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# Effect of Chitosan Nanocomposite and Chamomile Extract on the Chemical and Microbial Changes of Phytofag Fish Fillet (Hypophthalmichthys molitrix) Infected with Staphylococcus Aureus

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**Abstract:**

*The present study aims to measure the effects of antioxidant, antimicrobial properties of chitosan nanocomposite coating and chamomile extract in* ***Phytofag fish fillet*** *(Hypophthalmichthys molitrix) inoculated with Staphylococcus Aureus stored in the refrigerator (4 ±1°C). Phytofag fish fillet was divided into 8 groups .Then all samples were inoculated with Staphylococcus Aureus. Subsequently, considering the chemical parameters (active nitrogen base, peroxide number, PH), and the microbial parameters (total visible count (TVC) of bacteria, cold-free bacteria) and the samples were analyzed within 16 days (4 ±1°C). Based on the results, chitosan nanocomposite exhibited meaningful potential (P<0.05) for storage in the refrigerator for preventing Staphylococcus Aureus for chitosan1% treatments and chamomile extract 0.5% ,respectively, compared with the control chamomile at the end of the storage period .In terms of the chemical parameters , though nanocomposite coated samples had the lowest PH and the volatile nitrogen bases ,TVB-N (P<0.05),on the other hand, the chitosan nanocomposite coating delayed lipid oxidation by decreasing peroxide production in the samples compared to the control treatment at the end of the refrigerated storage period. Also, this coating reduced the total bacterial count number and cold-free bacteria in the fillets compared to the control sample. This study findings*

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*derived results indicated that chitosan nanocomposite coating increased the texture strength and penetrated force if the penetration depth increased from D0 to D12 and then decreased on day 16.*

**Key words:** chitosan nanocomposite, chamomile extract, chitosan, Staphylococcus Aureus, Phytofag fish fillet

## INTRODUCTION

Staphylococcus Aureus or golden Staphylococcus is of bacillus order and the Staphylococcus family and is spherical form seen as irregular as grape cluster. This gram-positive bacteria is immobile and non-sporaza with growth temperature ranging from 6 to 48°C but the suitable temperature for their growth is 37° C.This bacteria is an important pathogenic agent for a wide range of human and animal infections including toxin production induced diseases (Blake Bourne and Peter, 2002).In many countries, after Salmonella and Clostridium perfringens, this bacteria is of the pathogenic bacteria leading to the spread of food infections and poisoning .Humans and most domestic animals are the host of these bacteria( Munoz, 2006).

Undoubtedly, natural and harmless anti-microbial agents will be required in future. Also the global economic conditions under which we live cause the trade and transportation of foodstuff worldwide. Then worrying about the health of chemical preservatives and the negative reaction of the consumers to chemical and synthetic preservatives have promoted and increased the interest in natural substitutes to prolong the shelf-life of the product. Applying antimicrobial agents as a combination with each other and via traditional or new processing methods is very significant for determining the potential synergistic effects of these compounds (Burt 2004).

Regarding the criticality of the research, it has to be stated that since food is a fundamental issue, either from the food consumers' perspective or from the food industry owners' view and given the reports on various cases of contaminated food induced infections, paying attention to food health and presenting some strategies to preserve the health of the foodstuff as much as possible is expanding. In addition, due to the ever-increasing negative and unfavorable attitude of the consumers in using foodstuff in which chemical preservatives are used, on the one hand, and on the other hand, the numerous problems of authorities in the food control methods and systems as being expensive and time-consuming made the producers and health authorities to pay more attention to applying natural preservatives .The preservatives we analyze in this experiment are chamomile extract and chitosan nanocomposite .

**Problem Statement**:

Fish and fishery products play a remarkable role in the global food security and human nutritional needs in developed and developing countries (FAO, 2014). Consuming fish and its products is because of good digestibility and high content of polyunsaturated fatty acids (Gomez-Estaca et al., 2006).At the time being, fish is highly significant as a healthy foodstuff because many species are a source of high digestible proteins, including essential amino acids. In terms of therapeutics, fish contain essential polyunsaturated fatty acids plus calcium, iodine, vitamins, and many other nutrients (Wengopal, 2006).However, in addition to the mentioned advantages, fish are highly susceptible to adverse chemical and microbiological changes.

Today, in order to limit meat product corruption, phytoantimicrobials are applied which used to be employed as flavors .While at the moment, their antibacterial and

antioxidant traits have been proved (Hosseini et al., 2010). Chitosan is one of the natural preservatives and active coating (Lopez Caballero et al., 2004), focused on as one of the natural food additives due to its non-toxic nature, antioxidant and anti-bacterial activity, film formation, biocompatibility and biodegradability. Antimicrobial nanocomposite systems are highly appropriate for controlling pathogens. Since the materials in the nano-scale range have a higher surface-to-volume ratio compared to micro-scale counterparts, they are more effective nano-materials. Nano-scale materials act as an inhibitory agent for antimicrobial activity, fatal agents, or antibiotic carriers (Helander et al., 2001).Thus, in the present study, efforts are made to study the applicability of active packaging system containing chitosan nanocomposite as a base material and also as an antibacterial agent and chamomile extract as an antioxidant agent in Phytofag fish fillet during refrigerated storage period.

Silver carp or Phytofag, the main habitat of this fish in Siberia and China (Amur River), but due to rapid growth, flocking life, artificial reproduction and optimal meat quality, they are introduced as the most desirable breeding fish in the world and reproduced throughout the world. This fish belongs to the carp fish species and is considered as the main species in the common breeding system of carp in Iran. So that in Iran also according to the 2007 Fisheries Statistics of Iran, the production of carp fish was 97,262 tons, the main part of which includes Phytofag fish.

## METHODOLOGY

In this chapter, whatever done, whether providing and preparation method for chamomile extract ,or chitosan nanocomposite, the preparation of Phytofag fish and Staphylococcus Aureus bacteria for inoculation, and then their

measurement procedure using microbiological and chemical analysis have been described.

## MATERIALS AND EQUIPMENT

### Applied Material

Phytofag fish - chamomile leaf - ethanol alcohol - chitosan – acetic acid - glycerol - culture media – Ringer tablet- distilled water -Vial Lyophilized of Staphylococcus - buffer - physiologic serum - chloroform-Tween- Dimethyl sulfoxide - starch 1% - butanol- TBA reagent - magnesium oxide-octanol- methyl red reagent -boric acid.

### Non Applicable Devices and Equipment

Rotary-scales-decanter-Arlene Meyer-aluminum foil-filter paper-mechanical mixer- cleansing cloth pipette, desiccator - metal containers - oven – precise scales - electric furnace – porcelain crucible – round-bottom flask- Soxhlet device - heater apparatus –kjeldahl digestion and distillation apparatus- Spectrophotometer buret- Whatman paper Ultrasound device - incubator - centrifuge - plate - sampler - flask- Falcon-Ben-Marie tube - Brookfield device - digital pH meter-loop.

### 1-Chamomile Extract Preparation

First, 50 g of the dried plant was ground and 500 ml of ethanol alcohol 95% (i.e., to every 50 g, add10 times alcohol) was added. It was poured it in the Erlene and the whole Erlene was covered with an aluminum plate. It was put in Ben Marie 45°C for 24 h. High-colored alcohol extract (as the solvent indicating the release of effective ingredients) absorbed the color of the plant. It was passed through filter paper. The extract was concentrated in the 2nd stage .That is, dry material was added .This was done by rotary device through creating vacuum. The temperature between 40 °and 45°C leads to evaporation. This

operation was repeated until sufficient amount of extract was gained.

**2. Preparation of Chitosan 1%**

First, chitosan 1% solution (average molecular weight) was prepared from Sigma Company (the USA) and prepared in acetic acid 1% and after putting over the magnetic heater and setting the temperature at 40°C range , stirring was done so that chitosan dissolves completely. After 3 h, chitosan dissolved completely in acetic acid and a pale brown solution was yielded. In this stage, 0.75 mml of glycerol for every gram of chitosan was added as softener, the time period for complete glycerol dissolution was 30m.Then the question solution was 3 times vacuum-impregnated due to the presence of impurities with Whatman paper 3. Tween 80 was added as emulsifier for 0.2% of chitosan solution. Then stirring was done for 30 m at 40°C, so that the emulsifier uniformly spread throughout the solution and finally, it was used as a cover film (Tajkarimi et al., 2010).

**3. Preparation of Chitosan Nanocomposite**

To prepare the nanocomposite, chamomile extract was added to chitosan solution1% prepared above and the final solution was sonicated for 5 m at amplitude 100 and 20 kHz frequency (generated waves are called "sonication") by using an ultrasound device , which breaks the chitosan particles and the extract by probe placed inside the liquid and converts into the nano.

**4. Preparing Staphylococcus Aureus for Inoculation**

First off, the Lyophilized strain of Staphylococcus Aureus was purchased from Iran Scientific and Industrial Studies Institute and the vial containing the bacteria was decomposed in the vicinity of the flame and under sterile condition and its contents were transferred to the culture medium of cow's heart

and brain and incubated for 18 h at 35°C.After that the sample containing bacteria was centrifuged at 500 rpm for15 m and the supernatant was discarded and some phosphate buffer was added to the residual deposition and re-centrifuged. This was repeated 3 times and finally, and some phosphate buffer was added to the residual deposition and was compared with McFarlane 0.5m tube(10×1.5) and produced by preparing successive dilutions for inoculation, that is(10×1.5)( Alboofetile et a.,2014).

## 5. Preparation of Silver Carp (Phytofag)

### 5.1. Fish Preparation

The common Phytofag fish were purchased from the hydrothermal fish farms located in Sari and transferred to the laboratory in the vicinity of ice.After the initial washing and cutting the head and tail and taking the guts out and rewashing, they were prepared for the treatments selection.

## 6. Measurement Methods

### 6-1.Microbial Analysis

### 6-1-1.Determining Staphylococcus Aureus Bacteria

After preparing the dilution of the Phytofag fish in the ringer solution and also the preparing successive dilutions, the stuff Parker agar medium was used in order to count the staph and after incubation at 35°C for 48h, the colonies with transparent halo were randomly sampled and were analyzed for Coagulase test (causes diluted plasma to coagulate) and after this test getting positive, they were considered as the Staphylococcus Aureus.

### 6-1-2: Determining Total Visible Count (TVC)of Bacteria

To count TVC of the prepared samples, Tryptic Soy Agar (TSA) medium was prepared. After preparing the medium with microsampler, 0.1ml of the prepared samples was superficially spread on the medium .If necessary (the bacteria number being high in a plate), the dilution of the samples (talog 6) was done in the physiologic serum solution and the cultured plates related to total bacteria were counted after 48h incubation at 35°C.

### 6-1-3.Determining Cold-Free Bacteria (PTC)

To count **PTC** from the prepared samples, TSA medium was used .0.1 ml of the prepared samples were superficially spread on the culture medium and the plates related to the cold-free bacteria were counted after 10 days at 4° C(AOAC,2005).

### 6-2.Chemical Analysis

### 6-2-1.PV Measurement

In order to determine peroxide level, first 15 g well mixed Phytofag was added to 500 ml decanter ,then 30 ml chloroform was added and shaken a bit ,again 30 ml chloroform was added and then 60 ml methanol was added .After 12-24 h, 36 ml distilled water was added to the sample ,and it was left for 1-2 h so that phase 3 formed .With accuracy ,20 ml of the low-phase was transferred to 250 ml **Arlon Meyer** Flask,25 ml chloroform acetic acid (chloroform to acid citric ratio as 2:3) was added. Later 0.5 ml saturated potassium iodine solution (as fresh prepared and kept in dark) and 30 ml distilled water were added to the **Arlon Meyer** Flask contents ,its cap was fixed ,and left for 1 m in dark and then 0.5 ml starch reagent was added and the Arlo was capped, the solution was severely shaken.

The released iodine changed the solution color titrated with 0.01 thiosulfate solution until the solution decolorizatiion

or the emergence of a milky solution and the upper oil phase getting transparent .After using the following equation , the peroxide level is estimated :

$$PV = \text{thiosulfate consumption volume} \times \text{normality} \times 100$$

$$PV = \frac{\text{حجم مصرفی تیوسلفات} \times \text{نرمالیته} \times 100}{\text{وزن نمونه روغن}}$$

## Data Analysis

Ahead of examining the present study hypotheses, the applied test case and its conclusion is briefly discussed .As we know, in this study we are seeking to analyze the effect of chitosan nanocomposite and chamomile extract on the chemical and physical changes of Phytofag fish fillet infected with Staphylococcus Aureus. For this purpose, after collecting the study variables' related data, Shapiro-Wilk test (due to the observations in each group of the treatments being fewer than 25 individuals, it is better to use Shapiro-Wilk test rather than Kolmogorov-Smirnov )is run to establish if the data (the stated variables ) are normal. In case of the data normality, the parametric tests are applied to analyze and test the hypotheses, otherwise the non-parametric test is used

The following table presents the variables normality test results, the conclusion drawing in this test is this way that if the significance level is less than 0.05, the data are non-normal and if more than 0.05, they are normal.

**Table 1**: The study variables' normality test by group separation

| Groups | Staph | PTC | TVC | PH | PV | TVN |
|---|---|---|---|---|---|---|
| | P-value | P-value | P-value | P-value | P-value | P-value |
| Chamomile 0.5% | 0.100 | 0.083 | 0.080 | 0.668 | 0.055 | 0.062 |
| Chamomile 0.5%+ bacteria | 0.086 | 0.086 | 0.072 | 0.965 | 0.061 | 0.062 |
| Control | 0.057 | 0.116 | 0.116 | 0.760 | 0.024 | 0.061 |
| Control+ bacteria | 0.076 | 0.051 | 0.088 | 0.649 | 0.066 | 0.068 |
| Chitosan 1%+ bacteria | 0.065 | 0.058 | 0.061 | 0.242 | 0.053 | 0.063 |
| Chitosan 1% | 0.052 | 0.070 | 0.058 | 0.488 | 0.064 | 0.052 |
| Chitosan1%+chamomile0.5% | 0.052 | 0.058 | 0.055 | 0.306 | 0.061 | 0.054 |
| Chitosan 1%+ chamomile 0.5%+ bacteria | 0.058 | 0.063 | 0.056 | 0.070 | 0.059 | 0.058 |

As seen in the above tables, all the study variables are normal, as a result, we apply the parametric test to test the variables.

## Analyzing Study Hypotheses

1-Chitosan nanocomposite exerts effect on increasing the shelf life of Phytofag fish fillet.
2-Using chamomile extract influences the increase of Phytofag fish fillet.
3-Simultanously using chitosan nanocomposite and chamomile extract has more effect on increasing the shelf life of Phytofag fish fillet.
4- Chitosan nanocomposite inhibits the behavior of Staphylococcus Aureus bacteria in Phytofag fish fillet.
5- Chamomile extract inhibits the behavior of Staphylococcus Aureus bacteria in Phytofag fish fillet.

## Treatments Influencing the Behavior of Staphylococcus Aureus bacteria in Phytofag fish fillet

**Table (2): Treatments Influencing the Behavior of Staphylococcus Aureus bacteria in Silver Carp fish fillet**

| Time | Treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Chitosan 1%+ chamomile 0.5%+ bacteria | Chitosan 1%+chamomile 0.5% | Chitosan 1% | Chitosan 1%+ bacteria | Control+ bacteria | Control | Chamomile 0.5%+ bacteria | Chamomile 0.5% |
| 0 | $0.02\pm3.2^{Da}$ | $0.71\pm0.82^{Aa}$ | $0.76\pm0.44^{Aa}$ | $0.02\pm3.13^{Ea}$ | $0.04\pm3.24^{Fa}$ | $0.88\pm0.51^{Ca}$ | $0.02\pm3.19^{Ea}$ | $0.1\pm1.2^{Ba}$ |
| 4 | $0.03\pm3.79^{Db}$ | 0 | $1.51\pm0.87^{Ab}$ | $0.02\pm4.21^{Eb}$ | $0.02\pm5.3^{Fb}$ | $1.33\pm0.77^{Cb}$ | $0.02\pm4.3^{Eb}$ | $1.25\pm1.44^{Bb}$ |
| 8 | 0 | $0.03\pm5.23^{Ac}$ | $1.29\pm0.74^{Ac}$ | $0.03\pm5.67^{Ec}$ | $0.03\pm7.58^{Fc}$ | $0.09\pm3.33^{Cc}$ | $0.08\pm5.78^{Ec}$ | $0.06\pm2.23^{Bc}$ |
| 12 | $0.02\pm6.11^{Dc}$ | 0 | 0 | $0.02\pm6.69^{Ec}$ | $0.04\pm8.47^{Fc}$ | $0.05\pm4.16^{Cc}$ | $0.06\pm6.75^{Ec}$ | $1.82\pm1.05^{Bc}$ |
| 16 | $0.12\pm6.66^{Dd}$ | 0 | $0.04\pm2.59^{Ad}$ | $0.03\pm7.51^{Ed}$ | $0.13\pm9.91^{Fd}$ | $0.48\pm4.85^{Cd}$ | $0.04\pm7.69^{Ed}$ | $1.51\pm4.9^{Bd}$ |

Regarding the above table results, the two-way variance analysis and F statistics values and the significance level less than 0.05 related to it, it can be concluded that the significant group variable, time variable and the group interaction and time are meaningful, Duncan test results also indicated that there is a meaningful difference at the times 0, 4, 8 and 16 and there is no meaningful difference only at the times 8 and 16 and in the chitosan 0.5% group variable (the treatments' effect) and

chitosan 1% and chitosan 1% +chamomile 0.5%, no meaningful difference exists and there is a significant difference between chitosan 1% + chamomile 0.5% + bacteria(they are in one group), while there is no significant difference between the rest of the groups .Duncan test results in the capital letters in each row indicate the significance or lack of significance between the treatments at 0.05 level .(the effect of each treatment at various times) and the small letters in each column signify the significance or lack of significance between the treatments at 0.05 level.(the effect of the treatments at each time) have been given in each table above.

## Treatments Influencing PTS of Silver Carp Fish Fillet

**Table (3):** Treatments Influencing PTS of Silver Carp Fish Fillet

| Time | Treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Chitosan 1%+ chamomile 0.5%+ bacteria | Chitosan 1%+chamomile 0.5% | Chitosan 1% | Chitosan 1%+ bacteria | Control+ bacteria | Control | Chamomile 0.5%+ bacteria | Chamomile 0.5% |
| 0 | $0.03\pm3.41^{Ba}$ | $0.04\pm3.31^{Aa}$ | $0.03\pm3.38^{Ca}$ | $0.03\pm3.42^{Da}$ | $0.05\pm3.63^{Ga}$ | $0.04\pm3.29^{Fa}$ | $0.1\pm3.51^{Ea}$ | $0.02\pm3.34^{Ca}$ |
| 4 | $0.03\pm4.24^{Bb}$ | $0.05\pm4.07^{Ab}$ | $0.02\pm4.24^{Cb}$ | $0.04\pm4.4^{Db}$ | $0.02\pm5.31^{Gb}$ | $0.03\pm4.94^{Fb}$ | $0.03\pm4.47^{Eb}$ | $0.02\pm4.31^{Cb}$ |
| 8 | $0.02\pm5.09^{Bc}$ | $0.02\pm5.16^{Ac}$ | $0.02\pm5.53^{Cc}$ | $0.03\pm5.74^{Dc}$ | $0.02\pm7.64^{Gc}$ | $0.02\pm6.61^{Fc}$ | $0.03\pm5.69^{Ec}$ | $0.02\pm5.39^{Cc}$ |
| 12 | $0.03\pm6.33^{Bd}$ | $0.01\pm6.17^{Ad}$ | $0.03\pm6.53^{Cd}$ | $0.02\pm6.65^{Dd}$ | $0.02\pm8.31^{Gd}$ | $0.03\pm8.1^{Fd}$ | $0.51\pm7.14^{Ed}$ | $0.03\pm6.54^{Cd}$ |
| 16 | $0.02\pm6.95^{Be}$ | $0.03\pm6.64^{Ae}$ | $0.08\pm7.23^{Ce}$ | $0.01\pm7.33^{De}$ | $0.02\pm9.71^{Ge}$ | $0.02\pm9.55^{Fe}$ | $0.02\pm7.33^{Ee}$ | $0.02\pm7.33^{Ce}$ |

Considering the above table results, the two-way variance analysis and F statistics and the significance level less than 0.05 related to it, it is concluded that the meaningful group variable, time variable and the group interaction and time are meaningful, Duncan test results suggest that there is a significant difference at the times 0,4,8,12 and 16 and in the group variable (the effect of the treatments), chitosan 1% and chamomile 0.5%,the difference isn't significant (they are in one group),while there is a meaningful difference between the rest of the groups .Duncan test results in each row indicate the significance or lack of significance between the treatments at 0.05 level .(the effect of each treatment at various times, that is the group effect ) and the small letters in each column stand for the significance or lack of significance between the treatments

at 0.05 level.(the effect of the treatments at each time) are given in the above table.

## Treatments 'Effect on TVC in Silver Carp Fish Fillet

**Table (4): Treatments 'Effect on TVC in Silver Carp Fish Fillet**

| Time | Treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Chitosan 1%+ chamomile 0.5%+ bacteria | Chitosan 1%+chamomile 0.5% | Chitosan 1% | Chitosan 1%+ bacteria | Control+ bacteria | Control | Chamomile 0.5%+ bacteria | Chamomile 0.5% |
| 0 | $0.03\pm3.33^{Ba}$ | $0.04\pm3.29^{Aa}$ | $0.02\pm3.16^{Ca}$ | $0.03\pm3.39^{Ba}$ | $0.08\pm3.43^{Fa}$ | $0.08\pm3.29^{Ea}$ | $0.07\pm3.39^{Da}$ | $0.02\pm3.23^{Ca}$ |
| 4 | $0.02\pm4.12^{Bb}$ | $0.03\pm3.7^{Ab}$ | $0.01\pm4.21^{Cb}$ | $0.02\pm4.5^{Db}$ | $0.02\pm5.25^{Fb}$ | $0.05\pm4.85^{Eb}$ | $0.02\pm4.43^{Db}$ | $0.03\pm4.24^{Cb}$ |
| 8 | $0.03\pm4.88^{Bc}$ | $0.03\pm5.14^{Ac}$ | $0.03\pm5.4^{Cc}$ | $0.03\pm5.73^{Dc}$ | $0.02\pm7.24^{Fc}$ | $0.02\pm6.46^{Ec}$ | $0.03\pm5.6^{Dc}$ | $0.02\pm5.31^{Cc}$ |
| 12 | $0.02\pm6.25^{Bd}$ | $0.01\pm6.12^{Ad}$ | $0.02\pm6.55^{Cd}$ | $0.01\pm6.64^{Dd}$ | $0.02\pm8.22^{Fd}$ | $0.03\pm8.08^{Ed}$ | $0.02\pm6.7^{Dd}$ | $0.03\pm6.48^{Cd}$ |
| 16 | $0.04\pm6.88^{Be}$ | $0.02\pm6.55^{Ae}$ | $0.01\pm7.18^{Ce}$ | $0.03\pm7.26^{De}$ | $0.03\pm9.58^{Fe}$ | $0.02\pm9.48^{Ee}$ | $0.02\pm7.31^{De}$ | $0.02\pm7.22^{Ce}$ |

Concerning the above table results, the two-way variance analysis and F statistics and the significance level less than 0.05 related to it, it is concluded that the significant group variable, time variable and the group interaction and time are significant , and Duncan test results show that there is a significant difference at the times 0,4,8,12 and 16 and in the group variable (the effect of the treatments), chitosan 1% and chamomile 0.5% ,the difference isn't meaningful and chitosan 1% + bacteria and chamomile 0.5% +bacteria, there is no meaningful difference (they are in one group) ,while there is a meaningful difference between the rest of the groups.

Duncan test results in the capital letters in each row indicate the significance or lack of significance between the treatments at 0.05 level. (the effect of each treatment at various times, that is the group effect) and the small letters in each column stand for the significance or lack of significance between the treatments at 0.05 level. (the effect of the treatments at each time) are given in the above table.

## Treatments' Effect on PH in Silver Carp Fish Fillet

**Table (5): Treatments' Effect on PH in Silver Carp Fish Fillet**

| Time | Treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Chitosan 1%+ chamomile 0.5%+ bacteria | Chitosan 1%+chamomile 0.5% | Chitosan 1% | Chitosan 1%+ bacteria | Control+ bacteria | Control | Chamomile 0.5%+ bacteria | Chamomile 0.5% |
| 0 | $0.15\pm5.49^{Aa}$ | $0.08\pm5.97^{BCDa}$ | $0.08\pm5.74^{ABa}$ | $0.1\pm6.06^{CDa}$ | $0.1\pm5.85^{CDa}$ | $0.08\pm6.03^{Da}$ | $0.27\pm6.17^{Da}$ | $0.14\pm5.9^{BCa}$ |
| 4 | $0.03\pm6.13^{Ab}$ | $0.01\pm6.14^{BCDb}$ | $0.04\pm6.13^{ABb}$ | $0.03\pm6.13^{CDb}$ | $0.1\pm6.21^{CDb}$ | $0.02\pm6.2^{Db}$ | $0.03\pm6.16^{Db}$ | $0.02\pm6.1^{BCb}$ |
| 8 | $0.01\pm6.29^{Ac}$ | $0.01\pm6.28^{BCDc}$ | $0.02\pm6.27^{ABc}$ | $0.04\pm6.35^{CDc}$ | $0.04\pm6.33^{CDc}$ | $0.01\pm6.34^{Dc}$ | $0.02\pm6.33^{Dc}$ | $0.02\pm6.29^{BCc}$ |
| 12 | $0.02\pm6.46^{Ad}$ | $0.12\pm6.52^{BCDd}$ | $0.03\pm6.41^{ABd}$ | $0.02\pm6.5^{CDd}$ | $0.08\pm6.45^{CDd}$ | $0.04\pm6.51^{Dd}$ | $0.08\pm6.52^{Dd}$ | $0.02\pm6.47^{BCd}$ |
| 16 | $0.03\pm6.64^{Ae}$ | $0.02\pm6.63^{BCDe}$ | $0.05\pm6.73^{ABe}$ | $0.02\pm6.63^{CDe}$ | $0.06\pm6.76^{CDe}$ | $0.06\pm6.72^{De}$ | $0.1\pm6.66^{De}$ | $0.02\pm6.71^{BCe}$ |

Given the above table results, the two-way variance analysis and F statistics and the significance level less than 0.05 related to it, it can be concluded that the significant group variable, time variable and the group interaction and time are significant , and Duncan test results suggest that there is a significant difference at the times 0,4,8,12 and 16 and in the group variable (the effect of the treatments), chitosan 1% + chamomile 0.5% and bacteria + chitosan 1% in one group and the treatments of chitosan 1%, chamomile 0.5% and chitosan 1% +chamomile 0.5% in one group , the treatments of chamomile 0.5% and chitosan 1%+ chamomile 0.5% ,control + bacteria and chitosan 1% + bacteria in one group , the treatments of chitosan 1% + chamomile 0.5%,control+bacteria and chitosan 1% + chamomile 0.5% ,control +bacteria and chitosan 1%+ bacteria, control and chamomile 0.5%+bacteria in one group ( no significant difference has been found at error level 0.05)

Duncan test results in the capital letters in each row suggest the significance or lack of significance between the treatments at 0.05 level. (the effect of each treatment at various times, that is the group effect) and the small letters in each column signify the significance or lack of significance between the treatments at 0.05 level. (the effect of the treatments at each time) are given in the above table.

## Treatments' Effect on PV in Silver Carp Fish Fillet

**Table (6): Treatments' Effect on PV in Silver Carp Fish Fillet**

| Time | Treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Chitosan 1%+ chamomile 0.5%+ bacteria | Chitosan 1%+chamomile 0.5% | Chitosan 1% | Chitosan 1%+ bacteria | Control+ bacteria | Control | Chamomile 0.5%+ bacteria | Chamomile 0.5% |
| 0 | $0.02\pm0.9^{Ba}$ | $0.03\pm0.91^{Aa}$ | $0.01\pm0.86^{Ba}$ | $0.01\pm0.85^{Ba}$ | $0.001\pm0.92^{Ha}$ | $0.03\pm0.87^{Fa}$ | $0.02\pm0.89^{Ea}$ | $0.001\pm0.94^{Ca}$ |
| 4 | $0.06\pm1.77^{Bb}$ | $0.02\pm1.6^{Ab}$ | $0.07\pm1.71^{Bb}$ | $0.07\pm2.02^{Db}$ | $0.08\pm2.66^{Hb}$ | $0.14\pm2.52^{Fb}$ | $0.06\pm2.18^{Eb}$ | $0.16\pm2.04^{Cb}$ |
| 8 | $0.12\pm2.72^{Bc}$ | $0.03\pm2.39^{Ac}$ | $0.04\pm2.69^{Bc}$ | $0.09\pm3.47^{Dc}$ | $0.04\pm3.99^{Hc}$ | $0.03\pm3.79^{Fc}$ | $0.02\pm3.69^{Ec}$ | $0.2\pm3.05^{Cc}$ |
| 12 | $0.14\pm4.46^{Bd}$ | $0.18\pm4.03^{Ad}$ | $0.25\pm4.88^{Bd}$ | $0.03\pm5\,.\,7^{Dd}$ | $0.15\pm6.33^{Hd}$ | $0.23\pm6.17^{Fd}$ | $0.09\pm5.97^{Ed}$ | $0.15\pm4.99^{Cd}$ |
| 16 | $0.05\pm4.23^{Be}$ | $0.1\pm3.68^{Ae}$ | $0.07\pm4.3^{Be}$ | $0.05\pm5.51^{De}$ | $0.12\pm6.12^{He}$ | $0.06\pm6.13^{Fe}$ | $0.17\pm5.72^{Ee}$ | $0.08\pm4.61^{Ce}$ |

Concerning the above table results, the two-way variance analysis and F statistics and the significance level less than 0.05 related to it, it is concluded that the significant group variable, time variable and the group interaction and time are significant and Duncan test results indicate that there is a significant difference at the times 0,4,8,12 and 16 and in the group variable (the effect of the treatments), chitosan 1% + chamomile 0.5%+ bacteria and chitosan 1% in one group (there is no meaningful difference at error level 0.5), while other treatments revealed a meaningful difference.

Duncan test results in the capital letters in each row show the significance or lack of significance between the treatments at 0.05 level. (the effect of each treatment at various times, that is the group effect) and the small letters in each column stand for the significance or lack of significance between the treatments at 0.05 level. (the effect of the treatments at each time) are depicted in the above table.

## Treatments' Effect on TVN in Silver Carp Fish Fillet

**Table (7): Treatments' Effect on TVN in Silver Carp Fish Fillet**

| Time | Treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Chitosan 1%+ chamomile 0.5%+ bacteria | Chitosan 1%+chamomile 0.5% | Chitosan 1% | Chitosan 1%+ bacteria | Control+ bacteria | Control | Chamomile 0.5%+ bacteria | Chamomile 0.5% |
| 0 | $0.51\pm11.02^{Ba}$ | $0.15\pm11^{Aa}$ | $0.15\pm11\,.\,36^{Ba}$ | $0.54\pm11.32^{Da}$ | $0.44\pm11.05^{Ga}$ | $0.29\pm10.96^{Fa}$ | $0.05\pm11.41^{Ea}$ | $0.05\pm11.01^{Ca}$ |
| 4 | $0.4\pm12.76^{Bb}$ | $0.24\pm12.11^{Ab}$ | $0.21\pm12.56^{Bb}$ | $0.16\pm13.42^{Db}$ | $0.64\pm15.17^{Gb}$ | $0.93\pm14.53^{Fb}$ | $0.34\pm13.9^{Eb}$ | $0.64\pm13.68^{Cb}$ |
| 8 | $0.43\pm23.81^{Bc}$ | $1.15\pm21.45^{Ac}$ | $0.22\pm23.1^{Bc}$ | $0.45\pm25.42^{Dc}$ | $0.4\pm28.35^{Gc}$ | $1.22\pm26.88^{Fc}$ | $0.26\pm25.46^{Ec}$ | $0.25\pm23.43^{Cc}$ |
| 12 | $0.3\pm43^{Bd}$ | $1.21\pm43.02^{Ad}$ | $1.17\pm42.54^{Bd}$ | $0.61\pm45.12^{Dd}$ | $1.23\pm48.71^{Gd}$ | $1.17\pm48.67^{Fd}$ | $0.43\pm46.7^{Ed}$ | $0.48\pm43.77^{Cd}$ |
| 16 | $1.33\pm51.38^{Be}$ | $2.76\pm50.34^{Ae}$ | $1.16\pm54.46^{Be}$ | $0.96\pm60.57^{De}$ | $1.42\pm65.9^{Ge}$ | $1.75\pm63.94^{Fe}$ | $1.67\pm63.22^{Ee}$ | $1.38\pm58.86^{Ce}$ |

Considering the above table results, the two-way variance analysis and F statistics and the significance level less than 0.05 related to it, it is concluded that the significant group variable, time variable and the group interaction and time are significant.

Duncan test results denote that there is a meaningful difference at the times 0,4,8,12 and 16 and in the group variable (the effect of the treatments), chitosan 1% + chamomile 0.5%+ bacteria and chitosan 1% in one group (there is no meaningful difference at error level 0.5), while other treatments revealed a meaningful difference.

Duncan test results in the capital letters in each row signify the significance or lack of significance between the treatments at 0.05 level. (The effect of each treatment at various times, that is the group effect) and the small letters in each column stand for the significance or lack of significance between the treatments at 0.05 level. (The effect of the treatments at each time) are listed in the above table.

## DISCUSSION & CONCLUSION & SUGGESTIONS

- Microbial Factors (The Behavior of Staphylococcus Aureus, Total Count of Visible Bacteria, Cold-Free Bacteria)

The fresh and slightly preserved fish rotting causes the growth and activation of the specific organisms of the corrosive factor. The metabolites produced by these microorganisms create unpleasant odors and undesirable smells, resulting in this food not being accepted by the consumers. The level of the certain corrosive factor organisms has higher correlation with fresh fish shelf life than that of the total bacterial level (conventional bacterial level).

## Behavior of Staphylococcus Aureus

Staphylococcus Aureus grows well in fishery products stored in the refrigerator (Embarek, 1994).Given table (2-4), at day 0, the number of the staph in the samples of chamomile, chamomile and bacteria, control, control and bacteria, ,chitosan and chamomile, chitosan, chitosan and chamomile and bacteria got 0.1+1.2,0.02+3.19, 3.24+0.51,0.02+0.88, 0.02+3.13, 0.76+0.44,0.71+0.82 and 0.02+3.2. As the results in this study indicate ,the number of the staph increased in the control and chamomile sample on day 16 of storage. In the chitosan treated fillets, the number of the staph decreased on day 12 compared with the 1st day and then the number of this bacteria dropped until day 12 and on day 16, it increased relative to days 0, 4, 8 and 12.Also, the chitosan nanocomposite treated samples revealed the lowest staph number and the control samples showed the highest staph number. Generally speaking, on day 16, the highest staph was spotted except for the chitosan+chamomile treatment that both resulted in the staph growth drop.

As Rozman & Jersek (2009) reported, the herbal essential oils and extracts as the biological preservatives are capable to decrease the microbial load of the foodstuff and aquatics and are employed as the natural preservatives for reducing gram-positive bacteria such as Staphylococcus Aureus. In the present research, the fillets coated with nanocomposite showed meaningful difference (P>0.05) with other samples on days 4,8,12 and 16 and chitosan nanocomposite coating significantly (P>0.05) reduced the number of Staphylococcus Aureus bacteria. The reason behind this phenomenon is that chitosan possesses anti-staph property (Fernandez-Saiz et al., 2010) and chamomile extract owns good anti-microbial activity against Staphylococcus Aureus .In addition, chamomile extract is used as a foodstuff additive for preserving and preventing the foodstuff rotting. Moreover, this plant's extract is used in the

food contaminated with staph to control this bacteria. Generally, the combination of chitosan and chamomile as nanocomposite will have higher staph activity than that of chamomile and chitosan.

## Cold-Free Bacteria

The major groups of microorganisms rotting the fresh-frozen fish stored in the refrigerator and under aerobic conditions are gram-negative cold-free bacteria .Regarding table (3-4), the significant drop of the number of cold-free bacteria in the chitosan nanocomposite treated samples has been seen compared with other treatments.

According to the study by Ojagh et al.,(2010) on the effect of chitosan 2% w/v enriched with cinnamon essential oil 1.5%v/v and chitosan 2% w/v on the quality of rainbow trout fillet fish kept in the refrigerator (4±1°C) for 16 days ,it has been seen that the number of cold-free bacteria increased in all samples during the storage period and the lowest number of cold-free bacteria were in the cinnamon essential oil enriched chitosan treated samples.

In the study by Abdollahi et al., (2014) on the effect of chitosan/clay functional bionanocomposite activated with rosemary essential oil (chitosan/clay/rosemary essential oil ) on the shelf life of fresh silver carp stored for 16 days in the refrigerator (4±1°C) and as their report stated the coated samples led to decreased cold-free bacteria compared to the control samples .In addition, the nanocomposite activated with rosemary essential oil had the best inhibitory effect against cold-free bacteria compared with the control samples .

In this study, concerning table (3-4), chitosan is able to preserve Phytofag fish fillet against cold-free bacteria for 8 days .While chamomile extract was ineffective against PTC as the treated fillets bacteria number reaching from 3.33 log cfu/g on the first day of this study to 7.33 log cfu/g on day 16 of storage

at 4±1°C.Chitosan nanocomposite treatment exhibited higher inhibitory effect to chitosan and chamomile treatment.

**Total Visible Count (TVC) of Bacteria**

Due to the bacteria exerting influence on corrosion**,** TVC is considered as an acceptable indicator for fishery products (Ozogul et al., 2010).The initial number of TVC for the fillets ranged from 3.16 to 3.39 log cfu/g. This number in all samples increased significantly (P<0.05) compared to the initial number .The current research results are consistent with those found by Ojagh et al. (2010) and Jeon et al.(2002),so that based on the study by Ojagh et al.,(2010) on the effect of cinnamon essential oil 1.5%v/v enriched chitosan 2%w/v and chitosan 2%w/v on the quality of rainbow trout fish fillet kept in the refrigerator for 16 days at (4±1°C) ,it has been found that TVC increased in all samples during the storage period .Also Jeon et al.(2002) studied the effect of chitosan coating with various molecular weights on the shelf life increase of Atlantic cod(*Gadusmorhua) and Herring (Clupea harengu ) fillet fish* for 12 refrigerated stored days *(4±1°C) ,the results of which indicated that* chitosan coating meaningfully (P<0.05) increased the total TVC of the bacteria.

At the time of fishing, the fish muscle are free any pathogens but they immediately get infected by intestinal bacteria, contamination from water, equipment, human manipulation and storage conditions (Abdou et al., 2012).In this study, TVC increased in the untreated Phytofag fish fillet (the control samples) during the storage period and exceeded its admissible limit of raw fish (6 log cfu / g).Similar results like this one were reported by several researchers (Jeon et al.,2002, Kav et al.,2013 and Hu et al.,2015) that rosemary treatment on days 8,12 and 16 exceeded the admissible limit .Although in the present research ,the TVC of the chitosan nanocomposite treated samples was significantly lower than that of other

samples ,this coating indicates the chitosan nanocomposite bacterial inhibiting potential .Besides, TVC in the chitosan coated samples showed a trend similar to nanocomposite except for day 16 which exceeded the admissible limit.

The study results by Ozogul et al., (2010) on rosemary essential oil potential (1% and 2%) to inhibit the lipid oxidation of Sardine fish revealed that rosemary extract could upgrade sardine shelf life for 7 days through decreasing TVC.

Though Ozogul et al. considered 7 log cfu/g as the maximum TVC defined by the International Commission on Microbiological Specifications for Foods (ICMSF) (1986).Thus in the current research, concerning table (4-4), chamomile extract had the potential to keep Phytofag fish fillet for more than 12 days.

Moreover, the antibacterial traits of chitosan have been verified by many researchers .Identical results were gained by Fan et al., (2009), supporting the chitosan 2% treated silver carp fish fillet with antibacterial properties compared with the control samples (untreated) for 5 days of storage at -3°C.

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