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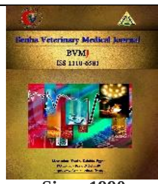

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Novel approaches with acetic acid, liquid smoke, and *Lactobacillus rhamnosus* for mitigating some heavy metals in Nile fish.

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ABSTRACT

Mercury and cadmium are toxic heavy metals that can bioaccumulate in freshwater fish, posing a health risk to human consumers. This study analyzed residue levels of these metals in 105 randomly sampled fish of three common species - *Clarias lazera, Oreochromis niloticus*, and *Bagrus bayad* - (35 each) from markets in Gharbia, Egypt. Concentrations were compared to national and international standards to evaluate suitability for human consumption. Further interventions using acetic acid, liquid smoke, and *Lactobacillus rhamnosus* probiotic cultures were tested for their efficacy in reducing or controlling these toxic residues in farmed fish. The results showed that 44.8% of all Nile fish samples exceeded mercury's maximum residue limit (MRL), while 25.7% exceeded the MRL for cadmium based on Egyptian standards. Single applications of 5% acetic acid and 3% liquid smoke reduced experimentally inoculated mercury by 52.8% and 64.2%, respectively. *L. rhamnosus* treatment achieved 71.6% reduction. For cadmium, the reduction was 43.5% for acetic acid, 56.0% for liquid smoke, and 61.5% with *L. rhamnosus*, respectively.

1. INTRODUCTION

Fish serves as the principal animal protein source for many populations globally, especially coastal communities and people living along major lakes and rivers where fishing resources are abundant (Akpalu and Okyere, 2022). For example, Egyptians obtain around 45% of their total animal protein intake from fish, as access to diverse low-cost fresh and processed fish is higher in its Nile-situated cities compared to inland regions (Mohamed et al., 2022).

Mercury (Hg) and cadmium (Cd) are toxic metals that can cause various adverse effects on human health, such as neurological disorders, kidney damage, cancer, and birth defects. The main source of exposure for most people is through food, especially fish and seafood (Balali-Mood et al., 2021)

The Nile River is the longest river in the world and a major source of freshwater fish for many African countries. However, the Nile River is also subjected to various anthropogenic activities, such as industrial effluents, agricultural runoff, mining, and urbanization, that can introduce Hg and Cd into the aquatic ecosystem (Goher et al., 2021)

One of the possible methods to reduce the levels of Hg and Cd in fish is to use natural preservatives, such as acetic acid and liquid smoke, that can inhibit the growth of microorganisms and enhance the sensory quality of fish. Acetic acid is a weak organic acid that can lower the pH of fish tissue and form complexes with metal ions, thus reducing their solubility and bio-accessibility (Chan et al., 2021). Liquid smoke is a complex mixture of phenolic compounds, organic acids, carbonyls, and other volatile substances that can impart antimicrobial, antioxidant, and flavoring properties to fish (Andhikawati and Pratiwi, 2021). However, the effects of acetic acid and liquid smoke on the levels of Hg and Cd in fish may depend on several factors, such as the concentration, time, and temperature of treatment (Swastawati et al., 2022)

Another potential method to control the levels of Hg and Cd in fish is to use probiotics, such as *Lactobacillus rhamnosus*, which can produce lactic acid and other metabolites to chelate metal ions, thus reducing their availability and toxicity depending on the strain, dose, and duration of administration (Abdel-Megeed, 2020).

This study aimed to assess the levels of mercury and cadmium residues in commonly consumed freshwater fish species and investigate the efficacy of interventions using acetic acid, liquid smoke, and *Lactobacillus rhamnosus* probiotic cultures in reducing or controlling the bioaccumulation of these toxic heavy metals in farmed fish, with the ultimate goal of providing insights into potential mitigation strategies for ensuring the safety of fish as a food source for human consumers..

2. MATERIAL AND METHODS

2.1. Collection of samples:

One hundred and five random samples of fresh fish represented by *Clarias lazera, Oreochromis niloticus,* and

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Bagrus bayad fillets (35 of each) were collected from different fish markets in Gharbia governorate, Egypt. The collected samples were kept in an ice box and transferred directly to the laboratory for determination of their residues of heavy metals (mercury and cadmium).

2.2. Sample preparation

2.2.1. Washing procedures (AOAC, 2006):

The equipment was cleaned with deionized water, soaked in hot diluted HNO_3 (10%) for 24 hours, rinsed with deionized water, and dried to remove metals. Digestion vessels were soaked in water and soap for 2 hours, rinsed with tap water, distilled water, a mixture of deionized water, conc. HCl, and H_2O_2, and 10% HNO_3, and then air-dried.

2.2.2. Digestion technique (Staniskiene et al., 2006):

Concerning cadmium determination, 1 g of each sample was digested in a mixture of 65% nitric acid and 40% perchloric acid. For mercury determination, 0.5 g of each sample was digested in concentrated H_2SO_4/HNO_3 solution (1:1). All samples were heated in a water bath for 4 hours to ensure complete digestion. The digestion tubes were shaken every 30 minutes during heating, then cooled and diluted with deionized water. They were reheated to ensure complete digestion and diluted to 25 ml. The digests were filtered, and the filtrates were collected for analysis.

2.2.3. Preparation of blank and standard solutions (Andreji et al., 2005):

The blank and standard solutions were prepared using the same method as the wet digestion method. The blank solution consisted of 10 parts of nitric acid and 1 part of H_2O_2, diluted with 25 parts of deionized water. The standard solutions were prepared using pure certified metal standards and diluted with the same mixture of nitric acid and H_2O_2.

2.3. Quantitative determination of heavy metal residues:

The digest, blanks, and standard solutions were aspirated by Atomic Absorption Spectrophotometer (VARIAN, Australia, model AA240 FS) and analyzed for mercury and cadmium concentrations.

2.4. Experimental part (Control of heavy metals):

2.4.1. Preparation of bacterial suspension:

The probiotic *Lactobacillus rhamnosus* strain was obtained from Food Center analysis, Faculty of Veterinary Medicine, Benha University. The strain was grown in Brain Heart Infusion (BHI) Broth for 24 hours at 37°C. The viable count of the strain was determined using the plate count method on BHI Agar (Halttunen et al., 2007).

2.4.2. Binding assay

The fish fillets (1 Kg) were mixed with 2×10^7 bacteria and one of these metal solutions: 50 mg/Kg mercury and 20 mg/ Kg cadmium. This was based on Halttunen et al. (2008). The mixture was shaken softly for 24 hours. The experiment compared fish fillets with metals only (control) and with metals and bacteria (test). The metal levels were measured by atomic absorption after acidifying the samples with ultrapure HNO_3.

2.5. Preparation of acetic acid:

Acetic acid was prepared at a concentration of 5% in which the fish fillet samples were soaked for 30 minutes and then examined for determination of its effect on the inoculated heavy metals. (Fronthea Swastawati et al.,2022)

2.6. Application of liquid smoke:

The concentration of liquid smoke was 3%. The tested samples of fish fillets were sprayed with liquid smoke and left for 6 minutes then examined for determination of its effect on the inoculated heavy metals. (Andhikawati and Pratiwi, 2021)

2.7. Statistical Analysis:

The obtained results were statistically analyzed using a using One-way analysis of variance (ANOVA) for heavy metals in the examined samples of Nile fish according to Feldman et al. (2003). $P<0.05$ was significant. Values were expressed as means ± standard Error (SE).

3. RESULTS

Table (1) showed the occurrence rates of mercury residues in 105 tested samples across three Nile fish species. Among these, more than half (56.2%) tested were positive for mercury contamination. Clarias lazera exhibited the highest occurrence rate (68.6%), followed by *Oreochromis niloticus* (54.3%), and Bagrus bayad (46.2%). In terms of average levels, Clarias lazera demonstrated significantly higher ($P< 0.05$) concentrations (1.28 mg/kg) compared to *Oreochromis niloticus* (0.94 mg/kg) and Bagrus bayad (0.67 mg/kg).

Table 1 Occurrence and levels of mercury residues in the examined samples (n=35).

Fish Species	Positive Samples No	%	Min.	Max.	Mean ± S.E
Clarias lazera	24	68.6	0.33	1.87	1.28 ± 0.02^{A}
Oreochromis niloticus	19	54.3	0.25	1.41	0.94 ± 0.01^{B}
Bagrus bayad	16	15.2	0.14	0.98	0.67 ± 0.01^{C}

Means with different superscript letters in the same column are significantly different at ($P< 0.05$).

Table (2) categorized the acceptance rates and percentages of the examined Nile fish samples based on Egyptian regulatory standards for allowable mercury residues (0.5 mg/kg). For *Clarias lazera*, 16 out of 35 samples (45.7%) were accepted and 19 (54.3%) were unaccepted. For *Oreochromis niloticus*, 20 samples (57.2%) were accepted and 15 (42.8%) were unaccepted, respectively. For *Bagrus bayad*, 22 samples (62.9%) were accepted and 13 (37.1%) were unaccepted.

Table 3 Acceptability of the examined Nile fish based on their residues of mercury (n=35).

Nile fish species	EOS (mg/Kg)*	Accepted samples No.	%	Unaccepted samples No.	%
Clarias lazera	0.50	16	45.7	19	54.3
Oreochromis niloticus		20	57.2	15	42.8
Bagrus bayad		22	62.9	13	37.1
Total (105)		58	55.2	47	44.8

* Egyptian Organization for Standardization "EOS" (2010).

On the other hand, table (3) recorded the occurrence and levels of Cd residues in the same 35 fish samples. *Clarias lazera* had the highest percentage of positive samples (45.7%) and the highest mean cadmium level (0.45 mg/kg). *Oreochromis niloticus* had lower values (37.1% and 0.27

mg/kg), and *Bagrus bayad* had the lowest (31.4% and 0.16 mg/kg).

Table 3 Occurrence and levels of cadmium residues in the examined samples (n=35).

Fish Species	Positive Samples No	%	Min.	Max.	Mean ± S.E*
Clarias lazera	16	45.7	0.06	0.69	0.45 ± 0.01^{A}
Oreochromis niloticus	13	37.1	0.05	0.39	0.27 ± 0.01^{B}
Bagrus bayad	11	31.4	0.03	0.24	0.16 ± 0.01^{C}

*Means with different superscript letters in the same column are significantly different at (P<0.05).

Table (4) presented the acceptance rates and percentages for Cd, with a regulatory limit of 0.1 mg/kg. For *Clarias lazera*, 24 out of 35 samples (68.6%) were accepted and 11 (31.4%) were unaccepted. For *Oreochromis niloticus*, 25 samples (71.4%) were accepted and 10 (28.6%) were unaccepted. For *Bagrus bayad*, 29 samples (82.9%) were accepted and 6 (17.1%) were unaccepted.

Table 4 Acceptability of the examined Nile fish based on their residues of cadmium (n=35).

Nile fish species	EOS (mg/Kg)*	Accepted samples		Unaccepted samples	
		No.	%	No.	%
Clarias lazera	0.10	24	68.6	11	31.4
Oreochromis niloticus		25	71.4	10	28.6
Bagrus bayad		29	82.9	6	17.1
Total (105)		78	74.3	27	25.7

* Egyptian Organization for Standardization "EOS" (2010).

As shown in table (5) the efficacy of different treatments (acetic acid 5%, liquid smoke 3%, *Lactobacillus rhamnosus*) in reducing experimentally inoculated Hg and Cd residues in fish fillets after specified contact times (30 minutes, 6 minutes, and 24 hours, respectively). For Hg, the control level was 50 mg/kg. After treatment with acetic acid, liquid smoke, and *L. rhamnosus*, the residue levels were reduced to 23.6 mg/kg (52.8% reduction), 17.9 mg/kg (64.2% reduction), and 14.2 mg/kg (71.6% reduction), respectively. For Cd, the control level was 20 mg/kg. After the same treatments, the residue levels were reduced to 11.3 mg/kg (43.5% reduction), 8.8 mg/kg (56% reduction), and 7.6 mg/kg (61.5% reduction) with acetic acid, liquid smoke, and *L. rhamnosus*, respectively.

Table 5 Effect of certain treatments on the concentration of mercury and cadmium experimentally inoculated to fish fillets (50 mg/Kg and 20 mg/Kg, respectively).

Treatment	Mercury		Cadmium	
	Levels after treatment (mg/Kg)	Reduction Percentages	Levels after treatment (mg/Kg)	Reduction Percentages
Control	50	-------	20	-------
Acetic acid 5% (30 min)	23.6	52.8	11.3	43.5
Liquid smoke 3% (6 min)	17.9	64.2	8.8	56.0
L. rhamnosus (24 hours)	14.2	71.6	7.6	61.5

4. DISCUSSION

One major consequence of elevated heavy metal concentrations is their accumulation in aquatic ecosystems, especially in fish and other organisms. This bioaccumulation occurs as metals are absorbed by aquatic organisms from water and sediment. Fish, being at the top of aquatic food chains, are particularly prone to accumulating high levels of heavy metals. This phenomenon poses a serious threat to both aquatic ecosystems and human populations dependent on fish for sustenance (Niu et al., 2020)

The observed average mercury level of 0.94 mg/kg in the examined *Oreochromis niloticus* proves lower than the 1.87 mg/kg mean Hussien et al. (2011) recently documented across retail Nile fish surveys, possibly reflecting seasonal biomagnification variations. However, study findings moderately exceed earlier contamination baselines in the same local water system with Madiha (2009) who quantified 0.49±0.05 mg/kg and 0.81±0.05 mg/kg mean residues for smaller and larger wild-sourced tilapia, respectively, sourced from the Nile several years prior.

The obtained results revealed concentrations notably higher than those documented by Marzouk et al. (2016), who reported a mean concentration of 0.105±0.005 ppm in tilapia and 0.115±0.004 ppm in *Bagrus bayad*. Similarly, the findings surpass the mean concentrations reported by Ali et al. (2016), which were 0.074±0.017 ppm in Nile catfish. Notably, Moustafa et al. (2011) recorded considerably elevated results, citing a mean concentration of 1.9 mg/kg for mercury in tilapia.

The findings of this study align with those reported by Hamada et al. (2018), who observed a mean mercury concentration of 0.94±0.10 mg/gm in Nile tilapia. This suggested that mercury contamination in Nile tilapia remains a persistent issue.

The examined Nile tilapia samples align closely with the 1.13 ± 0.01 mg/kg mean Bayoumi et al. (2023). However, study findings fall appreciably below the higher 1.44 ± 0.03 mg/kg baseline they established for catfish - possibly reflecting interspecies variability in bottom-feeding bioaccumulation pathways. The study also noted substantially higher tissue retention versus Nyantakyi et al. (2021), who reported average mercury concentrations of 0.58 ± 0.69 mg/kg for tilapia fillets sampled from Ghanaian aquaculture farms utilizing compound feeds. This hints at better water quality regulation and feed monitoring practices reducing bioaccumulation risks in the Ghanaian tilapia farms compared to the examined Egyptian fish from Nile waterways.

Airborne mercury pollution poses a significant environmental risk, particularly for aquatic ecosystems and human health. As mercury travels long distances and settles in rivers, it transforms into methylmercury, a more bioavailable form that accumulates in fish. To protect the safety of fish consumption, comprehensive control measures are necessary throughout the mercury's pathway, from air to water to fish and other organisms (Gworek et al., 2020)

The act of disposing of dead animals and birds in the Nile River becomes a source of concern due to its role in initiating the decay process. As these carcasses break down, they release a substantial load of heavy metals into the surrounding water. The organic matter of decaying animals interacts with the aquatic environment, facilitating the leaching of heavy metals such as mercury into the river and its branches (Al-Afify andAbdel-Satar, 2022)

Study findings quantified cadmium proved substantially higher than the 0.14±0.01 mg/kg and 0.203±0.044 mg/kg means Helmy (2018) and El-Kewaiey et al. (2011) had documented for the same Nile fish species sourced locally. This hints at intensifying environmental contamination over the past decade. However, our findings fall appreciably below the extreme 1.16-1.2 mg/kg averages. Hussien et al. (2011) and Lasheen et al. (2012) reported previously the possibly episodic industrial effluent dumps that year.

In comparison with the findings reported by Hamada et al. (2018), Clarias lazera exhibited a higher cadmium content in the examined samples, registering at 0.45±0.01 mg/kg in contrast to the previously reported 0.20 ± 0.03 mg/kg. Similarly, *Oreochromis niloticus* displayed an increased cadmium concentration of 0.27±0.01 mg/kg, differing from the previously documented 0.12±0.02 mg/kg. Bagrus bayad also demonstrated an elevated cadmium level of 0.16±0.01 mg/kg, diverging from the reported 0.07±0.01 mg/kg.

The deleterious impact of cadmium is intensified due to its exceptionally prolonged biological half-life, leading to an extended retention period within organisms following bioaccumulation. This extended duration of retention exacerbates the toxic effects of cadmium, underscoring the enduring threat it poses to living organisms (Wang et al., 2021).

Low concentrations of cadmium in fish pose a risk of adverse effects in humans, including kidney damage and severe bone pain. Despite their initial low levels, there is concern about the potential accumulation of more hazardous concentrations in individuals consuming contaminated fish. Monitoring and regulating cadmium levels in the food chain are crucial to mitigating potential health risks to humans (Peana et al., 2022)

Study findings showed over 50% mercury reduction in fish fillets after a 30-minute 5% acetic acid soak corroborating past research on employing this organic acid for fish and shellfish decontamination. This reduction is attributed to the chelating ability of acetic acid, which forms soluble complexes with heavy metals, allowing them to be removed during processing (Tonsy andAbdel-Rahman, 2012). Elnimr (2011) recorded 58.3% and 25% cadmium and mercury decrease, respectively, in intentionally contaminated tilapia fillets after rinsing in a 5% acetic acid solution. According to Oustan et al. (2011), ions in the acid will bind the metal so that it can remove the metal ions that accumulate in the shells. The higher the concentration of acid in a solution, the faster the solution will react with metal ions. Likewise with immersion time.

Aligning with study observations, past studies also demonstrate liquid smoke's promise for extracting heavy metal contaminants from aquatic foodstuff. Saloko et al. (2014) showed over 30% of cadmium reduction in blood cockles soaked in 5% smoke solutions, attributing chelation to carboxylic moieties from pyrolyzed lignin components. liquid smoke possesses antioxidant properties that could prove beneficial. The antioxidants present in liquid smoke may assist in neutralizing free radicals, potentially alleviating oxidative stress induced by heavy metals, and can bind with heavy metals, thereby reducing their bioavailability in the fish fillet (Suprapti et al., 2016)

The inclusion of probiotics like *Lactobacillus rhamnosus* is known for 00capacity to interact with heavy metals. This interaction can lead to the immobilization or transformation of heavy metals into less toxic forms, contributing to the heavy metal attached to the cell wall matrix of *Lactobacillus rhamnosus* (Zoghi *et al*., 2014). In comparison with the findings reported by Bayoumi et al. (2023), where the inoculation of *Lactobacillus rhamnosus* led to a 67.7% reduction in mercury levels (from 50 mg/kg to 16.1 mg/kg) and a 75% reduction in cadmium levels (from 10 mg/kg to 2.5 mg/kg) after dipping the fish fillets for 24 hours, while the present study observed comparable but slightly distinct outcomes, while our results exhibit a slightly lower reduction percentage for cadmium compared to the 72% reduction reported by Samir et al. (2021) within 24 hours of incubation.

5. CONCLUSIONS

The present study highlights the alarming contamination of fish samples from the River Nile with toxic heavy metals, specifically mercury and cadmium. The fish are accumulating metals in their organs, which poses a risk to consumers who eat the fish. These findings suggested that all three candidate methods significantly reduced initial mercury spike concentrations in fish tissues, but the *L. rhamnosus* probiotic achieved maximum reduction efficacy in this experimental setup.

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