ORIGINAL ARTICLE

Identification and Salt Survival of Multiple Antibiotic-Resistant Foodborne Bacteria Isolated from Market-Fresh Raw Chicken Breasts in Terengganu, Malaysia

Pui Xin Yap[1], Mohd Nizam Lania[1,2], Elham Taghavi[1], Zarizal Suhaili[3], Effarizah Mohd Esah[4], Suguna Migeemanathan[5], W. Widodo[6]

[1] Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu (UMT), 21030 Kuala Nerus, Terengganu, Malaysia
[2] Institute of Marine Biotechnology, Universiti Malaysia Terengganu (UMT), 21030 Kuala Nerus, Terengganu, Malaysia
[3] Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin, Besut Campus, 22000 Besut, Terengganu, Malaysia
[4] Food Technology Division, School of Industrial Technology, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia
[5] School of Hospitality, Tourism and Culinary Arts, UOW Malaysia, KDU Penang University College, Georgetown Campus, 32, Jalan Anson 10400 Penang, Malaysia
[6] Graduate School of Biotechnology, Universitas Gadjah Mada, Bulaksumur, 55281 Yogyakarta, Indonesia

ABSTRACT

Introduction: Chicken is a source of protein that becomes a reservoir for pathogens under poor hygienic environments. Uncontrolled use of antibiotics in the poultry industry may result in the development of multidrug-resistant (MDR) bacteria. This study aimed to assess the microbiological quality of raw chicken breasts sold in Terengganu, Malaysia, as well as the antibiotic profile of isolated pathogenic bacteria and their survival in high salt concentrations. **Methods:** Isolation of foodborne pathogens was performed via selective media according to the Food and Drug Administration's Bacteriological Analytical Manual. Phenotypic identification was carried out using the Analytical Profile Index (API) 20E Test Kit, followed by an antimicrobial susceptibility test using Kirby–Bauer methods. The growth of MDR bacteria was determined using optical density and was compared to that of the antibiotic susceptible isolates after 24 h incubation in high salt concentrations. **Results:** *C. youngae, E. aerogenes, E. coli, K. oxytoca* and *Salmonella* spp. were identified from the samples. All samples showed unacceptable microbial count limit. Out of eight bacteria isolates, six were MDR (75%) and three expressed resistance to all six antibiotics tested (37%). The bacteria isolates had a multiple antibiotic resistance (MAR) index of 0.33–1.0. *C. youngae, E. coli, K. oxytoca* and *Salmonella* spp. exhibited enhanced survival at 6% or 8% salt concentration. **Conclusion:** This study revealed the poor microbiological quality of raw chicken breasts due to contamination of MAR foodborne pathogens that developed cross-protection under high salt concentrations, thus indicating food safety risks and challenges.

Keywords: Microbiological quality, Antibiotic susceptibility, Raw chicken breast, Foodborne bacteria, Salt

Corresponding Author:
Mohd Nizam Lani, PhD
Email: nizamlani@umt.edu.my; nizamlani@gmail.com
Tel: +609-6684967/ +60124988040

INTRODUCTION

According to the Department of Statistics Malaysia (2018), consumption of chicken in Malaysia increased from the year of 2013 to 2017, from 46.0 to 52.0 kg, respectively, per year per capita consumption (1). The wide consumption of chicken is due to shifting dietary habits from traditional food staples towards livestock protein, since chicken is the cheapest protein-rich meat and is deemed acceptable by all races and beliefs (2).

Nevertheless, at fresh food markets, chickens are under constant exposure to the open environment and are thus exposed to insect vectors and other microorganisms (3). Raw chicken sold in the marketplace is often subjected to temperature abuse. Hence, the microbiological quality of chicken breast sold at various fresh food markets are of interest, as many studies have shown that chicken in Malaysia is a highly contaminated foodborne pathogen that can cause foodborne diseases (4).

Foodborne diseases are a prevalent cause of morbidity and mortality, which may obstruct socio-economic development worldwide. In Terengganu, where the research was conducted, food poisoning cases had increased from 592 cases in 2013 to 999 cases in 2014 (5). Our past studies revealed the prevalence of multiple antibiotic resistance in fresh 'ulam' (6) and keropok lekor (7, 8). According to the World Health Organisation

(WHO), enteropathogenic *Escherichia coli* and non-typhoidal *Salmonella* are among the main causes of diarrhoeal diseases worldwide (9). Foodborne disease cases are common in Malaysia, because Malaysia has a humid tropical climate that is suitable for the growth of foodborne pathogens.

Antibiotics are used to treat infection caused by bacteria. In 2017, 73% of the antibiotics used in livestock were administered to prevent disease or promote growth of livestock (10). Antibiotics such as chloramphenicol, erythromycin, penicillin and tetracycline have been used by local poultry farms to treat and prevent diseases caused by bacterial infection (11). However, the routine use of antibiotics in agriculture has raised the issue of drug-resistant bacterial pathogens in animals, which may transmit the antibiotic-resistant gene to another bacterium.

To produce food which is microbiologically safe and shelf-stable, food manufacturers often employ hurdle technology for food preservation during food processing to prolong the lag phase of bacteria and prevent bacterial growth. Salt is one of the most important preservation methods in food manufacturing, as it causes environmental stress to the foodborne pathogens.

Since chicken consumption is high in Malaysia, the product safety of chicken is a major concern. Studies that have reported the microbiological quality of chicken breast sold at fresh food markets in Malaysia are deemed insufficient for some parts of Terengganu. Furthermore, there is insufficient study on the antibiotic resistance of bacteria isolated from raw chicken breast in Malaysia. In addition, the ability of multiple antibiotic-resistant bacteria to tolerate environmental stress such as a high salt concentration is not fully understood. Therefore, the objectives of this study were to determine the microbiological quality of raw chicken breast sold at different fresh food markets in Kuala Nerus, Malaysia, as well as their antimicrobial profile and the ability of multiple antibiotic-resistant (MAR) bacteria to tolerate a high salt concentration.

MATERIALS AND METHODS

Sample collection

A total of nine samples of raw chicken breasts were purchased from three different fresh food markets around Kuala Nerus District, Terengganu, Malaysia, in three visits during the month of June 2018 to September 2018. One chicken sample was collected from each market per visit. The samples were kept in sterile polystyrene bags then stored in a sampling box and maintained at 4°C with ice pads during immediate transfer to the laboratory. The samples were analysed within 2 hours upon arrival at Food Microbiology Laboratory, Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu.

Isolation and identification of food pathogenic bacteria from raw chicken breast

The methods of isolation for foodborne pathogens were performed based on the Food and Drug Administration's Bacteriological Analytical Manual (BAM) method (2015) (12). Briefly, approximately 25 g chicken breast was weighed and added to 225 ml of 0.1% sterile buffered peptone water (Oxoid, UK) as a diluent. Those samples were fully homogenised using a stomacher (Interscience, France) for 90 seconds to obtain 10^{-1} dilution, which was then followed by a series of dilutions up to 10^{-3}. Then, 0.1 mL of aliquot from each dilution was spread onto four selective media: Bismuth-Sulphite Agar (BSA), Hektoen Enteric Agar (HEA), Xylose Lysine Deoxycholate (XLD) Agar and MacConkey agar. BSA, HEA and XLD media were used for *Salmonella* detection and MacConkey agar for Enterobacteriaceae. The spread plating for each dilution was triplicated. All plates were then inverted and incubated at 37 ± 2°C for 18 hours. All microbiological media were obtained from Merck, Germany, except for HEA, which was obtained from Oxoid, UK. In this study, enrichment was omitted because the chicken samples were found to be heavily contaminated, and direct detection was possible (14). Typical colonies of *E. coli* and *Salmonella* on the selective media were enumerated and selected for inoculation onto Tryptone Soy Agar (TSA) for biochemical tests according to Food and Drug Administration's BAM, including Gram staining, catalase, oxidase, triple sugar iron (TSI) agar, lysine iron agar (LIA) and oxidative fermentative of glucose and lactose. The presumptive positive colonies from the biochemical tests were subjected to phenotypic identification using the Analytical Profile Index (API 20E Test Kit) (Biomerieux, France) for confirmation. Briefly, a single colony of presumptive cultures grown on Nutrient Agar for 24 h was added in API NaCl 0.85% medium. Using a Pasteur pipette, filled both tube and cupule of tests for citrate, VP and gel with bacterial suspension. Then, filled only the tube but not cupule of other tests. Then, anaerobiosis was created for the following tests: L-arginine (ADH), L-lysine (LDC), L-ornithine (ODC), sodium thiosulfate (H2S) and urea (URE) by overlaying with mineral oil. Then, the incubation box was closed and incubated at 37°C for 18 to 24 h (14). The API was recorded and read through the database in the API web™ (Biomerieux, France), and the percentages of similarity among identified organisms were compared with records in the database; then those results were recorded.

Antimicrobial susceptibility test

An antibiotic resistance profile test for the isolates was performed using the Kirby–Bauer method on a Mueller–Hilton agar (MHA, Oxoid, UK) according to the Clinical and Laboratory Standards Institute Guidelines (15). Six antibacterial agents, including chloramphenicol (30 µg), erythromycin (15 µg), penicillin (10 µg), gentamicin (10 µg), trimethoprim-sulfamethoxazole (25 µg) and tetracycline (30 µg) were obtained from Oxoid, UK.

A twenty-four hour culture was added to sterile saline until the turbidity achieved 0.5 McFarland standard. Then, the bacterial suspension was streaked onto MHA using a sterile cotton swab. After air-drying, antibiotic discs were dispensed evenly onto agar plates. The plates were inverted and incubated aerobically at 35 ± 2°C for 16–18 h. Then, the zones of inhibition that formed around the antibiotic discs were measured, recorded and interpreted based on the 2017 Clinical & Labs Standards Institute Guidelines (16).

Exposure of bacteria to different salt concentrations

This experiment was conducted according to Abbas et al. (2014) (17). Media modification was made by the addition of sodium chloride in different concentration to Tryptone Soy Broth (TBS, Merck, Germany) as follows: TSB + 2%, 4%, 6%, 8% NaCl, respectively. A fresh bacterial culture with the optical density (OD600) of 0.5 was added into each broth media. Then, all inoculated broth media were incubated at 37 ± 2°C for 24 h, where the OD (OD_{600} = 0.5) was measured using a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis

All analyses were performed in triplicate. A T-test was performed using IBM SPSS Statistics Base Version 22 to analyse the significant differences in optical density between 2 groups (control and multiple antibiotic-resistant bacteria) at $p < 0.05$. The data obtained were presented as the mean ± standard deviation.

RESULTS

Isolation and enumeration of foodborne pathogenic bacteria in chicken breast

In this study, out of 41 presumptive cultures grown on all selective media (data not shown), only eight isolates including two isolates of *Escherichia coli*, two isolates of *Citrobacter youngae*, two isolates of *Salmonella* spp., one isolate of *Enterobacter aerogenes* and one isolate of *Klebsiella oxytoca*, were isolated from chicken breast samples collected from three premises in Kuala Nerus, Terengganu, Malaysia. Table I shows the mean count of each bacterial isolate that was isolated from different sampling locations, which was expressed in terms of $\log_{10}$ colony-forming units (CFU)/g. The mean count of all bacterial strains isolated from raw chicken breast exceeded the acceptable range (Table I) for microbial count as set by the Food and Agriculture Organisation (FAO) guidelines, which state that the count should be less than 2 $\log_{10}$ CFU/g (18).

Table I: Mean count of bacteria ($\log_{10}$ CFU/g) isolated from raw chicken breasts and the identification of organisms using API 20E as well as occurrence rate

Sample location	Mean count ($\log_{10}$ CFU/g)	Identified organism by API 20E	% Similarity in APIWeb database	Occurrence rate
Premise A*	3.39 ± 0.27	*E. coli*	99.5% (Excellent identification)	2/8 (25%)
Premise A	2.24 ± 0.34	*C. youngae*	97.5% (Excellent identification)	2/8 (25%)
Premise B	4.30 ± 0.00	*Salmonella* spp.	97.5% (Excellent identification)	2/8 (25%)
Premise B	3.61 ± 0.54	*C. youngae*	99.7% (Excellent identification)	2/8 (25%)
Premise B	4.21 ± 0.05	*E. aerogenes*	97.1% (Excellent identification)	1/8 (12.5%)
Premise C	3.80 ± 0.33	*E. coli*	99.5% (Excellent identification)	2/8 (25%)
Premise C	3.62 ± 0.18	*K. oxytoca*	97.2% (Excellent identification)	1/8 (12.5%)
Premise C	1.85 ± 0.21	*Salmonella* spp.	99.7% (Excellent identification)	2/8 (25%)

* For Premise A, only 2 out of 3 samples were acceptable results for API 20E.

Antibiotic susceptibility profile of identified foodborne pathogens isolated from raw chicken breasts

An antibiotic susceptibility test was carried out to determine the susceptibility of identified *C. youngae, E. aerogenes, E. coli, K. oxytoca* and *Salmonella* spp. towards chloramphenicol, erythromycin, penicillin, gentamycin, trimethoprim-sulfamethoxazole and tetracycline. Antibiotic-resistant and multidrug-resistant profiles of bacteria are presented in Table II.

The MAR index, which is the ratio of the number of antibiotics in which bacteria were resistant to the total number of antibiotics tested, was calculated and is presented in Table II. All identified bacteria have a MAR index ranging from 0.33 to 1.0. The reported average MAR index was 0.37. The result of the current

Table II. Antibiotic-resistant and multiple antibiotic-resistant (MAR) profile of bacteria isolated from raw chicken breast

Sampling location	Type of bacteria	Average inhibition zone (mm) and interpretation						No of antibiotic-resistant	No of antibiotic class resistant	MAR index
		Chloramphenicol (30 µg)	Erythromycin (15 µg)	Gentamicin (10 µg)	Penicillin (10 µg)	Trimethoprim-sulfamethoxazole (25 µg)	Tetracycline (30 µg)			
Premise A	*C. youngae*	22.0 (S)	7.0 (R)	17.5 (S)	7.0 (R)	7.0 (R)	8.5 (R)	4	3	0.67
Premise A	*E. coli*	7.0 (R)	7.5 (R)	8.0 (R)	7.0 (R)	7.0 (R)	7.0 (R)	6	3	1.00
Premise B	*C. youngae*	26.5 (S)	9.0 (R)	18.0 (S)	10.0 (R)	7.0 (R)	10.0 (R)	4	3	0.67
Premise B	*E. aerogenes*	7.0 (R)	8.5 (R)	15.00 (R)	7.0 (R)	7.0 (R)	8.5 (R)	6	3	1.00
Premise B	*Salmonella* spp.	26.0 (S)	7.0 (R)	17.0 (S)	7.0 (R)	19.5 (S)	13.5 (I)	2	1	0.33
Premise C	*E. coli*	7.0 (R)	7.0 (R)	9.0 (R)	7.0 (R)	7.0 (R)	7.0 (R)	6	3	1.00
Premise C	*K. oxytoca*	7.0 (R)	7.0 (R)	13.5 (I)	7.0 (R)	7.0 (R)	7.0 (R)	5	3	0.83
Premise C	*Salmonella* spp.	20.5 (S)	7.0 (R)	16.0 (S)	7.5 (R)	7.0 (R)	15 (S)	3	2	0.50

(R): Resistant (Inhibition zone (mm): Chloramphenicol: ≤ 12, erythromycin: ≤ 13, gentamicin: ≤ 12, penicillin: ≤11, tetracycline: ≤11, trimethoprim-sulfamethoxazole: ≤10)
(S): Susceptible (Inhibition zone (mm): Chloramphenicol: ≥ 18, erythromycin: ≥ 18, gentamicin: ≥ 15, penicillin: ≥22, tetracycline: ≥15, trimethoprim-sulfamethoxazole: ≥16)
(I): Intermediate (Inhibition zone (mm): Chloramphenicol: 13–17, erythromycin: 14–17, gentamicin: 13–14, penicillin: 12–21, tetracycline: 12–14, trimethoprim-sulfamethoxazole: 11–15)

study reported that *C. youngae, E. aerogenes, E. coli, K. oxytoca* and *Salmonella* spp. were resistant to multiple antibiotics. The spread of these multiple antibiotic-resistant bacteria will eventually cause the development of multi-resistant serotypes in bacteria in different parts of the world.

Survival of identified bacteria in high salt concentrations

Bacteria with high MAR index were cultured in Tryptone soy broth with 2%, 4%, 6% and 8% of NaCl. Then, the optical density at OD_{600} of 0.5 for each multiple antibiotic-resistant bacterium was compared with the antibiotic susceptible isolate which acts as a control to determine the presence of enhanced survival of bacteria under high salt concentration. Tables III to Table VII present the results for each type of bacteria.

Multiple antibiotic-resistant *K. oxytoca* with a MAR index of 0.83 exhibited significantly enhanced survival ($p < 0.05$) as compared to the control at a salt concentration ranging from 2% to 8%. Meanwhile, *E. coli* isolated from Premises A and C with a MAR index of 1.0 showed significantly enhanced survival ($p < 0.05$) at 8% salt concentration. In addition, *C. youngae* with a MAR index of 0.67 also showed significantly enhanced survival ($p < 0.05$) at 6%, whilst *Salmonella* spp. with a MAR index of 0.33 showed significantly enhanced survival ($p < 0.05$) at 4% and 8% of salt.

DISCUSSION

This study highlights the prevalence of foodborne bacteria in raw chicken breast sold in fresh markets in Terengganu, Malaysia. In this study, the occurrence rate of *C. youngae* was 25% (Table I). As natural microflora in human intestine, the presence of *C. youngae* indicates poor hygiene practiced during the handling of chicken breast at fresh food markets. A similar study was conducted by Tassew et al. (2010) who had collected carcasses (i.e., minced meat) swab samples of lean meat from butcher shops and slaughterhouses in Ethiopia (19). However, researchers reported that the contamination rate of *Citrobacter* spp. was 9% (20), which was significantly lower compared to the current study. As theorised by Cunha-Neto et al. (2018), high variation in contamination rates may be due to differences in chicken slaughtering procedures among various countries (20). In addition, data from the current study was supported by a meta-analysis conducted by Saba and Gonzalez (2012), who reported a high prevalence rate (50%) of *Citrobacter* spp. in Ghanaian food (21). It had been reported that foodborne disease is a major health hazard in countries that have insufficient food surveillance systems and ineffective enforcement of legislation and regulation (22).

In this study, the occurrence of *E. aerogenes* in raw chicken breast was 12.5% (Table I). However, the occurrence of *Enterobacter* spp. was lower as compared to meta-analysis data reported by Saba and Gonzalez (2012), who showed a 65% prevalence rate of *Enterobacter* spp. in the food samples that were analysed (21). Then combining all the data, *Enterobacter* spp. were found in seventeen (65%) of the twenty-six food samples (21). The most contaminated foods in their study were macaroni, salad and milk. The lower occurrence rate of *E. aerogenes* reported in this study indicates that raw chicken breast is not the main reservoir for *E. aerogenes*. This is supported by the fact that out of 11 analysed meta-analyses conducted by Saba and Gonzalez (2012),

Table IV: The optical density (600 nm) of the isolated *E. aerogenes* and control at different salt concentrations after incubation for 24 h

Source	Type of bacteria	2% salt (Mean OD)	4% salt (Mean OD)	6% salt (Mean OD)	8% salt (Mean OD)
Control	*E. aerogenes*	1.351 ± 0.0099[a]	1.077 ± 0.0184[a]	0.8945 ± 0.0134[a]	0.5925 ± 0.0247[a]
Premise B	*E. aerogenes*	1.0730 ± 0.0141[b]	0.9315 ±0.0219[b]	0.7910 ± 0.0099[b]	0.4685 ± 0.0205[b]

The data obtained were mean ± standard deviation (n = 3). The superscript of optical density of different bacteria at different salt concentrations in the same column with different letter was significantly different at $P < 0.05$.

Table V: The optical density (600 nm) of the isolated *E. coli* and control at different salt concentrations after incubation for 24 h

Source	Type of bacteria	2% salt (Mean OD)	4% salt (Mean OD)	6% salt (Mean OD)	8% salt (Mean OD)
Control	*E. coli*	0.9920 ± 0.0113[a]	0.8590 ± 0.0057[a]	0.631 ± 0.0014[a]	0.2660 ± 0.0071[a]
Premise A	*E. coli*	1.299 ± 0.0601[a]	1.1385 ± 0.0290[b]	0.6815 ± 0.0177[a]	0.4830 ± 0.0028[b]
Premise C	*E. coli*	0.9325 ± 0.0134[b]	0.8250 ± 0.0014[a]	0.6525 ± 0.0163[a]	0.5075 ± 0.0035[b]

The data obtained were mean ± standard deviation (n = 3). The superscript of optical density of different bacteria at different salt concentrations in the same column with different letter was significantly different at $P < 0.05$.

Table VI: The optical density (600 nm) of the isolated *K. oxytoca* and control at different salt concentrations after incubation for 24 h

Source	Type of bacteria	2% salt (Mean OD)	4% salt (Mean OD)	6% salt (Mean OD)	8% salt (Mean OD)
Control	*K. pneumonia*	0.4100 ± 0.0014[a]	0.3405 ± 0.0021[a]	0.2390 ± 0.0099[a]	0.1335 ± 0.0064[a]
Premise C	*K. oxytoca*	1.0625 ± 0.0304[b]	04435 ± 0.0049[b]	0.3270 ± 0.0014[b]	0.1825 ± 0.0064[b]

The data obtained were mean ± standard deviation (n = 3). The superscript of optical density of different bacteria at different salt concentrations in the same column with different letter was significantly different at $P < 0.05$.

Table VII: The optical density (600 nm) of the isolated *Salmonella* spp and control at different salt concentrations after incubation for 24 h

Source	Type of bacteria	2% salt (Mean OD)	4% salt (Mean OD)	6% salt (Mean OD)	8% salt (Mean OD)
Control	*K. pneumonia*	0.4100 ± 0.0014[a]	0.3405 ± 0.0021[a]	0.2390 ± 0.0099[a]	0.1335 ± 0.0064[a]
Premise C	*K. oxytoca*	1.0625 ± 0.0304[b]	04435 ± 0.0049[b]	0.3270 ± 0.0014[b]	0.1825 ± 0.0064[b]

The data obtained were mean ± standard deviation (n = 3). The superscript of optical density of different bacteria at different salt concentrations in the same column with different letter was significantly different at $P < 0.05$.

no raw chicken was found to be contaminated with *E. aerogenes* (21). The occurrence of *E. aerogenes* in raw chicken breast in the present study indicates possible faecal contamination of food due to poor hygiene of the workers or cross-contamination from the environment.

This study identified the occurrence rate of *E. coli* in retail chicken samples to be 25% (Table I). By comparison, the occurrence of *E. coli* in raw chicken meat in Egypt was 12%, 85.71% in Bangalore and 53.8% in Selangor, Malaysia (23, 24). The data reported in this finding varied in comparison with the findings reported by previous studies. The prevalence rate of *E. coli* is expected to be higher under the conditions of a hot and humid climate, since *E. coli* is mesophilic, with optimum growth at 37°C (25). Also, the prevalence rate can vary among different locations within a country, which depends in part upon the enforcement of food safety regulations by local authorities (25).

The current study also found that the contamination rate of *K. oxytoca* in raw chicken breast was 12.5% as mentioned in Table I, which supported a previous study by Fielding et al. (2012), who isolated *K. oxytoca* from chicken neck skin (26). However, data from the current study was significantly higher as compared to the study by Al-Mutairi (2011), which revealed that 10.66% of meat products sold in supermarkets and shops in Saudi Arabia, including sausage, kofta and shawarma, were contaminated with *Klebsiella* spp. (27). Lower prevalence of *Klebsiella* spp. in food reported by previous studies can be explained by the fact that *Klebsiella* spp. are mesophilic bacteria that are sensitive to temperature. Thus, cooked meat should have a lower contamination level of bacteria in comparison to raw meat such as chicken in the current study.

The occurrence rate (25%) of *Salmonella* spp. in raw chicken breast observed in this study was lower with that observed in a study conducted elsewhere in Malaysia. Shafini et al. (2017) documented that the occurrence rate of *Salmonella* spp. in raw chicken purchased from supermarkets, butcher shops and wet markets in Selangor, Malaysia, was higher as 72.2% compared to 25% in this study (4). As described by the researchers, raw chicken in Malaysia, always displayed without ice or merely with some ice flakes, was exposed to the ambient temperature of an open space when it was being sold. This increases the possibility of contamination from rodents and insect vectors, thus causing a high prevalence of bacteria in raw chicken. In Malaysia, chickens sold at retail outlets are mostly obtained from traditional slaughterhouses, which may have basic or unsatisfactory slaughtering and post-slaughtering conditions, limited water supply and use of recycled water (4). In addition, chicken carcasses are usually transported using unhygienic containers with insufficient low temperature. These common practices of handling chicken meat with a lack of awareness of food safety contribute to a high prevalence of *Salmonella* in raw chicken meat sold in Malaysia.

As set by the Hazard Analysis and Critical Control Point system, which was developed by the FAO and adopted by the Codex Alimentarius Commission, the acceptable food safety of pathogens range is 2 $\log_{10}$ CFU/g or less (18). The Table I shows that all retail chicken samples (100%) in this study were not within the acceptable range for *C. youngae*, *E. aerogenes*, *E. coli*, *K. oxytoca* and *Salmonella* spp. counts. This finding is in accordance with research by Odwar et al. (2014) who found that 60% of the retail chicken samples in Kenya were not within the acceptable range for *E. coli* counts, whereas 76% of the samples fell under the unacceptable range for total coliforms (28). Similarly, high percentages of retail chicken that fell under the range for acceptable food safety have been observed in studies in Vietnam (29). Besides, this observation could be attributed to inadequate compliance with food safety requirements for slaughterhouses, which increases the risk of contamination due to bio-security flaws. Moreover, the common practice of transporting a carcass in one large container or sack at ambient temperature also increases the risk of cross-contamination by enabling a transfer of bacteria between carcasses and subsequent microbial multiplication due to inadequate temperature settings (28).

A prior study noted that chicken meat with a level of *Salmonella* spp. contamination higher than 1 CFU/g can contribute to the increased risk of salmonellosis (30). In this study, the mean counts of *Salmonella* spp. from Premise B and Premise C were 4.30 ± 0 $\log_{10}$ CFU/g and 1.85 ± 0.21 $\log_{10}$ CFU/g, respectively. Even though *Salmonella* is heat sensitive, inadequate cooking may fail to lower the initial contamination level under the infectious dose. In most cases, the infection dose to cause an outbreak in healthy people is between 10^1 and 10^3 CFU/g (31). Salmonellosis can be fatal if the infection spreads from the intestines to the blood circulatory system (32).

Antibiotic resistance occurs when bacteria develop a mechanism of defence to survive and grow in the presence of antibiotics that generally inhibit or kill bacteria of the same species. Out of eight isolates as mentioned in Table II, six (75%) were resistant to at least three antibiotic classes. Resistance to more than one class of antibiotic is defined as multidrug-resistant bacteria. The multidrug-resistance occurs due to poor control of the use of antibiotics in the veterinary industry (33). Gentamicin, penicillin and trimethoprim-sulfamethoxazole were chosen in this study because they are commonly used in the treatment of a variety of bacterial infections for livestock (34). Moreover, chloramphenicol, erythromycin and tetracycline were chosen as they are commonly being used to treat various infections in human (35).

From Table II, *C. youngae* isolated from Premises A and B were resistant to erythromycin, penicillin, trimethoprim-sulfamethoxazole and tetracycline, but were susceptible to chloramphenicol and gentamicin. This result proposed that chloramphenicol and gentamicin have been used extensively in human medicine yet are of low prevalence of application in the poultry industry. Chloramphenicol is a broad-spectrum antibiotic approved for use in humans and is the first-line antibiotic used to treat typhoid fever, which makes it a commonly used medication in humans (36). Furthermore, various applications of chloramphenicol have been banned for human use in Malaysia since 1988 (37). Chloramphenicol has also been linked with dose-independent aplastic anaemia, which has a high mortality rate (38). It can pose risks for workers who handle the product whilst the antibiotic residues in food products could have threatened the life of the consumer.

Also, Table II shows that *E. aerogenes* was resistant to all of the antibiotics that were tested in this study. Multiple antibiotics resistance may develop due to the fact that large amounts of multiple antibiotics were used in the poultry environment (33). However, since *E. aerogenes* is not a major foodborne pathogen of concern, there is limited literature on the prevalence of antibiotic susceptibility of *E. aerogenes*. The findings of the current study were different compared to previous research on the antibiotic susceptibility of clinical isolates. Al-Tawfiq et al. (2009) reported that *Enterobacter* spp. isolated from a patient with an infection had low frequency of resistance towards gentamicin (1.6%–11.2%) and trimethoprim-sulfamethoxazole (5.5%–13.1%) (39).

E. coli isolated from Premises A and C were resistant to all six antibiotics. A similar finding was reported for previous studies in Malaysia, which found that *E. coli* isolated from raw chicken were resistant to erythromycin, penicillin, gentamicin, tetracycline and sulfamethoxazole (40). This observation indicates that a substantial amount of antibiotics used for human therapy has been administered to farm animals, thus leading to the development of MDR pathogenic bacteria (33). This finding also indicated that *E. coli* is resistant to gentamicin, penicillin and erythromycin. These antibiotics are listed as critically important antimicrobial drugs in human medicine by the WHO, whilst chloramphenicol, sulfamethoxazole and tetracycline are listed as highly important antimicrobial drugs (41). The ranking developed by the WHO was intended to support farm owners in risk management efforts for drugs used in food animals so that drugs that are medically important for humans remain effective in the treatment of bacterial infection (25). Infection caused by multiple antibiotic resistance was more difficult to treat and thus associated with a higher infection-related cost, longer duration of hospital stays and higher mortality rate.

Furthermore, *K. oxytoca* identified in this study was found to be resistant to all of the antibiotics tested except gentamicin. This finding was consistent with that of Zhang et al. (2018) (42), who demonstrated that retail food samples obtained from markets in China were resistant to gentamicin, chloramphenicol, trimethoprim-sulfamethoxazole and tetracycline. This finding proposes that massive use of multiple antibiotics has applied selective pressure on food pathogens, thus causing them to develop into multiple antibiotic-resistant bacteria (42).

Salmonella spp. isolated from raw chicken breast were resistant to erythromycin and penicillin. However, this finding contradicts previous studies that demonstrated that *Salmonella* spp. isolated from raw chicken breast was highly resistant to chloramphenicol, tetracycline and trimethoprim-sulfamethoxazole (43). This discrepancy indicates that different types of antibiotics were used by different poultry farms. In addition, Bilge et al. (2018) demonstrated that the antibiotic profile of bacteria varies widely within and between countries and over time (25). The MAR index, which was more than 0.2, confirmed that there was high antibiotics use and high selective pressure in the poultry environment from which the broiler was reared (44). Bacterial isolates from raw chicken with a high MAR index were documented by previous studies (25). Bilge et al. isolated *Salmonella* spp. from raw chicken wings in Turkey and found that all of the isolates were resistant to multiple antibiotics (25). Based on our finding, *C. youngae*, *E. aerogenes*, *E. coli*, *K. oxytoca* and *Salmonella* spp. were resistant to multiple antibiotics. Therefore, researchers urged local authorities to implement immediate action to ensure continuous effectiveness of the antimicrobial agents in treating bacterial infections in humans. The spread of these multiple antibiotic-resistant bacteria will eventually cause the development of multi-resistant serotypes in bacteria in different parts of the world.

After determination of multiple antibiotic-resistant in all isolates studied, the cultures were subjected to different salt concentrations as shown in Table III to VII. The results of this study explain that enhanced survival occurred under high environmental stress, which is the salt content that bacteria usually encounter in food processing. The findings were in consistent with Komora et al. (2017) who reported that multiple antibiotic-resistant *Listeria monocytogenes* were less susceptible to a high salt concentration (37%) (45). Similarly, this observation is consistent with Akhtar et al. (2016) who reported that the resistant strains showed higher resistance to sodium hypochlorite treatment (46). Pagedar et al. (2012) also demonstrated that the resistant strain underwent better adaption to environmental stress, since the resistant strain was found to have a stronger biofilm activity in the study (47). This observation indicated that the bacteria may induce mutagenesis in response to antibiotics.

To regulate stress responses, bacteria are induced to

activate an expression of code that either protects the cells from stress or repairs cellular damage. Therefore, exposure of bacterial cells to one type of stress may lead to the acquisition of tolerance against other stressors (48). In bacteria, stress responses are regulated by the sigma factor RpoH (σ32). Upon exposure to antibiotics, several proteins are induced in the bacteria under the transcriptional control of RpoH (48). Induction of stress shock proteins have been found to induce some tolerance against osmotic shock in bacteria (49). Furthermore, McGee (2003) reported possible cross-protection to environmental stress due to the acquisition of antibiotic resistance in bacteria (50). However, in this study, *E. aerogenes* with a MAR index of 1.0 were significantly less resistant ($p > 0.05$) to high salt concentrations as compared to the control. This suggests that this bacterium may not be able to express genetic code that enables bacteria to develop cross-protection against environmental stress.

CONCLUSION

In conclusion, this study phenotypically identified the presence of *C. youngae*, *E. youngae, E. coli*, *K. oxytoca* and *Salmonella* spp. in raw chicken breasts purchased from fresh food markets. The mean count of the foodborne bacteria isolated from all of the retail chicken samples (100%) in this study were not within the acceptable range of 2 $\log_{10}$ CFU/g. These results indicate poor personal hygiene of workers, poor hygiene processing and handling of chicken meat and food safety violations occurring at any step of the chicken supply chain. In addition, this study found that six (75%) out of eight bacteria were MAR bacteria. All isolates had a MAR index of more than 0.2. These data highlight the issue of massive use of multiple antibiotics in the poultry farm, which applied selective pressure on the bacteria, thus causing them to develop resistance. Lastly, the multiple antibiotic resistance of *C. youngae*, *E. coli*, *K. oxytoca* and *Salmonella* spp. were found to have enhanced survival as compared with the antibiotic susceptible strain at 6% or 8% salt concentration, which indicates the development of cross-protection against high salt concentrations.

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