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Influence of MAP and Multi-layer Flexible Films on growth of Anaerobic Bacteria of Smoked Kutum Fish (*Rutilus frisii kutum*)

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ABSTRACT

In this study the influence of different concentrations of three gas mixture (carbon dioxide, nitrogen,oxygen) ,and also vacuum conditions and flexible multi-layer films on growth of anaerobic bacteria of smoked kutum fish (Rutilus frisii kutum) at ambient condition (T= 25 ^{0}C) were evaluated. Ordinary condition as control packaging were compared with four kinds of modified atmosphere packaging: ($N_2$70%+ $CO_2$30%), ($N_2$30% + $CO_2$70%), (45%CO2+45%N2+10%O2),and vacuum conditions, in this study. These samples(smoked kutum fish) were packaged in 3 types of flexible multi-layer films under modified atmosphere packaging,3-layers($PET_{(12)}/AL_{(12)}/LLD_{(100)}$)and 4-layers ($PET_{(12)}/AL_{(7)}/PET_{(12)}/LLD_{(100)}$)and 3- layer($PET_{(12)}/AL_{(7)}/LLD_{(100)}$). Packed samples were performed microbial tests (Anaerobic 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 spolage but the spoilage process was delayed. The shelf life of smoked kutum fish(according to anaerobic bacteria count) in 4-layers , under conditions 1 , 2 & 3 were reported 60,58,45 days and in vacuum conditions about were 40 days, in 3-layers (AL:12) , under conditions 1,2 &3 and in vacuum conditions were 55,50,40,35 days, with 3-layers(AL:7),under conditions 1,2 &3 45,40,35 days and in vacuum conditions were 30 days. Anaerobic bacteria count showed that increasing CO2 concentration increased shelf life. According to these results could be concluded the best condition for anaerobic bacteria count belonged to treatment under modified atmosphere CO2 70% and 4-layers due to thickness (131 µ), low steam permeability in this 4-layer container and antibacterial properties of more amount of carbon dioxide.

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

INTRODUCTION

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) [4,12], smoking process have usually be done in cold system, as an economically efficiency and 30 % weight loss of initial products so must be considered that smoked kutum fishes are potential source of pathogenic microorganisms especially, the present of Anaerobic bacteria's in these smoked marine products which are too dangerous [4]. Smoked kutum fish be usually prepared by steam in short time or even serve raw, that can be poisoned people. These foods are ready to serve (ready-to-eat), which are not prepared by too much heat [4,12]. The modified atmosphere packaging (MAP) is well known that there is a non-thermal method for inactivation microorganism and is widely used for shelf-life prolongation and improvement the quality of perishable foods stored at refrigeration temperatures [6-8,19, 33-36]. Howere there is no degradation of flavor and taste with heat denaturation of objectives [14]. The ability of modified-atmosphere packaging for extending the shelf life of foods has been known for many years [1-3,19,33-36]. Modified atmosphere packaging is the enclosure of a 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 air is removed and replaced by a controlled mixture of gases [6,19,33-36]. MAP inhibits some microorganisms, so can increase the quality of variety foods [5]. Kutum fish with the scientific name, *Rutilus frisii kutum*, belongs to the Cyprinids family and has a high commercial value. The main distribution of this species from Turkmenistan to Azerbaijan along the Caspian Sea. It is one of the economically important fish in the region [9]. These products (smoked kutum fish) without an efficient processing are potential source of pathogenic microorganisms, since the low acidity (pH 4-5) and suitable water activity of these packed meals, create an ideal environment for rapid microbial spoilage in this package [13,14,33-36]. Although, thermal treatment (120 ^{o} C, 20 min) effectively destroys these microorganisms [7,33-36], 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 [15,19,33-36]. For that reason, significant efforts are leading to the development of novel processing such as MAP [18,19,34,36] ,which is proving to be able to inactivate spoilage microorganisms without significantly affect nutritional and sensory properties of several foods [2]. 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 [13,14]. 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 [5,8,21,33-36], 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 [7,20]. Nitrogen (N2) is a non-reactive gas that has no smell or taste, unlike carbon dioxide, is not absorbed in food or water [18,19,33-36]. It is used as a filler gas to replace oxygen and thus prevent spoilage or to replace carbon dioxide and prevent package collapse, and also oxygen (O2) prevents anaerobic bacteria growth [1,5,33-36]. Anaerobic bacteria count is 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 [2,15]. Other hand the multi-layer films have been used for packaging these meal are plastic films laminated with aluminum for packaging cooked meat and cooked poultry instead of can [16,17, 24-30]. These laminated packages with some metal component can considerably change the food temperatures and also microwave transparent with a high melting point [18, 24-32]. 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 [20, 17,23-28], and aluminum foil [18,22-24,31,32]. In this study, we investigate about the effects of modified atmosphere packaging with different concentrations of CO2/N2/O2, and the usage of three multilayer flexible pouches [18,22-24,31,32] on anaerobic bacteria count of smoked kutum fish during storage times (60 days) [21]. We try to prove MAP and these multilayer flexible films are able to control the growth of Anaerobic bacteria, and can extend the shelf life of seafood [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 from a distribution center of fish in Tehran were bought 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). 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 study. Samples were packed into three multilayer flexible pouch 3-layer ($PET_{(12)}/AL_{(12)}/LLD_{(100)}$), 4layer ($PET_{(12)}/AL_{(7)}/PET_{(12)}/LLD_{(100)}$) , and 3-layer($PET_{(12)}/AL_{(7)}/LLD_{(100)}$) under modified atmosphere [16,17,21]. After packaging, samples were put in at ambient condition (T= 25 ^{0}C) immediately, for anaerobic bacteria count in different times (19, 33-36).

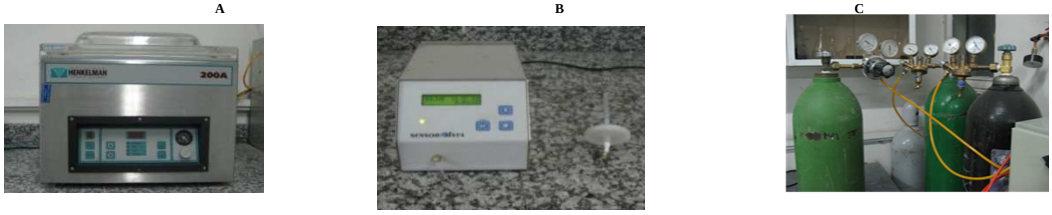

Fig 1.(A) Modified atmosphere packaging, (B) gas analyzer, (C) gas flash tank(Model: Boxer-200A) (19, 33-36)

Microbial Culture

Total count of anaerobic bacteria in PE 2 & BHI culture

BHI (Beef heart infusion solids 17.5 g/litre , Proteose peptone10.0 g/litre , Glucose2.0 g/litre , Sodium chloride5.0 g/litre , Di-sodium phosphate 2.5 g/litre , pH 7.4 ± 0.2 @25°C). BHI is a general media for anaerobic count.

PE 2(Peptone digest of animal extract 20 g/1000 ml, Yeast Extract 3g/1000 ml ,2%Alcoholic solution of bromocresol purple 0.04g/1000 ml,Cicer arietinum L450 no, Distillated water1000 ml) Peptone Yeast Extract Bromocresol Purple is an enrichment media for anaerobic bacteria(10,11,19,24-30,33-36).

First, 1 g sample under the hood of microorganisms in the laboratory was weighed and was crushed in 10 ml Ringer's solution, then PE 2 (10 ml) was added which was a culture for enrichment and with gas pack type A in anaerobic jar, for 3 days was incubated at 37 ° C, 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 double BHI culture ,too in order to count Anaerobic bacteria which was incubated for 4 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 [18, 19, 25]

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 anaerobic bacteria. Statistical analysis of data, was performed by software Statistical Analysis System (SAS : 9/1) with Anowa 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 anaerobic bacteria in different conditions

Analysis of variance (table 2) showed that the main factors between (layers, gas, time) of anaerobic bacteria had significant difference (P<0.01). The interaction of (gas × time) had also significant difference (P<0.01).

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

Total count of Anaerobic bacteria	Degrees of freedom	Variables
0.749**	2	**Layers**
1.889**	4	**gas composition**
0.0006^{ns}	8	**Layer*gas**
4.105**	3	**Time(days)**
0.0011^{ns}	6	**Layer* days**
0.146**	12	**gas*days**
0.001^{ns1}	24	**Layer*gas*days**
0.0008	120	**Errors**
3.429	-	**Variance Index (CV)**

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

Anaerobic bacteria	various films
3.704^{b}	Film1 3 layers(AL:12)
3.843^{a}	Film2 3 layers(AL:7)
3.613^{c}	Film3 4 layers

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

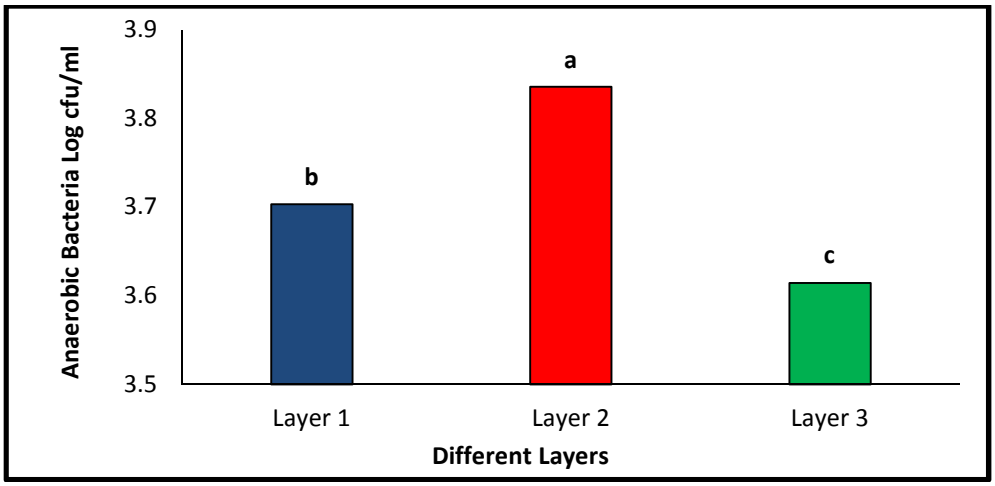

Fig 2-The effect of different layers on anaerobic count (log cfu/ml)

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

Anaerobic bacteria	various gases
3.390[d]	CO_2 70% +N_2 30%
3.805[b]	CO_2 30% + N_2 70%
3.572[c]	CO_2 45% + N_2 45%,+ 10% O_2
3.920[a]	vacuum
3.911[a]	control

Figure 3 and table 4 were shown, the effect of different gas compositions on anaerobic bacteria count of smoked kutum fish. Different gas composition were separated , e- CO2 70%,N2 30%{ red }; c-CO2 30%,N2 70%{ blue }; d - CO2 45%,N2 45%{green}; b-Vacuum{ pink }; a-control sample{ black }. The lowest amount of anaerobic bacteria belonged to gas combinations 1 (70% CO_2 + 30% N2), and then (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).

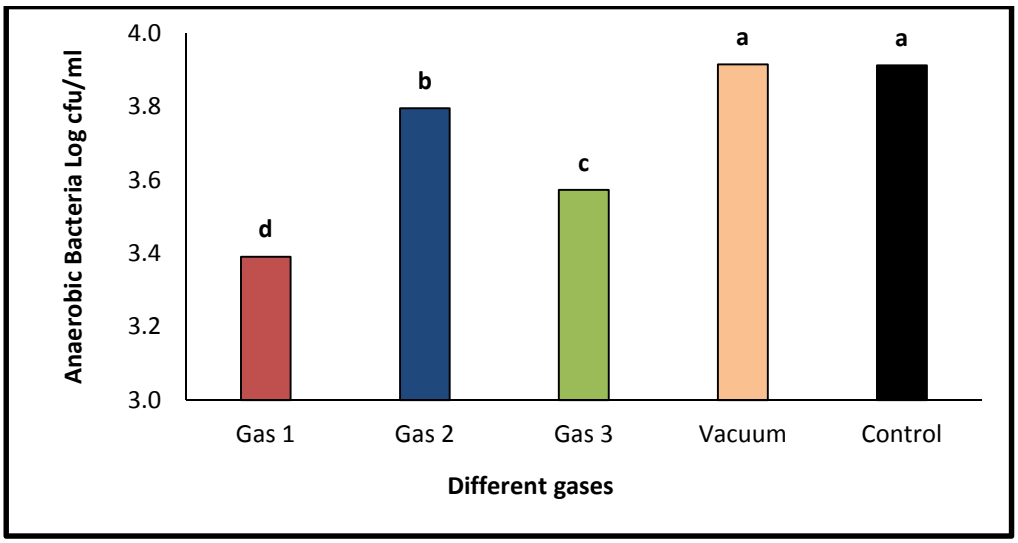

Fig 3-The effect of different gas compositions on anaerobic bacteria count (log cfu/ml)

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

Anaerobic bacteria	various days
3.411[d]	Day 15
3.532[c]	Day 30
3.863[b]	Day 45
4.081[a]	Day 60

Figure 4 and table 5 were shown, the effect of different days on anaerobic bacteria count of smoked kutum fish. Different days were separated in different color, day 15 {red}; day 30{blue}; day 45 {green}; day 60 {black}. the lowest amount of anaerobic bacteria in packed smoked kutum fish was observed after 15 days and highest after 60 days ,which caused bacteria increased rapidly by the times

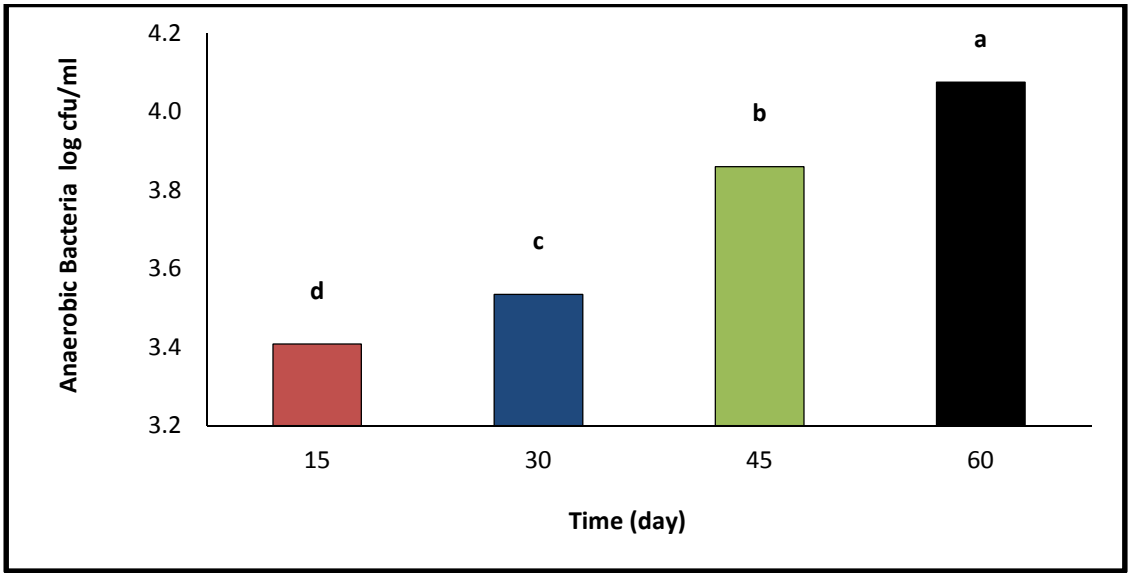

Fig 4-The effect of different days on anaerobic bacteria count (log cfu/ml)

According to figure 5, the effect of different gas compositions in different days on anaerobic bacteria count of smoked kutum fish were shown. Different gas compositions were separated in different color, CO2 70% , N2 30% { White }; CO2 30%,N2 70%{ red }; 45%CO2, 45% N2,10% O2{green}; vacuum{ Violet }; control sample{ blue }. The lowest amount of anaerobic bacteria count belonged to gas combinations (70% CO_2 + 30% N2), and then (45%CO2, 45% N2,10% O2) after 15 days but highest amount observed in ordinary condition and vacuum condition after 60 days.

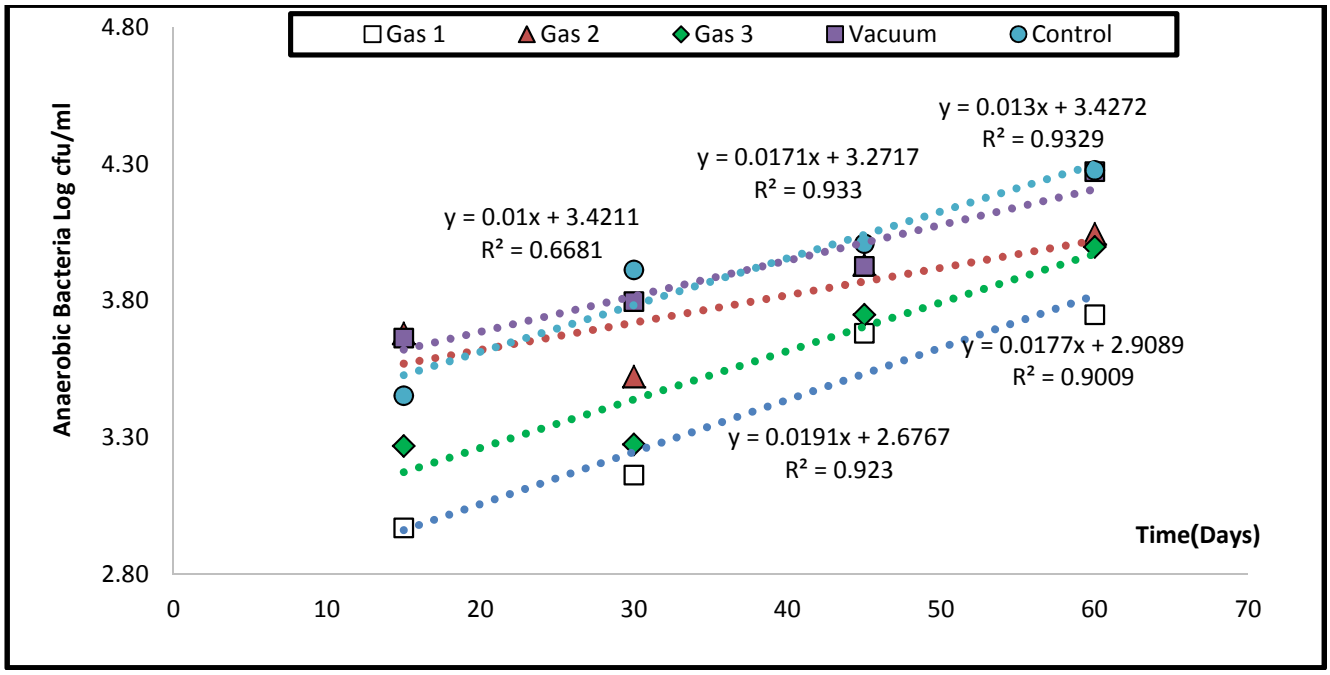

Fig 5-The effect of different gas combinations and different days on anaerobic bacteria count (log cfu/ml)

According to figure 6, the effect different gas combinations and different layers on anaerobic bacteria count of smoked kutum fish were shown. Different layer were separated in different color, {3-layers($PET_{(12)}$/$AL_{(12)}$/$LLD_{(100)}$)-blue,4-layers ($PET_{(12)}$/$AL_{(7)}$/$PET_{(12)}$/$LLD_{(100)}$)-green, and 3–layer ($PET_{(12)}$/$AL_{(7)}$/$LLD_{(100)}$)-red} The lowest amount of

anaerobic bacteria in packed smoked kutum fish belonged to layer:3(4-layers) under gas composition CO2 70%, highest amount belonged to layer:2(3-layers) under ordinary condition then vacuum condition.

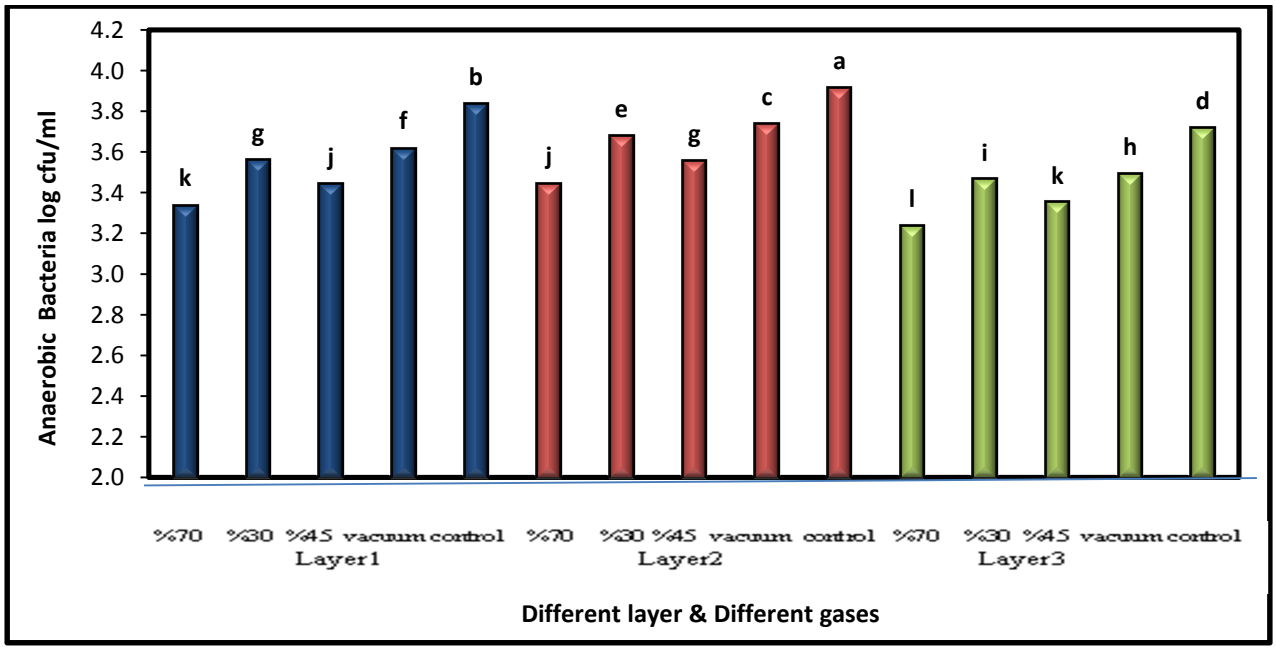

Fig 6-The effect of different gas combinations and different layers on anaerobic bacteria count(log cfu/ml)

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 anaerobic 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 anaerobic bacteria in packed smoked kutum fish belonged to layer:3 (4-layers) under gas composition CO2 70%,but highest amount belonged to layer :2(3-layers) under ordinary condition and then vacuum. Because of the thickness and of type gas atmosphere, which were prolonged the shelf life of smoked kutum fish till 60 days.

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

Nortje *et al*, 1989, due to research about the effect of againg treatment on the microbiology and storage characteristic of beef in modified atmosphere packs containing 25%CO2+75%O2 , the result were corresponded with these results. Blackistone, B.A. , 1998, due to research about principles and applications of Modified Atmosphere Packaging of different foods , the results on growth of anaerobic microorganisms , were similar to these results .Erkan *et al*., 2006 , indicated that due to effet of Modified Atmosphere Packaging on shelf life and growth of anaerobic bacteria in packed fresh sardines, the results were similar to 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 anaerobic 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, the results showed that the shelf life of ostrich meat under %60 CO2 prolonged till 7 days , the results on growth of anaerobic count were similar to these results. Zand & Allahyari , 2013, conducted due to the influence of MAP and different multilayer flexible films on shelf life extension of candy bread during 20 days , the results under CO2 %70 in 4-layer container ,were corresponded with these results.

Zand & Sotoudeh , 2013 , indicated that due to , effect of MAP and multilayer flexible pouch for shelf life prolongation of chicken meal , the results of anaerobic bacteria count ,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 was better than 3-layer during 20 days, best condition belonged to CO2 %70, that the results were similar to 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 during 60 days ,the results were corresponded with these results .Zand, 2013 , due to research about the shelf life prolongation of packed vegetables meal in multilayer flexible pouches 4-layer container was better than 3-layer during 60 days , the results were similar to this study.

CONCLUSION

The use of modified atmosphere packaging not lead to stop spoilage completely, but postponed it. The effect of MAP was not adequate but using this technique inactivated anaerobic microorganism of smoked kutum fish without a significant adverse effect on food properties. In the present study, it was concluded that, anaerobic microorganism of packed smoked kutum fish have been affected by different flexible multi-layer pouches and different concentrations of three gas mixture (carbon dioxide, nitrogen, oxygen), and also vacuum conditions during 60 days. Our results confirmed, substitution of MAP and these barrier containers instead of traditional packaging in seafood packaging industries, due to a lot of privilege of them for shelf life prolongation of smoked fish in long times.

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