Available online at **www.ijmrhs.com**

ISSN No: 2319-5886

International Journal of Medical Research & Health Sciences, 2016, 5, 11:191-198

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

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ABSTRACT

In this study the effect of different concentrations of three gas mixture (carbon dioxide, nitrogen, oxygen), and also vacuum conditions and flexible multi-layer films were evaluated on Clostridium count of smoked kutum fish (Rutilus frisii kutum) at ambient condition (T= 25 ^{0}C). Ordinary condition as control packaging were compared with four types of modified atmosphere packaging: ($N_2$70%+ $CO_2$30%), ($N_2$30% + $CO_2$70%), (45%CO2+ 45%N2+10%O2) and vacuum conditions, in this project. Smoked kutum fish were packaged into 3 kinds of flexible pouches {3-layers($PET_{(12)}/AL_{(12)}/LLD_{(100)}$), 4-layers ($PET_{(12)}/AL_{(7)}/PET_{(12)}/LLD_{(100)}$), and 3-layer ($PET_{(12)}/AL_{(7)}/LLD_{(100)}$)}. Packed samples were performed microbial tests (Clostridium 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 shelf life of Samples (according to Clostridium count) were reported in 4-layers , under conditions 1,2,3 and vacuum conditions, 60,58,45,40 days, in 3-layers (AL:12), under conditions 1,2,3 were 55,50,40 days and in vacuum conditions were about 35 days, with 3-layers(AL:7), under conditions 1,2,3 and vacuum conditions 45,40,35, 30 days. Clostridium count showed that increasing CO2 concentration prolonged shelf life. During the period of this experiment Clostridium count of samples in various conditions, had significant level. According to these results could be concluded the best condition belonged to treatment under modified atmosphere CO2 70% and also 4- layer container due to the thickness (131 µ), low permeability of water vapor in this 4-layer container and anti-microbial effect of more percentage of CO2.

Keywords: Modified atmosphere packaging (MAP), flexible multi-layer films, smoked kutum fish (*Rutilus frisii kutum),* Clostridium

INTRODUCTION

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 [4,9]. 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) [12], cold method have already been used as economically efficiency , and 30% weight loss in initial products . 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 [5,6,20,21]. However there is no degradation of flavor and taste with heat denaturation of objectives [13,14]. The ability of modified-atmosphere packaging for extending the shelf life of foods has been recognized for many years, over 100 years ago [1,5,20]. 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 [6-8,19,33-36]. MAP inhibits some microorganisms, so can increase the quality of variety foods. Smoked kutum fish without an efficient

processing is a potential source of pathogenic microorganisms, since this acidity (pH 5-6) and suitable water activity in cold smoke type of this fish, creates an ideal environment for rapid microbial spoilage in this package [13,14,19,33-36]. Although, thermal treatment effectively destroys these microorganisms [5,12,24-30], 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 [1-3,19,33-36]. For that reason, significant efforts are leading to the development of novel processing such as MAP [6-8,19,33-36], which is proving to be able to inactivate spoilage microorganisms without significantly affect nutritional and sensory properties of several foods [2,3]. 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 [20,21,19,33-36], 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 [19,33-36]. Nitrogen (N2) is a non-reactive gas that has no smell or taste, unlike carbon dioxide, is not absorbed in food or water [15]. 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 growth of anaerobic bacteria [5,21,33-36]. Clostridium 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 [18,15].Other hand the multi-layer films have been used for packaging these products 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 [17,18,22-24,31,32]. 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 [16,17,19,33-36] ,and aluminum foil [19,22-24,31,32]. In this study, we investigate about the effects of modified atmosphere packaging; different concentrations of CO2/N2/O2; Clostridium count (4), and the usage of three types of multilayer flexible pouch on smoked kutum fish during storage times (16,17,19,33-36). Smoked kutum fish due to high amount of microorganism especially Clostridium, which led to cause poisoning and even death of people. For this reason, we want to prove different gas mixture and vacuum in multilayer flexible pouch can substitute thermal processing in conservation industries in order to control the growth of Clostridium [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 [16,17,21]. After packaging, samples were put in at ambient condition (T= 25 ^{0}C) immediately, for Clostridium count during 60 days [19, 33-36].

A **B** **C**

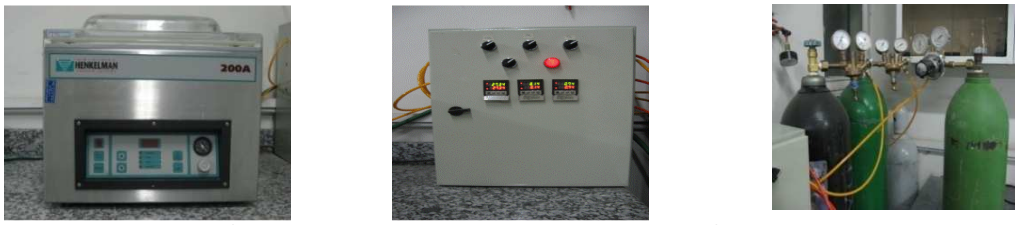

Fig 1.(A) Modified atmosphere packaging, (B) gas analyzer, (C) gas flash tank(Model: Boxer-200A) (19, 33-36).

Microbial culture

Total Clostridium count in RCM & PE 2 media.

RCM (Peptone from casein 10g/1000 ml; Meat Extract 10g/1000, Yeast Extract 3g/1000 ml, Starch 1g/1000 ml, glucose 5 g/1000 ml,l- cystein hydrochloride0.5g/1000 ml, Sodium acetate 3g/1000 ml, Sodium chloride 5 g/1000

ml, Agar12.5g/1000 ml, Distillated water 1000 ml)Rein Clostridia is a culture media for clostridium 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 RCM culture ,too in order to count Clostridium 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 (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 Clostridium. 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 Clostridium count in different conditions
Analysis of variance (table 2) showed that the main factors between (layers, gas, time) had significant difference (P<0.01). The interaction of (gas × time) had significant difference (P<0.01) , and also the effect of (layers ×gas) had significant difference (P<0.05).

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

Clostridium	Degrees of freedom	Variables
0.706**	2	**Layers**
1.220*	4	**gas composition**
0.0014*	8	**Layer*gas**
6.869^{ns}	3	**Time(days)**
0.0006^{ns}	6	**Layer* days**
0.16*	12	**gas*days**
0.0006^{ns}	24	**Layer*gas*days**
0.0006	120	**Errors**
2.896	-	**(CV) Variance Index**

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

Clostridium	various films
2.561^{b}	Film1 3-layers(AL:12)
2.670^{a}	Film 2 3-layers(AL:7)
2.452^{c}	Film 3 4-layers

Figure 2, and table 3 were shown, the effect of different layers on Clostridium count of smoked kutum fish. Different layers were separated in different color, 1 {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 Clostridium count of smoked kutum fish belonged to layers 3 (4-layers), and then layers 1(3-layers) , due to the thickness (131 µ), low permeability of water vapor in this container.

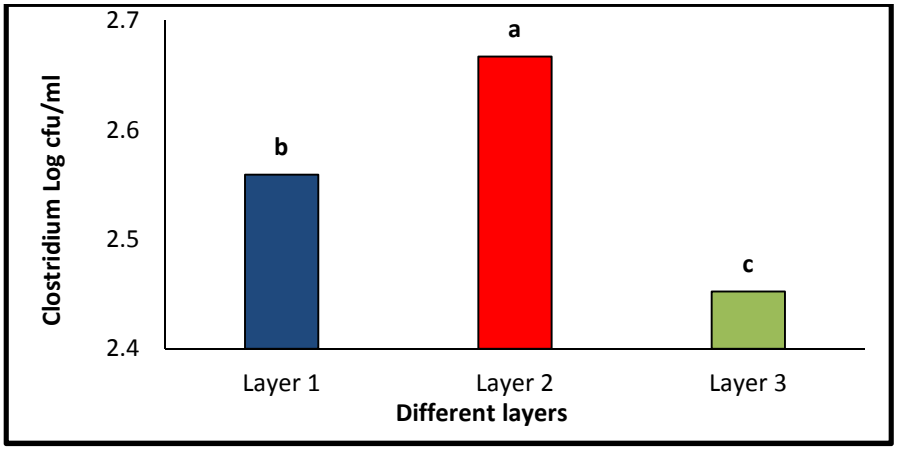

Fig 2-The effect of different layers on Clostridium count (log cfu/ml)

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

Clostridium	various gases
2.340[e]	CO_2 70% +N_2 30%
2.573[c]	CO_2 30% + N_2 70%
2.451[d]	CO_2 45% + N_2 45%,+ 10% O_2
2.624[b]	vacuum
2.820[a]	control

Figure 3 and table 4 were shown, the effect of different gas compositions on Clostridium count of smoked kutum fish. Different gas composition were separated in different color ,(CO2 70%,N2 30%){ red }; (CO2 30%,N2 70%){ blue }; (CO2 45%,N2 45%,O2 10%){green}; vacuum{pink}; control as ordinary condition{ black}. The lowest amount of Clostridium count belonged to gas combination 1 (70% Co_2 + 30% N2) and (45%CO2+ 45% N2+ 10% O2) too, and highest amount observed in ordinary condition because of gas atmosphere, and antibacterial properties of carbon dioxide gas.

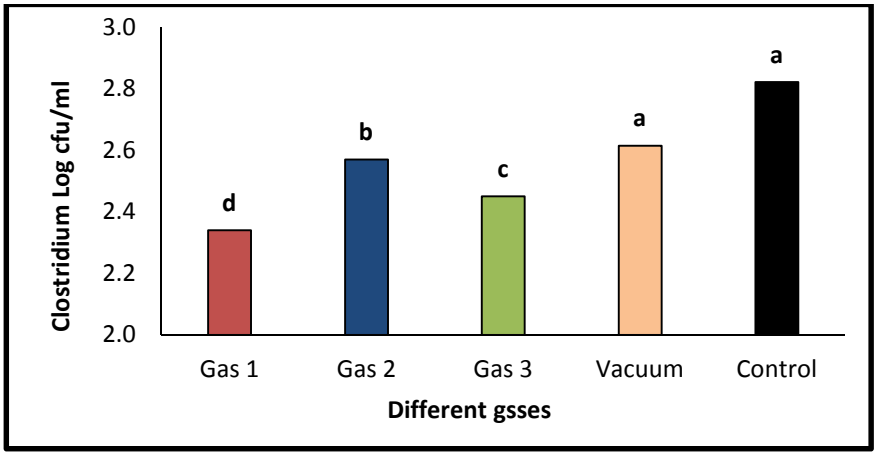

Fig 3-The effect of different gas compositions on Clostridium count (log cfu/ml)

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

Clostridium	various days
2.030[d]	Day 15
2.521[c]	Day 30
2.731[b]	Day 45
2.950[a]	Day 60

Figure 4 and table 5 were shown, the effect of different days on Clostridium 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 Clostridium count of smoked kutum fish was reported after 15 days and highest after 60 days ,which caused to grow Clostridium by the times rapidly.

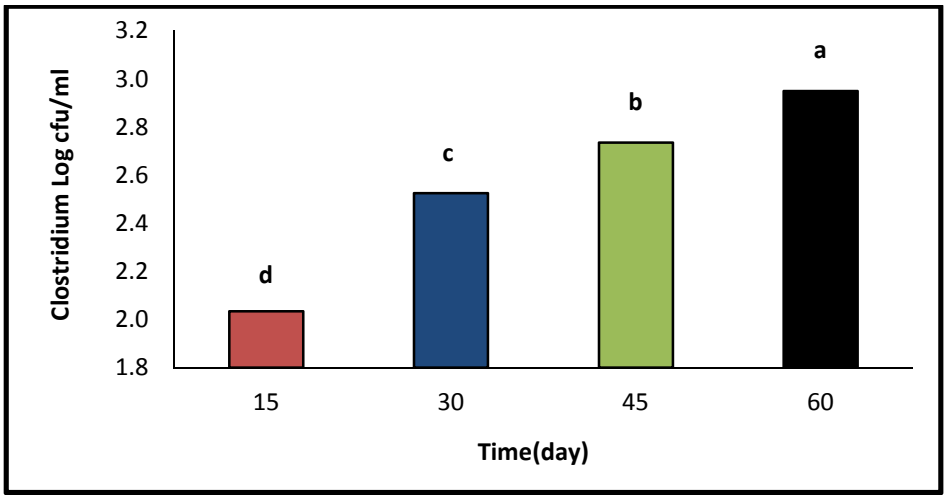

Fig 4-The effect of different days on Clostridium count (log cfu/ml)

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

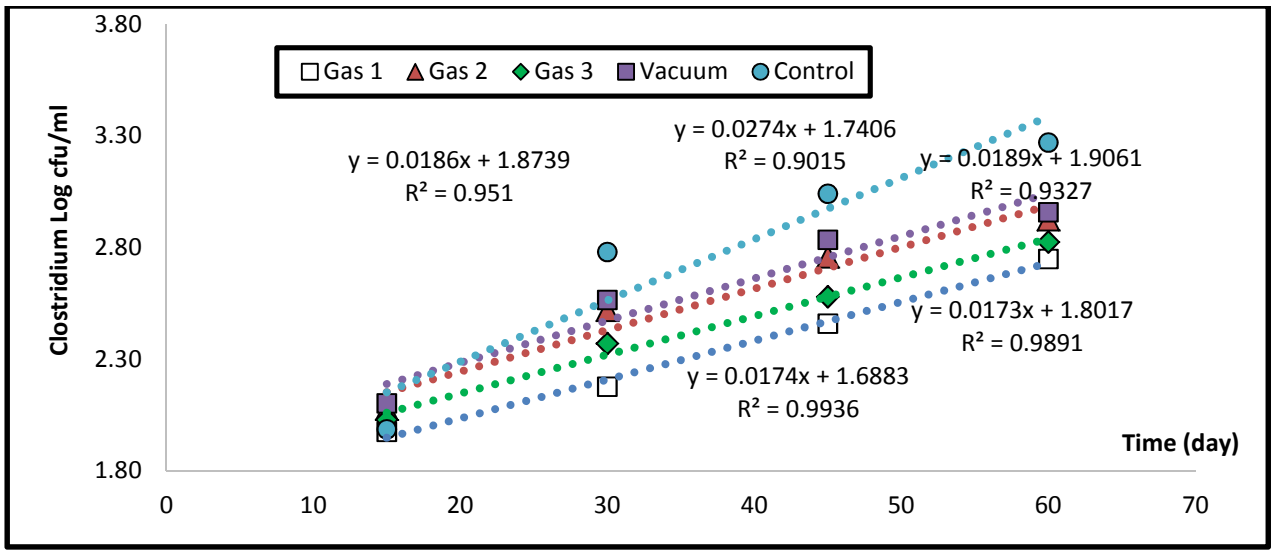

Fig 5-The effect different gas combinations and different days on Clostridium count (log cfu/ml)

According to Figure 6, the effect of gas combinations and different layers on Clostridium 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 smoked kutum fish belonged to layers:3(4-layers) under gas composition CO2 70%, but highest amount belonged to layers: 2(3-layers) under ordinary condition and also vacuum.

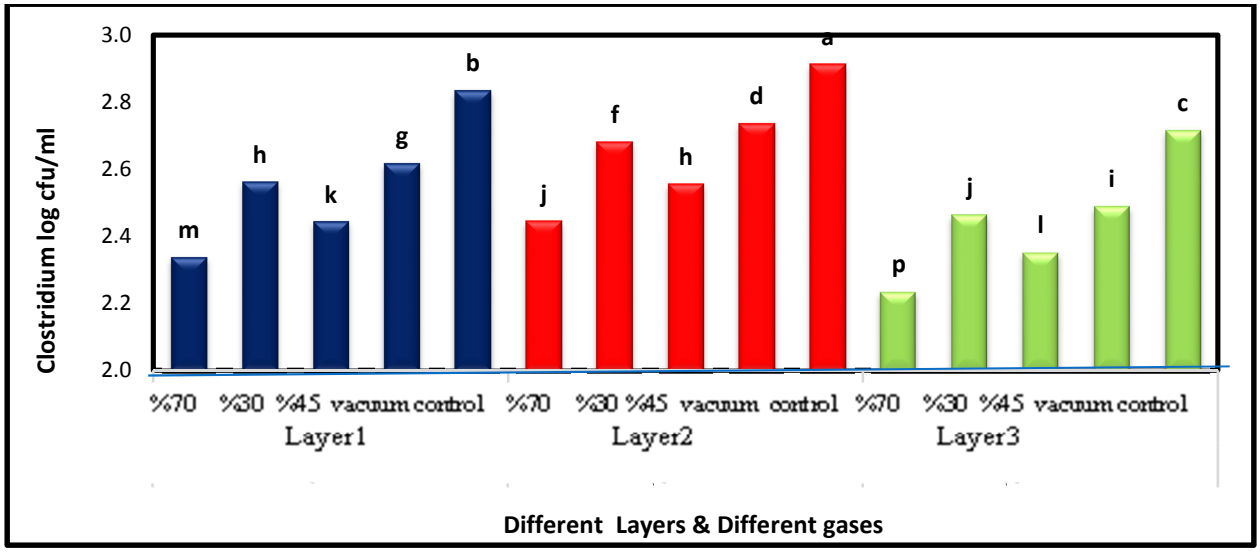

Fig 6-The effect of different gas combinations and different layers on Clostridium count (log cfu/ml)

DISCUSSION

1-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 produces 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 such as clostridium. The second role of nitrogen in modified atmosphere packaging, was a gas filler and protecting the development of flexible packaging against vacuum. The shelf life of smoked kutum fish (according to Clostridium count) were reported in 4-layers , under conditions (70% CO2 ,30% N2) & (45%CO2,45%N2,10%O2) & (30% CO2,70% N2) 60,58,45 days and in vacuum conditions were 40 days, in 3-layers(AL:12) , under conditions (70% CO2 ,30% N2)& (45%CO2,45%N2,10%O2) & (30% CO2 ,70% N2) 55,50,40 days and in vacuum conditions were about 35 days, with 3-layers(AL:7),under conditions(70% CO2 ,30% N2)& (45%CO2,45%N2,10%O2) & (30% CO2 , 70% N2) 45,40,35 days and in vacuum conditions were 30 days. As you see the best conditin belonged to samples in 4-layer packing , under (70% Co_2) because of the thickness and of type gas atmosphere, which were prolonged the shelf life of smoked kutum fish till 60 days. The population of clostridium under condition above were acceptable.

2- Clostridium count of samples in various conditions, had significant level between layers, gas and time ($P<0.01$), and there was significant level between treatments gas and time ($P<0.01$), and also had significant level between gas and layers ($P<0.05$).

Cai *et al*.,1997 , due to research about toxin prodaction of clostridium botulinum type E in packed catfish under 80% CO 2+ 20% N2, the results of clostridium count, were corresponded with these results. Blackistone, B.A., 1998, due to principles and applications of MAP in different foods, the results were corresponded with these results .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 results of microorganism count, were similar to these results. Erkan *et al*., 2006, indicated that due to effet of Modified Atmosphere Packaging on growth of anaerobic bacteria in packed fresh minced meat, the results were similar to these results. Athina *et al*., 2008, conducted due to formation of biogenic amines and relation to microbial flora and sensory changes in smoked turkey breast fillets stored under various packaging conditions , the results were similar to these results. Zand & Allahyari, 2013, due to research about the influence of MAP and different multilayer flexible films on shelf life extension of candy bread in 3 week days, the results of clostridium count under count 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 Clostridium count, were similar to these results.

Sotoudeh *et al*, 2013, conducted 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,and best condition belonged to CO2 %70, the results, were corresponded with these results. 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 similar to 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, results were corresponded with these results.

CONCLUSION

Our results confirmed, the modified atmosphere packaging (MAP) was not lead to stop spoilage completely but delayed it .The effect of MAP was not adequate, but using this technique inactivated microorganism without a significant adverse effect on clostridium growth of fish samples. In the present study, it was concluded that, the amount of Clostridium and shelf life of packed smoked kutum fish (*Rutilus Frisii Kutum)* have been affected by different flexible multi-layer containers and different concentrations of three gas mixture (carbon dioxide, nitrogen, oxygen), and also vacuum conditions during 60 days. These parameters could be promoted, substitution of MAP and these barrier containers instead of traditional packaging in seafood packaging, due to a lot of privilege of them for shelf life extension of smoked fish in long times.

Acknowledgements
The authors appreciate, Dr. Bijan khorshidpour (Department of Food Science and Technology, Faculty of Agriculture, Varamin-Pishva Branch, Islamic Azad University, Varamin, Iran), Eng. Hamid Reza Taheri and PMA Com (Plastic Machine Alvan, Tehran-Iran) for supplying equipment and materials.

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