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Influence of MAP and Multi-layer Flexible Pouches on Aerobic Bacteria Count of Smoked Kutum Fish (*Rutilus frisii kutum)*

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ABSTRACT

The usage of different concentrations of three gas mixture (carbon dioxide, nitrogen,oxygen) ,and also vacuum conditions and flexible multi-layer films were studied for determination of aerobic bacteria of smoked kutum fish (Rutilus Frisii Kutum) at ambient condition (T= 25 ^{0}C). Normal conditions as a control packaging were compared with four types of modified atmosphere packaging: ($N_2$70%+$CO_2$30%), ($N_2$30% + $CO_2$70%), (45%CO2+45%N2+10%O2) and vacuum conditions, in this project. These samples(smoked kutum fish) were packaged in 3 kinds of flexible multi-layer films 3-layer ($PET_{(12)}/AL_{(12)}/LLD_{(100)}$) , 4-layer ($PET_{(12)}/AL_{(7)}/PET_{(12)}/LLD_{(100)}$),and 3-layer ($PET_{(12)}/AL_{(7)}/LLD_{(100)}$). Packed samples were performed microbial tests (Aerobic bacteria count), in different times during 60 days, with 15 treatment ,3 run, statistical analysis and comparison of data, were done by software SAS (Ver:9/1) and Duncan's new multiple range test, with confidence level of 95% (P <0.05). The usage of MAP was not adequet for controlling spoilage but the spoilage process was delayed. The shelf life of smoked kutum fishes (according to Aerobic bacteria count) in 4-layer , under conditions 1,2,3 were reported 60,58,45 days and in vacuum conditions were 40 days, in 3-layer (AL:12) , under conditions 1,2,3 and in vacuum conditions were 55,50,40, 35 days, with 3-layer (AL:7),under conditions 1,2,3 were 45,40,35 days and under vacuum conditions were 30 days. Aerobic bacteria count showed that increasing CO2 concentration increased shelf life of smoked fish. According to these results could be concluded the best condition for controlling the growth of Aerobic bacteria belonged to treatment under modified atmosphere CO2 70% as an antibacterial properties of more carbon dioxide in container 4-layer, since steam permeability of 4-layer was less than 3-layer in order to maintain long-term shelf life of smoked kutum Fish.

Keywords: modified atmosphere packaging (MAP), flexible multi-layer films, smoked kutum fish *(Rutilus Frisii Kutum),* aerobic bacteria.

INTRODUCTION

Aerobic bacteria of smoked marine products are too dangerous, while smoking process have be done with cold method, must be considered [4]. Although, thermal treatment (120 ^{o} C, 20 min) effectively destroys these microorganisms [6-8,14,26-30], has been used widely, proteins and some other physiological substrates are inactivated, and consequently the sensory properties and contents of nutrients in foods are lost [1,20,34,36]. For that reason, significant efforts are leading to the development of novel processing such as MAP [3,19,33-36], which is proving to be able to inactivate spoilage microorganisms without significantly affect nutritional and sensory properties of several foods [2,19,33-36]. Kutum fish with the scientific name, *Rutilus frisii kutum*, is a cyprinid fish which is distributed from Turkmenistan to Azerbaijan along the Caspian Sea. It is one of the economically important fish in the region [9]. Kutum fish constituted about 78% of bony fish harvest and about 76.6% of the whole income of fishermen in the 2008-2009 fishing season in the southern part of Caspian Sea[9].Smoked fish, is a processed fish which prepared by two system , smoke in cold condition (25-30 ^{0}C) or smoke in hot condition (80 ^{0}C) ,cold type has short shelf life as a potential source of pathogenic microorganisms, so significant efforts are leading to the development of novel processing such as MAP [9]. The modified atmosphere packaging (MAP) is a technique, which is widely used for shelf-life extension and improvement the quality of perishable foods stored at refrigeration temperatures [3,5,19,33-36]. The ability of modified atmosphere

packaging for extending the shelf life of foods has been recognized for many years. Indeed, over 100 years ago [7,12,13,19]. Modified atmosphere packaging is an enclosure of food, in a package which the atmosphere has been changed by altering the proportions of carbon dioxide, oxygen, nitrogen, water vapor and trace gases. The process limits microorganism as well as biochemical activity. This modification is performed by gas flash packaging which oxygen is removed and replaced by a controlled mixture of gases [12,19]. MAP inhibits some microorganisms, so can increase the quality of variety foods. These products (smoked Kutum fish) without an efficient processing are potential source of pathogenic microorganisms, especially mesophile and thermophile aerobic , anaerobic , Clostridium and Bacillus, since the low acidity (pH 5-5.5) and high water activity of these packed sea food creates an ideal environment for rapid microbial spoilage container [26-30]. However the growth of microorganisms depends on temperature, pH and water activity as the main growth-determining factors, other factors can significantly influence the growth characteristics of the microorganism. All mentioned in this study include the initial CO2/ N2/ O2 concentration (%) in the head space of pouches as the independent variable for the gas atmosphere demonstrated that CO2 exerts as an antimicrobial effect in the water-phase of the food product [15,19-21,33-36], therefore except the effect of intrinsic, extrinsic and processing parameters on the CO2 solubility, the concentration of dissolved CO2 in the water-phase of the food product should be incorporated in this study as independent variable [1,15]. Nitrogen (N2) is a non-reactive gas that has no smell or taste, unlike carbon dioxide, is not absorbed in food or water [33-36]. It is used as a filler gas to replace oxygen and thus prevent spoilage or to replace carbon dioxide and prevent package collapse. Oxygen (O2) prevents anaerobic bacteria growth [19,20,33-36]. Anaerobic and aerobic microorganisms count are unequivocally assigning in the scientific methods. It is one of the oldest means of quality control, but in principle is an essential part of the mandatory assessment of food quality [15,20]. Other hand the multi-layer films have been used for packaging these food product are plastic films laminated with aluminum for packaging cooked meat and cooked poultry instead of can [17,18,22-24,31] .These laminated packages with some metal component can considerably change the food temperatures and also microwave transparent with a high melting point [16,22-26-30]. The most common packages that have been tried, are individual pouches made of microwave transparent rigid films such as polyethylene (LLD), and polyethylene terephthalate (PET), which are barrier films, and aluminum foil [18,22-24,31,32]. In this investigation, we investigate about the effects of modified atmosphere packaging; different concentrations of CO2/N2/O2; microbiological test (aerobic bacteria), and the use of three multilayer flexible pouches on smoked kutum fish during storage times [19,22-24,31,32]. We try to prove MAP can substitute thermal processing in conservation industries [19,22-24,33-36]. These flexible pouches can also improve the marketability of smoked kutum fish, for easy usage of the package, and great importance for best sell [16,17,19,33-36].

MATERIALS AND METHODS

Preparation of smoked kutum fish

5, smoked kutum fish (prepared by cold smoke recently) each weighing 1.5 kg were bought from a distribution center of fish in Tehran for this experiment. The head and tail of samples, were isolated and then samples of fish, were divided into small pieces (60 g) and placed under sterile conditions inside the containers. Temperature was controlled in order to decrease to ambient temperature (T=25 °C). Smoked fish were ready for gas injection. Analytical parameters such as pH (Crison 2001 pH meter; Crison Instruments, SA, Barcelona, Spain) soluble solid content (Atago RX-1000 refract meter; Atago Company Ltd., Japan), were measured according to the ISIRI regulation [19,24-30,33-36].

Modified Atmosphere Packaging

Henkelman packing machine, model Boxer-200A was used in this project. Samples were packed into three multilayer flexible pouches 3-layer ($PET_{(12)}/AL_{(12)}/LLD_{(100)}$), 4-layer ($PET_{(12)}/AL_{(7)}/PET_{(12)}/LLD_{(100)}$), and 3-layer ($PET_{(12)}/AL_{(7)}/LLD_{(100)}$) under modified atmosphere . After packaging, samples were put in at ambient condition (T= 25 ^{0}C) immediately, for aerobic bacteria count in different times (60 days) [19, 33-36].

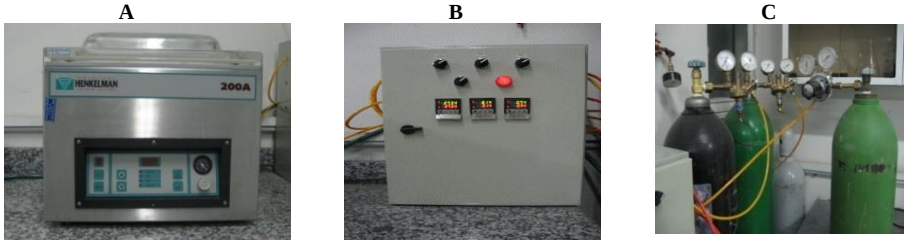

Fig 1. (A) Modified atmosphere packaging, (B) gas analyzer, (C) gas flash tank (Model: Boxer-200A)
[19, 33-36].

Microbial culture

Total count of aerobic bacteria in PCA & CMM media

PCA (Peptone from casein 5g/1000 ml; Glucose 1g/1000 ml, Yeast Extract 2.5 g/1000 ml, Agar 14g/1000 ml, Distillated water 1000 ml), Plate Count Agar is a general media for aerobic bacteria count. **CMM** (Beef heart 454g/1000 ,Protease peptone 20 g/1000 ml, Glucose 5 g/1000 ml, Sodium chloride 5g/1000 ml, Sodium hydrochloride ½ 454 g/1000, Distillated water 1000 ml).Cooked Meat Media is an enrichment media for aerobic bacteria [10,11,19,24-30,33-36].

First 1-2 g samples was put in CMM (3-4day) ,while 1 g of enriched sample was weighed under the microbial laboratory hood, and was crushed in 10 ml of ringer's solution. According to CFU method, divided into one series tube (six tubes) which contain 9 cc sterile distilled water. First 1 cc of the sample added to tube no one and transferred tube by tube, main sample was prepared by serial dilution (0.01,0.001…). Finally pour plate method were done in the PCA culture ,too in order to count aerobic bacteria which was incubated for 3 days at 37 ° C [19,24-30,33-36].

Samples packaging and storage

All pouches (smoked kutum fish), were put in at ambient condition (T= 25 ^{0}C). Analytical characteristics of these barrier containers were shown in table 1 [19,24-30,33-36].

Table 1- Analytical characteristics of containers [19,24-30,33-36].

Sample	Layers	Thickness (µ)	Tensile of sealing film (N)	O.T.R (ml/m^2.day)	W.V.T.R (g/ m^2.day)
PET/AL/LLD	100/12/12	124	58.88	0	0.11
PET/AL/LLD	100/7/12	119	48.89	0	0.50
PET/AL/PET/LLD	100/12/7/12	131	61.03	0	0.089

PET: Poly Ethylene Terephthalate; LLD: Low Density Poly Ethylene; AL: Aluminum

Statistical Analysis

In order to describe the variables of this experiment, we must design a model to analysis relationship between type of samples, type of treatments, and growth of aerobic bacteria. Statistical analysis of data, was performed by software Statistical Analysis System (SAS: 9/1) with ANOVA test, and comparison of data was done by Duncan's new multiple range test, with confidence level of 95% (P <0.05) [26-30, 33-36].

RESULTS

Total count of aerobic bacteria in different conditions

Analysis of variance was shown in table 2, that the main factors between(layer, gas ,time) for determination of aerobic bacteria had significant difference (P<0.01).The interaction of (gas , time) had a significant difference (P<0.01) too.

Table 2-Analysis of variance mean squares traits in response to treatments

Total count of Aerobic bacteria	Degrees of freedom	Variables
0.804^{**}	2	**Layer**
5.84^{**}	4	**Gas**
0.0043^{ns}	8	**Gas*layer**
16.395^{**}	3	**Time(day)**
0.0034^{ns}	6	**Layer*Time(day)**
1.258^{**}	12	**Gas*Time(day)**
0.003^{ns}	24	**Layer*Gas*Time(day)**
0.0043	120	**Errors**
4.172	-	**Variance Index (CV)**

Table 3-Comparison of the mean traits in response to different films

Aerobic bacteria	various films
6.283^{b}	Film 1 3-layer(AL:12)
6.411^{a}	Film 2 3-layer (AL:7)
6.182^{c}	Film 3 4-layer

Figure 2 and table 3 were shown, the effect of different layers on aerobic bacteria count of smoked kutum fish. Different layers were separated in different colors, layer: 1 ($PET_{(12)}/AL_{(12)}/LLD_{(100)}$) {blue}; layer:2 ($PET_{(12)}/AL_{(7)}/LLD_{(100)}$){red}; layer:3($PET_{(12)}/AL_{(7)}/PET_{(12)}/LLD_{(100)}$) {green}. The lowest amount of aerobic bacteria of smoked kutum fish belonged to layer:3 (4-layer) and then layer:1(3-layer), because of thickness (131 µ), low steam permeability in this container.

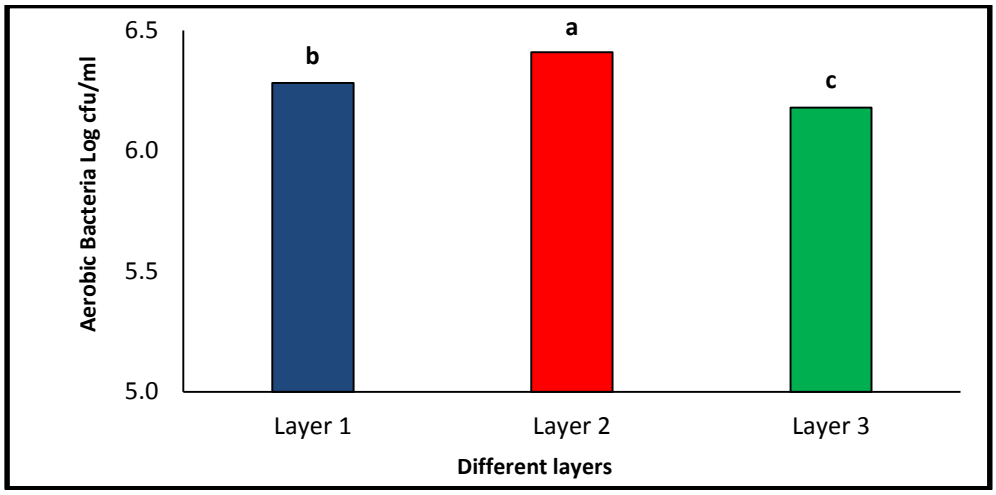

Fig 2-The effect of different layers on aerobic bacteria count (log cfu/ml)

Table 4-Comparison of the mean traits in response to different gas compositions

Aerobic bacteria	various gases
5.670[e]	CO_2 70% +N_2 30%
6.402[c]	CO_2 30% + N_2 70%
6.150[d]	CO_2 45% + N_2 45%,+ 10% O_2
6.722[a]	vacuum
6.512[b]	control

Figure 3 and table 4 were shown, the effect of different gas compositions on aerobic bacteria count of smoked kutum fish. Different gas compositions were separated in different colors, e- CO2 70%,N2 30%{ red }; c-CO2 30%,N2 70%{blue}; d - CO2 45%,N2 45%{green}; a-vacuum{yellow}; b-control sample{black}. The lowest amount of aerobic bacteria count belonged to gases combinations (70% CO_2, 30%), and (45% CO2, 45% N2 , 10% O2) and highest amount observed in vacuum and ordinary condition ,because of gas atmosphere (antibacterial properties of carbon dioxide gas).

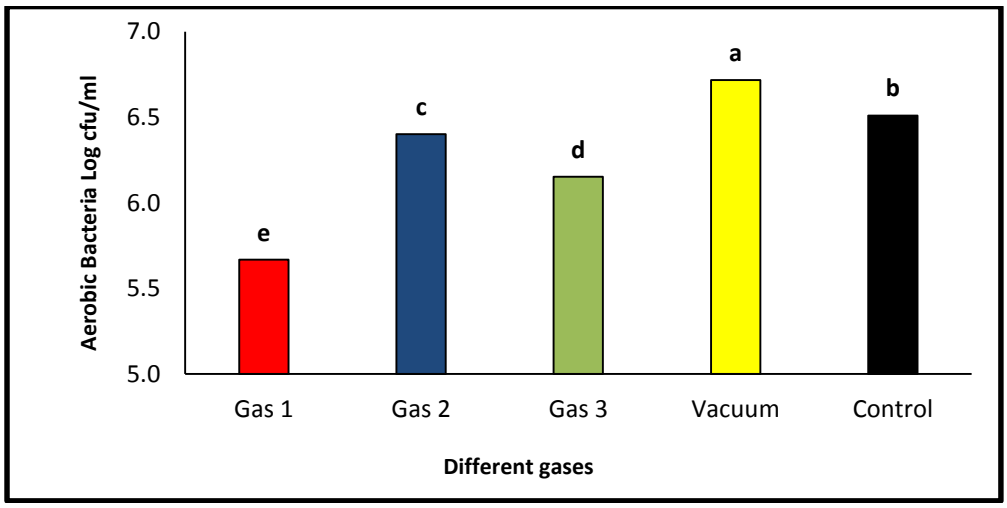

Fig 3-The effect of different gas compositions on aerobic bacteria count (log cfu/ml)

Table 5- Comparison of the mean traits in response to different days

Aerobic bacteria	various days
5.451[d]	Day 15
6.283[c]	Day 30
6.570[b]	Day 45
6.870[a]	Day 60

Figure 4 and table 5 were shown, the effect of different days on aerobic bacteria count of smoked kutum fish. Different days were separated in different colors , days15 {red}; days 30{blue}; days 45 {green}; days 60 {black}.The lowest

amount of aerobic bacteria in smoked kutum fish was reported after 15 days and highest after 60 days, which caused aerobic bacteria to grow by the times and increased rapidly.

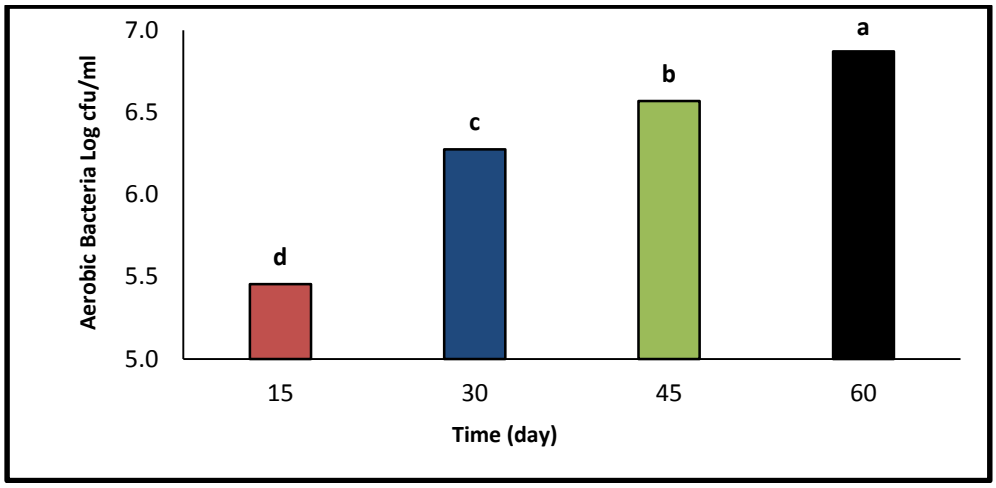

Fig 4-The effect of different days on aerobic bacteria count(log cfu/ml)

According to figure 5, the effects of different gas compositions in different times on aerobic bacteria count of smoked kutum fish were shown. Different gas compositions were separated in different colors, CO2 70%,N2 30%{ White }; CO2 30%,N2 70%{ red }; CO2 45%,N2 45%{green}; vacuum{violet}; control sample{blue}. The lowest amount of aerobic bacteria count belonged to gases combinations (70% CO2 ,30% N2) and then (45%CO2,45% N2,10% O2) after 15 days, but highest amount observed in ordinary condition and also vacuum after 60 days.

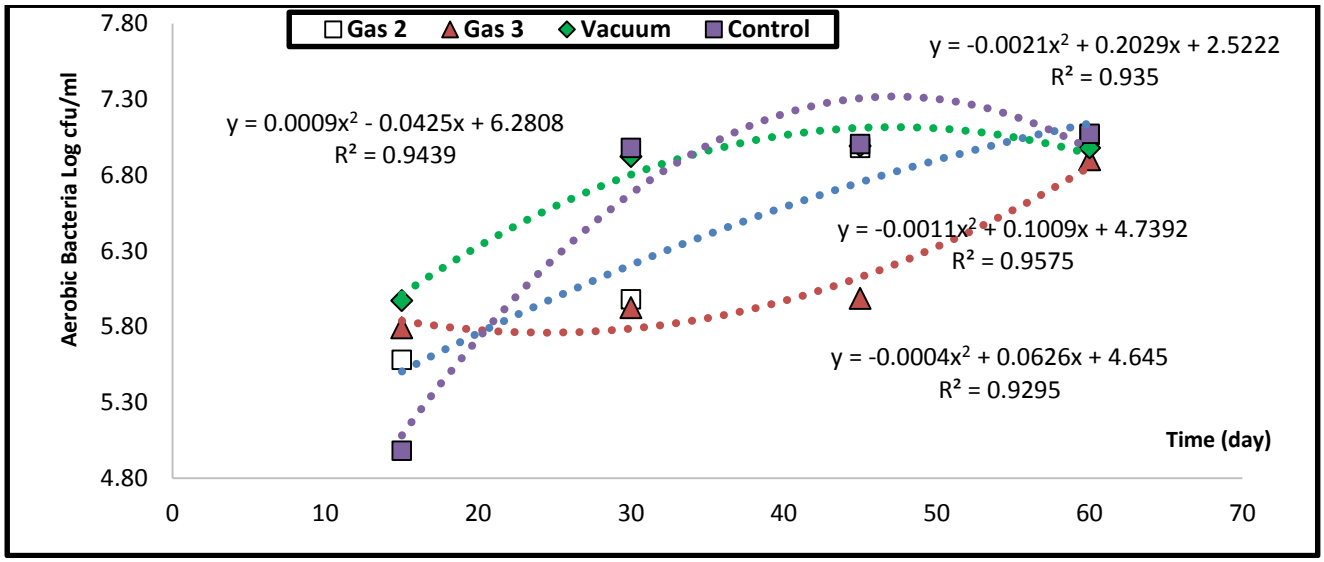

Fig 5-The effect of different gas combinations and different days on aerobic bacteria count (log cfu/ml)

According to figure 6, the effects of different gas combinations and different layers on aerobic bacteria count of smoked kutum fish were shown. Different layers were separated in different colors, {3-layer ($PET_{(12)}/AL_{(12)}/LLD_{(100)}$) -blue, 4-layer ($PET_{(12)}/AL_{(7)}/PET_{(12)}/LLD_{(100)}$)-green, and 3–layer ($PET_{(12)}/AL_{(7)}/LLD_{(100)}$)-red}. The lowest amount of smoked kutum fish belonged to layer: 3(4-layers) under gas composition CO2 70%, highest amount belonged to layer: 2 and layer: 1, under ordinary condition and then layer: 2 under vacuum condition.

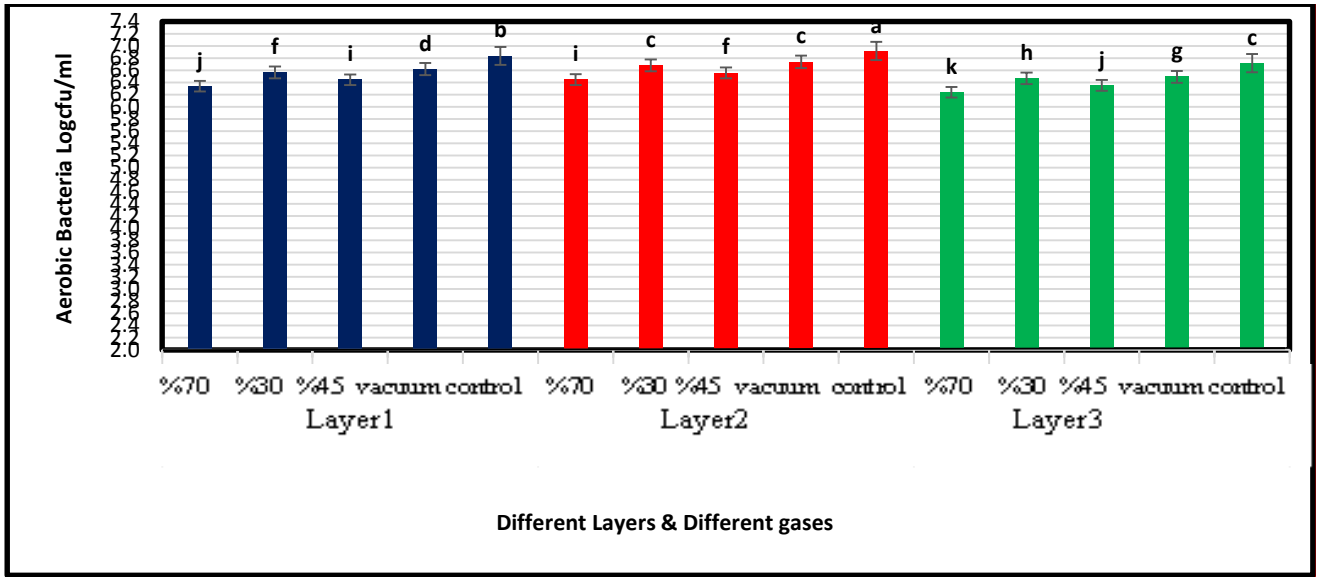

Fig 6-The effect of different gas combinations and different layers on aerobic bacteria count (log cfu/ml)

DISCUSSION

The results of this study showed that CO2 had antimicrobial effect and its mechanism could be described by its solution in water of food tissue and produced carbonic acid which the carbonic acid arrived to cell membrane of microorganisms and ionized into the cell and the collapsed electrical balance within the cell in order to killing microorganisms . The difference between microbial activities in the samples was significantly dependent on the concentration of nitrogen and carbon dioxide, as well as the role of nitrogen gas, indirectly could influenced perishable foods by decreasing the growth of aerobic micro-organisms. The second role of nitrogen in modified atmosphere packaging, was a gas filler and protecting the development of flexible packaging against vacuum.

1- The lowest amount of aerobic bacteria of smoked kutum fish belonged to container 3 (4-layer) under gas composition CO2 70%, and the highest amount belonged to container 2 (3-layer) under ordinary condition and then vacuum condition. Because of the thickness and type of gas atmosphere (antibacterial properties of carbon dioxide gas), which were prolonged the shelf life of smoked kutum fish till 60 days.

2-Aerobic bacteria count of samples in various conditions, had significant differences between (layer, gas, time) ($P<0.01$), and also there was significant differences between (gas, time) ($P<0.01$).

Blackistone, 1998, due to principles and applications of Modified Atmosphere Packaging of different food products, growth of aerobic bacteria in this investigation were evaluated reliable. Vanderzant *et al*., 2000 indicated that due to the shelf life and growth of aerobic bacteria of beef steak packaged under vacuum and MAP condition in different barrier containers during 30 days which were significant difference with ordinary condition as a control ,and the results were corresponded with these results. Chouliara & Karatapanis, 2007, indicated that due to combined effect of oregano essential oil and modified atmosphere packaging on shelf-life extension of fresh chicken breast meat, the results of aerobic bacteria count were corresponded with this study .Athina *et al.*, 2008, due to research formation of biogenic amines and relation to microbial flora and sensory changes in smoked turkey breast fillets stored under various packaging conditions at at 4°C ,the results of aerobic bacteria count were corresponded with these results. Bingol & Ergun, 2011, due to effect of two different type gas carbon dioxide and oxygen had been perform on ostrich meat, results showed that the shelf life of ostrich meat %60 CO2 prolonged till 7 days, the results were similar to these results. Zand & Sotoudeh, 2013, conducted due to effect of packaging chicken meal under gas combination (%30 N2 + %70 CO2) in 4-layer flexible films (131 µ), was better than 3-layer flexible films (124 µ) on shelf life and aerobic bacteria during 20 days , the results were similar to this investigation. Zand & Allahyari, 2013, indicated that due to effect of packaging under gas combination (%30 N2 + %70 CO2) in 4-layer flexible pouch (131 µ),was better than 3-layer flexible pouch (124 µ) on shelf life and aerobic bacteria of candy bread during 20 days, the results were corresponded with these results.

Sotoudeh *et al* , 2013, due to research about usage of MAP for shelf life extension of packed spicy chicken meal in multilayer flexible pouches ,4-layer container under CO2 %70 was better than 3-layer during 20 days, results were corresponded with this study .Zand, 2013, indicated that due to shelf life extension of mashroom meal in multilayer

flexible pouches, 4-layer container was better than 3-layer container during 60 days, the results were similar to these results. Zand, 2013,conducted due to shelf life prolongation of packed vegetables meal in multilayer flexible pouches ,4-layer container was better than 3-layer during 60 days, results were corresponded with this investigation.

CONCLUSION

In the present study, it was concluded that, shelf life and growth of aerobic bacteria of packed smoked fish have been affected by different flexible multi-layer pouches and different concentrations of different gases (carbon dioxide, nitrogen, oxygen), and also vacuum conditions during 60 days. Our results confirmed, the modified atmosphere packaging (MAP) was not lead to stop spoilage completely but delayed it .The effect of MAP was not sufficient but using this technique inactivated aerobic microorganism without a significant adverse effect on food properties of fish samples. These parameters could be promoted substitution of MAP and these barrier containers instead of other processing in fish packaging industries, due to a lot of privilege for shelf life extension of smoked seafood as a potential source of pathogen microorganisms in long times.

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