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Microbiological quality aspects of ready-to-eat foods with focus on antibiotic resistance and biofilm formation abilities of foodborne bacteria

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ABSTRACT

In this study, microbiological quality of 93 ready-to-eat food products was determined by enumeration of total aerobic bacteria and members of *Enterobacteriaceae*. Presence of *Staphylococcus aureus* and *Listeria monocytogenes* in the products were also investigated. Aerobic colony counts were between 1.9×10^2–3.4×10^8 CFU g^{-1} for 84.9% and *Enterobacteriaceae* counts were between 2×10^2 and 6.7×10^6 CFU g^{-1} for 43% of the samples. *S. aureus* was detected in 7.5% of the samples, but *L. monocytogenes* was not detected in any sample. 72.9% of the *Enterobacteriaceae* isolates showed resistance to at least one antibiotic tested, and 5 among the *S. aureus* isolates were found to be resistant to penicillin G and 2 against methicillin. Four *S. aureus* isolates produced only Staphylococcal enterotoxin A and 1 isolate produced both Staphylococcal enterotoxin A and B. Biofilm experiments revealed biofilm formation on polystyrene for 95.8%, 87.5%, and 91.6% of *Enterobacteriaceae* isolates at 4 °C, 22 °C, and 37 °C, respectively, whereas all *S. aureus* isolates formed biofilm at all temperatures.

KEYWORDS

ready-to-eat food, Staphylococcus aureus, Enterobacteriaceae, antibiotic resistance, biofilm, Staphylococcal enterotoxin

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1. INTRODUCTION

Ready-to-eat (RTE) food products are defined as foods that can be consumed immediately where purchased, without any cooling or heating pre-treatment. Easy accessibility and high nutritional value of these products provide a great advantage for consumers. With changing human lifestyles, the consumption rate of RTE foods increases, which in turn raises the question whether they are microbiologically safe and of the desired quality (Cho et al., 2011).

Foodborne diseases are considered to be an important cause of morbidity and mortality, especially in infants and immunocompromised people. In healthy adults, foodborne infections and poisoning are usually mild and self-limiting, but treatment with antimicrobials is necessary in invasive and complicated cases. Outbreaks caused by drug-resistant foodborne bacteria are no longer seen as an emerging problem; rather they form a settled situation. Therefore, antimicrobial resistance causes serious public health problems by making related antimicrobials ineffective in the treatment of such infections (Sergelidis and Angelidis, 2017). Biofilm structures formed by foodborne pathogens are one of the sources of food contamination. Environments in food processing processes create favourable conditions for microbial attachment and biofilm formation. The mature biofilm structure acts as a barrier against sanitisers and other agents. Food and microbial residues left as a result of inadequate and ineffective sanitation practices facilitate biofilm formation (Abebe, 2020).

This study was performed to investigate the microbiological quality of RTE food products and virulence factors of foodborne *Enterobacteriaceae* and *Staphylococcus aureus* isolates. Microbiological quality analysis was performed by enumeration of total aerobic bacteria and members of *Enterobacteriaceae* and by investigation of presence of *S. aureus* and *Listeria monocytogenes*. The antibiotic resistance patterns and biofilm forming capacities of *Enterobacteriaceae* and *S. aureus* isolates and toxin production of *S. aureus* isolates were also determined.

2. MATERIALS AND METHODS

2.1. Sample collection

Ninety-three RTE food products selected according to their availability and popularity of consumption were randomly purchased in Edirne, Turkey from October 2018 to September 2019. Samples were stored under food storage conditions during transportation and microbiological analysis was performed in a maximum period of 24 h from purchase.

2.2. Microbiological quality and safety analysis

Ten gram and 25 g (for *L. monocytogenes* detection) food samples were added to a culture medium (Half Fraiser broth for *L. monocytogenes*, Giolitti and Cantoni broth for *S. aureus* detection) or diluent (phosphate buffer saline for aerobic colony and *Enterobacteriaceae* counts) (1:10) and homogenised in a stomacher for 2 min. Enumeration of aerobic colonies and *Enterobacteriaceae* was performed according to the standard methods ISO 4833:2013-1 and ISO 21528-2:2004 (ISO 2004a, 2013), respectively. Oxidase test using oxidase strips (Sigma Aldrich, USA) and catalase test were performed for confirmation of one or two presumptive *Enterobacteriaceae* colonies. DE, SE, and AE codes were given to the *Enterobacteriaceae* isolates isolated from desserts, street food, and appetiser and salads groups, respectively.

Detection of *L. monocytogenes* and *S. aureus* was performed according to the standard methods ISO 11290-1:2004 and ISO 6888-3:2003 (ISO 2003; 2004b), respectively. Gram and catalase positive and oxidase negative presumptive colonies with typical aspects of *L. monocytogenes* on Palcam agar (Sigma Aldrich, USA) and Gram and catalase positive presumptive colonies of *S. aureus* on Baird-Parker agar (Sigma Aldrich, USA) were biochemically identified using the API Listeria and Staph system (BioMérieux, Marcy l'Etoile, France), respectively.

2.3. Virulence properties of *Enterobacteriaceae* and *S. aureus* isolates

Determination of SE production was performed by Reversed Passive Latex Agglutination method using the SET-RPLA kit (TD 9000, Oxoid) according to the manufacturer's instructions.

Disk diffusion method according to Clinical Laboratory Standards Institute guidelines (CLSI, 2018) was used for determination of the antibiotic resistance profiles of the isolates.

The method of Stepanović et al. (2004) was used for determination of biofilm formation abilities of *Enterobacteriaceae* and *S. aureus* isolates in 96-well polystyrene microplates (Grenier Bio-One). Eighteen hours old cultures were diluted with Luria Bertani broth without NaCl and Tryptic Soy Broth (TSB) with 0.4% glucose to $OD_{590} = 0.2$ for *Enterobacteriaceae* and *S. aureus* isolates, respectively. These broths were also used to determine biofilm formation of isolates. Microplates were incubated statically for 48 h at 4 °C, 22 °C, and 37 °C. The optical densities (OD) were measured at 590 nm using a Multiskan EX reader (Bio-Rad). Strains were classified as no, weak, moderate, and strong biofilm producers according to Stepanović et al. (2000).

2.4. Statistical analyses

The Kruskal-Wallis test was used to calculate the significance of the differences between aerobic colony counts (ACC) and *Enterobacteriaceae* counts of the food groups and the biofilm formation abilities at different temperatures ($P < 0.05$). The Pearson correlation test was performed to determine the correlation, if any, between antibiotic resistance patterns and SE production properties of *S. aureus* isolates. Analyses were performed with the GraphPad PRISM software (Intuitive Software for Science, San Diego, CA).

3. RESULTS AND DISCUSSION

3.1. Microbiological quality and safety analysis

Food products were assigned to 3 groups as desserts ($n = 33$), street foods ($n = 35$), and appetiser and salads ($n = 25$). ACC is commonly used to indicate the sanitary quality of foods and high *Enterobacteriaceae* counts are regarded as evidence for inappropriate processing, incomplete heating, or contamination after processing through contact with contaminated equipment (Wei et al., 2006). ACC were between 1.9×10^2–3.4×10^8 CFU g^{-1} for 84.9%, *Enterobacteriaceae* counts were between 2×10^2 and 6.7×10^6 CFU g^{-1} for 43% of the samples (Bacterial load values of each food samples were given in Supplementary Table 1, available online at Publisher). The results of the group-based analysis of microbiological evaluation of the food samples are given in Table 1.

The Kruskal-Wallis test showed that the food groups did not significantly differ from each other ($P < 0.05$) for *Enterobacteriaceae* counts, but only the appetiser and salads group significantly differed from each of desserts and street foods groups for ACC ($P < 0.05$).

Table 1. ACC and *Enterobacteriaceae* counts (CFU g^{-1}) and detection percentages in food groups

	ACC Detection%	*Enterobacteriaceae* count Detection%
Desserts	1.9×10^2–3.4×10^8 81.8%	2×10^2–1.72×10^5 54.5%
Appetiser and salads	3.2×10^2–2.5×10^8 100%	3.1×10^3–6.7×10^6 44%
Street food	5.6×10^2–1.9×10^8 74.2%	3×10^2–3.7×10^6 31.4%

Food samples were classified as satisfactory, acceptable, and unsatisfactory according to Gilbert et al. (2000). For ACC-based classification, samples such as sandwiches and filled rolls with salad and cheese and fermented products and for *Enterobacteriaceae* count-based classification, fresh fruits, vegetables, and sandwiches containing them were excluded from the evaluation, as suggested. Eighty and 89 food samples were evaluated for ACC and *Enterobacteriaceae* count-based classifications, respectively. The results of evaluations in terms of group-based classifications of the food samples are given in Fig. 1.

The appetiser and salads group was characterised with the highest number of unsatisfactory class food samples according to both ACC (57.1%) and *Enterobacteriaceae* counts (23.8%), whereas the highest numbers of satisfactory and acceptable class food samples, in total, according to ACC (84.6%) and *Enterobacteriaceae* counts (85.7%) were in the street foods group.

The evaluation of individual food samples in appetiser and salads group in terms of ACC showed that this group with the highest number of unsatisfactory class food samples was the most risky group. The high ACC values in this group (2.6×10^6 – 1.36×10^8 CFU g^{-1} for five samples) were determined in samples taken from a local market place, where products are sold at ambient temperature. In addition, most products in this group are prepared without pre-heat treatment. The results revealed the importance of the conditions in which RTE foods are prepared and sold for hygienic production in terms of sanitation and also heat treatment and temperature control during sales in terms of food safety.

The second group with a high ratio of unsatisfactory class food samples according to bacterial load was the desserts group (Fig. 2). The highest bacterial counts in this group were in

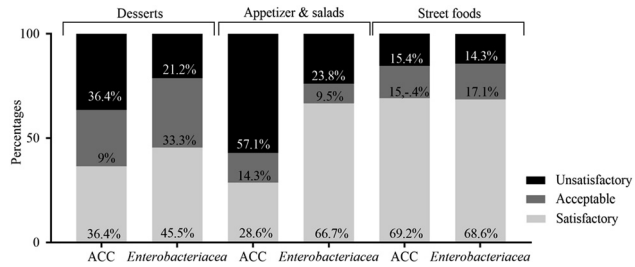

Fig. 1. Group-based classification of food samples as unsatisfactory, acceptable, and satisfactory based on ACC and *Enterobacteriaceae* counts

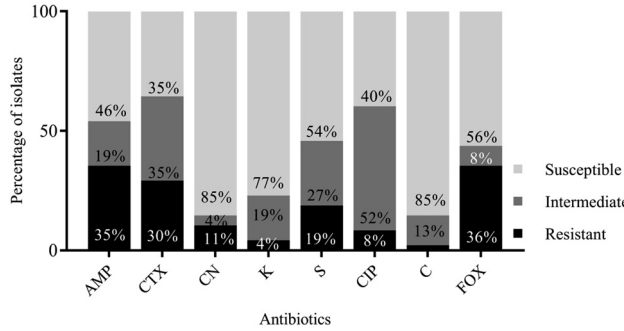

Fig. 2. Percentage of resistant, intermediate resistant, and susceptible *Enterobacteriaceae* isolates

milk-containing desserts, such as gullac, trilice, and éclair, showing that they are not microbiologically safe for consumers.

L. monocytogenes was not detected in any of the samples, whereas *S. aureus* was detected in 7 (7.5%) samples (Table 2).

The most important source of *S. aureus* is humans, and its presence is mainly associated with cross-contamination among food preparation surfaces, food handlers, and RTE foods. The main contamination cause is improper handling of cooked or processed food (Kadariya et al., 2014), highlighting the importance of training of the personnel working in food industry.

Although the obtained data on microbiological quality of the sampled foods are similar to previously reported (Öz et al., 2014; Gurler et al., 2015), the following results provide an additional insight into the antibiotic resistance and biofilm formation abilities of foodborne bacteria.

Table 2. The API codes, antibiotic resistance patterns, and SE production of *S. aureus* isolates

Strain code	API code	Source	Resistance pattern	SE	Biofilm formation (OD_{590} values)		
					4 °C	22 °C	37 °C
AS12	6736153	Mediterranean salad	–	–	0.077 ±0.003 weak	0.292 ± 0.095 strong	0.816 ± 0.146 strong
SS24	6736153	Chicken doner	PEN, CIP, RIF, FOX	SEA	0.074 ± 0.005 weak	0.26 ± 0.121 moderate	0.304 ± 0.124 moderate
DS16	6736113	Turkish delight	–	SEA	0.069 ± 0.005 weak	0.323 ± 0.046 moderate	1.16 ± 0.215 strong
DS70	6736153	Chicken tantuni	PEN	SEA, SEB	0.092 ± 0.012 weak	0.113 ± 0.009 weak	2.04 ± 0.522 strong
DS18	6736153	Banana pudding	PEN, FOX	SEA	0.088 ± 0.004 weak	0.14 ± 0.059 moderate	5.105 ± 0.766 strong
DS20	6336153	Ice cream	PEN	–	0.074 ± 0.006 weak	0.105 ± 0.032 weak	0.112 ± 0.018 weak
AS11	6736153	Mediterranean salad	PEN, CIP	SEA	0.087 ± 0.005 weak	0.201 ± 0.042 moderate	1.664 ± 0.837 strong

3.2. Virulence properties of *Enterobacteriaceae* and *S. aureus* isolates

Forty-eight *Enterobacteriaceae* strains, isolated from street food ($n = 16$), appetizer and salad ($n = 12$) and dessert ($n = 20$) groups, were investigated for their virulence properties. The disc diffusion test showed that 72.9% of all isolates showed resistance against at least one and 22.9% showed resistance against at least 3 of the antibiotics tested. Among the *Enterobacteriaceae* isolates, the highest rate (36%) of resistance was observed against cefoxitin, while there was no isolate resistant to tetracycline and trimethoprim-sulfamethoxazole (Fig. 2). It is also important that 62.5% of the *Enterobacteriaceae* isolates were resistant against at least one antibiotic including ampicillin, kanamycin, ciprofloxacin, cefotaxime, gentamicin, and streptomycin that are considered "critically important" antimicrobials for human medicine according to the World Health Organization (WHO, 2019). Resistance patterns of *Enterobacteriaceae* isolates are given in Table 3.

Five of the seven *S. aureus* isolates showed resistance against at least one of the antibiotics. No isolate was resistant against clindamycin, gentamicin, trimethoprim-sulfamethaxazole, kanamycin, chloramphenicol, and erythromycin (Table 3). These five isolates were resistant to penicillin G used in the treatment of *S. aureus* infections in many countries. Two *S. aureus* strains (DS16 and DS18) were found to be resistant to methicillin. These methicillin resistant *S. aureus* (MRSA) strains are very important for public health. With increased circulation of food products worldwide, problems arising in any country could soon become a global threat. Food-borne pathogens, commensals, and bacteria in food chain provide a genome pool or a

Table 3. Antibiotic resistance pattern of *Enterobacteriaceae* isolates

Resistance Pattern	Isolates
AMP, CTX, CN, CIP, C, FOX	SE1-2
AMP, CTX, CN, S, FOX	DE19
AMP, CTX, K, FOX	SE1
AMP, CN, S, FOX	DE20
CTX, CIP, FOX	SE3, DE17
AMP, K, FOX	SE13
AMP, CN, FOX	DE3
AMP, S, FOX	DE6, AE2, AE5-2
CTX, FOX	SE10
AMP, CTX	DE2
AMP, S	DE5
AMP, CIP	DE9
AMP, CN	DE15
CTX, S	AE10
CTX	SE5, DE3-2, DE8, DE18, AE1, AE4
FOX	SE4, SE11, SE13-2, DE11, DE12
AMP	SE15-2, DE1, DE10, AE7
S	DE16, AE3
CIP	SE15

AMP: ampicillin; CTX: cefotaxime; CN: gentamicin; CIP: ciprofloxacin; C: chloramphenicol; FOX: cefoxitin; S: streptomycin; K: kanamycin.

microbiome that may contain genes encoding resistance to resistome or antimicrobials. Resistance may result from a gene mutation or from horizontal gene transfer resulting from the large resistance gene pool found in the whole food chain resistome. Resistant bacteria in the food chain can become dominant in the bacterial population as a result of the selective pressure created by antimicrobials, disinfectants, and biocides (Cahill et al., 2017).

The RPLA method showed that four isolates produced only SEA and one isolate produced both SEA and B (Table 3). *S. aureus* produces SEs at high cell concentrations in foods, and SE production by *S. aureus* makes it one of the predominant foodborne problems worldwide causing gastroenteritis (Sergelidis and Angelidis, 2017). SEA and B are two of the most important gastroenteritis causing agents, and SEA is one of the most common toxins (Kadariya et al., 2014). The 2 MRSA isolates were also found to produce SEA. No correlation was found between the antibiotic resistance patterns and SE production properties of the isolates. However, the change in gut microbiota as a result of antibiotic therapy indicates that MRSA colonising the intestinal tract contributes to the expression of pathogenic properties such as enterotoxin production (Ortega et al., 2010).

95.8%, 87.5% and 91.6% of *Enterobacteriaceae* isolates produced biofilm on polystyrene plates at 4 °C, 22 °C and 37 °C, respectively. The average biofilm formation capabilities of the isolates were significantly different at the three incubation temperatures ($P < 0.05$), which were chosen to represent routine storage conditions of food (4 °C and 22 °C) and optimum growth condition of the isolates (37 °C). Polystyrene is a hydrophobic material and widely used in production of food contact, food packing, and food service materials and commonly used in microtiter plates as an *in vitro* mimic of the plastic surfaces (Borges et al., 2018). The percentages of weak, moderate, and strong biofilm producers at three temperature conditions with their corresponding OD_{590} values are given in Table 4 (OD_{590} values of each isolates are given in Supplementary Table 2).

All strong biofilm producer isolates were resistant to at least one antibiotic. Group-based analysis of biofilm formation of *Enterobacteriaceae* isolates showed that there was no significant difference between different incubation temperatures ($P < 0.05$).

Table 4. Biofilm production pattern of *Enterobacteriaceae* isolates

Biofilm production	Minimum and maximum OD_{590} values ± SD		
	Detection %		
	4 °C	22 °C	37 °C
Weak	0.071 ± 0.002 and 0.125 ± 0.002	0.178 ± 0.007 and 0.281 ± 0.191	0.105 ± 0.026 and 0.184 ± 0.026
	70.8%	41.6%	45.8%
Moderate	0.117 ± 0.004 and 0.267 ± 0.013	0.348 ± 0.116 and 0.634 ± 0.047	0.220 ± 0.134 and 0.409 ± 0.172
	22.9%	20.8%	29.1%
Strong	0.339 ± 0.038*	0.654 ± 0.094 and 1.619 ± 0.087	0.454 ± 0.016 and 1.025 ± 0.110
	2.1%	25%	16.6%

*Only AE5-2 was strong biofilm producer at 4 °C.

Biofilm formation was determined for all *S. aureus* isolates at all temperatures (Table 3). MRSA isolates were classified as weak and moderate biofilm producers at 4 °C and 22 °C, respectively, and as moderate (DS16) and strong (DS18) biofilm producers at 37 °C. Food and food production environments provide suitable conditions for biofilm formation. Inadequate and ineffective sanitation practices cause food residues to remain on the surfaces, and this can facilitate the attachment of bacteria to these surfaces and the formation of biofilms. This leads to post processing contamination, which reduces the quality and shelf life of food products and can be a vehicle for disease transmission. It is very difficult to properly sterilise these surfaces where the biofilm structure is formed, because the cells in the biofilm are more resistant to disinfectants (Abebe, 2020). In addition, biofilms create environments that facilitate the emergence and spread of antimicrobial resistance (Cahill et al., 2017).

4. CONCLUSIONS

The present study highlighted the potential of RTE food products to act as risk factor for public health and to be vehicles of antibiotic resistant and biofilm producing *Enterobacteriaceae* and clinically relevant antimicrobial resistant and enterotoxigenic *S. aureus*. High microbiological load of such food products reveals the necessity of improved sanitary conditions of food preparation surfaces and vendors. Inadequate sanitation methods used to reduce the bacterial load of these food create a selective environment for these resistant and biofilm-forming bacteria. The results also showed that new strategies are needed to monitor and control the microbiological quality of RTE foods.

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SUPPLEMENTARY MATERIAL

Supplementary data to this article can be found online at https://doi.org/10.1556/066.2021.00061.

REFERENCES

Abebe, G.M. (2020). The role of bacterial biofilm in antibiotic resistance and food contamination. *International Journal of Microbiology*, 2020: 1–10.

Borges, K.A., Furian, T.Q., Souza, S.N., Menezes, R., Tondo, E.C., Salle, C.T., Moraes, H.L.S., and Nascimento, V.P. (2018). Biofilm formation capacity of *Salmonella* serotypes at different temperature conditions. *Pesquisa Veterinária Brasileira*, 38(1): 71–76.

Cahill, S.M., Desmarchelier, P., Fattori, V., Bruno, A., and Cannavan, A. (2017). Global perspectives on antimicrobial resistance in the food chain. *Food Protection Trends*, 37(5): 353–360.

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., and Ha, S.D. (2011). Assessment of microbial contamination levels of street-vended foods in Korea. *Journal of Food Safety*, 31: 41–47.

CLSI (2018). *Performance standards for antimicrobial susceptibility testing*, 28th ed. CLSI supplement M100. Clinical and Laboratory Standards Institute, Wayne, PA.

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

Gurler, Z., Pamuk, S., Yildirim, Y., and Ertas, N. (2015). The microbiological quality of ready-to-eat salads in Turkey: a focus on *Salmonella* spp. and *Listeria monocytogenes*. *International Journal of Food Microbiology*, 196C: 79–83.

ISO (2003). *Microbiology of food and animal feeding stuffs – horizontal method for the enumeration of coagulase-positive staphylococci* (Staphylococcus aureus *and other species) – Part 3: Detection and MPN technique for low numbers*. ISO 6888-3:2003.

ISO (2004a). *Microbiology of food and animal feeding stuffs – horizontal method for detection and enumeration of* Enterobacteriaceae – *part 2: Colony count method.* ISO 21528-2:2004.

ISO (2004b). *Microbiology of food and animal feeding stuffs – horizontal method for the detection and enumeration of* Listeria monocytogenes – *part 1: Detection method amendment 1: Modification of the isolation media and the haemolysis test and inclusion of precision data*. ISO 11290-1:2004.

ISO (2013). *Microbiology of the food chain – horizontal method for the enumeration of microorganisms – Part 1: Colony-count at 30 degrees C by the pour plate technique.* ISO 4833:2013-1.

Kadariya, J., Smith, T.C., and Thapaliya, D. (2014). *Staphylococcus aureus* and staphylococcal food-borne disease: an ongoing challenge in public health. *BioMed Research International*, 2014: Article ID 827965. https://doi.org/10.1155/2014/827965.

Ortega, E., Abriouel, H., Lucas, R. and Galvez, A. (2010). Multiple roles of *Staphylococcus aureus* enterotoxins: pathogenicity, superantigenic activity, and correlation to antibiotic resistance. *Toxins*, 2: 2117–2131.

Öz, V., Karadayi S., Çakan, H., Karadayi, B., and Çevik, F.E. (2014). Assessment of microbiological quality of ready-to-eat foods in Istanbul, Turkey. *Journal of Food Agriculture and Environment*, 12(3&4): 56–60.

Sergelidis, D. and Angelidis, A.S. (2017). Methicillin-resistant *Staphylococcus aureus*: a controversial food-borne pathogen. *Letters in Applied Microbiology*, 64(6): 409–418.

Stepanović, S., Ćirković, I., Ranin, L., and Svabić-Vlahović, M. (2004). Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. *Letters in Applied Microbiology*, 38(5): 428–432.

Stepanović, S., Vuković, D., Dakić, I., Savić, B., and Švabić-Vlahović, M. (2000). A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of Microbiological Methods*, 40(2): 175–179.

Wei, Q.K., Hwang, S.L., and Chen, T.R. (2006). Microbiological quality of ready-to-eat food products in southern Taiwan. *Journal of Food and Drug Analysis*, 14(1): 68–73.

WHO (2019). *Critically important antimicrobials for human medicine*, 6th ed. World Health Organization, Geneva, Switzerland. Available at: https://apps.who.int/iris/bitstream/handle/10665/312266/9789241515528-eng.pdf?ua=1 (Accessed: 10 March 2021).