Survival of Vibrio cholerae O1 and Vibrio parahaemolyticus in fried and boiled Malaysian fish sausage

John Yew Huat Tang a,*, Nurul Hidayah Mohd-Noor a, Nurhidayah Mazlan a, Chew Chieng Yeo b, Che Abdullah Abu-Bakar a, Son Radu c

a Faculty of Agriculture, Biotechnology and Food Sciences, Universiti Sultan Zainal Abidin, Tembila Campus, 22200 Besut, Malaysia
b Faculty of Medicine and Health Sciences, Universiti Sultan Zainal Abidin, City Campus, 20400 Kuala Terengganu, Malaysia
c Food Safety Research Centre, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Malaysia

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A B S T R A C T
Survivability of Vibrio cholerae O1 and Vibrio parahaemolyticus in a popular Malaysian fish sausage snack, keropok lekor (either boiled or fried) in two conditions (closed or opened) was investigated by spiking ca. 8.00 log CFU/ml of each bacterial culture. Spread plate method onto TCBS agar was used to determine the numbers of inoculated vibrios that had survived as per tested condition. V. parahaemolyticus was more sensitive than V. cholerae O1 and lost viability in saline (control, closed condition). Both vibrios were not detected in the opened condition control after 1 h but keropok lekor proved to significantly support the viability of vibrios for up to 6 h. No significant growth or reduction (p > 0.05) was detected in an enclosed container but a significant reduction (p < 0.05) was observed in the opened condition over a 6 h incubation period. After 6 h incubation, V. cholerae survived at 7.95 ± 0.05 log CFU/g (boiled, enclosed), 6.11 ± 0.18 log CFU/g (fried, enclosed), 4.23 ± 0.48 log CFU/g (boiled, opened) and 3.08 ± 0.46 log CFU/g (fried, opened), respectively. Though V. parahaemolyticus showed poor viability in saline, they persisted well in keropok lekor with numbers recorded at 5.75 ± 0.09 log CFU/g (boiled, enclosed), 5.49 ± 0.59 log CFU/g (fried, enclosed), 2.65 ± 0.45 log CFU/g (boiled, opened) and 2.13 ± 0.52 log CFU/g (fried, opened), respectively. While vibrios are known to be easily killed by heat, post-cooking contamination might lead to food poisoning as a result from vibrios persistence in keropok lekor.

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1. Introduction

Keropok lekor is a popular Malaysian fish sausage generally consumed as snack. It is made from minced fish flesh mixed with starch, salt, crushed ice and monosodium glutamate (MSG) to improve texture and taste. The processing and production of keropok lekor may range from backyard to modern small- or medium-scale factory production (Nor-Khaizura, Loh, Zaiton, Jamilah, & Rusul, 2010; Omar et al., 2011). They are widely available at hawker stalls, night markets, canteens as well as restaurants and can be consumed either boiled or fried.

Vibrios are ubiquitous and widely distributed in aquatic environments ranging from brackish to deep-sea water (Thompson & Polz, 2006; Uraiwaka & Rivera, 2006). Vibrio cholerae and Vibrio parahaemolyticus are two important vibrios that cause foodborne illnesses in humans (Fujino, Sakazaki, & Tamura, 1974; Harth et al., 2009; Ottaviani et al., 2009; Zhao, Zhou, Cao, Ma, & Jiang, 2011). They have been frequently associated with seafood and seafood-related products for causing food poisoning (Fujino et al., 1974; Harth et al., 2009; Ottaviani et al., 2009; Zhao et al., 2011).

Over the past years, the domestic demand of keropok lekor had increased significantly and they have now been exported to overseas markets (Bernama, 2011). Due to the increasing popularity of keropok lekor, this study serves as a timely and preliminary investigation of the survivability of V. cholerae O1 and V. parahaemolyticus in boiled and fried keropok lekor. Keropok lekor is an easily perishable snack in which the uncooked sausage will spoil within 2 days if kept at room temperature. Cooked sausage (fried or boiled) might prolong the shelf life but keeping them for more than a day will be organoleptically unacceptable. It is however a common practice by some food handlers to prepare cooked keropok lekor earlier and then keeping them at ambient temperature for an extended period of time. Such practice was thought to be responsible for the choleran outbreak in Terengganu in 2009 (Bernama, 2009).

Our previous studies have shown that V. cholerae O1 is able to persist in Malaysian street foods (rice, coffee and tea) which were...
commonly prepared in bulk (Huat, Leong, & Lian, 2008; Tang et al., 2013). The current study is aimed to investigate the survivability of two important marine pathogens, *V. cholerae* and *V. parahaemolyticus*, in *keropok lekor* that has been kept under enclosed and opened conditions similar to what is common practiced by vendors of street foods in Malaysia.

### 2. Materials and methods

#### 2.1. Vibrio strains

*V. cholerae* O1 serotype Inaba was obtained from Kyoto University, Japan and *V. parahaemolyticus* ATCC 17802 (Microbiologics, USA) were used throughout this study.

#### 2.2. Preparation of *V. cholerae* and *V. parahaemolyticus* for experiments

*V. cholerae* and *V. parahaemolyticus* were prepared as described in our previous study (Tang et al., 2013) with minor modifications. Both Vibrio spp. from a stock culture were streaked onto TCBS agar and incubated at 37 °C for 24 h. Each isolated *V. cholerae* or *V. parahaemolyticus* colony was inoculated into alkaline peptone water (with 3% NaCl in the case of *V. parahaemolyticus*) and incubated in shaker incubator (150 rpm) (Infors HT Ecotron, Basel, Switzerland) at 37 °C for 22 h. The culture was centrifuged, and the bacterial pellet was resuspended in phosphate-buffered saline (PBS). Absorbance of the bacterial suspension at 625 nm wavelength was adjusted to a reading of 1.80, which corresponded to about 9 log CFU/ml.

#### 2.3. Enumeration of spiked *V. cholerae* and *V. parahaemolyticus*

The spread plate method was performed as described in our previous study (Tang et al., 2013). Each dilution was plated in triplicates, and the plates were incubated at 37 °C for 24 h. Yellow colonies on replicate plates were counted and expressed as mean *V. cholerae* CFU per (g/ml) while green colonies on replicated plates were counted and expressed as mean *V. parahaemolyticus* CFU per (g/ml).

#### 2.4. Preparation of boiled and fried *keropok lekor* for experiments

Boiled *keropok lekor* was prepared by boiling *keropok lekor* (20 g) in sterile distilled water (200 ml) for ca. 5 mins. Fried *keropok lekor* (20 g) was deep fried in 200 ml of palm oil for ca. 8 mins. Both samples were cooled to 28 °C using incubator (Infors HT Ecotron). A new batch was freshly prepared on the day of each experiment.

#### 2.5. Survival determination of spiked *V. cholerae* and *V. parahaemolyticus* on boiled and fried *keropok lekor* under closed condition

This experiment was designed to mimic the condition in which the prepared *keropok lekor* was kept in a lid-covered container. Three grams of prepared *keropok lekor* was transferred to each of four universal bottles meant for holding times of 0, 1, 3 and 6 h, respectively. An estimated 1 × 10^8 CFU of *V. cholerae* (or *V. parahaemolyticus*) in 20 μl of PBS was spiked onto the boiled (or fried) *keropok lekor* kept in the loosely capped universal bottles and incubated at 28 °C in incubator (Infors HT Ecotron). *V. cholerae* (or *V. parahaemolyticus*) counts were performed on TCBS agar using spread plate method. Three replicated experiments were done on boiled and fried *keropok lekor*.

#### 2.6. Survival determination of spiked *V. cholerae* and *V. parahaemolyticus* on boiled and fried *keropok lekor* under opened condition

This experiment is designed to mimic the condition in which the prepared *keropok lekor* was being kept in a container without lid. The same procedure was performed as described above but with the universal bottles left opened throughout the holding time. Three replicated experiments were done on boiled and fried *keropok lekor*.

#### 2.7. Determination of pH and a_w of boiled and fried *keropok lekor*

The pH and a_w of boiled and fried *keropok lekor* were taken using pH 211 Microprocessor pH meter (Hanna Instrument, India) and HygroLab 3 Bench (Rotronic Instrument Corp., NY, USA), respectively.

#### 2.8. Statistical analysis

Data collected during the experiment was analyzed using SPSS 17.0 software. The data were analyzed using ANOVA, Kruskal–Wallis and Mann–Whitney U test. The significance level was set at *p* < 0.05.

### 3. Results and discussion

The two important vibrios that were investigated in this study are known to be ubiquitous in characteristically different aquatic environments whereby *V. cholerae* can be found in both freshwater as well as coastal waters while *V. parahaemolyticus* is mainly distributed in coastal waters (Thompson & Polz, 2006; Urakawa & Rivera, 2006). Foodborne illness caused by these vibrios can be due to consumption of raw and undercooked seafood (Fujino et al., 1974; Harth et al., 2009; Ottaviani et al., 2009; Zhao et al., 2011) or through cross-contamination of cooked foods (Abdullah Sani, Ariyawansa, Babji, & Hashim, 2013; Center for Health, 2011; Center for Health Protection, 2010).

Survivability of *V. cholerae* and *V. parahaemolyticus* in cooked *keropok lekor* is summarized in Tables 1 and 2. For control, *V. parahaemolyticus* was unable to survive in PBS in both conditions.

**Table 1**

<table>
<thead>
<tr>
<th>Sample conditions</th>
<th>Incubation time</th>
<th>Log CFU/(g or ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>1 h</td>
</tr>
<tr>
<td></td>
<td>Log CFU/ml or g</td>
<td>Log CFU/ml or g</td>
</tr>
<tr>
<td>Control Closed</td>
<td>7.19 ± 0.36 a</td>
<td>6.99 ± 0.37 a</td>
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<tr>
<td>Condition</td>
<td>8.24 ± 0.42 a</td>
<td>ND b</td>
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<td>Opened Condition</td>
<td>9.01 ± 0.25 a</td>
<td>8.52 ± 0.11 a</td>
</tr>
<tr>
<td>Closed Condition</td>
<td>8.84 ± 0.52 a</td>
<td>6.02 ± 0.54 b</td>
</tr>
<tr>
<td>Fried</td>
<td>7.46 ± 0.06 a</td>
<td>6.91 ± 0.25 a</td>
</tr>
<tr>
<td>Closed Condition</td>
<td>7.85 ± 0.48 a</td>
<td>5.02 ± 0.62 b</td>
</tr>
</tbody>
</table>

Data in the same row with different letter is different significantly (*p* < 0.05).

ND, Not detected.

*a* Data represent mean ± standard deviation of three replications.
(closed or opened) while *V. cholerae* survived very well with no significant difference (*p* > 0.05) at the end of 6 h incubation in a closed condition but was not detected after 1 h in an opened container. No viable vibrios were found in opened container after 1 h in agreement with the finding in Huat et al. (2008) where they reported *V. cholerae* O1 was not recovered 30 min after inoculation inside opened universal bottle. It is noteworthy to highlight that the inoculums had dried up after 1 h in the opened container but not in the closed container and this was likely due to evaporation (Huat et al., 2008). In a closed environment, the inoculums for both vibrios were still observed in the empty universal bottle. *V. parahaemolyticus* is known to prefer higher salinity environments (2–4% NaCl) for optimal growth compared to *V. cholerae* in which the optimal growth was reported at 0.5–1.0% NaCl (ICMSF, 1996a, chap. 22, 1996b, chap. 23). In addition, *V. cholerae* was reportedly capable of growing in broth containing less than 0.5% NaCl but not in the case of *V. parahaemolyticus* (ICMSF, 1996a, chap. 22, 1996b, chap. 23). This might explain the reduced survivability of *V. parahaemolyticus* in PBS since PBS does not provide any nutrients and the 0.8% NaCl content is below its optimal growth condition.

This study showed that *keropok lekor* supported the survival of *V. cholerae* and *V. parahaemolyticus* whether in opened or closed conditions (Tables 1 and 2). *Keropok lekor* provided a suitable condition for microbial growth due to its pH, water activity and temperature (Nor-Khaizura, Zaiton, Jamilah, & Gulam Rusul, 2009). Since salt is part of the ingredient in *keropok lekor* (Omar et al., 2011) this favors the viability of halophilic bacteria such as *V. cholerae* and *V. parahaemolyticus*. Both boiled and fried *keropok lekor* has the pH and water activity that lies within the range which supports the survivability of vibrios (ICMSF, 1996a, chap. 22, 1996b, chap. 23; Nor-Khaizura et al., 2010). In a closed environment, the pH and water activity (aw) were consistent (*p* > 0.05) throughout the 6 h incubation with the pH for boiled *keropok lekor* at 6.52 ± 0.06 and for fried *keropok lekor* at 6.70 ± 0.24, while aw values were 0.97 ± 0.01 and 0.96 ± 0.01 for boiled and fried *keropok lekor*, respectively. However, in an opened environment, slight decreases in both the pH and aw were observed after the 6 h incubation period. For boiled *keropok lekor*, the pH decreased from 6.52 ± 0.06 to 6.42 ± 0.10 while for fried *keropok lekor* the pH was from 6.70 ± 0.24 to 6.58 ± 0.20. Likewise, the aw value for boiled *keropok lekor* decreased from 0.97 ± 0.01 to 0.95 ± 0.01 whereas for fried *keropok lekor* a decrease from 0.96 ± 0.01 to 0.94 ± 0.01 was recorded after 6 h incubation. The consistency of these properties of *keropok lekor* particularly in closed conditions during the experimental time frame could perhaps enable both vibrios to survive even in the case of *V. parahaemolyticus* which is known to be highly sensitive to drying (Lake, Hudson, & Cressy, 2003, pp. 4–5).

Fishes are recognized as reservoirs for *V. cholerae* (Saravanan, Kumar, Karunasagar, & Karunasaga, 2007), increasing the chances of vibrio contamination to the end products of fish. Most cholera outbreaks were mainly attributed to water contaminated with *V. cholerae* (Khunia, Samal, Kar, & Pal, 2010) which is the main route for the transmission of this bacterium. However, the presence of this pathogen can also be due to cross-contamination. It has been estimated that about 40–60% cases of foodborne disease were caused by improper handling practices such as cross contamination from the cutting board (Soares et al., 2012) and 25% of outbreaks were due to improper handling by the food handlers (Carrasco, Morales-Rueda, & García-Gimeno, 2012; Ravishankar, Zhu, & Jam-on, 2010). If the raw food is contaminated, the possibility for pathogens to transfer to cooked food is significant (Soares et al., 2012; Tang et al., 2011).

It is known that vibrios are heat sensitive bacteria (Johnston & Brown, 2002) but their contamination in seafood and processed seafood-related products has been frequently reported and cannot be ignored (Abd-Elyghany & Sallam, 2013; Aberoumand, 2010; Huang, Ghate, Phua, & Yik, 2012). A recent report by Abdullah Sani et al. (2013) estimated that 123 Malaysians (aged from 18 to 59 years old) will fall ill each year as a result of the consumption of cooked black tiger shrimps (*Penaeus monodon*) contaminated with *V. parahaemolyticus*. *V. parahaemolyticus* is the major contributor for cases of foodborne illness due to the consumption of raw, undercooked or contaminated shellfish (Harth et al., 2009; Ottaviani et al., 2009; Zhao et al., 2011).

This study proved that vibrios are capable of surviving when present in cooked Malaysian fish sausage (*keropok lekor*) despite their sensitive and fragile nature. Thus, it would be noteworthy to study other *Vibrio* spp. behavior in this increasingly popular fish sausage.

### Acknowledgments

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### References


### Table 2

<table>
<thead>
<tr>
<th>Sample conditions</th>
<th>Incubation time</th>
<th>Log CFU (ml or g)</th>
<th>Log CFU (ml or g)</th>
<th>Log CFU (ml or g)</th>
<th>Log CFU (ml or g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>1 h</td>
<td>3 h</td>
<td>6 h</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Closed condition</td>
<td>5.66 ± 0.19 a</td>
<td>4.91 ± 0.47 ab</td>
<td>4.02 ± 0.50 b</td>
<td>ND c</td>
</tr>
<tr>
<td></td>
<td>Opened condition</td>
<td>6.01 ± 0.32 a</td>
<td>ND b</td>
<td>ND b</td>
<td>ND b</td>
</tr>
<tr>
<td>Boiled <em>keropok lekor</em></td>
<td>Closed condition</td>
<td>5.96 ± 0.13 a</td>
<td>6.07 ± 0.15 a</td>
<td>5.72 ± 0.08 a</td>
<td>5.75 ± 0.09 a</td>
</tr>
<tr>
<td></td>
<td>Opened condition</td>
<td>6.25 ± 0.30 a</td>
<td>5.12 ± 0.35 ab</td>
<td>4.02 ± 0.26 bc</td>
<td>2.85 ± 0.45 c</td>
</tr>
<tr>
<td>Fried <em>keropok lekor</em></td>
<td>Closed condition</td>
<td>7.00 ± 0.37 a</td>
<td>7.17 ± 0.33 a</td>
<td>5.40 ± 0.15 a</td>
<td>5.49 ± 0.59 a</td>
</tr>
<tr>
<td></td>
<td>Opened condition</td>
<td>5.55 ± 0.22 a</td>
<td>4.02 ± 0.35 ab</td>
<td>2.28 ± 0.32 bc</td>
<td>2.13 ± 0.52 c</td>
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</tbody>
</table>

Data in the same row with different letter is different significantly (*p* < 0.05).

ND, Not detected.

* Data represent mean ± standard deviation of three replications.