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Survivability of *Vibrio parahaemolyticus* in satar and otak-otak, Malaysian fish-based street food

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Abstract

Street food is popular in Asia due to its availability, low price and good taste. The safety of street food has been always questionable due to its poor handling which probably leads to microbial contamination. The objective of this study was to determine the surviving quantities of *V. parahaemolyticus* under various conditions in street-vended food, namely satar and otak-otak after anticipated cross-contamination to support policy and regulatory documents. The satar and otak-otak were prepared from minced and unminced fish flesh, respectively, together with other ingredients. Each satar and otak-otak were prepared with 0, 0.5, 1.5 and 3% of sodium chloride (NaCl), respectively. *V. parahaemolyticus* inoculum at approximately 8.66 log CFU/ml were inoculated into the samples and incubated for up to 6 h. Samples were taken at 0, 1, 3 and 6 h for enumeration of *V. parahaemolyticus* using spread plate method on Thiosulphate Citrate Bile Salts Sucrose (TCBS) agar. For control samples, *V. parahaemolyticus* was not immediately inactivated in distilled water even though significant better survivability was observed in Phosphate Buffer Saline (PBS). The numbers of *V. parahaemolyticus* was found to decrease by varying amounts based on the salt content and duration of holding. However, significant amounts survived to indicate potential risk.

Introduction

Vibrio parahaemolyticus is a halophilic gram negative bacteria that naturally found in coastal marine waters and seafood throughout the world (Quiroz-Guzmán *et al*., 2013; Micky *et al*., 2014). *V. parahaemolyticus* is well-known as a leading causative agent of human acute gastroenteritis that associated with consumption of raw, undercooked, or mishandled seafood and related seafood products (Ottaviani *et al*., 2009; Zhao *et al*., 2011). The *V. parahaemolyticus* infection is characterized by diarrhea, headache, vomiting, nausea, abdominal pain and low fever.

Satar and otak-otak are popular fish-based street food product dishes among locals and tourists in the East Coast of Peninsular Malaysia. Satar is a blend of minced deboned fish mixed spices and wrapped with banana leaves into pyramid shape while otak-otak is an unminced deboned fish cake mixed with spices and wrapped with coconut leaves into thin stick and they are grilled over a flaming charcoal fire. Due to their unique taste and tantalizing aromas, these products have been well received and consider as one of the snack not to be missed by overseas visitors to Terengganu. Increasing demand and consumption of satar has made it to be recognized as one of the heritage food in Malaysia (Lani *et al*., 2014).

Satar and otak-otak are exposed to pathogenic microbial contamination if the fishes used as main ingredient are contaminated. *Vibrio* spp. has been recognised to inhabit coastal and aquatic environment (Alam *et al*., 2006; Vuddhakul *et al*., 2006; Yano *et al*., 2006a;) and it has been implicated with foodborne outbreaks in Taiwan, Japan and South East Asian countries (Wong *et al*., 2000).

Besides fish as the main ingredient, herbs and spices also added into satar and otak-otak to give distinctive flavor to the product. Studies have found these herbs and spices were thought to have antibacterial effect (Yano *et al*., 2006b; Shan *et al*., 2007; Zhang *et al*., 2009; Filipović *et al*., 2016). Some ingredients used in satar and otak-otak which is known to have antibacterial effect include garlic, ginger and chilli.

It is recognised that *Vibrio parahaemolyticus*

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requires 3% of sodium chloride for optimal growth (Kalburge *et al*., 2014) and it is readily killed in a broth without sodium chloride or distilled water (Covert and Woodburn, 1972; Lee, 1972). Many studies have reported occurrence of *V. parahaemolyticus* in fish, oysters and mussels (Terzi *et al.*, 2009; Yu *et al*., 2013). By contrast, few data are available for seafood related products though it is known to support growth of pathogenic *Vibrio* spp. (Tang *et al*., 2014). Fish product such as fish balls has been reported to be contaminated with *Vibrio* spp. and cause outbreaks due to undercooking and poor hygienic practices by food handlers (Tangkanakul *et al.*, 2000; Huang *et al.*, 2012).

Many studies reported on *Vibrio* spp. survival under different conditions or environments (Jiang and Chai, 1996; McCarthy, 1996; Jubair *et al*., 2012) but very few in the food matrix (Bernbom *et al*., 2009). Thus, this study aimed to investigate the survival pattern of *V. parahaemolyticus* in food matrix with formulation similar to those sold by the street vendor. The fish flesh in satar and otak-otak used in this study was substituted with different percentages of sodium chloride (0, 0.5, 1.5 and 3%).

Materials and Methods

Vibrio parahaemolyticus *culture*

V. parahaemolyticus ATCC 17802 (Microbiologics,USA) was used throughout this study. The bacterium was revived in the alkaline peptone water (APW) with 3% NaCl incubated at 37°C for 24 h. The purified *V. parahaemolyticus* was grown on Tryptic Soy Agar (TSA) slant with 3% NaCl as working culture.

Preparation of satar and otak-otak

In brief, four samples of satar were prepared as follows: 202.00 g of filleted and minced mackerel fish was mixed with 50.00 ml of tamarind juice, 1.10 g of ginger, 12.60 g of shallot, 2.90 g of garlic, 75.00 g of grated coconut, 2.60 g of sugar, and 3.80 g of shrimp paste. Sodium chloride (NaCl) was added to satar samples at 0, 0.5, 1.5 or 3%.

By contrast, four samples of otak-otak were prepared using 150.00 g of unminced mackerel fish fillet and added with mixture of other ingredients which include 100.00 g of grated coconut, 25.00 g of shallot, 6.00 g of garlic, 5.00 g of curry powder, and 4.00 g of chilli paste. Sodium chloride (NaCl) was added to otak-otak samples at 0, 0.5, 1.5 or 3%.

Satar and otak-otak with 1.5% NaCl provide the taste similar to those sold at food stall. Satar and otak-otak were grilled by using grill pan for 5 mins on each side. A new batch was used and freshly prepared for each experiment.

Preparation of V. parahaemolyticus *inoculum*

Preparation of *V. parahaemolyticus* were done as described in our previous study (Tang *et al*., 2014). *V. parahaemolyticus* from a working culture was inoculated into alkaline peptone water with 3% NaCl and incubated in a shaker incubator (150 rpm) (Infors HT Ecotron, Basel, Switzerland) at 37 ˚C for 22 h. The revived culture was then centrifuged at 5000 rpm for 10 mins using microcentrifuge (Sigma 1-14 microfuge, Germany) to pellet the bacteria cells, and the bacterial pellet was resuspended in phosphate-buffered saline (PBS). Absorbance of the bacterial suspension was adjusted to a reading of 1.13 at 620 nm wavelength, which corresponded to approximate 8.66 log CFU/ml.

Survival determination of V. parahaemolyticus *on satar and otak-otak*

Three grams of each prepared satar or otak-otak with different of salt content (0, 0.5, 1.5 and 3%) were placed in loosely capped universal bottles. Twenty microliter of inoculum was spiked onto the prepared samples (satar or otak-otak) in each loosely capped universal bottles with designated control with Phosphate Buffer Saline (PBS), control with distilled water (dH_2O), samples (without inoculum) and samples (with inoculum) and incubated at 28 ˚C in incubator (Infors HT Ecotron, Switzerland). Each experiments were carried out in three replicates and sampling times were carried out at 0, 1, 3 and 6 h. Enumeration of *V. parahaemolyticus* were performed on Thiosulfate Citrate Bile-salt Sucrose (TCBS) agar (Merck, Germany) using spread plate method and numbers of *V. parahaemolyticus* expressed as mean log CFU/g.

Statistical analysis

Data collected during the experiment were analyzed using SPSS statistics 17.0 (SPSS Inc., USA) using one-way ANOVA. The significance level was set at $p < 0.05$.

Results and Discussion

Vibrio parahaemolyticus is a halophilic and fragile microorganism that grow optimally at 3% NaCl (Kalburge *et al.*, 2014) and will lose viability at 0% NaCl (Covert and Woodburn, 1972; Lee, 1972). In uninoculated grilled satar and otak-otak samples, the *Vibrio parahaemolyticus* was not detected throughout the 6 h test period. For control samples, inoculum of

Table 1. Level of *Vibrio parahaemolyticus* in satar inoculated with 8.66 log CFU/ml inoculum and stored at 28°C.

Sample	Level of *Vibrio parahaemolyticus* (log CFU/g) after hour(s)			
	0 h	1 h	3 h	6 h
Control (PBS)	6.55 ± 0.02 ^{a,A}	6.34 ± 0.32 ^{a,A}	6.19 ± 0.15 ^{a,A}	5.98 ±0.48 ^{a,A}
Control (dH_2O)	6.41 ± 0.05 ^{a,A}	5.23 ± 0.25 ^{b,B}	5.12 ± 0.17 ^{b,B}	4.57 ±0.11 ^{c,B}
0 % NaCl	5.76 ± 0.02 ^{a,B}	4.04 ± 0.11 ^{b,D}	3.89 ± 0.12 ^{b,C}	4.03 ± 0.16 ^{b,C}
0.5 % NaCl	5.82 ± 0.09 ^{a,B}	5.36 ± 0.40 ^{a,B}	4.97 ± 0.24 ^{b,B}	5.65 ± 0.21 ^{a,A}
1.5% NaCl	5.82 ± 0.22 ^{a,B}	4.31 ± 0.10 ^{b,C,D}	4.31 ± 0.83 ^{b,B,C}	4.34 ± 0.10 ^{b,B,C}
3% NaCl	5.72 ± 0.02 ^{a,B}	4.19 ± 0.21 ^{b,C,D}	4.27 ± 0.16 ^{c,B,C}	4.40 ± 0.18 ^{b,B,C}

PBS, Phosphate Buffer Saline
dH_2O, distilled water
Data represent mean ± standard deviation of three replications.
^{a,b,c} Data in the same row with different letter is different significantly ($p < 0.05$).
^{A,B,C} Data in the same column with different letter is different significantly ($p < 0.05$).

8.66 log CFU/ml of *Vibrio parahaemolyticus* has shown good survivability in Phosphate Buffer Saline (PBS) and distilled water (dH_2O) (Table 1 and 2). In this study, *V. parahaemolyticus* in dH_2O was significantly ($p<0.05$) lower than *V. parahaemolyticus* in PBS but it survived throughout the 6 h incubation period at 4.57 log CFU/g and 5.98 log CFU/g, respectively. Our previous study reported *V. parahaemolyticus* was not detected after 1 h incubation. The *V. parahaemolyticus* was prepared in PBS and inoculated into uncapped universal bottles. *V. parahaemolyticus* was not detected after 1 h incubation and the inoculum was found dried up at the end of the experiment (Tang et al., 2014). This result is in line with the report that V. parahaemolyticus is very sensitive to drying (ICMSF, 1996; FAO/WHO, 2011) and present study indicated *V. parahaemolyticus* is more sensitive to drying than the presence of NaCl.

V. parahaemolyticus generally show decreasing pattern in satar samples (0, 1.5 and 3% NaCl) prepared in the lab and best survivability was found in satar with 0.5% NaCl (Table 1). Sodium chloride (NaCl) is essential for the growth of *V. parahaemolyticus* and concentration of 3% was reported to provide optimal growth (ICMSF, 1996). Survivability of *V. parahaemolyticus* in satar with 0% NaCl was significantly lower is in agreement with other studies which reported *V. parahaemolyticus* is readily inactivated in broth or fish homogenate without NaCl (Covert and Woodburn, 1972). Bernbom *et al.* (2009) reported that *V. parahaemolyticus* only grew in fish product preserved with NaCl concentration lower than 1% when combined with 0.5% garlic. The finding agreed with the current study in which *V. parahaemolyticus* decreased significantly at the end of 6 h incubation for satar with 1.5% and 3% of NaCl but not in satar with 0.5% NaCl. The ingredients such as garlic and ginger used for preparing the satar also contribute to the inhibitory effect on *V. parahaemolyticus* decreasing survival pattern. Ginger has been reported to exert anti-vibrio effect against *V. parahaemolyticus* (Yano *et al.*, 2006b; Filipović *et al.*, 2016). Inconsistent antibacterial results were found on garlic against *V. parahaemolyticus* in which Yano *et al.* (2006b) demonstrated weak anti-*vibrio* bacterial effect while study by Filipović *et al*. (2016) exhibited significant anti-vibrio effect. However, it is generally recognised ginger and garlic exhibited substantial antibacterial effect against many types of pathogens (Deans and Ritchie, 1987; Shan *et al.*, 2007; Lucera *et al*., 2012).

Similar decreasing survival pattern was observed for *V. parahaemolyticus* in otak-otak samples (0, 1.5 and 3% NaCl) prepared in the lab (Table 2) to those found in satar and *V. parahaemolyticus* survived best in otak-otak with 0.5% NaCl. The higher amount of garlic used (2.07%) in otak-otak as compared to satar (0.83%) might contribute to the continuous decrease ($p<0.05$) with regards to the number of *V. parahaemolyticus* in otak-otak with 3% NaCl. This study showed *V. pahaemolyticus* was not affected by the characteristic of fish flesh used either minced (satar) or unminced (otak-otak). The amount of fish flesh used in satar ranged from 55% to 58% and otak-otak ranged from 49% to 52%. Ground or minced meat are generally reported to be more susceptible to microbial contamination due to larger surface area as compared to solid cut of meat (Eisel *et al*., 1997). This is due to the increase in surface area and internalization of microorganism through mechanical forces during processing (Eisel *et al.*, 1997). Ground meat has been recognized to pose significant risk for foodborne outbreak (Kassenborg *et al.*, 2004; Bogard *et al.*, 2013). The result from this study suggest though ground or minced meat has high prevalence of pathogens and pose risk of foodborne outbreak, the presence of pathogens in these high risk food do not necessarily grow within the tested incubation time. *V. parahaemolyticus* had survived equally well in both satar and otak-otak throughout the 6 h incubation time.

Satar and otak-otak are made up of fish from

Table 2. Level of *Vibrio parahaemolyticus* in otak-otak inoculated with 8.66 log CFU/ml inoculum and stored at 28°C.

Sample	Level of *Vibrio parahaemolyticus* (log CFU/g) after hour(s)			
	0 h	1 h	3 h	6 h
Control (PBS)	6.55 ± 0.02 ^{a,A}	6.34 ± 0.32 ^{a,A}	6.19 ± 0.15 ^{a,A}	5.98 ±0.48 ^{a,A}
Control (dH_20)	6.41 ± 0.05 ^{a,A}	5.23 ± 0.25 ^{b,B}	5.12 ± 0.17 ^{b,B}	4.57 ±0.11 ^{c,B}
0 %	5.73 ± 0.02 ^{a,B}	4.52 ± 0.21 ^{b,B,C}	4.28 ± 0.07 ^{b,C}	4.53 ± 0.06 ^{b,B}
0.5 %	5.87 ± 0.29 ^{a,B}	5.16 ± 0.04 ^{a,B}	5.14 ± 0.21 ^{a,B}	3.95 ± 0.35 ^{b,B,C}
1.5%	5.74 ± 0.05 ^{a,B}	3.95 ± 0.05 ^{b,C}	3.82 ± 0.20 ^{b,D}	5.58 ± 0.34 ^{a,A}
3%	5.76 ± 0.17 ^{a,B}	5.41 ± 0.16 ^{b,B}	4.07 ± 0.14 ^{c,C,D}	3.73 ± 0.20 ^{d,C}

PBS, Phosphate Buffer Saline
dH_2O, distilled water
Data represent mean ± standard deviation of three replications.
^{a,b,c} Data in the same row with different letter is different significantly ($p < 0.05$).
^{A,B,C} Data in the same column with different letter is different significantly ($p < 0.05$).

mackerel family as main ingredient and they are popular in coastal areas in which fish supply is abundant and each street vendors have their own family recipe for preparation of satar and otak-otak that has been inherited by generations (Lani *et al*., 2014). They are known to be highly perishable food products which cannot be kept for extended period of time at ambient temperature. The high perishability of fish product may pose health risk from pathogens contamination (Reyhanath and Kutty, 2014). *V. parahaemolyticus* is ubiquitous in the estuarine and marine environments, frequently isolated from seawater, sediment, and a variety of aquatic products including fish, shellfish, and crustaceans (Farmer III and Janda, 2004; Jones *et al*., 2012). It is capable to grow at temperature from 10°C to 44°C and survive at pH ranges from 5 to 11 (Odeyemi and Stratev, 2016).

Fish and fish products are one of the source of pathogenic bacteria infection in human that could be transmitted to fish during processing under poor hygienic conditions (Stratev *et al*., 2015; Uddin *et al.*, 2013). In addition, *Vibrio* is thought to be capable of survival in fish based product that had been cooked and pasteurised, but cross-contaminated later due to poor handling, can possibly cause consumer illness (FDA, 2011). This study proved that recontamination of cooked satar and otak-otak with *V. parahaemolyticus* will pose significant risk of foodborne illness. Generally, the cooked status of satar and otak-otak are evaluated by observing the color changes of wrapper leaves from green to slightly burn. This cooking method may cause the filling of satar or otak-otak to be undercooked as internal temperature is not usually measured. Since commercial satar and otak-otak are produced in a large quantity at one particular time, it will be exposed to high possibility of cross-contamination during the preparation process. It has been estimated that 25% of foodborne outbreaks were due to improper handling by the food handlers (Carrasco and Morales-Rueda, 2012). According to Odeyemi and Stratev (2016), contamination may occur at various stages like processing, storage and distribution of seafood. Sources of contamination include water, facilities, equipment and handlers. The processing stage is the most important due to the high microbial load on the surface of processing facilities. Seafood has been described as a vehicle of transmission of food borne bacteria that cause human illness worldwide (Letchumanan *et al.*, 2015) and *V. parahaemolyticus* is one of the most important pathogens causing seafood-borne gastroenteritis associated with the consumption of raw, undercooked or poor handling of cooked seafood (Letchumanan *et al*., 2015).

This study showed that *V. parahaemolyticus* is susceptible to antibacterial effect from the spices used along with various salt concentrations. Since *V. parahaemolyticus* survived throughout the tested incubation time, significant risk of foodborne infection remained through cross-contaminated or undercooked satar and otak-otak consumption.

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