

Studies on Anaerobic Bacteria in Some Cheeses Sold in Assiut City, Egypt

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ABSTRACT

A total of 200 random samples of Kareish, Domiati, Ras and Processed cheese (50 samples each) were collected from different dairy shops and supermarkets in Assiut City-Egypt, from February 2011 to May 2012 for the presence of anaerobes. By using plate method technique their percentages were 92, 96, 100 and 90% with average counts of 4.1×10^3 , 1.3×10^3 , 2.7×10^3 and 4.5×10^2 /g, respectively. *Cl. species* as *Cl. ramosum*, *Cl. perfringens*, *Cl. difficile*, *Cl. innoculum*, *Cl. septicum*, *Cl. novyi* type A, *Cl. cadaveris*, *Cl. sporogenes*, *Cl. hastiforme*, *Cl. butyricum* and *Cl. subterminale* were identified in various percentages from the concerning samples. *Cl. perfringens* were recorded in percentages of 12, 2, 22 and 10% with average counts of 3.7×10 , <10 , 8.4 and 2.2×10^2 /g, respectively by using MPN technique however, *Cl. botulinum* failed to be detected. Moreover, *Cl. perfringens* type A enterotoxines were determined by using enzyme linked immunosorbent assay (ELISA). Two samples of Ras cheese were positive with optical density of 0.262 and 0.314. In addition, lysozyme (Delvozyme) at concentration of 250 IU was found to be highly effective on growth rate of the vegetative form of *Cl. perfringens*. Public health as well as economical importance of detected microorganisms in the locally manufactured cheeses was discussed.

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Introduction

Milk products provide a highly favourable media for multiplication of different types of food poisoning microorganisms from different sources. The presence of anaerobes in dairy products is no assurance that they will develop but it would appear that if large numbers were involved the chance for their growth would be better than if only small numbers were included. Their presence is more difficult to control in dairy industry due to several reasons. First it seems to be impossible to completely avoid the presence of anaerobic spores in all milk samples. Secondary, the spores are very hydrophobic and will attach to pipelines surface of dairy plant, where they multiply and resporulate. A third problem is that pasteurization heating is insufficient to kill the spores, while competition from other vegetative bacteria is eliminated (Andersson *et al.*, 1995).

The genus *Clostridium* comprises nowadays up to 200 species of gram-positive spore-forming anaerobic rods, which are distributed worldwide. Fortunately, most of the species are harmless non-pathogenic bacteria living in the environment,

on plants, on the skin and on the mucosa of animals and humans. Therefore, their presence in milk products is indicative of faecal or soil contamination and most of them produce enterotoxins, which are pathogenic to man and animals, while some others have been reported to induce defects in some dairy products (Ron, 1973; Willis, 1977). In the genus *Clostridium*, the species *butyricum*, *pasteurianum*, *tyrobutyricum*, and *sporogenes* are associated with late gas spoilage in cheese (late blowing). They all produced acetic and butyric acids and this spoilage is called butyric fermentation and can create considerable economic loss in brine-salted soft cheese and semi-hard cheese production which used in manufacturing of processed cheese (Bergere and Sivala, 1990). Some strains that grow in canned cheese, however, don't produce gas and therefore cause no abnormal appearance of the can; nevertheless, they cause spoilage of the product (Landry *et al.*, 2001). *Cl. difficile* has also emerged as a pathogen or commensal in various animal such as pigs, calves and chickens and describe its presence in soil and water (Gould and Limbgo, 2010; Rupnik and Songer, 2010). The two clostridia of most significance with respect to foodborne illness are *Cl. botulinum* and *Cl. perfringens*. Foodborne botulism is intoxication due to consumption of preformed neurotoxins of *Cl. botulinum* in foods. Although relatively rare, foodborne botulism is a serious disease that is often fatal, and treatment usually involves long-term hospi-

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talization. Infant botulism is an infection of the gut of infants less than 12 months of age. On the other hand, *Cl. perfringens* type A is associated with food poisoning outbreak in man (Peck et al., 2004). It is a gram-positive, sporeforming rod and it is an obligate anaerobe and is non-motile. It causes a mild but common type of food poisoning and its spores are heat resistant, surviving normal cooking. Primarily the formation of enterotoxins that cause the typical symptoms is associated with *Cl. perfringens*. The toxin is formed in the human intestine and has started to sporulate (Roberts et al., 1996). *Cl. perfringens* food poisoning typically causes severe abdominal pain and prolific diarrhoea, sometimes fever, nausea and even vomiting occur. Normally large number of cells need to be ingested to cause illness and recovery is usually rapid (24-48 hrs). Deaths in elderly and debilitated people have occurred from *Cl. Perfringens*, but are extremely rare. Their spores are widely spread in soil, dust and marine sediment and also found in faeces of many animals, including on occasions man (Bates, 1997). Incidence of *Cl. perfringens* outbreaks varies according to countries and cooking practices. A recent report from WHO shows that *Cl. perfringens* is the second or third cause of reported foodborne disease outbreaks representing 4-16%. In each country, outbreaks number has changed with time and this could be due to changes in cooking practices (Petit et al., 1999).

Pre-formed toxin of *Cl. perfringens* in food sometimes occurs but is not usually in sufficient quantities to cause illness and destroyed by heating at 60°C for 10 min (Roberts et al., 1996). Contamination with *Clostridium*, usually responsible for late gas formation in hard cheeses during late ripening, to control or prevent this defect, many attempts has been approached (Carminati et al., 1985). In Egypt, cheese makers used to add some preservatives to cheese milk, particularly, during the hot months of the year to extend its shelf life especially in Ras cheese, which is a hard cheese variety widely accepted by Egyptian consumers. Cow's milk or a mixture of cow's and buffalo's milk are used in manufacture and stored 4 to 6 months after processing for ripening (Zaki, 1988). The effect of these preservatives on limiting cheese blowing was investigated by Nassib et al. (1994).

Nowadays, lysozyme has been effectively destructive on vegetative forms of *Clostridium* bacteria, which found to survive the normal heat treatment of milk used in cheese production. This has led to the wide-spread use of lysozyme, which is an enzyme has the potency of lysis of bacterial cells and detected as widespread enzyme among animals and plants and involved as a natural defense against pathogenic bacteria (Chiang et al., 2006; Nakimbugwe et al., 2006; Scaman et al., 2006). Most of lysozyme is commercially produced from egg white and its enzymatic activity carried out through its lytic function on glycosidic bonds present between N-acetyl muramic acid and N-acetyl glucosamine of cell wall peptidoglycon (Gill and Holley, 2003; Chung and Hancock, 2005). Its activity is variable relatively, but in general gram positive bacteria are more sensitive than gram negative bacteria and among gram positive bacteria thermophilic spore formers are more susceptible (Nattress and Baker, 2003; Vannini et al., 2004).

Therefore, this work was planned to enumerate; isolate and identify *Clostridium* species as well as detection of *Cl. perfringens* type A enterotoxin by using enzyme linked immunosorbent assay (ELISA) in Kareish, Domiati, Ras and Processed cheeses to explore their public health and economic significance. Moreover, the effect of lysozyme on the growth rate of enterotoxigenic *Cl. perfringens* type A in Ras cheese was also determined.

Materials and methods

Part I: Detection of anaerobic bacteria in some cheeses

A total of 200 random samples of Kareish, Domiati, Ras and Processed cheeses, (50 samples each) were purchased from different dairy shops and supermarkets from February 2011 to May 2012 in Assiut city, Egypt. All samples were delivered promptly to the laboratory with a minimum of delay for bacteriological examination and prepared according to L.M.B.G. (1991).

Clostridium species were enumerated by plate method using Reinforced Clostridial agar (RCM) as described by I.C.M.S.F. (1978) and isolated according to Wen and McClane (2004). Moreover, *Cl. perfringens* were enumerated by MPN technique using lactose sulphite broth (LSB) (Beerens et al., 1980) and their counts were recorded by using MPN Tables. The isolates were identified by Morphological characters (Holt et al., 1994), Biochemical reactions (Krieg and Holt, 1984) and Reaction on egg yolk media as described by Walker (1990).

Part II: Detection the enterotoxigenicity of *Cl. perfringens* type A by using ELISA

This part has been done in the Genetic Engineering and Molecular Biology Research Centre in Assiut University, Egypt, using Bio-x Alpha toxin ELISA kit (Biok 084) (Bio-X Diagnostics-Belgique, 2008). Selected strains of the isolated *Cl. perfringens* were tested for their ability to produce enterotoxins.

Part III. Effect of lysozyme on the growth rate of enterotoxigenic *Cl. perfringens* inoculated in Ras chesse

Culture preparation

Enterotoxigenic *Cl. perfringens* strain used was previously isolated and well identified from the examined Ras cheese samples. The organism was propagated in thioglycolate broth at 37°C for 48hrs and its number /ml of broth was counted. The culture was kept in refrigerator till inoculation into pasteurized milk used for manufacture of Ras cheese. Enumeration of the viable cells was performed by pour plate count technique on sulphite - polymyxin - sulfadiazine agar (SPS agar).

Preparation of milk for manufacture of Ras cheese

Raw cow's milk (3-5% fat) was pasteurized at 80°C for 15 sec, then tempered at 32°C and inoculated with 10⁶ cfu /ml of *Cl. perfringens* culture. The inoculated milk was divided into three equal portions (each of 20 litres). Lysozyme obtained from Delvozyme-DSM Food Specialties-Holland, was added to the first portion to give a concentration 250 IU/ml milk as a treatment one (T1), and to the second portion to give a concentration 500 IU/ml milk as a treatment two (T2). Third portion was left as a control without addition of lysozyme. Ras cheese was made from the three treatments according to Hofi et al. (1970) with a slight modification in a dairy plant in Assiut City.

Examination of inoculated Ras cheese during manufacture and storage

Samples were taken from inoculated milk, curd, curd after scalding, from end product and then from the cheese after 24 and 72 hrs to determine *Cl. perfringens* count. Samples were prepared according to A.P.H.A. (1992).

Enumeration of enterotoxigenic strain of *Cl. perfringens* during manufacture and storage of Ras cheese

plates of SPS agar medium. All plates were incubated at 46°C for 24hrs and the cfu /g was calculated and recorded.

From the prepared samples, 10 fold serial dilutions were prepared using sterile 0.1% peptone water for determination of *Cl. perfringens* count using spread method on SPS agar. 0.1 ml of each dilution was carefully transferred into duplicate

Results

The obtained results were recorded in Tables 1-5 and Fig.1.

Table1. *Clostridium species* count / g of some cheeses using surface plating technique

Examined samples	Positive samples		Count / g		
	No./ 50	%	Min.	Max.	Average
Kareish cheese	46	92	*<10 ²	8.0 ×10 ³	4.1 ×10 ³
Domiaty cheese	48	96	*<10 ²	6.6 ×10 ³	1.3 × 10 ³
Ras cheese	50	100	*<10 ²	1.2 ×10 ⁴	2.7 ×10 ³
Processed cheese	45	90	*<10 ²	3.4 ×10 ³	4.5 ×10 ²

*Colonies could not be detected on the plates.

Table 2. Incidence of different *Clostridium species* in the examined cheese samples using surface plating technique

<i>Clostridium species</i>	Kareish cheese		Domiaty cheese		Ras cheese		Processed cheese	
	Positive samples		Positive samples		Positive samples		Positive samples	
	No./50	%	No./50	%	No./50	%	No./50	%
<i>Cl. ramosum</i>	8	16	11	22	16	32	10	20
<i>Cl. perfringens</i>	6	12	1	2	11	22	5	10
<i>Cl. difficile</i>	5	10	4	8	5	10	10	20
<i>Cl. innoculum</i>	4	8	1	2	-	-	-	-
<i>Cl. septicum</i>	2	4	11	22	11	22	11	22
<i>Cl. novyi type A</i>	-	-	6	12	1	2	1	2
<i>Cl. cadaveris</i>	15	30	5	10	2	4	1	2
<i>Cl. sporogenes</i>	-	-	2	4	3	6	-	-
<i>Cl. hastiforme</i>	1	2	3	6	1	2	-	-
<i>Cl. butyricum</i>	5	10	2	4	-	-	-	-
<i>Cl. subterminale</i>	-	-	2	4	-	-	7	14

Table 3. *Clostridium perfringens* count in the positive samples using MPN technique/g.

Examined samples	Positive samples		Count / g		
	No./50	%	Min.	Max.	Avg. of +ve samples
Kareish cheese	6	12	<10	1.5×10 ²	3.7×10
Domiaty cheese	1	2	<10	<10	<10
Ras cheese	11	22	<10	2.8×10	8.4
Processed cheese	10	20	<10	1.1×10 ³	2.2×10 ²

Table 4. Detection of *Clostridium perfringens* type A α-Toxin by Elisa in cheeses samples.

Examined samples	No. of examined samples	Positive α-Toxin		Negative α-Toxin	
		No.	%	No.	%
Kareish cheese	6	0	0	6	100
Domiaty cheese	1	0	0	1	100
Ras cheese	11	2	18.2	9	81.8
Processed cheese	10	0	0	10	100
Total	28	2		26	

Table 5. Effect of lysozyme on growth rate of enterotoxigenic *Cl. perfringens* during manufacture and storage of Ras cheese.

Examined samples	Treatment		
	T1*	T2*	C*
Milk	10 ⁶	10 ⁶	10 ⁶
Curd	6.1×10 ⁴	1.1×10 ³	4.5×10 ⁵
Curd after scalding	9.8×10 ³	1.2×10 ²	3.5×10 ⁴
Cheese at 0h	5.2×10	NC	1.2×10 ⁴
Cheese at 24hrs	*NC	NC	3.6×10 ⁵
Cheese at 72hrs	NC	NC	7.2×10 ⁶

*T1: treatment with 250 IU lysozyme /ml milk; *T2: treatment with 500 IU lysozyme /ml milk; *C: Control; *NC: non counted

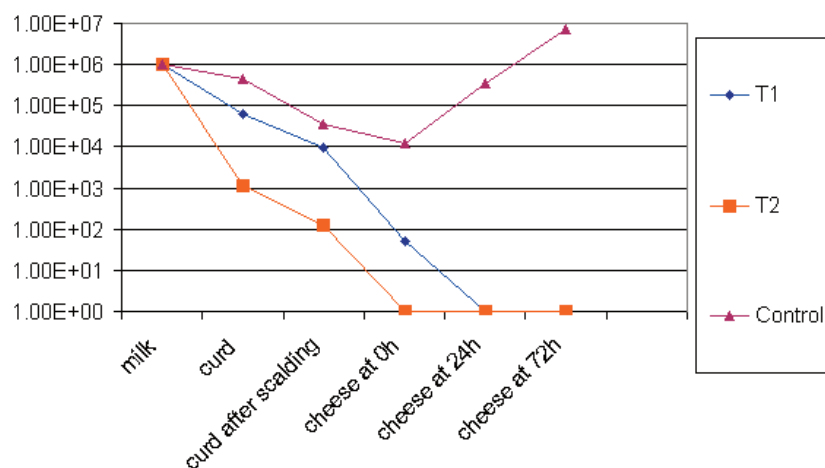


Fig 1. Effect of lysozyme on growth rate of enterotoxigenic *Cl. perfringens* during manufacture and storage of Ras cheese.

Discussion

From data represented in Table 1, it is evident that 46 (92%) of examined Kareish cheese samples contained *Clostridium* organisms. The counts ranged from $<10^2$ to 8.0×10^3 /g with an average count of 4.1×10^3 . The achieved results were higher than that obtained by Amer (1982); Amer *et al.* (1986); Wahba (1997). However, lower incidence was recorded by Abo-Donia *et al.* (1975).

In Domiati cheese the counts were ranged from $<10^2$ to 6.6×10^3 /g, with an average count of 1.3×10^3 /g. This finding was higher than that reported by Amer (1982). Moreover, all of examined Ras cheese samples were contaminated in counts ranged from $<10^2$ to 1.2×10^4 with an average of 2.7×10^3 /g. This result was in coinciding with that recorded by Nazem and Saleh (1994), while lower value was postulated by Varga (2007) in hard cheeses.

Concerning processed cheese, it evident that *Clostridium* organism was detected in 45 (90%) of samples, in count varied from $<10^2$ to 3.4×10^3 with an average count of 4.5×10^2 /g (Table 1). This result was higher than those obtained by Amer *et al.* (1986); Nazem (1992); Dardir (2005); Lycken and Borch (2006), but lower finding was detected by Al-Ashmawy *et al.* (1977).

The presence of anaerobes in cheese is considered to be indicative of manure and soil contamination. Several factors may account of the presence of *Clostridium* in both types of Domiati and processed cheese, including lack of maximum limits for spore number in raw milk. Moreover, the pH of final product was conducive for the viability of this pathogen (Bergere and Sivela, 1990).

Results recorded in Table 2 showed that *Cl. ramosum*, *Cl. perfringens*, *Cl. difficile*, *Cl. innoculum*, *Cl. speticum*, *Cl. novyi* type A, *Cl. cadaveris*, *Cl. sporogenes*, *Cl. hastiforme*, *Cl. butyricum* and *Cl. subterminale* were recovered from all of the evaluated samples in various percentages ranged from 1 to 32%.

Higher findings were indicated by El-Bassiony (1980); El-Leboudy (1985); El-Sayed (1985); Abd El-Hakiem (1992) for *Cl. perfringens* in case of Kareish cheese samples.

These findings support concerns about foodborne acquisition of *Cl. difficile* through consumption or handling of contaminated food product (de Boer *et al.*, 2011).

As regard the results of Domiati cheese, nearly similar species of *Clostridium* could be isolated from soft cheese examined by Saudi (1980). However, higher findings for *Cl. perfringens* were detected in feta cheese examined by El-Bassiony (1980); Abd El-Hakiem (1992); Ahmed and Abd El-Gaber

(1994).

In case of Ras cheese, Matteuzzi *et al.* (1972); Korhonen *et al.* (1977) detected higher incidence for *Cl. butyricum* and *Cl. sporogenes* in Grana cheese (hard cheese) and Emmental cheese, respectively. However, lower result for *Cl. perfringens* was demonstrated by El-Bassiony (1980) in hard cheese. Furthermore, lower incidence for *Cl. perfringens* were indicated by El-Bassiony (1980); Sallam (1981); Amer *et al.* (1986); Ahmed and Abd El-Gaber (1994); Dardir (2005) as comparing with the results of Processed cheese.

Although cooking of food will destroy the vegetative cells yet the spores will survive in processed cheese and indicate unsatisfactory processing, mishandling faecal or soil contamination. *Cl. sporogenes*, *Cl. butyricum* and *Cl. bifermentans* may occasionally produce gassy defects, purification and spoilage of processed cheese when held at favourable environmental conditions. Therefore, their presence may be responsible for its inferior quality resulting in economic losses (Willis, 1977; Frazier and Westhoff, 1978; I.C.M.S.F., 1978).

The results outlined in Table 3 indicated the incidence and count of *Cl. perfringens* using MPN Technique. They could be detected in 6 (12%) of examined Kareish cheese samples in a range between <10 and 1.5×10^2 with an average count of 3.7×10 /g. Nearly similar count was obtained by Wahba (1997) while lower results were recorded by El-Bassiony (1980); Abd El-Hakiem (1992). However, higher incidences were recorded by Abo-Donia *et al.* (1975); El-Bassiony (1975); Shelaih (1979); El-Leboudy (1985); El-Sayed (1985); Abd El-Hakiem (1992).

Cl. perfringens was detected in one sample of Domiati cheese (2%) and had count < 10 /g. Higher counts were recorded by El-Bassiony (1980), Abd El-Hakiem (1992), Ahmed and Abd El-Gaber (1994); Dardir (2005). On the contrary, Wahba (1997) failed to detect *Cl. perfringens* in the examined samples of Domiati cheese.

Concerning Ras cheese, *Cl. perfringens* was detected in 11(22%) samples with a minimum of <10 , a maximum of 2.8×10 and an average of 8.4 organisms/g. These findings were lower than that postulated by El-Bassiony (1980); Varga (2007) in hard cheese. Furthermore, the min., max. and average counts of Processed cheese samples were < 10 , 1.1×10^3 and 2.2×10^2 /g, respectively. These results were higher than that recorded by El-Bassiony (1980); Ahmed and Abd El-Gaber (1994) but agreed to a certain extent with that indicated by Dardir (2005).

Detection of *Cl. perfringens* Alpha Toxin in culture supernatants, using Bio-X Alpha Toxin Elisa Kit (BioK084), is produced in varying amount by all biotypes (A, B, C, D and E) and considered a primary virulence factor involved in *Clostridial myonecrosis*.

Data postulated in Table 4, indicated that 2(18.2%) samples of Ras cheese only was positive for Alpha Toxin. The positive samples have optical density 0.262 and 0.314 where the limit of positively for antigen is 0.150.

Alpha Toxin is the only lethal protein produced during vegetative growth of type A strains, the most ubiquitous *Cl. perfringens* biotype that is commonly found in soil and normal intestinal flora of humans and animals. Due to its role in gas gangrene, food poisoning and animal enterotoxigenicity *Cl. perfringens* type A strain, particularly the Alpha Toxin have been the subject of intense investigations over the past 60 years (McDonel, 1986).

It is evident from Table 5 and Fig. 1, that *Cl. perfringens* numbers decreased suddenly in Ras cheese prepared from milk containing a concentration of 250 IU/ml added lysozyme from 10^6 /ml to reach 6.1×10^4 cfu/g of curd before scalding. The count of *Cl. perfringens* reached to 5.2×10 by the end of cheese making and could not be counted thereafter.

Bester and Lombard (1990) detected the same effect of lysozyme at concentration of 250 IU/ml on *Cl. tyrobutyricum* in Gouda cheese, while Zhang et al. (2006) obtained the same effect at concentration 156 IU/ml.

While in Ras cheese prepared from milk containing 500 IU/ml added lysozyme and inoculated with 10^6 *Cl. perfringens* /ml, the organism lost its viability and decreased sharply to reach 1.1×10^2 cfu/g of curd after scalding. *Cl. perfringens* could not be counted by the end of the preparation and thereafter (Table 5 and Figure 1). The same effect was recorded on *Cl. tyrobutyricum* by Wasserfall and Teuber (1979).

In case of cheese prepared from milk inoculated with 106 *Cl. perfringens* /ml without addition of lysozyme, the organism decreased very slowly and gradually to reach 1.2×10^4 cfu/g of prepared Ras cheese at 0 times. The organisms started to increase in number to reach 3.6×10^5 after 24h and 7.2×10^6 after 72 hrs of its preparation (Table 5 and Fig. 1). These results were in agreement with that recorded by Ghita et al. (1998).

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