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Prevalence and Characterization of Aerobic Spore Forming Bacteria in Raw Milk and Some Cheeses

Khater, K. A. A. and S. A. S. Abdella
Dairy Department, Faculty of Agriculture, Al Azhar University, Cairo, Egypt.

ABSTRACT

Prevalence of aerobic spore forming bacteria in raw milk, Ras cheese and Domiati cheese was investigated. Total bacterial and total aerobic spore forming counts were enumerated by using tryptone soy agar medium. All isolated aerobic sporeformers were identified on genus and species levels. The minimum, maximum and the means of total aerobic sporeformers counts in raw milk samples were 3.2×10^3, 2.7×10^4 and 1.5×10^4 cfu/ml, respectively. Counts in Ras cheese samples were, 2×10^2, 3.5×10^4 and 1.8×10^4 cfu/g, and in Domiati cheese samples being, 1.2×10^3, 2.3×10^4 and 1.2×10^4 cfu/g, in the same order. A total of 60 *B. cereus* group of bacterial cultures isolated from market dairy products were identified as *B. anthracis* 39(65%), *B. cereus* 6(10%), *B. mycoides* 13(21.7%) and *B. thuringiensis* 2(3.3%). Results also show that all tested 60 isolates could be plotted in different patterns from A to G, e.g. *B. cereus* (A), *B. mycoides* (B, C and D), *B. thuringiensis* (E) and *B. anthracis* (F and G).
Keywords: aerobic spore forming bacteria, milk products, physiological properties, prevalence, identification.

INTRODUCTION

Spore forming bacteria are usually isolated from silage, bedding, water, manure and paper towels. They exist in a dormant state as spores, which can survive many of the unfavorable conditions such as high heat (e. g., pasteurization), drying, acidity and radiation. Sporeformers belonging to the aerobic Bacillus and anaerobic Clostridia usually found in multitude of farm sources, from which they could infect the raw milk, and consequently germinate, and hence infect the cheese being made of this milk. Spore forming bacteria are usually heat-resistant, and when present in a variety of foods, resulting in their deterioration. Therefore, they could be easily isolated from spoilage foods(Rodriguez-Lozano *et al.,* 2010Martin, 2014). *et al.,* 2010Martin, 2014).. Seven closely related species of B. cereus group were identified by (Guinebretiere *et al.,* 2013).

Therefore, prevalence, identification and characterization of aerobic spore forming bacteria isolated from Raw milk and some cheeses were studied in the present work.

MATERIALS AND METHODS

Milk and some dairy products samples (cheeses) were collected from Cairo markets in sterile glass containers or sterilized polyethylene bags. Samples were transferred to laboratory in ice box and analyzed at the same day. 1ml of each market milk sample was added to sterilized tubes with 9ml of sterilized physiological solution, and the serial dilution was applied. And then heated at 80 ºC for 10 min and cooled in melting ice preior to plating. Heating was applied within 5 min after blending in order to prevent germination of spores during sample preparation.

Total bacterial counts, total spore counts and the identification of the isolated cultures were determined using the tryptone Soy Agar (TSA), followed by incubation for 24 hr at 30 ºC under aerobic condition (APHA,1992). Voges-Proskaur test, nitrate reduction, starch hydrolysis, acid and gas production by the isolated cultures from glucose, motility, hemolysis, rhizoid growth, production of toxin crystals and sensitivity to penicillin were all carried according to (APHA,1992).

RESULTS AND DISCUSSION

Prevalence of aerobic spore forming bacteria in studied Raw milk and some cheeses:

The incidence of spore forming bacteria in raw milk, Ras cheese and Domiati cheese were investigated. Besides, comparative study of the total bacterial and total aerobic spore forming counts was performed. Tables (1and 2) show the total bacterial and total aerobic spore forming counts in 30 samples of milk and cheeses by using tryptone soy agar medium (TSA).

Table 1. Total bacterial and aerobic spore forming counts (cfu/ml) in studied Raw milk, Ras and Domiati cheeses by using tryptone soy agar.

Samples	TBC	ASFC	Samples	TBC	ASFC	Samples	TBC	ASFC
Rm1	2.5×10^9	2.7×10^4	Rc1	4.2×10^7	3.2×10^3	Dc1	4×10^8	1.5×10^4
Rm2	4.2×10^8	5×10^3	Rc2	5.3×10^7	4×10^3	Dc2	6×10^8	2.3×10^4
Rm3	2.9×10^9	1.2×10^4	Rc3	2.2×10^8	6.2×10^3	Dc3	5×10^6	1.2×10^3
Rm4	5.3×10^9	1.7×10^4	Rc4	3.6×10^8	3.5×10^4	Dc4	1.1×10^7	4.2×10^3
Rm5	2.8×10^8	6.3×10^3	Rc5	8.5×10^6	2.2×10^3	Dc5	3.9×10^7	2.8×10^3
Rm6	7.5×10^8	7×10^3	Rc6	2.5×10^7	5×10^3	Dc6	2.5×10^8	4.5×10^3
Rm7	1.4×10^9	2.2×10^4	Rc7	3.4×10^7	1.2×10^3	Dc7	2.9×10^8	1.2×10^4
Rm8	3.7×10^8	4.2×10^3	Rc8	1.5×10^8	3.2×10^4	Dc8	4.2×10^7	6.5×10^3
Rm9	8.2×10^7	3.2×10^3	Rc9	3.8×10^7	2×10^2	Dc9	7.3×10^6	2.2×10^3
Rm10	5×10^8	3.5×10^3	Rc10	5.4×10^7	1×10^3	Dc10	2.8×10^7	7.7×10^3

Rc= Ras cheese Dc= Domiati cheeseRm= Raw milk TBC= Total bacterial counts ASFC= Aerobic spore forming counts

Table 2. Minimum, maximum and mean of total bacterial and total aerobic spore forming counts in studied dairy samples.

Samples	Total bacterial counts			Total spore forming counts Aerobic		
	Min	Max	Mean	Min	Max	Mean
Raw milk	8.2×10^7	5.3×10^9	2.7×10^9	3.2×10^3	2.7×10^4	1.5×10^4
Ras cheese	8.5×10^6	3.6×10^8	1.8×10^8	2×10^2	3.5×10^4	1.8×10^4
Domiati cheese	5×10^6	6×10^8	3×10^8	1.2×10^3	2.3×10^4	1.2×10^4

The total bacterial counts in raw milk samples on tryptone soy agar medium showed similar minimum and maximum log counts of 108 and 109 except, Rm9 (107). While the minimum and maximum log counts of aerobic

spore forming bacteria grown on the same medium were mostly similar (103 to 104). Nearly similar findings were reported by Abdallah. 1997, Ghellai and Moussaboudjemaa. 2013, and Saad and Ahmed. (2013).

The minimum, maximum and mean counts of total bacterial in Ras cheese samples grown on tryptone soy agar medium being, 8.5×106, 3.6×108 and 1.8×108 cfu/ml, respectively. In addition, total aerobic spore forming counts grown on the same medium also showed minimum, maximum and mean counts being, 2×102, 3.5×104 and 1.8×104 cfu/ml, respectively. The obtained results are lower than those reported by Khater. (2001).

Also, total bacterial counts in Domiati cheese samples on tryptone soy agar medium showed minimum, maximum and mean counts, being 5×106, 6×108 and 3×108 cfu/ml, respectively. In addition, total aerobic spore forming counts showed minimum, maximum and mean counts, being 1.2×103, 2.3×104 and 1.2×104 cfu/ml, respectively. Similar results were reported by Khater. 2001 Saad and Ahmed. (2013) for the mean values of total aerobic spore counts in soft Dommietta cheese.

Identification and characterization of aerobic spore forming bacteria.

In this study we used Bacillus Cereus Selective Agar (BCSA) medium (Oxoid) which based on the highly specific, diagnostic and selective Polymyxine Egg yolk Mannitol Bacillus Agar (PEMBA) medium. All Bacillus isolates from BCSA medium were confirmed by appropriate tests described by APHA. (1992), Andrews. (1992), Iso – 7932. (1993), Giffil et al. (1995) and Logan *et al.* (2011). Previously, Harmon. (1982), Harmon and Goepfert. (1984) and Bergey's manual. (1986) B. mycoides, B. thuringiensis and B. anthracis to be differentiated.

A total of 60 Bacillus spp. gave typical growth on BSCA, isolated from market milk and some dairy products were studied for some physiological properties. These tests were recommended for identification of Bacillus group and being, Starch hydrolysis (SH), Voges proskaur reaction (VP), Nitrate reduction (NR), Glucose fermentation (GF), Motility (M), Egg yolk reaction (EY), Hemolytic activity (HA), Rhizoid growth (RG), Toxin crystal produced (TC) and Sensitivity to penicillin (SP). Untabulated results showed that all isolated cultures gave Catalase positive, Gram positive and were Spore forming bacteria. It is clear also from literature that this group can be differentiated from B. cereus by one or more characteristics.

Absolute separation of these four B. cereus groups into distinct species is not possible in all instances (APHA, 1992). However, typical characteristics of B. cereus are quite stable, where the other three biotypes usually differentiated. Gordon *et al.* (1973) have classified B. mycoides, B. anthracis and B. thuringiensis as closely related Bacillus spp. to B. cereus, Ash et al. (1991) and Carlson. (1994 Hsieh *et al.* (1999) B. anthracis, B. mycoides and B. thuringiensis could be considered as subspecies of B. cereus based on phenotype and genetic properties.

The preliminary identification of aerobic spore forming isolates was performed and the results indicated that morphological characteristics of isolates were bacilli. The Gram staining techniques showed that all isolates were gram positive and from the Catalase test all isolates were found to be positive.

As shown from Table (3) in Raw milk samples, the biochemical tests results revealed that all isolates gave positive reactions with starch hydrolysis, voges proskaur and acid from glucose tests and gave negative reactions with gas from glucose, motility and toxin crystal produced. In addition, 19(95%), 18(90%) and 12(60%) gave positive reactions with egg yolk reaction, sensitivity to penicillin and nitrate reduction, respectively. However, only 2(10%) of isolates were positive reactions with hemolytic activity and had typical rhizoid growth.

Table 3. Number and percentage of some biochemical tests of aerobic spore forming bacteria isolated from Raw milk, Ras and Domiati cheeses.

Tests	Raw milk				Ras cheese				Domiati cheese			
	No.P	%	No.N	%	No.P	%	No.N	%	No.P	%	No.N	%
Starch hydrolysis	20	100	0	0.0	20	100	0	0.0	20	100	0	0.0
Voges proskaur	20	100	0	0.0	20	100	0	0.0	20	100	0	0.0
Nitrate reduction	12	60	8	40	13	65	7	35	14	70	6	30
Acid from glucose	20	100	0	0.0	20	100	0	0.0	20	100	0	0.0
Gas from glucose	0	0.0	20	100	0	0.0	20	100	0	0.0	20	100
Motility	0	0.0	20	100	5	25	15	75	3	15	17	85
Egg yolk reaction	19	95	1	5	20	100	0	0.0	19	95	1	5
Hemolytic activity	2	10	18	90	10	50	10	50	9	45	11	55
Rhizoid growth	2	10	18	90	5	25	15	75	6	30	14	70
Toxin crystal produced	0	0.0	20	100	2	10	18	90	0	0.0	20	100
Sensitivity to penicillin	18	90	2	10	10	50	10	50	11	55	9	45

No.P= Number of positive isolates No.N= Number of negative isolates

Results of Table (3) show that all isolated Bacillus spp. from Ras cheese samples gave positive reactions with starch hydrolysis, voges proskaur, acid from glucose and egg yolk reactions tests and gave negative reaction with gas from glucose test. In addition, 13(65%), 10(50%), 10(50%), 5(25%) and 5(25%) were positive reactions with nitrate reduction, hemolytic activity, sensitivity to penicillin, motility and had typical rhizoid growth, respectively. However, only 2(10%) were positive reaction with toxin crystal produced.

It could be extracted from Table (3) that all isolated Bacillus spp. from Domiati cheese samples gave positive reactions with starch hydrolysis, voges proskaur and acid from glucose tests and gave negative reactions with gas from glucose and toxin crystal produced. Also, 19(95%), 14(70%), 11(55%), 9(45%) and 6(30%) were positive reactions with egg yolk reactions, nitrate reduction, sensitivity to penicillin, hemolytic activity and had typical rhizoid growth, respectively. However, only 3(15%) were positive reaction with motility.

It is still possible to discriminate the B. cereus group strains, on the basis of various phenotypical traits and found that B. mycoides exhibit a typical rhizoid growth whereas the rest of the species have round to irregular colonies. B. thuringiensis strains produce para – sporal toxin crystals with insecticidal properties. Finally, B. anthracis strains are non-hemolytic on sheep blood agar (SBA) plates and sensitive to penicillin as shown in Table (4). Nakamura. 1998, Nour *et al.* 2002, Papaparaskevas *et al.* 2004, and Vachon *et al.* 2012).

Table 4. Identification patterns of aerobic spore forming bacteria isolated from milk and some dairy products.

Species	Phenotypic characteristic											No. of Isolates	Patterns
	SP	TC	RG	HA	EY	M	GF		NR	VP	SH		
							Gas	Acid					
B.cereus	-	-	-	+	+	+	-	+	+	+	+	6	A
	-	-	+	+	-	-	-	+	-	+	+	2	B
B.mycoides	-	-	+	+	+	-	-	+	-	+	+	7	C
	-	-	+	+	+	-	-	+	+	+	+	4	D
B.thuringiensis	-	+	-	+	+	+	-	+	+	+	+	2	E
B.anthracis	+	-	-	-	+	-	-	+	-	+	+	12	F
	+	-	-	-	+	-	-	+	+	+	+	27	G

SH = Starch hydrolysis **VP = Voges proskaur** **NR = Nitrate reduction** **M = Motility**
GF = Glucose fermentation **EY = Egg yolk reaction** **HA = Hemolytic activity** **RG = Rhizoid growth**
TC = Toxin crystal produced **SP = Sensitivity to penicillin**

All of the isolated spores (60) from BSCA medium from different sources (Table 6) were identified as B. cereus (6), B. mycoides (13), B. thuringiensis (2) and B. anthracis (39).

Table (6) shows identification of Bacillus spp. isolated from different sources. Results of this table show that all isolated aerobic spore formers were B. cereus group. Of isolated spores from Raw milk and dairy product samples, 6(10%) were identified as B. cereus, 13(21.7%) were B. mycoides, 2(3.3%) were B. thuringiensis and 39(65%) were B. anthracis.

It could be extracted from Tables (5 and 6) that out of 20 isolated aerobic spore formers from Raw milk samples 18(90%) were B. anthracis and 2(10%) were B. mycoides (Rm5-2 and Rm10-2). In this respect, Raw milk was free from B. cereus and B. thuringiensis. Out of 20isolated aerobic spore formers from Ras cheese samples 10(50%) were B. anthracis, 3(15%) were B. cereus (Rc5-1, Rc5-2 and Rc6-1), 5(25%) were B. mycoides (Rc2-1, Rc3-1, Rc3-2, Rc4-1 and Rc8-1) and 2(10%) were B. thuringiensis (Rc1-1 and Rc1-2). Also, out of 20 isolated aerobic spore formers from Domiati cheese samples 11(55%) were B. anthracis, 3(15%) were B. cereus (Dc8-1, Dc10-1 and Dc10-2) and 6(30%) were B. mycoides (Dc1-1, Dc1-2, Dc2-1, Dc5-2, Dc7-1 and Dc9-2). In this respect, Domiati cheese was free from B. thuringiensis.

Table 5. Distribution of identified *B. cereus* group among tested Raw milk, Ras and Domiati cheeses.

Species	Strains		
	Raw milk	Ras cheese	Domiati cheese
B.anthracis	Rm1-1, Rm1-2, Rm2-1, Rm2-2, Rm3-1, Rm3-2, Rm4-1, Rm4-2, Rm5-1, Rm6-1, Rm6-2, Rm7-1, Rm7-2, Rm8-1, Rm8-2, Rm9-1, Rm9-2, Rm10-1	Rc2-2, Rc4-2, Rc6-2, Rc7-1, Rc7-2, Rc8-2, Rc9-1, Rc9-2, Rc10-1, Rc10-2	Dc2-2, Dc3-1, Dc3-2, Dc4-1, Dc4-2, Dc5-1, Dc6-1, Dc6-2, Dc7-2, Dc8-2, Dc9-1
B.cereus	-	Rc5-1, Rc5-2, Rc6-1	Dc8-1, Dc10-1, Dc10-2
B.mycoides	Rm5-2, Rm10-2	Rc2-1, Rc3-1, Rc3-2, Rc4-1, Rc8-1	Dc1-1, Dc1-2, Dc2-1, Dc5-2, Dc7-1, Dc9-2
B.thuringiensis	-	Rc1-1, Rc1-2	-

Based on studied physiological properties all identified 60 B. cereus group, could be plotted in different physiological patterns. Table (4) shows the physiological patterns of B. cereus group, which are plotted from A to G e.g. B. cereus (A), B. mycoides (B, C and D), B. thuringiensis (E) and B. anthracis (F and G). All B. cereus strains, being 6 (10 %) out of the 60 gave positive with starch hydrolysis, voges proskaur, nitrate reduction, acid from glucose, motility, egg yolk reaction and hemolytic activity and gave negative with gas from glucose, rhizoid growth, toxin crystal produced and sensitivity to penicillin (pattern A).

Three different characteristic patterns (B, C and D) were recognized for B. mycoides. Contrary to B. cereus, B. mycoides strains were non motile and gave obvious rhizoid growth. This is in agreement with data in Table (4) as recorded by APHA. (1992) and Andrews. (1992). It could be seen from this table that the three B. mycoides patterns, are differentiated by their ability to produce nitrate and egg yolk reaction. B. mycoides strains, being 7 out of the 13 (21.7%) gave positive with starch hydrolysis, voges proskaur, acid from glucose, egg yolk reaction, hemolytic activity and rhizoid growth (pattern C). however, only 4 strains (pattern D) gave positive with nitrate reduction and egg yolk reaction, while two strains of pattern (B) gave negative with nitrate reduction and egg yolk reaction.

It could be extracted from Table (4) that all B. thuringiensis strains, being 2 (3.3%) out of the 60 gave positive with starch hydrolysis, voges proskaur, nitrate reduction, acid from glucose, motility, egg yolk reaction, hemolytic activity and toxin crystal produced (pattern E). Contrary to B. cereus, B. thuringiensis strains were toxin crystal produced.

Table (4) also, shows that B. anthracis was differentiated by two patterns, F and G. Differences were observed only with nitrate reduction test. B. anthracis strains, being 27 out of the 39 (65%) gave positive with starch hydrolysis, voges proskaur, nitrate reduction, acid from glucose, egg yolk reaction and sensitivity to penicillin (pattern G). However, only 12 strains (pattern F) gave positive with the same previous tests except nitrate reduction. Contrary to B. cereus, B. anthracis strains were non motile, non-hemolytic activity on sheep red blood cells and were sensitive to penicillin.

Table 6. Number and percentage of identified *B. cereus* group.

Species	No. of isolates						Total	
	Raw milk		Ras cheese		Domiati cheese			
	No	%	No	%	No	%	No	%
B.anthracis	18	90	10	50	11	55	39	65
B.cereus	0.0	0.0	3	15	3	15	6	10
B.mycoides	2	10	5	25	6	30	13	21.7
B.thuringiensis	0.0	0.0	2	10	0.0	0.0	2	3.3

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تواجد الجراثيم البكتيريه الهوائيه وخصائصها فى اللبن الخام وبعض الجبن

خاطر عبدالفتاح أحمد خاطر و شريف عادل سعد عبدإللا

قسم الألبان- كلية الزراعة - جامعة الأزهر

تم دراسة تواجد البكتيريا المكونه للجراثيم الهوائيه فى ١٠ عينات من كلا من اللبن الخام والجبن الراس والجبن الدمياطى من حيث العد الكلى . حيث تم عمل مقارنه للعد الكلى للبكتيريا وعد البكتيريا المكونه للجراثيم الهوائيه باستخدام وسط التربتون صويا اجار. من ناحية أخرى تم تعريف كل عزلات البكتيريا المكونه للجراثيم الهوائيه الى مستوى الجنس والنوع. أظهرت النتائج أن الحد الأدنى والحد الأقصى ومتوسط العدد للبكتيريا المكونه للجراثيم الهوائيه فى عينات اللبن الخام هو 3.2×10^{3}, 2.7×10^{4}, 1.5×10^{4} وحده مكونه للمستعمره وفى عينات الجبن الراس هو 2×10^{2}, 3.5×10^{4}, 1.8×10^{4} وحده مكونه للمستعمره وفى عينات الجبن الدمياطى هو 1.2×10^{3}, 2.3×10^{4}, 1.2×10^{4} وحده مكونه للمستعمره. لقد تم تعريف ودراسة الخصائص الفسيولوجيه لمجموعه من ٦٠ عزلـه من عينات لبن السوق وبعض منتجاتـه حيث أظهرت النتائج ان ٣٩ (٦٥ %) تابعه للنوع *B. anthracis* , ٦ (١٠%) تابعه للنوع *B. cereus* , ١٣ (٢١.٧ %) تابعه للنوع *B. mycoides* , ٢ (٣.٣ %) تابعه للنوع *B. thuringiensis* . وبناءا على الصفات الفسيولوجيه أمكن تعريف هذه المجموعه الى ٧ أنماط فسيولوجيه رقمت من A وحتى G حيث كانت *B. cereus* تمثل النمط (A) , *B. mycoides* تمثل النمط (B, C, D) , *B. thuringiensis* تمثل النمط (E) , *B. anthracis* تمثل النمط (F, (G).