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Control of Halotolerant Bacteria in Salted Fish (Faseikh) Using Trisodium Phosphate

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Abstract: Trisodium Phosphate (TSP) as a food additive was studied for its effects on controlling the growth of halotolerant bacteria in the traditional Egyptian salted fish, faseikh. The experimental design included dividing the fish samples to 5 groups. First salted fish group was randomly purchased from the retail market as represent commercial product on retail sale. The other 4 groups were laboratory processed like simulated commercial condition using dry salting technique; in which two groups treated by addition of 0.5% TSP (w/w) with the salt mixtures and the other two groups were kept as control. Treated and control groups were stored at room (22°C) and chilling (4°C) temperatures for 60 day till ripening. When the obtained results were statistically compared to the markets group, 0.5% TSP significantly reduced ($p<0.05$) the counts of halotolerant bacteria by 2.5 and 3.7 logs in the treated fish groups stored at 22 and 4°C, respectively. Significant reduction ($p<0.05$) in the halotolerant bacterial counts was obtained in that control group stored at 4°C by 1.55 log10 CFU g^{-1}. Combined reductions in the frequency of halotolerant strains by 2.58 were observed, when addition of 0.5% TSP with storage of salted fish groups at 4°C. Moreover, decreasing the storage temperature from 22 to 4°C resulted in complete reduction (100%) of the halotolerant pathogens in treated salted fish group. New regulations for salted fish products are required in order to establishing microbiological performance standards for halotolerant bacteria contamination and advice for the addition of 0.5% TSP as food additives to the products.

Key words: Halotolerant, Trisodium Phosphate (TSP), salting, salted fish

INTRODUCTION

Faseikh, a traditional Egyptian salted fish, has been considered as a popular part of the Egyptian diet especially in certain celebration times as spring day. Since the WHO/FAO[1] recorded in 1991, the first documented outbreak of food poisoning in Egypt due to consumption of uneviscerated salted fish, faseikh, pressure has been put on the government, industry and the scientific community to seek new safe alternatives to control the microbial growth in uneviscerated salted fish.

Salt (NaCl) has been used for preservation and as a flavoring agent since ancient times. Proper salting is essential for processing the salted fish products to reduce the moisture content, imparts essential flavors, work as a preservative or inhibitor of microbial growth and prolong the shelf life[2]. Nevertheless, outbreaks of food poisoning diseases due to consumption of the uneviscerated salted fish products have been reported over 30 years ago[3,4]. Bacterial halophiles are abundant in environments especially in salt lakes, saline soils and salted food products[5]. In the last century, Hof[6] described the halophiles as groups of bacteria with the ability of live in concentrations up to about 15% salt and that many groups are physiologically active even at much higher salt concentrations. Halotolerant bacteria constitute a heterogeneous physiological bacterial group which includes spoilage microorganisms as *Bacillus* sp. and *Micrococcus* sp.[7] and food-borne pathogens as *Clostridium botulinum*, *Staphylococcus aureus* and *Vibrio parahemolyticus*[8]. Phosphates have been approved for use in the United States by ood and Drug Administration Department as food additives especially in a wide variety of meat products[9]. Trisodium phosphate (TSP, Na_3PO_4), is Generally Recognized As Safe (GRAS) food additive[10]. The antimicrobial effect of TSP has been examined in several types of foods as beef[11], chicken[12], fish[13] and mutton[14]. TSP kills the microorganisms through permeabilizing and disrupting the cytoplasmic and outer membranes of the bacterial cell because of its alkaline pH, which in turn leads to release of intracellular contents and eventual cell death[15]. There is a lack of

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literature concerning addition of TSP as a preservative agent to salted fish and their effects on the products shelf-life[16]. Therefore, the objective of this study was to evaluate the effect of 0.5% TSP to control the growth of halotolerant bacteria in the uneviscerated salted fish under the usual commercial conditions.

MATERIALS AND METHODS

Experimental design: The study was carried out during the period of June, 2004 untill January, 2005. The experimental design was based on dividing the fish samples, *Mullet cephalus*, to five groups. The first group (C) was 40 uneviscerated salted fish samples; each weigh 120±5 g, un-packaged, soaked in its brine and 60 days old (ripened under dry method salting at temperature of 22±5°C). The samples were randomly purchased from local salted fish market in Ismailia city, Egypt and represent the commercial product on retail sale. This group soon after purchase was packed in sterile polyethylene bag and transferred to laboratory for microbiological analysis without delayed.

The other four fish groups were laboratory prepared using standard practices and recipes like simulated commercial condition. Each group contained at least forty samples, each weigh 120±15 g. The used fish, *M. cephalus* for salting were fresh of good keeping quality according to the Egyptian Organization for Standardization for Salted fish EOS[17]. The following dry method recipe was used: for one-kilogram fish, 150 g salt, 65 g sugar and 10 g spice. The spice contained sodium nitrite as commercial procedures. The preliminary work using panelist tests selected concentration of Trisodium Phosphate (TSP) not more than 0.5% added to ripened fish for taste to be acceptable, therefore two groups (T_1 and T_2) were treated by the addition of 5 g TSP for each kilogram fish (0.5% w/w TSP) other two groups without treatment keep as control groups (C_1 and C_2). The fish were mixed thoroughly with the dry materials and placed into small barrels. The barrels were allowed to stand overnight so the fish could settle. The barrels were then filled with salt brine and closed. The C_1 and T_1 groups were aerobically stored at room temperature, 22±1°C like commercial condition. The other C_2 and T_2 groups were aerobically stored at recommended chilling temperature 4±1°C. The barrels were rolled each week during the first 6-week of storage. This was done to ensure an even salt uptake and good ripening. The total ripening period was 60 days. Triplicate microbiological measurements were carried out on each sample. The four groups were randomly duplicated sampling (15 each) and microbiologically analyzed on day 60 after ripening.

Preparation of serial dilutions: The technique recommended by APHA[18] was used for preparation of samples homogenate and serial dilutions. The fish flesh with skin was removed aseptically from each fish by using sterile knife and scissors then thoroughly mixed in sterile mortar. Ten gram of prepared flesh and 90 mL of 0.1% sterile synthetic sea water (3% NaCl) were homogenized in a Seward Stomacher (400^R/UK) for 2 min to a 1:9 dilution (Wt/Vol). One milliliter of the original homogenate was transferred serially into test tubes containing 9 mL of 0.1% sterile peptone water (Oxoid) to final dilution of 10^{-7}.

Enumeration of total halotolerant bacterial ounts: The recommended method described by APHA[18] was used to determine the total halotolerant bacterial counts. One milliliter from each prepared dilution was inoculated in duplicated plates containing sea water agar contain 3% NaCl using spread plate technique. All plates were incubated at 32°C for 2 days and the total halotolerant counts were expressed as CFU g^{-1}.

Identification of isolates: Five similar representative colonies were randomly picked from each plate and purified for further identification by using biochemical tests according to MacFadyean[19].

Statistical analysis: The effects of the treatment of fish groups by 0.5% TSP on the reduction of mean halotolerant bacteria were analyzed by 2-way ANOVA of a Completely Randomized auctorial Design and t-test for significance. Statistical calculations were processed using SAS statistical analysis software program, Version 6.11, significance was defined at $p<0.05$.

RESULTS AND DISCUSSION

Nowadays decontamination technologies are used extensively all over the world to reduce microbial contamination of foods. Thus in this study, the uneviscerated salted fish were decontaminated by addition of 0.5% TSP in order to optimize products quality and minimize potential risks from halotolerant bacteria.

Effect of 0.5% TSP on halotolerant bacterial counts: The average values of total halotolerant bacterial counts for market group (C) collected from the retail market was 6.84 $\log_{10}$ CFU g^{-1}. Halotolerant bacteria can grow in salted fish under wide range of salt concentrations. The initial microbial load, hygienic measures during manufacture and storage temperature are playing an important role in the counts of halotolerant bacteria in salted fish products[20].

The average values of total halotolerant bacterial counts for control (C_1) and treated groups (T_1) fish groups stored at room temperature (22°C) were 5.72 and 4.34 log_{10} CFU g^{-1}, respectively. On the other hand, for those groups (C_2 and T_2) stored at chilling temperature (4°C), the counts were 4.17 and 3.14 log_{10} CFU g^{-1}, respectively (Table 1). Data from another experiments counting the halotolerant and halophilic bacteria in uneviscerated salted fish by different values, Ahmed and Saad[21] recorded the mean value of 4.53x10 ^{4}CFU g^{-1}, while Baze[22] mentioned that the halophilic counts ranged from 10^5 to 10^6 CFU g^{-1} in salted fish products. Whatever, Egyptian Organization for Standardization for Salted fish[17] listed a microbiological performance standards for some bacterial groups, meanwhile excluding yet the microbiological performance standards for halotolerant bacteria in uneviscerated salted fish. When the obtained results were statistically compared to the markets samples, 0.5% TSP significantly reduced ($p<0.05$) the counts of halotolerant bacteria by 2.5 (36.5%) and 3.7 (54.1%) logs in both treated salted fish groups (T_1 and T_2) stored at 22 and 4°C, respectively (Table 2). It is worthy to mention that the significant reduction ($p<0.05$) in halotolerant bacterial counts for control group (39%) stored at 4°C is related to the inhibitory effect of chilling on microbial growth during storage[23], with other factors included raw fish and salt quality[24].

Effect of 0.5% TSP between examined groups: The results showed that the addition of 0.5% TSP to fish group (T_1) stored at 22°C was successful in significant reducing ($p<0.05$) the halotolerant bacterial counts by 1.38 (24.1%) log_{10} CFU g^{-1}. There was additional advantage in combining chilling storage (4°C) of salted fish (group T_2) with this 0.5% TSP treatment. A significant reduction ($p<0.05$) in the halotolerant bacterial count was obtained in fish group (T_2) stored at 4°C by 2.58 (45.1%) log10 CFU g^{-1} (Table 3). The uses of good quality fish and salts during fish procssesing[25] and the synergistic effect of TSP[26] with chilling[27] could also be interpreted the obtained results.

There was observed significance difference ($p<0.05$) in the total halotolerant bacterial counts of control group (C_2) stored at 4°C when compared to the other control group (C_1) that was stored at 22°C. This was due fundamentally to the fact that the growths of microbial halophiles on control samples stored at 4°C are suppressed because of chilling[28].

Meanwhile, salting as a preservative agent is not enough to completely inhibit the growth of halotolerant and halophiles in salted fish products[29]. In the same time, chilling one side and 0.5% TSP treatment on the other side could interprets the non

Table 1: Mean values (log_{10} CFU g^{-1}) of halotolerant bacteria for market and salted fish groups treated with 0.5% TSP stored at 22 and 4°C

		Treated groups			
22°C		22°C		4°C	
Time (day)	Market group C	C_1	T_1	C_2	T_2
60 day	6.84±3.86*	5.72±2.61	4.34±2.30	4.17±1.50	3.14±1.30

*±Means Standard Error

Table 2: Means halotolerant counts differences of log_{10} reductions between markets group (C) and treated fish groups stored at 22 and 4°C

Fish groups	Mean	*Mean difference with market group (C)	(%)	±SE
C_1	5.72^a	1.12^a	16.4	±1.25
T_1	4.34^a	2.5^b	36.5	±1.56
C_2	4.17^a	2.7^b	39.0	±2.36
T_2	3.14^a	3.7^b	54.1	±2.56

*Means followed by a different letter in the same row are significantly different ($p>0.05$)

Table 3: Means halotolerant counts significance differences of log_{10} reductions within and between fish groups stored at 22 and 4°C

Treated group				*Mean differences	(%)	±SE
C_1	5.72^a	T_1	4.34^b	1.38^c	24.10	±0.31
C_1	5.72^a	T_2	3.14^b	2.58^c	45.10	±1.31
C_1	5.72^a	C_2	4.17^b	1.55^c	27.10	±1.11
C_2	4.17^a	T_1	4.34^a	0.17^a	0.04	±0.80
C_2	4.17^a	T_2	3.14^a	1.03^a	0.25	±0.70
T_1	4.34^a	T_2	3.14^b	1.20^c	27.60	±1.00

*Means followed by a different letter in the same row are significantly different ($p>0.05$)

significance difference ($p>0.05$) in log mean values of halotolerant bacteria between C_2 and T_1 groups. Decreasing the storage temperature could inhibit the microbial growth in the seafood. Storage temperatures below the growth minimum result in a continued extension of the lag-phase until multiplication ceases and stops the growth of the microorganisms[30]. Hence, significance reduction ($p<0.05$) was observed by 1.20 (27.6%) log_{10} CFU g^{-1} between T_1 and T_2 fish groups (Table 3).

Effects of 0.5% TSP on isolated halotolerant strains: 0.5% TSP reduced the frequency of halotolerant bacteria by reduction level of 40 (39.2%) for the group stored at room temperature (22°C). Combined reductions in the microbial frequency by 32 (55.2%) were observed when addition of 0.5% TSP followed by storage of salted fish groups at chilling temperature (Table 4). Genus of *Bacillus*, *Flavobacterium*, *Micrococcus*, *Pediococcus*, *Proteus*, *Staphylococcus* and *Vibrio* were recorded as a halotolerant species isolated from the salted fish groups. The most predominated halotolerant strains in all examined groups were *Staphylococcus* sp. and *Bacillus* sp., which are gram-positive bacteria. These could interpret as salting processes typically results in microbial population shifting of the salted fish dominated by gram-negative bacteria to one in which gram-positive organisms

Table 4: Frequency distribution of halotolerant bacteria isolated from salted fish groups after 60 day salting

	Storage temperatures						
	22°C	22°C			4°C		
Groups	C	C_1	T_1	**D	C_2	T_2	D
Halotolerant strains	*F(%)	F(%)	F(%)	(%)	F(%)	F(%)	(%)
Bacillus sp	24	21	15	6	14	10	4
	(16.9)	(20.0)	(24.2)	(28.6)	(24.1)	(38.5)	(71.4)
Flavobacterium sp.	11	6	3	3	2	0	2
	(7.8)	(5.9)	(4.8)	(50)	(3.5)	(0)	(100)
Micrococcus sp.	16	15	9	6	8	4	4
	(11.3)	(14.7)	(14.5)	(66.7)	(13.8)	(15.4)	(50)
Pediococcus sp.	8	4	0	4	6	0	6
	(5.6)	(3.9)	(0)	(100)	(10.3)	(0)	(100)
Proteus sp.	19	16	10	6	5	3	2
	(13.3)	(15.7)	(16.2)	(37.5)	(8.6)	(11.5)	(40)
Staphylococcus sp.	61	39	25	14	23	9	14
	(43)	(38.2)	(40.3)	(35.9)	(39.7)	(34.6)	(39.1)
Vibrio sp.	3	1	0	1	0	0	0
	(2.1)	(1)	(0)	(100)	(0)	(0)	(0)
Total	142	102	62	40	58	26	32
	(100)	(100)	(100)	(39.2)	(100)	(100)	(55.2)

*F means the frequency, **D means the difference between the two frequencies

dominate[31]. Gram-negative bacteria are considered more salt sensitive than gram-positive bacteria. Gram-negative bacteria have previously been shown to be more susceptible to salt and TSP. This might be due to the cells toxicity to chloride ions and their very thin (2 to 3nm) peptidoglycan layer[32,33].

There are observed reduction in the frequency of the isolated halotolerant bacteria in both treated groups by 0.5% TSP. The frequency of *Bacillus* sp., *Flavobacterium* sp., *Micrococcus* sp., *Pediococcus* sp., *Proteus* sp., *Staphylococcus* sp. and *Vibrio* sp. were reduced by 28.6, 50, 66.7, 100, 37.5, 35.9 and 100%, respectively in the T_1 group stored at 22°C, while the reduction percentages for the other group (T_2) stored at 4°C were 71.4, 100, 50, 100, 40 and 39.1%, respectively (Table 4). Halotolerant bacteria are usually classified by their salt requirements needed for growth and as spoilage microorganisms. Spoilage halophiles have the ability to break down flesh of salted fish by their extracellular enzymes[34].

Effects of 0.5% TSP on pathogenic halotolerant strains: In that randomelly collected salted fish group from the retail markets, we were able to isolate *B. cereus, P. vulgaris, S. aureus, V. parahaemolyticus* were correlated by frequency of 5 (9.3%), 9 (16.6%), 39 (72.2%) and 1 (1.9%), respectively. Whereas for those samples stored at 22°C, the frequency of theses halotolerant pathogens were 3 (9.7%), 5 (16.1%), 23 (74.2%) and 0% for group C_1 and 1 (6.7%), 3 (20%), 11 (73.3%) and 0% for group T_1, respectively (Table 5).

However in this study, decreasing the storage temperature of salted fish groups from 22 to 4°C resulted in reducing the frequency of halotolerant food-borne

Table 5: Incidence of isolated halotolerant pathogens from the salted fish after 60 day salting

	Storage temperatures						
	22°C	22°C			4°C		
Groups	C	C_1	T_1	**D	C_2	T_2	D
Halotolerant pathogens	*F(%)	F(%)	F(%)	(%)	F(%)	F(%)	(%)
B. cereus	5	3	1	2	1	0	1
	(9.3)	(9.7)	(6.7)	(66.7)	(100)	(0)	(100)
Proteus vulgaris	9	5	3	2	0	0	0
	(16.6)	(16.1)	(20)	(40)	(0)	(0)	(0)
S. aureus	39	23	11	12	0	0	0
	(72.2)	(74.2)	(73.3)	(52.2)	(0)	(0)	(0)
V. parahaemolyticus	1	0	0	0	0	0	0
	(1.9)	(0)	(0)	(0)	(0)	(0)	(0)
Total	54	31	15	16	1	0	1
	(100)	(100)	(100)	(51.6)	(100)	(0)	(100)

*F means the frequency, **D means the difference between the two frequencies

pathogens (100%) in the treated salted fish group (T_2). These results in microbial reduction in the product were related to the inhibitory effect of chilling temperature and salting on the pathogens with addition of the bactericidal effect of TSP. The food-borne diseases acquired by human due to consumption of salted fish contaminated by *B. cereus*[35], *S. aureus*[36] and *V. parahaemolyticus*[37] are well known. TSP was studied by many investigators as preservative agents and succeeded in inhibiting the growth of food borne pathogens[38] in foods of animal origin. However, there is no evidence from literatures for use of TSP as a food additive or preservative in uneviscerated salted fish.

The results presented in this study indicate that 0.5% TSP act as a preservative agent when added to uneviscerated salted fish as food additives. The effect of TSP was observed at both salted fish groups stored at room and chilling temperatures. The addition of 0.5% TSP to uneviscerated fish as additive during salting processes and storing at 4°C along during period may help in reduction of halotolerant pathogens in this product. It should be consider that other numerous factors play a role in the microbial counts of the uneviscerated salted fish including the quality of raw fish, water and salt. New regulations are required for salted fish products to establish microbiological performance standards for halotolerant bacteria contamination and advice for the addition of 0.5% TSP as fish additives to the salting fish products during processing.

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