Vitik Detection of Aerobic Spore-Forming Bacteria Isolated from Raw Milk, Skim Milk Powder and UHT Milk

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Abstract

Background Aerobic spore-forming bacteria can be found in a wide range of environmental niches such as food production. Among them, the aerobic spore-forming bacteria like *Bacillus ceruse*, pose a risk of causing dairy product poisoning by the production of toxins.

Materials and Method: Out of 40 samples, 10 from each of Raw Milk, Skim Milk Powder, UHT Milk and Wight chees were collected to isolate aerobic spore-forming bacteria by morphological, physiological and biochemical tests.

Conclusion: A total of 25 isolates of heat resistant bacteria, 10(40%), 8(32%), 3(12%), 4(16%) from "RM, white chees, SMP and UHT milk respectively," were purified and characterized. Various spore forming bacteria belonging to Bacilli spp. which include pathogenic were biochemically identified using vitek 2 systems, the result showed the highest rate of *Bacillus subtilis* (28%) followed by *Bacillus ceruse* and *Bacillus thuringiensis* (24%).

Keywords: *Vitik Detection, Aerobic Spore-Forming Bacteria, Raw milk, Skim milk Powder, UHT milk.*

Introduction

Spore-forming bacteria is gram-positive microorganisms aerobic or anaerobic, ubiquitous in nature. Bacterial spores are common contaminants of food products, and their outgrowth may cause food spoilage or food borne diseases. Bacilli and Clostridia remain the most important classes relevant to the dairy industry(1). The *Bacillus* genus, part of the Bacillaceae family, have been recognized as major contributors to dairy product quality issues over the past 2 decades. They have a remarkable range of physiological characteristics that renders appropriate categorization and generalizations impossible(2,3). They are a primary cause of concern for the international dairy industry because of the pervasive and resistant nature of their spores in comparison to vegetative cells, surviving environmental challenges, such as heat, desiccation, freezing, thawing, presence of organic solvents and oxidizing agents, and UV irradiation, as well as predation by protozoa(4).

Spore-forming bacteria pose the greatest spoilage threat to dairy products, causing severe economic losses, equipment impairment and/or reputational damage of food companies. Bacilli and related genera are responsible for spoilage problems in milk and dairy products such as bitty cream, sweet curdling, off flavor, flat sour, non-sterility, bitterness, ropiness, interference with cheese production, and cheese blowing(5,6,7,8,9).

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Material and Method

Sample Collections: A total of forty samples, including: 10 samples from each of Raw Milk, Skim Milk Powder, UHT Milk and Wight cheese samples were collected during the period from September to December

2019 from Basrah city markets according to standard Method for the Examination of Dairy Products[10].

Enumeration of total viable and aerobic spore-forming bacteria: Total viable counts of all samples and aerobic spore-forming bacteria were enumerated using the nutrient agar mediums. The bacterial and spore bacterial count was expressed as cfu/ml or g milk and cheese[10]. However, the samples of RM and SMP and cheese after preparation were heated in water bath at 80oC for 10 minimums then they cooled suddenly to the room temperature before transferring one ml aliquots in petri dishes[11].

Isolation of aerobic spore-forming bacteria: Some of the colonies, which suspected to be *Bacillus* spp. according to the colony morphology on Nutrient Agar and blood agar were identified by using Gram's staining, motility, spore staining, catalase test, hemolytic activity on blood agar[12], Casein hydrolysis test [11] and Lactose fermentation test [12].

Biochemical identification of Bacteria using Vitek-2 System: VITEK-2 system imparts an automated, computer-based technique of species identifications, relies on advanced colorimetry technology, the measurement of light attenuation associated with each biochemical reaction in VITEK cards.

Antibiotic susceptibility test: The antimicrobial susceptibility testing was determined by the disk agar diffusion method [13]. It tested for susceptibility to 7 antimicrobial disks. Erythromycin E (15mg), Cadazolid (10mg), chloramphenicol C (30mg), Cephalexin CN (10mg), Ciprofloxacin CIP (30mg) and Tetracycline TE (30 mg).

Results and Discussion

Enumeration of total viable and aerobic spore forming bacteria in raw milk, cheese, skim milk powder and UHT-milk

The results in Table (1) shown that all RM and white cheese samples contain highly bacterial levels or counts according to the ESS (No, 0154-01/2005) the average of the total number of micro-organisms should not exceed 100.000 per ml (log 5 cfu/ml) of raw cow's milk from primary production. Hence, the raw milk samples and locally synthesize white cheese may be considered of bad quality. While the skim milk powder and UHT milk contain fewer amounts of microbial count, the current results also were compatible with the study of (14) who reported the presence of bacteria isolated from raw milk, skim milk powder and UHT milk in Egypt and found that the mean counts of total viable count ranged between logs 5.06 and 8.03 cfu/ml in raw milk samples, RM samples were containing highly bacterial levels, also as shown in Table (2) aerobic spore-forming bacteria count ranged between 1.1*103 cfu/ml in in skim milk powder to 6.7*103 and 3.7*103 cfu/ml in white cheese and raw milk samples respectively. *Bacillus* contamination has been demonstrated to be a problem in the dairy manufacturing process, affecting the quality and safety of the final product. Second, the issue of secondary contamination of heat-treated milk products by spoiling bacteria is now widely known [15, 16, 17]. Also the present study is in line with study of (14) who recorded the aerobic spore-forming bacteria count ranged between <10 and log 3.53 cfu/ml in raw milk samples

Table 1. Total bacterial counts for raw milk (RM), White cheese (Wch), skim milk powder (SMP) and UHT milk

Sample	TBC	Sample	TBC	Sample	TBC	Sample	TBC
RM1	$10.1*10^5$	Wch1	$7.12*10^5$	SMP1	$2.3*10^5$	UHT1	$1.1* 10^4$
RM2	$9.8 * 10^5$	Wch2	$8.1*10^5$	SMP2	$1.1*10^5$	UHT2	$2* 10^4$
RM3	$2.44*10^6$	Wch3	$2.02*10^6$	SMP3	$2.5*10^5$	UHT3	$1.3* 10^4$
RM4	$7.33*10^6$	Wch4	$7.3*10^5$	SMP4	$2.2*10^5$	UHT4	$1* 10^4$
RM5	$8.5*10^5$	Wch5	$10.1*10^5$	SMP5	$3* 10^4$	UHT5	$1.1* 10^4$
RM6	$1.11* 10^6$	Wch6	$5* 10^6$	SMP6	$1.1*10^5$	UHT6	$2* 10^4$
RM7	$2.97*10^6$	Wch7	$3.33* 10^6$	SMP7	$2.7*10^5$	UHT7	$2.01* 10^4$

Sample	TBC	Sample	TBC	Sample	TBC	Sample	TBC
RM8	$1.4*10^6$	Wch8	$8.1*10^5$	SMP8	$1.6*10^5$	UHT8	$1.6* 10^4$
RM9	$3.43*10^5$	Wch9	$9.2*10^5$	SMP9	$4* 10^4$	UHT9	$2.5* 10^4$
RM10	$3.11*10^5$	Wch10	$6.3*10^5$	SMP10	$6.1* 10^4$	UHT10	$1.1* 10^4$

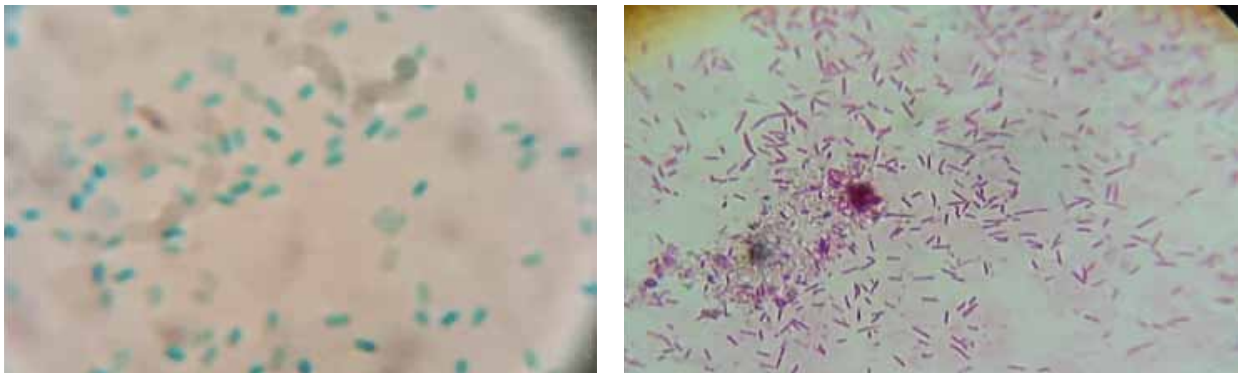
TBC: Total Bacterial Count

Table 2. Total aerobic spore forming cell for raw milk (RM), White chees (Wch), skim milk powder (SMP) and UHT milk.

Sample	ASFC	Sample	ASFC	Sample	ASFC	Sample	ASFC
RM1	2.1*103	Wch1	2.6*103	SMP1	1.2*103	UHT1	-
RM2	2.2* 104	Wch2	3.7*103	SMP2	1.1*103	UHT2	5*103
RM3	1.7* 104	Wch3	3*103	SMP3	-	UHT3	2.1*103
RM4	3.1* 104	Wch4	5*103	SMP4	2*103	UHT4	-
RM5	2.7* 104	Wch5	1* 104	SMP5	1.5*103	UHT5	3.1*103
RM6	3* 104	Wch6	4.3* 104	SMP6	2*103	UHT6	3.6*103
RM7	3.7*103	Wch7	3.6*103	SMP7	-	UHT7	-
RM8	2.8*103	Wch8	6.7*103	SMP8	2.1*103	UHT8	-
RM9	2,8*103	Wch9	1.2*103	SMP9	-	UHT9	-
RM10	3.1*103	Wch10	2*103	SMP10	-	UHT10	-

ASB: Aerobic Spore Forming Cell

Characterization and Enzymatic properties of aerobic spore-forming isolates: A total of 25 isolates 10, 8, 3, 4 from "RM, white chees, SMP and UHT milk respectively", were purified and characterized by gram positive, spore forming and motile bacteria, Fig 1 (a, b, c). Biochemical tests were used for detection the enzymatic properties of the of aerobic spore-forming isolates isolated bacteria; the isolates were shown different enzymatic properties. According to the results illustrated in fig (2), it was revealed that the proportion of 96% for total *Bacillus* Spp. isolates were able to hydrolyze the casein, the results were determined as clearing of the agar around the bacterial growth while there are 4% of the isolates was not able to hydrolyze the casein. Furthermore, 60 % of the isolates were ferment lactose, and 40% were determined as lactose non fermenter. The study of enzymatic properties of aerobic spore-forming isolates showed that the isolates have the applet to hydrolyze the casein and fermented lactose, these can cause spoilage in sterilized milk due to their production of proteolytic and lipolytic enzymes or recontamination during the filling of sterilized milk[18]. A finding in accordance with the study of (19) who reported that contamination with spore formers can lead to spoilage of milk and dairy products, mainly caused by enzyme deterioration (proteolytic and lipolytic activity by Bacillus species), acid production, (i.e. lactic, butyric and acetic acid. Moreover, (20), mentioned that the presence of *B. cereus* in UHT milk and 91.67% of mesophilic *Bacillus ssp*. isolates were able to hydrolyze the casein and 36.36% of isolates able to hydrolyze the casein were able to ferment the lactose. The UHT processing of milk destroys all microorganisms that can grow under normal storage conditions[21,22]. Almost all enzymes are also inactivated by UHT processing because the most enzymes in milk are inactivated at temperatures below 100 °C, but some bacterial proteinases and lipases needs temperatures above 150°C for inactivation[23].



a **b**

c

Fig. 1: a: Gram staining, b: spore staining, c: motility test

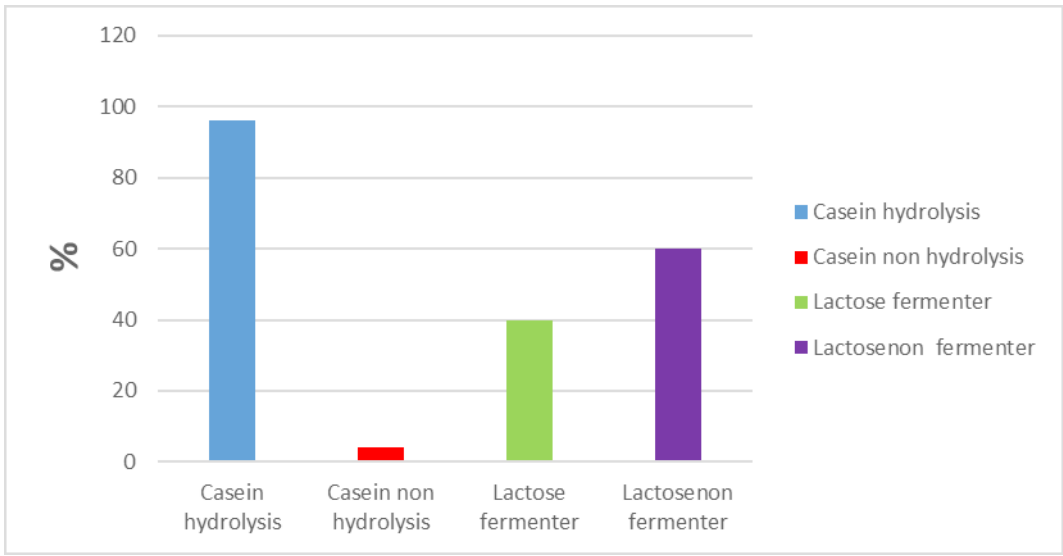


Fig (2): The Percentages of Casein hydrolyses and lactose fermenter isolates

The results showed that all the isolates were positive for Catalase test and have variable activity for blood hemolysis activity 44% blood hemolysis, 48% non-blood hemolysis and 8 % variable in hemolysis activity.

Biochemical identification of Bacteria using Vitek-2 System: Aerobic spore forming isolates were biochemically identified using vitek-2 system version: 07.01 according to the following test, AMY, APPA, LeuA, AlaA, Drib, NOVO, Draf, OPTO, PIPLC, CDEX, ProA, TyrA, ILATk, NC6.5, O129R, Dxyl, AspA, BGURr, Dsor, LAC, d MAN, SAL, ADH, BGAR, AGAL, URE, NAG, dMNAE, SAC, BGAL,

AMAN, PyrA, POLYB, dMAL, MBdG, dTRE, AGLU, AMAN, PyrA, POLYB, dMAL, MBdG, dTRE, AGLU, PHOS, BGUR, dGAL, BACI, PUL, ADH2s. The result for 25 isolates were presented in figure (3), there are 28% isolates were identified *Bacillus subtilis,* 24% isolates were identified as *Bacillus ceruse* and *Bacillus thuringiensis,* 8% isolates were identified as *Bacillus licheniformis* while 16% isolates were unidentified.

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. The selected organisms were identified using VITEK 2 system imparts an automated, computer based technique of species identifications, relies on advanced colorimetry technology, the measurement of light attenuation associated with each biochemical reaction in VITEK cards. The reagent cards have 63 wells and each well contain an individual test substrate. Substrates assess various metabolic activities such as alkalinisation, acidification, enzyme hydrolysis, and growth in the presence of inhibitory compounds. The VITEK-2 compact system combines several advantages like rapid identification, a simple methodology, high level of automation and taxonomically updated databases.

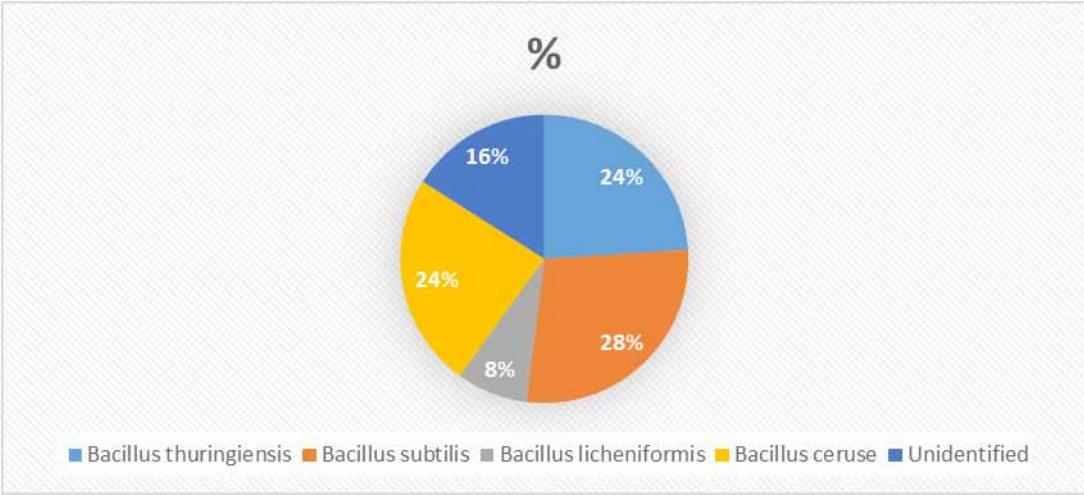


Figure (3) Results of microbial identification using VITEK 2 system

Antibiotic susceptibility test: Among the variety of antibiotics tested, the aerobic spore-forming isolated bacteria show resistance to Cadazolid (CDZ) and Chloramphenicol (C) and Show medium sensitivity for Tetracycline (TE), Erythromycin (E) and Vancomycin (VA), its show highest sensitivity to Ciprofloxacin (CIP) and Cephalexin (CN) (Table 3).

Table (3) Antibiotic susceptibility test of the isolated bacteria

Identified species	Mean of Diameter of inhibition						
	CDZ	C	VA	CN	TE	E	CIP
Bacillus thuringiensis	0	0	±13.2	±25.66	±15	±18.9	±27.66
Bacillus subtilis	0	0	±16.1	±27	±15	±20.2	± 25
Bacillus licheniformis	0	0	±19	±28.33	±20	±22.3	±31.66
Bacillus ceruse	0	0	±13	±26.32	±10.9	±13	±25.3

In conclusion, raw milk, skimmed milk powder and some of UHT milk samples were contaminated with aerobic spore forming bacteria. *Bacillus subtilis* was the highest isolates from Bacillus species. There were differences among isolates in ability to hydrolyze the casein and ferment lactose.

Conflict of Interest: None

Funding: Self

Ethical Clearance: Not required

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