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Survey of Hygiene Level by Indicator Bacteria in Some Cheese from Port-Said City

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ABSTRACT

The indicator bacteria are the gauge of the presence possibility of fecal substances which may relate to fecal pathogens and consider being responsible of food quality because they are not usually the cause of disease but sometimes related to foodborne organisms. In this research, sixty samples of milk products were collected from various areas in Port-Said city including Romi cheese (Ras/dry cheese), mozzarella cheese (shredded cheese), triangle cheese (processed cheese), Tallaga cheese (low salt/soft cheese) and feta cheese (tetrapacked cheese). Indicator bacteria were detected by the cultural traditional techniques (cultivation on general and selective media) and by advanced (PCR techniques) multiplex polymerase chain reaction. This study developed multiplex PCR targeting specific genes for differentiation of the most popular indicator bacteria and simultaneous detection for the bacteria which indicate the pathogen presence *Escherichia coli* (*uidA*), *Enterobacter* sp. (*16S rRNA*) and *Enterococcus* sp. (*tuf*) from targeted food samples. Generally, the 3plex-PCR assay revealed that the most common indicator found in most samples of milk products with high frequency was *Escherichia coli* which was distributed within most of the samples having the maximum occurrence frequency reached 60% followed by *Enterobacter* sp. reached 24% then *Enterococcus* sp. reached 16%. The aim of using rapid 3 multiplex polymerase chain reaction for detecting indicator bacteria in cheese samples is providing more food safety for consumers and rapid action against violating food suppliers.

Keywords

Multiplex PCR, Indicator organisms, Dairy products, Food pathogens.

1. INTRODUCTION

Detecting the quality of food is related to indicator bacteria although not all of the indicator bacteria are causing disease some of them are responsible for transmitting foodborne diseases. The higher- level of indicator bacteria, the lower the likelihood of food safety. Most of pathogenic bacteria can be gotten from animals wastes. Diseases such as (typhoid, cholera, fever, dysentery and gastroenteritis) can be happened due to eating food that containing some pathogenic bacteria as (*Vibrio*, *Salmonella* and *Shigella*). The forthright proof on contamination with fecal materials is *Escherichia coli* presence and

that is leading to the possibility of pathogens presence. The most known indicator bacteria are (fecal streptococci groups; as: *Enterococcus* sp., fecal coliform; as: *Escherichia coli* and total coliform. the food organizations used bacteriological experiments to estimate food hygiene and quality also limit potential risk from diseases occurring by foodborne pathogens[1].

Death-rate and disease-rate are caused by Foodborne disease. infection and poisoning are the most common diseases caused by (harmful bacteria, worms, moulds, protozoa and viruses) This, in turn, leads to gastrointestinal tract irritations or infections [2]. There are two ways for pathogens to be harmful to humans, first: the human may ingest the pathogen itself through the contaminated food that is called (food infection), second: the human may ingest the poison produced by the pathogen itself that is called (food intoxication) also that is leading to Poisoning Syndrome [3]. Foodborne diseases provenance a many such as (soil, water, sewage, animals or from the hands of worker people through food manufacturing) [4].

Coliform bacteria can originate from fecal contamination. They are theorized as an indicator of food contamination provenance or through packaging [5]. In addition, coliform bacteria should be absent immediately after pasteurization or heat processing, and these organisms in food shows inadequate treatment. Enterobacter species are vastly spreader in most areas of nature as an example (dairy products, water, soil and intestines of humans and animals) [6]. Enterococci (which are a sub-group of fecal streptococci) they tend to survive longer than *E. coli* and coliforms in food in addition water environment and because of that, it considers as an additional indicator of contamination with fecal matters [5]. Furthermore, Enterobacteriaceae members can form scombrotoxin (histamine) like scombroid cheeses and that happens in case of the inadequate temperature of refrigeration or improperly processing [7].

The demand for rapid and precise detection methods for these pathogens in both various clinical samples stays high, given their continued impact on human and animal health. Furthermore, identifying the most common indicator species, such as E. coli, Enterobacter sp., and Enterococcus sp., is critical for food contamination surveillance, prevention, and control. An accurate and quick identification technique would aid in identifying the sources of their spread across the food chain. Culturing, selective enrichment, and biochemical testing are common procedures for detecting indicator organisms, but they are laborious, inefficient, labour expensive, and time taking [8]. Advanced DNA-based technologies, such as DNA hybridization and PCR(Polymerase chain reaction)-based assays (mono and multiplex PCR, real-time PCRs), fluorescent DNA probes, microarrays, and DNA fingerprinting techniques, and ELISA(enzyme-linked immunosorbent assay) -based tests, on the other hand, are fast and sensitive. Because they rely on the nucleic acid makeup of the bacteria rather than their phenotypic expressions, which can be varied under culture conditions, those techniques could be used to identify indicator organisms worldwide [9, 10].The PCR is a faster process with diagnostic accuracy for the detection and characterization of specific indicator organisms from diverse food matrices as individual detection (monoplex PCR) and (multiplex PCR)[11, 12] and [13, 14].

The major objective of detecting indicator bacteria is to detect the hygienic quality of food [7]. Therefore, this study was planned to examine some of the dairy products to detect the most popular indicator bacteria by DNA-based methods, such as PCR-based assays and by classical methods for detection of pathogen based on selective enrichment and culturing in cheese samples and then identification of the isolated bacteria.

2. MATERIAL AND METHODS

2.1 Cheese samples, bacterial isolation and culturing

Randomly 60 samples comprising 3 samples of each kind of these cheeses; Romi cheese (Ras/dry cheese), mozzarella cheese (shredded cheese), triangle cheese (processed cheese), Tallaga cheese (low salt/soft cheese) and feta cheese (tetrapacked cheese) were collected from various tradesmen during the four seasons (winter, spring, summer and autumn) in Port-Said City (Egypt) for Physico – chemical estimation such as (Ph, moisture, dry matter, fat and salt) and microbiological estimation. One ml of cheese sample was added to (9 ml) of (2% saline solution) to be homogenized and be ready for cultivation [15]. The ready samples were submitted to the next examination:

- Aerobic plate count (APC) using standard plate count agar (APHA) (Pediatrics).
- Enterobacteriaceae count using MacConkey agar medium.
- *E. coli* count using tryptone bile x-glucuronide medium (TBX).

The colonies which come out from examination were isolated as a single colony and then purified for more identification by using Bergey`s Manual of (Determinative Bacteriology). the PCR analysis and chromosomal DNA extraction is done by preparing 200mL of the sample and centrifuged then kept in the freezer with a degree (-20°C) [16].

2.2 Molecular Identification of Bacterial Pathogens

The Microbiology Laboratory in the Faculty of Science, Damietta University contributed to giving us the reference bacterial strains (*Enterobacter* sp., *E. coli* and *Enterococcus* sp.)

2.3 The Extraction of DNA

The extraction of bacterial DNA genome was made by using the technique of (phenol/chloroform) by centrifugation of 1.5mL of cell suspension of bacteria at (6.500rpm for 5min). 1ml SET buffer which is compose of (pH 7.6, 50mM Tris-HCl, 20% sucrose, 50mM EDTA) was used to suspend the pellet. 100µL buffer consist of lysozyme and SET buffer used to resuspend the pellet and then vortex and add 2µL of RNase then incubate it for 10min at 37oC. After the incubation 500µL of TE buffer which consists of (10mM Tris-HCl, pH 7.6, 0.2mM EDTA) was added beside 70µL of sodium dodecyl sulfate (10%). DNA was extracted with (500µL) of (phenol/chloroform/isoamyl alcohol solution) with (25:24:1) and then centrifuged for 5min at (6.500rpm) then precipitated with isopropanol. The centrifuge was used to harvest The DNA for 10min at (6.500rpm), and then washed by 75% cold ethanol (500µL). The pellet of DNA was suspended in TE buffer (1/10) then stored at the temperature of (-20oC) till we use in amplification for PCR [17].

2.4 The primers and PCR conditions

Table 1 represented the sequence of oligonucleotide for the primers with the predictable sizes for each pathogen. Regarding the amplification of multiplex PCR, extracted chromosomal DNA (2µL), and 25µL 2x MyTaq Red Mix (BIOLINE) and 30 pmole of each primer were used, water free nuclease was used to adjust the quantum of the mixture of the reaction to 50µL. the thermal cycler (TECHNE TC-312, UK) was used with the first denaturation for five minutes at (95°C), then with 35 cycles at (95°C) for thirty seconds to perform the multiplex PCR reactions, at (55°C) for one minute primer annealing and at (72°C) for 1:30 minute primer extension. The last extension at (72°C) for 10 minutes.

2.5 The agarose gel electrophoresis and the DNA detection

By using agarose gel electrophoresis, the size of PCR products and genomic DNA were detected. In TAE buffer pH 8.0 (0.001M EDTA and 0.04M Tris-acetate) 3% of agarose was dissolved. For DNA staining we add 2µL of ethidium bromide 10 mg/mL. Loading buffer (70% glycerol, 0.25% Bromophenol blue, 10 mM Tris-HCl pH 7.0) with 2µL amount was added to the sample. For molecular marker (50bp) DNA Ladder was used. UV transilluminator was used to visualize the DNA on the gel [17].

2.6 Sensitivity of multiplex PCR primers

To detect the sensitivity of the prior sets of the primers towards the minimal cell count, 1ml of reference bacterial strains with serial dilutions (10^0 to 10^{-4}) from their overnight cultures on the media (nutrient broth medium) were set. from each dilution in triplicates (100µl aliquots) were cultured on the media (nutrient agar) plates for (CFU) colony-forming unit count, Other 100µl aliquots from the same dilutions used for bacterial cell collection were centrifuged. From each pellet The DNA was extracted and then subjected to be analyzed by PCR. The resulting products from PCR were contrasted with correspondent CFU count for each dilution [21].

Table (1): List of the primers and the expected amplicon sizes for each target gene and pathogen.

Primer	Sequence (5'-3')	Amplicon size (bp)	Target gene	Bacterial strain	Reference
Ent1-F Ent2-R	F: TACTGACAAACCATTCATGATG R: AACTTCGTCACCAACGCGAAC	112	*tuf*	*Enterococcus* sp.	[18]
16S rDNA-F 16S rDNA-R	F: ATGTCTGGGAAACTGCCTGATG R: CGGGTAACGTCAATAGACAAGG	372	*16S rRNA*	*Enterobacter* sp.	[19]
UidA-F UidA-R	F: GTCACGCCGTATGTTATTG R: CCAAAGCCAGTAAAGTAGAAC	530	*uidA*	*E. coli*	[20]

3. RESULTS AND DISCUSSION

3.1 Physico – chemical result of cheese samples

Each sample was chemically and physically examined, the result of each type has been recorded as shown below:

- The pH of the collected Tallaga cheese samples ranged from 5.71 to 6.42 which not acidic enough for inhibition or elimination of most species, in addition to the high moisture content (52.05 to 60.70), fat content (19.5 to 34) and dry matter content (39.30 to 47.95) with low salt content (1.05 to 2.83) which allow most of microorganisms to grow.
- The pH of the collected feta cheese samples ranged from 2.92 to 4.18, which was acidic enough for inhibition or elimination of most species of microorganisms if present with moisture content of collected samples (62.30 to 66.45), salt content (2.10 to 3.40), fat content (23 to 27) and dry matter content (33.55 to 37.70).
- The pH of the collected triangle cheese samples ranged from 5.43 to 5.82, The reason for obtaining the appropriate pH value is due to the appropriate salt content, which is (0.06 to 0.81), as any increase in the amount of salt will lead to an increase in the pH value, which will lead to an increase in the moisture in the product by increasing the absorption of protein molecules to water. As reported moisture content in triangle cheese (55.21 to 59.49), dry matter content (40.51 to 44.79) and fat content (15 to 25.5).
- The pH of the collected mozzarella cheese samples ranged from 5.32 to 6.42 with salt content ranged from 1.00 to 1.75 and it found that the product with low pH had low salt with high melting and

starching properties, while the product with high pH had high salt with low melting and starching properties. The moisture content of collected samples (49.20 to 52.98), fat content (21 to 24.5) and dry matter content (47.02 to 50.80).

- Letting the Romi product in ripening rooms without waxing for long 4 – 6 months during the manufacturing process leads to moisture content losing reaching to this moisture (22.62 to 38.78), that is leading to high in dry matter content (61.22 to 77.38), fat content (31.5 to 45.5), salt content (2.38 to 4.60) and pH rang (5.00 to 5.30).

3.2 Identification and distribution of the foodborne pathogens

After isolation each sample on selected medium (Tryptone bile x-glucuronide medium (TBX), Standard plate count agar medium (APHA) and MacConkey agar. *E. coli* appeared as a blue colony on tryptone bile x-glucuronide medium (TBX), as a creamy colony on standard plate count agar medium (APHA) and as a pink colony on MacConkey agar medium. *Enterobacter* sp. appeared as a white colony on tryptone bile x-glucuronide medium (TBX). *Enterococcus* sp. appeared as creamy to a yellowish colony on standard plate count agar (APHA). The classically identified indicator bacteria which isolated from the different sources (Tallaga cheese, feta cheese, triangle cheese, mozzarella cheese and Romi cheese) were distributed based on its frequencies % occurrence with in these types of cheese as represented Table (2).

Table (2): The occurrence frequency (%) and distribution of the identified indicator bacteria found in the different kinds of dairy product samples.

Types of cheese		Romi	Mozzarella	Tallaga	Grand Total
Indicator organisms	*E. coli*	48.00%	8.00%	4.00%	60.00%
	Enterobacter sp.	16.00%	0.00%	8.00%	24.00%
	Enterococcus sp.	12.00%	0.00%	4.00%	16.00%
Grand Total		76.00%	8.00%	16.00%	100.00%

The most predominant genus is *E. coli*, which spread within most of many of cheese having the maximum occurrence frequency reached 48% in Romi samples followed by 8% in mozzarella samples and then 4% in Tallaga samples. The occurrence frequency of *Enterobacter* sp. was found to be 16% in Romi samples and then 8% in Tallaga samples. The occurrence frequency of *Enterococcus* sp. was found to be 12% in Romi samples and then 4% in Tallaga samples. The triangle cheese (processed cheese) and feta cheese (tetrapacked cheese) was free from all indicator bacteria.

3.3 Sensitivity of the primers used in multiplex PCR

The sensitivity of the PCR assay for each primer used in this study was examined as shown in Figure (1). The primers *Tuf* and *UidA* were able to detect the *Enterococcus* sp. and *E. coli* strains, respectively, at dilution reached 10^{-3} which corresponding to about 5-10 CFU (Figure 1 A & C). On the other hand, *16S rDNA* primer was able to detect *Enterobacter* sp. up to dilution10^{-4} with about 5-10 CFU (Figure 1, B).

3.1 The multiplex PCR

As aforesaid in the material and methods segment by using reference bacterial strains, the 3 groups of the oligonucleotide primers were separately tested. As shown in Figure (2) the agarose gel electrophoresis was used to examine each strain for detecting the fragments of amplified DNA of the expected molecular. Lane (1-3) represented individual PCR products of primer sets *Tuf, 16S rDNA* and *UidA*, respectively. Lane (4) represented the multiplex PCR for the three pair sets of primers giving the expected Mut. For each one Figure (2).

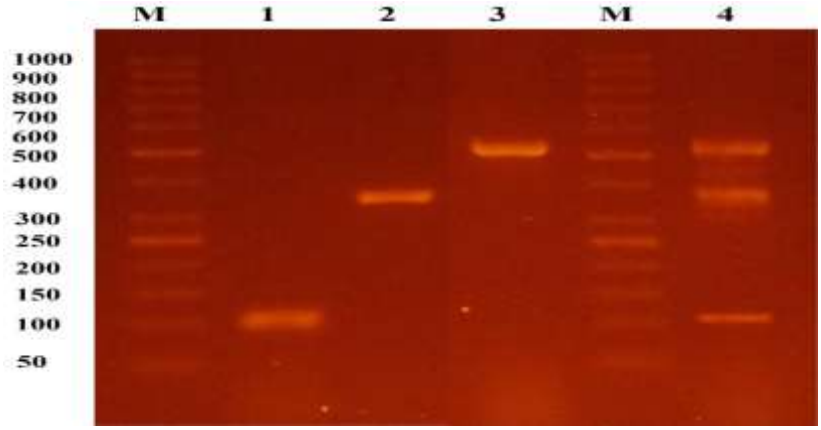

Figure (1): Sensitivity of the primers used in PCR applied to target genes of the standard pathogens. Lanes 1 to 5, bacterial dilutions, 10^{0}-10^{-4}, respectively. Lane M, 50 bp DNA marker. (A) *Tuf* primers for *Enterococcus* sp., (B) *16S rDNA* primers for *Enterobacter* sp., and (C) *UidA* primers for *E. coli*.

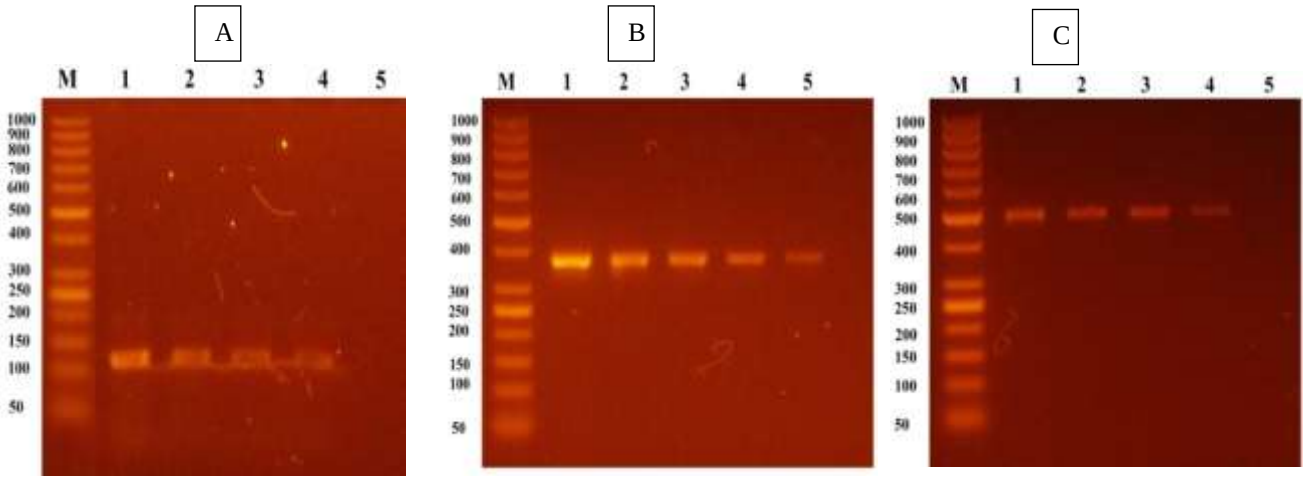

Figure (2): Agarose gel electrophoresis for the individual PCR amplification of the three target gene from purified DNA of the standard microbial pathogens (lane 1, *Enterococcus* sp.*;* lane 2, *Enterobacter* sp.; lane 3, *E. coli*) and the multiplex PCR for the all three gene targets (lane 4). Lane M is the 50 bp DNA marker.

3.2 Detection of indicator bacteria by multiplex PCR in cheese samples

A total of 12 Romi cheese (Ras/dry cheese) were collected during the four seasons 3 samples in each season and examined by multiplex PCR assay where their patterns are represented in Table (3). , And in Figure (3). (Part A) includes (lanes 1-6). As detected by the multiplex PCR, out of 12 Romi samples, only 4 were contaminated with *E. coli*. Two of Romi samples were contaminated with *Enterococcus* sp., Two of Romi samples were contaminated with *Enterobacter* sp. and two of Romi samples were contaminated with both *Enterobacter* sp. and *E. coli*. The occurrence frequency of *E. coli* was the highest (48%) then the occurrence

frequency of *Enterobacter* sp. was set to be 16% in and then *Enterococcus* sp. was found to be 12% in Romi samples.

A total of 12 Tallaga cheese (soft cheese) were collected during the four seasons 3 samples in each season and examined by multiplex PCR assay where their patterns are represented in Table (3), and Figure (3), part (B) including Lanes 1-3. As detected by the multiplex PCR, out of 12 Tallaga samples, only 1 sample was contaminated with *E. coli*, one of Tallaga samples was contaminated with Enterococcus sp., Two of Tallaga samples was contaminated with *Enterobacter* sp. The occurrence frequency of *Enterobacter* sp. was the highest (8%) then occurrence frequency of *Enterococcus* sp. and *E. coli* was set to be 4% in Tallaga samples.

A total of 12 mozzarella cheese (shredded cheese) were collected during the four seasons 3 samples in each season and examined by multiplex PCR assay where their patterns are represented in Table (3), and Figure (3), part (B) including lane 4. As detected by the multiplex PCR, out of 12 mozzarella samples, only 2 samples were contaminated with *E. coli*. The occurrence frequency of *E. coli* was the highest (8%), while *Enterococcus* sp. and *Enterobacter* sp. could not be detected within any mozzarella samples.

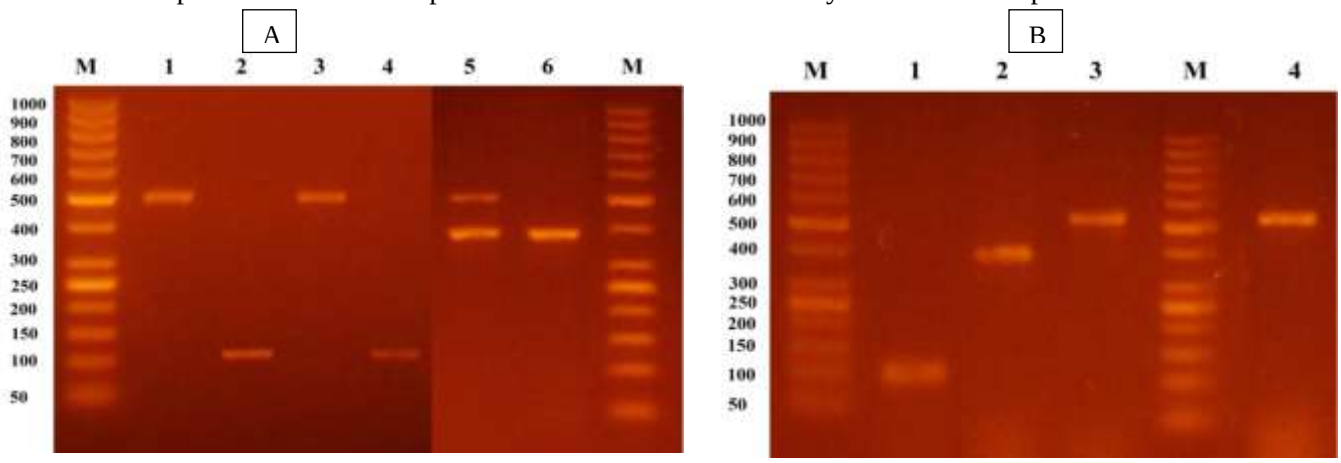

Figure (3): Agarose gel electrophoresis pattern for the multiplex PCR of positive contaminated cheese samples. (A) Romi cheese samples including lane 1-6. (B) Tallaga samples including Lane 1-3, and Mozzarella cheese lane 4. Lane M is the 50 bp DNA marker.

Despite, the refinement of hygiene in food processing in the last few years, the occurrence of indicator organisms is still commonplace. For that, disease control ought to be progressed for mankind health and for providing safe food for consuming [22]. the classical methods which used in the accurate identification of the targeted pathogen cost lots of time, effort and money [23], whereas using the strategy of multiplex PCR is trustworthy with low cost and rapid [24-27].

in this study, a simultaneous and rapid detection by using 3-plex PCR assay of three indicator bacteria for five different dairy products including Romi cheese (Ras/dry cheese), mozzarella cheese (shredded cheese), triangle cheese (processed cheese), Tallaga cheese (low salt/soft cheese) and feta cheese (tetrapacked cheese) were performed. The determination of the sensitivity of (multiplex PCR assay) was done. according to the result of multiplex PCR assay, it is showed that in the culture dilution (10^{-4}) of *Enterobacter* sp. the bacterial count cultivable pathogens up to 5-10 CFU and it is showed that in the culture dilution (10^{-3}) of *Enterococcus* sp. and *E. coli* strains the cultivable pathogens up to 5-10 CFU bacterial count. Comparison of indicator bacterial occurrence frequencies in the dairy product samples between the use of cultural traditional and advanced multiplex techniques revealed the same result. This might be attributed to the high viability of bacterial strains that could be grown, isolated and identified classically giving positive detection results by multiplex PCR [28, 29].

The invention of a PCR detection approach for more than one indicator species in dairy products was a major benefit of this work. We believe that this method can be used for the simultaneous detection of other microorganisms by using appropriate primers. We can use this procedure as a daily microbiological

analysis of food products. The high sensitivity and specificity can make this an ideal test for screening possible contaminated food samples.

Table (3): Distribution of and multiplex PCR detection of the bacterial pathogens within the different cheese samples through the four seasons.

Cheese type		Romi cheese								ND	Tallaga cheese							ND	Mozzarella cheese					ND
Season		1st		2nd		3rd		4th			1st		2nd	3rd		4th			1st	2nd	3rd		4th	
No. of samples		2	1	2	1	2	1	2	1	2	1	2	3	2	1	1	2	8	3	3	2	1	3	10
Bacterial pathogens	*E. coli*	+	-	+	-	+	-	-	-		-	-	-	-	-	+	-		-	-	+	-	-	
	Enterobacter sp.	-	-	-	-	+	-	+	-		-	-	-	+	-	-	-		-	-	-	-	-	
	Enterococcus sp.	-	+	-	+	-	-	-	-		+	-	-	-	-	-	-		-	-	-	-	-	

Legend: + _ positive PCR results, - _ negative PCR results, ND _ not detected samples.

The result revealed that the highest frequency percentage of the detected indicator occurred in the Romi samples that are due to inefficient pre-heating process during manufacturing and poor hygiene on storage. Followed by Tallaga samples, since it is not exposed to enough heat during processing and a small amount of salt is added to it, which in turn helps in bacterial growth. The mozzarella cheese had the least bacterial growth that is returning to the storage temperature -18°C which kill all non-spore forming bacteria. Furthermore, this probably was due to many reasons including the low level of hygiene during the handling procedures during the transportation or food processing, the inadequate biosafety storage and shelf life. Therefore, the highest frequency percentage of the bacterial occurrence was recorded for *E. coli*, which is the most common indicator organism. Triangle cheese (Processed cheese) and feta cheese (tetrapacked cheese) are free from any indicator organism that is due to high temperature using in manufacturing processed cheese and closed system and not using human hands during packed cheese manufacturing.

4. CONCULSION

The developed multiplex PCR targeting specific genes for differentiation of the most popular indicator bacteria and simultaneous detection for the bacteria which indicate the pathogen presence *Escherichia coli* (*uidA*), *Enterobacter* sp. (*16S rRNA*) and *Enterococcus* sp. (*tuf*) from targeted cheese samples. The multiplex PCR detected *Escherichia coli* (*uidA*) in Romi cheese, Tallaga cheese and mozzarella cheese, *Enterobacter* sp. (*16S rRNA*) in Romi cheese and Tallaga cheese, *Enterococcus* sp. (*tuf*) in Romi cheese and Tallaga cheese. This study recommends the use of 3plex-PCR detection system for the rapid, accurate

and unambiguous identification and detection of indicator bacteria *E. coli*, *Enterobacter* sp., *Enterococcus* sp. to control the food safety beside good storage conditions. Also Continuous control over food is required by the competent authorities and the application of strict regulations and laws on anyone who violates proper manufacturing procedures, in addition to preventing the supply of food from unknown suppliers and unlicensed facilities to provide safe and healthy food to all consumers.

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