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Analysis of the Microbial Load and the Amount of Acryl Amide in Heated Sausages Using Microbiological Methods and HPLC Chromatography in Gilan Province of Iran

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Abstract: Due to having meat protein and carbohydrates sausages are suitable environment for the growth of microorganisms. Many studies have been conducted on reducing the microbial load. Also acryl amide is a toxic and carcinogenic chemical produced in sausages heated >100-120°C. The present research studied the presence of some indicator activated pathogenic microorganisms with the ability to withstand cooking temperatures and the production of acrylamide. The aim of the study was to measure microbial contamination as well as acrylamide in sausage comparing with the standard rate and the aggravating factors. Sausage samples preparation and dilution (10^{-1}-10^{-5}) was conducted using maximum recoveri diluent to evaluate microbiological tests and to count various microorganisms. To prepare and measuring acrylamide, degreasing by Sox let extraction apparatus, sonickitation by ultrasonic lab device, condensation byvacuum distillation and chromatography was performed by HPLC. Sausage samples after the thermal process was collected randomly from food shops and fast food restaurants in the city of Rasht. Colonies of growth medium of microorganisms were counted and growth charts were plotted. Sausage microbial contamination was significantly higher than the permissible contamination ($p<0.05$) and growth of bacteria also significantly showed higher than the standard ($p<0.05$). The charts and the chromatograms for acryl amid production in micrograms per kilogram (ppb) also demonstrated an over production of the toxic and dangerous to human health. The maximum production rate of acrylamide was in fried, grilled and cooked sausages, respectively. It should be noted that the amount of acrylamide in sausages differed in different brands, because of the different amount of carbohydrates in their production.

Key words: Sausage, microbial contamination, acrylamid, chromatograms, demonstrated

INTRODUCTION

Sausage is a staple food, especially by the younger generation. Due to changing lifestyles and the growing consumption of fast foods in the community, it is extremely important to analyze the sausage in terms of materials used in its production and in particular to determine the microbial load and harmful, toxins and carcinogens substances in it. In terms of raw materials, types of sausages are qualitatively different (Boughton *et al*., 2004). The most important ingredients of sausages are minced meat (40-90%), salt (for flavoring and as preservative), spices, nitrite and its derivatives (antibacterial, preservative and antioxidant property), powdered milk, flour, starch, soy protein, bread crumbs, potato flour, rice, liquid oil, sugar, ascorbic acid, natural and artificial veneer, etc (Boughton *et al*., 2004) Considering the sausage ingredients, the growth of microorganisms such as Staphylococcus, psychrophilic bacteria, coliforms (specially *E. coli*), Salmonella, *Clostridium perfringens*, mold and yeast, before and after the cooking process is important and should not be greater than the allowable standard. Especially in a product that is ready to use and has passed thermal processing (frying, grilling or baking). Adams and Moss (2011) on the other hand the raw material ingredients of sausage like bread crumbs are rich in amino acids as asparagine. Maillard reaction (non-enzymatic browning reaction) occurs at temperatures above 100°C between certain free amino acids (e.g., asparagine) with glycosidic hydroxyl group of reducing sugar (such as dextrose, glucose and fructose) or carbonyl compounds (such as formaldehyde and ketones that arise as a result of lipid oxidation) (Tareke *et al*., 2002). This reaction as well as changes resulting from it, are responsible to degradation of nutrients, reducing its nutritional quality, destruction of essential amino acids, the decline in digestibility and most importantly to produce toxic compounds such as acrylamide (which is classified in the group of carcinogenic compounds that at high levels can damage the immune system) (Muttucumaru *et al*., 2006). Its risk is crucial because acrylamide-containing foods is a major contribution to people's daily food basket (Becalski *et al*., 2003).

The aim of the study: To analyze the microbial load and chemical contamination (the amount of acryl amide) in heated or ready-cooked sausages collected from restaurants in Rasht.

MATERIALS AND METHODS

In this study, twenty different samples of sausage were obtained and analyzed. MRD of QLAB Company used for diluting, PCA, EMB AGAR, RVS, XLD, TSC, PDA culture medias of MERCK company from Germany and MSA, BPW of SCHARL Company from Spain applied to count and check different colonies of bacteria: also HPLC chromatography equipment of WATMAN from Germany and SIGMA from America was prepared and used.

Sausage samples preparation for microbiological tests: For preparing MRD (Maximum Recoveri Diolent) medium, after resolving 9 g of MRD in 1 L of distilled water the erlenmeyer flask containing it was placed in autoclave (121°C for) 15 min. After cooling, the samplesdiluted up by MRD. To assess the microbial contamination load, all sausage samples were cut into pieces weighing 10 g and dilutions of 10^{-1}-10^{-5} wereprepared using MRD. (Merck Group, 2010).

To examine and total plate count of bacteria in samples of sausages: The total plate count of bacteria was monitored using PCA (Plate Count Agar) culture medium. After passing the required time from exposure to flame under the laboratory fume hood, two plates of each 5 dilution (10 plates for each sample) were cultured in a linear fashion. Plates were then transferred to an incubator 37°C and the grown colonies were counted after 24 h (Merck Group, 2010).

To examine and counting of psychrophilic bacteria in samples: For counting psychrophilic bacteria, culturing was done for dilutions of 1-3 and of each dilution in two plates containing culture medium PCA. Then samples were placed in the refrigerator 4°C for 48 h and then colonies were counted (Merck Group, 2010).

Review and counting staphylococcus bacteria in sausages samples: MSA (Manitol Salt Agar) medium was used to examine the staphylococcus. Exposure to flame and under the laboratory fume hood culturing was done for dilutions of 1-3 and of each dilution in two plates. Plates were then transferred to an incubator 37°C and after 48 h the necessary investigations took place (Merck Group, 2010).

Detecting coagulase-positive staphylococci in samples of sausages: After 48 h, sampling was made of the yellow colonies with bright halo around them, by sterile loop and they were cultured in nutrient broth and incubated for 24 h was at 37°C. After 24 h 1cc of rabbit serum mixed with 4cc sterile physiological saline and 500 Landa of this mixture was poured into a small test tube. Then 100 Landa of bacteria grown in nutrient broth medium (opaque) added into the test tube containing serum, mixed and incubated 37°C. Every half hour, tubes were monitored for Coagulase test and evaluate and at half hourly intervals for a period of twenty four hoursevery sample clotted was reported aspositive (Merck Group, 2010).

To examine and counting coliforms in samples: To Count the coliforms (especially Escherichia coli), EMB AGAR medium was used.Diluted samples 1-5 and of each dilution in two platescontaining the above mentioned culture 100 Lambda poured and cultured by spreader. The plates incubated at 37°C and colonies including metallic luster were counted after 20 h (Merck Group, 2010).

Checking and counting of Salmonella in samples of sausages: To measure Salmonella, 25 g of sausage chopped and weight in a sterile condition, added to 225 mL of sterile medium (Buffered peptone water) BPV inside the flask and incubated 37°C for 24 h. Then 100 lambda of each medium (containing sausages samples) cultured in two tubs containing RVS (Rappaport and Vassiliadis broth) both incubated 37 and 41.5°C for 24 h, respectively. After 24 h, XLD medium was used to continue the examination and incubated 37°C for 24 h (Merck Group, 2010).

Review and counting *Clostridium perfringens* sausage samples: *Clostridium perfringens* bacteria forms spores so must activate spores to vegetative state. Flask containing Sausage sample dissolved in 90 mL of MRD, heated in bain marie up to 80°C then rapidly cooled (this process was repeated three times), so if the sausage samples containing spore bacteria, the spores transform to actively growing. Then 100 lambdas of the dilutions of 1-3 (of any dilution in two plates) cultured in TSC (Tryptose Sulfite Cyclosrine agar base) medium by the sampler exposure to flame by glass rod in a linear fashion. *Clostridium perfringens* bacteria are anaerobic. So, the plates put into anaerobic jar and a candle was lit (the candle consumed oxygen inside the jar and turned it off) and jar doors tightly closed with gauze Pack A of the Merck company. Anaerobic jar containing plates incubated 37°C for 48 h and colonies were then checked and counted (Merck Group, 2010).

Detecting mold and yeast in samples of sausages: The 100 Lambda of five dilution sausages (of 10^{-1}-10^{-5}) (two plates for every dilution) were cultured in PDA (Potato Dextrose Agar) in a linear fashion. Plates were incubated 30°C and after 24 h colonies were counted for review yeasts. To investigate the molds, plates incubated 30°C for 5 days and then mold colonies were counted (Merck Group, 2010).

The method of preparation and measurement of the amount of acrylamide in samples of sausages: 10 grams of very finely chopped sausage pieces were packed inash less filter study with cotton thread. Then, it degreased (defatted) for 3 h with 25 mL of hexane in sox let apparatus. Then hexane phase was discarded and sample sausages moved into a beaker. After adding 30 mL dichloromethane were sonicated for 4 min in a bain marie ultrasonic device manufactured by TECHNO GAS (Italy) with a frequency of 28.6 kHz and were soxlet again for 2 h. Dichloromethane phase in a vacuum distillation (rotary evaporator) concentrated. The vacuum distillation device was manufactured by IKA, Germany. Then the sample was transferred to a beaker and the solvent dichloromethane gently evaporates. The remaining sample resolved in 1 mL of methanol and the solution passed through the filter C18 (with a diameter of 1 cm). The 20 μL were injected to HPLC (High Performance Liquid Chromatography) device (with carrier phase speed of 1 mL min^{-1}, detection wavelength 210 nm, Column ODS 5 μm, carrier phase include: methanol 60% (CH_3OH, acetonitrile 38% (CH_3CN), water 2% (H_2O)). HPLC device used in these experiments was manufactured by AJILLENT, USA. Quality and quantity measuring were done by standard adding (some basic standard was added to the initial sausages sample, so that standard concentrations were equal to 50 ppb and after all the mentioned steps acrylamide levels were measured along with recording the chromatographs).

RESULTS AND DISCUSSION

Counting the colonies of microorganisms: Sausage samples after preparation and cultivation were evaluated in terms of growth of microorganisms, diagrams and the following results were obtained.

The growth and total plate count of bacteria in samples of sausage: Figure 1 shows the overall growth of bacteria in examined sausage samples. As can be seen, the growths of bacteria in the studied samples were higher than the standard.

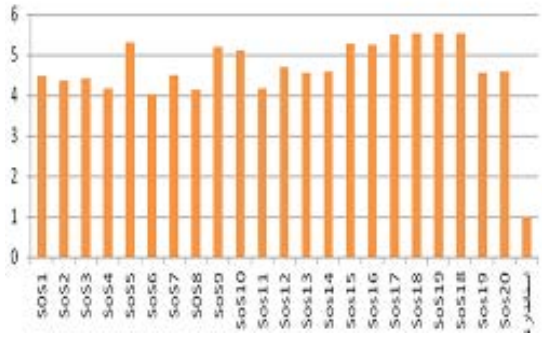

Fig. 1: Overall growths of bacteria in sausage samples. Vertical columns:logarithm of values obtained; horizontal columns: samples

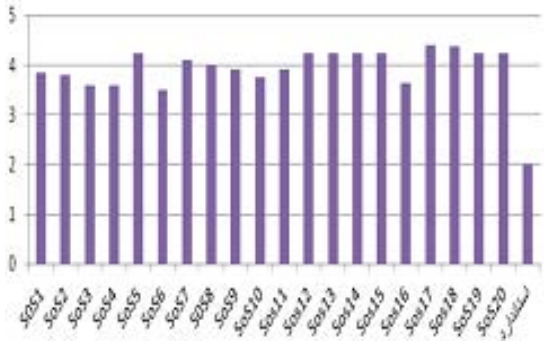

Fig. 2: Overall growths of psychrophilic bacteria in sausage samples. Vertical columns: logarithm of values obtained; horizontal columns: samples

The growth and counting of bacteria in samples: Figure 2 shows psychrophilicbacteria intested sausage samples. According to Fig. 2, the growths of these bacteria in the samples were 100% higher than the standard.

The growth and counting staphylococcus bacteria in sausages samples: Figure 3 shows the growth of Staphylococcus in tested sausage samples. Based on the chart the growth of Staphylococcus in 95% cases were higher than the standard.

The growth and counting of coliforms in samples of sausages: Figure 4 shows the growth of coli forms in tested sausage samples. As can be seen, in all samples coli forms have been growing more than the standard.

The growth and counting of *E. coli* in samples of sausages: Figure 5 shows the growth of *E. coli* in tested sausage samples. According to the chart, in all samples growths of *E. coli* exceed the standard.

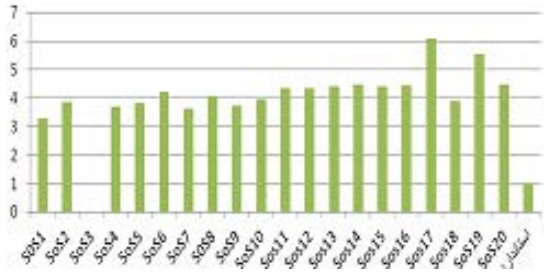

Fig. 3: Growth of staphylococciin sausage samples. Vertical columns: logarithm of values obtained; horizontal columns: samples

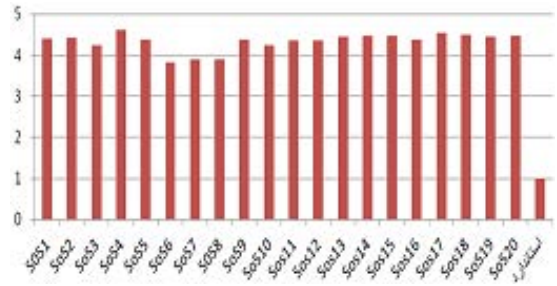

Fig. 4: Growth of coliforms in sausage samples. Vertical columns: logarithm of values obtained; horizontal columns: samples

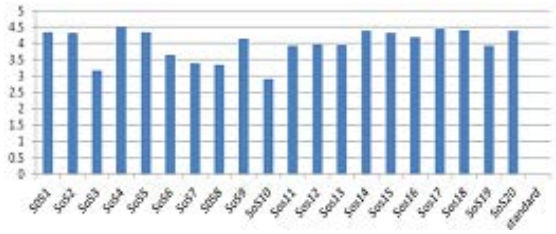

Fig. 5: Growth of *E. coli* in sausage samples. Vertical columns: logarithm of values obtained, horizontal columns: samples

The growth and counting of yeast in samples of sausages: Figure 6 shows the growth of yeast in tested sausage samples. As it is seen the yeast growth in the studied sample was higher than the standard allowed level.

The growth and counting of mold in samples of sausages: Figure 7 shows the growth of mold in tested sausage samples. Based on the chart the growth of molds in 20% of cases were higher than the standard.

Table oftotal counting colonies of microorganisms grown in culture media: Sausage samples obtained from

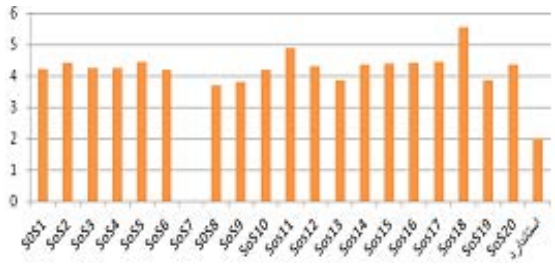

Fig. 6: Growth of yeast in sausage samples. Vertical columns: logarithm of values obtained; horizontal columns: samples

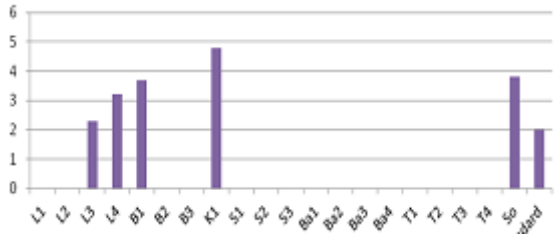

Fig. 7: Growth of mold in sausage samples. Vertical columns: logarithm of values obtained; horizontal columns: samples

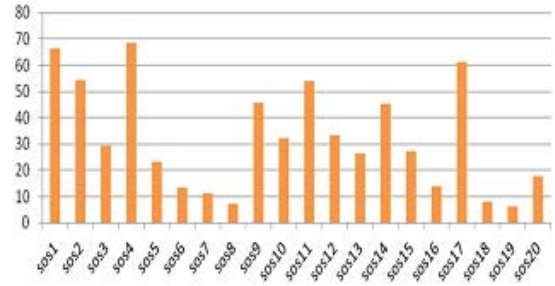

Fig. 8: The amount of acrylamide in sausage samples examined in terms of micrograms per kilogram (ppb). Vertical columns: acrylamide; Horizontal columns: samples

different restaurants or supermarkets after preparation and cultivation were evaluated in terms of growth of microorganisms and the grown colonies were counted.According to the above descriptions Table 1 was drawn. Table 1 shows high pollution in sausages. These pollutionsare more than the allowable amount. This infection can be due to the contaminated sausage products on the one hand (not gone even after frying) and on the other hand be related to the polluted equipment of restaurants or workers hands. It is clear that sandwiches prepared in various restaurants are far more

Table 1: The growth of pathogens in examined sausage samples cfu/gr

Coagulase-positive	Staphylococcus	Mold	Yeast	Klstrdyvm Prfrjnz	Salmonella	*E. coli*	Cold-loving Coliforms	Total bacterial bacteria	Bacteria count	Samples
-	$1/9\times10^3$	-	$1/7\times10^4$	-	-	$2/2\times10^4$	$2/6\times10^4$	$7/2\times10^3$	3×10^4	S0S1
+	$7/6\times10^3$	-	$2/6\times10^4$	-	-	$2/1\times10^4$	$2/7\times10^4$	$6/5\times10^3$	$2/3\times10^4$	SoS2
-	-	-	$1/8\times10^4$	-	-	$1/5\times10^3$	$1/7\times10^4$	4×10^3	$2/7\times10^4$	SoS3
+	$5/1\times10^3$		$1/8\times10^4$	-	-	$3/3\times10^4$	$3/9\times10^4$	$3/9\times10^3$	$1/5\times10^4$	SoS4
+	$6/9\times10^3$	-	$2/9\times10^4$	-	-	$2/2\times10^4$	$2/4\times10^4$	$1/7\times10^4$	$2/1\times10^5$	SoS5
+	$1/7\times10^4$	-	$1/6\times10^4$	-	-	$4/5\times10^3$	$6/7\times10^3$	$3/2\times10^3$	$10/7\times10^3$	SoS6
-	$43/5\times10^2$	-	-	-	-	$25/5*10^2$	$79/5\times10^2$	$1/3\times10^4$	$3/2\times10^4$	SoS7
+	$1/2\times10^4$	-	$5/1\times10^3$	-	-	$22/5\times10^2$	$79/5\times10^2$	1×10^4	$1/4\times10^4$	S0S8
-	$5/5\times10^3$	-	$66/7\times10^2$	-	-	$1/4\times10^4$	$2/4\times10^4$	$8/5\times10^3$	$1/6\times10^5$	SoS9
-	$9/1\times10^3$	-	$16/3\times10^3$	-	-	$8/5\times10^2$	$1/8\times10^4$	$5/8\times10^3$	$1/3\times10^5$	SoS10
-	$2/2\times10^4$	-	$7/6\times10^4$	-	-	$8/7\times10^3$	$2/3\times10^4$	$8/5\times10^3$	$14/6\times10^3$	Sos11
-	$22/9\times10^3$	-	2×10^4	-	-	$9/6\times10^3$	$2/3\times10^4$	$1/7\times10^4$	$4/9\times10^4$	Sos12
+	$2/6\times10^4$	-	$76/5\times10^2$	-	-	$8/9\times10^3$	$2/8\times10^4$	$17/5\times10^3$	$3/8\times10^4$	Sos13
+	3×10^4	$1/5\times10^3$	$2/3\times10^4$	-	-	$2/4\times10^4$	3×10^4	17×10^3	$3/9\times10^4$	Sos14
-	$2/6\times10^4$	-	$25/5\times10^3$	-	-	$2/1\times10^4$	$2/9\times10^4$	$1/7\times10^4$	$1/9\times10^5$	Sos15
-	$2/8\times10^4$	-	$2/6\times10^4$	-	-	$1/6\times10^4$	$2/4\times10^4$	$44/5\times10^2$	$1/8\times10^5$	Sos16
+	$1/3\times10^6$	-	$2/9\times10^4$	-	-	$2/9\times10^4$	$3/5\times10^4$	$2/5\times10^4$	$3/1\times10^5$	Sos17
+	$1/3\times10^5$	-	$2/9\times10^5$	-	-	$2/5\times10^4$	$3/1\times10^4$	$2/4\times10^4$	$3/4\times10^5$	Sos18
+	8×10^3	$2/9\times10^3$	$3/6\times10^5$	-	-	$2/5\times10^4$	3×10^4	$2/4\times10^4$	$3/4\times10^5$	SoS19
+	$3/6\times10^5$	8×10^4	$3/6\times10^5$	-	-	$2/6\times10^4$	$3/5\times10^4$	$2/8\times10^4$	$3/5\times10^5$	SoS18
+	$2/6\times10^4$	-	$76/5\times10^2$	-	-	$8/9\times10^3$	$2/8\times10^4$	$17/5\times10^3$	$3/8\times10^4$	Sos19
+	3×10^4	$1/5\times10^3$	$2/3\times10^4$	-	-	$2/4\times10^4$	3×10^4	17×10^3	$3/9\times10^4$	Sos20
-	aEN CO 10	100	100	50	-	-	10	100	10	COECaICNI

Table 2: Test result

The amount of acrylamide in processed samples mean of three replicates	Sample
66.4 ppb	Sos1
54.3 ppb	Sos 2
29.4 ppb	Sos3
68.4 ppb	Sos4
23.3 ppb	Sos5
13.3 ppb	Sos6
11.2 ppb	Sos7
7.2 ppb	Sos8
45.5 ppb	Sos9
32.3 ppb	Sos10
54.1 ppb	Sos11
33.3 ppb	Sos12
26.5 ppb	Sos13
45.4 ppb	Sos14
27.3 ppb	Sos15
13.7 ppb	Sos16
61.4 ppb	Sos17
8.0 ppb	Sos18
6.1 ppb	Sos19
17.7 ppb	Sos20

polluted than the sausages fried in the laboratory. That level of microbial contamination in various sausage products in addition to the damages of preservatives in sausages could be threatening the health of consumers. (SOS equivalent sausage).

The amount of acrylamide in samples of sausages: The amount of acrylamide in sausage samples examined in terms of micrograms per kilogram (ppb). Table 2 and Fig. 8 were drawn to it. Also the chromatograms from some sausage samples were obtained. The amount of acrylamide in samples 1, 4 and 17 were higher than the other samples. Meat is one of the suitable sources for the growth of microorganisms. Given that the main ingredients of sausages are meat proteins and carbohydrates, the food can be a good source for the growth of bacteria, mold and yeast. Various researches conducted on the microbial factors of sausages all over the world. But, there is a significant difference between Iranian sausages and European and American ones in production lines. In European and American factories sausages are made in so-called fermentation method (lactic bactria group). In Iran the method is completely different “and only raw materials are mixed together” and practically nofermentationtake place. this difference in the production practically led tothe difference in the burden of bacterial contamination presented in the final product Extensive studies were done on microbial factors remaining in the sausage, all of which indicate that pathogens such as staphylococci and coliforms, *E. coli* and mold easily can remain inside sausage dough. Sosausages should be used in the form of cooked and raw sausage consumption must be avoided. Studies show that cooked sausages preserved for the duration of 18 days at 2 and 8°C as well as sausages that heated at 80-85°C for 15-20 min had far less bacterial contamination than sausages heated 65°C for 10 min. These studies reported that Enterobacteriaceae and listeria bacteria were easily destroyed at high temperatures but for the complete elimination of Staphylococcus aureus enterotoxigenic more temperature and time is needed. According to the results in this study, amounts of coliforms, *Escherichia coli*, psychrophilic bacteria and Staphylococcus (65% coagulase-positive) (Table 1) in all

tested samples were positive and higher than the standard. The levels of pollutions in samples taken from restaurants bought in form of sandwiches are higher than the sausages bought in vacuum from and fried in oil. This contamination can be due to the restaurant staff and/or kitchen equipment as well asthe other sandwich ingredients such as lettuce, tomatoes, pickles and bread. Filling and packing sausages also have significant impact on the growth of microorganisms in the sausage. A study conducted in the United Kingdom to test the vacuum-packed sausages show, if the sausages are in perfect condition and package vacuum-sealing there is still the possibility of remaining some pathogenic bacteria inside the sausages. In their study, various sausages after being fried or grilled held for 28 days at a temperature of 6°C and at this time were microbiologically tested (Greenwood *et al*., 1984). In the present study also sausages of well-known brands obtained that all of them have had health certificates. Considering all sausage samples examined in this study had vacuumsacceptable for IRIFDO (Islamic Republic of Iran Food and Drug Organization) but still according to the results tested samples show bacterial growth. So that, all vacuum samples after being fried in total count of bacteria, psychrotrophic bacteria, coliform and *E. coli*, yeast and staphylococcus were in terms of microbial contamination higher than the standard. Therefore, health certificates for products such as sausages must not only guarantee the product quality but also ensure the correct product packaging. It can be alarming to consumers because in addition to risks of preservatives, nitrites and nitrates can be found in sausages, bacterial contamination of this popular food may be led to gastrointestinal diseases as well as various other illnesses. The use of fast foods often occurs in restaurants and certainly consumer don't know the sausage brands cooked in fast food restaurants and may be some restaurants to benefit from sausages that have low-quality products in terms of health and nutrition. There are many problems and diseases that psychrophilic bacteria bring to the human health. These bacteria because of their specific cellular wall have ability to grow in the refrigerator, so taken after moving foods out of the refrigerator should be immediately warmed up to eliminate psychrophilic bacteria. A study merely conducted on psychrophilic bacteria in cooked sausages reported the growth of these bacteria up to 73/4%. The amount of psychrophilic bacteria in all samples analyzed in the paper show positive (100%) which could cause different digestive problems in consumers (Table 1) (Gupta *et al*., 1988). Given that the sausages can be very good reservoirs for the growth of microorganisms, it should be note that one way of transmitting germs could be factories equipment. According to a study on handmade and manufacturing sausages (small factories with small-scale production and factories with large-scale production) the health of the environment and sausage processing equipment play essential role in the microbial stability as well as safety in the final product. The purpose of the present study was to count psychrophilic bacteria, Enterobacteriaceae, *E. coli*, Salmonella and staphylococcus. The presence of all these bacteria in sausages production sites have been proved. Therefore, amounts of these pollutions can be transferred to final product. At the end, it is recommended to cook the sausages in adequate heat to eliminate all polluting germs. Sometimes evenunusual serotypes including Salmonella Golcoast (SGC) find opportunities to growth in the sausage. The research conducted in Germany in 2001 with the aim of study on unusual Salmonella serotypes in sausages including SGC Salmonella serotype, of 521 samples, 19 cases reported positive. During studies conducted in this research also one examined bacteria was Salmonella. As we found the growth of Salmonella bacteria in all tested samples were negative. Different ways of cooking as well baking time have a great impact on the elimination of all microorganisms present in the sausage. A study in the UK donewith the aim of comparing different methods of cooking to destroy as many microorganisms in sausage. In this study, three different methods of grilling, frying and cooking at different time intervals were used. Survey was conducted on 162 samples of sausages and each sample was prepared in three ways listed above at different time intervals. Findings show that eliminating all microbial load in sausage need to high temperature and adequate time. Minimum temperature to destroy microorganism is 85°c and minimum time is 15 min. Type of sausage and parts cooking are also effective on the time and temperature. It is important to note that our study show that sausages before frying chopped into smaller pieces have less microbial load than the grilled hot do gwithout chopping purchased from the restaurants.

Acrylamide is a potentially carcinogenic substance discovered in 2002 by Swedish researchers in the food received 120°C temperature. So, far acrylamidehas not been observed in foods that are cooked at a temperature of 100°C. It has been observed only at high heated foods in particular rich in sugar and the amino acid asparagine (Becalski *et al*., 2003). The more the amount of protein and a variety of sugars, particularly glucose and fructose in food, higher temperatures and less water, the more possibility and the more speed up the formation of acrylamide and increasing the Maillard reaction, inversely the lesser the cooking time and the lower PH lower, the lower acrylamide formation (Muttucumaru *et al*., 2006).

CONCLUSION

In our studies, we found that acrylamide is formed in foods such as sausages made of a type of flour or bread crumb.

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