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Microbiological assessment and the potential antimicrobial activities of nisin and\or garlic extract in commercial fish

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ABSTRACT

Fish is considered as a major source of animal-derived protein. However, fish is a perishable food that rapidly decomposed and its initial microbial load determines the extent of spoilage. The objectives of this study were firstly to screen the microbial status of three commonly consumed fish species in Egypt, namely, tilapia, mullet and catfish via estimation of total plate counts, total psychrophilic counts, most probable number of coliforms and total mould counts. Secondly, an experimental trial to investigate the antimicrobial potentials of either nisin, garlic extract or an equal mixture of them using tilapia fish as a food matrix. The results achieved in this study declared that, the insufficient hygienic measure adopted during handling, marketing and storage of fish, in terms of the high microbial load. Catfish had the highest bio- indicator parameters followed by tilapia and mullet. Both of nisin and garlic extracts showed significant antimicrobial properties in a concentration- dependent manner. In particular, the synergistic effects of the co-treatment of both nisin and garlic extract were documented. Thus, efficient hygienic handling, rapid cooling of fish can reduce the initial microbial load of fish. In addition, we highly recommend soaking of fish in nisin, garlic extract or both as an efficient strategy in reducing the microbial load of raw fish. Furthermore, efficient cooking of fish can prevent the intake of food-borne pathogens.

1. INTRODUCTION

Fish supplies humans with a major part of their needs from protein, vitamins, minerals and omega-3-fatty acids, however, on the same time, fish is a rapidly decomposed food if not properly handled and stored.

Microbial spoilage of fish is considered the most rapid fish spoilage as once the fish died, the immune response breakdown, bacterial populations near gills and in the intestinal tracts start to invade the muscular tissue and signs of decomposition start to begin (Ashie et al., 1996). It was reported that warm water fish is mainly invaded by mesophilic Gram's positive organisms such as *Micrococcus, coryneforms* and *Bacilli*, while cold-water fish is mainly invaded by psychrophilic Gram's negative organisms such as *Moraxella/Acinetobacter, Pseudomonas, Flavobacterium,* and *Vibrio* genera. Subsequently, indicators of the microbial status of fish include total mesophilic (plate) count, total psychrophilic counts, most probable number of coliforms (MPNC) and total mould and yeast counts (Liston 1980). Thus, there is a great need for proper handling and preservation of this precious food source. Using food preservatives to extend the shelf life of fish and to control microbial growth is another alternative way for fish preservation.

Nisin is an antibacterial peptide that is secreted by several strains of *Lactococcus lactis*, it has a well-documented bactericidal effects against several Gram's positive bacteria (Abts et al., 2011). Nisin was used as a food preservative for many kinds of food matrices such as meat, fish, milk and dairy products. However, there is no evidence of bacterial resistance (Enserink, 1999). There is two bactericidal mode of action for nisin. The first mechanism is via binding to lipid II, an essential cell-wall precursor, thus prevents cell wall synthesis (Hasper et al., 2004). This nisin-lipid II complex perforate the membrane thus increases the permeability of the membrane of the target cells as a second mechanism of bactericidal action of nisin (van Heusden et al., 2002).

Garlic (*Allium sativum*) is commonly used in the traditional medicine for prevention and as a cure for many diseases. Garlic contains sulfur-containing compounds such as cysteine sulfoxide that when chopped is converted by allinase enzyme into thiosulfinates, that possess a well-documented antibacterial and antifungal activities (Block et al., 1992; Whitemore and Naidu, 2000). There were and still ongoing several research efforts to investigate the antimicrobial effects of garlic and its possible use to extend shelf life of several meat and fish products.

In sight of these factors, the objectives of this study were firstly to screen the microbial status of three commonly consumed fish species in Egypt, namely, tilapia (*Tilapia nilotica*), mullet (*Mugil cephalus*) and catfish (*Clarias lazera*) via estimation of total plate counts, total psychrophilic counts, MPNC and total mould counts. Secondly, an experimental trial to investigate the antimicrobial potentials of either nisin, garlic extract or an equal mixture of them using tilapia fish as a food matrix.

2. MATERIALS AND METHODS

2.1. Collection of Samples:

Ninety ungutted apparently normal fish samples were collected randomly and equally from three fish markets in Dakahlia governorate, Egypt. The examined fish species were tilapia, mullet and catfish (n = 30 each). The collected samples were transferred cooled directly without delay to the educational veterinary hospital, Faculty of Veterinary Medicine, Mansoura University for bacterial isolation and identification.

2.2. Microbiological examinations:

Samples were prepared according to the technique recommended by APHA (2001) as follows: under aseptic conditions, 25 g of each sample weighed and homogenized with 225 ml of 0.1% of sterile buffered peptone water (LAB104 , LAB M , UK) for 1-2 minutes at 2000 rpm using sterile homogenizer (type M-p3- 302, mechanic, precyzina, Poland), such homogenate represents the dilution of 10^{-1}. The homogenate was kept for 5 minutes at room temperature. One ml from the original dilution (10^{-1}) was aseptically transferred to another sterile tube containing 9 ml of sterile 0.1% buffered peptone water and further tenfold decimal serial dilutions were prepared.

2.3. Total Plate Count (TPC):

Total plate count was performed as the method described by APHA (2001). In brief, one ml from each dilution was transferred to a clean and sterile Petri dish. Add 12-15 ml of plate count agar (Difco Laboratories, Detroit, Michigan, USA), cooling to 45 ± 1 ºC for each Petri dish, mix well, then left to solidify and incubated for 48 h at 35 ± 2 ºC. All colonies including pinpoint size colony forming units were recorded as TPC.

TPC/g = average No. of colonies × reciprocal of dilution Counted colonies expressed as log cfu/g.

2.4. Determination of total psychrophilic count (TPC):

The pour plate technique recommended by **APHA (2001)** was applied using standard plate count agar medium and incubated at 7 ºC for 10 days. Results were calculated in the same way as TPC. Counted colonies expressed as log cfu/g.

2.5. Determination of most probable number (MPN) of Coliforms:

One ml of each dilution was used to inoculate separately into three test tubes containing MacConkey broth with inverted Durham's tubes. The inoculated tubes were incubated at 37 ºC for 24-48 hrs. Positive tubes showing acid (yellow color) and gas production in inverted Durham's tubes were recorded as the method described by (APHA, 2001). The most probable number of coliforms was calculated according to the recommended tables.

2.6. Determination of the total mould counts:

The total mould counts were determined by culturing duplicate plates on Sabouraud's dextrose agar media (Oxoid, Basingstoke, UK) supplemented with chloramphenicol 100 mg/L followed by dark incubation at 25 ˚C for 5-7 days. During the incubation time, the plates were examined daily for the fungal growth. Estimation of total mould was obtained by direct counting of the cultured agar plates (APHA, 2001).

2.7 Reduction of the microbial load of tilapia fish:

In a trial for reduction of the microbial load of tilapia fish samples, diluted solutions of both nisin (Food grade, SIDLEY chemical, Linyi city, China) and garlic extracts (Food commercial grade) were used under 0.5, 1.5 and 2.5% separate or in combination. A total number of 50 tilapia fish (weigh about 200 g/each) were divided to 10 groups (n= 5 fish). The first group was soaked in distilled water for 30 min and kept as a control (non-treated) group. The second, third and fourth groups were soaked for 30 min at room temperature in nisin 0.5%, 1.5% and 2.5% solutions, respectively. The fifth, sixth and seventh groups were soaked for 30 min at room temperature in garlic extract 0.5%, 1.5% and 2.5% solutions, respectively. The 8^{th} , 9^{th}, and 10^{th} groups were soaked for 30 min at room temperature in nisin/garlic extract (1:1) 0.5%, 1.5% and 2.5% solutions, respectively. Total plate count, total psychrophilic count, MPN of coliforms and total mould count were conducted as mentioned before.

2.8. Statistical analysis:

All values were expressed as (means ± SE), and all measurements were carried out in duplicates. Bacterial counts were converted into base logarithms of colony forming units per g (log cfu/g). Statistical significance was evaluated using the Tukey–Kramer HSD test. In all analyses, $P < 0.05$ was taken to indicate statistical significance using JMP statistical package; SAS Institute Inc., Cary, NC.

3. RESULTS AND DISCUSSIONS

4. 3.1.Surveillance of the microbial status of tilapia, mullet and catfish

Fish carries microorganisms on their skin surfaces, in the intestine and in the gills. In many parts of Egypt other developing countries, fish is sold in the fish markets in open air, kept in wooden boxes and covered with ice. Rush handling of the fish might lead to rupture of the intestinal tract and spoiling of the fish body with the fish intestinal contents. Thus, in the first part of this study, the microbial status of the examined fish species was investigated using the microbial indicators of the hygienic measures including TPC, TPC, MPNC and TMC.

The recorded results indicated that catfish had significantly ($p<0.05$) the highest TPC followed by tilapia and finally mullet. The mean values of the total plate counts were 8.5 ± 0.8,

6.3 ± 1.5 and 4.2 ± 0.5 log cfu/g in the examined catfish, tilapia and mullet, respectively (Fig.1). Similarly, catfish had significantly the highest total psychrophilic count (6.2 ± 0.4 log cfu/g) followed by tilapia and mullet (4.7 ± 1.3 and 3.4 ± 0.5 log cfu/g), respectively (Fig.2). These achieved results go inagreement with other reports which indicated high total bacterial counts and poor hygienic measures in fish marketed in Portugal, Greece, Botswana and Malawi (Da Silva et al., 2002; Mhango et al., 2010; Tzikas et al., 2007). The total mesophilic and psychrophilic counts give an indication about the sanitary status of the fish and the spoilage degree of fish (Reij and Den Aantrekker, 2004). The high total bacterial count in this study indicates the poor hygienic conditions adopted during handling, storage and processing of fish. It might also indicate cross contamination of the fish flesh from the viscera, anglers, hands, storage boxes, water and ice used during storage and washing.

Coliform bacteria are considered significant microbiological sanitary indicators, which emphasizes hygiene in all steps of preparation and handling of food subjects (Darwish et al. 2015). Most probable number of coliforms had the same trend, in terms of catfish had the highest MPNC followed by tilapia and finally mullet. The recorded mean MPN values of in the examined samples were 5.2 ± 0.5, 4.5 ± 0.8 and 3.5 ± 0.6 log MPN/g in the examined catfish, tilapia and mullet, respectively (Fig.3). In agreement with the results of the study of Arannilewa et al. (2005) an increasing coliform count with a prolonged frozen storage of tilapia fish (*Sarotherodun galiaenus*), they argued that, possible temperature fluctuations of the freezers, time taken during the processing, time taken to transport and trade the fish might have contributed to the high coliform counts. Furthermore, Mhango et al. (2010) investigated that, the higher most probable number of coliforms found in catfish rather than tilapia in fish markets in Botswana and Malawi. In addition, comparable MPNC values were reported in mullet and tilapia sampled from Nigeria and Sudan, respectively (Okoro et al., 2010; Goja, 2013). Thus, the coliform counts may be used as an indicator of fecal contamination of fish through either inadequate processing or post processing recontamination. (ICMSF, 1996).

Regarding to the total mould counts, their mean values were 4.8 ± 0.6, 3.8 ± 0.8 and 2.6 ±

0.4 log cfu/g in the examined catfish, tilapia and mullet, respectively (Fig.4). In agreement with the results of this study, Efiuvwevwere and Ajiboye, (1996) recorded higher total mould counts was found in catfish *(Clarias gariepinus)*,where the Aspergillus and Penicillium were the dominated mould genera. Furthermore, Adebayo-Tayo *et al.* (2012) recorded high mould counts in catfish collected from different markets in Akwa Ibom State, Nigeria. They added that, the dominant mould genera were *Mucor*, *Rhizopus*, *Penicillium* and *Aspergilli*. Fungal contamination of fish in this study indicates inadequate sanitary measures performed starting from the point of catching. The conditions of the environment in the refrigerators, cooling boxes, anglers' hands and clothes are very suitable for the development of mould spores (Mizakova et al., 2002; Reij and Den Aantrekker, 2004). Fungal contamination of fish may lead to their spoilage and production of mycotoxins with potential health hazards to human due to their carcinogenic effects, liver diseases and organ damage (Darwish et al., 2014).

In general, it is clear from the achieved results in this study, the lack of proper hygienic measures adopted during handling and marketing of the examined fish species. Furthermore, catfish had the highest contamination levels followed by tilapia and mullet. This phenomenon may be reasonable as catfish lives in the bottom of the river Nile and water canals and the feeding habits of the catfish is usually regarded as the scavenger fish and bottom feeder. This speculation goes in agreement with other previous reports (Efiuvwevwere and Ajiboye, 1996; Mhango et al., 2010).

Table 1: Antimicrobial activities of nisin under different concentrations in Tilapia fish

	TPC Log cfu/g	Reduction (%)	Psy. count Log cfu/g	Reduction (%)	MPNC MPN/g	Reduction (%)	TMC Log cfu/g	Reduction (%)
Nisin 0%	6.3 ± 1.6^{a}	0.00%	4.7 ± 1.35^{a}	0.00%	4.5 ± 0.8^{a}	0.00%	3.8 ± 0.8^{a}	0.00%
Nisin 0.5%	5.8 ± 1.1^{a}	7.94%	4.1 ± 1.71^{b}	12.77%	3.8 ± 0.72^{b}	15.55%	3.5 ± 1.1^{ab}	7.89%
Nisin 1.5%	4.6 ± 1.8^{b}	26.98%	3.3 ± 0.88^{c}	29.79%	3.2 ± 0.85^{c}	28.88%	3.0 ± 0.55^{bc}	21.05%
Nisin 2.5%	3.5 ± 0.9^{c}	55.55%	2.7 ± 0.95^{d}	42.55%	2.8 ± 0.66^{c}	37.77%	2.8 ± 0.92^{c}	26.32%

TPC: Total plate count; Psy: psychrophilic; MPNC: Most probable number of coliforms; TMC: Total mould count Means in the same column carrying different superscript letter are significantly different at $p<0.05$
Reduction % = Control – After treatment / Control x 100

Table 2: Antimicrobial activities of garlic extract under different concentrations in Tilapia fish

	TPC Log cfu/g	Reduction (%)	Psy. count Log cfu/g	Reduction (%)	MPNC MPN/g	Reduction (%)	TMC Log cfu/g	Reduction (%)
Garlic ex. 0%	6.3 ± 1.6^{a}	0.00%	4.7 ± 1.35^{a}	0.00%	4.5 ± 0.8^{a}	0.00%	3.8 ± 0.8^{a}	0.00%
Garlic ex. 0.5%	6.1 ± 1.4^{ab}	3.17%	4.2 ± 1.61^{b}	10.64%	3.6 ± 0.98^{b}	20.00%	3.1 ± 0.6^{b}	18.42%
Garlic ex. 1.5%	5.5 ± 1.3^{b}	12.7%	3.7 ± 0.71^{c}	21.28%	3.1 ± 0.75^{c}	31.11%	2.6 ± 0.75^{c}	31.58%
Garlic ex. 2.5%	4.6 ± 1.1^{c}	26.98%	3.0 ± 1.05^{d}	36.17%	2.5 ± 0.81^{d}	44.44%	2.1 ± 0.55^{d}	44.74%

TPC: Total plate count; Psy: psychrophilic; MPNC: Most probable number of coliforms; TMC: Total mould count Means in the same column carrying different superscript letter are significantly different at $p<0.05$

Reduction % = Control – After treatment / Control x 100

Table 3: Synergistic antimicrobial activities of nisin and garlic extract under different concentrations in Tilapia fish

	TPC Log cfu/g	Reduction (%)	Psy. count Log cfu/g	Reduction (%)	MPNC MPN/g	Reduction (%)	TMC Log cfu/g	Reduction (%)
Nisin + Garlic ex. 0%	6.3 ± 1.6^{a}	0.00%	4.7 ± 1.35^{a}	0.00%	4.5 ± 0.8^{a}	0.00%	3.8 ± 0.8^{a}	0.00%
Nisin + Garlic ex. 0.5%	5.5 ± 1.2^{b}	12.17%	3.7 ± 0.66^{b}	21.28%	3.5 ± 0.6^{b}	22.22%	3.0 ± 0.66^{b}	21.05%
Nisin + Garlic ex. 1.5%	4.1 ± 1.1^{c}	34.92%	2.8 ± 0.78^{c}	40.43%	2.6 ± 0.77^{c}	42.22%	2.5 ± 0.81^{bc}	34.21%
Nisin + Garlic ex. 2.5%	3.1 ± 0.7^{d}	50.79%	2.0 ± 0.55^{d}	57.45%	2.2 ± 0.44^{c}	51.11%	2.1 ± 0.1^{c}	44.74%

TPC: Total plate count; Psy: psychrophilic; MPNC: Most probable number of coliforms; TMC: Total mould count Means in the same column carrying different superscript letter are significantly different at $p<0.05$

Reduction % = Control – After treatment / Control x 100

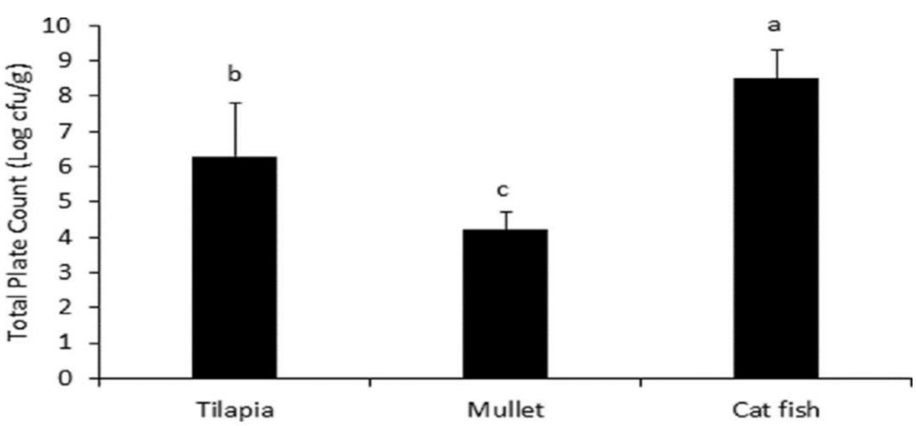

Figure 1: Total plate count in examined fish samples

Values represent means ± SE (Log cfu/g) of thirty samples from each of tilapia, mullet and catfish. Columns carrying different superscript letter differ significantly among examined samples at $P < 0.05$.

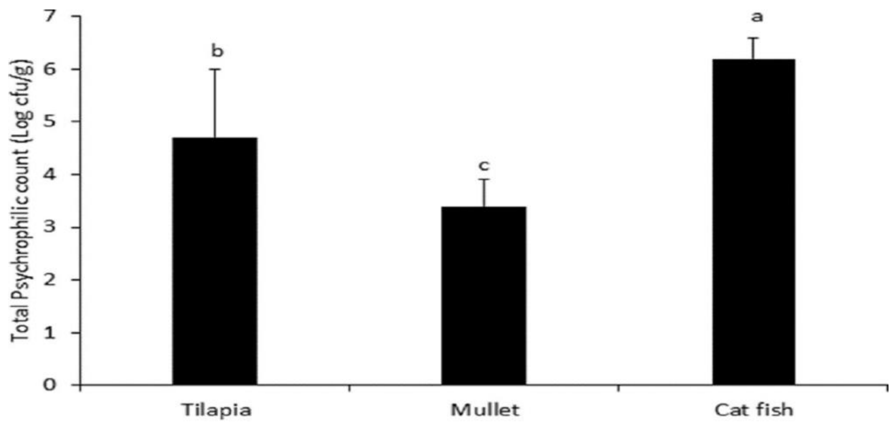

Figure 2: Total psychrophilic count in examined fish samples

Values represent means ± SE (Log cfu/g) of thirty samples from each of tilapia, mullet and catfish. Columns carrying different superscript letter differ significantly among examined samples at $P < 0.05$.

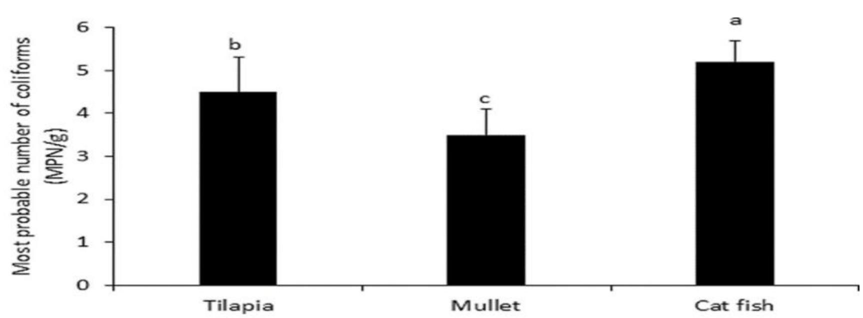

Figure 3: Most probable number of coliforms in examined fish samples

Values represent means ± SE (Log MPN/g) of thirty samples from each of tilapia, mullet and catfish. Columns carrying different superscript letter differ significantly among examined samples at $P < 0.05$.

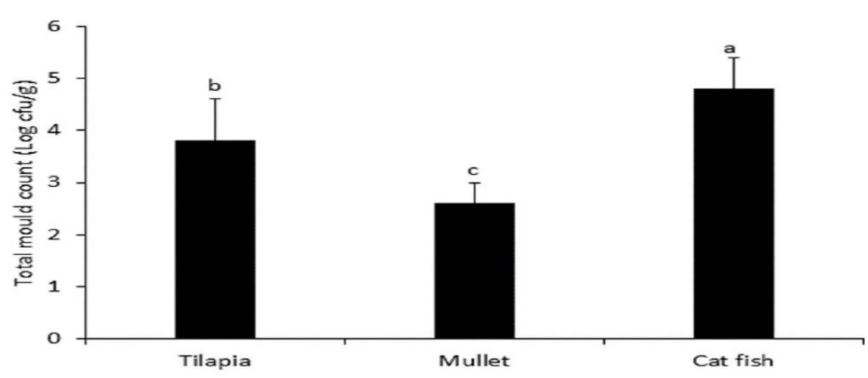

Figure 4: Total mould counts in the examined fish samples

Values represent means ± SE (Log cfu/g) of thirty samples from each of tilapia, mullet and catfish. Columns carrying different superscript letter differ significantly among examined samples at $P < 0.05$.

3.2. Reduction trials of the microbial load of tilapia

Microbial contamination of fish had several implications starting from organoleptical changes such as off-flavor, slime formation over the skin, softening of the muscular tissue, or extend to economic losses due to unmarketability of such spoiled fish and the most hazardous implication is the related food-poisoning if such contaminated fish is consumed without efficient cooking. Thus, one major task of the food hygienists and microbiologists is to find ways to extend the shelf life of fish and to decontaminate or reduce microbial contamination levels of fish. Thus, in the second part of this study, trials to investigate the potential antimicrobial effects of nisin and or aqueous garlic extract in reducing contamination of tilapia fish.

The results revealed that, nisin could significantly reduce the bio-indicator parameters of fish spoilage in a concentration-dependent fashion as clear in table 1. For instances, nisin 2.5% significantly reduced total plate, psychrophilic, MPNC and mould counts with 55.55%, 42.55%, 37.77% and 26.32%, respectively (Table 1). In correspondence to the findings of this study, Elotmani and Assobhei (2004) declared the antimicrobial activity of nisin especially against Gram's positive bacteria (in vitro study) in Sardine. In a recent study, Chaves López et al. (2017) confirmed the preservative effects of nisin and extension of the shelf life of vacuum packaged rainbow trout (Oncorhynchus mykiss) fillets. Nisin is considered as a promising preservative in the fish industry as so far there is no microbial resistance was reported against nisin. Similar to nisin antimicrobial properties, garlic extract 1.5% could significantly ($p<0.05$) reduce total plate counts (12.7%), total psychrophilic counts (21.28%), most probable number of coliforms (31.11%) and total mould counts (31.58%). These reduction percentages were 26.98%, 36.17%, 44.44% and 44.74% respectively, after immersion in garlic extract 2.5% for 30 min (Table 2). In agreement with our results, antifungal activity of seven Allium plants was reported by Yin and Tsao (1997), they observed that, garlic had antifungal activity against three tested Aspergillus species. Furthermore, Benkeblia (2004) confirmed the antimicrobial activity of the essential oils extracted from garlic against Salmonella Enteritidis and Staphylococcus aureus. In addition, Ranjan et al. (2012) recorded a strong antimicrobial activity of aqueous garlic extract against Bacillus cereus, Staphylococcus aureus, Enterococcus faecalis, E. coli and Proteus mirabilis. They added that, garlic was used efficiently for preservation of fish. The antimicrobial effect of garlic is attributed to its content of thiosulfinates (Whitemore and Naidu, 2000). There is a lack of information about the synergistic antimicrobial action of both nisin and garlic especially in the fish industry. Thus, to investigate the synergistic antimicrobial effects of both nisin and garlic extracts, tilapia fish was immersed for 30 min in different concentrations of equal volumes of these solutions. The obtained results demonstrated that, nisin-garlic extracts significantly reduced total plate counts, total psychrophilic counts, MPNC and total mould counts in a concentration-dependent manner. The reduction percentages of nisin-garlic extract 0.5% for these parameters were 12.17%, 21.28%, 22.22% and 21.05%, respectively. However, at 2.5%.these parameters were strongly reduced with 50.79%, 57.45%, 51.11% and 44.74%, respectively (Table 3). In agreement with these results, the synergistic effects of nisin and garlic was confirmed via their antimicrobial activity against Listeria monocytogens in milk (Kim et al., 2008).

4. CONCLUSION

In conclusions, efficient hygienic handling, rapid cooling of fish, using of clean water during fish wash could reduce the initial microbial load of fish. In addition, we highly recommend soaking of fish in nisin, garlic extract or both as an efficient strategy for reduction of the microbial load of raw fish. Furthermore, efficient cooking of fish could prevent the food-borne pathogens.

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