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Research Article

Chemical and microbial properties of a fermented fish sauce in the presence of *Lactobacillus plantarum* and *Paenibacillus polymyxa*

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Abstract

Mahyaveh is an Iranian fermented fish sauce produced from fish, salt, spices and water during fermentation period. The main problem related to this product is very high histamine content of it. Effects of starter cultures (*Lactobacillus plantarum* and *Paenibacillus polymyxa*) on reduction of histamine in mahyaveh, as "Southern Iran Fermented Fish Sauce" were investigated. This study was performed in 4 experimental groups, including control and treatments on 30th and 45th days of the product. Chemical analysis of treated and control samples revealed that pH, salt percentage, TVB-N content decreased significantly (p<0.05), and acidity increased significantly (p<0.05) in treated samples compared to control samples. Also inoculate starter culture (*Lactobacillus plantarum* and *Paenibacillus polymyxa*) reduced the amount of histamine content to about 0.347 and 0.326 mg mL^{-1} from 0.473 and 0.368 mg mL^{-1} in control and treated samples, respectively. Results of microbial evaluation revealed that populations of *Halophiles*, *Enterobacteriaceae* and fungi decreased significantly in treated samples compared to those of control samples (p<0.05), but colony populations of *Bacillus* and LAB increased significantly in treated samples compared to those of control samples (p<0.05). In general, addition of starter culture had a positive effect in reducing the amount of histamine and improving other chemical properties and microbial quality of the fermented fish sauce.

Keywords: Fish sauce, Histamine, *Lactobacillus plantarum*, Mahyaveh, *Paenibacillus polymyxa*

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Introduction

Mahyaweh is a traditional fermented fish sauce that is used extensively in southern parts of Iran, especially in Fars and Hormozgan provinces. This product is mostly made based on family tradition, access to raw materials, consumer preference and climatic condition of the area. Despite popularity of fish sauce in south of Iran, this product is traditionally prepared, marketed and there is no standard for it (Zarei *et al.,* 2012; Taheri *et al.,* 2014). Fish sauce is considered as a rich source of protein and contains essential amino acids like lysine. Lots of vitamins and minerals are found in fish sauce and this product is a rich source of B_{12} vitamin, sodium, calcium, magnesium, iron, manganese and phosphorus. During fermentation process fish proteins are converted to small peptides by the influence of microbial enzymes. Chemical composition and its changes (protein content, amount of free volatile bases and salt content) is considered in various types of fish sauce (Ranjbar *et al.,* 2017). Biogenic amines are one of the anti-nutritional factors in fish sauce. Presence of high amounts of protein, transaminase and decarboxylase bacterial enzymes intensifies biogenic amines production from amino acids in fish sauce (Zarei *et al.,* 2012). The most important biogenic amines that formed in fish sauce, including tyramine, cadaverine, Putrescine, histamine, spermine and spermidine, but histamine is the predominant biogenic amine in these products (Mah and Hwang, 2009). Histamine as a toxic biogenic amine in fish and seafood is produced due to presence of bacterial histidine decarboxylase and environmental conditions such as high temperature (Biji *et al.,* 2016). Some methods, like irradiation, high pressure process, controlled atmosphere packaging and adding preservative has been suggested to control and eliminate biogenic amines in foods products (Naila *et al.,* 2010).

Zarei *et al.* (2012) evaluated the microbial and chemical properties of mahyaveh samples in southern Iran and reported that histamine is the main biogenic amine in this Iranian fish sauce. Also Ranjbar *et al.* (2017) studied the microbial and chemical properties of mahyaveh fish sauce in Zarindasht and reported high flora of coagulase-positive staphylococci and fungi in mahyaveh samples. Moayedi and Moosavi-Nasab (2013) revealed positive relationship between total amount of bacteria and tri-methylamine concentration in the fish sauce. Taheri *et al.* (2014) identified *Staphylococcus* as the only pathogenic bacteria in mahyaveh and recommended standardization, monitoring and hygienic control over production. Recently, using Lactic acid bacteria (LAB) starter culture in fermented food is recommended as a new method to reduce biogenic amines (Niu *et al.,* 2019). Tapingkae *et al.* (2010) reported decreasing histamine content in presence of halophilic starter culture and Zaman *et al.* (2011) reported reducing biogenic amines with

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Staphylococcus carnosus FS19 and *Bacillus amyloliquefaciens* starter cultures. *Paenibacillus polymyxa* and *Lactobacillus plantarum* are Gram-positive, non-pathogenic bacteria with the ability of biogenic amine degradation, so they can be used as starter in production of mahyaveh sauce (Lee *et al.*, 2015; Taoka *et al.*, 2019). Although high amount of mahyaveh is consumed in southern Iran, limited research has been done on mahyaveh characteristics. The purpose of this study was to investigate chemical and microbial properties of mahyaveh in the presence of *P. polymyxa* and *L. plantarum* as starter culture during 45 days fermentation.

Material and methods

Materials

In this study raw materials were used, including sardine (*Sardinella sindensis*), coriander, fennel, cumin, barley. Coarse sea salt was obtained from Hormozgan, histamine assay kit 'Check color Histamine', L-histidine hydrochloride monohydrate and histamine de-chloride were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sulfuric acid, sodium hydroxide, hydrochloric acid, boric acid and nitric acid were supplied by Merck (Darmstadt, Germany). Cultures of *L. plantarum* (PTCC 1896, Iran) and *P. polymyxa* (previously *Bacillus polymyxa*) (ATCC 21830, Iran) came from head office of Iranian Research Organization for Science and Technology (IROST) in Tehran.

Methods

Production of Mahyaveh

Mahyaveh production was carried out according to the diagram presented in Figure 1. Sardines with mean weight of 25g and length of 12 cm were bought from local market. Heads and scales were removed and entrails were evacuated and sardines were used as fillets. Dried sardines (1000g) were washed, smashed and heated for 15 minutes according to local recipes. Heated fish were poured in to a clay pot then water (3000 mL), salt (500g) and barley (300g) was added to the mixtures. Samples was placed at ambient temperature for 30 days to ferment, then filtered and mixed with spices including coriander (200g), mustard (200g), barley (300g), fennel (200g) and cumin (200g). Then we placed the clay pot in room temperature for 15 days to continue the fermentation process. The overnight culture of *L. plantarum* and *P. polymyxa* were centrifuged at 10,000×g for 10 min. In addition to the raw materials used in the production of the control samples, *L. plantarum* and *P. polymyxa* cell biomass (10^7 cfu/mL) were used in the preparation of the treated samples. The experimental groups include control (C) and treatment (T) groups which were examined on the 30^{th} day (CD30 and TD30) and 45^{th} day (CD45 and TD45) after preparation of the samples.

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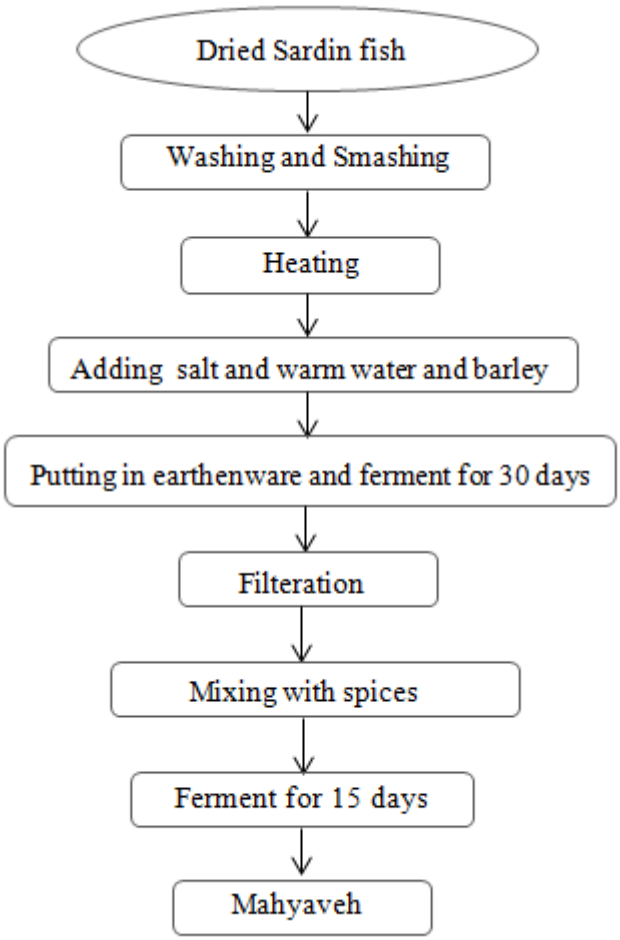


Figure 1: Diagram of mahyaveh sauce production.

Chemical analysis

pH of mahyaveh samples were measured using a standard pH meter (Mettler, Switzerland) by placing the probe into diluted samples. Total acidity was determined by titration with 0.1 M NaOH (Zarei *et al.*, 2012). Crude protein was determined by Kjeldahl method using a 6.25 Kjeldahl conversion factor (AOAC, 1984). Total volatile basic nitrogen (TVB-N) was determined through direct distillation into boric acid using a Kjeldahl-type distillatory. The acid was titrated with 0.1 N of H_2SO_4 solutions. Salt content of samples was measured by the method of AOAC (2000). Histamine concentration in mahyaveh was determined by an enzymatic assay kit (Check Color Histamine, Kikkoman Co., Noda, Japan) according to Kuda *et al.* (2007).

Microbial analysis

To perform microbiological analyzes, 10g of sample was homogenized into sterile glass with 90 mL of sterilized saline solution (0.95% w/v) to gain the initial dilution (10^{-1}). From this dilution, a number of decimal dilutions

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were prepared using the same diluent. Total viable counts (TVC) were determined by pour plate method using Trypticase soy agar, MRS agar, Tryptic soy agar containing 10% NaCl, and Nutrient agar (Merck, Germany) for cultivation of *Bacillus*, LAB, halophilic bacteria and Enterobacteriaceae with plate incubation at 30°C for 24-48h (Kilinc *et al.,* 2006). For enumeration of LAB, the dilutions were plated in depth in MRS agar (Merck, Germany) and plates were placed for 72h at 37°C in a CO_2 incubator. Potato dextrose agar (PDA) was used to cultivate mold and yeast, which were incubated for 5 days at 30°C. The results were expressed as colony forming units per gram of product (log CFU g^{-1}). (Zarei *et al.,* 2012; Colombo *et al.,* 2014).

Statistical analysis

Experiments were performed in triplicates, and the significant differences among the means were analyzed using One way- ANOVA and LSD post hoc test at $p<0.05$ (SPSS, version 24, 2016).

Results

The pH values and acidity of control and treated samples with *L. plantarum* and *P. polymyxa* (10^7 CFU g^{-1}) can be observed in Figures 2 and 3. The pH value and acidity of mahyaveh samples were in the range of 5.08-5.15 and 2.33-2.53 (mL $100mL^{-1}$) and there was significant difference in pH value and acidity among different mahyaveh samples ($p<0.05$). As the pH values of mahyaveh samples reduced significantly during fermentation (Fig. 2), acidity of samples revealed significant increase during 45 days of fermentation (Fig. 3). Control samples on 30^{th} day revealed the most pH value and treatment samples on 45^{th} day showed the least pH value. Results showed that presence of *L. plantarum* and *P. polymyxa* reduced the pH values of the treated mahyaveh samples compared to those of the control samples ($p<0.05$).

Salt as a main component in fish sauce making was evaluated during fermentation. Figure 4 shows that mean NaCl concentrations in tested samples were in the range of 13.17% to 18.83%. Control samples on 30^{th} day had the most NaCl concentrations and treatment samples on 45^{th} day had the least NaCl concentrations. Figure 4 reveals that NaCl concentrations of mahyaveh treated samples reduced significantly during fermentation ($p<0.05$).

TVB-N content of mahyaveh samples during 45 days fermentation can be seen in Figure 5. Mean TVB-N content of mahyaveh samples were in the range of 240.12- 344.21 mg $100g^{-1}$ and the TVB-N index increased significantly during fermentation ($p<0.05$). Our results cleared that TVB-N levels decreased significantly ($p<0.05$) in the treated samples (with *L. plantarum* and *P. polymyxa*) compared to those of control samples at each sampling time (Fig. 5).

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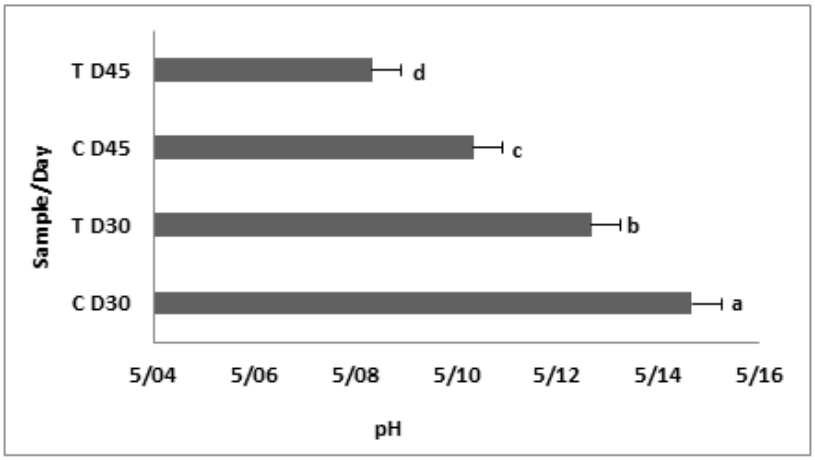


Figure 2: pH value changes in mahyaveh samples. CD30: control samples on 30th day, TD30: treatment samples on 30th day, CD45: control samples on 45th day and TD45: treatment samples on 45th day. Superscripts on each column with different letters are significantly different (p<0.05). Error bars show standard deviation.

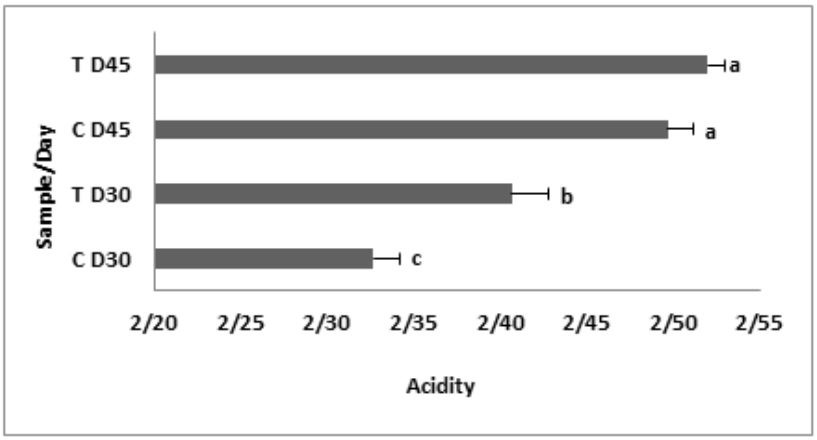


Figure 3: Acidity changes in mahyaveh samples. CD30: control samples on 30th day, TD30: treatment samples on 30th day, CD45: control samples on 45th day and TD45: treatment samples on 45th day. Superscripts on each column with different letters are significantly different (p<0.05). Error bars show standard deviation.

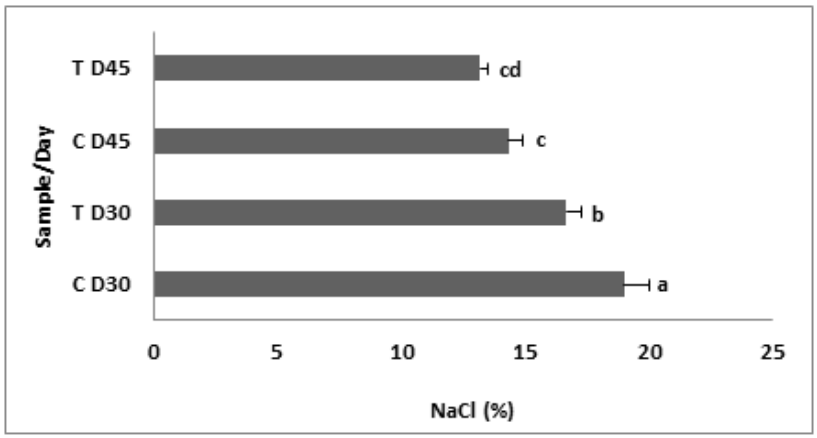


Figure 4: NaCl concentrations in mahyaveh samples. CD30: control samples on 30th day, TD30: treatment samples on 30th day, CD45: control samples on 45th day and TD45: treatment samples on 45th day. Superscripts on each column with different letters are significantly different (p<0.05). Error bars show standard deviation.

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Table 1 presents the amount of histamine in control and treated samples on 30th, 45th and 60th day of fermentation. Control samples on 30th day revealed most histamine concentration and treatment samples on 60th day had least histamine concentration. Samples treated with *L. plantarum* and *P. polymyxa* revealed decreasing histamine trend compared to that of control samples on test days.

Table 1: Histamine concentration in mahyaveh samples during fermentation.

Day 30		Day 45		Day 60	
CD 30	**TD 30**	**CD 45**	**TD 45**	**CD 60**	**TD 60**
44. 73 ± 0.58[a]	38.67± 0.51[b]	39.23 ± 0.96[b]	35.33 ± 0.56[c]	34. 7 ± 0.26[c]	32.66 ± 0.58[d]

Each number is average of three replicates ± standard deviation.
Lowercase letters indicate significant difference among experimental groups at $p<0.05$ level.

Tables 2 present *Bacillus* and Halophilic bacterial population of control and treated mahyaveh samples during fermentation. Significant statistical differences were observed in *Bacillus* and halophilic bacterial populations of mahyaveh samples during fermentation ($p<0.05$). *Bacillus* populations decreased progressively from 2.66 (log CFU g^{-1}) on 30th day to 2.54 (log CFU g^{-1}) on 45th day in control samples. Halophilic bacterial population gradually increased from 4.58 (log CFU g^{-1}) on 30th day to 4.84 (log CFU g^{-1}) on 45th day in control samples. Treated samples with *L. plantarum* and *P. polymyxa* showed increasing trend in *Bacillus* population compared to that of control samples but treated samples reviled a decreasing trend in halophilic bacteria population compared to that of control samples.

Table 2: Results of microbial tests of mahyaveh samples during fermentation.

Microorganism	Viable bacteria count (Log cfu g^{-1})			
	Day 30		Day 45	
	C D30	**T D30**	**C D45**	**T D45**
Bacillus	2.66 ± 0.04[b]	3.99 ± 0.03[a]	2.54 ± 0.04[c]	3.81± 0.06[a]
Halophile	4.58 ± 0.01[b]	3.55 ± 0.02[c]	4.84 ± 0.01[a]	3.29 ± 0.03[c]
Enterobacteriaceae	4.32 ± 0.02[b]	3.63 ± 0.03[c]	4.68 ± 0.02[a]	3.67 ± 0.01[c]
Mold and yeast	2.43 ± 0.05[a]	2.27 ± 0.03[bc]	2.30 ± 0.00[b]	2.04 ± 0.04[d]
Lactic Acid	3.69 ± 0.01[c]	4.84 ± 0.01[a]	3.91 ± 0.02[b]	4.96 ± 0.01[a]

Each number is average of three replicates ± standard deviation.

Table 2 presents LAB count of different mahyaveh samples in the range of 3.69 to 4.96 (log CFU g^{-1}). There was significant difference ($p<0.05$) in the number of LAB of mahyaveh samples. Although LAB counts of treated samples increased during fermentation, the number of LAB of control samples decreased in this period. The results showed that numbers of LAB of treated samples on 30th and 45th days (4.84 and 4.96 log CFU g^{-1}) were higher compared to those of control samples (3.96 and 3.91 log CFU g^{-1}).

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Table 2 presents Enterobacteriaceae and fungi populations of control and treated mahyaveh samples during fermentation. There was significant difference in the Enterobacteriaceae and fungi populations of different mahyaveh samples (p<0.05). Control samples on 30th and 45th days had the most fungi and Enterobacteriaceae populations respectively, while treated samples on 45th and 30th days had the least numbers of these microorganisms respectively. Results showed less population of Enterobacteriaceae and fungi in treated samples compared to those on control samples during fermentation.

Discussion

Acidity and pH value of mahyaveh are critical factors in evaluation of product quality. The quality of fish sauce is representative of the raw fish quality which is used for its production. Accepted pH range for fish sauce products by Codex Alimentarius Commission (2013) is 5.0-6.5 and pH of mahyaveh samples produced in this study correspond with the range recommended by Codex. The pH value of mahyaveh samples in this study were in the range of pH reported by Zarei *et al.* (2012) and was more similar to the values reported by Park *et al.* (2001) for fish Sauces produced in southeast and East Asian countries. It seems that reduction of pH during fermentation in mahyaveh samples was due to LAB activity and producing organic acid. Park *et al.* (2001) reported lactate, pyroglutamate, and acetate as predominant organic acids in most fermented fish sauces. Similarly, Kilinc *et al.* (2006) observed a significant decrease in pH of fish sauce with glucose after 22 days of fermentation. This finding is in contrasts with the finding of Mueda (2015) who reported increasing trend in pH of anchovy fish sauce from 14th to 28th day of fermentation process. It seems that if putrefying bacteria overcome, basic nitrogen compounds are produced and pH values are increased, but if fermentative bacteria overcome, organic acids are formed and pH values are decreased (Kilinc *et al.*, 2006). In this research pH values of treated mahyaveh samples decreased significantly in presence of *L. plantarum* and *P. polymyxa* compared to control samples (p<0.05). This reduction in pH values of treated samples may be due to promotion of fermentative bacteria growth that produced more organic acids in treated samples. Dissimilar to the present study Lee *et al.* (2015) revealed no significant difference (p>0.05) between pH of treated samples with *B. polymyxa* and control samples during 120 days of fermentation. Also Zaman *et al.* (2011) reported no significant difference between the pH of control and treated samples inoculated with *staphylococcus carnosus* and *bacillus amyloliquefaciens FS05* during fermentation.

Salt is a major component in producing fish sauce because it prevents growth of spoilage bacteria

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and forms proper condition for fermentative bacteria (Puat *et al.*, 2015). Consistent with our results, Zarei *et al.* (2012) reported that salt concentration is an effective factor for microbial growth in mahyaveh and samples originated from five different locations in southern part of Iran had 7.48-17.1% NaCl. Dissimilar to the present study Puat *et al.* (2015) reported higher concentrations of salt (26.66% to 27%) in Indonesian fish sauces. In this study salt concentrations of mahyaveh samples reduced significantly during fermentation (p<0.05). Dissimilar to our study Mueda (2015) revealed no significant change in salt content of fish sauce during fermentation (p>0.05) and values were above our results within range of 18.51 to19.74%.

The TVB-N index of fish sauce is indicator of proteolysis intensity, autolytic activity, amino acid deamination, and nucleotide devastating reactions. TVB-N is mainly composed of ammonia, di-methylamine and tri-methylamine and this index is often applied in commercial rules as a rejection factor (Mueda, 2015). In this study TVB-N index of control and treated samples significantly increased during fermentation (p<0.05). TVB-N values of mahyaveh were not within permissible limits, which are at 100-200 mg $100g^{-1}$ for salted and dried fish products (FDA, 2002). The presence of proteolytic bacteria in the fermented fish sauce samples lead to increase in the amount of TVB-N during fermentation. The same as this trend, Besas and Dizon (2012) revealed increasing pattern in the TVB-N content of fish sauce samples during fermentation. This finding is in agreement with those of Zarei *et al.* (2012) who reported high amount of TVB-N in their mahyaveh samples (mean 309.8 mg 100 g^{-1}). Contrary to our studies Kuda *et al.* (2007) and Mueda (2015) reported less amount of TVB-N in mackerel-nukasuke (41-76 mg 100 mL^{-1}) and salt fermented anchovy sauce (21.99-147.35 mg 100 mL^{-1}) which were within the FDA acceptable limits (FDA, 2002).

Our results cleared that TVB-N levels decreased significantly (p<0.05) in treated samples compared to those in control samples. It seems that presence of *L. plantarum* and *P. polymyxa* may reduce growth of proteolytic bacteria producing endogenous catalytic enzymes. This finding is similar to the results of Lee *et al.* (2015) who reported less TVB-N content in inoculated samples with *B. polymyxa* compared to those of control. Zaman *et al.* (2011) observed higher count of proteolytic bacteria in control samples compared to that of treated samples with starter cultures of *staphylococcus carnosus* and *bacillus amyloliquefaciens.* It seems that reduction of TVB-N content in treated samples compared to that in control samples in this study is related to less proteolytic bacteria content and their proteolytic enzyme.

Histamine is a biogenic amine and is formed in sea foods by microbial

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decarboxylation of histidine or by transamination of aldehydes and ketones by amino acid transaminases (Biji *et al.* 2016). Histamine is the main biogenic amine in fermented fish sauces and scombroid fish poisoning is related with consumption of fish products with high histamine levels (>500 mg L^{-1}). Although Food and Drug Administration (FDA) recommended 50 mg Kg^{-1} level for histamine in seafood products (FDA, 2004), the amount of histamine in control and treated samples during fermentation were higher than the maximum permitted level. This finding is in agreement with those of Zarei *et al.* (2012) who reported histamine in mahyaveh samples more than the maximum permitted level (50 mg Kg^{-1}). In this study, we observed higher concentration of histamine in control samples than that in treated samples containing *L. plantarum* and *P. polymyxa* during fermentation. It represents that starter culture of *L. plantarum* and *P. polymyxa* presumably serves as competing organisms possessing less efficient histidine decarboxylase than strong histamine producers. Inoculating this starter culture in treated samples may be an effective way to inhibit histamine production and they probably can decompose histamine produced by strong histamine producers (Mah and Hwang, 2009). Similarly, Lee *et al.* (2015) reported that application of *B. Polymyxa DO5-1* starter culture was impressive in reducing histamine accumulation in salted fish products.

In this study *bacillus* bacteria identified as one of the effective bacteria in mahyaveh production. We found higher population of *Bacillus* in treated samples than in control samples during fermentation. It represents that inoculating samples with starter culture of *L. plantarum* and *P. polymyxa* intensified growth of *bacillus* bacteria in treated samples. Similarly, Taheri *et al.* (2014) reported that *bacillus licheniformis and bacillus subtilis* were the dominant *bacillus* species in mahyaveh samples. This is consistent with findings in the study of Faisal *et al.* (2015) who reported *bacillus* as the main type of bacteria during fermentation period of fish sauce because of spore forming and salt tolerant ability.

High level of NaCl in fish sauce is likely to have a substantial effect on microbial growth, fermentation progress, safety and sensory properties of the product (Paludan- Müller *et al.*, 2002). Based on the results halophilic bacteria were impressive bacteria in mahyaveh production. Results showed less population of halophilic bacteria in treated samples than that in control samples during fermentation. It seems that starter culture of *L. plantarum* and *P. polymyxa* reduce the growth of halophilic bacteria in treated samples. This observation is comparable to the findings of Tanasupawat *et al.* (2009) who reported the important role of moderately and extremely halophilic

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bacteria in Thai fish sauce production. Similar to our results, Zarei *et al.* (2012) reported a range of 3.19 - 4.03 log CFU g^{-1} halophilic bacteria in mahyaveh samples.

LAB is generally predominant microorganisms in producing fermented fish sauces. These bacteria effect is on extend organoleptic properties of fermented foods and have an important effect on improving their qualification and safety (Karparvar *et al.*, 2019). The LAB counts (log CFU g^{-1}) of different mahyaveh samples in this study were similar with the findings of Zarei *et al.* (2012) that levels of LAB were in a range of 3-4.80 (log CFU g^{-1}) in mahyaveh samples. Although number of LAB of treated samples inoculated with *L. plantarum* and *P. polymyxa* increased during fermentation, control samples without inoculation showed significant decrease in number of LAB during this time. The viability of LAB is influenced by some factors such as Low pH, organic acid, high redox potential, hydrogen peroxide, molecular oxygen, bacterial competition, relatively high temperatures during fermentation. It seems that LAB survival condition in control samples during fermentation was not proper but presence of *L. plantarum* and *P. polymyxa* in treated samples had an improving effect on LAB growth and viability (Trisnawita *et al.*, 2018). Similar increasing pattern in the number of LAB in treated samples with *Lactobacillus plantarum IFRPD P15* and *Lactobacillus reuteri IFRPD P17* were revealed in the study of Saithong *et al.* (2010) wherein the viability in Thai fermented fish was evaluated during 5 days.

Enterobacteriaceae and fungi are major cause of spoilage of most seafood products like fish sauces (Anihouvi *et al.*, 2019). In this study, the populations of Enterobacteriaceae and fungi of treated samples were less than those of control samples during fermentation. It seems that starter culture of *L. plantarum* and *P. polymyxa* had suppressive effect on Enterobacteriaceae and fungi growth in treated samples compared to those in control samples. Inhibitory effects are related to organic acid production by LAB and higher acid content in treated samples than those in control samples (Zarei *et al.*, 2012). This is in agreement with the findings of Saithong *et al.* (2010) who reported the ability of LAB as a starter culture in Thai fermented fish to eliminate pathogenic bacteria and yeast. It represents that high amount of LAB in treated samples restrict the growth of halophilic bacteria, Enterobacteriaceae and fungi compared to that in control samples.

Based on the results, in presence of starter cultures and production of organic acids, pH values of samples decreased and acidity increased. Smaller levels of TVB-N in treated samples are related to the presence of *L. plantarum* and *P. polymyxa* bacteria that reduce the growth of proteolytic bacteria that produce endogenous catalytic enzymes. *L. plantarum* and *P.*

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polymyxa are considered as important culture in controlling the growth of undesirable microorganism such as Enterobacteriaceae and fungi, and in maintaining acidification of mahyaveh. The treated samples containing *L. plantarum* and *P. polymyxa* presented a significant decrease in histamine concentrations. This reduction was consisted with reduction in total viable count of bacteria and increase in viability of total LAB count. From human safety point of view, using treatments like adding LAB for reducing histamine is necessary for mahyaveh production.

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