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Citrus reticulata flavonoids as a valuable source for reducing meat-borne *Aeromonas hydrophila*

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ORIGINAL ARTICLE

Abstract

Meat products are one of the nutritious diet options available to consumers. However, meat products have the potential to serve as a reservoir of food-borne infections, such as those caused by *Aeromonas* species, which pose a significant risk to public health. A total of 270 samples, 30 of each minced beef, meatball, beef burger, chicken nuggets, chicken pane, chicken burger, canned anchovy, canned mackerel, and canned sardine, were obtained from supermarkets in Aswan Province, Egypt, to analyze the occurrence and virulence features of *Aeromonas hydrophila.* Influence of *Citrus reticulata* peel extract on the *A. hydrophila* count and its virulence capacity after different marinating periods was also analyzed. The obtained results revealed that the *Aeromonas* counts ranged from 2.02±0.30 to 4.21±0.27 $\log_{10}$ CFU/g and 37% of the analyzed samples were contaminated with *Aeromonas* spp. The main strains discovered were *A. hydrophila* (13.3%), *A. sobria* (9.6%), *A. caviae* (7.4%), *A. veronii* (4%), and *A. fluvialis* (2.6%). It was found by polymerase chain reaction that all tested strains (n = 36) belonged to *Aeromonas* spp., and 89% were identified as *A. hydrophila*. In contrast, virulence genes aerolysin (*aerA*) and cytotoxic enterotoxin (*act*) were found in 61.1% and 50% of the tested isolates, respectively, with a wide range of antibacterial resistance. Additionally, the influence of *Citrus reticulata* peel extract on *A. hydrophila* counts at different marinating periods declined significantly with increased concentration without major changes in the sensory criteria.

Keywords: *Aeromonas hydrophila*; antibiotics; *Citrus reticulate*; flavonoids; meat-borne pathogens

Introduction

Meat products are in great demand and are viewed as more enticing to customers because of their excellent taste, fair pricing, high nutritional value, ease of preparation, and ease of serving. Although the value of meat products to customers is under question, they are thought to be the ideal culture medium for the growth of a wide variety of microbes because of their humidity, nitrogenous contents, abundant mineral sources, glycogen, and promising pH for most of bacteria (Elbayoumi *et al.,* 2021). The family Aeromonadaceae includes the emerging food-borne pathogen *Aeromonas* species. These are Gram-negative microbes established in food,

aquatic environments, and soil. *Aeromonas* spp. proliferate and survive at a temperature of 2–10°C, which is typically used for keeping food chilled (Schoch *et al.,* 2020). It is an indication of the species potential to pose a public health risk, as they are responsible for a wide range of infections, such as infections of the digestive tract, intestines, liver, meninges, soft tissues, and wounds (Bravo and Figueras, 2020). The most prevalent *Aeromonas* spp. in meat and meat products are *A. hydrophila*, *A. caviae*, *A. sobria*, and *A. veronii* (Elbehiry *et al.,* 2019). The pathogenicity of *Aeromonas* spp. depends on virulence factors, such as their ability to produce biofilms to stick, attack, and destroy host cells while evading the host's immune reaction (Hidalgo and Figueras, 2013).

Antimicrobial resistance among enteric bacteria, including *Aeromonas* spp., is a serious global problem. There is a possibility that this could be due to a wide use or misuse of antimicrobial agents in treating different bacterial infections or for their use as sub-therapeutic growth promoters in animal feeds (El-Ghareeb *et al.,* 2019). Natural antimicrobial agents possess the following two primary attributes: (i) their organic nature ensures greater safety for both consumers and the environment, and (ii) their composition comprises a variety of compounds that ostensibly exhibit distinct antimicrobial mode of action, thereby posing a lower risk of developing a microbial resistance (Saleh *et al.,* 2017).

Citrus reticulata peel extract has been extensively studied as an organic supply of bioactive ingredients with useful effects, such as antibacterial, antioxidant, antidiabetic, anti-inflammatory, and neuroprotective (Lin *et al.,* 2021). Furthermore, citrus by-products are a beneficial source of phytochemicals (essential oils, phenolic acids, d-limonene, vitamins, carotenoids, minerals, and flavonoids). The synthesis of these compounds may exhibit an extensive range of biological mechanisms, many of which are beneficial for the food industry (Magalhães *et al.,* 2023). Therefore, citrus-based components may function as natural preservatives because their notable antibacterial efficacy against microorganisms found in food and their antioxidant activity, which prevents the influence of oxidation (Manzur *et al.*, 2023). The aim of the present study was to determine the prevalence of virulent antibiotic-resistant *A. hydrophila* and to create a novel strategy for reducing the pathogen's ability to spread through meat by employing alternative natural antibiotic flavonoids found in *Citrus reticulata*.

Materials and Methods

Collection of samples

A total of 270 products, 90 beef products (30 of each minced beef, meatball, and beef burger), 90 chicken products (30 of each chicken nugget, chicken pane, and chicken burger), and 90 fish products (30 of each canned anchovy, canned mackerel, and canned sardine) were randomly collected from the supermarkets of Aswan, Egypt, during November–December 2022. The samples were purchased in their original packing and represented various brands, manufacturing dates, lot numbers, and sell-by dates. For further analysis, all samples were transported in cold storage (4 °C) within <2 h to the meat laboratory of the Veterinary Medicine College, Aswan University, Egypt.

Samples preparation

Under aseptic conditions, 225 mL of alkaline peptone water (APW; Oxoid CM1028) was mixed with a 25-g sample and homogenized at 14,000 rpm for 3 min. The contents were left to rest for 5 min at room temperature; 1 mL of homogenate was added to 9 mL of APW (0.1%) for serial dilution.

Aeromonas spp. count, isolation, and identification procedure

Aeromonas isolation medium base (HiMedia-M884) supplemented with ampicillin was streaked with 0.1 mL of initial dilution and incubated for 24 h at 35°C (Austin and Austin, 2016). Biochemical identification and purification were performed on presumed *Aeromonas* colonies (pale green translucent colonies with a darker core) (Carnahan and Joseph, 2015).

Molecular identification and virulence genes detection of *A. hydrophila*

Polymerase chain reaction (PCR) was used to identify the presence *A. hydrophila* at genus- and species-specific levels by analyzing virulence genes, *16S* rRNA (genus-specific), *16S* rRNA (species-specific) as well as aerolysin (*aerA*) and cytotoxic enterotoxin (*act*), using the primers shown in Table 1. DNA extraction was carried out according to manufacturer's protocol using the Gene JET Genomic DNA purification kit (Catalogue No. #K0721; Thermo Scientific, Waltham, MA, USA). The PCR analysis comprised both positive control, supplied by Animal Health Research Institute (AHRI), Dokki, Egypt, and negative control (DNA-free). The reactions comprised 12.5-µL deionized water, 1.25 µL of each 20 pmol/µL primer (forward and transverse), 5 µL of DNA, and 5 µL of 5X master mix (TaQl/high yield; Jena Bioscience, Jena, Germany). The PCR amplification protocols were as follows: 1 cycle at 94°C/4 min; 30 cycles of denaturation at 94°C/30 s; annealing at 60°C/30 s for *16S*

rRNA (genus-specific), 45°C/60 s for *aerA*, and 42°C/60 s for *act*; extension at 72°C/30 s for *16S* rRNA (species-specific) and 72°C/60 s for *aerA* and *act*, and final extension at 72°C/7 min. The amplified PCR products were separated using an electrophoretic technique on a 1.5% agarose gel. Using gene ruler and a 1-Kb plus DNA ladder (Cat. No. N3236S; Biolabs, New England), the size of the amplified product was determined.

Antimicrobial susceptibility test for the isolated *A. hydrophila*

The isolated *A. hydrophila* was assessed for antimicrobial susceptibility using the Kirby-Bauer disc diffusion technique (Clinical and Laboratory Standards Institute [CLSI], 2020). After achieving a McFarland's turbidity of 0.5 (2×10^8 CFU/mL), the isolated and purified *A. hydrophila* inoculum was introduced into Muller Hinton broth (Oxoid CM0405B). Turbid broth, 100 µL, was incubated on Mueller–Hinton agar (MHA) with antibiotic discs (Oxoid CM0337B) for 24 h at 37°C. Antibiotic discs include streptomycin (SM; 10 µg), tetracycline (TE; 30 µg), gentamicin (G; 10 µg), chloramphenicol (CM; 30 µg), ampicillin (AP; 10 µg), kanamycin (K; 30 µg), nalidixic acid (NA; 30 µg), sulfamethoxazole (ST; 25 µg), amoxicillin (AX; 25 µg), ciprofloxacin (CP; 5 µg), norfloxacin (NR; 10 µg), amikacin (AK; 30 µg), imipenem (IP; 10 µg), penicillin (P; 10 µg), cefotaxime (CX; 30 µg), and ceftazidime (CZ; 30 µg). Furthermore, the multiple antibiotic resistance (MAR) index was calculated by dividing the number of antibiotics to which the microorganisms were resistant by the total number of antibiotics tested (Morshdy *et al.*, 2022).

Effects of *Citrus reticulata* peel extract on marinated chicken fillet

Preparation of Citrus reticulata *peel extract*

Citrus reticulata (Mandarin) fruit was rinsed under running tap water and skinned. The edible pieces were cautiously detached, sliced into little pieces, and dried at 40±2°C for 48 h in an air oven; these were subsequently crushed into a fine powder using a mechanical mincer. Then, the resulting powder was filtered through a 35-mesh sieve (0.425 mm), sealed in polyethylene bags, and kept at 4±1°C until usage. Peel powder (4 g) was mixed with 20 mL of water and ethanol in conical flasks (sealed with non-absorbent cotton and encased in aluminum foil). The flasks were centrifuged in a shaker incubator, 130 rpm/36 h, at 30°C. After taking out the flasks from incubator, their contents were placed into tightly closed centrifuge tubes, and the tubes were centrifuged at 4,200 rpm at 10°C. The acquired transparent fluids were promptly moved to clean and dehydrated Petri dishes and put in a tray dryer set to 80% concentration at 35°C to ensure the dispersion of extraction solvent. The pellet-filled centrifuge tubes were discarded. The extracts were transferred to Eppendorf tubes and kept at 10°C until most of the solvents evaporated (Yadav *et al.*, 2015).

Total phenolic content of Citrus reticulata *peel*

Gallic acid standard solution with concentrations of 0.2, 0.4, 0.6, 0.8, and 1 mg/mL was prepared in methanol. The extract (0.5 mL) was then combined with 4.5 mL of distilled water and 0.5 mL of 10-fold diluted Folin–Ciocalteau combination. Following this, combined distilled water (2 mL) and 7% Na_2CO_3 (5 mL) were left at room temperature for 90 min. Gallic acid was utilized as a positive control, and each assay was carried out in triplicate. The total phenolic content of citrus peel powder was measured at 760 nm as milligram of gallic acid equivalent (GAE) per 100 g of dry powder weight (Cicco *et al.*, 2009).

Total flavonoid content of Citrus reticulata *peel*

A mixture of 0.5 mL of extract (1 mg/mL) and 1.5 mL of methanol was used. To this mixture, distilled water (2.8 mL), potassium acetate (0.1 mL), and aluminium chloride ($AlCl_3$) 10% (0.1 mL) were added. The mixture was left at room temperature for 30 min. Absorbance was measured at 420 nm using a spectrophotometer (CECIL, CE 7200, UK). The results were expressed as

Table 1. Oligonucleotide primer sequences for isolated *A. hydrophila*.

Genes	Primer sequence (5'→3')	Size (bp)	References
16S rRNA (genus-specific)	CTA TGA AAA AAC TAA AAA TAA CTG CAG TAT AAG TGG GGA AAT GGA AAG	953	El-Hossary *et al.*, 2023
16S rRNA (species-specific)	CAC AGC CAA TAT GTC GGT GAA G GTC ACC TTC TCG CTC AGG C	625	
aerA	AACCGAACTCTCCAT TTGTCCGGGTTGTACTCGTC	301	Hu *et al.*, 2012
act	GAGAAGGTGACCACCAAGAAC AACTGACATCGGCCTTGAACTC	232	

equivalent of quercetin in milligram per gram of dry weight (Ebrahimzadeh *et al.,* 2008).

Phenolic component of Citrus reticulata peel

The following high-performance liquid chromatography (HPLC) equipment was used for analytical procedures: a degasser, a Zorbax OD, and a stainless-steel column (4×250 mm)C18 reverse-phase, and a Malti wavelength detector calibrated at 280 nm and 330 nm to detect phenolic and flavonoid chemicals. A 4.6×250-nm column was used for fractionation, and the mobile phase flow rate was 1 mL/min for the duration of experiment. HPLC was started with a linear inclination at a flow rate of 1.0 mL/min using water–acetic acid (98:2 v/v; solvent A) as a mobile phase and methanol–acetonitrile (50:50 v/v; solvent B). Starting with 5% of solvent B, the proportion increased to 30% at 25 min, 40% at 35 min, 52% at 40 min, 70% at 50 min, and 100% at 55 min (Goupy *et al.,* 1999).

Antimicrobial efficacy of Citrus reticulata *peel extract*

With some minor modifications, a positive *A. hydrophila* strain, harboring both *aerA a*nd *act* genes, was used in the experimental trial, as reported by Elbarbary and Abdelmotilib (2023). For each experiment, two groups of five raw chicken fillets (weighing 100 g each) were used, one for sensory evaluation and another for determining the antimicrobial effect of *Citrus reticulata* flavonoids against *A. hydrophila*. Chicken fillets were cleaned by immersing them in 70% ethyl alcohol for 3–5 min and allowing them to dry. Each fillet was artificially contaminated with *A. hydrophila* broth (1 mL) according to a 0.5 McFarland standard and kept at 35°C for 60 min. To determine the antimicrobial effect of *Citrus reticulata* flavonoids against *A. hydrophila* count over 36 h at 4°C, the following five groups were prepared: the untreated control group only got a microorganism inoculation, while the following groups were immersed in *Citrus reticulata* peel extract solutions: 25% (T1), 50% (T2), 75% (T3), and 100% (T4).

Organoleptic assessment

In all, 53 panelists used scoring tests to assess the sensory qualities of chicken fillet samples prepared with varying concentrations of *Citrus reticulata* marination. The fillet samples were randomly encoded with specific codes and the panelists were not informed about the study methods. They were asked to score for appearance, odor, texture, flavor, and overall satisfaction (Kilinc and Cakli, 2004).

Data analysis

The findings were presented as mean and standard error (SE) using the SPSS software (2001), with $p < 0.05$ being the level of significance.

Results and Discussion

Aeromonas spp. count, isolation, and identification

Meat products are among the most valued nourishments for human intake because of their high nutritional value, delicious flavor, ease of preparation, and ease of serving. However, during handling, processing, and preparation, they are contaminated by various food-borne pathogens, such as *Aeromonas* spp., from various sources, thus posing a serious risk to public health (Elbayoumi *et al.,* 2021). Results given in Table 2 showed that the incidence of *Aeromonas* counts ($\log_{10}$ CFU/g) in the studied samples was highest in beef burger (4.21±0.27) followed by bee kofta (3.74±0.63) and chicken burger (3.68±0.64). At the same time, canned anchovy recorded the lowest value (2.02±0.30 $\log_{10}$ CFU/g), with significant differences between other samples ($p < 0.05$). The outcomes were consistent with those of Sheir *et al.* (2020) but not coordinated with the findings stated by Ramadan *et al.* (2018) and Tawfik *et al.* (2022), who recorded the *Aeromonas* counts of 3.35 $\log_{10}$ CFU/g and 1.70, respectively. Meat products may be contaminated with *Aeromonas* because of inadequate industrial sanitation protocols, increased reliance on human labor for packaging and handling, and the absence of tunnel freezing, which could have prevented the multiplication of pathogens during processing (Bayoumi *et al.,* 2023). *Aeromonas* spp. are classified as zoonotic pathogens because of their potential to induce self-limiting diarrhea, gastroenteritis, and septicemia, particularly in children (Elbarbary *et al.,* 2024).

The acquired data (Figure 1) demonstrated that *Aeromonas* spp. were detected in 37% of the analyzed samples. The highest level of *Aeromonas* spp. was found in beef burger (56.7%) and beef kofta (46.7%) samples, while the lowest levels were observed in chicken pane (23.3%)

Table 2. The mean values of *Aeromonas* spp. counts ($\log_{10}$ CFU/g) in the examined samples.

Samples	Minimum	Maximum	Mean ± SE
Minced beef	2.26	3.81	2.68 ± 0.33^{b}
Beef kofta	2.41	4.19	3.74 ± 0.63^{a}
Beef burger	2.43	4.4	4.21 ± 0.27^{a}
Chicken nuggets	2.35	3.88	3.38 ± 0.13^{b}
Chicken pane	2.27	3.73	$2.58 \pm 0.35^{b,c}$
Chicken burger	2.42	4.17	3.68 ± 0.64^{a}
Canned anchovy	1.88	2.21	2.02 ± 0.30^{c}
Canned mackerel	1.82	2.45	2.05 ± 0.12^{c}
Canned sardine	1.89	2.39	2.19 ± 0.24^{c}

Data followed by different superscript alphabets ($^{a–c}$) are significant by ANOVA test, $p < 0.05$.

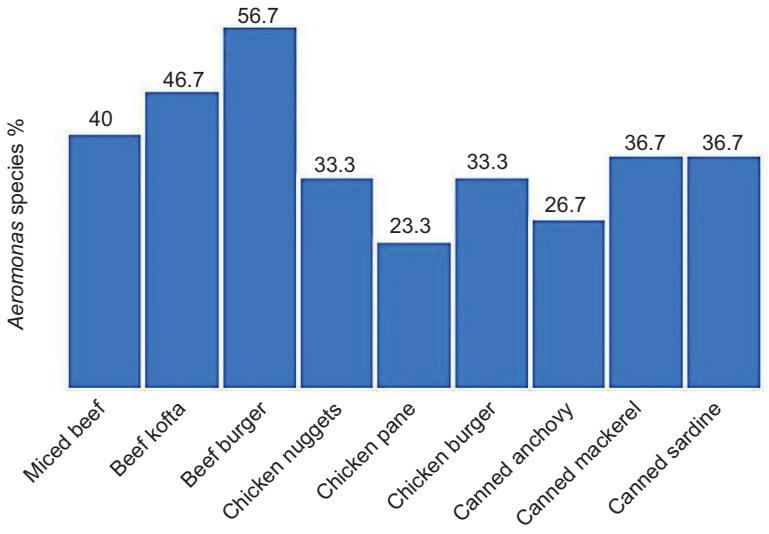

Figure 1. Percentage of *Aeromonas* spp. in the examined samples.

and anchovies (26.7%) samples. The results match the findings of Fauzi *et al.* (2021), Morshdy *et al.* (2022), and Tawfik *et al.*(2022), who discovered levels of *Aeromonas* spp. as 27.6%, 33.3%, and 39.3%, respectively, in the examined samples. Relatively lower frequencies were recorded by Elbayoumi et al. (2021) and Elbarbary *et al.* (2024), who isolated lower levels of *Aeromonas* spp. with proportions of 21.1% and 20%, respectively, while higher levels were documented by Kishk *et al.* (2020) and Sheir *et al.* (2020), who discovered higher levels of *Aeromonas* spp. as 64% and 53.3%, respectively, in the examined samples.

Aeromonas spp. were recognized in 37% of the analyzed samples in the existing study, and the most prevalent *Aeromonas* spp. (Table 3) were *A. hydrophila* (13.3%), *A. sobria* (9.6%), and *A. caviae* (7.4%), and the most contaminated products with *A. hydrophila* were beef burger (23.3%), minced meat (16.7%), and beef kofta (13.3%), which presented a perfect medium for the growth of several microbes that caused food-borne diseases in humans, economic losses, and deterioration of the food (Ercolini *et al.,* 2009). These findings matched the results of Manna *et al.* (2013) and Stratev and Odeyemi (2015), who found *A. hydrophila, A. sobria,* and *A. caviae* as the most discovered species in beef and chicken meat samples. In contrast, Sheir *et al.* (2020) found that *A. sobria* (41.3%), *A. caviae* (22.5%), *A. hydrophila* (15%), *A. veronii* (11.3%), and *A. fluvialis* (3.8%) were the most prevalent species. At the same time, Dhanapala *et al.* (2021) identified *A. veronii* (75.8%), *A. hydrophila* (9.3%), *A. caviae* (5%), and *A. sobria* (0.6%). Furthermore,

Table 3. The proportion of *Aeromonas* spp. in the examined samples (n = 270).

Samples	Positive samples		*Aeromonas* species									
			A. hydrophila		*A. fluvialis*		*A. sobria*		*A. veronii*		*A. caviae*	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Minced beef	12	4.44	5	16.7	0	0	3	10	1	3.3	3	10
Beef kofta	14	5.2	4	13.3	2	6.7	3	10	2	6.7	3	10
Beef burger	17	6.3	7	23.3	1	3.3	5	16.7	2	6.7	2	6.7
Chicken nuggets	10	3.7	4	13.3	0	0	3	10	2	6.7	1	3.3
Chicken pane	7	2.6	2	6.7	1	3.3	3	10	0	0	1	3.3
Chicken burger	10	3.7	3	10	1	3.3	2	6.7	2	6.7	2	6.7
Canned anchovy	8	2.9	3	10	0	0	2	6.7	0	0	3	10
Canned mackerel	11	4.1	4	13.3	1	3.3	2	6.7	1	3.3	3	10
Canned sardine	11	4.1	4	13.3	1	3.3	3	10	1	3.3	2	6.7
Total	100	37	36	13.3	7	2.6	26	9.6	11	4	20	7.4

Bayoumi *et al.* (2023) reported that minced beef and burgers were the most contaminated samples with various *Aeromonas* spp., the most common of which was *A. sorbia*. Changeable counts and occurrences among researchers was attributed to variations in the studied samples, the state of affairs prior to the time and location of the sampling, and the geographic range. Furthermore, meat products could be polluted with many pollutants throughout production, transportation, and retail due to poor hygiene and the lack of monitoring procedures (Hafez *et al.,* 2018).

Molecular identification and virulence genes detection of *A. hydrophila*

Aeromonas hydrophila exhibits a multifactorial mode of virulence characterized by synthesizing of various virulence factors (Sheir *et al.,* 2020). These factors include lipases, cytotoxins, hemolysins, adhesins, and proteases. Furthermore, the bacteria form biofilms via a distinct metabolic pathway and a mediator of virulence factor expression. Various virulence factors of *A. hydrophila* are identified to understand these infections' pathogenesis in a better manner. The existence of *Aeromonas* genus- and species-specific genes was checked using a PCR for molecular identification. Figure 2 shows the confirmed identification of genus-specific *16S* rRNA genes of *Aeromonas* spp. (36 isolates) at 953 bp. Following this, these isolates were tested using PCR for the gene-specific *16S* rRNA of *A. hydrophila* with an amplicon size of 625 bp. This revealed that 32 isolates were positive for *A. hydrophila* (Figure 3), suggesting that some strains were not recognized as *A. hydrophila*, and *16S* rRNA gene specific could serve as unique molecular fingerprints for bacterial species. In addition to highly conserved primer binding sites, *16S* rRNA gene sequences contain hypervariable regions that could provide species-specific signature sequences useful for identification of bacteria (Pereira *et al.,* 2010).

In this study, the most characteristically demonstrated genes were *aerA* (61.1%) and *act* (50%), which caused hemolysis and proteolytic activities in the host (Figure 3). Additionally, researchers identified *aerA* as a virulence factor that contributed to the development of *A. hydrophila* infections. We compared the obtained results with those of Ahangarzadeh *et al.* (2022), Elbarbary *et al.* (2024), and Praveen *et al.* (2016), who identified *aerA* and *act* genes in 58.06%, 60.0%, and 50.0% of *A. hydrophila*, respectively. Elbayoumi *et al.* (2021) and Sheir *et al.* (2020) detected the *aerA* gene in 63.1% and 75% of the analyzed isolates, respectively. Furthermore, Nhinh *et al.* (2021) recorded that the genes *aerA* (80.5%) and *act* (80.1%) were discovered commonly. Meanwhile, Hafez *et al.* (2018) and Osman *et al.* (2012) discovered *aerA* gene in 17% and 20% *A. hydrophila* isolates, respectively. Variations in the virulence of particular strains, primer design divergence, and the small number of strains may cause variations in the discovery of *aerA* and *act* genes. This present study demonstrated that a significant proportion of *A. hydrophila* isolates generated enterotoxins and hemolysins, which were enterotoxic, hemolytic, and lethal. Their presence in food signifies potential public health issues.

Antimicrobial susceptibility

Multiple antibiotic resistant (MAR) index resistance profile is a growing concern to track bacterial infections and drug resistance in order to evaluate the efficacy of *citrus reticulate* peel on antibiotic resistant genes on treated samples, resulting in treatment failure and adverse public

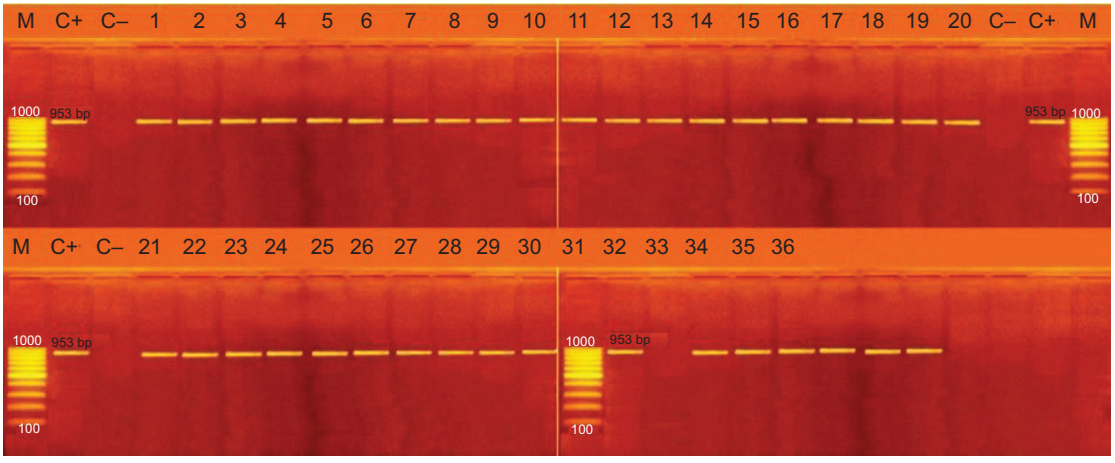

Figure 2. Electrophoretic profile of amplification products of *16S* rRNA *Aeromonas* spp. (genus-specific *geneat* 953 bp). M: marker (50 bp), C+: positive control, C–: negative control.

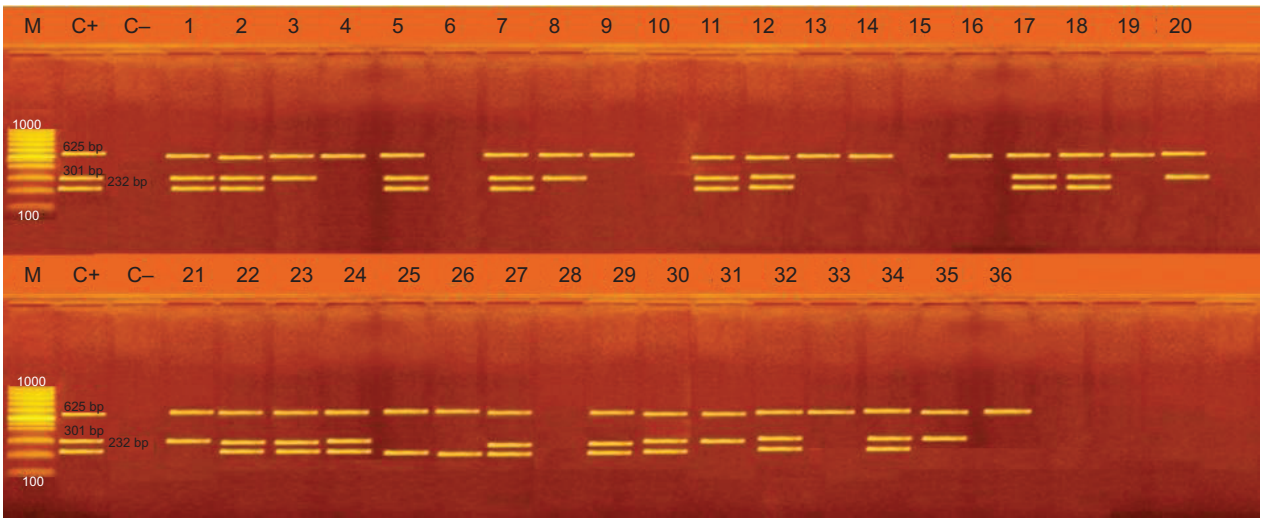

Figure 3. Electrophoretic profile of amplification products of *16S* rRNA gene specific of *A. hydrophila* at 625 bp, *aerA* gene at 301 bp, and *act* gene at 232 bp in *A. hydrophila*. M: marker (50 bp), C+: positive control, C–: negative control.

health consequences. As a result, a decreasing ability to treat bacteria due to the emergence of antibiotic-resistant bacterial pathogens is observed and reported more frequently (Fauzi *et al.,* 2021). In the existing study (Table 4), *A. hydrophila* isolates recorded 100% resistance to tetracycline and amoxicillin and were extremely resistant to ampicillin (91.7%) and penicillin (88.9%) as well; this was similar to the results of earlier studies because of their intrinsic resistance (Fauzi *et al.,* 2021; Morshdy *et al.,* 2022; Thaotumpitak *et al.,* 2023). Higher resistance for *A. hydrophila* was recorded by Hafez *et al.* (2018) against streptomycin (100%); cefotaxime and sulfamethoxazole (80% each); and chloramphenicol, oxytetracycline, amikacin, and cephalothin (60% each). Additionally, Dhanapala *et al.* (2021) found that antimicrobial resistance was 92.5% (amoxicillin), 63.4% (nalidixic acid), 23.6% (tetracycline), 18% (imipenem), 16.8% (trimethoprim-sulfamethoxazole), and 16.8% (gentamicin).

Table 4. Interpretation of the antimicrobial resistance of *A. hydrophila* isolates (n = 36).

Antimicrobial agents	Conc. (µg)	Resistance		Intermediate		Sensitive	
		No.	%	No.	%	No.	%
Tetracycline	30	36	100	0	0	0	0
Streptomycin	10	22	61.1	8	22.2	6	16.7
Ampicillin	10	33	91.7	3	8.3	0	0
Chloramphenicol	30	17	47.2	10	27.8	9	25
Kanamycin	30	9	25	6	16.7	21	58.3
Sulfamethoxazole	25	18	50	8	22.2	10	27.8
Gentamicin	10	7	19.4	3	8.3	16	44.4
Nalidixic acid	30	11	30.6	5	13.9	20	55.6
Ciprofloxacin	5	7	19.4	4	11.1	25	69.4
Norfloxacin	10	9	25	2	5.6	25	69.4
Amikacin	30	9	25	4	11.1	23	63.9
Imipenem	10	2	5.6	1	2.8	33	91.7
Penicillin	10	32	88.9	4	11.1	0	0
Amoxicillin	25	36	100	0	0	0	0
Cefotaxime	30	14	38.9	7	19.4	15	41.7
Ceftazidime	30	8	22.2	5	13.9	29	80.6

Table 5. Antimicrobial resistance profile of *A. hydrophila* (n = 36).

Species	Isolates No.	Antimicrobial resistance profile	No. of antibiotics	MAR index
A. hydrophila	12	T, SM, AX, ST, CM, K, G, CP, NT, AK, P, AX, CX, CZ	14	0.875
	7	T, SM, AX, ST, CM, K, G, CP, NR, IP	10	0.625
	6	T, SM, AX, CM, K, NA, AK, P, AX	9	0.562
	5	T, SM, AX, ST, G	5	0.312
	3	T, AX, P, AP	4	0.25
	3	T, AX, AP	3	0.188
Average				0.469

MAR: multiple antibiotic resistant index. T: tetracycline; SM: streptomycin; AP: ampicillin; ST: sulfamethoxazole; CM: chloramphenicol; K: kanamycin; G: gentamicin; NA: nalidixic acid; CP: ciprofloxacin; NR: norfloxacin; AK: amikacin; IP: imipenem; P: penicillin; AX: amoxicillin; CX: cefotaxime; CZ: ceftazidime.

The broad-spectrum resistance exhibited by *A. hydrophila* in this investigation could be due to bacteria's ability to endure elevated levels of antimicrobial agents, which in turn stimulated the activation of the efflux pump and generation of antioxidant compounds (Thaotumpitak *et al.,* 2023).

Concerning the resistance exhibited by *A. hydrophila* isolates (Table 5), most of the isolates demonstrated resistance to more than three antibiotics. Furthermore, the MAR index values for *A. hydrophila* ranged from 0.875 to 0.188, with an average of 0.469 (≥0.2), which indicated that the analyzed samples were contaminated at sources that presented a significant risk to human health and public safety (Morshdy *et al.,* 2022). The MAR index ≥0.2 for *A. hydrophila* was recorded by Ahmed *et al.* (2018) and Dhanapala *et al.* (2021), who indicated that MAR index ranged from 0.11 to 0.88, with an average of 0.489, while Hafez *et al.* (2018) and Morshdy *et al.* (2022) reported that MAR index ranged from 0.614 to 0.571, respectively, proving that the products were from a hazardous source of pollution. Volatility in MAR index was attributed to differences in sample sources, geographic dispersion, and methodology. The growing prevalence of germ resistance to medications made the quest for novel antimicrobial treatments a significant and continuous endeavor. Plant-based bioactive natural compounds greatly benefited drug design and discovery (Oikeh *et al.,* 2020).

Total phenolic and flavonoid contents of *Citrus reticulata* peel

Citrus reticulata peel, a by-product, is a significant source of naturally occurring compounds, especially polyphenols, that promote health and have a considerable potential to enhance the functional characteristics of food as a dietary additive (Fathy *et al.,* 2022). Polyphenolic compounds, including flavonoids and phenolic acids, because of their antioxidant properties, are essential fruit phytochemicals. The fruit peel, which makes up about half of the fruit weight, has the highest concentrations of naturally occurring products, such as sugars, flavonoids, carotenoids, folic acid, vitamin C, pectin, and volatile oils, all of which are extremely beneficial for human fitness as well as for the food industry (Zaki and Naeem, 2021).

Citrus peel is rich in phenolic compounds, which are used to create functional foods or as natural antioxidants to prevent oxidation in certain foods (Albishi *et al.,* 2013). Multiple studies have demonstrated the correlation between bacterial count and phenolic compounds' antioxidant properties. This correlation is accredited to the high redox potential of phenolic contents, which enable them to function as reducing agents, hydrogen donors, and singlet oxygen quenchers (Radha *et al.,* 2014).

The outcomes of this research (Figure 4) indicated that the *Citrus reticulata* peel extract had significantly higher total phenol content (946 mg GAE/100 g) and total flavonoid content (433 mg quercetin/100 g). The outcome was corroborated by the results of Oikeh *et al.* (2020) and Zaki and Naeem (2021). Numerous biological processes, such as antibacterial, antioxidant, and inflammatory processes, have been attributed to flavonoids. It is also known that they suppress cell division and alter enzymatic activity (Oikeh *et al.,* 2020). The major polyphenol compounds identified in *Citrus reticulata* peels (Table 6) were naringenin (3.327±0.15 mg/g) and catechin (1.192±0.04 mg/g). *Citrus reticulata* peels contain large flavonoids and several polyethoxylated flavones, uncommon in other plants (Rekha and Bhaskar, 2013).

Antimicrobial efficacy of *Citrus reticulata* peel extract

These peels could be suitable for incorporation into food products as functional additives (Abd El-ghfar *et al.,* 2016; Zaki and Naeem, 2021). In the current study, *Citrus*

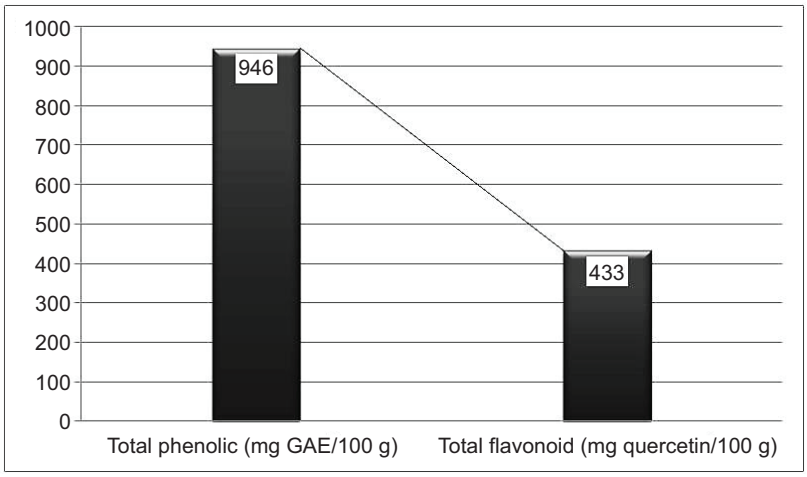

Figure 4. Total phenolic and flavonoid contents of *Citrus reticulata* peel.

Table 6. Total phenolic and flavonoid concentrations of *Citrus reticulata* peels.

Components	Concentration (mg/g)		
	Min.	Max.	Mean ± SE
Gallic acid	0.925	0.983	0.961 ± 0.02^{c}
Caffeine	0.022	0.029	0.028 ± 0.01^{e}
Coffeic acid	0.75	0.87	0.82 ± 0.03^{c}
Catechin	1.152	1.25	1.192 ± 0.03^{b}
Coumaric acid	0.053	0.073	0.064 ± 0.01^{e}
Chlorogenic acid	0.488	0.538	0.533 ± 0.02^{d}
Cinnamic acid	0.012	0.015	0.0146 ± 0.01^{e}
Dihydroxyiso Flavone	0.073	0.088	0.081 ± 0.01^{e}
Ellagic acid	0.011	0.019	0.014 ± 0.01^{e}
Ferulic acid	0.222	0.274	0.257 ± 0.03^{d}
Kaempferol	0.043	0.053	0.048 ± 0.01^{e}
Naringenin	3.196	3.488	3.327 ± 0.15^{a}
Propyl gallate	0.321	0.387	0.361 ± 0.02^{d}
Rutin	0.721	0.759	0.741 ± 0.01^{c}
Querectin	0.222	0.284	0.257 ± 0.02^{d}
Syringic acid	0.101	0.232	0.151 ± 0.04^{f}
Vanillin	0.000	0.050	0.021 ± 0.01^{e}

Values are expressed as mean ± standard error (SE) of three determinations.
Values with different superscript alphabets ($^{a–f}$) are significantly different by Tukey's test ($p < 0.05$).

reticulata peel was observed to lower *A. hydrophila* counts after 72 h of marination of citrus peel extract and the rate of decline of *A. hydrophila* increased from 18.7, 35.4, 54.3, and 61.9 log10 CFU/g after 0, 12, 24, and 48 hours, respectively, to 67.6% after 72 h of sinking at 100% Citrus reticulata peel extract concentration (Table 7).

The current investigation reveals that the *Citrus reticulata* peel extract may possess powerful antimicrobial activity against *A. hydrophila* because of it having the maximum concentrations of phenolics and flavonoids ($p < 0.05$). The potential synergistic properties of these phytochemical groups could account for the observed antimicrobial effects in this research. Their capacity to form by mixing with soluble and extracellular proteins and bacterial cell walls may be the source of their activity (Oikeh *et al.,* 2020; Singh *et al.,* 2020). Earlier research has demonstrated the antibacterial benefits of adding *Citrus reticulata* peel to a variety of meat products (Fathy *et al.,* 2022; Nishad *et al.,* 2018; Oikeh *et al.,* 2020; Zaki and Naeem, 2021). Functional foods possess biologically active components that supply essential nutrients and mitigate risk of diseases within the body. *Citrus reticulata* peel has the potential to act as a source of useful chemicals and preservatives in modern food items (Singh *et al.*, 2020).

Organoleptic assessment

A sensory assessment of chicken fillets prepared with altered ratios of *Citrus reticulata* peel during cold storage for 60 min at 4°C is shown in Table 8. The study confirmed that 50% and 75% of *Citrus reticulata* peel extract concentrations possessed the superior sensory criteria, with no significant variance in-between but significantly varied in comparison to the control, 25%, and 100% of *Citrus reticulata* peel extract concentrations. It was suggested that 100% of *Citrus reticulata* peel extract concentrations provided a more acidic environment to the samples. It could be the reason for the changes in sensory scores noted by panelists. The results are also supported by the research done by Gahruie *et al.* (2015) and Zaki and Naeem (2021).

Table 7. Effect of *Citrus reticulata* peel extract on *A. hydrophila* count (log_{10} CFU/g) at different marinating periods.

Treatment		Exposure time (h)				
		0	12	24	48	72
Control		6.26 ± 0.47[a]	6.88 ± 0.73[a]	7.37 ± 0.67[a]	8.23 ± 0.62[a]	9.12 ± 0.62[a]
T1	Mean ± SE	6.17 ± 0.28[b]	5.98 ± 0.34[b]	5.73 ± 0.28[b]	5.66 ± 0.72[b]	5.77 ± 0.25[b]
	R. count	0.03	0.13	0.22	0.31	0.36
	R. %	6.67	25.9	39.7	51.0	56.3
T2	Mean ± SE	5.92 ± 0.23[b]	5.84 ± 0.18[b]	5.52 ± 0.51[c]	4.88 ± 0.18[c]	4.72 ± 0.38[c]
	R. count	0.05	0.15	0.25	0.41	0.48
	R. %	10.9	29.2	43.7	61.1	66.8
T3	Mean ± SE	5.86 ± 0.37[b]	5.65 ± 0.75[c]	5.48 ± 0.24[c]	4.87 ± 0.52[c]	4.82 ± 0.72[c]
	R. count	0.06	0.17	0.26	0.41	0.47
	R. %	12.9	32.3	45.0	61.1	66.1
T4	Mean ± SE	5.68 ± 0.28[c]	5.54 ± 0.36[c]	4.87 ± 0.22[d]	4.76 ± 0.57[c]	4.65 ± 0.35[d]
	R. count	0.09	0.19	0.34	0.42	0.49
	R. %	18.7	35.4	54.3	61.9	67.6

The untreated control group only got a microorganism inoculation, while the following groups were immersed in *Citrus reticulata* peel extract solutions: 25% (T1), 50% (T2), 75% (T3), and 100% (T4). Values within the same column with different superscript alphabets ([a–d]) are significantly different at $p < 0.05$. Values are expressed as mean ± standard error (SE) of three determinations. R.: reduction.

Table 8. Effect of *Citrus reticulata* marination on organoleptic properties of chicken fillets.

Treatment	Sensory criteria					
	Appearance (5)	Odor (5)	Texture (5)	Flavor (5)	Overall (20)*	Grade
Control	4	4.2	5	5	18.2[b]	Very good
T1	4	4	4	4	16.0[c]	Good
T2	5	5	5	5	20.0[a]	Excellent
T3	5	5	5	5	20.0[a]	Excellent
T4	5	4.4	4.6	4.3	18.3[b]	Very good

*>5: spoiled, 5–10: poor, 10–15: middle, 15–18: good, 18–20: very good, and 20: excellent.
The untreated control group only got a microorganism inoculation, while the following groups were immersed in *Citrus reticulata* peel extract solutions: 25% (T1), 50% (T2), 75% (T3), and 100% (T4).
Values within the same column with different superscript alphabets ([a–b]) are significantly different at $p < 0.05$.

Since the panel members were not the same for each test, we suspected that personal rating differences could also contribute to the difference in the average of overall scores between tests. In addition, the samples that panelists assessed after each storage period could have differed sufficiently to affect their scores.

Conclusions

The results of this study demonstrated that antibiotic-resistant *Aeromonas*, particularly *A. hydrophila*, contaminate a wide range of meat products, posing a health risk for food-borne diseases and potentially facilitating their transmission. Therefore, it is crucial to apply measures that are more hygienic during all processing steps. Furthermore, the marination process with polyphenolic compounds, such as *Citrus reticulata* peel, can retard growth and virulence capacity of *A. hydrophila*, enhance sensory qualities, extend product's shelf life, and be a safe method of preserving meat products. Further research is required to detect the optimum conditions of marination for the maximum prolongation of product's shelf life.

Author Contributions

Nady Elbarbary and Reda Gomaa developed concept, design, and methodology of this research. Maha

Abdelhaseib and Marwa Ali collected and analyzed the material. Nermeen Malak and Mohamed M. Salem wrote, processed the data statistically and edited the manuscript. Ayman M. Al-Qaaneh and Mounir M. Bekhit performed supervision and investigation. All the authors approved the final version of the article.

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Declaration of Competing Interest

The authors disclosed no conflict of interests.

Data Availability

All data are provided in the manuscript.

Ethical Approval

The Scientific Research Committee and Bioethics Board of Aswan University, Faculty of Veterinary Medicine (10/2022), reviewed and approved the protocols used for this study.

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