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Validity of Cooking in Microwave and Gamma-irradiation on Highly Virulent *Aeromonas hydrophila* Isolates in Basa Fish Fillet

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Abstract

The purpose of the study was to verify the existence and pathogenicity of *Aeromonas hydrophila* (*A. hydrophila*) in fish by validating the bactericidal effects of microwave and Gamma radiation on infected fish fillets. A total of 100 frozen Basa fish fillet samples were collected randomly from different markets in Zagazig, Sharkia Governorate, Egypt, and subjected to microbiological examination. The results revealed a 14% prevalence rate of *A. hydrophila* in fish fillets, which were tested for the presence of seven virulence genes: *aerA*, *act*, *ast*, *alt*, *hyl*, *ahhR*, and *ahh1*. All isolates exhibited traits related to virulence. The most predominated gene was *ast* (64.2%), followed by *aerA*, *act*, *hyl*, and *ahhR* (57.14% for each). Then, an experimental protocol for several treatments showed that Gamma radiation at a dose of 1 kGy decreased the count of *A. hydrophila* in fish fillets by 4.4 $\log_{10}$ CFU/g whereas doses of 2 and 3 kGy eradicated the pathogen. The same positive effect was recorded towards the microwave after cooking for 1, 2, and 3 minutes. Therefore, using microwave cooking and Gamma-irradiation alone and in combination with other decontamination methods may be more efficient in lowering the pathogen counts in fish meat.

KEYWORDS
Aeromonas hydrophila, Fish fillets, Gamma radiation, Microwave, Undeniable virulence

INTRODUCTION

Recently, fish has received more attention in human nutrition all over the world (Ahern *et al*., 2021). Due to its palatability, nutritional, and healthful animal protein, as well as its role in resolving the issue of animal meat shortages, especially in the last ten decades. Fish is widely consumed by humans because it contains highly digestible protein, essential amino acids, minerals, and a respectable amount of B vitamins, all of which help to maintain excellent health.

Fish also has two different levels of omega-3 polyunsaturated fatty acids, which are essential for healthy growth and lower cholesterol and heart disease risk (Al-Bader, 2008).

Concerns have been raised about *Aeromonas* species as potential causative agents in both immunocompetent and immunocompromised people, as well as lower vertebrates, particularly fish (Nagar and Bandekar, 2011).

Aeromonas hydrophila (*A. hydrophila*) is regarded as a serious zoonotic foodborne pathogen. Septic arthritis, meningitis, skin and wound infections, diarrhea (traveler's diarrhea), and fulminating septicemia were just a few of the terrible effects of *A. hydrophila* on humans (Salunke *et al*., 2015).

A. hydrophila possesses a comprehensive virulence system that includes the ability to form biofilms, exhaust specific metabolic pathways, control virulence factor expression by quorum sensing, and produce and exude virulence factors such adhesins, cytotoxins, hemolysins, lipases, and proteases (Beaz-Hidalgo *et al*., 2013). Moreover, virulence capacity helps *Aeromonas* species grow and produce toxins in refrigerated conditions as it cannot effectively control the microorganisms. Radiation treatment is an applicable method of removing foodborne pathogens in meat products (Meng and Doyle, 2002).

Gamma radiation is highly penetrating and can inactivate pathogens that may have entered meat tissue (Abu-Tarboush *et al*., 1997). The organoleptic or nutritional qualities of poultry and meat products are unaffected by irradiation, which guarantees microbiological safety. Food can be exposed to microwave and gamma-irradiation to eradicate disease-causing microorganisms and reduce the frequency of foodborne diseases (Roberts, 2014). Irradiation is a secure and reliable method that can stop numerous foodborne illnesses and microbiological spoilage while preserving nutritional value and sensory appeal (Mohamad *et al*., 2016). Nagar and Bandekar (2011) proved that Gamma radiation prevents infection by dissolving chemical bonds in molecules that could otherwise break DNA and destroy cells when they encounter bacteria and other pathogens, all without raising the temperature of the product. Thus, Gamma radiation can be applied to prepare deep-frozen and chilled items without causing sensory or quality alterations. As a result, fishers and consumers would benefit from the widespread use of seafood irradiation.

Consequently, the objectives of the current investigation were to ascertain the frequency and kinds of *Aeromonas* spp. in

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fish fillets in Egypt and reveal virulence-related genes in the isolated bacteria. In addition, evaluate how microwave heating and Gamma radiation can affect the count of this pathogen.

MATERIALS AND METHODS

Collection of Basa fish samples

During the period from February 2022 to August 2022, 100 frozen fish fillet samples were selected at random from several fish markets in Zagazig city, Sharkia Governorate, Egypt. The samples were categorized, separated into sterile plastic bags, and immediately transported to the lab for aseptic bacterial isolation and identification. Preparation of fish samples

A hot spatula was used to sterilize and remove the surface. About 25 grams of back muscles were aseptically homogenized for 2.5 minutes with 225 ml of 0.1% sterile alkaline peptone water and then left to stand for 5 minutes (ICMSF, 1996).

Isolation and identification of Aeromonas hydrophila

Isolation of *A. hydrophila* was performed according to the protocol previously established (Aboyadak *et al*., 2015). In brief, a loopful of the specimen was transferred to tubes of tryptic soy broth (TSB, Oxoid®, USA) and incubated for 18–24 hours at 37 °C. For isolation of typical *A. hydrophila* colonies, a loopful was sub-cultured with streaking on a Rimler-Shotts medium (HiMedia, India) with novobiocin (Oxide®, USA) supplement and Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar (Oxoid-CM333), and then incubated for 24 hours at 37 °C. Typical colonies were sequentially undergone comprehensive biochemical analyses comprising oxidase, triple sugar iron agar (TSI), urea, indole, motility, Voges Proskauer, inositol, and rhamnose.

Molecular investigation and characterization of isolates

The manufacturer's instructions were followed to extract the bacterial DNA using a QIAamp DNA Mini kit from Qiagen GmbH in Hilden, Germany. In the Biotechnology Laboratory of the Animal Health Research Institute, Zagazig Branch, Egypt, PCR amplifications were conducted using the oligonucleotide primers and cycling conditions listed in Table 1. The standard strain (ATCCTM 49140TM) was used as a positive control and the negative control was a PCR mixture without a DNA template.

The PCR products were separated using electrophoresis on 1.5% agarose gel (AppliChem, Germany, GmbH). Alpha Innotech, Biometra's gel documentation framework displayed images of the gel, and automatic image captures software protein simple, formerly cell Bioscience, USA, evaluated the data.

Evaluation of the efficacy of irradiation on the inoculated fillet samples with the most virulent *A. hydrophila* isolates

Preparation of samples and inoculum

Another 140 Basa fish fillet samples were brought from the fish market in Zagazig City and free from *A. hydrophila* to be applied in the experiment. The most virulent *Aeromonas hydrophila* isolate was used as the inoculum. The isolate was transferred to test tubes containing 10 ml of tryptic soy broth using the sterile loop and incubated for 24 hours at 37 °C. After incubation, bacterial cultures were transferred to sterile tubes and centrifuged at 5000xg for 10 minutes. The pellets were re-suspended in 10 ml of 0.1% peptone water, and serially diluted to concentrations of 10^{-1} to 10^{-8} after removing the supernatant (Doyle *et al*., 2020).

Treatment of samples

According to Mohamad *et al*. (2016), seven groups of fillet samples were prepared after aseptically weighing. *A. hydrophila* isolate was injected into ten grams per each to generate a concentration of 10^6 CFU/g. The first group served as a control group without any treatment. Gamma-ray treatments of 1, 2, and 3 kGy were given to the second, third, and fourth groups, respectively. The irradiation was carried out using the Indian Gamma Cell (Ge 4000A), which is located at the National Center for Radiation Research and Technology (NCRRT), in Nasr City, Cairo, Egypt. The source was intended to be Cobalt 60 Gamma-ray, with a dosage rate of irradiation of six kGy/hour throughout the protocol period.

During 1, 2, and 3 minutes, respectively, the fifth, sixth, and seventh groups were subjected to microwave cooking. In a home microwave oven, microwave radiation was used (Daewoo, KOG185H, 50 Liter Capacity, with a rotating glass plate, power of 1000W).

Physical analysis of the irradiated samples

The panelists evaluated the color, texture, and smell of fish

Table 1. Oligonucleotide primer sequences, annealing conditions, and product size of *A. hydrophila* target genes.

Target gene	Primers sequences	Annealing (T°C)	Product size (bp)	Reference
16S rRNA	F; GGGAGTGCCTTCGGGAATCAGA R; TCACCGCAACATTCTGATTTG	55	356	Wang *et al*. (2003)
aerA	F; CAAGAACAAGTTCAAGTGGCCA R; ACGAAGGTGTGGTTCCAGT	55	309	Wang *et al*. (2003)
act	F; AGAAGGTGACCACCACCAAGAACA R; AACTGACATCGGCCTTGAACTC	55	232	Nawaz *et al*. (2006)
ast	F; TCTCCATGCTTCCCTTCCACT R; GTGTAGGGATTGAAGAAGCCG	55	331	Sen and Rodgers (2004)
hyl	F: GGCCGGTGGCCCGAAGATACGGG R: GGCGGCGCCGGACGAGACGGG	60	600	Zhou *et al*. (2013)
alt	F: TGACCCAGTCCTGGCACGGC R: GGTGATCGATCACCACCAGC	60	442	Zhou *et al*. (2013)
ahyR	F: TCTTGACGTGATGGGGTTGG R: GGCGGTGATGAACGACAGTA	60	106	Sun *et al*. (2021)
ahyI	F: CAGATGGGAGGTAGAAAACGAG R: TGGGTATCAGGGGTATCGAAA	60	123	Sun *et al*. (2021)

fillets that had undergone Gamma radiation treatment. The standard fillet was non-irradiated. The panelists had access to this standard alongside the treats and may compare it. A 5-point scale was used by the panel to evaluate each sample: Numbers 1 through 5 are very poor, poor, common, good, and very good (Aziz *et al*., 2002).

Bacterial Count

The 225 ml of 0.1% peptone water and 25 grams of each sample were aseptically transferred to a sterile stomacher bag and stirred for 60 seconds. An aliquot of each mixture was transferred aseptically, dispersed on the surface of selective medium plates, and incubated for a whole night at 37 °C. After incubation, colony-forming units (CFU) from each sample were counted, documented, averaged, and estimated as colony-forming units (CFU) per gram (Stratev *et al*., 2012).

Statistical analysis

The consequence of Gamma-irradiation on the physical parameters of fish fillets was determined by one-way ANOVA with the degree of significance set at α = 0.05. Meanwhile, the effects of microwave cooking and Gamma-irradiation on the enumeration of *A. hydrophila* in fish fillets were examined by Kruskal–Walli's test. Virulence genes pattern of *Aeromonas hydrophila* isolated from fillet fish were studied by chi-square test. Statistical significance between means was set at a p-value less than 0.05. Figures were fitted by the GraphPad Prism software 9.0 (GraphPad, US).

RESULTS

Occurrence and characteristics of isolates

In the current investigation, fourteen *Aeromonas* isolates were identified in the fish fillet samples and all of them were confirmed to be *A. hydrophila*, giving a prevalence rate of 14%. (14 of 100). Typical yellow colonies were Gram-negative, rod-shaped, and positive to oxidase, indole, motility, Triple Sugar Iron, Voges Proskauer, and rhamnose. Moreover, they had adverse reactions to urea and inositol.

PCR-based verification and identification of virulence genes

As shown in Fig. 1, all fourteen isolates tested positive for *Aeromonas hydrophila* (16S rRNA) at 356 bp. Figs. 2-3 detail the distribution of virulence genes across all *A. hydrophila* isolates.

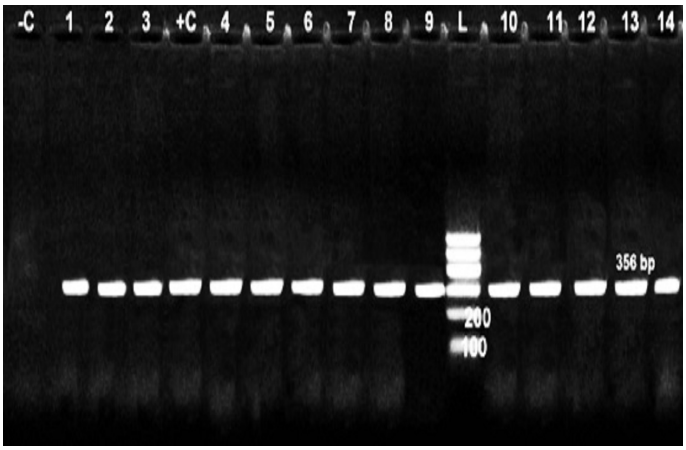

Fig. 1. Agarose gel electrophoresis of PCR for amplification products of 16S r RNA among 14 *Aeromonas* isolates; Lane +C: Control positive, Lane L: 100-bp ladder (marker); Lane -C: Control negative.

The results of the current research indicated that virulence gene associations were detected in all obtained isolates. One out of 14 isolates was positive for all examined genes giving their characteristic bands. Moreover, four isolates (28.5%) had five tested genes. Even though non-significant differences between the positive and negative cases (p = 0.620), the prevalence of positive cases was higher than the negative ones for all consid-

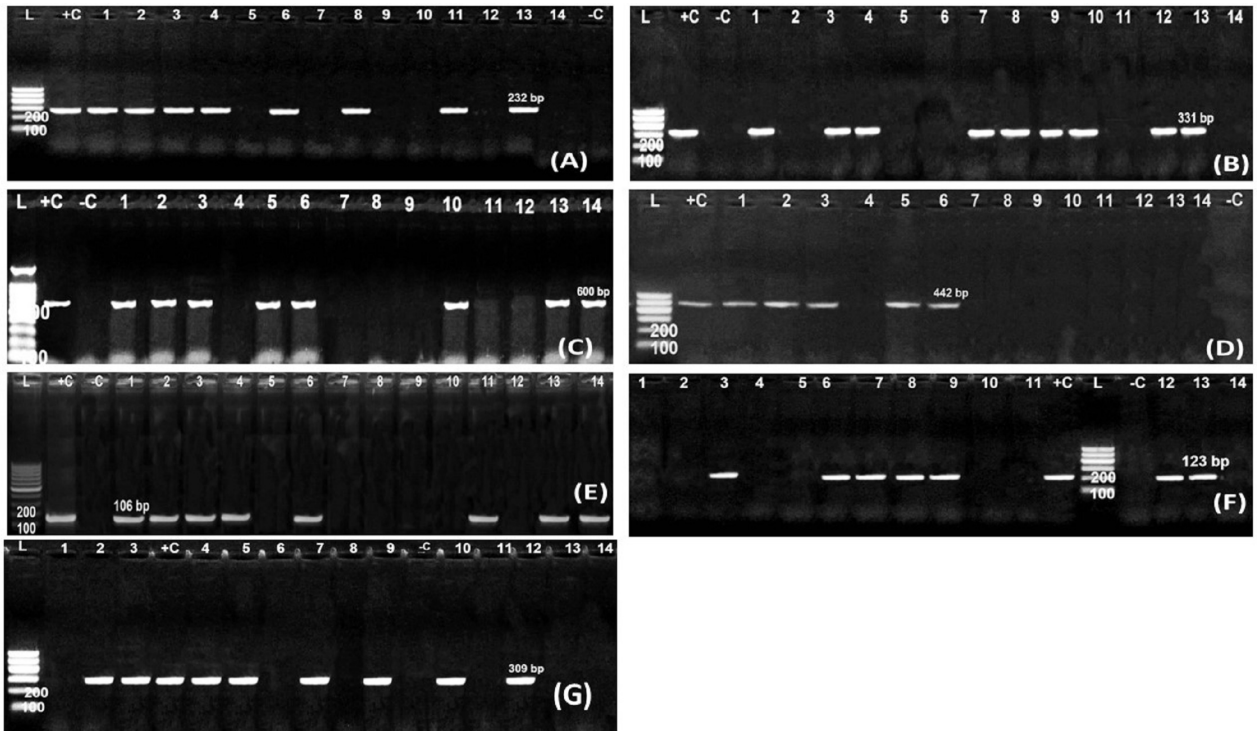

Fig. 2. Agarose gel electrophoresis of PCR for amplification products of different tested virulence genes among 14 *A. hydrophila* isolates at specific molecular sizes; Lane +C: Control positive, Lane L: 100-bp ladder (marker); Lane -C: Control negative; (A) *act*, (B) *ast*, (C) *hyl*, (D) *alt*, (E) *ahyR*, (F) *ahyI*; and (G) *aerA*.

ered genes except the *alt* gene, whereas equal values were perceived for the *ahyI* gene.

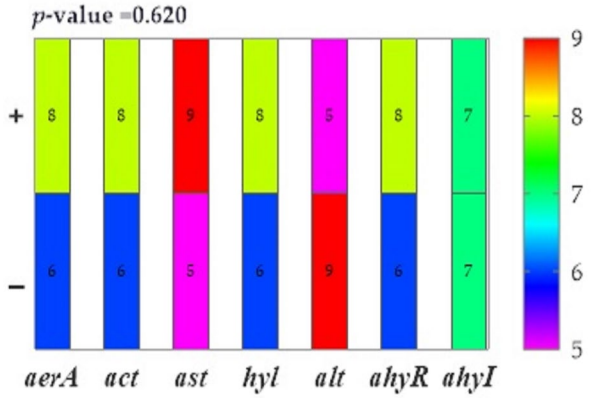

Fig. 3. Virulence genes pattern of *Aeromonas hydrophila* isolated from 100 fillet fish samples.

Effect of Gamma radiation and microwave cooking

There were significant effects of Gamma-irradiation on *A. hydrophila* count (log_{10} CFU/g), being in significantly decreases with increased levels of Gamma-irradiation doses ($p < 0.0001$; Fig. 4A). Concerning log_{10} reduction, the maximum values were observed with the dose of 1kGy ($p < 0.0001$; Fig. 4-B). The results showed that Gamma radiation treatment reduced the amount of *A. hydrophila* in fish fillets by 4.4 log_{10} CFU/g, while radiation doses of 2 and 3 kGy eliminated the organism from these groups when compared to the untreated group (control), where there were 10^6 CFU/g of *A. hydrophila*.

Figure 5 depicts how microwave cooking affected the quantity of *A. hydrophila* in fish fillets. *A. hydrophila* level in fish fillets peaked in the control group before starting to sharply diminish with prolonged microwave heating times (p= 0.0001).

Sensory evaluation

Fish exposed to Gamma radiation at doses of 1, 2, and 3 kGy exhibited no differences compared to the untreated control group. Even so, significant differences were found between the groups of 2 and 3 kGy and the control group (p 0.05; Fig. 6) despite statistical analysis showing that the effects of Gamma-irradiation on physical characteristics including the odor and color were not significant ($p > 0.05$).

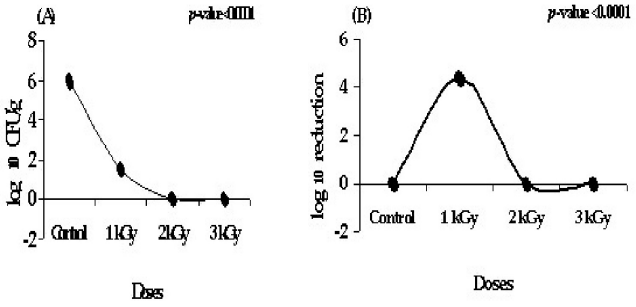

Fig. 4. Effect of Gamma-irradiation on *A. hydrophila* count (log_{10} CFU/g) of the examined fish fillet.

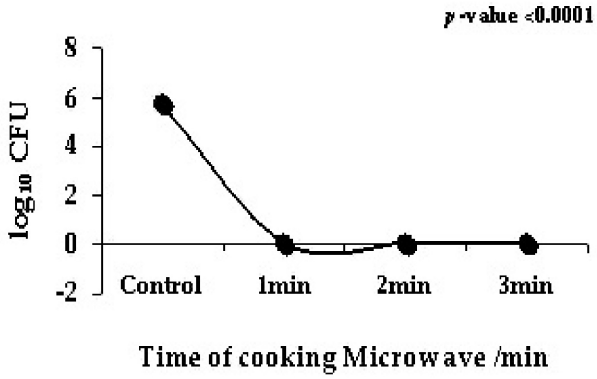

Fig. 5. Influence of microwave cooking on the enumeration of *A. hydrophila* in fish fillets.

DISCUSSION

The inquiry of microbial quality in fish is a significant issue for public health. Raw fish fillets may serve as a carrier for the spread of some pathogens, including *Aeromonas* species which

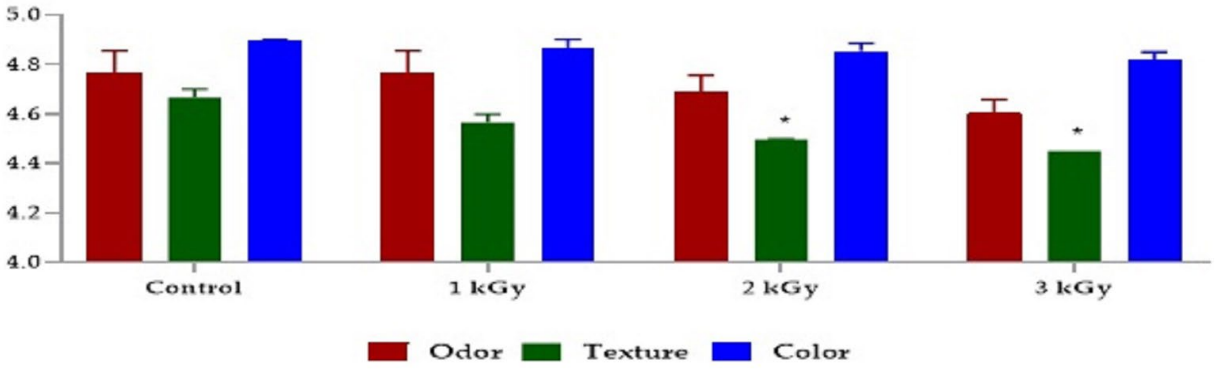

Fig. 6. Effect of Gamma-irradiation on physical parameters of fish fillets.

is regarded as one of the developing foodborne microorganisms (Massa *et al*., 2001).

Herein, the study assessed the existence of *Aeromonas* spp. in frozen fillet fish consumed in Sharkia governorate, Egypt. Relatively high contamination (14%) of the inspected fish by *Aeromonas hydrophila* was distinguished. This outcome signified that the frozen fish under investigation had been contaminated during storage, shipping, and marketing. In fish markets, keeping fish at room temperature encourages the growth of *Aeromonas* spp. (Hafez *et al*., 2018). Moreover, unsanitary practices of fish handlers and contaminated equipment during the filleting fish process can increase the bacterial burden of such products.

These findings agreed with the information provided by other nations on feedback regarding *Aeromonas*. For example, in India, *Aeromonas* spp. was isolated from 22.22% of fish samples (Praveen *et al*., 2016); while in Vietnam, it was isolated from 46.6% of the examined samples (Nhinh *et al*., 2021).

Aeromonas hydrophila is known to cause a variety of diseases in humans, including food poisoning, gastroenteritis, septicemia, skin conditions, soft tissue infections, and muscle infections (Batra *et al*., 2016).

The data acquired by PCR amplification of targeted 16S rRNA sequences supported the findings obtained by preliminary isolation using conventional methods. As 14 isolates harbored bands at 356 bp that are specific for *A. hydrophila*. These are consistent with the results of previous investigations (Aboyadak *et al*., 2015; El-Demerdash and Raslan, 2019; Omar *et al*., 2016).

The recognition of virulence genes can greatly aid our comprehension of *A. hydrophila*'s potential pathogenicity.

The aerolysin (*aerA*), cytotoxic enterotoxin (*act*), and hemolysin (*hlyA*) genes were perceived in 57.14% of isolates. Aerolysin is a toxin that forms pores and binds to particular receptors on the target cell membrane. After activating proteolytic pores or channel formation, this toxin impairs membrane permeability, leading to osmotic lysis and cell death. Enterotoxic, hemolytic, and cytotoxic characteristics are all displayed by cytotoxic enterotoxin (*act*). As a result, this protein is thought to be the most influential virulence gene that *A. hydrophila* possesses (Xu *et al*., 1998). Emeish *et al*., (2018) and Omar *et al*. (2016) proved the functional activity of the *act* gene in the pathogenicity of *A. hydrophila* in fish. In intestinal epithelial cells, *act* causes fluid exudation and is involved in tissue damage (Sha *et al*., 2002). Hemolysis is known to be initiated by hemolysin (*hlyA*), which culminates in anemia by damaging the RBC cell membranes (Tomás, 2012).

Cytotonic enterotoxins that are heat-labile (*alt*) and heat-stable (*ast*) are what cause *A. hydrophila* to promote gastroenteritis in infected consumers (Albert *et al*., 2000; Sha *et al*., 2002) and distinguished in 64.2 % and 28.5 % of the 14 obtained isolates.

Biofilm synthesis is crucial for infection and illness progress by pathogenic microorganisms. The lower metabolic state and decreased therapeutic penetration in the biofilm matrix make bacteria harboring biofilm more resistant to all treatments (Salehzadeh *et al*., 2016; Sharaf *et al*., 2022). Additionally, the creation of biofilms in the aquatic environment promotes bacterial survival, contaminates the entire ecosystem, and consequently makes aquatic animals more susceptible to the pathogen (Packiavathy *et al*., 2013). The *ahyI*/R genes are primarily responsible for the variety of planktonic growth to biofilm growth in *A. hydrophila*.

In this study, about 85.7% of isolates harbored biofilm-involved genes where *ahyR* was detected in 57.14%, *ahyI* in 50%, and both in 21.4%. Similar results were obtained from previous studies (Simon *et al*., 2016; Mzula *et al*., 2020; Tanhay Mangoudehi *et al*., 2020).

The existence of virulence factors in examined *A. hydrophila* isolates suggests that these factors play a predominant part in the pathogenesis of the microorganism (El-Bahar *et al*., 2019). According to the results of Sha *et al*. (2002), they found a considerable rise in the pathogenicity of *A. hydrophila* with several virulence genes. Therefore, we apply the experiment with the most virulent isolates that harbor all tested virulence genes.

The treatment evaluation results agreed with Mohamad *et al*. (2016), who showed that Gamma-irradiation diminished the quantity of *A. hydrophila* by 10^4 CFU/g in fish fillets and eliminated it at doses of 2 and 3 kGy. Moreover, Nagar and Bandekar (2011) noted that fish samples comprising 10^5 CFU/g of *A. hydrophila* were eradicated by 1.5 kGy of Gamma radiation. According to a study by Özbacs *et al*. (1996), the amount of *A. hydrophila* in meat samples decreased as the irradiation level increased, and a dose of 0.75 kGy was sufficient to kill roughly 10^4 CFU/g of *A. hydrophila* in a meatball. Arslan and Küçüksari (2015) studied the responses of mola fish to 2.5, 5.0, 7.5, and 10 kGy of Gamma radiation and discovered that 2.5 kGy completely removed the *Aeromonas* spp. where *A. hydrophila*, like all Gram-negative bacteria, was incredibly vulnerable to Gamma radiation (Mahin *et al*., 2011).

Regarding microwave treatment, our findings agreed with those obtained by Huang *et al*. (1993) and Mohamad *et al*., (2016) who noticed that the catfish fillets that had been microwave-cooked to a temperature of 70°C and then injected with 10^8 CFU/ml of *A. hydrophila* became devoid of the microbe. In their investigations of the impact of microwave cooking on bacterial survival in various products of animal origin, Aziz *et al*., (2002) discovered that longer exposure times were able to eliminate the pathogenic bacteria effectively. Bacterial enumerations were reduced by 1 log cycle in 20 seconds and by 2 log cycles in a 30-second exposure, showing that the contaminated bacteria may be successfully eliminated by using a lengthy contact period. Additionally, Quesada *et al*. (2003) showed that the degree of bacterial obliteration was significant (p 0.005) after injecting minced meat with 10^7 and 10^9 CFU/ml of *A. hydrophila* and heating it in the microwave for 30, 60, 90, and 120 seconds.

Examinations results of the impact of Gamma-irradiation on the physical parameters of fish fillets were in line with Mohamad *et al*. (2016) and Mohamed and El-Mossalami, (2009). They observed no alterations in the sensory properties of tilapia fish fillets at doses of 1, 2, and 3 kGy. Aworh *et al*. (2002)suggested that this low Gamma radiation dose, up to 6 kGy, suppressed microbiological development and did not significantly influence the appearance, taste, texture, and general acceptability of Nigerian fish products. Özden *et al*. (2007)found that Gamma radiation of 2.5 and 5 kGy had no impact on the sensory quality of sea bass. Chen *et al*. (2007) detected that low-dose Gamma rays up to 2 kGy critically decreased all bacteria in crab meat without altering their physical characteristics.

CONCLUSION

According to the results of the current investigation, *Aeromonas hydrophila* was found to be present in the majority of the Basa fish fillet samples, which may have contributed to the spread of a potential foodborne disease. Further isolated *A. hydrophila* carried virulent genes that are extremely dangerous to mental livelihoods. Additionally, all isolates of *Aeromonas* are very sensitive to Gamma radiation dosages of 2 and 3 kGy, as well as 1, 2, and 3 minutes of microwave cooking, which are effective means of reducing and averting the threat without any changing in its sensory qualities. It is recommended that use Gamma-irradiation alone or in combination with microwave cooking to eliminate *A. hydrophila*. Sanitation precautions should also be applied when handling, preparing, processing, and storing food to reduce the presence of pathogens.

CONFLICT OF INTEREST

The authors affirm that they have no conflicts of interest.

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