Tropical Biomedicine 37(1): 103–115 (2020)

Survival of antibiotic resistant *Escherichia coli* in vacuum-packed keropok lekor: Food safety alert among SME keropok lekor producers

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Received 27 February 2019; received in revised form 8 December 2019; accepted 9 December 2019

Abstract. The microbiological quality of thirty ready-to-eat (RTE) keropok lekor (a sausage shape Malaysian fish product) was evaluated in comparison to microbiological guidelines for ready to eat foods. The two *E. coli* isolates were subjected to DNA sequencing, identified and tested for their resistance towards fifteen different antibiotics. The survival and growth of the isolated *E. coli* strains inoculated in keropok lekor at atmospheric air and vacuum packaging were also evaluated. Results revealed that four samples (13.33%) contained Enterobacteriaceae counts that exceeded the recommended allowable counts of 4.0 $\log_{10}$ CFU/g. Unsatisfactory level of coliforms (> 1.7 $\log_{10}$ CFU/g) was also observed in ten of the samples; two of which contained *E. coli* (2.1 ± 0.17 and 3.7 ± 0.02 $\log_{10}$ CFU/g), suggesting of poor hygiene and sanitation practices. While the 'Possible E10' *E. coli* strain was observably resistant towards Nalidixic acid (30µg) alone, B10 *E. coli* isolate was worryingly resistant towards Ampicillin (10µg), Ceftazidime (30µg), Ciprofloxacin (5µg), Ceftriaxone (30µg), Nalidixic acid (30µg) and Tetracycline (30µg). This study also revealed that the growth and survival of the 'Possible E10' and B10 *E. coli* strains were not significantly affected by vacuum packaging when stored at both 4°C and 28°C. Therefore, intervention programmes to alert and educate small-medium enterprisers (SMEs) of keropok lekor producers on food safety as well as potential health risks that can be associated due to inappropriate handling procedures of such product, merits consideration.

INTRODUCTION

Keropok lekor is one of the famous traditional Malaysian cuisine, widely sold at hawker stalls, night markets, canteens and restaurants, and it is considered as the local heritage and tourism icon for the state of Terengganu, Malaysia. The dough is prepared by mixing fish flesh with sago starch, ice, salt and monosodium glutamate, which then rolled into sausage-like shape and boiled, prior to serving. Keropok lekor is primarily produced by small- or medium-scale (SME) enterprises, and remains as the main economic activity for local people as well as it is known as one of tourism

attraction (Noraien, 2015). Despite the high demand throughout Malaysia, the distribution process can be challenging as keropok lekor is an easily perishable product that may become organoleptically unacceptable after keeping at room temperature for more than a day (Tang *et al.*, 2014). Recently, prevalence of microbiologically assessment of keropok lekor in Kuala Nerus (Lani *et al.*, 2017), Kuala Terengganu and Marang (Wan-Hamat *et al.*, 2019) have been reported. In order to overcome this barrier, the use of vacuum packaging (VP) has been acquiring popularity among keropok lekor producers, attributable to its efficiency during transportation. In general, utilization of VP followed by storage at a strictly controlled temperature of -1.5°C can significantly reduce the growth of aerobic bacteria; however, this approach may also unwantedly support the growth of pathogenic anaerobic and microaerophilic organisms (Balamurugan *et al.*, 2013).

Antimicrobial resistance has become a serious global public health and food safety concern. Previous researchers (Kirbis & Krizman, 2015) have reported that multidrug resistance (MDR) bacteria (e.g. *Escherichia coli*) may spread from hospitals, nursing homes and livestock animals to healthy humans, foods as well as pets animals, leading to serious infections and providing treatment can be an uphill challenge. The situation is further exacerbated by the fact that antimicrobial resistant genes can rapidly move through bacterial populations, and emerge in pathogenic bacteria via horizontal gene transfer (Wright, 2012). Being a common inhabitant of gastrointestinal tract of humans and animals (Costa *et al.*, 2009), *E. coli* is easily disseminated through the food chain and water (Ryu *et al.*, 2012). It is known to transfer its resistant genes to transient bacterial pathogens that can cause diseases in humans (Alexander *et al.*, 2010; Kirbis & Krizman, 2015). Since foods derived from animals may become potential sources of antimicrobial resistant and virulent bacteria, studies focusing on the antibiotic resistance of non-pathogenic bacteria, especially *E. coli* have acquired considerable attention (Kirbis & Krizman, 2015).

Based on previous studies (Lani *et al.*, 2017; Wan-Hamat *et al.*, 2019), it has been found that, source of contamination of keropok lekor production was due to post-boiling contamination. Keropok lekor is normally boiled at 100°C for 10 min and this process is sufficient to kill Gram-negative bacteria (Lani *et al.*, 2017). However, there is no apparent study that focused on the survival of *E. coli* in vacuum packed keropok lekor. Vacuum packaging has lack of oxygen that is normally used by SME keropok lekor for extending the shelf life of the product, however, study on the safety of vacuum-packed keropok lekor is not seemingly visible. Therefore, this study was carried out to determine the microbiological quality and antibiotic resistance as well as to evaluate the survival of *E. coli* in vacuum-packed keropok lekor.

MATERIALS AND METHODS

Sampling and general microbiological quality assessment

Thirty samples (250 g each) of ready-to-eat (RTE) keropok lekor purchased from the different production premises in Kuala Terengganu, Malaysia during December 2017 were transported separately to the laboratory in a sterile cold box that contained packaged ice cubes and immediately analysed. About 25 g of each sample placed into a sterile stomacher bag (225 mL of 0.1% peptone water, Oxoid CM0009) was homogenized in a paddled laboratory blender (BagMixer® 400, Interscience) for 1 min. Following the Silbernagel and Lindberg (2003), Silbernagel (2003) and AOAC Official Methods 991.14 (AOAC, 2002), an aliquot of the individual homogenate (1 mL) was inoculated onto a 3M™ Petrifilm™ Count Plate and incubated at 37°C (Memmert INE 400) for 24 h for Enterobacteriaceae, Staph Express as well as *E. coli* and coliform assessments, respectively. The detection of *Salmonella*, *Vibrio cholerae* and *Vibrio parahaemolyticus* was also performed for

each sample using the International Standard Protocol ISO 6579 (2002) and ISO/TS 21872-1 (2007), respectively. The *E. coli* isolates were identified *via* DNA sequencing analysis, and their survivals at atmospheric air packed (AP) and vacuum-packed (VP) keropok lekor were evaluated.

Identification of E. coli isolates and their antimicrobial resistance

Prior in evaluating antimicrobial resistance, genomic DNA of both the *E. coli* isolates from two separate samples were extracted using the QIAamp DNA Mini kit (Qiagen Inc., Valencia, CA). The purity of extracted DNA was verified by comparing the SYBR (Invitrogen, USA) stained agarose gel electrophoresis (0.8% agarose in 1x Tris-borate-EDTA buffer) visualised under Blue-light LED Transilluminator (JUNYI, JY-ERV-01) with that of 1 kilobase DNA ladder (Promega, USA). Upon completion, the purified samples were outsourced to Apical Scientific Sdn. Bhd for DNA sequencing. To identify the strains of the *E. coli* isolates, the DNA sequences were aligned and compared with those maintained by the GenBank database using the Basic Local Alignment Search Tool (BLAST) program available at the National Centre for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Both of the *E. coli* isolates were also submitted to the Public Health Laboratory, Ministry of Health Malaysia for serotyping analysis to confirm their serotypes.

The *E. coli* isolates were tested for their antimicrobial susceptibility against 15 antimicrobial agents as follows: Amikacin 30µg (AK 30), Amoxycillin/Clavulanic acid 30µg (AMC 30), Ampicillin 10µg (AMP 10), Chloramphenicol 30µg (C 30), Ceftazidime 30µg (CAZ 30), Cefoperazone 75µg (CFP 75), Ciprofloxacin 5µg (CIP 5), Gentamicin 10µg (CN 10), Ceftriaxone 30µg (CRO 30), Kanamycin 30µg (K 30), Cephalothin 30µg (KF 30), Nalidixic acid 30µg (NA 30), Streptomycin 10µg (S 10), Ampicillin/Sulbactam 20µg (SAM 20) and Tetracycline 30µg (TE 30) disks. The standard Kirby-Bauer disk diffusion method was used following the standards and interpretive criteria described by the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2012) with *E. coli* ATCC 25922 as the reference strain.

Survival and growth of E. coli isolates at atmospheric air and vacuum packed keropok lekor

The survival rate of *E. coli* was evaluated by performing inoculation in the rolled of keropok lekor dough which was bought from a production premise in Kuala Terengganu. Prior to the inoculation, the dough was boiled in sterile distilled water for 10 min and aseptically cooled to 28°C in an incubator (Memmert IPP 400). Then, the absences of aerobic plate count, coliforms and *E. coli* in the prepared dough were ascertained using the 3M™ Petrifilm™ Aerobic Count (AC) based on AOAC Official Method 990.12 (AOAC, 2002) and 3M™ Petrifilm™ *E. coli* and Coliform Count (ECC) plates, respectively as stated in (mention the part here). The prepared dough (25 g) was placed into a sterile stomacher bag containing 225 mL of sterilized peptone water (0.1%), and homogenized. Both the AC and ECC plates were then inoculated separately with 1 mL aliquot of the homogenate, and incubated at 37°C for 48 h.

Preparation of E. coli cell suspensions

In this study, the stock cultures for the *E. coli* isolates were maintained at -20°C in Tryptic Soy Broth (Oxoid CM0129) containing 20% glycerol. Both cultures were streaked onto nutrient agar (Merck 1.05450.0500) and incubated for 24 h at 37°C. Upon completion, they were inoculated into Brain Heart Infusion (BHI) broth (Oxoid CM1135) followed by further incubation for approximately 18 h at 37°C. Each culture was then harvested and washed twice by centrifugation at 3000 x g for 15 min at 4°C (Hettich Universal 32 R), and resuspended in 5 mL of sterile peptone water (0.1%).

Inoculation of E. coli

The prepared keropok lekor (25 g) sample was transferred aseptically into the commonly used low density polyethylene pouch (PETLIN™ LD, FMP 1632), and

inoculated with approximately 8 $\log_{10}$ CFU in 1 mL culture suspension of inoculant (*E. coli* isolates). The inoculated pouches were either packed under atmospheric air (AP, by sealing only) or vacuum (VP, at 1.0 Kpa, Hualian DZ-400/2E). While samples packed under atmospheric air were stored at 28 ± 1°C and analysed at 0, 1, 2, 3, 5, 8, 12, 18, 21 and 24 h, the same was done at 0, 6, 12, 18, 24, 48, 72, 96, 120 and 168 h for VP (4 ± 1°C, scientific chiller, Protech). The control samples of keropok lekor (uninoculated with bacteria) were also packed under AP and VP, in which these samples were incubated and analysed alongside the test samples.

Data analysis

The individual and interaction effects of packaging methods and durations of storage (hours) on the growth of *E. coli* strains inoculated in keropok lekor at 4°C and 28°C were analysed using the two-way ANOVA with interaction (Minitab® 14). The level of significance of 0.05 ($\alpha < 0.05$) was utilised in order to determine the significant differences among groups.

RESULTS AND DISCUSSIONS

General microbiological quality assessment

For assessing Enterobacteriaceae in the RTE keropok lekor samples, the prevailing guidelines used are as follows: guidelines for the microbiological quality of some RTE foods sampled at the point of sale (Gilbert *et al.*, 2000), guidelines for the microbiological examination of ready-to-eat foods (Food Standards Australia New Zealand, 2001) and microbiological guidelines for food, for ready-to-eat food in general and specific food items (Hong Kong Food and Environment Hygiene Department, 2014). The microbiology standard prescribed by the Malaysian Food Act 1983 (Act 281) & Regulations (Law of Malaysia, 2016) is used as guidelines for coliforms and *E. coli* assessment. Although *Staphylococcus aureus*, *Salmonella*, *Vibrio cholerae* and *Vibrio parahaemolyticus* were undetected in all the 30 RTE keropok lekor samples, Enterobacteriaceae (1.62 – 4.60 $\log_{10}$ CFU/g) and coliforms (2.12 – 4.31 $\log_{10}$ CFU/g) were observed in samples, respectively; including *E. coli* has been detected in the samples, number 2 and 16 (Table 1). Alarmingly, 4 out of 11 samples were found positive for Enterobacteriaceae (13.33%) which had exceeded the recommended allowable counts for Enterobacteriaceae (4.0 $\log_{10}$ CFU/g) (Gilbert *et al.*, 2000; Food Standards Australia New Zealand, 2001; Hong Kong Food and Environment Hygiene Department, 2014). Similarly, all 10 samples (33.33%) with detectable coliforms had surpassed the 1.7 $\log_{10}$ CFU/g recommended allowable counts prescribed by the Malaysian Food Act 1983 & Regulations (Law of Malaysia, 2016). It is pertinent to mention that, Enterobacteriaceae and coliforms are useful indicators in assessing sanitation practices, whereby their presence would be undesirable and indicative for the possible presence of unwanted enteropathogenic bacteria (Tortorello, 2003). Therefore, the detection of Enterobacteriaceae and coliform counts above the recommended allowable counts in the RTE keropok lekor may indicate the possibility of post processing contamination, probably during handling, cooling and/or displaying of the product. This is consistent with the reports by previous researchers (Cho *et al.*, 2011; Mamun *et al.*, 2013; Manguiat & Fang, 2013) indicating that the use of poor-quality raw materials, and poor handling practices (including hygiene and/or temperature and time control) may possibly cause high Enterobacteriaceae and coliform counts in RTE foods.

E. coli (3.74 ± 0.02 $\log_{10}$ CFU/g and 2.12 ± 0.17 $\log_{10}$ CFU/g, Table 1) was also found in two of the samples (No. 2 and 16) indicating that it is positive for Enterobacteriaceae and coliforms, which has exceeded the recommended allowable count of 1.7 $\log_{10}$ CFU/g based on Malaysian Food Act 1983 & Regulations (Law of Malaysia, 2016). Considering that *E. coli* is an indicator for faecal contamination (Health Protection Agency, United Kingdom, 2009; Cho *et al.*, 2011), its detection in the RTE keropok lekor that has been observed here can be a serious

Table 1. Microbiological profiles of the 30 RTE keropok lekor samples

Samples	Enterobacteriaceae (Log_{10} CFU/g)	Coliforms (Log_{10} CFU/g)	*E. coli* (Log_{10} CFU/g)	*S. aureus* (Log_{10} CFU/g)	*Salmonella* (per 25 g)	*V. cholerae* (per 25 g)	*V. parahaemolyticus* (per 25 g)
1	ND	ND	ND	ND	ND	ND	ND
2	4.20 ± 0.05	4.00 ± 0.01	3.74 ± 0.02	ND	ND	ND	ND
3	4.60 ± 0.05	4.31 ± 0.09	ND	ND	ND	ND	ND
4	4.33 ± 0.04	2.12 ± 0.07	ND	ND	ND	ND	ND
5	ND	ND	ND	ND	ND	ND	ND
6	ND	ND	ND	ND	ND	ND	ND
7	ND	ND	ND	ND	ND	ND	ND
8	ND	ND	ND	ND	ND	ND	ND
9	ND	ND	ND	ND	ND	ND	ND
10	ND	ND	ND	ND	ND	ND	ND
11	ND	ND	ND	ND	ND	ND	ND
12	1.62 ± 0.13	ND	ND	ND	ND	ND	ND
13	ND	ND	ND	ND	ND	ND	ND
14	ND	ND	ND	ND	ND	ND	ND
15	ND	ND	ND	ND	ND	ND	ND
16	2.31 ± 0.10	2.20 ± 0.15	2.12 ± 0.17	ND	ND	ND	ND
17	3.00 ± 0.11	2.49 ± 0.19	ND	ND	ND	ND	ND
18	2.54 ± 0.21	2.47 ± 0.07	ND	ND	ND	ND	ND
19	ND	ND	ND	ND	ND	ND	ND
20	ND	ND	ND	ND	ND	ND	ND
21	3.93 ± 0.03	3.71 ± 0.45	ND	ND	ND	ND	ND
22	3.82 ± 0.56	3.81 ± 0.04	ND	ND	ND	ND	ND
23	ND	ND	ND	ND	ND	ND	ND
24	ND	ND	ND	ND	ND	ND	ND
25	ND	ND	ND	ND	ND	ND	ND
26	ND	ND	ND	ND	ND	ND	ND
27	3.87 ± 0.03	3.77 ± 0.03	ND	ND	ND	ND	ND
28	4.08 ± 0.04	3.58 ± 0.35	ND	ND	ND	ND	ND
29	ND	ND	ND	ND	ND	ND	ND
30	ND	ND	ND	ND	ND	ND	ND

Data are expressed as mean ± standard deviation. Limit of quantification: 1 Log_{10} CFU/g; Limit of detection: 1 CFU/25 g; ND: Not Detected.

public health concern. In addition to poor handling practices by food handlers, Christison *et al.* (2008) indicated that cross contamination from food contact surfaces may also lead to the presence of *E. coli* in RTE foods. Since certain *E. coli* strains are capable to acquire specific virulence genes that subsequently induce their ability to cause diseases (Ahmed *et al.*, 2008), isolating *E. coli* from these two RTE keropok lekor samples (No. 2 and 16) appears pertinent to reveal its real health threat. Kuhnert *et al.* (2000) demonstrated that (1) high plasticity of the genome and the fact that (2) most of the virulence genes are encoded in mobile elements such as plasmids, phages or transposons being the two factors that allow *E. coli* to acquire and lose those virulence genes at a relatively high frequency. In addition, gene transfer can occur in numerous environments, and this may lead to the production of strains with new combinations of virulence genes (Kaper *et al.*, 2004; Kelly *et al.*, 2009a, 2009b, Hwang *et al.*, 2017).

Identification of E. coli isolates and their antimicrobial resistance

The similarity search for the DNA sequences obtained for the two *E. coli* isolates derived from samples no. 2 and 16 has revealed that the closest similarities with *E. coli* strains E10 (87% relative identity, KY780345.1) and B10 (97% relative identity, KU870318.1) maintained by the GenBank database, respectively. Considering the small amount of dissimilarity (3%) between the *E. coli* isolate from Sample No. 16 with that of KU870318.1, the fact that such isolate of *E. coli* strains B10 can be construed. On the other hand, because substantial dissimilarity percentage (13%) between DNA sequences of the *E. coli* strain isolated from sample no. 2 with the closest *E. coli* strain E10 are available in the GenBank database, further clarification appears necessary. However, for the purpose of discussion, the *E. coli* strain isolated from sample no. 2 was considered as the 'possible E10'. Results of serotyping analysis obtained from the Public Health Laboratory, Ministry of Health Malaysia revealed that both of the *E. coli* strains ('possible E10' and B10) were not of pathogenic serotypes.

Table 2 represents results on the antimicrobial susceptibility of the two *E. coli* isolates ('possible E10' and B10) tested on the different types of antibiotics. While the 'possible E10' *E. coli* strain was observably resistant towards Nalidixic acid (30µg) alone, B10 *E. coli* isolate was worryingly resistant towards Ampicillin

Table 2. Antibiotic resistance profile of 'possible E10' and B10 *E. coli* isolates

Antibiotics	*E. coli*	
	'possible E10'	B10
Amikacin 30µg (AK 30)	S	S
Amoxycillin/Clavulanic acid 30µg (AMC 30)	S	S
Ampicillin 10µg (AMP 10)	S	R
Chloramphenicol 30µg (C 30)	S	I
Ceftazidime 30µg (CAZ 30)	S	R
Cefoperazone 75µg (CFP 75)	S	S
Ciprofloxacin 5µg (CIP 5)	S	R
Gentamicin 10µg (CN 10)	S	S
Ceftriaxone 30µg (CRO 30)	S	R
Kanamycin 30µg (K 30)	S	S
Cephalothin 30µg (KF 30)	S	I
Nalidixic acid 30µg (NA 30)	R	R
Streptomycin 10µg (S 10)	S	S
Ampicillin/Sulbactam 20µg (SAM 20)	S	S
Tetracycline 30µg (TE 30)	S	R

I: Intermediate, R: Resistant, S: Susceptible.

(10µg), Ceftazidime (30µg), Ciprofloxacin (5µg), Ceftriaxone (30µg), Nalidixic acid (30µg) and Tetracycline (30µg). The B10 *E. coli* isolate was evidently resistant to more than three classes of antimicrobial agents and therefore, considered as an MDR. A MDR *E. coli* is a serious public health concern as the strain (despite being non-pathogenic) may act as a reservoir of resistant genes that could be easily exchanged among the members of the Enterobacteriaceae family (Singh *et al.*, 2005; Ahmed & Shimamoto, 2015). In this context, Nagy *et al.* (2015) reported that the various antimicrobial resistance determinants, especially those carried by mobile genetic elements (e.g. plasmids and integrons) contribute to the spread of resistance among *E. coli*. Similarly, Balis *et al.* (1996) in their *in vivo* study reported on the possible transfer of a plasmid harbouring antimicrobial resistant from the food contaminant *Salmonella enterica* serovar Enteritidis to the indigenous *E. coli* in the gastrointestinal tract of humans.

While resistance of *E. coli* isolates towards tetracycline, sulphonamides, ampicillin and streptomycin are commonly observed (Kelly *et al.*, 2009a, 2009b), commensal *E. coli* strains are typically considered as indicators of antibiotic resistance because of the genetic flexibility and ability of these organisms to adapt to constantly changing environments (Szmolka & Nagy, 2013). Nagy *et al.* (2015) found that most of *E. coli* isolated from various foods of animal origin exhibited high frequency of resistance towards ampicillin, tetracycline, streptomycin and sulphonamide compounds; lower frequency of resistance towards chloramphenicol, florfenicol and sulfamethoxazole/trimethoprim. Strains of *E. coli* are also reported to be resistant to clinically relevant, front-line antimicrobials such as fluoroquinolones and extended-spectrum β-lactams (including extended-spectrum cephalosporins) (Schroeder *et al.*, 2002). In this aspect, food remains as a vehicle of resistant bacteria and/or anti-microbial resistant genes to humans. Therefore, continuous improvement in food hygiene and sanitation practice for small - medium industrial products like the RTE keropok lekor proves imperative to combat the issues of antimicrobial resistance.

Survival and growth of E. coli isolates at atmospheric air packed (AP) and vacuum packed (VP) keropok lekor

Figures 1 (A) and (B) represent the survival and growth of 'possible E10' and B10 *E. coli* isolates at 28 ± 1°C (A) and 4 ± 1°C (B) in RTE keropok lekor samples stored under AP and VP, respectively. In addition, the individual and interaction effects from packaging methods and durations of storage (hours) on the growth of *E. coli* strains inoculated in keropok lekor at 4°C and 28°C are presented in Table 3. It was observed that the survival of the inoculated 'possible E10' *E. coli* strain had increased significantly ($P < 0.05$) from its initial count (3.34 $\log_{10}$ CFU/g) to 9.55 and 9.22 $\log_{10}$ CFU/g in AP and VP, respectively after 24 h of storage at 28°C (Figure 1A, Table 3). Upon storing the *E. coli* B10 inoculated keropok lekor at the same condition, significant increase in its survival ($P < 0.05$) from 3.25 $\log_{10}$ CFU/g to the final counts of 9.32 and 9.62 $\log_{10}$ CFU/g in AP and VP, respectively was observed (Figure 1A, Table 3). For all the cases, comparisons made between the under AP versus those of VP did not reveal any statistical differences ($P > 0.05$, Table 3) in the survival of both *E. coli* strains. However, the interaction effect between packaging methods and storage time for the 'possible E10' *E. coli* and B10 strains stored at 28°C was observably significant ($P < 0.05$, Table 3).

By taking consideration that the increase in survival rate for both the *E. coli* strains remained gradual during the first 3 hours of incubation period prior to intensifying tremendously, such period can be considered as the critical length of time for keeping the RTE keropok lekor at 28°C. The findings were consistent with the study conducted by previous researchers (Heinrich *et al.*, 2016) that *E. coli* multiplies strongly at 21°C to 37°C. These findings substantiated that RTE keropok lekor should be consumed immediately or packed within 3 hours upon boiling to ensure its safe consumption. Due to the fact that keropok lekor is made up of

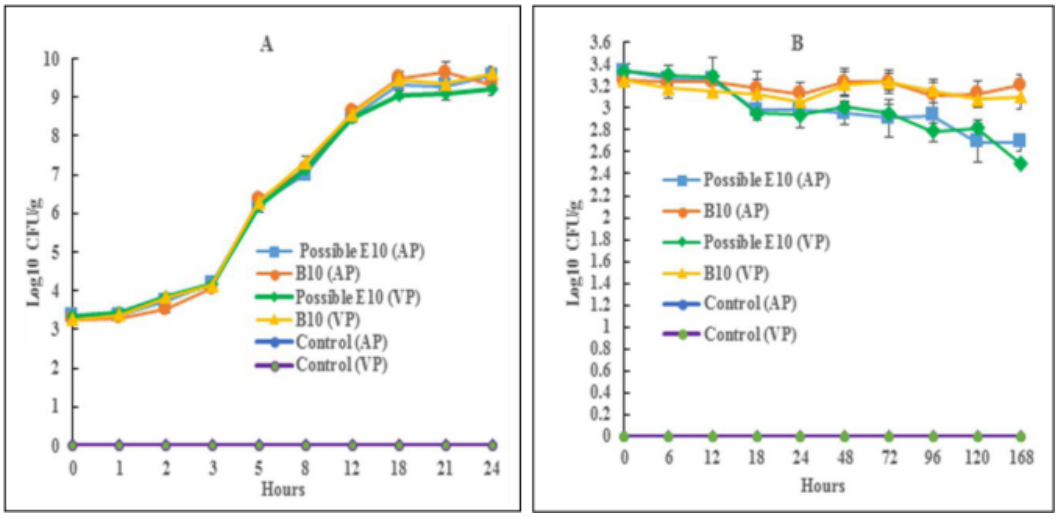

Figure 1. Survival and growth of 'possible E10' and B10 *E. coli* isolates at 28 ± 1°C (A) and 4 ± 1°C (B) in RTE keropok lekor stored under atmospheric air (AP) and vacuum packaging (VP).

Table 3. Effects of packaging methods and durations of storage (hours), as well as their interactions (method*hours) on *E. coli* strains inoculated in keropok lekor at 4°C and 28°C

E. coli strains/storage temperature	Method	Hours	Interaction
'possible E10' / 28°C	$P > 0.05$	$P < 0.05$	$P < 0.05$
'possible E10' / 4°C	$P > 0.05$	$P < 0.05$	$P > 0.05$
B10 / 28°C	$P > 0.05$	$P < 0.05$	$P < 0.05$
B10 / 4°C	$P > 0.05$	$P > 0.05$	$P > 0.05$

The individual and interaction effects of packaging methods and durations of storage (hours) on the *E. coli* growth were analysed using the two-way ANOVA. Level of significance was set at 0.05.

highly nutritious fish and starch, when combined with high pH, water as well as exposed to hot tropical temperatures, the conditions may prove suitable for spurring rapid microbial growth (Nor-Khaizura *et al.*, 2009).

It was observed that storing (at 4°C for 7 days) packed keropok lekor inoculated with the 'possible E10' *E. coli* strain in either AP or VP had significantly ($P < 0.05$) decreased its total counts from 3.34 $\log_{10}$ CFU/g to 2.69 and 2.49 $\log_{10}$ CFU/g, respectively (Figure 1B, Table 3). Being a mesophilic microorganism with the minimum growth temperature of 7.1°C (Walker & Betts, 2008), observing the decreased growth for the 'possible E10' *E. coli* strain in keropok lekor stored at 4°C was within expectation. Although *E. coli* B10 strain survived better than the 'possible E10' *E. coli* strain at 4°C in keropok lekor samples packed in both the AP and VP; the difference between the uses of both packaging methods was insignificant (Figure 1B, Table 3, $P > 0.05$). The total count only decreased marginally from 3.25 $\log_{10}$ CFU/g to 3.21 and 3.10 $\log_{10}$ CFU/g for AP and VP, respectively (Figure 1B, $P > 0.05$). Nonetheless, insignificant interaction effect between packaging methods and storage time for both *E. coli* strains in keropok lekor stored at 4°C was observed (Table 3, $P > 0.05$).

It has been well documented that *E. coli* as well as related enteric pathogens like verotoxigenic *E. coli* (VTEC) do not grow substantially at temperatures below 7°C (Visvalingam *et al.*, 2017). Moreover, Vardaka

et al. (2016) reported that *E. coli* did not even grow in turkey samples stored at 4°C. Previous researchers also indicated that the storage temperature of 4 ± 2°C may inhibit growth of *E. coli* (Leistner & Gorris, 1995; Scheutz & Strockbine, 2005). Notwithstanding, survival of *E. coli* including that of VTEC can vary greatly among strains when exposed to food processing related stresses (Li *et al.*, 2015; Liu *et al.*, 2015; Visvalingam *et al.*, 2012, Visvalingam *et al.*, 2013). Storage of VP under chilled condition (4°C) has been reported as effective for extending the shelf life of various perishable foods by limiting the availability of oxygen that is necessary for the growth of aerobic bacteria, and hence, facilitating long term storage and intercontinental transport of such food products (Pennacchia *et al.*, 2011; Gen *et al.*, 2013). Interestingly, this present study revealed that there was no significant difference in the survival and growth for both 'Possible E10' and B10 *E. coli* strains in the VP keropok lekor stored at 4°C when compared with that of 28°C (Table 3, $P > 0.05$). This was because *E. coli* is a facultative anaerobe that does not require oxygen although it can grow better in the presence of oxygen (Unden *et al.*, 1993). In the absence of oxygen, *E. coli* also has the capability to switch to fermentation or anaerobic respiration (Unden *et al.*, 1993). Such facultative anaerobe characteristic would probably be the reason of *E. coli's* survival and growth in a vacuum environment observed in this study. Yamamoto & Droffner (1985) suggested that the reduced effectiveness of VP on *E. coli* at 4°C may be due to the facultative anaerobic nature of this organism, possessing enzyme-mediated protective mechanisms to survive under stressful conditions. Having said that, the fact that the MDR *E. coli* B10 able to survive in keropok lekor stored at 4°C for both packaging methods (AP and VP), indicating that there are possible health threats on such RTE food as keropok lekor which cannot be ignored.

The acquisition of antibiotic resistance may also influence the behaviour of such microorganisms during food processing. It has been suggested that antibiotic resistant bacteria may display different growth kinetics in laboratory media as well as different resistance to stresses like acid and heat (Duffy *et al.*, 2006). Blackburn & Davies (1994) reported that antibiotic resistant strains of *E. coli* O157:H7 grew at a slower rate as compared to antibiotic sensitive strains. However, study conducted by Duffy *et al.* (2006) revealed no significant differences between the survival rates of antibiotic sensitive and resistant strains of either *E. coli* O157:H7 or O26 in yoghurt and juice. However, in this present study, it was unclear whether the *E. coli* B10 strain had naturally high capability of survival, or resultant from a phenomenon related to its MDR, as only one MDR strain was available for analysis in this study. The capability of *E. coli* B10 to survive in keropok lekor during storage in AP and VP at 4°C for 7 days may indicate its potential role as a vector for transferring antibiotic resistant genes in food, and subsequently to humans.

CONCLUSION

The assessment on the survival/growth of 'possible E10' and B10 *E. coli* strains in keropok lekor which was packed in AP or VP stored at 4°C and 28°C has been carried out. Our study has revealed that *E. coli* B10 was an MDR strain, exhibiting its resistance towards Ampicillin (10µg), Ceftazidime (30µg), Ciprofloxacin (5µg), Ceftriaxone (30µg), Nalidixic acid (30µg) and Tetracycline (30µg), as well as has the ability to survive in storage at 4°C for 7 days. Since the MDR strain of *E. coli* B10 has been found in the RTE keropok lekor with the capability to survive at AP and VP stored at chilled temperature (4°C), further investigations to understand its characteristics/ability to survive at various environmental conditions, especially during storage of RTE foods may prove to be necessary. The findings reported here also has accentuated the need for educating small-medium scale RTE producers on food safety, potential health risks that can be associated with

inappropriate handling procedure of such products as well as improvements in terms of storage and packaging conditions for this product.

Acknowledgements. This research was financially supported by the Food Safety and Quality Programme, Ministry of Health Malaysia. The authors sincerely thank the Food Safety and Quality Laboratory, Terengganu State Health Department for providing technical support.

Conflict of interest

The authors declare that they have no conflict of interests.

REFERENCES

Ahmed, N., Dobrindt, U., Hacker, J. & Hasnain, S.E. (2008). Genomic fluidity and pathogenic bacteria: applications in diagnostics, epidemiology and intervention. *Nature Review Microbiology* **6**: 387-394.

Ahmed, A.M. & Shimamoto, T. (2015). Molecular analysis of multidrug resistance in Shiga toxin-producing *Escherichia coli* O157:H7 isolated from meat and dairy products. *International Journal of Food Microbiology* **193**: 68-73.

Alexander, T.W., Inglis, G.D., Yanke, L.J., Topp, E., Read, R.R., Reuter, T. & McAllister, T.A. (2010). Farm to fork characterization of *Escherichia coli* associated with feedlot cattle with a known history of antimicrobial use. *International Journal of Food Microbiology* **137**: 40-48.

AOAC Official Methods of Analysis. (2002). Official Method 990.12 Aerobic plate count in foods, dry rehydratable film (Petrifilm™ Aerobic Count Plate) method. AOAC International Gaithersburg, MD.

AOAC Official Methods of Analysis. (2002). Official Method 991.14 Coliform and *Escherichia coli* counts in foods, dry rehydratable film (Petrifilm™ *E. coli*/ Coliform Count Plate) methods. AOAC International Gaithersburg, MD.

Balamurugan, S., Ahmed, R. & Chambers, J.R. (2013). Survival of *Arcobacter butzleri* on vacuum packaged chill stored beef. *Food Research International* **52**: 503-507.

Balis, E., Vatopoulos, A.C., Kanelopoulou, M., Mainas, E., Hatzoudis, G. & Kontogianni, V. (1996). Indications of *in vivo* transfer of an epidemic R plasmid from *Salmonella enteritidis* to *Escherichia coli* of the normal human gut flora. *Journal Clinical Microbiology* **34**: 9-977.

Blackburn, C. de W. & Davies, A.R. (1994). Development of antibiotic-resistant strains for the enumeration of food-borne pathogenic bacteria in stored food. *International Journal of Food Microbiology* **24**: 125-136.

Cho, J.-I., Cheung, C.-Y., Lee, S.-M., Ko, S.-I., Kim, K.-H., Hwang, I.-S., Kim, S.-H., Cho, S.-Y., Lim, C.-J., Lee, K.-H., Kim, K.-S. & Ha, S.-D. (2011). Assessment of microbial contamination levels of street vended foods in Korea. *Journal of Food Safety* **31**: 41-47.

Christison, C.A., Lindsay, D. & von Holy, A. (2008). Microbiological survey of ready-to-eat foods and associated preparation surfaces in retail delicatessens, Johannesburg, South Africa. *Food Control* **19**: 727-733.

CLSI (Clinical and Laboratory Standards Institute). (2012). Performance standards for antimicrobial susceptibility testing, Twenty-Second informational supplement. M100-S22. CLSI, Wayne, PA.

Costa, D., Vinue, L., Poeta, P., Coelho, A.C., Matos, M., Saenz, Y., Somalo, S., Zarazaga, M., Rodriguez, J. & Torres, C. (2009). Prevalence of extended-spectrum beta-lactamase producing *Escherichia coli* isolates in faecal samples of broilers. *Veterinary Microbiology* **138**: 339-344.

Duffy, G., Walsh, C., Blair, I.S. & McDowell, D.A. (2006). Survival of antibiotic resistant and antibiotic sensitive strains of *E. coli* O157 and *E. coli* O26 in food matrices. *International Journal of Food Microbiology* **109**: 179-186.

Food Standards Australia New Zealand (2001). Guidelines for the microbiological examination of ready-to-eat foods; Available at: (https://www.foodstandards.gov.au/code/microbiollimits/documents/Guidelines%20for%20Micro%20exam.pdf).

Gen, I.Y., Esteves, E., Aníbal, J. & Diler, A. (2013). Effects of chilled storage on quality of vacuum packed meagre fillets. *Journal of Food Engineering* **115**: 486-494.

Gilbert, R.J., de Louvois, J., Donovan, T., Little, C., Nye, K., Ribeiro, C.D., Richards, J., Roberts, D. & Bolton, F.J. (2000). Guidelines for the microbiological quality of some ready-to-eat foods sampled at the point of sale. PHLS Advisory Committee for Food and Dairy Products. *Communicable Disease and Public Health / PHLS* **3**: 163-167.

Health Protection Agency (HPA) United Kingdom. (2009). Guidelines for assessing the microbiological safety of ready-to-eat foods placed on the market, London: Health Protection Agency, November 2009.

Heinrich, V., Zunabovic, M., Nehm, L., Bergmair, J. & Kneifel, W. (2016). Influence of argon modified atmosphere packaging on the growth potential of strains of *Listeria monocytogenes* and *Escherichia coli*. *Food Control* **59**: 513-523.

Hong Kong Food & Environmental Hygiene Department. (2014). Microbiological guidelines for food (for ready-to-eat food in general and specific food items); Available at: (https://www.cfs.gov.hk/english/food_leg/files/food_leg_Microbiological_Guidelines_for_Food_e.pdf

Hwang, D., Kim, S.M. & Kim, H.J. (2017). Modelling of tetracycline resistance gene transfer by commensal *Escherichia coli* food isolates that survived in gastric fluid conditions. *International Journal of Antimicrobial Agents* **49**: 81-87.

ISO 6579. (2002). Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp. Geneva: International Organisation for Standardization.

ISO/TS 21872-1. (2007). Microbiology of food and animal feeding stuffs – Horizontal method for the detection of potentially enteropathogenic *Vibrio* spp. – Part 1: Detection of *Vibrio parahaemolyticus* and *Vibrio cholerae*. Geneva: International Organisation for Standardization.

Kaper, J.B., Nataro, J.P. & Mobley, H.L. (2004). Pathogenic *Escherichia coli*. *Nature Review Microbiology* **2**: 123-140.

Kelly, B.G., Vespermann, A. & Bolton, D.J. (2009). Gene transfer events and their occurrence in selected environments. *Food and Chemical Toxicology* **47**: 978-983.

Kelly, B.G., Vespermann, A. & Bolton, D.J. (2009). Horizontal gene transfer of virulence determinants in selected bacterial foodborne pathogens. *Food and Chemical Toxicology* **47**: 969-977.

Kirbis, A. & Krizman, M. (2015). Spread of antibiotic resistant bacteria from food of animal origin to humans and vice versa. *Procedia Food Science* **5**: 148-151.

Kuhnert, P., Boerlin, P. & Frey, J. (2000). Target genes for virulence assessment of *Escherichia coli* isolates from water, food and the environment. *FEMS Microbiological Reviews* **24**: 107-117.

Lani, M.N., Ai Peng, T., Suhaili, Z. & Hassan, Z. (2017). Microbiological evaluation on raw materials and food contact surfaces of 'keropok lekor' premises in Kuala Nerus, Terengganu and their prevalence of antibiotic resistant bacteria. *Malaysian Applied Biology* **46**(3): 71-80.

Law of Malaysia. (2016). Food Act 1983 (Act 281) and Regulations. International Law Book Services, Petaling Jaya, Malaysia.

Leistner, L. & Gorris, L.G.M. (1995). Food preservation by hurdle technology. *Trends in Food Science & Technology* **6**: 41-46.

Li, S., Kundu, D. & Holley, R.A. (2015). Use of lactic acid with electron beam irradiation for control of *Escherichia coli* O157:H7, non-O157 VTEC *E. coli* and *Salmonella* serovars on fresh and frozen beef. *Food Microbiology* **46**: 34-39.

Liu, Y., Gill, A., McMullen, L. & Gänzle, M.G. (2015). Variation in heat and pressure resistance of verotoxigenic and non-toxigenic *Escherichia coli*. *Journal of Food Protection* **78**: 111-120.

Mamun, M.A., Rahman, S.M.M. & Turin, T.C. (2013). Microbiological quality of selected street food items vended by school-based street food vendors in Dhaka, Bangladesh. *International Journal Food Microbiology* **166**(3): 413-418.

Manguiat, L.S. & Fang, T.J. (2013). Microbiological quality of chicken- and pork-based street-vended foods from Taichung, Taiwan, and Laguna, Philippines. *Food Microbiology* **36**: 57-62.

Nagy, B., Szmolka, A., Smole Mo•ina, S., Kovaè, J., Strauss, A., Schlager, S., Beutliche, J., Appele, B., Lušicky, M., Aprikiang, P., Pászti, J., Tótha, I., Kugler, R. & Wagner, M. (2015). Virulence and antimicrobial resistance determinants of verotoxigenic *Escherichia coli* (VTEC) and of multidrug-resistant *E. coli* from foods of animal origin illegally imported to the EU by flight passengers. *International Journal of Food Microbiology* **209**: 52-59.

Noraien, M. (2015). Keropok lekor Terengganu. Terengganu: Penerbit Universiti Malaysia Terengganu, pp.1-35.

Nor-Khaizura, M.A.R., Zaiton, H., Jamilah, B. & Gulam Rusul, R.A. (2009). Microbiological quality of *keropok lekor* during processing. *International Food Research Journal* **16**: 215-223.

Pennacchia, C., Ercolini, D. & Villani, F. (2011). Spoilage-related microbiota associated with chilled beef stored in air or vacuum pack. *Food Microbiology* **28**: 84-93.

Ryu, S.H., Park, S.G., Choi, S.M., Hwang, Y.O., Ham, H.J., Kim, S.U., Lee, Y.K., Kim, M.S., Park, G.Y., Kim, K.S. & Chae, Y.Z. (2012). Antimicrobial resistance and resistance genes in *Escherichia coli* strains isolated from commercial fish and seafood. *International Journal of Food Microbiology* **152**: 14-18.

Schroeder, C.M., Zhao, C.W., DebRoy, C., Torcolini, J., Zhao, S.H., White, D.G., Wagner, D.D., McDermott, P.F., Walker, R.D. & Meng, J.H. (2002). Antimicrobial resistance of *Escherichia coli* O157 isolated from humans, cattle, swine, and food. *Applied and Environmental Microbiology* **68**: 576-581.

Silbernagel, K.M. & Lindberg, K.G. (2003). 3M™ Petrifilm™ Enterobacteriaceae count plate method for enumeration of Enterobacteriaceae in selected foods: Collaborative study. *Journal of AOAC International* **86**(4): 802-814.

Silbernagel, K.M. (2003). 3M™ Petrifilm™ Staph express count plate method for the enumeration of *Staphylococcus aureus* in selected types of processed and prepared foods: Collaborative study. *Journal of AOAC International* **86**(5): 954-962.

Singh, R., Schroeder, C.M., Meng, J., White, D.G., McDermott, P.F., Wagner, D.D., Yang, H., Simjee, S., Debroy, C., Walker, R.D. & Zhao, S. (2005). Identification of antimicrobial resistance and class 1 integrons in Shiga toxin-producing *Escherichia coli* recovered from humans and food animals. *Journal of Antimicrobial Chemotherapy* **56**(1): 216-219.

Scheutz, F. & Strockbine, N. (2005). Genus *Escherichia*. In: Bergey's Manual of Systematic Bacteriology, Brenner, D.J., Krieg, N.R., Staley, J.R. & Garrity, G. (editors) 2nd edition. USA: Springer, pp.607-624.

Szmolka, A. & Nagy, B. (2013). Multidrug resistant commensal *Escherichia coli* in animals and its impact for public health. *Frontier Microbiology* **4**: 258.

Tang, J.Y.H., Nurul Hidayah, M.N., Nurhidayah, M., Yeo, C.C., Che Abdullah, A.B. & Son, R. (2014). Survival of *Vibrio cholera* O1 and *Vibrio parahaemolyticus* in fried and boiled Malaysian fish sausage. *Food Control* **41**: 102-105.

Tortorello, M.L. (2003). Indicator organisms for safety and quality – uses and methods for detection: minireview. *Journal of AOAC International* **86**(6): 1208-1217.

Unden, G., Becker, S., Bongaerts, J., Schirawski, J. & Six, S. (1993). Oxygen regulated gene expression in facultatively anaerobic bacteria. *Antonie van Leeuwenhoek Journal of Microbiology* **66**(1-3): 3-22.

Vardaka, V.D., Yehia, H.M. & Savvaidis, I.N. (2016). Effects of citrox and chitosan on the survival of *Escherichia coli* O157:H7 and *Salmonella enterica* in vacuum-packaged turkey meat. *Food Microbiology* **58**: 128-134.

Visvalingam, J., Gill, C.O. & Holley, R.A. (2012). The viabilities of cells in cultures of *Escherichia coli* growing with formation of filaments at 6°C. *International Journal Food Microbiology* **153**: 129-134.

Visvalingam, J., Gill, C.O. & Holley, R.A. (2013). Morphological and viability changes in *Escherichia coli* and *E. coli* O157:H7 cells upon rapid shift from 6°C to 37°C. *Food Microbiology* **34**: 95-99.

Visvalingam, J., Liu, Y. & Yang, X. (2017). Impact of dry chilling on the genetic diversity of *Escherichia coli* on beef carcasses and on the survival of *E. coli* and *E. coli* O157. *International Journal of Food Microbiology* **244**: 62-66.

Walker, S.J. & Betts, G. (2008). Chilled foods microbiology. In: Chilled Foods: A comprehensive guide, Brown, M. (editor) 3rd edition. Cambridge: Woodhead Publishing, pp.445-476.

Wan-Hamat, H., Lani, M.N., Hamzah, Y., Alias, R. & Hassan, Z. (2019). Microbiological assessment of keropok lekor production in Kuala Terengganu and Marang, Malaysia. *Asian Journal of Agriculture and Biology* **7**(1): 74-85.

Wright, G.D. (2012). The origins of antibiotic resistance. *Handbook of Experimental Pharmacology* **211**: 13-30.

Yamamoto, N. & Droffner, M.L. (1985). Mechanisms of determining aerobic or anaerobic growth in the facultative anaerobe *Salmonella* Typhimurium. *Proceedings National Academy Sciences* **82**: 2077-2081.