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Comparative Investigations for Detection of Foodborne Microorganisms in Egyptian Hard Cheese "Ras" Using Conventional and Fast Biochemical Tests

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Abstract: A rapid microbiological method (Food system kit, Liofilchem Diagnostic, Italy) was evaluated under the Egyptian conditions for testing the quality and safety of Ras cheese in Cairo and Giza markets. Investigation were carried out to study the incidence and recovery rate for a number of gram positive and negative foodborne bacteria as well as mold and yeast, via FS Kit and the traditional methods. Results revealed good reactivity of the FS with mold and yeast, with weak reaction for *E. coli* due to the wide difference in the incidence of detection by FS and the classic methods, (3 Vs 21% of total samples). The rest of gram negative pathogens have the similar frequency percentages by both methods.. Concerning gram positive pathogens,*B.cereus* and *Listeria spp.* were not detected by FS,despite they were detected by the classic methods in7 and 17%of the total samples, respectively. Results of *S.aureus* were very close by the two methods. Moreover,recovery study of these foodborne bacteria in Ras cheese by the two techniques revealed similarity at the high levels of contamination.Thus, FS test is recommended for routine microbiological analysis of Ras cheese. On the other hand, the microbiological quality of Ras cheese in this study indicated insufficient process and sanitation during manufacture and handling, especially for the group sold by vendors which did not meet the Egyptian standards.

Key words: Bacteria · Molds · Yeast · Foodborne microorganisms · Egyptian hard cheese (Ras)

INTRODUCTION

The issue of food borne pathogens has captured the attention and concern of the scientific community, food industry, governmental agencies and consumers lately. So, there is a great need to apply methods posses rapidity, specificity and sensitivity.

For a long time, industrial as well as research laboratories have been interested in the possibility of replacing reference methods which are often time consuming and expensive, by alternative rapid methods. These methods are supposed to present many advantages as rapidity, less costly and allow examining a large number of samples at the same time [1-3].

The concept of rapid and miniaturized techniques has gained popularity since wide variety of commercial kits and mini-systems covering many different purposes are available. These purposes range from the enumeration of bacteria in samples to the more complex task of identification of species based on other intrinsic parameters [2] and time required to obtain results are usually too long and,in many instances,food will be consumed before laboratory tests are completed [4].

Ras cheese is the only Egyptian hard cheese variety manufactured either at industrial scale under sanitary conditions or at small scale in private laboratories under poor manufacturing conditions ;storage and handling practices.It is made under certain condition from raw cow's and buffalo's milk. But in 2001, the Egyptian Organization for Standardization and Quality Control recommended pasteurization of cheese milk to produce constant and high quality cheese to protect the health of consumers [5].

Ras cheese is consumed after more than three months of ripening [6-8] and its manufacture is still primitive and some steps need to be modified. Specific starter cultures are not used despite the importance of starter organisms for enhancement of cheese quality and safety. Thus, increased attention should be taken for the routine microbiological examination of Ras cheese in the market

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for assessment of safety and conformation with standards.

This study aimed to determine the microbiological quality of Ras cheese collected from different districts in relation to the Egyptian standards. Also comparison of Food-system (FS) as a rapid, easy, specific and sensitive method (Liofilchem-kits) with the traditional methods was another target.

MATERIALS AND METHODS

Materials

Ras Cheese Samples: Thirty Ras cheese samples (250 g each) were collected from Cairo and Giza markets in sterile plastic bags. Samples were transferred to the laboratory in ice box and refrigerated until analysis.

Tested Microbial Strains and Cheese Contamination: Food borne bacteria (as reference strains) of *Salmonella typhimrium, Escherichia coli, Proteus mirabilis, Pseudomonas aeroginosa, Staphylococcus aureus, Listeria monocytogenes* and *Bacillus cereus* were obtained from Central Public Health laboratories (Ministry of Health). Fresh cultures were suspended, separately, in physiological saline solutions with decimal dilutions up to 10^8 cfu/ml. Ras cheese portions (3×100g/each) were mixed well with 1 ml of each culture preparation to get ~ 10^6, 10^3 and 10 cfu/g of high, medium and low levels of cheese contamination, respectively. In addition moulds and yeasts as *Penicillium spp.* and *Saccharomyces spp.* were used together to contaminate 3 portions of Ras cheese. Hence, 24 contaminated cheese samples were analysed by FS and the traditional methods for testing FS accuracy and foodborne recovery, respectively.

Food System Kits: Miniaturized biochemical food-system, kits (microtiter plates) for identification of pathogenic germs were delivered from Liofilchem., via Scozia-Zona Ind. Le-64026 Roseto D.A. (TE) Italy.

Methods of Analysis

Food-system Method

Preparation of the Sample: An appropriate quantity of Ras cheese (10g) was homogenized in buffered peptone water (90 ml) and incubated at 36°C, 24 hrs. Aliquot of 0.2 ml of the sample was dispensed into the vial of the physiological solution contained in the kit and 0.2 ml (4 drops) of the sample suspension was transferred into each well of the system. The first wells 1-LDS, 2-H2S and 3-UR was covered with 2 drops of Vaseline oil and the system was covered with the lid and incubated at 36°C for 18-24 hrs.

Presumptive Identification of Microorganisms: *Salmonella* spp. is detected by a change in color from yellow to red in the well 1-LDS, by the change in colour from yellow to black in well 2-H2S and by the yellow color in well 3-UR. *Citrobacter spp.* is detected by the yellow colour of well 1-LDC, by the change in color from yellow to black in well 2-H2S and by the yellow colour in well 3-UR. Proteus/Providence spp. is detected by the yellow color of well

1-LDC,by the change in from Yellow to red-fuchsia of well 3-UR. Confirmation of *Proteus/Providencia spp.* is provided by the change in colour from yellow to brown-black of well 4-PRO. *Pseudomonas spp.* is detected by the change in colour from yellow to turbid green of well 5-PSE. *Staphylococcus aureus* is revealed by the appearance of a black ring in the bottom of well 6-STA. *Escherichia coli* is apparent by the change of red to blue colour in well 7-ESC and by the appearance of a pink-red ring following the addition of kovac's reagent to well 8-IND. *Bacillus cereus* is detected by the change from yellow to turbid green colour in well 9-BCE. *Listeria spp.* is apparent from the change yellow to black color in well 10-Lis and by the development of bubbles following addition of H_2O_2 reagent to well 11-CAT. Yeasts and moulds are detected by the change from green to yellow colour in well 12-Y/M and observation under the microscope of mycelial strands (hyphae) and chlamydospores.

Calculation of FS (LiofilChem. Ltd.) Accuracy: Accuracy was defined by Fung [9] as to express the test specificity, sensitivity and versatility. In the current study, accuracy percentages were calculated throughout postulations [10] depending upon the features or reactions responded by the test as follow:

Specificity: The typical colours indicated by the FS test to a certain organism according to the producer instruction. Specificity (SP) scoring out of 50 points due to the colour or colors (1 to 4) needed for identification.

Sensitivity: The dense and distinction of the colors and time required for reaction end-point were represented by FS sensitivity (Sn) and scored out of 50 points.

Versatility: The frequency of a test (number of replicates or kinds of foods) indicates the versatility (Vr) which is 3 in the present study. Accuracy is calculated as follow:

$$\text{Accuracy (\%)} = \frac{Sn + Sp \times Vr}{100}$$

Interpretation: Due to accuracy % the reaction could be classified as high (>75%), moderate (>50 %), low (>30%) and unsatisfactory (<30%).

Methods of Traditional (T.M) Microbiological Analysis

Aerobic Colony Bacterial Count: The aerobic colony count (ACC) was carried out as the conventional method [11] using plate count agar (Oxoid). After 48±2h incubation at 35±1°C colony forming units were accounted and calculated per gram of sample.

Molds and Yeasts Count: Enumeration and count of yeasts and molds were carried out in the samples using the media of acidified potato dextrose agar (Oxoid). The method recommended by FDA [11] was followed up. Plates were incubated at 22-25°C for 3-5 days and colonies of yeasts and molds were accounted and calculated per gram of sample.

Detection of *Listeria monocytogenes*: Each sample (25g) was homogenized and mixed with 225 ml tryptose soy broth (fluka, Switzerland) supplemented with yeast extract and listeria selective enrichment supplement (Oxoid), in 500 ml flasks [12]. Flasks incubated at 30°C for 7 day. Every day a plate of oxford agar base (Oxoid) supplemented with listeria supplement was streaked from each of an enrichment flask and incubated at 35°C for 48h as reported by *El-Ashmawy et al.* [22]. Suspected colonies were picked up and propagated for further specific morphological, biochemical and serological tests as recommended by FDA [11].

Enumeration of *Staphylococcus aureus*: Enumeration of *S. aureus* in the samples was carried out by spreading 0.1 ml of each of sufficient (expected) dilution onto the surface agar media. Baird Parker media (Fluka, Switzerland) supplemented with egg yolk and potassium telurite solution was used for enumeration as the method and media were recommended byFDA [11] and APHA[13].

Enumeration of *Bacillus cereus*: *Bacillus cereus* was determined by the surface plating technique onto the manitol egg yolk-polymyxin ager (MYBA,Oxoid 2005). The suspected colonies peacock blue-coloured and surrounded by a zone of precipitation of egg yolk [14] were further tested for specific identification according to FDA [11].

Determination of Coliforms and *Escherichia coli*: Coliform group was determined using solid medium method onto plates of violet red bile agar (VRBA) (Difco) according to the method reported by FDA [11]. Plates were incubated 24hrs at 32-35°C. A portion of purple red colonies (5/a plate) per each plate was transferred (loopful) into tubes of MacConkey broth medium (Oxoid,England.) which were incubated at 35°C. Positive acid and gas tubes, after 24 and/or 48h, where further transferred into EC broth which in turn are incubated at 45.5°C for 48h. Positive tubes were streaked onto MacConkey agar (Merck, Germany) according to APHA [13]. Suspected red colonies were tested for IMVIC test (++--)for typical *E.coli*. Enteropathogenic & enterotoxigenic *E.coli* identification within the (+) IMVIC test isolates were examined using the serological reactions and indicators.

Detection of *Escherichia coli O157 : H7*: Samples dilutions were spread onto plates of medium Sorbitol Mac Conkey agar (Oxoid, England). After 18-24hrs at 35°C incubation, sorbitol negative colonies (pale-coloured, typical E. coli 0157: H7) were serologically tested, as outlined by FDA [11].

Isolation and identification of *Salmonellae*: Aseptically 25g of each sample was mixed with 225 ml of sterile lactose broth and incubated at 35°C for 24hrs.A 1 ml to 10 ml mixture was transferred to selenite cystein broth (SC) (Oxoid) and incubated at 35°C for 72hrs. Plates of *Salmonella & Shigella* ager (SS) were streaked every day and incubated at 35°C for 24hrs. Lactose negative suspected *Salmonella or Shigella spp.* were biochemically and serologically identified according to FDA [11] and APHA [13] using the recent reagent kits

Isolation and Identification of other Members of Gram-Negative Bacilli: The non-lactose ferementers of the gram negative bacilli : *Citrobacter spp, Pseudomonas spp., Proteus/Providencia spp.* were isolated onto MacConkey agar and SS agar as described for Salmonella onto TSI agar and the other biochemical Tests [15].

RESULTS

Table 1 compare the differences between food system and classic methods for recovery of mold & yeast and aerobic colony counts in retail Egyptian Ras cheese.

Table 1: Food system and classic methods for mold & yeast and aerobic colony count in retail Ras cheese

		FS* %			Classic Mold (cfu/g)				Classic Yeast (cfu/g)				Classic ACC (cfu/g)		
Products/Markets	No. Samples	Mold	Yeast	Total	Min	Max	Avrg	%	Min	Max	Avrg	%	Min	Max	Avrg.
Group I/factory	10	100	100	100	2×102	6×103	2×103	100	1×102	1.0×105	3×103	100	3×104	5×107	6×106
Group II/vendors	20	100	100	100	8×102	2×105	5×104	100	2×104	5×105	6×104	100	4×106	3×107	5×106
Total	30	100	100	100	5×102	9×104	1×104	100	1×102	5×105	2×104	100	1×106	4×107	5×106

* Food system, miniaturized microbial test, liofilchem Co., Italy. Note % approx. whole number

Table 2: Rapid Food-system and classic methods for coliforms and *E. coli* in retail Ras cheese

			Classic Coliforms (cfu/g)				*E. coli* (cfu/g)				*E. coli*** 0157 : H7	
Products/Markets	No. Samples	FS* % *E. coli* (+)	Min	Max	Avrg	%	Min	Max	Avrg	%	%Samples	% Strains
Group I factory	10	0	3×10	3×10^4	3×10	10	Nil	Nil	Nil	0	0	0
group II/vendors	20	5	2×10^2	5×10^4	3×10^4	60	2×10	1×10^4	1×10^3	30	10	33
Total	30	3	3×10	5×10^4	4×10^3	46	2×10	1×10^4	2×10^3	21	7	33

* FS: Food system miniaturized microbial test, liofilchem Co., Italy
** E. *coli* O157: H7, Latex test., Oxiod Itd England.
Note: % approx whole numbers

Table 3: Food system and classic methods for *Salmonella*, *Citrobacter*, *Pseudomonas* and *Proteus*/*Providencia* spp. in retail Ras cheese

		FS**				Classic			
Products/Markets	No. Samples	Sal %	Cit %	PS %	Prot/Prov %	Sal %	Cit %	PS %	Prot/Prov %
Group I/factory	10	0	0	0	20	0	0	0	30
Group II/vendors	20	10	0	60	25	10	0	50	25
Total	30	7	0	40	23	7	0	33	26

*FSS: Food system, miniaturized microbial test, liofilchem Co., Italy. Note: % approx whole numbers.

Table 4: Food system and classic methods for *Staphylococcus aureus*, *Bacillus cereus* and *Listeria spp.* in retail Ras cheese

		FS*			Classic *S. aureus* (cfu/g)				Classic *B. cereus* (cfu/g)				
Products/Markets	No. Samples	*S. aureus* %	*B. cereus* %	List %	Min	Max	Avrg	%	Min	Max	Avrg	%	List %
Group I/factory	10	20	0	0	2×10^2	2×10^6	7×10^4	20	Nil	Nil	Nil	0	10
Group II/vendors	20	20	0	0	5×10^2	5×10^6	3×10^4	30	1×10	5×10^2	3×10^2	10	20
Total	30	20	0	0	2×10^2	5×10^6	4×10^4	26	1×10^2	5×10^2	3×10^2	7	17

* Food System, miniaturized microbial test, liofilchem Co., Italy.
Note: % approx

Table 5: Recovery of foodborne microorganisms and FS accuracy in Ras cheese

	Recovery levels (average counts) (CFU/gm)			FS* accuracy % at levels		
Microorganism	1h	2 m	3L	h	m	L
Salmonella spp.	4×10^6	6×10^3	< 10	66	30	15
E. coli	2×10^7	3×10^4	< 10	85	50	15
Proteus spp.	10^7	10^5	10^2	75	30	15
Pseudomonas spp.	10^{10}	10^6	10^3	75	75	30
S. aureus	9×10^8	10^4	< 10	100	75	15
B. cereus	1×10^7	4×10^3	< 10	75	75	15
Listeria spp.	10^6	2×10^4	< 10	50	30	15
Yeast/Molds	2×10^5	3×10^3	20	100	100	30

* FS: Foodsystem (Liofilchem, Co., Kit) accuracy % evaluated, sensitivity, specificity and versatility (Fung, 1992) H. high,-m, medium-L, low

Table 2, 3 and 4 Show the incidence and recovery rate of coliforms and *E. coli*, *Salmonella*, *Citrobacter*, *Pseudomonas* and *Proteus/Providencia* spp. and *Staphylococcus aureus*, *Bacillus cereus* and *Listeria spp.* in Egyptian Ras cheese respectively.

Table 5 Shows the recovery rate of food borne microorganisms and FS accuracy in Ras cheese by the tow used methods.

DISCUSSION

Results of the microbiological analysis of Ras cheese revealed a strict confirmatory between FS and classic methods for testing mold and yeast in Ras cheeses. Therefore, FS has been respected as a reliable (accurate, sensitive and rapid,2days) method than the classic method (>5 days) for mold and yeast detection in

Ras cheese as previously determined [10]. Meanwhile, FS test did not share the classic method for testing ACC (which reflect the hygienic conditions prevailing during cheese production, ripening period).

ACC by the classic method revealed lower averages as previously reported in Ras cheese [16, 17] (28.4 and 8.7×10^7cfu/g, respectively). While,[18] give higher values. These variations may be due to the differences in production,storage or handling conditions and/or in salt, moisture contents and titratable acidity of the tested cheese.

Results of applying F.S with the classic methods for Coliforms,*E. coli* and *E. coli* 0157: H7 in Ras cheese indicated that.Coliforms and *E. coli* sero type 0157 have not been announced by FS, due to its lack in the test.Data indicated that Group 2(vendors) samples were heavily contaminated with coliforms (the E.S recomended that cheese must be free from coliforms), 60%of the samples were positive with an average of 3×10^3 cfu/g,for coliforms by the classic method. Also, incidene of *E. coli* in Group1 (factory), Gruop2 (vendors) samples was higher than Group1 in which *E. coli* was not detected in all the samples which reflect good hygienic conditions during production and handling of this group.

Results for detecting *E. coli* in Ras cheese using FS showed also weak reactions due to the big differences between FS and classic method. Once again, FS is not highly recommended for *E. coli* in Ras cheese as a reliable routine test. Similar results were obtained by El Knoly [10] for *E. coli* in Tallaga cheese.

E. coli 0157:H7 is considered as an important causative agent of diarrhea, hemorrhagic colitis and hemolytic uremic syndrome[19] and it was not detected in Group1(factory),while it was found in 10% 0f Group2 samples and in 7% of the total samples. However *E. coli* 0157:H7was previously reported in the Egyptian Ras cheese by *Sharaf* [20] and *ElAshmawy et al.* [21].

In respect of the gram negative pathogens by F S and classic methods in Ras cheese, results revealed that FS gave similar incidence of *Salmonella spp.*(7%)in the total samples.Thus, FS test is recommended for *Salmonella spp.* detection (2days) when compared with the classic method(3 days,two enrichment media,two isolation media,and biochemical tests for identification). On the other hand, the incidence of this dangerous pathogen in the present study was lower than that previously reported [17].

Both FS and classic methods indicated the absence of *Citrobacter* spp.in all the samples.

Pseudomonas spp. and *Proteus & Providence* spp.have similar frequency percentage around 40-33 and 23-26% of the total samples, respectively by either of F S and classic methods. Thus, FS test is highly recommended for routine and quick analysis of Ras cheese for gram negative pathogens.

The other side of the FS test is the indication of gram positive foodborne bacteria. *B. cereus* and *Listeria* spp were not detected by FS, while they were 7 and 17 % of the total samples by classic method, respectively. Contrarily, results of *S. aureus* by both of F S and classic were very close.However,FS could be recommended for gram positive pathogens for routine analysis where a high number of samples is supposed. Also, in group 1 (factory)samples where they present high microbiological quality,result are close to the classic method. But,in the case of high microbial counts (more than10^6 cfu/g), it would be advisable to apply the classic method,since the staphylococcal enterotoxins may persist in the cheese.

The reason for high counts of *S.aureus* and coliforms in Ras cheese could be due to the use of raw milk and/or insufficient hygienic condition during manufacturing, storage and/or handling.

Generally, the obtained results showed poor microbiological quality of the tested Ras cheese, particularly when sold at vendors scale (Group 2) with high risk of potential hazards as *Salmonella* spp. and *E. coli* 0157:H7 which were detected only in these group of samples. Also, due to the presence of the most common pathogens of food born bacteria as indicated by F.S or classic methods.

It is worthy to mention that FS fulfill most of general requirements [22] to accept this test for Ras cheese and related food category 2 [23].

The Egyptian standard [24] stated that microbial characteristic of Ras cheese must be free from pathogenic microorganisms,coliform,yeasts and moulds. Although, all the cheese samples gained higher mould and yeast counts than that allowed by the legal standards. However, samples of Group 2, failed to comply with Egyptian standard largely due to the elevated counts of coliforms& *E. coli* and,to a lesser extent, to aerobic colony counts and the presence of other foodborne bacteria.This reflect the bad hygienic conditions of Ras cheese production and handling at the common,popular and not authorized scale.

It is obvious that FS accuracy depended on the type of microorganisms and its contamination level. At high contamination levels(10^6-10^7cfu/g) of Ras cheese, FS test

showed high accuracy 66-85% and 50-100 %for gram negative and gram positive bacteria. Moderate levels of cheese contamination (10-10^4cfu/g)showed moderate FS accuracy 30-75% for the different tested organisms. The lowest accuracy was obtained by the low level of contamination.While,molds and yeasts showed the highest FS accuracy as previously reported [10].

In conclusion, FS test is recommended as an useful and alert tool in the dairy quality control laboratory and will likely help to more easily deal and detect of some foodborne bacteria in Ras cheese. But viable cell count (total aerobic colony count,coliform, *E. coli* count and other pathogenic counts) will remain an important parameter to assess the potential safety and hygienic quality of food which are not designated in FS test (kits).

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