MICROBIOLOGICAL SAFETY ASSESSMENT AND RISK MITIGATION OF INDIAN ROJAK (DEEP FRIED READY-TO-EAT FOOD) IN SINGAPORE

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Abstract. We conducted a microbiological assessment of Indian Rojak, a popular deep fried food in Singapore to evaluate its overall microbial quality, assess the effectiveness of reheating and identify key food items that could contribute to the microbial load of the dish. In 2009, an outbreak of foodborne illness associated with this food led to 154 reported cases of acute gastroenteritis, 48 were hospitalized and 2 died. *Vibrio parahaemolyticus* was isolated from the patients. We evaluated 455 Indian Rojak ingredients from 35 stalls; no *Salmonella* spp, *Vibrio cholerae/parahaemolyticus* or *Escherichia coli* O157:H7 were recovered from the studied samples. The reheating by the food handlers significantly reduced the overall median Standard Plate Count (SPC) of food from 4.5 to 2.7 log colony forming units (CFU)/g ($p<0.05$). The cooked ingredients with the highest microbial loads were tofu and fish cake, with those purchased from wet markets having significantly higher bacterial loads than those purchased from supermarkets ($p<0.05$). The Rojak gravy had the lowest median bacterial load (1.9 log CFU/g). Raw, ready-to-eat vegetables, namely green chillis, cucumbers and onions had higher levels ranging from 5.9 to 6.1 log CFU/g. Contamination with *E. coli*, *Staphylococcus aureus*, and *Bacillus cereus* was seen with some of the ready-to-eat raw vegetables. Repeated education of food handlers with emphasis on good hygiene practices should be conducted to reduce the risk of foodborne illnesses.

Keywords: microbiological quality, risk mitigation, ready-to-eat, foodborne pathogen

INTRODUCTION

Foodborne illnesses are a public health concern worldwide and have significant economic and health impacts (Kaferstein *et al*, 1997). Foodborne ill-nesses may be caused by ingestion of pathogens (bacteria, fungi, viruses and parasites), biological toxins or chemicals (Tauxe *et al*, 2010). Food from unsafe sources, poor personal hygiene, inadequate cooking, improper holding temperatures and contaminated equipment or poor protection from contamination, have been identified as common contributory factors in foodborne illness (US Food and Drug Administration, 2009). This high-

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lights the importance of good hygiene practices in the handling and preparation of food, especially in the retail sector, as it is the final step before the food reaches the consumer.

In Singapore, four out of five Singapore residents eat at local hawker centers/food courts/coffee shops at least twice a week; while nearly half of Singapore residents eat at these outlets more than 5 times a week (Health Promotion Board Singapore, 2010). With such habits and with over 34,000 retail food outlets catering to the public in Singapore, much emphasis must be placed on food hygiene and the safety of retail food to protect the public from foodborne illnesses. Singapore's national food hygiene management framework, along with stringent control of food sources, has helped to keep the numbers of cases of notifiable water- and food-borne diseases at low levels, with an overall morbidity rate of 41.2 per 100,000 population (Ministry of Health Singapore; 2011, 2012, 2013).

However, Singapore has had food poisoning outbreaks in the past. In 2009, there were 154 cases of foodborne illness and 2 fatalities associated with consumption of Indian Rojak, a local deep-fried ready-to-eat (RTE) food (Ministry of Health Singapore, 2009). *Vibrio parahaemolyticus* was isolated from 13 of 27 stool samples obtained from the cases, including one of the fatalities. A similar *V. parahaemolyticus* food poisoning outbreak associated with the consumption of Indian Rojak was described in 1983, with 34 cases reported. Investigation of the 1983 outbreak found it was likely due to cross-contamination of the gravy by raw cuttlefish drippings; the events that led to the contamination of the Rojak in the 2009 outbreak remain obscure (Ministry of Health Singapore, 2009).

Indian Rojak is a popular local dish composed of a mixture of deep-fried food items and raw vegetables, served with a thick peanut gravy. Common Rojak items available in food stalls in Singapore include cuttlefish, eggs, fishcakes, prawn fritters, sausage and tofu, which are usually pre-cooked before being displayed in opened cabinets at room temperature. Upon receiving the customer's order, the food handler then reheats the selected items through deep frying in oil for 1 to 2 minutes before cutting them into bite-size pieces and serving them with raw vegetables, such as cucumbers, onions and green chillis. The thick gravy that accompanies the dish is usually kept warm throughout the day.

Our study aimed to determine the presence of bacteria in the Indian Rojak, assess the bactericidal effectiveness of re-heating the Rojak food items after prolonged displaying at room temperature, and identify the key food items that could contribute to the bacterial load in the dish.

MATERIALS AND METHODS

Sample collection and preparation

A total of 455 samples of Indian Rojak food items were collected from 35 different Indian Rojak stalls across Singapore from September 2011 to January 2012. The samples were comprised of 321 cooked items with or without re-heating, 99 serving portions of raw vegetables and 35 serving portions of gravy. All collected samples had been at room temperature for at least 4 hours at the time of collection with the exception of the gravy, which was usually held at warm temperature on the stove throughout the day. To assess the impact of the traditional brief re-heating step practiced by food handlers, we collected two pieces of each fried item from

the same batch, one prior to and the other after reheating. Each sample was placed in a sterile re-sealable plastic bag upon purchase and transported promptly to the laboratory on ice for immediate processing or stored temporarily at 4°C. All the samples were processed in the laboratory within 2 hours of purchase. All 455 samples were cultured to determine the Standard Plate Count (SPC), *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus* counts, as well as presence of *Salmonella* spp, *Vibrio cholerae/parahaemolyticus* and *E. coli* O157:H7, as described below.

To assess the initial microbial quality of raw ready-to-eat (RTE) ingredients, additional raw cucumber (*n*=20), onion (*n*=20) and green chilli (*n*=20) samples were purchased from wet markets and supermarkets, and cultured to determine the Standard Plate Count after undergoing prior rinsing and scrubbing under running tap water for 10-15 seconds. Similarly, additional raw tofu (*n*=40) and fishcake (*n*=39) samples were purchased from wet markets and supermarkets, and cultured to determine the Standard Plate Count (SPC) and detect *E. coli.*

A ten gram sample of each food item was obtained and placed in a sterile stomacher bag and homogenized with 90 g of Universal Pre-enrichment Broth (UPB) (Acumedia Manufacturers, Lansing, MI) using a stomacher (Seward Stomacher® 400 Circulator, Seward, West Sussex, UK) at 230 rpm for 30 seconds. Serial 10-fold dilutions were prepared using 9 ml Butterfield's buffer (3M, St Paul, MN). The diluted homogenized samples were then processed as described below.

Standard plate count

For each sample, one milliliter (1 ml) of 10- to 1,000-fold diluted samples was inoculated onto a Petrifilm™ Aerobic Plate Count (3M, St Paul, MN) and distributed evenly using a spreader. The Petrifilm was then incubated at 37°C for 48 hours before quantification.

Detection and quantification of *Escherichia coli*

For each sample, 1 ml of 10-fold diluted sample (dilution performed as described above) was inoculated onto a Petrifilm™ *E. coli*/Coliform Count Plate (3M). The Petrifilm was then incubated at 37°C for 48 hours. Presumptive *E. coli* colonies (dark blue color) were counted, streaked onto Levine Eosin Methylene Blue (LEMB) agar (Acumedia Manufacturers) and incubated at 37°C for 24 hours for further confirmation. Presumptive *E. coli* colonies identified from the LEMB agar (dark blue color) were then inoculated into 5 ml Tryptone water (Acumedia Manufacturers) and incubated at 37°C for 24 hours. Indole testing was carried out using Kovacs' reagent (Remel, Lenexa, KS) for confirmation.

Detection and quantification of *Bacillus cereus*

A hundred microliters of 10-fold diluted sample (dilution performed as described above) were spread onto mannitol-egg yolk-polymyxin (MYP) agar (Oxoid, Hamshire, UK) and incubated at 30°C for 24 hours. Presumptive *B. cereus* colonies (bright pink color surrounded by a zone of precipitation) were counted and streaked onto tryptic soy-sheep blood agar (SBA) (Oxoid) and incubated at 37°C for 24 hours. The colonies were subsequently tested using API® 50 CHB (bioMérieux, Marcy l'Étoile, France).

Detection and quantification of *Staphylococcus aureus*

One milliliter of 10-fold diluted sample (dilution performed as described above) was equally distributed between

two plates of Baird-Parker agar (Oxoid) before incubation at 37°C for 48 hours. Presumptive *S. aureus* colonies (grey-black colonies with a narrow white margin surrounded by a zone of clearing) were counted, tested for a catalase reaction using 3% hydrogen peroxide (ICM Pharma, Singapore), and confirmed using coagulase rabbit plasma (Remel).

Detection of *Salmonella* species

For *Salmonella*, no quantification was done but enrichment was carried out to isolate *Salmonella* strains. After 18-24 hours of incubation at 37°C in Universal Pre-enrichment Broth (Acumedia Manufacturers), the enriched samples were streaked onto xylose lysine desoxycholate (XLD) agar (Oxoid) using sterile 10 μl loops, and incubated at 37°C for 24 hours. Presumptive *Salmonella* colonies (red colonies with black centers) were inoculated onto triple-sugar iron (TSI) and lysine-iron agar (LIA) slants (Oxoid) and incubated at 37°C for 24 hours. Isolates with positive slant reactions were confirmed to be *Salmonella* species using API® 20E (bioMérieux) and serological latex agglutination test (Oxoid).

Detection of *Vibrio cholerae* and *V. parahaemolyticus*

For *Vibrio*, no quantification was done but enrichment was done to isolate *Vibrio* strains. After 18-24 hours of incubation at 37°C in Universal Pre-enrichment Broth (Acumedia Manufacturers), 10 μl loopful of the enriched samples was streaked onto CHROMagar™ Vibrio (Oxoid) and incubated at 37°C for 24 hours. Presumptive *Vibrio* colonies (blue-green to turquoise blue or mauve color) were further tested for positive oxidase reaction (Oxoid) and with the API® 20E test (bioMérieux).

Detection of *E. coli* O157:H7

For *E. coli* O157, no quantification was done but enrichment was performed to determine strains. After 18-24 hours of incubation at 37°C in Universal Pre-enrichment Broth (Acumedia Manufacturers), 10 μl enriched sample was streaked onto HiCrome™ EC O157 agar (Sigma-Aldrich, St Louis, MO) and incubated at 37°C for 24 hours. Presumptive *E. coli* O157 colonies (dark purple to magenta color) were sub-cultured onto LEMB agar, sorbitol MacConkey (Oxoid) and incubated at 37°C for 24 hours. The suspected colonies were subsequently confirmed to be *E. coli* with the indole test using Kovacs' reagent (Remel™) and identification of *E. coli* O157:H7 was performed with a latex agglutination kit (Oxoid).

Molecular characterization of specific microorganisms

Of the pathogens tested for, only *Bacillus cereus* and *Staphylococcus aureus* were detected. Molecular characterization was performed on the isolates.

Molecular characterization of *Bacillus cereus*

DNA was extracted from pure colonies grown on TSA (Acumedia) using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) (Gram-positive bacteria protocol) with Calbiochem® Lysozyme Chicken Egg White (Merck, Darmstadt, Germany). The isolates were confirmed to be *B. cereus* by PCR using published primers (Yamada *et al*, 1999). The presence of virulence factors among the *B. cereus* isolates was determined using PCR with published primers for *ces, hbla, hblc, hbld*, and *cytK* genes (Mantynen and Lindstrom, 1998; Rowan *et al*, 2003; Nakano *et al*, 2004; Guinebretiere *et al*, 2006). Amplification by PCR was performed by initial strand denaturation for 30 seconds at 98°C; followed by 35 cycles of 10 seconds at 98°C, 30 seconds at 60°C, 30 seconds at 72°C; and final extension for

10 minutes at 72°C. The amplified products were mixed with 6x DNA loading dye (Thermo Fisher Scientific, Rockford, IL) and visualized on 2% agarose gel stained with GelRed (Biotium, Hayward, CA). Detectable PCR bands were regarded as confirmation of isolates and/or the virulence genes.

Molecular characterization of *Staphylococcus aureus*

DNA was extracted from pure colonies on Tryptic Soy Agar (TSA) (Acumedia, Baltimore, MD) using the method stated above. The presence of enterotoxic genes, SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, SEJ and SEL (Cremonesi *et al*, 2005; Rosec and Gigaud, 2002); and the *mecA* gene characteristic of Methicillin Resistant *Staphylococcus aureus* (MRSA) (Strommenger *et al*, 2003); was determined using multiplex PCR. Amplification was performed using the following parameters: initial strand denaturation for 30 seconds at 98°C; followed by 30 cycles of 10 seconds at 98°C, 30 seconds at 61°C, 30 seconds at 72°C; and final extension for 10 minutes at 72°C. PCR-positive MRSA samples were confirmed with a latex agglutination test (PBP2) (Oxoid) and a cefoxitin disc (Oxoid) using the disc diffusion method following Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2007). Molecular typing targeting the Staphylococcal Protein A gene (spa typing) was done on MRSA isolates using published primers (Spa 1113f and 1514r) (Strommenger *et al*, 2006) with the following PCR conditions: initial strand denaturation for 30 seconds at 98°C; followed by 35 cycles of 10 seconds at 98°C, 30 seconds at 61°C, 30 seconds at 72°C; and final extension for 10 minutes at 72°C. The amplified products were mixed with 6x DNA loading dye (Thermo Fisher

Scientific) and visualized on 2% agarose gel stained with GelRed (Biotium, Hayward, CA). Detectable PCR bands were regarded as confirmation of isolates and/or the virulence genes. PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced by capillary electrophoresis using BigDye Terminator chemistry (AIT Biotech, Singapore). Sequences were analyzed using BioNumerics 7.1 to determine spa types and repeats.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Statistics Software, version 21.0 (IBM, Armonk, NY). Descriptive analysis was done using the Mann-Whitney test to compare median microbial counts. A p-value < 0.05 was considered statistically significant.

RESULTS

Absence of tested foodborne pathogens in Indian Rojak samples

No *Salmonella* spp, *Vibrio* spp or *E. coli* O157:H7 were recovered from any of the samples tested in this study.

Microbial quality of cooked ingredients before re-heating

In Singapore, the microbial standards for RTE food are found in the Sale of Food Act, which sets the acceptable limits for total microbiological counts at 5.0 log CFU/g and *E. coli* counts at 1.3 log CFU/g (Agrifood and Veterinary Authority singapore, 2005). The Sale of Food Act also states that no pathogen should be detected in RTE food. In our study, the median Standard plate count (SPC) among all 161 Indian Rojak items (prawn fritters, tofu, fishcake, eggs and cuttlefish) before re-heating was 4.5 log CFU/g (Fig 1). However, only 59.6% of the samples complied with local
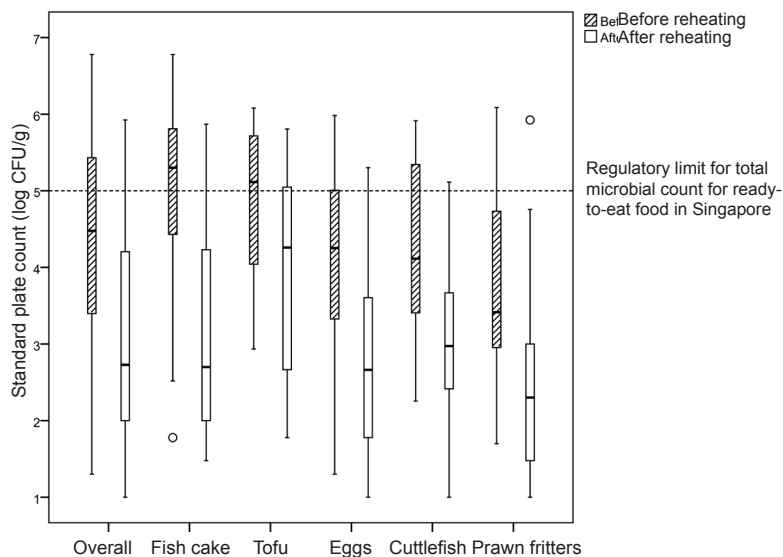
Fig 1–The box plot represents the median standard plate counts (SPC) for the cooked Indian Rojak ingredients, before (shaded box plots) and after reheating (blank box plots). The dotted line at 5.0 log represents the regulatory limit for the total microbial count for ready-to-eat food in Singapore. The horizontal line in the box plot represents the median value. The box plot is drawn from the 25th to 75th percentile and the whiskers show the minimum and maximum values. Circles represent outliers.

regulatory requirements for total microbial loads (Fig 1).

Among the cooked samples collected before undergoing the re-heating step, fish cake and tofu had the highest bacterial loads (Fig 1) and the largest proportion of items contaminated by *E. coli* (Table 1). Interviews with food operators revealed raw fishcake and tofu ingredients tend to be purchased from wet markets rather than supermarkets. Further investigation revealed raw fishcake and tofu samples collected from wet markets had significantly greater bacterial loads ($p<0.05$) than those collected from supermarkets (Fig 2). *E. coli* was detected in 25% of fish cake samples and 35% of tofu samples collected from wet markets, whereas none of the

supermarket samples was positive for *E. coli*.

Effectiveness of reheating

After brief reheating, the overall median SPC of the cooked items decreased significantly by 2.0 log CFU/g ($p<0.05$), bringing it down to 2.7 log CFU/g (Fig 1). Although the bacterial loads of the cooked fish cake and tofu prior to reheating were relatively higher than the other ingredients, the reheating step was successful in reducing these microbial loads by 99.2% and 97.4% for fishcake and tofu, respectively (Figs 1 and 2).

The re-heating process also brought down the overall percentages of contamination with *S. aureus* and *B. cereus* from 5.0% and 6.8%, respectively, to 0%, and decreased the rate of contamination with *E. coli* from 14.9% to 3.1% (Table 1). Among the re-heated items, 5 (3.1%) (tofu: *n*=3; egg *n*=1; cuttle fish: *n*=1) still bore detectable *E. coli* counts, including 4 (2.5%) (tofu: *n*=3; eggs: *n*=1) with *E. coli* counts above the regulatory limit of 1.3 log CFU/g (Table 1).

Microbial quality of Rojak gravy

In our study, of all the ingredients, the Rojak gravy had the lowest bacterial load, with a median SPC of 1.9 log CFU/g, and no pathogens were detected.

Microbial quality of raw vegetable ingredients

In contrast to the gravy, bacterial loads measured on raw vegetables reached high
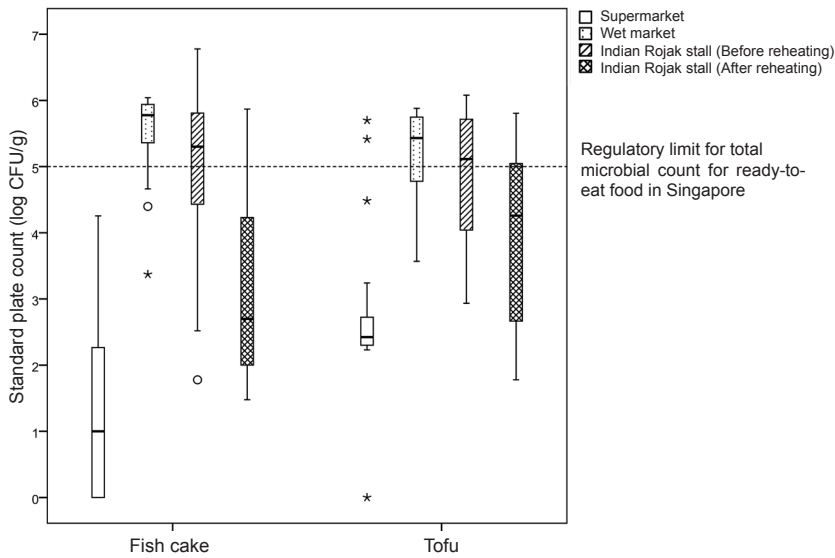
Fig 2–The box plot represents the median standard plate counts (SPC) for the fish cake and tofu samples collected from supermarkets (raw) (blank box plots), wet markets (raw) (dotted box plots) and Indian Rojak stalls (cooked, before reheating shown as the left-shaded box plots and after reheating shown as crisscrossed box plots). The dotted line at 5.0 log represents the regulatory limit for the total microbial count for ready-to-eat food in Singapore. The horizontal line in the box plot represents the median value. The box plot is drawn from the 25th to 75th percentile and the whiskers show minimum and maximum values. Circles and asterisks represent outliers.

median SPC values of 6.1, 5.9 and 5.9 log CFU/g for green chillis, cucumbers, and onions, respectively (Fig 3), with an average of 23.2% of raw vegetables being contaminated with either *E. coli*, *S. aureus* or *B. cereus* (Table 2). No co-contamination was observed. These results amount to 91.9% of raw vegetables not complying with Singapore microbial standards for RTE food (data not shown).

Molecular characterization of *Bacillus cereus* and *Staphylococcus aureus* isolates from Indian Rojak samples

Bacillus cereus. Out of 455 Rojak samples, 14 samples (3.1%), including three raw RTE vegetables and 11 cooked ingredients,

were positive for *B. cereus* with an overall median value of 2.9 log CFU/g (Table 2). Of the 14 *B. cereus* positive samples, 13 had *B. cereus* isolates. Of these 13 isolates, three were from raw RTE vegetables and ten were isolated from cooked ingredients. No emetic toxin (cereulide) gene was detected by PCR. Four of the 13 isolates (30.8%) (fish cake *n*=1, prawn fritters *n*=3) (Table 3) were positive for *hbla*, *hblc* and *hbld* genes, encoding for the B (binding), L1 and L2 (lysis) components of the hemolytic BL diarrheal toxin (Hbl), respectively (Beecher *et al*, 1995). The gene encoding the single-component diarrheal toxin, *cyt*K, was also detected in 8 isolates (61.5%) (eggs: *n*=1; green chilli: *n*=1; onions: *n*=2; prawn fritters: *n*=4) (Table 3).

Staphylococcus aureus. *S. aureus* was isolated from 2.4% (*n*=11) of the 455 samples tested in this study, specifically from three raw RTE vegetables and 8 cooked ingredients, with an overall bacterial load of 2.5 log CFU/g (Table 2). Of the 11 *S. aureus* isolates, 5 (45%) were found to carry at least 1 enterotoxin gene. Specifically, 3 tested positive for the enterotoxin B gene (SEB), one carried the enterotoxin genes SEC and SEL and one carried the enterotoxin genes SEG and SEI (Table 4). Three
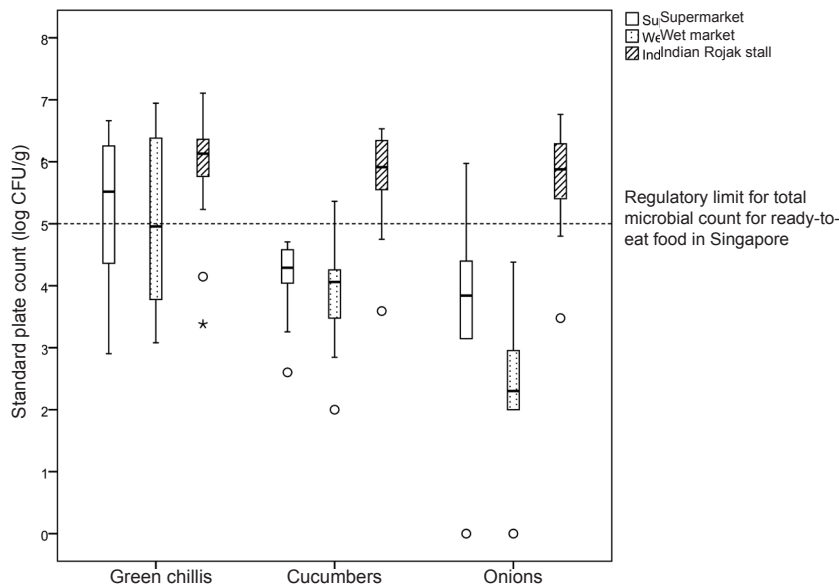
Fig 3–Median standard plate counts (SPC) for the raw ready-to-eat vegetables (green chilli, cucumber and onion) collected from supermarkets (blank box plots), wet markets (dotted box plots) and Indian Rojak stalls (left-shaded box plots). The dotted line at 5.0 log represents the regulatory limit for the total microbial count for ready-to-eat food in Singapore. The horizontal line in the box plot represents the median value. The box plot is drawn from the 25th to 75th percentile and the whiskers show minimum and maximum values. Circles and asterisks represent outliers.

of these 11 isolates, collected from onions, prawn fritters and eggs from the same Rojak stall, that were positive for the enterotoxin B gene (SEB) were confirmed to be methicillin resistant *S. aureus* (MRSA) (Table 4). They were determined to be of closely related *spa* (Staphylococcal protein A) types, with a single repeat difference observed between types (data not shown).

DISCUSSION

In our study, we did not detect *Salmonella*, *E. coli* O157:H7, *Vibrio cholerae* or *V. parahaemolyticus* in any of the samples tested. *Vibrio parahaemolyticus* was the pathogen incriminated in earlier outbreaks associated with Indian Rojak in Singapore (Ministry of Health Singapore, 2009). *V. parahaemolyticus* poisoning is most commonly associated with the consumption of uncooked seafood, or food that has been contaminated with seafood products during processing. This bacteria is destroyed by most heat treatments (Health Protection Agency, 2009). Our data suggest the incidents reported in 1983 and 2009 were isolated events and contamination of Rojak with *V. parahaemolyticus* is not common.

Approximately 40% of the samples tested prior to reheating were found to be with a high bacterial load, above 5.0 log CFU/g (Fig 1). These food items had undergone a pre-cooking step followed by displaying at room temperature for over 4 hours. The high proportion of samples with bacterial loads exceeding 5.0 log CFU/g prior to reheating underscores the importance of the reheating step prior to serving. The finding is consistent with an earlier study from Taiwan: RTE food kept at room temperature had a higher incidence of aerobic plate counts exceeding 5.0 log CFU/g than food stored in the refrigerator or at hot temperatures (Wei *et al*, 2006).

A higher percentage of tofu samples

Table 1

Prevalences of *E. coli*, *B. cereus* and *S. aureus* among Indian Rojak samples, before and after reheating.

	Before re-heating[a]				After re-heating[b]			
	Total number of samples	No. of samples with *E. coli*	No. of samples with *B. cereus*	No. of samples with *S. aureus*	Total number of samples	No. of samples with *E. coli*	No. of samples with *B. cereus*	No. of samples with *S. aureus*
Fish cake	35	8	2	1	34	0	0	0
Tofu	33	11	0	2	33	3	0	0
Eggs	32	1	3	2	31	1	0	0
Cuttlefish	29	3	0	2	29	1	0	0
Prawn fritters	32	1	6	1	33	0	0	0
Total	161	24 (14.9%)	11 (6.80%)	8 (5.0%)	160	5 (3.1%)	0 (0.0%)	0 (0.0%)

[a]Deep-fried once at the start of the day and displayed at room temperature for ≥4 hours before collection (no reheating process).
[b]Deep-fried once at the start of the day, displayed at room temperature for ≥4 hours and re-deep-fried for 1-2 minutes before collection.

Table 2

Prevalence of *E. coli*, *B. cereus* and *S. aureus* among cooked and raw Indian Rojak ingredients (*N*=455).

Total number of samples (*N*=455)	Cooked ingredients (*n*=321)					Raw vegetables (*n*=99)			Rojak gravy (*n*=35)	Total number of positive samples	Median count of pathogens in positive samples (log CFU/g)
	Fish cakes (*n*=69)	Tofu (*n*=66)	Eggs (*n*=63)	Cuttlefish (*n*=58)	Prawn fritters (*n*=65)	Green chillis (*n*=33)	Cucumbers (*n*=34)	Onions (*n*=32)			
E. coli	8	14	2	4	1	5	7	5	0	46 (10.1%)	2.2
B. cereus	2	0	3	0	6	1	0	2	0	14 (3.1%)	2.9
S. aureus	1	2	2	2	1	0	1	2	0	11 (2.4%)	2.5

Table 3
Molecular characterization of *B. cereus* isolates (*n*=13).

	Virulence genes					
	Emetic toxin	*hbla*	*hblc*	*hbld*	*hbla+hblc+hbld*	*cytK*
RJ17 (Green chillis)	-	+	-	+	-	+
RJ36 (Fish cakes)	-	+	+	+	+	-
RJ74 (Prawn fritters)	-	-	-	-	-	+
RJ78 (Fish cakes)	-	+	-	+	-	-
RJ88 (Prawn fritters)	-	+	+	+	+	-
RJ100 (Onions)	-	-	-	-	-	+
RJ158 (Prawn fritters)	-	+	+	+	+	+
RJ164 (Eggs)	-	-	-	-	-	-
RJ368 (Prawn fritters)	-	+	+	+	+	+
RJ416 (Eggs)	-	-	-	-	-	+
RJ424 (Prawn fritters)	-	-	-	-	-	-
RJ438 (Prawn fritters)	-	-	-	-	-	+
RJ464 (Onions)	-	-	-	-	-	+
Number (%) of isolates with virulence genes	0 (0.0%)	6 (46.2%)	4 (30.8%)	6 (46.2%)	4 (30.8%)	8 (61.5%)

from wet markets had *E. coli* than from supermarkets in our study. This differs from a study from Taiwan in 2006 showing detection of *E. coli* in RTE foods sold in supermarkets but not in those sold in traditional markets (Wei *et al*, 2006). In Singapore, fish cake and tofu sold in super markets are displayed in chilled packed form while those sold in wet markets are usually displayed unpacked at room temperature. These storage conditions and possibly more direct handling may explain the significantly higher bacterial loads found in fish cake and tofu.

In our study, reheating significantly decreased the initial bacterial load and risk for foodborne illness. However, some reheated items were still found to be contaminated with *E. coli*. Although the proportion of ingredients in our study contaminated with *E. coli* was low, it is evident reheating is not effective enough to destroy all the pathogens present. After

reheating, the median SPC value in tofu (4.3 log CFU/g) was still slightly higher than all the other reheated items (Fig 1), but not significantly. In a previous study (Baik and Mittal, 2005), the temperatures of the different parts of the tofu were measured at intervals during deep-frying. While the surface of the tofu reached high temperatures rapidly, the maximum temperature reached at the centre part was lower than that of the surface of the tofu. The lower heat in the center of the tofu may explain the relatively high SPC observed in tofu compared to the other ingredients after reheating.

Rojak gravy was found to have the lowest bacterial load of all the Rojak ingredients tested. When Indian Rojak was incriminated in the 1983 food poisoning incident in Singapore (Ministry of Health Singapore, 2009), *Vibrio parahaemolyticus* was isolated from raw cuttlefish drippings as well as from the gravy, suggesting the

Table 4
Molecular characterization of *S. aureus* isolates (n=11)

					Virulence genes						
	mecA	SEA	SEB	SEC	SED	SEE	SEG	SEH	SEI	SEJ	SEL
RJ122 (Eggs)	-	-	-	-	-	-	-	-	-	-	-
RJ124 (Cuttlefish)	-	-	-	-	-	-	-	-	-	-	-
RJ142 (Onions)	+	-	+	-	-	-	-	-	-	-	-
RJ144 (Prawn fritters)	+	-	+	-	-	-	-	-	-	-	-
RJ148 (Fish cakes)	-	-	-	-	-	-	-	-	-	-	-
RJ150 (Eggs)	+	-	+	-	-	-	-	-	-	-	-
RJ198 (Onions)	-	-	-	-	-	-	-	-	-	-	-
RJ208 (Cuttlefish)	-	-	-	-	-	-	-	-	-	-	-
RJ267 (Cucumbers)	-	-	-	-	-	-	+	-	-	-	-
RJ384 (Tofu)	-	-	-	+	-	-	-	-	+	-	-
RJ468 (Tofu)	-	-	-	-	-	-	-	-	-	-	+
Number (%) of isolates with virulence genes	3 (27.3%)	0 (0.0%)	3 (27.3%)	1 (9.1%)	0 (0.0%)	0 (0.0%)	1 (9.1%)	0 (0.0%)	1 (9.1%)	0 (0.0%)	1 (9.1%)

contamination of the latter by the former. Our findings suggest, unless such cross-contamination occurs, the bacteriological quality of the gravy is not a concern. The continuous heating of the gravy throughout the day seems to be effective in keeping this ingredient free of pathogens and with a low bacterial load.

Raw RTE vegetable ingredients such as green chillis, cucumbers and onions had high bacterial loads in our study. Similar findings of high microbial counts (>5.0 log CFU/g) were recently reported in fresh vegetables and fruits sold in Singapore (Seow *et al*, 2012). Previous surveillance data (unpublished data) shows up to 71.4% of Indian Rojak samples (n=91) bore standard plate counts that exceeded regulatory standards (data not shown). Our findings suggest these high bacterial loads may be from the raw vegetables. While some countries, such as Hong Kong, New Zealand and the United Kingdom, take into consideration the high inherent bacterial loads found in raw RTE vegetables and do not include these food items when determining bacterial count limits in this food type (Centre for Food Safety, 2007; Food Standards Australia New Zealand, 2001; Health Protection Agency, 2009), Singapore's regulations regarding RTE foods do not differentiate food types and impose the same limits on all mixed RTE food types (Agrifood and Veterinary Authority Singapore, 2005). The failure

rates of the raw vegetable ingredients to meet local regulations should be interpreted with care.

However, comparison of the bacterial loads on cucumbers and onions found the bacterial loads of these items in Indian Rojak stalls were higher than on these items obtained from supermarkets and wet markets ($p<0.05$) (Fig 3) suggesting prolonged storage of the cut and prepared raw vegetables at room temperature may facilitate bacterial growth and cross-contamination. Thus, there is a need to educate food handlers about the importance of hygienic preparation and storage of vegetables at cold temperatures, particularly if the vegetables are to be eaten raw.

Prawn fritters ($n=6$) had the highest contamination rates with *B. cereus* in this study. Since flour is a main component of prawn fritters, our findings are compatible with those of an earlier report that reported similarly high counts of *B. cereus*-like organisms in heat-treated starchy foods, following improper cooling after heat-treatment (Rosenquist *et al*, 2005). Rosenquist *et al* (2005) in Denmark reported 75% ($n=30/40$), 67.5% ($n=27/40$) and 2.5% ($n=1/40$) of *B. cereus*-like organisms isolated from RTE food were positive for *hblA*, *cytK* and emetic toxin, respectively. In comparison, our proportions were slightly lower, with *hblA, cytK* and emetic toxin genes detected in 46.2% ($n=6/13$), 61.5% ($n=8/13$), 0.0% ($n=0/13$) of *B. cereus* isolates, respectively (Table 3A).

B. cereus and its spores are abundant in soil (Tewari and Abdullah, 2015); hence all kind of foods are likely to be contaminated. However, not all *B. cereus* strains produce toxins (Granum and Lund, 1997). The emetic syndrome is usually associated with starchy foods (pasta, rice) while a wide range of foods, such as meat products, soups, vegetables, sauce and pudding, have been implicated with *B. cereus*-linked diarrheal syndrome (Health Protection Agency, 2009). Although we did not verify the ability of isolates to produce toxins in our study, the presence of toxigenic *B. cereus* strains in Rojak samples is a concern. The prolonged storage of contaminated food at room temperature, as typically practiced with Indian Rojak, may favor the proliferation of *B. cereus* and toxins. Time-temperature control is essential to reduce the risk of food poisoning.

We detected the presence of enterotoxin genes in some of the *S. aureus* strains isolated from the Rojak samples. Although gene expression was not determined in this study, the presence of toxigenic *S. aureus* strains suggests the potential for food poisoning, particularly if the food is stored at room temperature for prolonged periods. If the bacterial load of *S. aureus* exceeds 5.0 log CFU/g during food preparations, it may produce a sufficient amount of heat-resistant enterotoxin to cause illness, regardless of the final bacterial load (Health Protection Agency, 2009).

Our finding of genetically associated MRSA isolates ($n=3$) in the Rojak samples from the same food stall suggests these isolates may have been introduced into the food through a common source of contamination, possibly via a human source, such as a food handler. However, due to the lack of additional environmental and clinical samples, no definitive conclusion can be drawn regarding the source of contamination. Most MRSA outbreaks are nosocomial infections; MRSA is not usually considered as a foodborne pathogen. However, food-related incidents involving MRSA have been reported (Lee, 2003; Wendlandt *et al*, 2013). For instance, a foodborne MRSA outbreak was pre-

viously reported in the University Hospital of Rotterdam, Dijkzigt, The Netherlands, between November 1992 and April 1993 (Kluytmans *et al*, 1995). The likely source of the outbreak was determined to be a dietary worker who prepared food for patients and was found to carry the epidemic strain. Another study investigated a food poisoning outbreak caused by MRSA, in which samples from a food handler, food specimen and three patients were positive for the same toxin-producing strain of community-acquired MRSA (Jones *et al*, 2002). Good hygiene practices ought to be observed by food handlers to prevent transmission of MRSA via contaminated food.

To our knowledge, this is the first microbiological quality assessment study of Indian Rojak. Our data show the traditional re-heating step as practiced at local Indian Rojak stalls in Singapore can significantly lower the bacterial loads of ingredietns. *Salmonella* spp, *E. coli* O157:H7, *Vibrio cholerae* and *V. parahaemolyticus* were not detected in any of the samples tested, though 15.2% of the samples tested positive for the presence of either non-pathogenic *E. coli*, *S. aureus* or *B. cereus*. Additionally, the gravy, which was incriminated in an earlier food poisoning outbreak in Singapore, was found to be of satisfactory bacterial quality in all the Indian Rojak stalls tested. These findings suggest Indian Rojak may not pose a high microbiological risk to consumers. Yet, the detection of *S. aureus* and *B. cereus* isolates with toxin genes and the detection of MRSA, points to the need for food handlers to continuously exercise care and vigilance in hygiene practices. Continuous education of food handlers with emphasis on good hygiene practices, to prevent microbial growth and cross-contamination, will help Indian Rojak sellers in their efforts to reduce risk of bacterial contamination of RTE food.

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