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Original Article

Antimicrobial Activity of Lemon and Peppermint Essential oil in Edible Coating Containing Chitosan and Pectin on Rainbow Trout (*Oncorhynchus mykiss*) Fillets

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Introduction: Essential oils are used as flavoring agents in various foods. Layer-by-Layer (LBL) technique is a method in which the material is dipped into a series of different solutions containing oppositely charged polyelectrolytes. The aim of this study is to investigate the effectiveness of a multilayered edible coating with an antimicrobial compound (Lemon and Peppermint) in enhancing the quality and shelf-life of rainbow trout (*Oncorhynchus mykiss*) during refrigerated storage (4 ± 1˚C) over a period of 16 days. **Methods:** In this study, multilayered coating was used with two concentrations of Lemon (LEO) and Peppermint (PEO) essential oils (0.5 and 1%). Antibacterial effect of these treatments was evaluated by enumeration of bacteria in special culture media. The control and the coated fish samples were analyzed periodically for pH and microbiological (total viable count, psychrotrophic count, lactic acid bacteria, Enterobacteriaceae, and coliform) characteristics. **Results**: The results obtained in this study demonstrate that multilayered coating in combination with Lemon and Peppermint essential oils can significantly decrease the number of bacteria and delay the spoilage of the samples (p<0.05). **Conclusion:** Multilayered edible coating with an antimicrobial compound can properly delay the growth of spoilage microorganisms and prolong the shelf life of meat products. *J Med Microbiol Infec Dis, 2015, 3 (1-2): 38-43.*

Keywords: Lemon, Peppermint, Rainbow Trout, Essential Oil.

INTRODUCTION

Edible coatings from polysaccharides, proteins, and lipids can increase the shelf life of the foods due to their functioning as solute, gas, and vapor barriers [1]. Edible coatings can help prevent physical damage, enhance appearance, and reduce microbial growths so that they can be a cost-effective alternative to the modified atmosphere packaging [2, 3]. Among the polysaccharides that have been successfully used as film-forming compounds, Chitosan (poly-b-(1–4)-D-glucosamine) is a cationic polysaccharide, which is mainly made by deacetylation of chitin, the major constituent of crustaceans shell [3, 4, 5]. Chitosan which is a natural food additive has become popular because of its non-toxic nature, anti-oxidant and antibacterial activity, biodegradability, and the ability to form film [4]. The antimicrobial activity of chitosan against a wide range of foodborne agents including filamentous fungi, yeast, and bacteria has made this compound a useful preservative in the food industry [6, 7]. Pectin is an important polysaccharide which is naturally occurring from underutilized agricultural waste material and can form excellent films through demethylation [3, 8]. Pectin is classified as Generally Recognized As Safe (GRAS) food substance [9].

Due to their biological composition, the fresh fish are highly perishable [10, 11]. In recent years, there has been increased consumption of seafood [12]. Quality deterioration in fish is a major concern to industry and consumers [13]. The loss of quality is a result of changes made by biological reactions such as lipids oxidation, metabolic activities of microorganisms, and reactions arising from activities of the fish's enzymes. These activities finally cause shortening of shelf life in fish and seafood products and may result in disease following the consumption of products that could potentially cause a toxic reaction [14, 15].

By classification, Rainbow trout (*Oncorhynchus mykiss*) is a fatty fish, and the loss of quality of fatty fish species is mainly due to microorganisms and lipid oxidation. The main reason of quality problem is lipid oxidation [16]. Many reports indicate that use of chitosan can lead to decreased microbial counts in fish and their products, and thus, it has been widely used in fish products [17, 18, 19, 20, 5]. Several antimicrobials can be incorporated into edible coatings. Considering their availability, less side effect or toxicity, and better biodegradability of natural antimicrobials such as plant extracts or essential oils are preferred for use compared to the available preservatives [2]. Many essential oils are known as GRAS food flavoring, but their utilization in foods are limited due to their effect on the flavor of foods [3].

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Lemon (*Citrus limon*) is classified in the citrus family (Rutaceae), and it is the third most important species of citrus after orange and mandarin [21]. Lemon is among the essential oils that have been successfully used as antimicrobial agent [22].

Peppermint (Mentha piperita) is widely used in food, cosmetics, and medicine. Many studies have been reported the antimicrobial activity of peppermint oil [23, 24, 25]. Only a few studies are reporting the effectiveness of these compounds when incorporated into edible coatings applied to fresh fish [26, 27, 28]. Dipping is a common method to coat food, which may not stick to the surface of the fish. Therefore, there is more interest in the use of multilayered edible coating using Layer-by-Layer (LbL) electro-deposition technique, which could solve the problem related to the adhesion of materials to the surface of the fish [2].

The present study was aimed to evaluate the viability of LbL incorporation of essential oils (Lemon and Peppermint) into a multilayered edible coating to enhance the shelf life and quality of rainbow trout fillet.

MATERIAL AND METHODS

Preparation of coating solutions and treatment of fish fillet. Medium molecular weight chitosan (450 kDa) was purchased from Sigma-Aldrich Chemical Co. Chitosan solution (medium MW, deacetylated chitin, Poly (D-glucosamine), 448877; 95e98%; viscosity_30 mPa. s, Sigma-Aldrich) was prepared with 2 g chitosan and 1 g acetic acid (Sigma-Aldrich) in 100 g distilled water. To achieve complete dispersion of chitosan, the solution was stirred for 3 h at room temperature. Chitosan received an addition of Glycerol as a plasticizer at 0.75 ml/g concentration and was beated for 10 min. In the next step, essential oil, mingled with Tween 80 (Aldrich Chemical Co., Steinheim, Germany) was added to the chitosan solution to help distribute and completely incorporate the essential oil. The final coating solution was formed including chitosan (2%), acetic acid (1%), glycerol (0.75%), Tween 80 (0.2%), and each essential oils (0.5, 1%). The final coating solution was homogenized under aseptic conditions at 21600 rpm for 1 min (Polytron Kinamatica Inc., PV, Cincinnati, OH, USA). The control solution was made in the absence of Lemon and Peppermint oil as described by others [1].Pectin solution (obtained from citrus peel, 2g/100g, galacturonic acid ≥74.0% (anhydrous basis), P8471, Sigma-Aldrich) was dissolved in distilled water and stirred in a hot plate (70°C) for 1 h to achieve complete dispersion of Pectin [3].

Calcium chloride (2g/100g, C5670, Sigma-Aldrich) was made by using distilled water and applied in order to crosslink the polymers [3].

Distillation of oil. Hydrodistillation was conducted through a standard procedure (Clevenger apparatus) in order to provide essential oils [29].

Preparation and treatment of fish samples

Fish sample preparation. Fresh water rainbow trout, varying from 200 g to 300 g in weight, were purchased alive from a public market and were transferred to the Laboratory in ice slurry. After gutting and washing the fishes, two fillets were obtained from each fish.

Coating method (LbL technique). A five-step procedure was used to ensure suitable coating of the fish fillets, as part of the LbL deposition process. In this method, fish fillets are dipped into series of different solutions that contain oppositely charged polyelectrolytes, and the excess of coating material from the fillet surface is allowed to be removed by a drying step between each dipping steps [30]. Fillet samples were randomly assigned into six treatment lots consisting of one control lot (uncoated) and five lots treated with the following coating solutions: chitosan + pectin (ch+ p), chitosan + pectin + 0/5% LEO, chitosan + pectin + 1% LEO, chitosan + pectin+ 0/5% PEO, and chitosan + pectin + 1% PEO. The fillets were dipped into each coating solution for 2 min and the excess coating was allowed to drip off for 2 min before the samples were submerged into the next solution. The order of the coating solutions was calcium chloride, chitosan plus antimicrobial, calcium chloride, pectin, and a third dipping into calcium chloride. Control samples were only dipped into sterile distilled water for 2 min and then allowed to drip off for 2 more minutes. After 8 min of drying at room temperature, the treatment fillets were placed into plastic containers (Ziploc Brand with Smart $Snap^{TM}$ Seal, 591- ml) with polyethylene lid, and stored at 4±1˚C for 16 days. For subsequent quality assessment microbiological analyses were performed at 4-day intervals to determine the overall quality of the fish [30].

Determination of pH. For determination of pH, 10 g specimen of the fish muscle was homogenized in 100 ml of distilled water. The pH of fish sample was measured using a digital pH meter after the mixture was filtered (Cyberscan PC 510 UK) [26].

Microbial analysis. The microbiological counts were specified by placing 10 g fish specimen in 90 ml of 0.9% Nacl solution and were homogenized in a stomacher for 1 min (Stomacher 400 Lab Blender; Seward Medical, London, UK). Other decimal dilutions were prepared from this dilution and plated in the suitable media total viable counts (TVC) and Psychrotrophic count were determined via surface plate method, using plate count agar (PCA, Merk, Darmstadt, Germany). The cultures were incubated at 37°C for 3 days for TVC, and at 10°C for 7 days for psychrotrophilic counts.

Lactic acid bacteria (LAB) were put one by one on de Man, Rogosa, and Sharpe (MRS) agar (MRS, Merck) incubated at 35°C for 2-3 days in anaerobic jars with one-use Anaerocult C bags (Merck, Darmstadt, Germany) for the generation of an anaerobic medium.

Violet Red Bile Glucose Agar (VRBGA, Merck) was used to determine the Enterobacteriaceae. The plates were incubated at 37°C for 24 h after being overlaid with a virgin layer of the same growth medium.

Coliforms were determined in Violet Red Bile Lactose Agar (VRBA) by pour plate method and incubated at 37°C for 24 h. All enumerations were triplicate and expressed as log_{10} CFU/g and performed in duplicate [31, 32].

Statistical Analysis. Each treatment underwent three replicated measurements for which the mean values ±

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standard deviation were obtained. Analysis of variance (ANOVA), least significant difference (p<0.05), and Duncan's test were performed to evaluate the significance of difference among mean values, using SPSS version 20.0.

RESULTS

pH value. Variations in the value of pH during the refrigerated storage are presented in (Figure 1a). The initial pH of the control fish sample was found to be 6.45.

At the end of storage period, pH values reached up to 6.95, 6.09, 5.44, 5.67, 5.17, and 5.43 in control, treated sample with 0% EO, 0.5% LEO, 1% LEO, 0.5% PEO, and 1% PEO, respectively. The variation of pH value in fish meat is usually between 5.7 and 6.6. Fresh fish is of neutral pH, but due to the formation of lactic acid following the fish death, the pH value falls and then rises again with spoilage [33, 23], where we observe an initial decrease and then an increase in pH value. No significant difference (p> 0.05) was observed between treated sample with 0.5% LEO and treated sample with 1% PEO at the end of storage time. All the treatments except control group were significantly prevent from increasing pH values. This means that the lower pH value might prevent microbial growth and hamper the activity of the endogenous proteases at different degree, leading to the extened preservation of fish.

Bacteriological analysis

Total viable count. Changes in the value of TVC during the refrigerated storage are shown in Figure 1b. An important factor for quality evaluation of fresh fish is TVC. The initial TVC of control samples was 3.87 log_{10} CFU/g, and the low initial TVC indicated very good fish quality. The bacterial values of all samples increased progressively with the storage time. However, by the day 8 of storage, for all different treatments, the TVC in trout fillet was still below 7 log_{10} CFU/g, whereas that of controls reached a count of 8.12 at day 12, which is higher than the maximal recommended limit of 7 log_{10} CFU/g for TVC in raw fish [1], indicating a microbiological shelf life of about 9-10 days for the control samples. At the end of storage period, treated sample with ch + p reached to 7.04 log_{10} CFU/g which is upper than microbiological limit for fresh fish. It should be mentioned that at the end of storage period, treated samples with 0.5% LEO, 1% LEO, 0.5%PEO, and 1% PEO were still considered to be acceptable for human consumption, while the final bacterial load in these samples were close to the limited level.

At the end of storage time, there were no significant difference between treated sample with 1% LEO, 0/5% PEO, and 1% PEO (P>0.05).

Compared with the control, all treatments significantly inhibited the growth of bacteria during the storage time. Bacterial growth was inhibited in samples with increased concentration of lemon and peppermint oil due to the antimicrobial activity of them.

Psychrotrophic bacteria. In this study, the initial PTC (log_{10} CFU/g) in trout fillet ranged from 2.11 in treated sample with 1% PEO to 3.86 in controls (Figure 1c). Control also being the highest at day 16 (10 log 10 CFU/g), followed by treated sample with 1% LEO (5.46 log10 CFU/g), and a lowest count (5.34 log_{10} CFU/g) was detected in treated sample with 1% PEO. At the end of storage time, there were no significant differences between treated samples with 1% LEO and 1% PEO (P>0.05). But there were significant differences between control, treated sample with ch + p and treated samples with 0.5%, 1% LEO, 0.5% , 1% PEO, which indicates that treated samples with different concentration of essential oil strongly inhibited the growth of PTC.

As a major group of microorganisms, the gram-negative psychrotrophic bacteria (PTC) spoil aerobically stored fresh fish during the refrigerated storage [32]. Moreover, the growth pattern of PTC revealed the same behaviour as that of TVC which means that PTC increased during the storage time.

At the end of storage time control and treated sample with ch + p were not acceptable for human consumption.

Coliform account. *Escherichia coli*, the fecal indicator organism, were part of the microflora of fresh rainbow trout [34]. The differences between bacterial loads are shown in Figure 1d. Many researchers believe that presence of coliform bacteria is a direct consequence of fecal contamination [35]. The initial coliform (log_{10} CFU/g) in control samples of rainbow trout fillets was 0.97 which is below the 2.3 or 2.4 (log_{10} CFU/g) known as maximum allowable bacterial load for coliform [35]. Additionally, the growth pattern of coliform showed the same behaviour as that of TVC and PTC and increased during storage time in all groups. Control being the highest at day 16 (7.66 log_{10} CFU/g), whereas lower count (3.99 log_{10} CFU/g) was detected in treated sample with 1% PEO (P>0.05).

Lactic acid bacteria. Lactic acid bacteria is also part of the natural microflora of fresh rainbow trout fillets (Figure 1e). In our study, the initial LAB (day 0) of sample was 1.52 log cfu/g. At the end of storage time, Lactic acid bacteria populations amounted up to 4.96 log_{10} CFU/g in control sample and 2.1 log_{10} CFU/g in the sample treated with 1% PEO. The value of LAB increased during the storage time in all groups.

At the end of storage time, there were no significant differences between treated samples with 0.5% LEO, 1% LEO and 0.5% PEO (P>0.05). But there were significant differences between control and the treated sample with 1% PEO, which indicates that compared to the control, the treated samples with 1% PEO strongly inhibited the growth of LAB.

Enterobacteriaceae. As a hygiene indicator, Enterobacteriaceae was also part of the microflora of fresh rainbow trout. The first Enterobacteriaceae count was 1.35 log_{10} CFU/g (Figure 1f), while this value increased during storage time in all groups. The results of the present study showed that at the end of storage time, Enterobacteriaceae counts reached to 5.01 log_{10} CFU/g in control sample and 1.65 in treated sample with 1% LEO.

At the end of storage time, there were no significant differences between treated samples with 1% LEO and 1% PEO. It shows that both treatments had the same inhibitory effect on the growth of Enterobacteriaceae.

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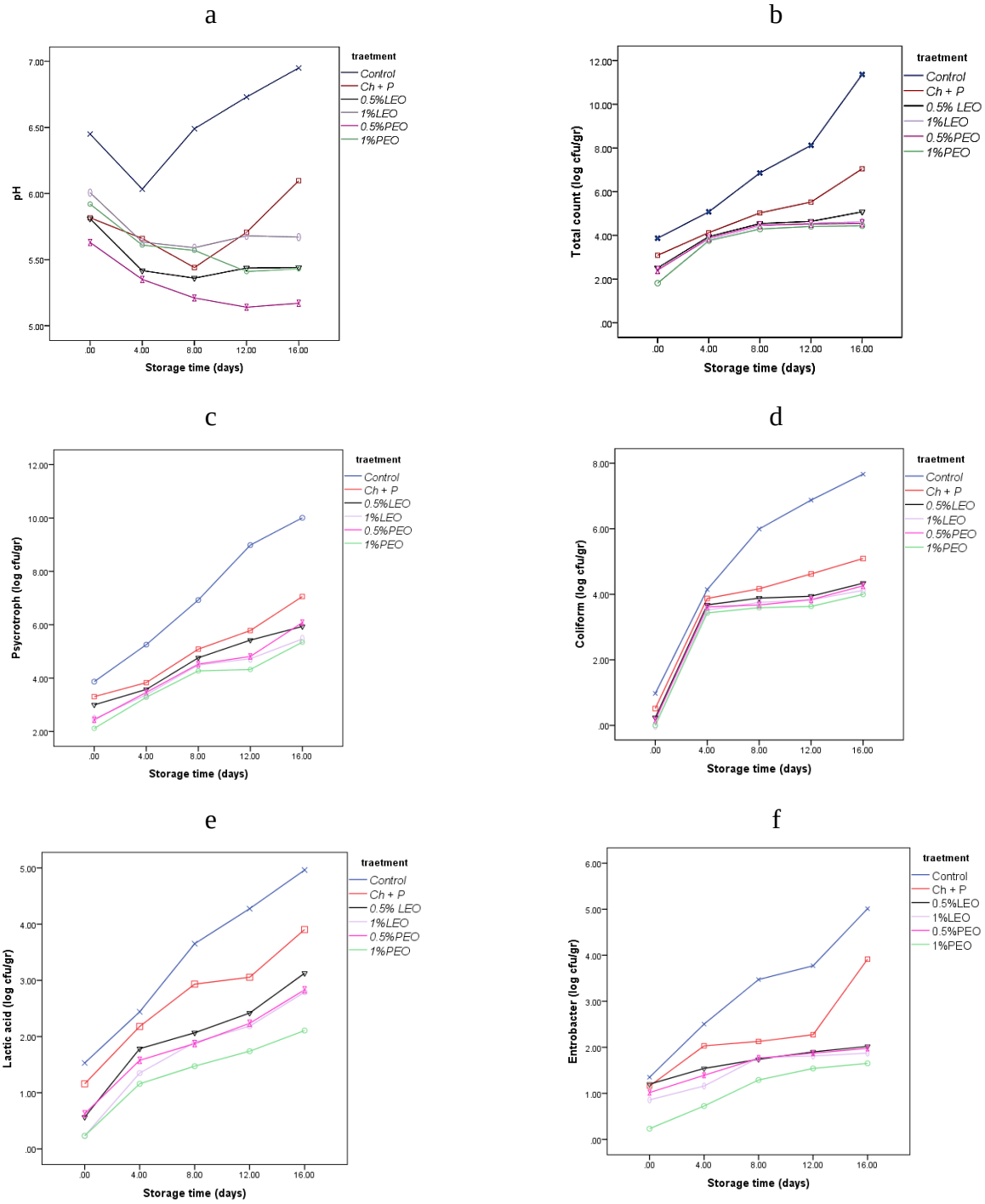


Fig. 1. pH (a), TVC (b), PTC (c), Coliform (d), Lactic acid bacteria counts (e), Enterobacteriaceae (f) changes of different treatments of fish samples during refrigerated storage at 4℃

DISCUSSION

In this study, the main objective was to assess the effectiveness of a multilayered antimicrobial edible coating using the LbL technique to extend the shelf life of rainbow troat fillets in refrigerated storage.

The initial pH in this study was 6.45, which was sparingly lower than that reported by Volpe *et al.* (2005). According to their study performed in Italy the effect of carrageenan based biocomposites on shelf life of rainbow trout fillets during refrigerated storage were investigated which show similar result for pH [27]. Many researchers demonstrate that the initial decrease of pH value may be related to the dissociation of carbonic acid in the fish samples for fish fillets [31]. Chaijan *et al.* (2005) believed that increased volatile bases that were produced by either endogenous or microbial enzymes led to an increase in pH [36]. Benjakul, Visessanguan, Riebroy, Ishizaki, & Tanaka

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(2002) reported that the decomposition of nitrogenous compounds caused an increase in pH in fish flesh [37].

The data of a study in Turkey in 2004 indicated that the value of TVC increased during refrigerated storage in all groups [31]. Similar results has been reported about kilka [38], also Oguzhan reported similar results for rainbow trout fillets in 2013 [39]. Another study in 2005 showed that the growth pattern of PTC is as same behaviour as TVC, and increased during storage time in all groups.

In another study in 2004, the effect of filleting on microbiological fresh water trout stored in ice was studied which showed that Enterobacteriaceae in lower counts were found in the spoilage microflora of trout. The result of the present study are in accordance to it [40]. Also similar results were found for rainbow trout [41], and 42sardine [42]. A survey performed in 2014 in Iran to evaluate the relationship between histamine content and microbial load of refrigerated rainbow trout fillet during storage time, showed that coliform counts increased in all groups during storage time [43].

In this study, it is obvious that bacterial growth was inhibited in samples with increased concentration of Lemon and Peppermint essential oil due to the antimicrobial activity of them. The results were consistent with observations of other researchers [33].

The present study shows that multilayered edible coating in combination with Lemon and Pepper mint essential oil effectively lead to the extended shelf life of fresh rainbow trout fillets.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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