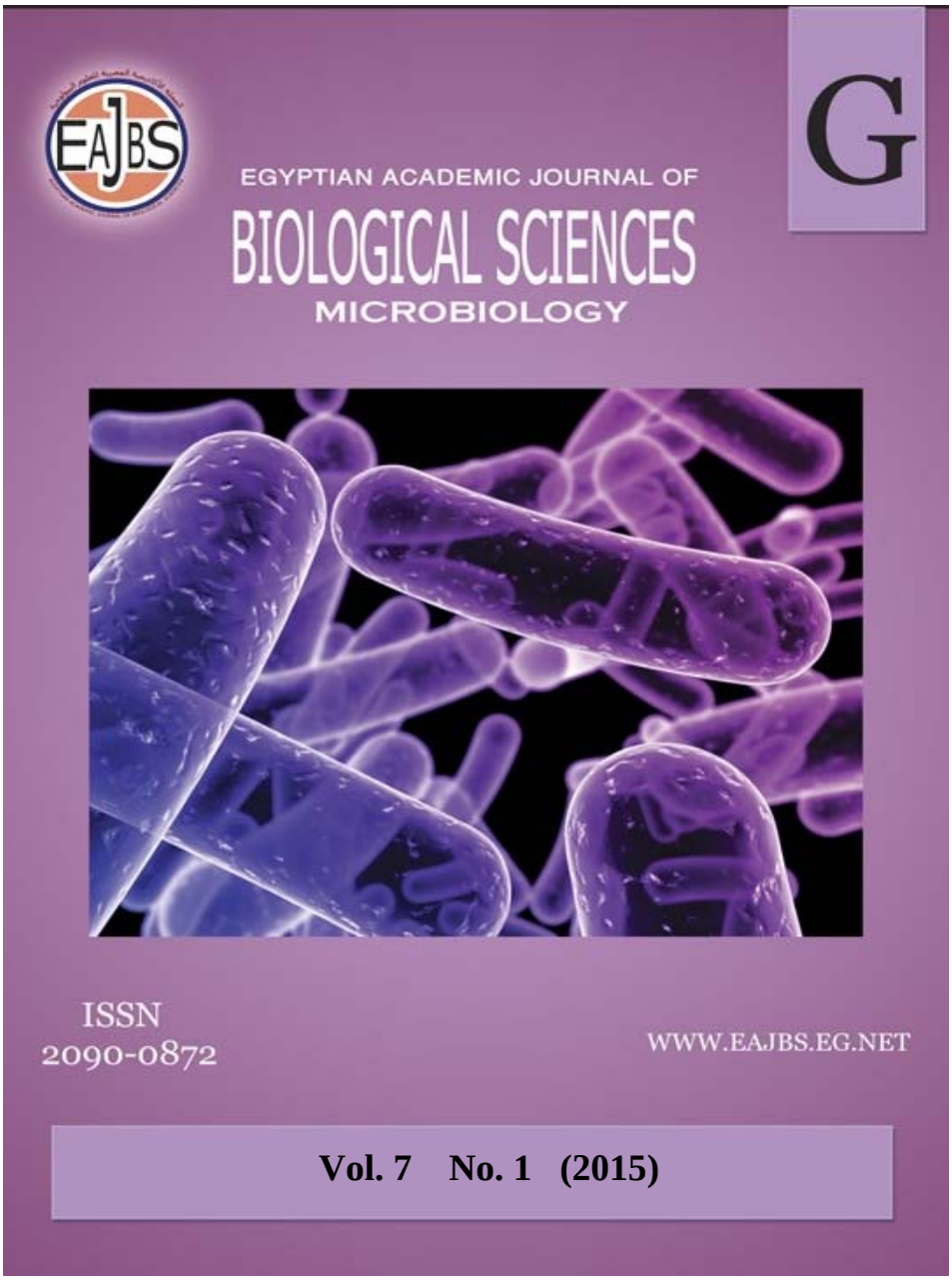

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Microbiological Study On Children Biscuits in Saudi Arabia

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ABSTRACT
Biscuits foods are a variety of quick breads popular in different forms throughout the Kingdom. This study aims to verify if the biscuits can cause food poisoning for children in the Kingdom of Saudi Arabiaor not. Therefore, a four samples of Biscuits were stored for few days. It was noticed that there are bacteria and fungi in all collected biscuits brands. Also, there is an increase in bacterial growth rate in Hein's biscuits in compressing to the other brands. Moreover, some biscuits caused food poisoning for children in the Kingdom of Saudi Arabia. It was found Biscuits foods contaminated with pathogenic bacteria (*Staph aureus*; *Salmonella* sp; *E. coli*, and *P. saeruginosa*) and fungi (*Aspergillus; Alternaria* and *Fusarium*). Finally, the recommendation that Saudi authorities must turn its attention to this problem to solve the food poisoning for children in Kingdom of Saudi Arabia.

INTRODUCTION

Biscuits are a variety of quick breads popular in different forms throughout the Kingdom Saudi Arabia. They are made from a combination of flour, shortening, leavening and milk or water. This simple dough is generally rolled out, cut into small rounds, baked and served hot. Food preferences and ingredients in various regions of the country often determine what type of biscuit is preferred. Some people enjoy tall, tender flaky biscuits; other people from the South like biscuits with a soft, tender crumb.

The original biscuit was a flat cake that was put back in the oven after being removed from it's tin, hence the French name *"bis"* (twice) *"cuit"* (cooked). This very hard, dry biscuit was the staple for sailors and soldiers for centuries. During the time of Louis XIV, soldiers' biscuits were known as "stone bread." "Animalized" biscuits were introduced later. They were considered very nutritious because meat juices were used as the liquid base. In the 19thcenturies, travelers' biscuits were hard cakes that kept well wrapped in a kind of tin foil. On the other hand, feathery, light biscuits originated in Southern plantation kitchens but now are popular throughout the United States. Rolled biscuits were a staple at most meals, but beaten biscuits became another Southern favorite. They are made light by beating air into the dough with a mallet or a rolling pin (up to 100 strokes "or more for company"). Beaten biscuits are typically thinner and crispier than the baking powder biscuits.

Biscuits are high in fat, which makes them flaky, tender and delicious. The average home recipe has 50% of calories from fat, so budget fat calories accordingly. The average recipe calories consist of 43% carbohydrates and 7% protein. This study aims to know if the biscuits causes food poisoning for children in Kingdom of Saudi Arabia or not

METHODS AND MATERIALS

Study Area and Sampling: Four different types of biscuits were collected randomly from pharmacy shops in the capital city of Saudi Arabia Riyadh. Packaged biscuit samples were taken to microbiological laboratory in the Department of Biology. PNU. All samples collected were bacteriological analyzed in the laboratories.

Preparation of Media: All dehydrated media (nutrient agar for bacteria; Czapek dox agar and potato dextrose agar for fungi) were prepared according to manufacturer's instructions. They were mixed with distilled water and dissolved by gentle heat to boil. The media were sterilized in an autoclave (LTE J7090 model, LTE Scientific Ltd, England) at 121°C for 15 minutes. The sterile media were dispensed or poured into sterilized Petri dishes and allowed to cool. The sterility of the prepared media was checked by incubation of blindly selected plates at 37°C for 24hrs.

Isolation of Pathogenic Bacteria: The method of Collinse *et al. (*1984) was used with some modifications. A stock solution of the each sample was prepared by weighing one gram (1g) of the sample into 9 ml of sterile water and shaken thoroughly. A 4-fold serial dilution of the bacterial suspension was made. This was done until 10^{-4}dilution was achieved. 1ml of each biscuit types was pipetted from the 10^{-4} dilution onto the surface Petri dishes of nutrient agar medium and incubated aerobically at 37^{o}C for maximum up to 48 hours , and repetition three time to ensure and counted the bacterial colonies One ml of the sample was placed on each of media, M-PAC agar medium petri plate and incubated at 41.5^{0}C for 72 h. colonies were flat in appearance with light outer rims and brownish to greenish- black centers (*Pseudomonas aeruginosa*). Brilliant green agar on which typical well-isolated *Salmonella* colonies are pinkish white with a red background *Salmonella* spp.).Using multiple tube technique was used for further *E. coli* confirmation, Tubes showing gas production with growth is considered a positive indication of *E. coli* presence, from which several loopfulls were streaked on Mac Conkey agar plates and suspected colonies showing pink to red color surrounded by red zone (Pettibone, 1992). The medium used was Baired Parker (BP) agar medium, Petri plate incubated at 37^{0}C for 48h, the counted colonies were black shining colonies, narrow with white edge surrounded by a clear zone. (*Staphylococcus aureus*) Results were recorded as CFU/ 100 ml .

Identification of Bacterial Isolates: A pure culture for each isolate sample was grown on nutrient agar medium for maintenance, as well as, for cultural and morphological characterization and then placed into genera or groups, after that they will be subjected to a scheme of biochemical tests either to complete or confirm their identification (Bergey's Manual,1994) .

Congo Red Dye Agar Test (CR Test): The test was carried out according to Berkhoff and Vinal, (1986). The colonies were streaked on Congo red agar (Soybean-casein digest agar; 890.0 ml, Hemoglobin solution; 100.0 ml, Supplement solution; 10.0 ml and Congo red 0.01% solution) as described in hand book of microbiological media, USA and incubated for 72 hours at 25^{o}C. Reaction was recorded at 18, 24, 48 and 72 hours. Appearance of red colonies within 72 hours was recorded as a positive reaction. Negative colonies did not bind the dye and remained white or grey even after 72 hours and were declared negative.

Isolation of fungi: One gm from each biscuit sample was inoculated onto Petri dishes of two media Czapek dox agar and potato dextrose agar and incubated at 25^{o}C

for 4 days. Three replicates were maintained. The plates were incubated at 24°C and examined daily for growth and sporulation for 5 days. After 5 days of incubation the different fungal colonies were transferred into fresh Czapec (dox) plates. The fungi isolated were identified by the fungal colony was taken and placed on the slide.. The slide is then observed under 40x power in microscope and identified based on morphological characteristic. The method of Smith and Onions, (1994) and Sekar *et al*., (2008) were used for fungi isolation.

Data statistical analysis: The obtained data were statistically analyzed using the Analysis of Variance (ANOVA) ONE WAY WITH THE MSTAT-C statistical package. The Least Significant difference procedure (LSD) was used at 0.05 level of probability (Fisher, 1948).

RESULTS AND DISCUSSION

Answering on study questions:

The first question: what is the rate of bacteria in children biscuits?

The aforementioned Table (1) shows bacteria concentrations in certain types of children biscuits with different rates:

Batman biscuits 1, the arithmetic average was 7.33 CFU g^1 biscuit sample.(Table 1 and Fig. 1) and the standard deviation was 6.429 which means the rate of bacteria existence is few compared to biscuit type, by diluting the concentration to 1/100. The bacteria concentration reaches 12 CFU g^1biscuit sample which means decreasing in bacteria rate but while reducing the dilution rate to 1/1000, the bacteria was not existed.(Table 1).

Table 1: The bacteria concentrations rate contaminated four different types of biscuitsamples on nutrient agar mediumplate.

Biscuit types	Dilution rate	Bacteria count ($CFUm^1$)	Dilution rate	Bacteria count ($CFUm^1$)	Dilution rate	Bacteria count ($CFUm^1$)	Average (CFU g^1)	Standard deviation
Batman biscuit 1	1/10	10	1/100	12	1/1000	0	7.33	6.429
Hein's biscuit	1/10	90	1/100	110	1/1000	13	71.00	51.215
Batman biscuit 2	1/10	150	1/100	20	1/1000	10	60.00	78.102
Farley's biscuit	1/10	3000	1/100	80	1/1000	0	1026.6	155.34

Hein's biscuits, the arithmetic average was 71.00 CFU g^1 biscuit sample. (Table 1and Fig. 1) and the standard deviation was 51.215 which means the rate of bacteria existence is few, by diluting the concentration to 1/10, the bacteria concentration in the first sample was 90 CFU g^1 biscuit sample, but while diluting concentration to 1/100, the bacteria concentration rate reached 110 CFU g^1 biscuit sample which indicate increasing in bacteria rate in Hein's biscuits, while decreasing the dilution rate to 1/1000, the bacteria rate was 13 CFU g^1 biscuit sample. (Table 1)

Batman biscuits 2, the arithmetic average was 60.00 CFU g^1 biscuit sample (Table 1and Fig. 1). and the standard deviation was 78.05, which means the rate of bacteria existence is high, by diluting the concentration to 1/10, the bacteria concentration on the first sample was 150 CFU g^1 biscuit sample, by diluting the concentration to 1/100, the bacteria concentration rate in the sample Batman biscuit 2 reaches 20 g^1 biscuit sample, which means that the bacteria rate reduced in the Batman biscuit 2, while diluting the rate to 1/1000, the bacteria rate reaches 10 CFU g^1 biscuit sample, which means that the less

dilution rate the less bacteria rate in Batman biscuit 2. (Table 1)

Farley's biscuits, the arithmetic average in Farley's biscuit was 1026.6 CFU g^1 (Table 1) biscuit sample and the standard deviation was 155.34 which means that the bacteria existence is high, by diluting the concentration to 1/10, the bacteria concentration in the first sample reached 3000 CFU g^1 biscuit sample, while diluting the concentration to 1/100, the bacteria concentration in the Farley's biscuit sample reached 80 CFU g^1 biscuit sample, which means that the bacteria rate reduced in Farley's biscuit, by diluting the concentration to 1/100, the bacteria rate reached to 0, which means the less dilution rate, the less bacteria rate in Farley's biscuit.(Table 1)

Isolation and Purification of Pathogenic Bacteria:

In the present study , different selective media were used as mentioned in material and methods to isolate and purify the most indicator bacteria . Bacteria isolates were subjected to cultures physiologically and microscopic examination. The bacterial isolates were found to be belong to four genes which included *Staph aureus*; *Salmonella sp. E. coli*, *P. saeruginosa*, and at biscuit samples (Table 2). *Staph. aureus* count was found in biscuit samples 10; 90; 75; 250 cfu ml^1, *Salmonella* sp. count was decreased 12;110;20;80 CFU/100mL. *E. coli* count was 8; 14; 16; 5 CFU/1ml, and *P. aeruginosas* was count 0;15; 40;0 cfu ml^1 in Batman biscuit 1; Hein's biscuit Batman biscuit 2 and Farley' biscuit samples respectively. The Frequency% of *Staph. aureus Salmonella* sp. *E. coli* and *P. aeruginosa* contaminated biscuit samples was 22.59%; 40.98 %; 28.93% and 7.50 respectively.

Reaction of Congo red dye agar test with *Staph aureus*; *Salmonella* sp. *E. coli*, ,and *P. saeruginosa* was recorded at 18, 24, 48 and 72 hrs. Appearance of red colonies within 72 hrs was recorded as a positive reaction. Negative colonies did not bind the dye and remained white or grey even after 72 hrs and were declared negative.

Table 2: The bacteria concentrations and contaminated Frequency four different types of biscuit samples on nutrient agar mediumplate.

Biscuit types	***Staph. aureus*** CFUm^1)	***Salmonella* sp.** (CFUm^1)	***E. coli*** (CFUm^1)	***P. aeruginosa*** (CFU g^1)
Batman biscuit 1	10	12	8	0
Hein's Biscuit	90	110	14	15
Batman biscuit 2	75	20	16	40
Farley's biscuit	250	80	5	0
Frequency**%**	22.59 %	40.98 %	28.93 **%**	7.50 %

The second question: what is the rate of fungi in children biscuit?

The aforementioned Table (3) showed the fungi concentration ratein CZAPEK of children biscuits, as follows:

Batman biscuit, the arithmetic average was 78.00 CFU $g^1$and the standard deviation was 105.773, which means that there is a very high rate of fungi in CZAPEK. The fungi concentration in the first sample reached 200 CFU g^1, while the fungi concentration in the second sample reached 12 CFU g^1 which means that the fungi rate reduced. The fungi concentration in the third sample reached 22 CFU g^1 which means that the fungi concentration rate increased.

Hein's biscuit, the arithmetic average was 9.33 CFU g^1 and the standard deviation was 5.132, which means that the fungi concentration rate was few in samples CZAPEK, the fungi concentration in the first sample reached 8 CFU g^1, while in the second sample the fungi concentration reached 15 CFU g^1 which means that the

fungi concentration increased, but in the third sample the fungi concentration reached 5 CFU g^1 which means the fungi concentration reduced in samples CZAPEK.

Batman biscuit, the arithmetic average was 7.00 CFU g^1 and the standard deviation was 5.686, which the fungi concentration in the first sample no detected any fungi colony , while the fungi concentration in the second sample reached 5 CFU g^1which means that the fungi concentration rate increased, while the fungi concentration in the third sample reached 16 CFU g^1, which means the fungi concentration increased in CZAPEK

Farley's biscuit, the arithmetic average was 11.67 CFU g^1 and the standard deviation was 9.815 which means that there is a few rate of fungi in CZAPEK, the fungi concentration in the first sample reached 6 CFU g^1, while the fungi concentration in the second sample reached6 CFU g^1, which means that the fungi rate is equal in both samples, the fungi concentration reached 33 CFU g^1 in the third sample which means that the fungi concentration is increased.

Table 3: The fungi concentrations contaminated four different types of biscuiton Czapexagar mediumplate.

Biscuit types	First sample (CFU g1)	Second sample (CFU g1)	Third sample (CFU g1)	Average (CFU g^1)	Standard deviation
Batman biscuit 1	200	12	22	78.00	105.773
Hein's biscuit	8	15	5	9.33	5.132
Batman biscuit 2	0	5	16	7.0	5.686
Farley's biscuit	6	6	33	15.0	9.815

The aforementioned Table (4) showed the fungi concentration rate in POTATOS of children biscuits, as follows:

Batman1 biscuit, the arithmetic average was 24.33 and the standard deviation was 12.055, which means that there is a very high rate of fungi in POTATOS, the fungi concentration in the first sample reached 23, while the fungi concentration rate in the second sample reached 37 which means that the fungi rate increased, finally the fungi concentration in the third sample reached 13 which means that the fungi concentration rate decreased in POTATOS.

Table 4: The fungi concentrations contaminated four different types of biscuiton potato agar mediumplate.

Sample name	Firstsample (CFU g^1)	Second sample (CFU g^1)	Third sample (CFU g^1)	Average (CFU g^1)	Standard deviation
Batman biscuit 1	23	37	13	24.33	12.055
Heins biscuit	15	15	11	13.67	2.309
Batman biscuit 2	10	11	0	7.00	6.083
Farleys biscuit	14	25	25	21.33	6.351

Hein's biscuit, the arithmetic average was 13.67 and the standard deviation was 2.309 which means that the In fungi concentration rate was few in POTATOS, the fungi concentration in the first sample reached 15, while in the second sample the fungi concentration reached 15 which means that the fungi concentration is equal in both samples, but in the third sample the fungi concentration reached 11 which means the fungi concentration reduced in POTATOS.

Batman 2 biscuit, the arithmetic average was 7.00 and the standard deviation was 6.083 which means that the fungi concentration rate was few in POTATOS, the fungi concentration in the first sample reached 10, while in the second sample the fungi concentration reached 11 which means that the fungi concentration is increased, but in the third sample the fungi concentration reached 0 which means the fungi concentration is not found in POTATOS.

Farleys biscuit, the arithmetic average was 21.33 and the standard deviation was 6.351 which means that the fungi concentration rate was very high in POTATOS, the fungi

concentration in the first sample reached 14, while in the second sample the fungi concentration reached 25 which means that the fungi concentration is increased, in the third sample the fungi concentration reached 25 which means the fungi concentration is equal in both samples in POTATOS.

The results of isolation of some species of fungi from Four different types of biscuits were collected randomly from pharmacy shops in Riyadh city belong to Kingdom of Saudi Arabia . Atotal of three genera offungi were isolated (Tables 5and 6) Fungi are remarkable organisms that readily produce a wide range of natural products called secondary metabolites. Some are deleterious (e.g. mycotoxins). Fungi that exhibit filamentous growth and have a relatively complex morphology produce most secondary metabolites. The production of these secondary metabolites usually commences late in the growth of the fungus, often upon entering the stationary phase (Sekar *et al*., 2008)The collected samples were grown on Czapec (dox) plates. After 5 days incubation, The results of inoculation of the sample on Czapec (dox) plates are tabulated on Table 5. It is shown that some of the following biscuit samples contain fungi above or below the permissible number in terms of PFUs per gram of sample. For identification by morphology, LCB wet mount was prepared and the following morphologies were observed. The results of LCB wet mount preparation are shown on Table 3. Fungi propagules get on wheat grain or flour in different ways, most often with dust from soil, from the surface of plant remnants during harvesting, transportation, storage, and processing (Klich, 2002). Mold spores present in biscuit survive for several years, and therefore, care should be taken in the storage of biscuit (Christensen and Cohen, 1950).

Table 5: The frequency percentge of fungal concentrations contaminated four different types of biscuiton Czapexagar mediumplate.

Fungi	frequency percentage
Aspergillus sp	25 72. %
Alternariasp	65.05%
Fusarium sp	9.23.9%

Table 6: Morphology characters of isolated fungi on Czapec (dox) plates and results of wet mount microscopic observation

Probable organism	Colony morphology	Colony morphology		
		Mycelium	Spores	Conidiophores/ Sterigmata
A. flavus	Blue mycelia Woolly a tfirst, white to yellow, then turn dark brown to black	Blue mycelia Woolly a tfirst, white to yellow, then turn dark brown to black	Blue spores	Conidiophores variable in length, rough, spiny; sterigmata single and double, pointed in all directions
A. niger	Blue/brown mycelium	Blue/brown mycelium	Blue spores	Sterigmata double, cover entire vesicle, form radiate head
fusarium	White/pink mycelium	White/pink mycelium	Black spores	Conidiophores may be single or branched with conidia
Alternaria	usually starts white before changing to a darker color	usually starts white before changing to a darker color	dark brown to black spores	Conidiophores Pale brown to olive brown Straight or flexuous

Table 6 shows the mean values of total fungal counts obtained with the direct plating technique. These results are in agreement with the results reported by Cabanas *et al*.

(2008) in their work on wheat flour from Spanish markets. Dilution plating is the technique recommended for fungal enumeration in flours and direct plating is considered to be the more effective technique for mycological examination of particulate foods such as grains or nuts and wheat samples (ICFM, 2006; Cabanas *et al*., 2008). Cabanas *et al*. (2008) reported that the total mold counts obtained from wheat flour samples in Spain are similar to those reported by other authors. In Malaysia, total fungal count in wheat flour samples ranged from 102 cfu=g sample to slightly more than 104 cfu=g sample (Abdullah *et al*., 1998). In Spain, the maximum mold count limit for wheat flour for human consumption is 1_104 cfu=g (Real Decreto 1286=1984).

The third question: Are there any statistically significant differences (Table 7) in respect of answers of respondents according to the three groups?

The aforementioned results shows that there are not statistically significant difference at the level 0.05 or less between three groups (NUTRIENT-POTATOS-CZAPEK) in F-B biscuit sample-H biscuit sample-Batman sample) which means that there are bacteria and fungi in all biscuits types, thus there are not differences in both bacteria and fungi.

Table 7: Statistically significant differences in respect of answers of respondents according to the three groups

biscuit types		Sum of squares	D f	Mean Square	F	Significant
Batman biscuit 1	Between groups	442.889	2	221.444	3.095	0.119
	Within groups	429.333	6	71.556		
	Total	872.222	8			
Hein's biscuit	Between groups	7108.667	2	3554.333	4.017	0.078
	Within groups	5309.333	6	884.889		
	Total	12418.00	8			
Batman biscuit 2	Between groups	5312.667	2	2656.333	1.293	0.341
	Within groups	12325.33	6	2054.22		
	Total	17638.00	8			
Farleys biscuit	Between groups	23604.667	2	11802.333	1.450	0.306
	Within groups	48833.33	6	813.889		
	Total	72438.00	8			

CONCLUSION

Upon completing this research, I found that there are bacteria and fungi in all biscuits types. Moreover, there is an increase in bacteria rate in H biscuits. So this biscuits might cause poisoning for the children, in Saudi schools therefore, the Saudi authorities must turn its attention to this problem to solve the food poisoning for Saudi children in Kingdom of Saudi Arabia.

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