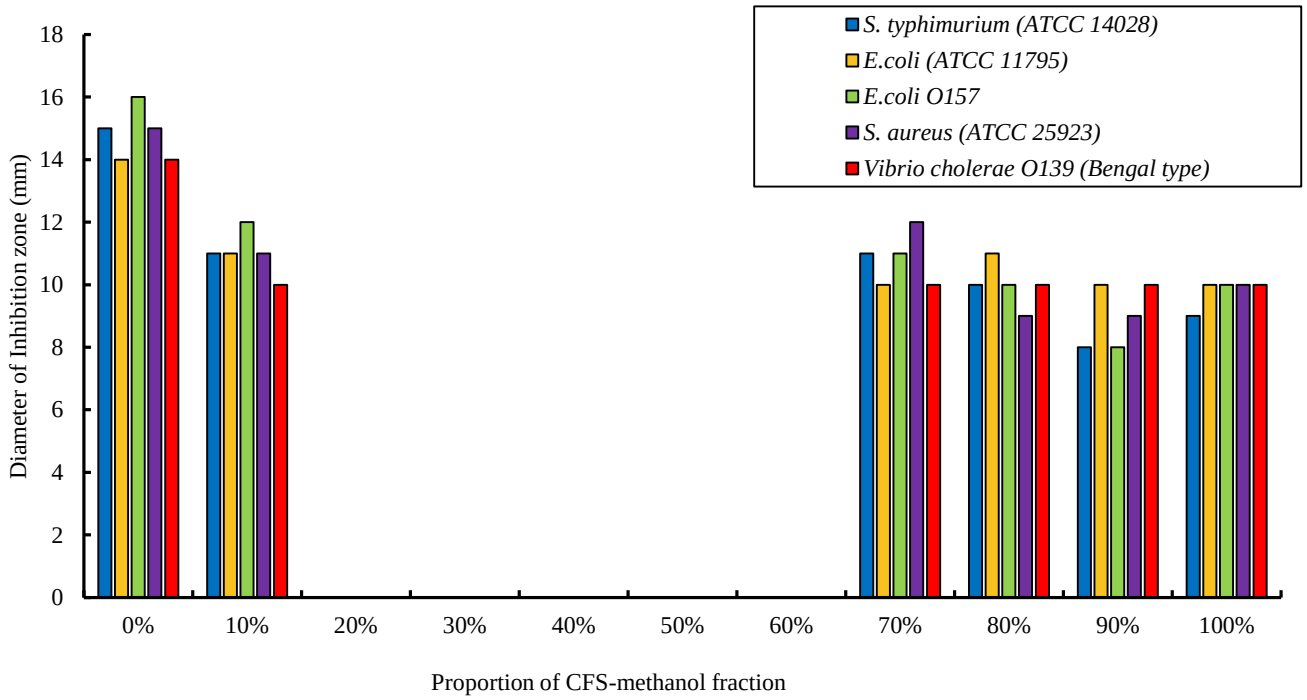

Figure 2. Effects of various proportion of CFS-methanol fraction isolated from *Lactobacillus buchneri* (V8) DSM 20057T against foodborne pathogenic bacteria

Table 6. Assessments of the antimicrobial compounds produced by *Lactobacillus buchneri* DSM 20057T (V8) after semi-purification in 70–80% methanol fractions using HPLC

Retention time (min)	Area (mAU*s)	Amount (mg l^{-1})	Compound name
8.502	64.598	3.396	Benzoic Acid
10.957	106.822	6.475	Sorbic Acid

Since discrepancy occurred in biochemical identifications using API 50 CHL Kit and MALDI-TOF/MS (particularly for LAB strains of V1, V11 and V14), molecular techniques such as 16sRNA sequencing are needed to verify the specific strains. Favourable conditions in tempoyak for the growth of four strains of LAB (*L. plantarum*, *L. buchneri*, *L. brevis* and *L. acidophilus*) were due to the chemical composition of the durian fruit with total sugar of 15-20% [39] and saccharose of 17% [11]. Based on the biochemical assay of API 50 CHL Kit, *L. plantarum* utilised the largest composition of sugars (25 types), followed by *L. brevis*, *L. acidophilus* and *L. buchneri* with 23, 15 and 12 types of sugars, respectively. These findings were supported by Leisner et al. [11], who reported *L. plantarum* as the predominant LAB due to the lack of acid production from glycerol and D-xylose. In general, roles of *L. plantarum* and *L. brevis* are well-known in acid-fermented durian [40].

In this study, *L. buchneri* DSM 20057T produced a greater yield of benzoic acid at 6.5 mg/kg, compared to sorbic acid at 3.4 mg kg^{-1}. This finding was contrary to that reported by Salomskiene et al. [41], who isolated a various strain of LAB, *L. helveticus* R, isolated from sour creams and breads using spontaneous fermentation as the best producer of benzoic acid (1.80 mg/L). The *L. buchneri* DSM 20057T produced the highest quantity of sorbic and benzoic acids, compared to that reported by Salomskiene et al. [41]. Salomskiene et al. reported that 12 LAB strains of *L. lactis* 140/2, *L. lactis* 57, *L. lactis* 768/5, *E. faecium* 59-30, *E. faecium* 41-2, *L. helveticus* 14, *L. helveticus* 3, *L. helveticus* 148/3, *L. helveticus* R, *S. thermophilus* 43, *L. reuteri* 3 and *L. reuteri* 7 produced benzoic acid at quantities of 0.20-1.8 mg l^{-1}. Out of these 12 LAB strains, only *L. lactis* 140/2 produced sorbic acid at 1.20 mg/L concentration [42], which was relatively lower than that from this study. It is noteworthy that methanol fractions (70, 80, 90 and 100%) were the most effective extraction solvents to produce CFS of *L. buchneri* DSM 20057T because it included resistance activity against *S. typhimurium* ATCC 14028, *E. coli* ATCC 11795, *E. coli* O157, *S. aureus* ATCC 25923 and *V. cholerae*. These results were similar to those by Amrutha et al. [42], who reported that three types of organic acids (acetic, citric and lactic acids) were able to act as potent inhibitors to inactivate biofilm formation of Gram-negative bacteria such as *E. coli* and *Salmonella* spp. from fresh

vegetables and fruits. The mechanism induced antimicrobial actions of Gram-negative bacteria explained by several steps of 1) weak acids polarized into ionised and non-ionised forms leading to balances, which then enabled these ions infiltrated into bacterial membranes, 2) the hydrophobic membrane initiated proton gradients via free diffusion to generate ATP, and 3) the electron transport chain pumped out free anions and periplasmic protons and transported them back across the membrane without moving through the two subunits of ATP synthase (F1 and F0) [43, 44]. Potential of *L. buchneri* for use as food preservative in foods should be investigated further since it demonstrated bactericidal effects on foodborne pathogens. Recent studies have revealed probiotic properties of tempoyak with significant effects in decreasing growth of harmful micro-organisms (foodborne pathogens) to prolong shelf-life of the fermented foods and to increase human health and safety [45]. Based on their potential merits as probiotics, further studies are necessary to verify health benefits and safety aspects.

4. Conclusion

This study has successfully identified eight isolates of LAB strains through phenotypic characterization using API-50 CHL Kit. Eight LAB isolates were identified with similarities of 95–99.9%. These isolates included *L. plantarum* (one isolate), *L. acidophilus* I (one isolate), *L. brevis* (one isolate) and *L. buchneri* (five isolates). Mass spectrum identification using MALDI-TOF/MS biotyper has successfully identified five species of *L. buchneri*, including *L. buchneri* DSM 20057T, *L. paracasei* DSM 2649, *L. parabuchneri* DSM 57069, *L. paracasei* DSM 20020 and *L. farciminis* CIP 103136T. Four LAB strains of *L. plantarum*, *L. buchneri*, *L. brevis* I and *L. acidophilus* were able to inhibit various types of *Salmonella* food poisoning and other non-*Salmonella* foodborne pathogens. Crude extracts of LAB produced two major compounds of benzoic and sorbic acids, including potentials to be developed as chemical preservative agents.

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6. Conflict of Interest

The authors report no conflicts of interest.

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باکتری‌های لاکتیک اسید تولید کننده ترکیبات سوربیک اسید و بنزوییک اسید از گوشت تخمیره شده میوه دوریان (تمپویاک) و فعالیت ضدباکتریایی آنها در برابر باکتری‌های بیماری‌زای منتقل شده از طریق غذا

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چکیده

سابقه و هدف: اعتقاد بر این است که ترکیبات ضدباکتریایی تولید شده توسط باکتری‌های لاکتیک اسید می‌توانند جایگزین عملکرد نگهدارنده‌های شیمیایی باشند. هدف مطالعه حاضر شناسایی باکتری‌های لاکتیک اسیدی است که از گوشت تخمیرشده دوریان (تمپویاک)، ترکیبات ضدباکتریایی تولید کنند و بررسی فعالیت ضدباکتری انواع جداسازی-شده می‌باشد.

مواد وروش ها : روش مورد استفاده برای شناسایی ترکیبات ضدباکترایی، کیت API 50 CHL به‌همراه محیط کشت مکمل و زمان یونیزاسیون دفع لیزر به‌همراه ماتریکس در طیف سنجی جرمی (MALDI-TOF/MS) بود.

یافته‌ها و نتیجه‌گیری چهار سویه[1] باکتری لاکتیک اسید شامل *لاکتوباسیلوس بوچرنی*، *لاکتوباسیلوس پلانتاروم*، *لاکتوباسیلوس برویس* I و *لاکتوباسیلوس اسیدوفیلوس* I با استفاده از کیت API 50 CHL و پنج گونه دیگر شامل *لاکتوباسیلوس پاراکازئی* DSM 2649، *لاکتوباسیلوس بوچرنی* DSM 20057T، *لاکتوباسیلوس پارابوچرنی* DSM 2649، *لاکتوباسیلوس پاراکازئی* DSM 20020 و لاکتوباسیلوس فارسیمینی CIP 103136T با استفاده از MALDI-TOF/MS شناسایی شدند. روماند[2] بدون سلول استخراج شده از *لاکتوباسیلوس پلانتاروم*، *لاکتوباسیلوس بوچرنی*، *لاکتوباسیلوس برویس* I و *لاکتوباسیلوس اسیدوفیلوس* I دارای اثرات مهاری قوی در برابر *ویبریو کلرا* O1 (نوع Inaba)، *ویبریو کلرا* O139(نوع Bengal)، *ویبریو پاراهمولیتیکوس* ATCC 17802، *اشرشیا کلی* ATCC 1179، *اشرشیا کلی* O139، *سالمونلا تیفیموریوم* ATCC 14028 و ۲۳ سروتایپ گونه سالمونلا مسئول شیوع مسمومیت غذایی ناشی از نمونه‌های گوشت خام مرغ، پوسته تخم مرغ و آب داشتند. تنها *لاکتوباسیلوس بوچرنی* DSM 20057T توسط MALDI-TOF/MS به‌عنوان سویه تولید کننده اسیدهای سوربیک و بنزوییک شناسایی شد. این سویه توانایی کاربرد به‌عنوان نگهدارنده مواد غذایی برای کاهش رشد باکتری‌های بیماریزای منتقل شده از طریق غذا را دارد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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[1] strain
[2] supernatant