



Effect of Marination on *Vibrio Parahaemolyticus* in Tilapia Fillets

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ABSTRACT

Foodborne diseases affect millions of people each year. To reduce the incidence of bacterial foodborne pathogens such as *V. parahaemolyticus*, more effective treatment methods are needed. Accordingly, the effect of marinating fish fillets with lemon juice 50% , thyme powder 4g/kg and pomegranate peel extract (PPE) (1% v/w) against *V. parahaemolyticus* were studied . The prepared marinades were artificially added to fish fillet samples inoculated with *V. parahaemolyticus* then hold in refrigerator at 4°C and examined every day. Results indicated that the lemon juice decreased the count of *V. parahaemolyticus* from log 8.99±0.02 cfu/g to log ≤1 cfu/g at fourth day by reduction % 100, while thyme powder reduced the count to 4.86±0.51 log cfu/g at fifth day by reduction % 88.5. Finally, PPE decreased the count to log 4.89±0.38 cfu/g at fifth day by reduction % 76.3 . Thus, lemon juice was the most effective agent against *V. parahaemolyticus* as killing all the bacteria within four days of incubation.

Keywords: *Vibrio parahaemolyticus*., marination, lemon, thyme, Pomegranate

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1.INTRODUCTION:

Fish and seafood constitute an important food component for a large section of world population. They come after meat and poultry as stable animal protein food where fish forms a cheap source of protein (Bakr *et al.*, 2011).

Long cooking periods (roasting and frying) can cause the formation of tricyclic amines in the foodstuffs. These

chemicals are considered as carcinogenic and may be dangerous for public health. In order to prevent the occurrence of this carcinogenic substances in fish meat, it can be processed with organic acids and salt either during cooking at reduced cooking temperatures or without any heat application. This widely accepted hygienic preservation method is known as marination (Collignana *et al.*,2001)

The marinating process is one of the oldest methods of preservation of fish and other sea products (Giuffrida *et al.*, 2007). Marinades are solutions, including sugar, spices, oil, acids (from vinegar, fruit juice, wine) and they are used to improve tenderness, juiciness, flavour and aroma and to extend shelf life of seafood, meat, poultry and vegetables (Cadun *et al.*, 2005).

Marinated fish are typically inspired as ready-to-eat products with no heat treatment (Gram and Huss, 1996). Marinades are semi-preserves; the preserving principal is the combination of acetic acid and salt. The inhibitory effects of these substances on bacteria and enzymes increase with concentration. The aim is not only to hold up the action of bacteria and enzymes, but also to tenderize or to change the taste, textural and structural properties of raw material, resulting in a product with a characteristic flavour and an extended shelf life. Marinades are stored at cooler temperatures (4-6°C) keep for a long time (Gokoglu *et al.*, 2004).

Lemon juice was very effective in inhibiting *Vibrio*, *Enterobacter*, *Citrobacter* spp. and *E. coli* (Jayana *et al.*, 2010). Lemon essential oils also showed antioxidant activity (Kim *et al.*, 2008).

Thyme oil is a tonic stimulant and stomachic and digestive relieves gastritis, enterocolitis and mouth thrush. It is useful for respiratory infections, asthma and bronchitis. It is effective for treating swelling provoked by gout or rheumatic problems, for joint pains, backache and sciatica. Thyme oil is also useful for urinary and vaginal infections, endometritis (*candida*), prostrates and vaginitis (Ratan, 2006). Thyme oil exhibits antibacterial activity and has been useful in dental practice.

Pomegranate was reported in numerous studies to have excellent antifungal, antiprotozoal, antioxidant, anticarcinogenic, anti-inflammatory and antibacterial properties (Wang *et al.*, 2011). Pomegranate (*Punica granatum* L.) is known as one of the healthiest fruits due to its high phenolic content and antioxidant activity (Tabaraki *et al.*, 2012).

Pomegranate have been reported in many studies that have antimicrobial activity against a wide range of microorganisms including Gram positive and Gram-negative bacteria (Naz *et al.*, 2007).

The present work was planned out to study the effect of different marinades (lemon juice, thyme powder and pomegranate peel extract) on safety and shelf life of tilapia fillets samples artificially inoculated with *Vibrio parahaemolyticus*.

2. MATERIAL AND METHODS:

2.1. Preparation of bacterial strain: -

Vibrio parahaemolyticus (NCTC10885) pure strain was obtained from the Food Microbiology Laboratory, Animal Health Research Institute, Dokki, Egypt. The strain was maintained on tryptic soy agar slants contain 3% NaCl at 4°C. Loopful of *Vibrio parahaemolyticus* was transferred aseptically into 10 ml of sterile alkaline peptone water with 3% NaCl and then incubated at 37°C for 24hrs. After that *Vibrio parahaemolyticus* was counted by using spread plate method (FDA, 2001) to determine the concentration. The count was adjusted to 10⁷ cfu/ml (Shirazinejad and Ismail, 2010) with tube dilution methods. The number of cfu/ml was considered as infective dose to be inoculated into fish fillet samples.

2.2. Preparation of fish fillet Samples:

A grand total of 1Kg of fresh Tilapia fillets were purchased directly from local markets in Tanta, Gharbia governorate, Egypt. The samples were taken and transferred directly to the laboratory in an ice box under complete aseptic conditions without delay. The samples were cut into pieces and divided into five equal groups (200g of each) and placed in aseptic polypropylene trays designed for disposable food packaging.

2.3. Preparation of Marinades:

Marined I consists of mixture of (100ml fresh lemon juice, 5.9g garlic powder, 9.23g table salt, 0.5g turmeric, 0.15g hot chili powder, 0.5g black pepper and 200ml distilled water) were mixed well according to Siavash *et al.* (2016).

Marined II as Marined I but addition of thyme powder 4g/kg instead of lemon juice according to Daniela Istrati *et al* (2011).

Marined III as Marined I but addition of pomegranate peel extract (PPE) (1% v/w) supplied from National Research Center, instead of lemon juice according to Unalan *et al.*, (2011).

2.4. Artificial contamination of fish fillet samples with *Vibrio parahaemolyticus*:

The tilapia fillets samples (200g) were inoculated with *V. parahaemolyticus* 10^7 cfu/ml (Shirazinejad and Ismail, 2010) by pipetting the inoculum drop by drop as evenly as possible across the samples and mixed well with sterile glass rod for distribution of the inoculum and gentle rocking the samples immediately after inoculation. The inoculated tilapia fillets samples were left for 30 min. at room temperature to allow attachment and absorption of the inoculated bacteria (Dubal *et*

al., 2004). The contaminated samples were stored at ambient temperature ($30\pm 2^\circ\text{C}$). *V. parahaemolyticus* count in the sample was enumerated to get the initial load before addition of marinades (Terzi and Gucukoglu, 2010).

2.5. Challenge trials:

The samples were divided into five groups. Group one (control -ve) in which the samples were immersed in 300ml distilled water only. Group two (control +ve) the samples were inoculated with 10^7 cfu/ml *V. parahaemolyticus* strain only and immersed in 300ml distilled water. Group three the samples were inoculated with 10^7 cfu/ml *V. parahaemolyticus* strain and immersed in 300ml of marinade I at a ratio of 1.5:1 (marinade: fish fillet, ml/g). For group four, the samples were inoculated with 10^7 cfu/ml *V. parahaemolyticus* strain and immersed in 300ml of marinade II at a ratio of 1.5:1 (marinade: fish fillet, ml/g). For group five, the samples were inoculated with 10^7 cfu/ml *V. parahaemolyticus* strain and immersed in 300ml of marinade III at a ratio of 1.5:1 (marinade: fish fillet, ml/g). Marinades covered all surface of tilapia fillets samples. The tilapia fillets samples were gently agitated or pressed with a sterile spatula to ensure immersion in the marinade. Care was taken to ensure that the solid components of the marinades were distributed as evenly as possible both within and between trays.

All the trays were properly labelled, stored at 4°C and examined every day for sensory analysis (colour, odour and texture) according to (Hemin, 2013) and *Vibrio parahaemolyticus* count until spoilage of the examined samples occurred, according to (FDA, 2001). All the above experiment was performed in triplicate (three times).

2.6. Sensory examination (colour, odour and texture): -

It was conducted during storage according to Hemin (2013).

2.7. Bacteriological Analysis:

Ten grams from each sample were taken under aseptic condition to sterile homogenizer containing 90 ml peptone water with 3% NaCl then the contents were homogenized at 4000rpm for 2.5 min. after that the mixture was allowed to stand for 6 min. at room temperature under aseptic condition. The content of the flask was thoroughly mixed and 1ml was transferred into separated tubes containing 9ml of sterile alkaline peptone water with 3% NaCl, from which tenfold serial dilutions up to 10^{-10} were prepared. From the prepared samples 0.1 ml from each prepared serial dilutions were spread plated with a glass spreader on Thiosulfate citrate bile and sucrose agar (TCBS) then incubated at 37°C for 24hrs (Thatcher and Clark, 1978). The rounded colonies 2-3mm in diameter, with green and/or blue centers were counted and expressed as colony forming units (log cfu/g).

2.8. Statistical analysis:

The data was statistically treated by one-way ANOVA using SPSS program for windows (Version 17) (SPSS Inc. Chicago, IL and USA) and Duncan's post hoc test with $p < 0.05$ considered to be statistically significant (Knapp and Miller 1992).

3. RESULTS:

The results obtained in table (1) showed that the scores of overall acceptability in case of using lemon juice 5; 4.66±0.33; 4.33±0.33; 3.66± 0.33; 3.33±0.33; 3.16±0.16 and 2.66±0.33 at zero day, 1st day, 2nd day, 3rd day, 4th day, 5th day and 6th day of

the storage period at 4°C, respectively. While, in case of using thyme powder the scores was 5; 4.33±0.33; 3.66±0.33; 3.33±0.33; 2.83±0.16; 2.50±0.50 and 1.66±0.33 at the same period of storage, respectively. In case of using PPE, the score was 5; 3.66±0.33; 3.33±0.33; 2.66±0.33; 1.66±0.33 and 1.33±0.33 at the same period of storage, respectively. Comparing with the score of overall acceptability in the control sample which was 5, 3 and 1 after zero day, 1st day and 2nd day of the storage period at 4°C respectively.

Table (2) revealed *V. parahaemolyticus* counts (log cfu/g) in tilapia fillet samples treated with different marinades. The initial counts of *V. parahaemolyticus* in tilapia fillet samples was 8.99± 0.02 log cfu/g. Such count of *V. parahaemolyticus* slightly decreased to 6.78±0.78; 7.94±0.53 and 8.37±0.11 log cfu/g respectively, after treatment with lemon juice, thyme powder and PPE, respectively. Comparing with *V. parahaemolyticus* count in the control-ve and +ve samples which were 3.28. ±1.76 and 9.14±0.49 log cfu/g, respectively. On the 1st day at refrigeration storage (4±1°C) the mean value of *V. parahaemolyticus* in control -ve and +ve samples was 2.9±1.45 and 9.58±0.34 log cfu/g, respectively. The mean value of *V. parahaemolyticus* were 4.33±0.33; 6.54±0.53 and 6.99±0.19 log cfu/g, respectively. On the 2nd day the mean counts of *V. parahaemolyticus* were 3.30±0.30; 6.19±0.41 and 6.31±0.17 log cfu/g, respectively. On the 3rd day the mean value of *V. parahaemolyticus* were 3.20±0.20; 5.03±0.22 and 5.40±0.11 log cfu/g, respectively. On the 4th day the mean counts of *V. parahaemolyticus* were not detected; 4.86±0.51 and 4.89±0.38 log cfu/g, respectively. By the 5th day the mean value of *V. parahaemolyticus* were not detected;

1.03±0.37 and 2.13±0.81log cfu/g for samples treated with lemon juice, thyme powder and PPE, respectively.

Table (3) showed that the reduction percent of *V. parahaemolyticus* in tilapia fillet were 24.58%; 51.83%; 63.29%; 64.40%; 100% and 100% during refrigerated storage, respectively in case of treatment with lemon juice. While marinating with thyme powder

reduced the growth of *V. parahaemolyticus* by 11.67%; 27.20%; 31.14%; 44.04%; 45.93% and 88.50% after zero day, 1st day, 2nd day, 3rd day, 4th day, 5th day of refrigerated storage, respectively. Meanwhile fish fillet samples treated with PPE recorded reduction in *V. parahaemolyticus* by 6.80%; 22.24%; 22.90%; 39.45%; 45.60% and 76.30% after zero day, 1st day, 2nd day, 3rd day, 4th day, 5th day of refrigerated storage, respectively.

Table (1): The effect of different marinades (lemon, thyme and pomegranate peel extract) on overall acceptability of artificially inoculated fish fillet samples with *V. parahaemolyticus* during cold storage at 4 °C.

Group Period	Control	Marined I (lemon juice)	Marined II (Thyme powder)	Marined III (PPE)
Zero day	5	5	5	5
1 st day	3.33±0.43	4.66±0.33	4.33±0.33	3.66±0.33
2 nd day	-	4.33±0.33	3.66±0.33	3.33±0.33
3 rd day	-	3.66±0.33	3.33±0.33	2.66±0.33
4 th day	-	3.33±0.33	2.83±0.16	1.66±0.33
5 th day	-	3.16±0.16	2.50±0.50	1.33±0.33
6 th day	-	2.66±0.33	1.66±0.33	-

5 = very acceptable 4 = Acceptable 3 = Middle 2 = Unacceptable 1 = Rejected - =spoilage

The values represent Mean ± SEM of three experiments.

Table (2): The effects different marinades (lemon, thyme and pomegranate peel extract) on *V. parahaemolyticus* count (log cfu/g) artificially inoculated in fish fillet samples

Groups	Zero day	1 st day	2 nd day	3 rd day	4 th day	5 th day
Control -ve	3.28±1.76 ^{ab}	2.9±1.45	-	-	-	-
Control +ve	9.14±0.49 ^{ab}	9.58±0.34 ^{abc}	-	-	-	-
Marined1(lemon)	6.78±0.78 ^a	4.33±0.33 ^{abc}	3.3±0.3 ^b	3.2±0.2 ^{bc}	ND	ND
Marined 2(Thyme)	7.94±0.11	6.54±0.53 ^{ab}	6.19±0.41 ^b	5.03±0.22 ^b	4.86±0.51	1.03±0.37 ^a
Marined3(PPE)	8.37±0.53 ^a	6.99±0.19 ^{ab}	6.31±0.17 ^b	5.40±0.11 ^c	4.89±0.38	2.13±0.81 ^a

Initial load of *V. parahaemolyticus* 8.99± 0.02 log cfu/g

The values represent Mean ± SEM of three experiments.

Means within a column followed by different letters are significantly different ($P < 0.05$).

N.B.: (-) means samples rejected

(ND) means microorganism not detected.

Table (3): Reduction % of *V. parahaemolyticus* count artificially inoculated into fish fillet samples treated with different marinades (lemon, thyme and pomegranate peel extract)

Groups	Zero day	1 st day	2 nd day	3 rd day	4 th day	5 th day	6 th day
Marined I (lemon juice)	24.58	51.83	63.29	64.4	100	100	-
Marined II (Thyme powder)	11.67	27.2	31.14	44.04	45.93	88.5	-
Marined III (PPE)	6.8	22.24	22.9	39.45	45.6	76.3	-

N.B.: (-) means samples rejected.

4. DISCUSSION:

The demand for safe foods, coupled with the preference by consumers for foods free of synthetic additives, has increased the interest for natural preservatives in recent years (Moreir, *et al.* 2007).

Improvements in the shelf-life of product have an important economic impact by reducing losses attributed to spoilage and by allowing the products to reach distant and new markets. In a broad sense the ultimate goal of sensory evaluation was to predict its acceptance. Sensory analysis has become popular marketing research as well as in quality assurance and in research and development (Gouin, 2004).

The results obtained in table (1) showed that the scores of overall acceptability in case of using lemon juice was $5; 4.66 \pm 0.33; 4.33 \pm 0.33; 3.66 \pm 0.33; 3.33 \pm 0.33; 3.16 \pm 0.16$ and 2.66 ± 0.33 at zero day, 1st day, 2nd day, 3rd day, 4th day, 5th day and 6th day of the storage period at 4 °C respectively. While, in case of using thyme powder the scores was $5; 4.33 \pm 0.33; 3.66 \pm 0.33; 3.33 \pm 0.33; 2.83 \pm 0.16; 2.50 \pm 0.50$ and 1.66 ± 0.33 at the same period of storage, respectively.

Moreover, concerning PPE, the score was $5; 3.66 \pm 0.33; 3.33 \pm 0.33; 2.66 \pm 0.33; 1.66 \pm 0.33$ and 1.33 ± 0.33 at the same period of storage, respectively. Comparing with the score of overall acceptability in the control sample which was 5, 3 and 1 after zero day, 1st day and 2nd day of the storage period at 4°C, respectively. From the obtained results there was a decline of sensorial characters after the first day of storage with clear reduction of overall acceptability values in the control samples and showed complete spoilage at 2nd day of the storage period at 4°C.

Furthermore, the obtained results indicated that only marination by lemon juice combated against the artificially inoculated *V. parahaemolyticus* till the 6th day of the study and revealed the highest improvement of sensory attributes, this is because lemon contain organic acid which have antioxidative action, acid taste production, decrease of the protein content and the increase of the acidity and the free amino-acid content give the marinated fillets a characteristic texture and aroma.

So, this could be considered of highly importance indication and also a solution for the rapid decomposition of fresh fish and meat products especially those preserved by refrigeration While the sensory quality obtained by thyme powder indicated that shelf life prolonged to 5th day, this may be due to thyme is traditionally used as flavouring agents in fish and thyme essential oil contains many flavonoids, like apigenin, aringenin, luteolin and thymonin (Penalever *et al.*, 2005).

On the other hand, Fathy *et al.* (2015) reported that thyme extract has strong antimicrobial and antioxidant activity and can maintain the quality parameters and extend the shelf life of refrigerated Nile tilapia fillet for 9 days longer than control one.

While, fish fillet group treated with PPE showed somewhat acceptable improvement of sensorial characters and extended the shelf life till the 4th day compared with control group during storage period.

Generally, sensorial characters of different treated tilapia fillet samples during cold storage (4°C) were improved by using lemon juice then thyme powder and at least PPE as compared with the control ones during the same storage period.

Spoilage characteristics develop in food as microorganisms digest the sugars, complex carbohydrates, proteins and fats of food producing undesirable effects in the food if the spoilage microorganisms grow to significant levels. Typically, the threshold level for observation of food spoilage by odour, taste or sight is not reached until the spoilage microflora exceeds about 10^7 organisms/g of food. Fish microflora includes bacterial species such as *Vibrio* species (Gram and Huss, 2000). Microbial growth and metabolism is a major cause of fish spoilage which produce amines, biogenic amines such as putrescine, histamine and cadaverine, organic acids, sulphides, alcohols, aldehydes and ketones with unpleasant and unacceptable off-flavours (Dalgaard *et al.*, 2006).

Many extracts and essential oils have been derived from plants and found to have antibacterial, fungicidal and insecticidal properties (Hänsel *et al.*, 1999).

Table (2) revealed *V. parahaemolyticus* counts (log cfu/g) in tilapia fillet samples treated with different marinades. The present data exhibited the potential of marinades as natural food preservative against *V. parahaemolyticus* in tilapia fillet samples. The initial counts of *V. parahaemolyticus* in tilapia fillet samples was 8.99 ± 0.02 log cfu/g. Such count of *V. parahaemolyticus* slightly decreased to 6.78 ± 0.78 ; 7.94 ± 0.53 and 8.37 ± 0.11 log cfu/g respectively, after treatment with lemon juice, thyme powder and PPE, respectively. Comparing with *V. parahaemolyticus* count in the control -ve and +ve samples which were 3.28 ± 1.76 and 9.14 ± 0.49 log cfu/g, respectively. The means within a column followed by different letters are significantly different ($P < 0.05$).

These finding was nearly similar to that reported by Prateek and Donald (2013)

who found that reduction levels of *V. parahaemolyticus* inoculated into tilapia (*Oreochromis niloticus*) fillet samples and marinated with lemon juice and incubated 4°C for 30 or 120 min from >7 log cfu/g to 5 log cfu/g.

By the 1st day at refrigeration storage ($4 \pm 1^\circ\text{C}$) the mean value of *V. parahaemolyticus* in control -ve and +ve samples was 2.9 ± 1.45 and 9.58 ± 0.34 log cfu/g, respectively. The mean value of *V. parahaemolyticus* were 4.33 ± 0.33 ; 6.54 ± 0.53 and 6.99 ± 0.19 log cfu/g for samples treated with lemon juice, thyme powder and PPE, respectively, the means within a column followed by different letters are significantly different ($P < 0.05$).

On the 2nd day of refrigeration storage at ($4 \pm 1^\circ\text{C}$) of treated tilapia fillets samples, the mean counts of *V. parahaemolyticus* were 3.30 ± 0.30 ; 6.19 ± 0.41 and 6.31 ± 0.17 log cfu/g respectively, as well as they were acceptable from aesthetic points without off odour or discoloration. compared with the control -ve and +ve. samples which showed extreme discoloration and off-odour in this day of storage indicating complete spoilage.

By the 3rd day of refrigeration storage of treated tilapia fillets samples with different marinades (lemon juice, thyme powder and PPE), the mean value of *V. parahaemolyticus* were 3.20 ± 0.20 ; 5.03 ± 0.22 and 5.40 ± 0.11 log cfu/g for samples treated with lemon juice, thyme powder and PPE, respectively, the means within a column followed by different letters are significantly different ($P < 0.05$).

At the 4th day of refrigeration storage at ($4 \pm 1^\circ\text{C}$) of treated tilapia fillets samples the mean counts of *V. parahaemolyticus* were not detected; 4.86 ± 0.51 and 4.89 ± 0.38 log cfu/g, respectively.

By the 5th day of refrigeration storage of treated tilapia fillets samples the mean value of *V. parahaemolyticus* were not detected; 1.03 ± 0.37 and 2.13 ± 0.81 log cfu/g for samples treated with lemon juice, thyme powder and PPE, respectively. The means within a column followed by different letters are significantly different ($P < 0.05$).

Also, at the six day the treated samples with lemon juice become yellow in colour while samples treated with thyme powder and PPE became darkened showed extreme discoloration, off-odor indicating complete spoilage.

These results were nearly similar to those reported by Daniela *et al.*, (2011) who found that marinating by lemon enhanced shelf life at 4°C by preventing protein degradation and delay microbial spoilage.

The results showed growth of *V. parahaemolyticus* in marinated samples. As illustrated in table (3). lemon juice reduced the growth of *V. parahaemolyticus* by 24.58%; 51.83%; 63.29%; 64.40%; 100% and 100% during refrigerated storage, respectively.

Lemon contain high concentrations of citric acid, the major organic acid present in these juices, which is partly responsible for the antibacterial activity of these fruits due to contain a group of flavonoids including polymethoxy flavones, flavone glycosides and limonoids which enhance antimicrobial activity (Ladaniya, 2008).

Another study in Nigeria also showed that lemon juice (both aqueous and ethanol extracts) killed most gram positive and gram-negative bacteria at a concentration of 256 mg/mL (Aibinu *et al.*, 2006).

While, marinating with thyme powder reduced the growth of *V. parahaemolyticus* by 11.67%; 27.20%; 31.14%; 44.04%; 45.93% and 88.50% after zero day, 1st day, 2nd day,

3rd day, 4th day, 5th day of refrigerated storage, respectively.

Meanwhile, fish fillet samples treated with PPE recorded reduction in *V. parahaemolyticus* by 6.80%; 22.24%; 22.90%; 39.45%; 45.60% and 76.30% after zero day, 1st day, 2nd day, 3rd day, 4th day, 5th day of refrigerated storage, respectively.

So, the present results showed that marinating with lemon juice showed maximum activity against *V. parahaemolyticus* compared with the control on fifth day of refrigerated storage at 4°C.

In this study, the information given by the obtained results revealed that using marinating by lemon juice on tilapia fillets can improve its safety and extended its shelf life by enhancing sensory characters and due to their antibacterial effect against Gram negative bacteria especially *Vibrio species*.

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