Official Journal Issued by Faculty of Veterinary Medicine

Benha Veterinary Medical Journal

Journal homepage: https://bvmj.journals.ekb.eg/

Since 1990

Original Paper

Assessment of marination with Kefir milk for reducing the development of aflatoxin B1 in Nile Tilapia fillets by acting against *A. flavus*

Reem, M. Elbehary[1], Nermeen, F. Elshopary[2], Abo Bakr, M. Edris[1]

[1]*Department of Food control, Faculty of Veterinary Medicine, Benha University, Egypt*

[2]*Department of Food Hygiene, Animal Health Research Institute, Tanta Branch, Agriculture Research Center, Egypt.*

ARTICLE INFO

Keywords

Tilipia fillets

Aspergillus flavus

Kefir milk

total aflatoxins.

Received 11/05/2024
Accepted 24/06/2025
Available On-Line 01/07/2025

ABSTRACT

Food contamination by mycotoxigenic fungi poses a risk to human health and the safety and quality of both human and animal feed. Accordingly, the purpose of this investigation was to assess the effects of Kefir milk (KM) at concentration (3%) and (5%) on the shelf life, quality and safety of chilled Tilipia fish (*Oreochromis niloticus*) fillets. Sensory attributes (colour, texture, odour, and overall acceptability), as well as the chemical indices (pH and TVB-N), count of *Aspergillus flavus* and aflatoxins production in marinated and control groups. A significant difference ($P< 0.01$) was recorded. Among the marinated groups, Kefir milk (5%) treated group showed the highest reduction in *Aspergillus flavus* count and aflatoxin production, as the mean count of *Aspergillus flavus* and aflatoxins level decreased from 6.11 ± 0.01 to 4.56 ± 0.08 (log cfu/g) and 0.78 ± 0.02 to 0.59 ± 0.01(µg/kg), respectively, at the end of storage followed by Kefir milk (3%). They extended the shelf-life of the Tilapia fillets that held under proper refrigeration conditions up to 10^{th} days compared to the un- marinated groups that was completely spoiled at the 6^{th} day of storage.

1. INTRODUCTION

Fish is a very nutrient-dense food that is a great source of important fatty acids, proteins, vitamins, and minerals. Because of its comparatively low-calorie content (10%), its nutritional value is acknowledged (Rybicka et al., 2022). Nile tilapia considered one of the major species cultivated in aquaculture, with a global production of 4407.2 tonnes, with increase from 4000.9 tonnes in 2015 (FAO., 2022). Food spoilage is a major concern worldwide, as it leads to the loss of nearly 5–10% of all food produced (Pitt and Hocking, 2009). Fungi cause major spoilage of foods, so, proliferation of various fungi in food products leads to a reduction in yield and their quality with massive financial losses (Awuchi et al., 2021). Molds are common in nature and produce mycotoxin, which deteriorates food (Schnürer and Magnusson, 2005). Many pathogenic strains develop and create harmful metabolites, such as aflatoxins, which are produced by some *Aspergillus species* and have been shown to be mutagenic, teratogenic, and carcinogenic to a variety of experimental animal species (Navale et al., 2021). *Aspergillus flavus, A. nomius, and A. parasiticus* are the primary producers of aflatoxins (Egal et al., 2005). According to reports, the most prevalent and dangerous mycotoxin is aflatoxin B1 (AFB1) (Taheur et al., 2019b). These mycotoxins have significant thermal stability during food processing and can seriously impair both human and animal health (Taheur et al., 2019a). Thus, a variety of chemical and physical techniques have been used to stop fungal contamination and lower mycotoxin levels, extending the shelf life of foods (Ade-bayo and Aderiye, 2011; Aziz et al., 2007). The development of safer and more efficient methods, such as marination, a processing method that involves submersion or addition of cooked or uncooked seasoned liquid marinades that may include different additions such acids, enzymes, and spices (Lopes et al., 2022). It is also a method for tenderizing meat using physical, chemical, and biological methods (Gómez et al., 2020). Various biological marinades, such as kefir, yogurt, and butter used to treat meat (Latoch., 2020). Kefir is one of the most popular consumed functional foods because it has health-promoting properties (Cufaoglu and Erdinc., 2023). Milk kefir is produced using different gelatinous particles containing species of probiotic microorganisms, known as “milk kefir grains”, and this beverages fermented from these grains have different physical, chemical and microbiological characteristics (Guzel-Seydim et al., 2021). The primary microorganisms found in kefir are often yeasts, acetic acid bacteria, and lactic acid bacteria, despite their varying microbiological compositions. Additionally, several of these species offer probiotic properties (Spizzirri et al., 2023). The probiotic qualities and high nutritional content of kefir provide a number of health benefits (Perna et al., 2019). It has been declared that kefr has inhibitory effect against a variety of fungi (Taheur et al., 2020). Indeed, Taheur et al. (2020) shown that A. *favus*'s growth and aflatoxin production are inhibited by kefir. The study's objective was to determine the efficiency of kefr (milk fermented with kefr grains) in reduction of *A. favus* growth and aflatoxin production in Nile Tilapia fillets.

2.1. MATERIAL AND METHODS

2.1.1Collection of samples:

4 Kg. of Fresh Nile Tilapia fillets (*Oreochromis niloticus*) samples were purchased from fish markets markets in El-

* Correspondence to: rm5986447@gmail.com

Menoufia governorate and packed in sterile polyethylene bags. They were transported directly as rapidly as possible in an insulated ice container to microbial lab. Fish fillets samples sterilized using UVA in (320–400 nm) (Wang et al. 2023) for 15 min. to every side (Morsy et al. 2018) in lab before further treatment and analysis.

2.1.2. Aspergillus flavus strain:
Aspergillus flavus strain (ATCC 22546) was supplied by the Food Safety Reference Lab, Animal Health Research Institute (AHRI), Dokki, Egypt.

2.1.3. Kefir Milk Preparation:
Fresh cow milk was pasteurized for 30 minutes at 80˚C. Milk was fermented by adding kefir grains at a ratio of 1:50 (w/v) at a temperature between 8 to 25˚ C after the pasteurized milk had cooled to 25˚ C. At the end of fermentation, the kefir grains were removed by filtration from the kefir product (Rosa et al., 2017).

2.2.4. Experimental Design (Elaksiry et al., 2024)
Four groups of fish fillets were made. The 1st groups were control negative that uninoculated fish fillets dipped and soaked in sterile distilled water only. The other three groups were injected with one milliliter of *A. flavus* spore suspension (10^6 spore/mL) with mixing of fish fillets with the culture solution to uniformity spreading. The inoculated fish fillets were kept at 25˚ C for 1 hr. to allow the inoculum to be absorbed. The second groups, the inoculated fish fillets were divided to three groups ; ; dipped and soaked in sterile distill water only(2nd group ,control positive), dipped and soaked in sterile distill water and Kefir milk (3%)(3rd groups) and dipped and soaked in sterile distill water and Kefir milk (5%)(4th groups). Each group soaks the fish fillet in marinade for 24 hours at 4±1˚ C. Then, all group samples were removed from marinated solution and samples were analyzed for *Aspergillus flavus* growth and aflatoxins levels as well as sensory, shelf life and physicochemical properties at zero, 2nd, 4th, 6th, 8th and 10thdays of the chilling storage .The experiment was conducted in triplicate.

2.2.5. Aspergillus flavus count
By pour plating method at Sabouaud dextrose agar according to ISO (21527- 1:2008).

2.2.6. Physical-chemical assessment
Values the pH were calculated using the procedure outlined in Zenebon et al. (2008), and the TVB-N content was assessed using the procedure outlined in Shokri et al. (2015) and reported as mg N/100 g

2.2.7. Sensory Evaluation performed according to ISO 13299 (2003)
Each sample has been evaluated by nine highly experienced panelists. Participants were given 100 ± 10 g meatball samples of each plant extract and asked to rate their sensory attributes (color, odor, and texture). Samples were coded with random numbers; panelists were unaware of the experimental approach. They were asked to rate color, odor, and texture of each sample. A ten-point descriptive scale was employed; score 10 was the highest, while score 1 was the lowest.

2.2.8 HPLC determination of total Aflatoxins according to Sebaei et al. (2020)
High performance liquid chromatography was supplied from Agilent, 1200 model series equipped with quaternary pump (G1311A), vacuum degasser (G1379A), autosampler (G1313A), fluorescence detector (G1321A) and chromatographic column: Agilent Eclipse Plus C18 5 µm × 150 × 4.6 mm. The pump flow rate was 1 mL/min with mobile phase composition of water: methanol: acetonitrile (65: 23: 12 v/v/v) and the fluorescence detector wavelengths were at 340 nm excitation and 440 nm emission.

2.2.10. Statistical analysis
The graph pad prism tool for Windows was used to evaluate the data. All data were subjected to analysis of variance (ANOVA) (Version 8.0.2). Values were reported as means and SD. At $P < 0.01$ significant F-values were recorded.

3. RESULTS

The results presented in table (1) showed, significant differences ($P < 0.01$) in pH values between different group samples in both control and treated ones as the mean values were 5.82, 5.87, 5.75 and 5.72 in control (–ve), Control (+ve) , KM(3%) and KM (5%)groups at zero day, respectively. With the increase of storage period time, the pH values increased in control groups while, it decreased gradually in treated ones. The pH values of control(–ve) and control(+ve) groups were 6.85 ± 0.07 and 7.12 ± 0.07 at 4th day, while, they were 5.51 ± 0.01 and 5.22 ± 0.01in KM 3% and KM 5% at 6th day of storage of refrigerated storage period, respectively ($P < 0.01$).

Table (1)Mean values of pH in untreated and treated fish fillets with kefir milk at different concentrations during cold storage at 4˚C.

groups / storage period	Control -ve	Control +ve	KM 3%	KM 5%
zero day	5.82 ± 0.10^{ab}	5.87 ± 0.07^{a}	5.75 ± 0.07^{b}	5.72 ± 0.03^{b}
2nd day	6.32 ± 0.03^{a}	6.45 ± 0.14^{b}	5.67 ± 0.03^{c}	5.57 ± 0.03^{d}
4th day	6.85 ± 0.07^{a}	7.12 ± 0.07^{b}	5.24 ± 0.03^{c}	5.12 ± 0.02^{d}
6th day	S	S	5.01 ± 0.02^{a}	4.95 ± 0.01^{b}
8th day	S	S	5.51 ± 0.01^{a}	5.22 ± 0.01^{b}
10th day	S	S	6.84 ± 0.01^{a}	6.72 ± 0.03^{b}

S= Spoiled The results are considered significant (P< 0.01) when the same row contained different small letters.

Moreover, the data in table (2) achieved that, at zero day of the study the TVB-N(mg/100mg) values in control –ve and control +ve were 14.55 ± 0.49 and 18.3 ± 0.28, respectively. While, they were 10.30 ± 0.14 and 10.11 ± 0.14 in KM (3%)and KM(5%) treated groups, respectively. There were significant different between all examined groups ($P < 0.01$) as the TVB-N value of control –ve and control +ve increased rapidly till 4th day of storage they became 35.61 ± 0.56 and 40.75 ± 0.35 (mg/100mg) in control –ve and control +ve, respectively. While, the value of groups treated with KM (3%)increased gradually till 10th day of storage with significance difference from KM (5%)treated groups ($P < 0.01$).

Table (2)Mean values of TVB-N (mg/100mg) in untreated and treated fish fillets with kefir milk at different concentrations during cold storage at 4˚C.

groups / storage period	Control –ve	Control +ve	KM 3%	KM5%
zero day	14.55 ± 0.49^{a}	18.3 ± 0.28^{b}	10.30 ± 0.14^{c}	10.11 ± 0.14^{c}
2nd day	18.80 ± 0.14^{a}	21.61 ± 0.28^{b}	14.45 ± 0.21^{c}	12.20 ± 0.14^{d}
4th day	35.61 ± 0.56^{a}	40.75 ± 0.35^{b}	18.30 ± 0.30^{c}	15.65 ± 0.07^{d}
6th day	S	S	21.35 ± 0.10^{a}	18.86 ± 0.11^{b}
8th day	S	S	28.91 ± 0.30^{a}	22.40 ± 0.28^{b}
10th day	S	S	35.05 ± 0.21^{a}	31.30 ± 0.11^{b}

S= Spoiled The results are considered significant (P< 0.01) when the same row contained different small letters.

Also, as showed table (3) results revealed, control –ve groups were free from *A. flavus* growth throughout the storage period. The initial count of *A. flavums* (log cfu/g) in Tilapia fillet of control +ve groups was 6.30 ± 0.07. That slightly increased to 6.85 ± 0.06 and 7.76 ± 0.07 at 2nd and 4th day at refrigerated storage, respectively. While, the mean counts of treated groups with kefir milk with

concentrations (3% and 5%) were 6.17 and 6.11, respectively. These counts decreased gradually during the refrigerated storage (4±1˚C). The mean values of *A. flavus* count in treated groups with KM (3%) at 2nd, 4th, 6th, 8th and 10th day of refrigerated storage were 5.96, 5.54, 5.07, 4.82 and 4.93(log cfu/g), respectively. Furthermore, The mean values of *A. flavus* count in treated groups with KM (5%)at 2nd, 4th, 6th, 8th and 10th day of storage were 5.84, 5.43, 4.86, 4.38 and 4.56 (log cfu/g), respectively, with significant differences between treated groups as well as control ones.

Table (3)Effect of different concentrations of kefir milk on *A. flavus* count (log cfu/g) in untreated and treated fish fillets stored at 4˚C.

groups / storage period	Control -ve	Control +ve	KM 3%	KM5%
zero day	ND*	6.30 ± 0.07^{a}	6.17 ± 0.03^{b}	6.11 ± 0.01^{b}
2nd day	ND*	6.85 ± 0.06^{a}	5.96 ± 0.06^{b}	5.84 ± 0.02^{c}
4th day	ND*	7.76 ± 0.07^{a}	5.54 ± 0.02^{b}	5.43 ± 0.01^{c}
6th day	S	S	5.07 ± 0.04^{a}	4.86 ± 0.08^{b}
8th day	S	S	4.82 ± 0.03^{a}	4.38 ± 0.04^{b}
10th day	S	S	4.93 ± 0.03^{a}	4.56 ± 0.08^{b}

ND=Not Detected S= Spoiled The results are considered significant (P< 0.01) when the same row contained different small letters.

Recovered data in table (4) indicated that, presence of aflatoxins in control (+ve)and KM treated groups. The concentration level of aflatoxins increased in control(+ve) while decreased in treated groups during the storage period. Aflatoxins average level(µg/kg) was 0.87 ± 0.03 at zero day of storage and increased to 1.04 ± 0.02 at 4th day at refrigerated storage. In marinated groups aflatoxins levels in KM (3%) and KM (5%) were 0.81 ± 0.01 and 0.78 ± 0.02 at zero day then decreased to 0.62 ± 0.01 and 0.54 ± 0.01 at 8th day of storage, respectively, but, it seemed to be increased at 10th day of storage.

Table (4) Mean values of Aflatoxins concentration levels (µg/kg) in untreated and treated fish fillets with kefir milk in different concentrations during cold storage at 4˚C.

groups / storage period	Control -ve	Control +ve	KM 3%	KM5%
zero day	ND*	0.87 ± 0.03^{a}	0.81 ± 0.01^{b}	0.78 ± 0.02^{b}
2nd day	ND*	0.96 ± 0.04^{a}	0.76 ± 0.02^{b}	0.70 ± 0.02^{c}
4th day	ND	1.04 ± 0.02^{a}	0.71 ± 0.01^{b}	0.64 ± 0.03^{c}
6th day	S	S	0.67 ± 0.02^{a}	0.59 ± 0.01^{b}
8th day	S	S	0.62 ± 0.01^{a}	0.54 ± 0.01^{b}
10th day	S	S	0.65 ± 0.01^{a}	0.59 ± 0.01^{b}

S= Spoiled The results are considered significant (P< 0.01) when the same row contained different small letters.

The sensory evaluation of this study based on evaluation of color, odor, texture and over all acceptability, while data in table (5) revealed that the samples of control groups spoiled rabidly than that treated groups as they spoiled at 4th day. While, the treated ones spoiled at 10th day of refrigerated storage. Ten points hedonic scale is used to determine the sensory evaluation of examined.

Table (5) Sensory evaluation scores of untreated and treated fish fillets with different concentrations of kefir milk during cold storage at 4˚C.

groups / storage period	Control -ve	Control +ve	KM 3%	KM5%
Colour				
zero day	9.5 ± 0.2^{a}	9.5 ± 0.2^{a}	9.7 ± 0.2^{a}	9.7 ± 0.2^{a}
2nd day	5.0 ± 0.5^{a}	4.5 ± 0.3^{b}	8.0 ± 0.5^{c}	8.5 ± 0.5^{d}
4th day	3.6 ± 0.3^{a}	3.1 ± 0.4^{b}	7.0 ± 0.5^{c}	7.5 ± 0.5^{d}
6th day	S	S	6.0 ± 0.3^{a}	6.5 ± 0.4^{b}
8th day	S	S	5.0 ± 0.2^{a}	5.5 ± 0.2^{b}
10th day	S	S	3.5 ± 0.1^{a}	3.5 ± 0.1^{a}
Odour				
zero day	9.8 ± 0.2^{a}	9.8 ± 0.2^{a}	9.8 ± 0.2^{a}	9.8 ± 0.2^{a}
2nd day	5.5 ± 0.2^{a}	4.5 ± 0.5^{b}	9.0 ± 0.2^{c}	9.5 ± 0.2^{d}
4th day	3.5 ± 0.2^{a}	3 ± 0.5^{b}	7.5 ± 0.1^{c}	8.5 ± 0.1^{d}
6th day	S	S	6.5 ± 0.1^{a}	7.0 ± 0.1^{b}
8th day	S	S	5.5 ± 0.2^{a}	6.0 ± 0.2^{b}
10th day	S	S	3.5 ± 0.1^{a}	3.9 ± 0.1^{a}
Texture				
zero day	9.8 ± 0.2^{a}	9.8 ± 0.2^{a}	9.8 ± 0.2^{a}	9.8 ± 0.2^{a}
2nd day	5.0 ± 0.2^{a}	4.5 ± 0.5^{b}	8.5 ± 0.2^{c}	9.0 ± 0.1^{d}
4th day	3 ± 0.2^{a}	2.5 ± 0.5^{b}	7.5 ± 0.1^{c}	8.0 ± 0.2^{d}
6th day	S	S	6.5 ± 0.1^{a}	7.0 ± 0.5^{b}
8th day	S	S	5.0 ± 0.2^{a}	6.0 ± 0.2^{b}
10th day	S	S	3.0 ± 0.1^{a}	3.5 ± 0.2^{b}
Over all acceptability				
zero day	9.7 ± 0.3^{a}	9.7 ± 0.3^{a}	9.7 ± 0.3^{a}	9.7 ± 0.3^{a}
2nd day	5.0 ± 0.5^{a}	4.0 ± 0.5^{b}	8.5 ± 0.5^{c}	9.5 ± 0.2^{d}
4th day	3.0 ± 0.5^{a}	2.5 ± 0.5^{b}	7.5 ± 0.1^{c}	8.5 ± 0.3^{d}
6th day	S	S	6.5 ± 0.5^{a}	7.0 ± 0.3^{b}
8th day	S	S	5.5 ± 0.5^{a}	6.0 ± 0.5^{b}
10th day	S	S	3.5 ± 0.5^{a}	3.9 ± 0.3^{a}

S= Spoiled 9- 10: Excellent 8-7: Very Good 7-6: Good 4-5: Acceptable
2-3: Unacceptable 1-2: Bad S: Spoiled

4. DISCUSSION

Fish is one of the most nutritious foods and highly perishable and has a limited shelf life; therefore, it needs to be handled and stored carefully to prevent degradation and to guarantee microbiological safety and a marketable shelf life (Tavares et al., 2021). Nowadays the usage of natural additives rather than synthetic ones is becoming more common (Naveena et al., 2008). So, Marinades used as a natural preservative technique to improve palatability, tenderness, color, flavor and/or texture of meat and meat products, including but not limited to chicken, fish, beef, steaks, chops, and seafood. Marinates not only enhance the sensory qualities but also have the ability to deactivate food microorganisms (Lopes et al., 2022).

The spoiling processes linked to these deteriorations are divided into three categories: chemical oxidation of lipids, endogenous enzymatic activities, and microbial metabolic activities. All of these processes reduce the shelf life of seafood (Mahmud et al., 2018). According to Gram and Huss (1996) the main factors causing seafood to spoil are its low acidity (pH>6) and high content of nitrogenous non-protein components, which encourage the growth of spoiling microbes.

Results were recorded the pH value in Tilapia fillets for ten days in kefir milk treated groups was lower($P < 0.05$) than that of the control ones. As the marinating time increased, the pH value of the Tilapia fillets dropped ($P < 0.05$) and subsequently started to rise. This was mostly because lactic bacteria are still alive throughout marinating days and create chemicals, primarily acids, as they develop, raising the environment's acidity then the endogenous enzymes affect fish protein and produce alkaline products that increase pH till spoilage at 10 days.

Kefir, the oldest fermented product of kefir grains, is an acidic dairy beverage with mild alcoholic content act as a starter it composed of a microbial symbiotic mixture of lactic acid bacteria (10^8 CFU/g), acetic acid bacteria (10^5 CFU/g), yeast (10^6–10^7 CFU/g) and that stick to a protein polysaccharide (mainly kefiran) matrix. Also, a complex microbial composition, including Lactobacillius, Leuconostoc, Lactococcus, Kluyver omyces, Kazachstania, Candida, and occasionally Acetobacter (Gao and Zhang, 2019). These bacteria fermented lactose in milk to lactic acid caused a decrease in pH (FAO/WHO, 2011). Because kefir milk contained organic acids, ethanol, CO2, and other volatile substances, its pH was approximately 4.2 (Zajšek, and Goršek, 2010).

Ammonia, methylamine, dimethylamine, trimethylamine, and other volatile chemicals created by microbial activity during meat storage in a refrigerator are all included in TVB, a crucial sign of fresh meat detection (Rodríguez et al., 2008).

According to table (2), TVB-N levels increased significantly ($P < 0.01$) with storage time. The lowest alteration in TVB values was noticed in treated groups with kefir milk (5%) while the highest alteration was recorded in control positive ones. Control negative and control positive groups were unaccepted at 4th day of storage as the permitted range with TVB levels of 30 mg N/100 g EOS (3494, 2005), while the treated groups were un accepted at the end of the 10th day.

The data in the study revealed that the count of inoculated *A. flavus* in control positive group increase throughout the refrigerated storage period while, *A. flavus* count decreased in different groups that treated with kefir milk. Similar results reported with Ismaiel et al. (2011) and Londero et al. (2014) as the authors found that Kefir milk suppress the

growth of *A. flavus.* The lactic and acetic acid mixture that kefir milk produces works in concert to suppress fungus growth (Gamba et al. 2016).

Mycotoxins are resistant to modern antimicrobial methods such high pressure and temperature (even at pasteurization and sterilization levels), low pH, and other food manufacturing process, it is nearly impossible to eradicate mycotoxins from food (Touranlou et al., 2023). Therefore, innovative approaches—particularly those with a natural and biological foundation—are urgently needed to prevent the economic losses and the health risks caused by mycotoxins (Hathout and Aly, 2014). Kefir milk can be used for antitoxic purposes since it contains probiotics. The volume of kefir, the duration of treatment, and the toxin concentration all affect how well kefir probiotics work against mycotoxins. (Du et al., 2022 and Touranlou et al., 2023).

The color, odor, texture, and general acceptability of controls and treatments were significantly reduced in sensory scores after storage; nevertheless, the control groups quickly spoiled on the fourth day of refrigeration. Using kefir milk improved all sensory attributes and decreased the rate of spoilage till 10^{th} day.

The antibacterial effect and the constituents present in kefir milk both are responsible for retaining the quality and sensory attributes of Tilapia fillets.

5. CONCLUSIONS

Deterioration in quality of fish and its products are numerous. The challenge of the preservation of such delicate and nutritious product so, marination with kefir milk is used as multidimensional as antifungal and aflatoxin scavenging agent. Furthermore, it improved the sensory characters and delayed the spoilage of Tilapia fillets.

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