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Occurrence of Staphylococcus aureus in some traditional dairy desserts with special reference to their virulent genes

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Abstract

A total of 100 samples of rice pudding, Mehalabeia, chocolate pudding and Om Ali (25 of each) were collected from Alexandria Governorate. Collected samples were examined for prevalence, enumeration and isolation of *Staphylococcus aureus (S. aureus)*. Further molecular detection of pathogenic (virulent) genes, those responsible for enterotoxins production, in five randomly selected *S. aureus* isolates was done by using convenient PCR technique. The obtained results showed that the highest prevalence percent of *S. aureus* was found in examined chocolate pudding (84%) and Om Ali (75%) samples followed by rice pudding (60%) and the lowest incidence percent was found in Mehalabeia whereas it could be detected in 40% of samples. Meanwhile, mean counts of *S. aureus* were found to be 2.32 x 10^3, 3.2 x 10^2, 2.22 x 10^3 and 3.1 x 10^3 cfu/ g in the examined samples, respectively. Regarding the Egyptian Standard; 66 (66%) of all examined dairy dessert samples were not complying with the safety requirement and considered unfit for human consumption. In addition, further molecular identification could detect four pathogenic genes in the isolated *S. aureus* strains from which three genes (*Hlg, coa and icaD) were* found in all examined *S.aureus* isolates while *seb* pathogenic gene was found only in Mehalabeia isolate. The public health significance of the isolated *S. aureus* and their responsible gens for entertoxins production as well as suggested control measures for improving the safety and quality of the traditional Egyptian dairy desserts were fully discussed.

Keywords*: Rice pudding, Mehallabeia, chocolate pudding and Om Ali, Traditional Egyptian dairy desserts, S. aureus, pathogenic genes, PCR.*

1-Introduction

Dairy products have generally been considered as an excellent source of high-quality protein, calcium, potassium, phosphorus, magnesium, Zinc, and many vitamins as riboflavin, niacin, vitamin B-6 and B-12 **(Buttriss, 1997).**

Sweetened dairy products are most palatable nutrient, healthful dairy foods. Their current consumption is relatively high and is expected to increase steadily during the next two decades **(Gerosa and Skoet, 2013).**

Rice pudding, Mehalabeia, chocolate pudding and Om Ali are the most popular traditional Egyptian dairy desserts that based on milk as the basic constituent and may serve alone or after meal.

Rice pudding may not be of Egyptian origin, but it is surely pretty popular around Egypt. It found in different areas of the world, it is made by mixing rice, water and milk. Optional ingredients include raisins, nuts and cinnamon. Recipes vary depending on the location. In Egypt, the ingredients are boiled and put in the fridge to come together. Toppings include ice cream, pistachio and nuts, giving the dessert an extra edge. Rice pudding isn't expensive, which makes it one of the best cheap meals in Egypt **(Shohyayeb, 2018).**

Mahalabia (Egyptian milk pudding) is a popular dessert in Egypt and other Middle Eastern

countries. This rich version features both milk and heavy cream. It made by combining of milk, cream, vanilla sugar, and cornstarch in a saucepan and whisked until well combined. The combined then boiled over medium heat and cooked, stirred constantly, until thickened and mixture coats the back of a spoon, 10 to 15 minutes. It removed from heat and poured into bowls to cool for about 30 minutes then refrigerated until chilled and sprinkle with cinnamon to serve **(Allrecipes, 2019).**

Chocolate pudding prepared by mixing sugar, cocoa, starch and milk in a pot over medium heat with stirring until the mixture boils and the strength slightly thickens. The mixture removed from the fire then vanilla will be added and it served with grated chocolate or nuts. Om Ali as a must-try dessert even if you set foot in Egypt for a minute. Om Ali, meaning Ali's mother, is made of layers of puff pastry soaked in milk and mixed with nuts, raisins, coconut flakes and sugar, then thrown into the oven to bake. The cooked pastry with the hot milk, complemented by various different ingredients, makes this a dish to remember **(Shohyayeb, 2018).**

Food borne diseases are of major concern worldwide. To date, around 250 different food borne disease have been described, and bacteria are the causative agents of two third of food borne disease outbreaks. *S. aureus* is among the predominant bacteria involved in these food borne diseases as a leading cause of gastroenteritis resulting from the consumption of contaminated food (**Hennekinne *et al,* 2012).**
S. aureus is an important etiologic agent of food-borne diseases due to its ability to produce heat-resistant staphylococcal enterotoxins (SEs) when it grows in foods. In fact some *S. aureus* strains may produce up to 20 serologically distinct SEs, which could be responsible for food poisoning **(Le Loir *et. al,* 2003)**. SEs have been divided initially into serological types ranged from SEA through SEE, and recently the existence of new types of SEs has also been reported (**Chiang *et al,* 2008**).
S. aureus causes a wide range of major and minor infections in man and animals and is characterized by its ability to clot blood plasma by the action of the enzyme coagulase. Staphylococcal food poisoning is a gastrointestinal illness. It is caused by eating foods contaminated with toxins produced by *S. aureus*. The most common way for food to be contaminated with Staphylococcus is through contact with food workers who carry the bacteria or through contaminated milk and cheeses. The emerging drug resistant strain of S. aureus associated with risk factors such as drug resistant in Intensive Care Unit, changes in blood cells etc., in children **(Kapil, 2015).**
S. aureus produces many important virulence factors including Staphylococcal enterotoxins (SEs) which are responsible for Staphylococcal food poisoning (SFP), a major type of foodborne illness Classical SEs have been divided into five serological types (SEA through SEE) on the basis of their antigenicities Staphylococcal enterotoxins are resistant to inactivation by gastrointestinal proteases such as pepsin. In addition, they displayed strong thermo resistance, for example, SEA retains some biological activity after 28 min at 121°C **(Nazari *et al,* 2014).**
The Egyptian Organization for Standardization and Quality for milk ice and other dairy products **(ES 1- 1185/2005)** stated that they must be free from pathogenic bacteria and their toxin.
Due to continuous demand for these traditional dairy desserts and the increase of consumer's awareness of the product safety used for as well as to assure a safe supply, it is extremely necessary not only to increase the production of this important product but also to ensure the bacteriological safety of the product to safeguard consumers against possible health hazard. Therefore, the present study was planned out to investigate the prevalence and count of *S. aureus* as well as detection of possible virulent genes responsible for toxins production by using PCR, in four most popular ready to eat dairy dessert; rice pudding, Mehalabeia, chocolate pudding and Om Ali. Meanwhile to suggest specific recommendations needed for controlling this dangerous pathogens and the required safety measures for producing safe and high quality Egyptian dairy desserts.

Materials and Methods

2.1. Collection of samples

One hundred random samples of dairy desserts including rice pudding, Mehalabeia, chocolate pudding and Om Ali (25 of each) were collected, at level of serving to consumers, from dairy shops, primitive restaurants, and supermarkets at Alexandria Government. The samples were directly transferred under cooling condition, at 4ºC to the Alexandria Provincial laboratory of AHRI with a minimum of delay for bacteriological examination.

2.2. Preparation of samples

The samples were prepared according to the technique recommended by (**APHA, 2004).** Twenty five gm of the examined samples of dairy desserts were transferred into a blender jar on which 225 ml of 0.1% sterile buffered peptone water were added under complete aseptic conditions. Each sample was then homogenized separately in the blender at 2000 rpm for 1-2 minutes to provide a homogenate for further bacteriological examinations.

2.3. Preparation of serial dilutions: by using 0.1 buffered peptone water **(APHA, 2004)**

2.4. Enumeration, Isolation and identification of *Staphylococcus aureus*: were performed on Mannitol Salt Agar **(Oxoid, 2006)** according to **Singh and Prakas (2008)** with slight modification. Mannitol Salt Agar is used as a selective and differential medium for the isolation and identification of *Staphylococcus aureus* from clinical and non-clinical specimens. It encourages the growth of a group of certain bacteria while inhibiting the growth of others. It is a selective medium prepared according to the recommendations of Chapman for the isolation of presumptive pathogenic staphylococci.

We aseptically spread 0.1 ml of previously prepared serial dilutions on the surface of Mannitol Salt agar plate and incubated at 37 ºC for 48 hours, then examined for presence of yellow colonies that surrounded by a yellow halo zone.

2.5.1. Morphological characters; Staining and microscopic examination:

Films were made from the pure culture of isolated organisms then stained by Grams stain and examined microscopically. Gram +ve, grape like clusters of cocci were considered as positive Staphylococcal strains.

2.5.2. Biochemical examinations: were performed according **to AOAC, (1995):** Typical biochemical identification of all S. aureus strains isolated from examined dairy desserts samples were;

Biochemical test	*S. aureus*
Catalase activity	+
Coagulase production	+
Anaerobic utilization of glucose	+
Mannitol fermentation	+

2.6. Molecular examination of *S. aureus* isolates was kindly performed in Biotechnology and PCR unit of National Laboratory for Poultry Quality Control of AHRI by using convenient PCR technique according to **Kapil A.(2015), Condera *et al* (2004), Gerosa and Skoet (2013)and Buttriss (1997):** Five positive *S. aureus* isolates recovered from rice pudding, Mehalabeia and chocolate pudding (one isolate of each) and two isolates of om Ali, were used for the detection of enterotoxigenic associated genes using convenientent PCR

2.6.1. DNA extraction. DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56OC for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manu-

facturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primer: The used Primers were supplied from Metabion (Germany) are listed in table (2).

2.6.2. PCR amplification. Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

2.6.4. Analysis of the PCR Products.

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. Gelpilot 100 bp ladder (Qiagen, Germany, GmbH) and a generuler 100 bp ladder (Fermentas, Germany) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table (1). Primers sequences, target genes, amplicon sizes and cycling conditions

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
Hlg	GCCAATCCGTTA TTAGAAAATGC CCATAGACGTAG CAACGGAT	937	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 50 sec.	72˚C 10 min.	**Kumar *et al*., (2009)**
icaD	AAA CGTAAG AGA GGT GG GGC AAT ATG ATC AAGATA	381	94˚C 5 min.	94˚C 30 sec.	49˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	**Ciftci *et al*., (2009)**
Seb	GTATGGTGGTG-TAACTGAGC CCAAA-TAGTGACGAGTT AGG	164	94˚C 5 min.	94˚C 30 sec.	57˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**Mehrotra *et al*., (2000)**
Coa	ATA GAG ATG CTG GTA CAG G GCT TCC GAT TGT TCG ATG C	Four different types of bands may be detected 350 bp 430 bp 570 bp 630 bp	94˚C 5 min.	94˚C 30 sec.	54˚C 30 sec.	72˚C 30 sec.	72˚C 10 min.	**Iyer and Kumosani, (2011)**

Results

Table (2). Prevalence and counts of *S. aureus* in examined dairy desserts (N= 25 samples of each):-

Criteria	Positive samples		Count of *S. aureus;* cfu/ 1gm		
Product	**No. of +ve samples**	**Percent**	**Min.**	**Max.**	**Mean**
Rice pudding	15	60%	2×10^2	1.9×10^4	2.32×10^3
Mehalabeia	10	40%	1×10^2	2×10^3	3.2×10^2
Chocolate pudding	21	84%	2×10^2	1.1×10^4	2.22×10^3
Om Ali	20	75%	1×10^2	3.3×10^3	3.1×10^3

So 66 sample from 100 examined dairy dessert samples were contaminated with *S.aureus*
CFU = Colony Forming Unit, Min. = Minimum, Max = Maximum

All positive *S. aureus* samples were not complied with Egyptian Standards for dairy product which stated that the product should be free from pathogenic microorganisms or their toxins.

Table (3). Results for molecular detection of some pathogenic genes of *S. aureus* strains isolated from examined dairy desserts (in 5 random selected isolates):

Pathogenic gene → / **Dairy dessert ↓**	***Hlg***	***coa***	***icaD***	***seb***
Mehalabeia (1 isolate)	+	+	+	+
Chocolate pudding (1 isolate)	+	+	+	-
Rice pudding (1 isolate)	+	+	+	-
Om Ali (2 isolates)	+	+	+	-

Photo (1). Agarose gel electrophoresis of cPCR amplified of (*icaD)* and *(seb) S. aureus* genes;

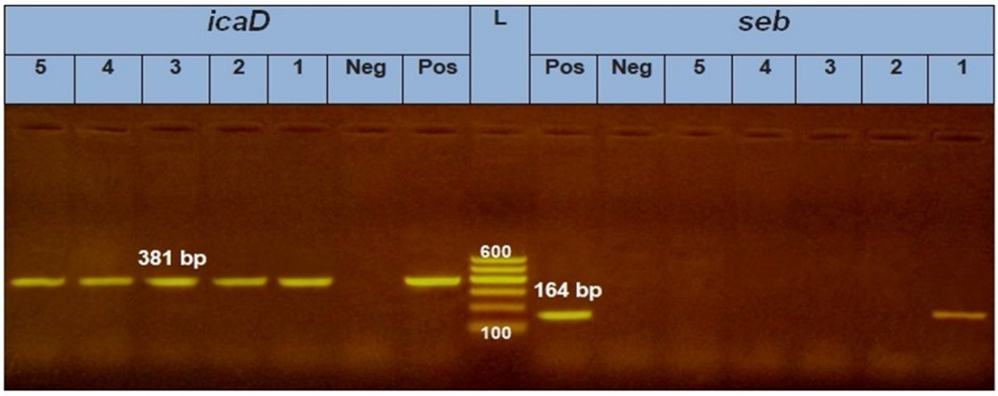

Photo. (1): Agarose gel photo documentation for detection of virulence factor encoding genes (***icaD*** *"Left side"* ***seb*** *"Right side"*) of *S . aureus* as a genotyping identification of the isolates. Lane L: molecular weight marker (100 – 600 bp). Results are as follow:
Lanes 1, 2, 3, 4, and 5 of the left side were positive samples for (***icaD***) gene with amplicon size of 381 bp.
Lane 1 of right side was positive while Lanes 2, 3, 4 and 5 for (**seb**) gene were negative with amplicon size of 164 bp.
Lanes Neg. of both sides: negative control. Lanes Pos. of both sides: positive control

Photo. (1): Agarose gel electrophoresis of cPCR amplified of (*hlg)* and *(coa) S. aureus* genes;

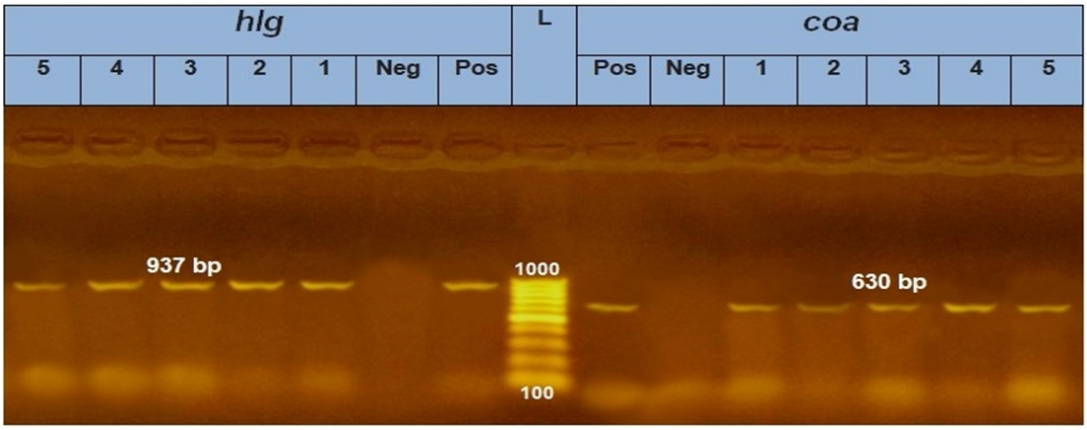

Photo. (2): Agarose gel photo documentation for detection of virulence factor encoding genes (***hlg*** *"Let side"* and ***coa*** *"Right side"*) of *S .aureus* as a genotyping identification of the isolates. Lane L: molecular weight marker (100 – 1000 bp). Results are as follow:
Lanes 1, 2, 3, 4, and 5 of the left side were positive samples for (***hlg***) gene with amplicon size of 937 bp.
Lanes 1, 2, 3, 4 and 5 of right side were positive for (**coa**) gene with amplicon size of 630 bp.
Lanes Neg. of both sides: negative control. Lanes Pos. of both sides: positive contro

Discussion

In our research; we investigated the prevalence of *S. aureus* in four most popular traditional Egyptian dairy desserts. The obtained results (Table, 2) showed that the highest prevalence percent of *S. aureus* was found in examined chocolate pudding (84%) and Om Ali (75%) samples followed by rice pudding (60%) and the lowest incidence percent was found in Mehalabeia whereas it could be detected in 40% of samples.

Meanwhile, from overall examined samples, 66% were positive for *S. aureus* and considered unfit for human consumption and not complied with Egyptian Standards for milk ice and other milk products **(ES, 1-1185/2005)** which stated that they must be free from pathogenic bacteria and their toxins.

The same table, our results showed that *S. aureus* counts in the examined rice pudding, Mehallabeia, chocolate pudding and Om Ali samples were ranged from 2 x 10^2 to 1.9 x 10^4, 1 x 10^2 to 2 x 10^3, 2 x 10^2 to 1.1 x 10^4 and from 1x10^2 to 3.3 x $10^3$with mean counts of 2.32x10^3, 3.2x10^2, 2.22x10^3 and 3.1x10^3 respectively. Although all these dairy desserts items are exposed to relatively high heat temperature during their processing, *S. aureus* could be detected in them with different high counts. These high levels of incidence and counts may be due to inadequate heat processing of contaminated raw milk and/ or the large diversity of raw ingredients or post-processing contamination due to unhygienic practices and poor hygienic conditions of processing facilities and equipment.

S. aureus prevalence in dairy products was common mainly from contaminated hides, food, and poor personal hygiene of the workers who deal with these products until served to consumer causes more than 90% of the sanitation problems in food service industry that handler's human contact as a result of coughing, sneezing or human contact with arm or hand lesion **(APHA, 2004)**.

Many studies on ready to eat dairy desserts have recorded various incidence percent and count levels of *S. aureus* contamination. **Hassan and Afifi (2016)** conducted a bacteriological quality assessment on some Egyptian dairy desserts in Beni-Suef city and recorded lower incidence percent of *S. aureus* in Rice and Mehalabia with nearly similar count of the pathogen in both products regarding our results.

Hussein *et al* (2015) could find lower incidence percent of *S. aureus* in for each of ice cream and rice with milk samples (in 15% of each) with an average of 6.7 X 10^5 and 2.7 X 10^7 cfu/g, respectively while they could not

detect it in Mehallabeia samples.

Ertas *et al* (2010) investigated the presence of *S. aureus* and staphylococcal enterotoxins (SEs) genes in sheep cheese and dairy dessert samples by multiplex PCR (mPCR) technique and could detect the pathogen in slight lower incidence percent, in 26 (52 %), dairy desserts samples.

Also, during 10-year inspection survey (2001-2010), a microbiological study of ready-to-eat foods from 15 canteens of the university campus was undertaken to determine their microbiological quality. The highest percentages of food borne pathogens were 12.5% *S. aureus* in desserts with dairy cream **(Kotzekidou, 2013)**.

In Konya, the microbiological quality of eighty units' milky desserts experimentally produced milky desserts have been analyzed in terms of *S. aureus* and it could be detected in 47 samples (58.57%) with average values $\log_{10}$ 0.87-3.67 cfu/gm. **(Yilmaz and Gurkan, 2014).**

The preparation and handling of foods may constitute the most direct and harmful source of microbiological contamination. The risk of contamination increased by storage of food at ambient temperature, by adding contaminated ingredients at stages were no further heat treatment was applied **(Ehirl *et al.,* 2001)**.

Staphylococcus aureus is one of the important causes of food-borne diseases in humans, *S. aureus* is commonly associated with intoxications due to its ability to produce a variety of potent enterotoxin **(Le Loir *et al.,* 2003).**

Molecular identification using **cPCR** technique on five positive *S. aureus* isolates recovered from rice pudding, Mehalabeia and chocolate pudding (one isolate of each) and two isolates of Om Ali, were used for the detection of classical enterotoxigenic associated genes (*Hlg, coa and icaD* and *seb*).

We could detect four pathogenic genes in the isolated *S. aureus* strains from which three genes (*Hlg, coa and icaD) were* found in three products isolates, Rice Pudding, Pudding and Om Ali, while *seb* pathogenic gene was detected only in Mehallabeia isolate.

These finding of *S. aureus* pathogenic genes clarify that even these detected low counts of the pathogen in examined dairy desserts can grow whereas a favourable growth conditions of temperature (above 10°C for 20 minutes), pH (ranged 4-9.5) and Wa (85-99%) . *S. aureus* can grow and produces their enterotoxins and poses their enterotoxins and can cause staphylococcal food poisoning to consumers of such products. **Ertas *et al* (2010)** concluded that even when the staphylococci count less than 10^5 cfu/g food, the organism was capable to produce enterotoxins in food.

As most of the dairy desserts consumers are children of vulnerable age groups, it is required to be microbiologically safe. Growth of enterotoxigenic *S. aureus* in both raw milk and dairy products poses a potential health hazard to consumers **(Pilar *et al,* 2009)**. *Staphylococcus aureus* produces many important virulence factors including Staphylococcal enterotoxins (SEs) which are responsible for Staphylococcal food poisoning.

Staphylococcal toxins are fast acting, sometimes causing illness in as little as 30 minutes after eating contaminated foods, but symptoms usually develop within one to six hours. Patients typically experience several of the following: nausea, retching, vomiting, stomach cramps and diarrhea. In more severe cases, dehydration, headache, muscle cramping, and changes in blood pressure and pulse rate may occur **(CDC, 2015).**

Other studies have reported that most *S. aureus* strains isolated from milk and dairy products harbored more than one toxin gene **(Katsuda *et al.,* 2005)**. **Ertas *et al.,* (2010)** could detect *sea*, *sec* and *sed* S. aureus enterotoxins genes in 3 (2.3%), 1 (0.76%), 1 (0.76%) of dairy desserts isolate they examined, respectively. They emphasized that the presence of *S. aureus* and their SEs genes in dairy desserts may be regarded as a potential risk for human health.

Different findings were reported by **Mathenge *et al*., (2005)** who concluded that out of the screened *S. aureus* isolates, *sea* was the most frequently detected gene, followed by *see.* Also, **Madahi *et al.,* (2014)** reported that the most commonly detected gene in *S. aureus* isolates was *sea*.

Food poisoning affects the public health and the economy in a negative way. In addition to its negative effects on human health, there are

work hour losses related to inefficient working or not being able to work. The health costs can cause bigger economical losses and may result in deaths **(Sahingoz and Sahin, 2009).** It is widely agreed that there has been a genuine increase in food poisoning. It is likely that a combination of

Conclusion and recommendations

We could conclude that the most popular examined Egyptian dairy desserts; Rice pudding, Pudding, Mehallabeia, and Om Ali could be contaminated with *S. aureus* either from raw milk and/ or the added different ingredients or post processing contaminations. This pathogen may carry different pathogenic genes that capable of growing, whereas a favourable growth conditions, and producing staphylococcal enterotoxins in such products and can constitute a public health hazards.

So, we could recommend that dairy desserts must be produced from safe and high quality raw milk and ingredients as well as should be processed under good hygienic practices. Also, the following control measures for improving the safety and quality of the traditional Egyptian dairy desserts must be followed:

• Implementation of good hygiene practices in dairy farms.

• Enforcement of food safety legislation on dairy shops are crucial as training of dairy and kitchen workers on good hygienic and processing practices including personal hygiene and sanitation rules. Food safety training to workers should strictly highlighted on washing hands and cleaning under fingernails vigorously with soap and water then sanitizing before handling and preparing food. Workers should not prepare food if they have a nose or eye infection, wounds or skin infections on their hands or wrists. In addition; store cooked food in a wide, shallow container and refrigerate as soon as possible and if food is to be out longer than two hours and keep hot foods hot (over 60°C) and cold foods cold (4°C or under).

Also, we could recommend that food safety training program should be obligatory applied for processing and selling workers of dairy shops, restaurants and supermarkets to be aware and educated on personal hygiene and sanitation rules during dealing with such products.`

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