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Antimicrobial Activity of Cell Free Supernatant of Lactic Acid Bacteria Isolated from Fermented Durian Flesh against Multiple Antibiotic Resistance's *Salmonella* Associated with Food Poisoning Cases in Malaysia

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Abstract: *Epidemic of food poisoning cases and outbreaks associated with Salmonella spp. have been increased all over the world. Most of food poisoning cases in Malaysia related to Salmonella are associated with contaminated water compared to other sources. Finding alternative treatment to reduce foodborne cases becomes crucial, and bio-preservation using lactic acid bacteria (LAB) is one of strategy that can be used in food industries. The objective of this study was to determine the antimicrobial activities in vitro of cell free supernatant from eight LAB strains isolated from fermented durian flesh, which were 5 strains from Lactobacillus buchneri, 1 strain from each culture, Lactobacillus plantarum, Lactobacillus brevis 1 and Lactobacillus acidophilus 1 against 23 species of various serotypes of pathogenic Salmonella obtained from food poisoning cases in Malaysia. The antimicrobial activity of crude bacteriocins from cell free supernatant (CFS) of LAB strains were tested using well diffusion method. Susceptibility test of eight selected Salmonella strains was done using disk diffusion method (DDT) against twelve selected antibiotics. Results showed that crude bacteriocins of Lb. plantarum, Lb. buchneri, Lb. brevis 1 and Lb. acidophilus 1 from fermented durian flesh had strong inhibitory effect against most of 23 species of MAR's Salmonella associated with food poisoning cases with intermediate to high inhibitory zone by average mean from 16 mm to 22 mm diameter zone. All eight Salmonella strains were resistant to bacitracin, nalidixic acid and vancomycin and some strains were also resistant to ampicillin, penicillin, streptomycin and tetracycline. Further study could be utilised to understand the mechanism of selected LAB bacteriocin producer from fermented durian flesh as antimicrobial substance.*

Keywords*: antimicrobial activity, bacteriocin, fermented durian flesh, Lactic Acid Bacteria, Salmonella*

I. Introduction

Food poisoning and outbreaks associated with Gram negative bacteria such as *Salmonella* spp. had been increased recently in Malaysia. The genus *Salmonella*, which belongs to the family of Enterobacteriaceae, is an important foodborne pathogen. There are two species in the genus *Salmonella*: *S. enterica* (six subspecies) and *S. bongori* (one subspecies). Members of the seven subspecies can be serotyped into one of more than 2500 different serovars based on somatic (O) and flagellar (H) antigens (Popoff, 2001). Non-typhoidal *Salmonella* remains the leading cause of bacterial food-borne infections and continue to be a main problem, in terms of both morbidity and economic costs (Modarressi & Thong, 2010). *Salmonella* serovars *typhimurium* and *enteritidis* are the supreme public source of human salmonellosis globally (Archambault et al., 2006), other *Salmonella* serovars associated with food poisoning cases are becoming important in recent years.

Malaysia produces several kinds of fermented foods which have been known for their antimicrobial activity. Fermented durian flesh which is also known as 'Tempoyak', is one of the valuable heritage food product that previously had been proven to have a diversity of lactic acid bacteria microflora with superior properties in inhibiting the growth and multiplication of pathogenic bacteria. Fermented durian flesh is a traditional Malaysian fermented condiment made from the pulp of the durian fruit (*Durio zibethinus*) and indeed is an economically significant fruit in the region of South-East Asia (Leisner et al., 2001). It has a unique durian smell and a creamy yellow colour and is commonly consumed in both Malaysia and Indonesia as side dish and condiment (Battcock and Ali, 1998; Irwandi and Che-Man, 1996).

Numerous kind of Lactic Acid Bacteria (LAB) strains were isolated from fermented durian flesh from all over the South East Asia region. From the past studies, *Lactobacillus* species have been the predominant LAB microflora in tempoyak produced in Indonesia and Malaysia. However, the type of LAB microflora is

influenced by the place where the product is prepared (Yuliana & Dizon, 2011). For example, *Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus mali, Lactobacilus fermentum* were found in tempoyak from Malaysia (Issa 2000, Leisner et al., 2001), while Wirawati (2002) and Ekowati (1998) isolated *Lactobacillus plantarum, Lactobacillus casei, Lactobacillus corynebacterium*, and *Lactobacillus casei*, from tempoyak in Indonesia. It is acknowledged that lactic acid bacteria produce antimicrobial compounds particularly bacteriocins with high antimicrobial activity (Kormin et al., 2001).

The occurrence of Multiple Antibiotic Resistant (MAR) bacterial strain is of public health concern because the bacteria are not easily killed by normal antibiotics used for health therapy (Aween et al., 2012). Previous study by Aween et al., 2012 had demonstrated that both cells and supernatants of LAB isolated from honey samples marketed in Malaysia could inhibit the growth of the MAR's bacteria. *Lb. acidophilus* supernatant showed bactericidal effect against the MAR target bacteria especially *Staphylococcus aureus*. However, no study has been reported on the ability of LAB isolated from 'tempoyak' against MAR's bacteria.

Although the use of LAB as probiotics has received well attention, the application of LAB to reduce food poisoning cases is still underexplored. The infection of foodstuff with foodborne and pathogenic bacteria are global issue and it is severe hazard for the health of the human (Muhialdin et.al, 2012). Therefore, the objective of this study was to determine the antimicrobial activities of crude bacteriocins from eight LAB strains isolated from fermented durian flesh, which were five strains from *Lactobacillus buchneri*, one strain from each culture of *Lb. plantarum*, *Lb. brevis* 1 and *Lb. acidophilus* 1, against Multiple Antibiotic Resistant (MAR)'s *Salmonella* strains associated with food poisoning cases in Malaysia.

II. Materials And Methods

2.1 Source of Microorganisms

Salmonella typhimurium ATCC 14028 culture was obtained from Microbiological Unit, Terengganu Food Safety and Quality Control Laboratory, Health Department, Ministry of Health, Malaysia. For food poisoning *Salmonella* cultures, 23 serotypes of *Salmonella* strains associated with food poisoning cases and outbreaks were obtained from several laboratories under Food Safety and Quality Division, Ministry of Health, Malaysia.

2.2 Susceptibility of *Salmonella* strains towards antibiotics

Eight selected *Salmonella* strains were tested for their resistance and susceptibility to selected antibiotics were tested by using disc diffusion method (Harrigan, 1998). Eleven antibiotics used were ampicillin (AM 10µg), bacitracin (10 IU), cephalothin (30 µg), chloramphenicol (30 µg), gentamycin (120 µg), nalidixic acid (30 µg), penicillin (10 IU), polymycin B (300 IU), streptomycin (10 µg), tetracycline (21 µg) and vancomycin (30 µg). These antibiotics were commonly used to treat infection caused by food poisoning cases.

After an overnight incubation at 37ºC, the diameter of each zone of inhibition was measured with a ruler or caliper. In all measurements, the zones of inhibition are measured from the edges of the last visible colony-forming growth. The ruler was positioned across the centre of the disc to make these measurements. The results were recorded in millimetres (mm) and interpretation of susceptibility was obtained by comparing the results to the standard zone sizes. Inhibition zone diameters were measured inclusive of the diameter of the discs. Results were expressed as sensitive, S ($\geq$ 21 mm); intermediate, I (16-20 mm) and resistant, R ($\leq$ 15 mm), respectively according to that described by Vlková et al., (2006).

2.3 Preparation of Cell Free Supernatant from LAB isolated from fermented durian flesh

Eight of LAB strains isolated from fermented durian flesh that previously have been identified using phenotypic identification system (API 50 CHL) and other biochemical testes were used in this study (Salleh et al., 2014). Five of these LAB strains were *Lb. buchneri*, and the rest was each LAB strains from *Lb. plantarum*, *Lb. brevis* 1 and *Lb. acidophilus* 1. Cell free supernatant from each LAB strain were prepared by centrifugation at 10,000 rpm for 5 minutes after all LAB colonies cells were harvested anaerobically at 30°C for 24 h in MRS (de Man, Rogosa and Sharpe) broth until the cultures reached about 10^7 CFU/ml. The cell free supernatants were then be used for antimicrobial activity using agar well diffusion assay. The microbiological media was purchased from Merck, Germany.

2.4. Evaluation of Antimicrobial Activity against *Salmonella* spp. using Agar Well Diffusion Assay

Targeted colony of 23 strains of food poisoning *Salmonella* spp and *Salmonella typhimurium* ATCC 14028 was diluted using 0.1% peptone water (PW) to get 0.5 McFarland Turbidity Standard. All targeted gram negative pathogenic bacteria being used were freshly streaked onto Muller Hilton Agar (Merck, Germany) respectively using Kirby Bauer technique. Then, 5 mm diameter size of well were immediately made up in each plates and 20 µl molten agar be poured to each well until solidified. Immediately, 80 µl of CFS from each LAB strains were transferred to each well separately. Each plate was controlled by adding with sterilized PW. All

plates were aerobically incubated at 37^0C for 24 h. For measurements, the zones of inhibition were measured from the edges of the last visible antimicrobial inhibition growth without deducted with the size of the LAB well (Kirby Bauer). The ruler was positioned across the centre of the well to make these measurements. The pathogenic bacteria without LAB cultures were used as control of experiments. The experiments were conducted twice in order to obtain the average mean of diameter of inhibitory zone.

III. Results And Discussion

3.1 Susceptibility of *Salmonella* strains associated with food poisoning cases in Malaysia towards antibiotics

The occurrence of multiple antibiotic resistant among *Salmonella* strains associated with food poisoning in Malaysia is summarised in Table 1. These selected strains were found to be resistant to bacitracin, nalidixic acid and vancomycin, while some strains such as *S. adamstua* (fp 021/K) was resistant to ampicillin and *S. albany* (fp 020/K) and *S. adamstua* (fp 021/K) were resistant to penicillin. At the same time, *S. albany* (fp 020/K), *S. adamstua* (fp 021/K) and *S. corvalis* (fp 02/K) were also resistant to tetracycline. Similarly, Bouchrif et al. (2009) observed that *S. typhimurium* phage type DT104 is a multiple antibiotic resistant strain to ampicillin, tetracycline, chloramphenicol, sulfamethoxazole and streptomycin. To the best of our knowledge, this is the first report on the occurrence of MAR among *Salmonella* strains associated with food poisoning cases in Malaysia. This present study highlights that these *Salmonella* associated with food poisoning cases may easily become multiple antibiotic resistant (MAR).

Table 1: Susceptibility of *Salmonella* spp. from food poisoning isolates towards antibiotics using disk diffusion test (DDT)

Antibiotics	*S.typhimurium* (fp 001/K)	*S.enteritidis* (fp002/K)	*S.paratyphi B* (fp 003/K)	*S.richmond* (fp 006/K)	*S.corvalis* (fp 019/K)	*S.albany* (fp 020/K)	*S.adamstua* (fp 021/K)	*S.corvalis* (fp 023/K)
Ampicillin (10ug)	21	21	22	21	19	21	0	21
Bacitracin (10 IU)	0	0	0	0	0	0	0	0
Cephalothin (30ug)	22	22	23	20	20	29	23	23
Chloramphenicol (30ug)	30	30	30	30	22	29	30	25
Gentamycin (120ug)	26	24	22	21	20	25	27	24
Nalidixic acid (30ug)	0	0	0	0	0	0	0	0
Penicillin (10IU)	12	13	12	10	9	0	0	7
Polymycin B (300 IU)	16	16	16	15	16	16	16	17
Streptomycin (10ug)	16	16	17	19	21	7	9	0
Tetracyclenes (21ug)	21	20	20	24	20	0	0	0
Vancomycin (30ug)	0	0	0	0	0	0	0	0

Control were = Blank disk (Sterilized) = 0mm, fp=food poisoning/Outbreak
Diameter of antimicrobial activity zone measured in mm after 24 h incubation period at 37°C in duplicates, n=2

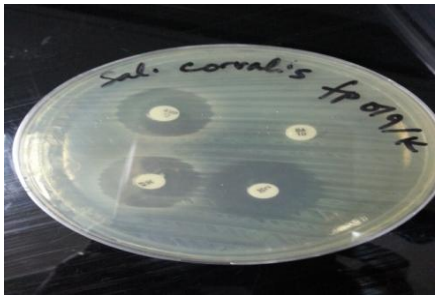

Figure 1: Antibiotic susceptibility of *S. corvalis*

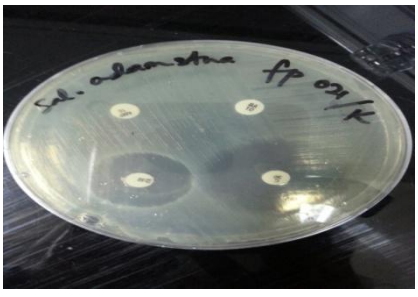

Figure 2: Antibiotic susceptibility of *S.adamstua*

Figure 1 and 2 show the inhibition growth of *S. corvalis* (on the left) and *S. adamstua* (on the right) tested against selected antibiotics using DDT method

3.2 Antimicrobial activity of LAB isolated from fermented durian flesh against Multiple Antibiotic Resistant (MAR) of *Salmonella* strains associated with food poisoning cases in Malaysia

Malaysian fermented durian flesh has been reported to have antimicrobial activity against microorganisms (Issa, 2000, Leisner et al., 2001). However, limited study has been focusing on evaluating the antimicrobial activity of LAB isolated from fermented durian flesh against *Salmonella* food poisoning strains. Salmonellosis which caused by Salmonella strains showed significant effect to human health and death, where

the most significant sources of foodborne gastroenteritis world-wide came from *Salmonella* (Nillian, 2011). The main factors that contribute to *Salmonella* pathogenesis are its capability to attack epithelial cells and affect the cellular destruction (Abdel-Daim et al., 2013).

In Malaysia, *S. enteritidis* (28.1%), *S. weltevreden* (25.7%), *S. corvallis* (10.3%) and *S. typhimurium* (6.7%) were the most common non-typhoidal *Salmonella* reported for the period 2003 – 2005 (National Public Health Laboratory, 2005). In some regions, *S. weltevreden, S. anatum* and *S. derby* have important to be of superior (Bangtrakulnonth et al., 2004). It has been proved that *S. enterica* are common food-borne bacterial pathogens and they are most widespread in chicken, eggs and beef (Lim et al., 2005; Mare et al., 2001; Ray et al., 2007).

All *Salmonella* strains used in this study showed resistant to bacitracin, nalidixic acid and vancomycin (Table 1). People who suffer from infection related to *Salmonella* food poisoning were commonly treated by antibiotics by medical practitioners. Nevertheless, this study has confirmed that these three antibiotics; bacitracin, nalidixic acid and vancomycin were not suitable to inhibit the growth of *Salmonella*. Finding alternative treatment to inhibit these MAR's *Salmonella* strains is crucial for medicinal purposes. Interestingly, this study demonstrated that several isolates of LAB strains, namely as *Lactobacillus plantarum* (V1), *Lactobacillus buchneri* (V9) and *Lactobacillus brevis* (V11) had strong antimicrobial activity against MAR's *Salmonella* strains associated with food poisoning cases in Malaysia as shown in Table 2.

Table 2: Antimicrobial activity of crude bacteriocins from cell free supernatant (CFS) of LAB strains against various types of *Salmonella* spp. from food poisoning isolates using well diffusion method

	ID	Source	V1	V3	V7	V8	V9	V11	V13	V14
Targeted										
Salmonella strains										
(ATCC and various serotypes)		**(Water and food contamination)**	**Diameter of inhibition growth (mm)**							
S. typhimurium	ATCC 14028	American type culture collection	18	18	17	18	20	20	19	20
S. typhimurium	FP 001/K	*raw chicken*	18	19	18	17	20	20	21	19
S. enteritidis	FP 002/K	*water*	18	16	18	14	16	18	18	20
S. paratyphi B	FP 003/K	*water*	18	12	16	11	15	13	12	14
S. chester	FP 004/K	*water*	14	10	12	12	18	13	12	14
S. sarajane	FP 006/K	*water*	13	12	12	11	16	15	14	15
S. richmond	FP 006/K	*water*	14	9	12	13	18	15	17	18
S. chingola	FP 007/K	*water*	13	11	11	9	19	12	12	15
S . paratyphi A	FP 008/K	*water*	16	10	12	12	17	15	13	15
S . louga	FP 009/K	*water*	11	7	12	12	18	15	15	16
S .mountpleasant	FP 010/K	*water*	14	12	6	10	17	14	13	14
S . sandiago	FP 011/K	*water*	18	15	17	16	18	16	21	21
S . winslow	FP 012/K	*water*	17	16	15	16	19	17	16	17
S . borbeck	FP 013/K	*water*	15	13	13	13	17	15	17	18
S . rhydyfellin	FP 014/K	*coconut milk seive*	17	16	16	17	18	16	17	18
S . weltevreden	FP 015/K	*water*	20	19	17	21	21	22	22	22
S . bareilly	FP 016/K	*water*	20	19	18	20	20	20	21	20
S . panama	FP 017/K	*water*	21	17	21	17	20	18	20	21
S . farsta	FP 018/K	*chicken dish*	20	21	20	19	19	19	17	19
S . corvalis	FP 019/K	*raw chicken*	20	18	18	17	21	20	20	20
S . albany	FP 020/K	*water*	19	17	16	14	18	17	17	18
S .adamstua	FP 021/K	*water*	18	18	18	17	19	18	18	18
S .tsevie	FP 022/K	*water*	14	16	14	15	20	16	17	18
S . corvalis	FP 001/T	*raw chicken*	20	20	20	20	18	20	18	22
S . livingston	FP 002/T	*egg shell*	20	20	20	20	20	22	20	22

Control of each plates= 0 mm, Fp= food poisoning
Mean diameter of antimicrobial activity zone measured in mm after 24 h incubation period at 37°C

Previously, Oh et al. (2000) reported that crude bacteriocin from *Lb. acidophilus* 30SC were not capable to inhibit the growth of Gram negative bacteria, including *Klebsiella pneumoniae*, *E. coli* and *S. typhimurium*. However, in this study, all four strains of LAB tested namely as *Lb. plantarum, Lb. buchneri, Lb. brevis* 1 and *Lb. acidophilus* 1 were highly capable to inhibit numerous kind of Gram negative bacteria of *Salmonella* spp. as shown in *Table 2*. These four strains of LAB are potential strains to be used to fight food poisoning cases in Malaysia since those targeted pathogens were purely isolated from food poisoning cases happened in Malaysia from different sources, including various kinds of water sources and meal dishes (chicken meals).

Earlier reports showed that isolate of *Lb. pentosus* G004 had very strong activity against all the tested bacteria except for *S. typhimurium* and *Bacillus subtilis*, which the isolate only had a moderate inhibition activity (Muhialdin et al., 2012). The present finding highlights the potential LAB strains from the Malaysian fermented durian flesh to inhibit *Salmonella* strains that associated with food poisoning cases in Malaysia. Although the exact mechanism for this antimicrobial activity was not further elucidated, it is well established that cell free supernatant of LAB consists of inhibitory compounds, such as organic acids, oxygen catabolites, proteinaceous compounds, fat and amino acids metabolites and other compounds (Helander eat al., 1977, Valerio et al., 2004). Figure 3 (A-F) exhibits the antimicrobial activity of crude bacteriocin of eight LAB against *Salmonella* spp. (food poisoning isolates).

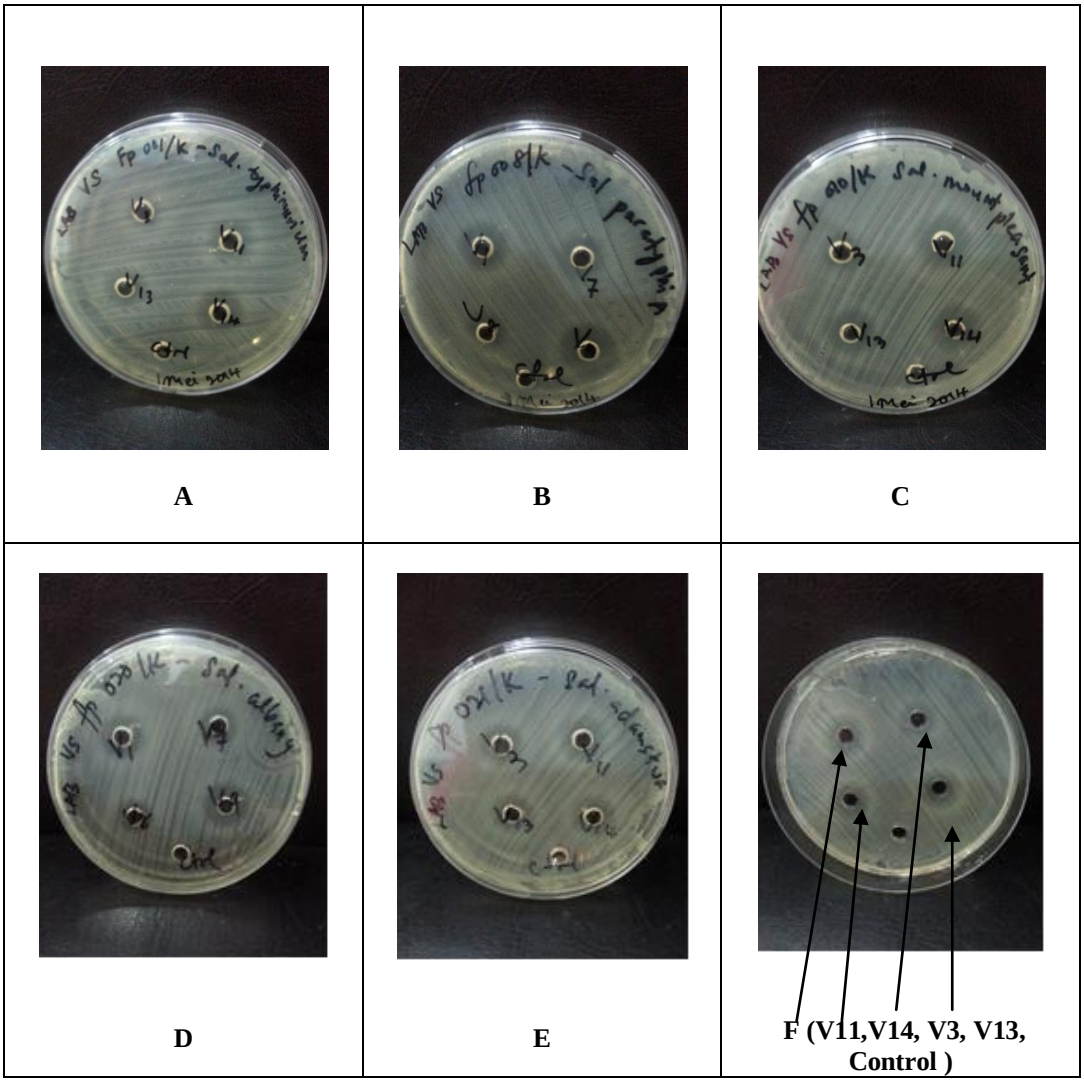

Figure 3 (A – F): Some pictures of antimicrobial activity of crude bacteriocin of eight LAB against Salmonella spp. (food poisoning isolates): A- *S. typhimurium*, B- *S. paratyphi* A, C- *S. mountpleasant*, D – *S. albany*, E – *S. adamstua* and F – *S. corvalis* by well diffusion method

IV. Conclusion

Eight isolates of Lactic Acid Bacteria strains namely as *Lb. plantarum*, *Lb. acidophilus* 1, *Lb. brevis* and *Lb. buchneri* had proven to inhibit abundant serotypes of MAR's *Salmonella* food poisoning strains from Malaysia. All eight LAB strains showed strong antimicrobial activity with broad spectrum (18–20 mm) diameter inhibition against *S. typhimurium* ATCC 14028. Besides that, cell free supernatant of eight LAB isolates (V1, V3, V7, V8, V9, V11, V13 and V14) showed strong antimicrobial activity against other 23 serotypes of *MAR's Salmonella* associated with food poisoning cases in Malaysia with almost 100% broad spectrum antimicrobial activities. This finding highlights the potential of fermented durian flesh to be used as bio-preservative substance in providing the solution in reducing the food poisoning and foodborne diseases cases in Malaysia and

all over the world. Further investigation will be done to understand the mechanism of related bacteriocins from these LAB strains in controlling the cycle of food poisoning incidences.

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