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INCEDENCE OF THERMODURIC ANTIMICROBIAL-RESISTANT *Bacillus* AND *Enterococcus* SPECIES IN SOME DAIRY PRODUCTS

Samah A. Sakr[1,2], Magdy S. Elsaid[2], Manal I. ElShaer[1], Mohamed A. Bayoumi[2]*

[1]Control Department, Animal Health Research Institute, El-Mohafza Street, 44516 Zagazig, [2]Food Control Department, Faculty of Veterinary Medicine, 44519 Zagazig, Egypt

*Corresponding author, E-mail: mohammad.bayoumi@gmail.com

Abstract: Thermoduric bacteria can survive pasteurization and subsequently grow in pasteurized or in contaminated products. Hazards associated with these organisms vary from health to spoilage issues. This study aimed to determine the microbial load, species identification, and antimicrobial resistance profile of some thermoduric bacillus and enterococcus isolates. Samples of raw, pasteurized and UHT milk, Ras cheese, and dairy desserts were collected and tested for the presence of antimicrobial-resistant *Bacillus* and *Enterococcus* species, which were identified using the Vitek 2 system. The most predominated strains were *B. subtilis* and *E. faecium* (100%, each). Different antimicrobial resistance patterns were observed. Antimicrobial resistance rate of *B. subtilis* was 100% to cefepime, ampicillins, oxacillin, amoxicillin/Clavulanic acid, vancomycin and chloramphenicol. Antimicrobial resistance of *E. faecium* was 100% to clindamycin. Multidrug-resistance (MDR) was observed in *B. subtilis* (25%) and *E. faecium* (12%) isolates. The most common antibiotic resistance patterns observed in *B. subtilis* isolates were AMP/CLI/AMC/VAN and FEP/OXA/CHL (12.25% each), while the most common antimicrobial resistance patterns observed in *E. faecium* isolates were GHL/CLI/DOX and SHL/CLI/TET (6% each). Accordingly, strict hygienic measures should be applied and followed during manufacture of heat treated dairy products to avoid contamination with those antimicrobial-resistant potential pathogens.

Key words: *Bacillus cereus; Enterococcus faecium*; antimicrobial resistance; thermoduric; dairy products

Introduction

Mesophilic thermoduric bacteria have an optimum temperature of about 30 °C, however, they can grow in the range 5 to 50 °C. These include spore-forming and non-spore-forming bacteria as *Bacillus*, *Enterococcus*, *Streptococcus*, and *Arthrobacter* species, which can withstand harsh environments (1).

Bacillus cereus (*B. cereus*) group classically consists of Gram-positive, rod-shaped, spore-forming bacteria, which can grow in aerobic or facultative anaerobic conditions. It is characterized by positive-lecithinase activity and do not produce acid from mannitol (2). Pasteurization temperature, rather than destroying the spores of *B. cereus*, it triggers spores' germination in the pasteurized product (3). *B. cereus* is considered the most important bacillus contaminant of dairy products and became dangerous owing to the ability of some strains to induce illness. Toxin-producing strains of *B. cereus* cause two types of food poisoning, emetic and diarrheal (4).

In Egypt, there is a shortage of high-quality raw milk. As a result, some dairy factories use low-quality raw milk or milk powder (skimmed or full cream) or a mix of raw milk with milk powder. Low quality of raw ingredients could increase

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the defects in milk or its products during production and/or during storage (5). The spores of *B. cereus* and *B. mycoides* cause a sweet curdling defect in pasteurized milk (5).

Another important group of the thermoduric bacteria is genus *Enterococci*. They are Gram-positive, catalase-negative, non-spore-forming, facultative anaerobic bacteria, of which *Enterococcus faecium* (*E. faecium*) can withstand heating at 60 °C for 30 min (6, 7). Interestingly, enterococcal drug resistance is prevalent in the food industry and has public health importance due to its ability to transfer resistance not to other strains of the same species but to other species as well as other genera by conjugation (8, 9).

Due to the excessive spread of foodborne pathogens among even heat-treated dairy products, the present study aimed to evaluate the microbiological quality of Egyptian boiled raw, UHT and pasteurized milk, Ras cheese, and chilled dairy desserts to identify and evaluate the incidence of some thermoduric bacteria. Additionally, the antimicrobial resistance of the isolated organisms was evaluated..

Material and methods

Sampling

Random samples of raw boiled (n = 21), UHT (n = 19) and pasteurized milk (n = 15), Ras cheese (n = 30), and dairy-based desserts (n = 7) were collected from different supermarkets, street vendors, and dairy shops in El-Sharkia Governorate, Egypt, over one year (from January 2020 to December 2020). These samples were immediately placed on ice for transportation.

Isolation and identification of Bacillus and Enterococcus species

Ten-fold dilutions of the samples were prepared in buffered peptone water (Oxoid, UK). For the isolation of *Bacillus* species, 0.1 mL of each dilution was inoculated onto duplicated agar plates containing *B. cereus* agar base (Oxoid, UK) supplemented with 100 mL/L of egg yolk emulsion (Oxoid, UK) and 5 mL/L of polymyxin B selective supplement (Oxoid, UK) as described by Owusu-Kwarteng et al. (2). For the isolation of *Enterococcus* species, a loop full of the prepared samples was inoculated onto the surface of Slanetz and Bartley agar plates (HiMedia, India) according to Slanetz and Bartley (10) guidelines. Colonies with typical phenotypic appearance were enumerated then further identified into species using the automated Vitek 2 according to Chatzigeorgiou et al. (11) and Pincus (12).

Antimicrobial Susceptibility testing of Bacillus and Enterococcus species

For *Bacillus* species, susceptibility of the highly prevalent strains to 10 antimicrobial agents was determined using a Vitek AST-GN-73 card (bioM´erieux, France). Tested antimicrobials were cefepime (FEP), ampicillins (AMP), chloramphenicol (CHL), clindamycin (CLI), amoxicillin/clavulanic acid (AMC), vancomycin (VAN), oxacillin (OXA), erythromycin (ERY), tobramycin (TOB) and rifampicin (RIF).Regarding the*Enterococcus* species, susceptibility of highly prevalent isolates to 14 antibiotics was determined using a Vitek AST-GP75 card (bioM´erieux, France) including ampicillin (AMP), gentamicin high-level (GHL), streptomycin high level (SHL), ciprofloxacin (CIP), levofloxacin (LVX), moxifloxacin (MXF), erythromycin (ERY), clindamycin (CLI), linezolid (LZD), vancomycin (VAN), doxycycline (DOX), tetracycline (TET), tigecycline (TGC) and nitrofurantoin (NIT). Isolates were considered susceptible, intermediate, or resistant according to the guidelines of the Clinical Laboratory Standards Institute (13). Multidrug-resistance (MDR) was defined as resistance to at least three different antimicrobial classes, as previously described (14).

Data analysis and interpretation

Data were statistically analyzed using SPSS program version 23. The one-way ANOVA test was performed for comparison between *Bacillus* and *Enterococcus* species load in examined dairy products. Post hoc test was performed using Duncan multiple range (DMR) test (15) for comparisons between means of bacterial count among milk products. Statistical significant level was expressed as $P<0.05$. The numbers of bacteria were converted to logarithmic values (log CFU/mL rinse solution) before calculating means and performing statistical analysis.

Results

Overall, 42.39% of the examined samples were positive for *Bacillus* species with the highest count mean in raw milk (3.2 logs CFU/mL) (Table 1). There was no statistically significant difference ($P > 0.05$) in the load proportions of *Bacillus* species in examined dairy samples.

The recovery percentage of *Enterococcus* species was 40.2% among the examined samples, while in Ras cheese samples was 96.6%, which was more than that recorded in pasteurized milk (53.3%) as present in Table- 2. There was statistically significant difference ($P < 0.05$) in the count means of enterococci in examined dairy samples.

The predominant *Bacillus* species in Ras cheese and dairy desserts was *B. subtilis* (100%), while *B. cereus* was the predominant species in raw milk and UHT milk samples recorded as 75% and 72.7%, respectively. *B. mycoides* was recorded as 16% and 18.1% in raw milk and UHT milk, respectively. While *B. thurigiensis* was found as 8.3% and 9% in raw milk and UHT milk, respectively (Figure 1).

Enterococcus faecium was the predominant species in pasteurized milk and Ras cheese samples representing 100% and 86.2%, respectively. On the other hand, *E. durans* was recorded as 13.7% in Ras cheese samples (Figure 2).

Herein, *B. subtilis* isolated from cheese and dairy desserts samples showed high resistance levels (100%) to most tested antimicrobials as FEP, OXA, AMP, CHL, CLI, AMC, and VAN (Table3).

Enterococci isolated from pasteurized milk samples showed 100% resistance to clindamycin only. On the other side, High-level resistance (HLR) of 52% to aminoglycosides (gentamicin and/or streptomycin) and tetracycline was detected for *E. faecium* of cheese origin (Table 4). None of the isolates showed full resistance to vancomycin or linezolid.

Of note, Table-5 shows that 25% of *B. subtilis* isolates were identified as MDR, which were isolated from Ras cheese samples, with most common antibiotic resistance patterns AMP/CLI/AMC/VAN (12.5%) and FEP/OXA/CHL (12.5 %). In addition, 12% of the *E. feacium* isolated from Ras cheese were identified as MDR isolates.

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Table 1: Bacillus incidence and CFU/mL count in raw milk and other dairy products

Sample type	Raw milk (n = 21)	UHT milk (n = 19)	Pasteurized milk (n = 15)	Ras cheese (n = 30)	Dairy desserts (n = 7)
No. of positive samples (%)	12 (57.14)	11 (57.8)	8 (53.3)	2 (6.6)	6 (85.7)
Mean (log CFU/mL ±SE)	3.24±0.15^{a}	2.91±0.18^{a}	2.66±0.15^{a}	2.54±0.17^{a}	2.86±0.18^{a}

^{a}: Values with the same superscripts within the same row are not significantly different ($P > 0.05$).

Table 2: Enterococcus incidence and CFU/mL count in raw milk and other dairy products

Sample type	Raw milk	UHT milk	Pasteurized milk	Ras cheese	Dairy desserts
No. of positive samples/no. of tested samples (%)	0/12 (0.0)	0/19 (0.0)	8/15 (53.3)	29/30 (96.6)	0/7 (0.0)
Mean (log CFU/mL ±SE)	00	00	2.27±0.26^{b}	3.88±0.21^{a}	00

^{ab}: Values with different superscripts within the same row are significantly different ($P < 0.05$)

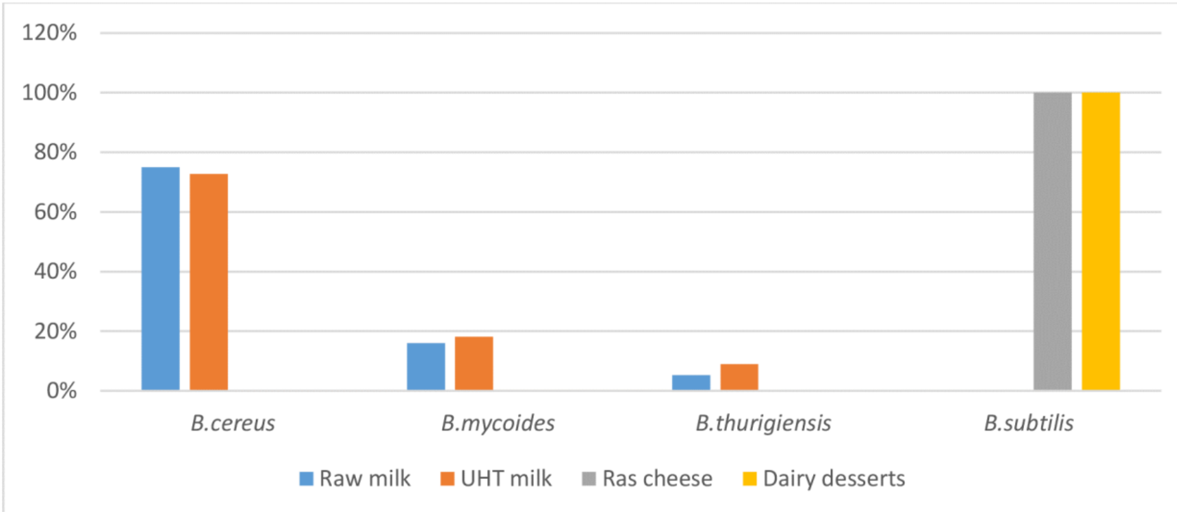

Figure 1: Recovery percentage of 39 *Bacillus* species isolated from raw milk and other dairy products using Vitek 2 system

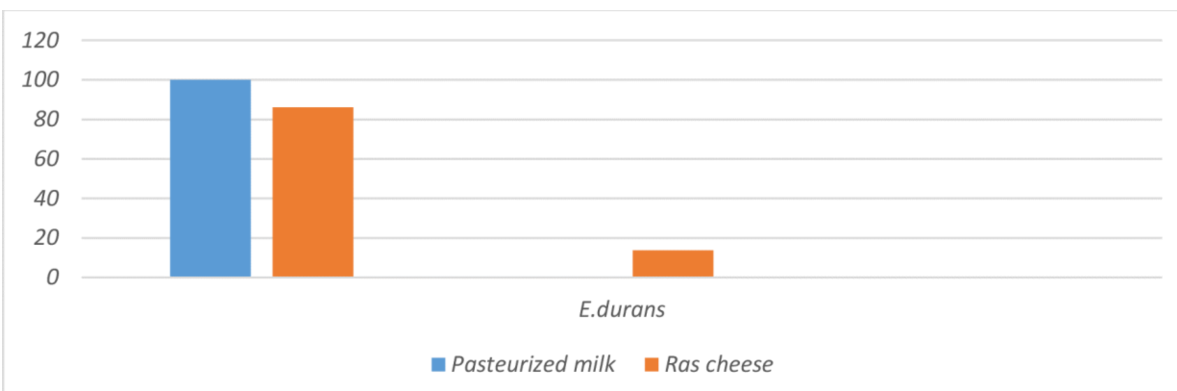

Figure 2: Distribution of 37 *Enterococcus* species among isolates from pasteurized milk and Ras cheese using Vitek 2 system

Table 3: The susceptibility pattern of *B. subtilis* isolates from Ras cheese and dairy desserts

Antimicrobial[a]	Susceptible No. (%)		Intermediate No. (%)		Resistant No. (%)	
	Ras cheese (n = 2)	Dairy desserts (n = 6)	Ras cheese (n = 2)	Dairy desserts (n = 6)	Ras cheese (n = 2)	Dairy desserts (n = 6)
FEP	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100)	6 (100)
OXA	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100)	6 (100)
AMP	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100)	6(100)
CHL	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100)	6 (100)
TOB	2 (100)	6 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
ERY	2 (100)	6 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
CLI	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2(100)	6 (100)
AMC	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100)	6 (100)
VAN	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100)	6 (100)
RIF	2 (100)	6 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

[a] Abbreviations: cefepime (FEP), ampicillins (AMP), chloramphenicol (CHL), clindamycin (CLI), amoxicillin/clavulanic acid (AMC), vancomycin (VAN), oxacillin (OXA), erythromycin (ERY), tobramycin (TOB) and rifampicin (RIF)

Table 4: The susceptibility pattern of *E. faecium* isolates from pasteurized milk and Ras cheese

Antimicrobials[a]	Susceptible No. (%)		Intermediate No. (%)		Resistant No. (%)	
	Pasteurized milk (n = 8)	Ras cheese (n = 25)	Pasteurized milk (n = 8)	Ras cheese (n = 25)	Pasteurized milk (n = 8)	Ras cheese (n = 25)
AMP	8 (100)	25(100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
GHL (synergy)	8 (100)	12(48)	0 (0.0)	0 (0.0)	0 (0.0)	13(52)
SHL (synergy)	8 (100)	12(48)	0 (0.0)	0 (0.0)	0 (0.0)	13(52)
CIP	8 (100)	25 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
LVX	8(100)	25 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
MXF	8 (100)	25 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
ERY	8 (100)	25 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
CLI	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	8(100)	25(100)
LZD	8 (100)	25 (100)	0(0.0)	0 (0.0)	0 (0.0)	0 (0.0)
VAN	8 (100)	25 (100)	0(0.0)	0 (0.0)	0 (0.0)	0 (0.0)
DOX	8 (100)	13 (52)	0(0.0)	0 (0.0)	0 (0.0)	12 (48)
TET	8 (100)	12 (48)	0(0.0)	0 (0.0)	0 (0.0)	13 (52)
TGC	8 (100)	25 (100)	0(0.0)	0 (0.0)	0 (0.0)	0 (0.0)
NIT	8 (100)	25 (100)	0(0.0)	0 (0.0)	0 (0.0)	0 (0.0)

[a] Abbreviations: Ampicillin (AMP), Gentamicin high-level (GHL), Streptomycin high level (SHL), ciprofloxacin (CIP), Levofloxacin (LVX), Moxifloxacin (MXF), Erythromycin (ERY), Clindamycin (CLI), Linezolid (LZD), Vancomycin (VAN), Doxycycline (DOX), Tetracycline (TET), Tigecycline (TGC) and Nitrofurantoin (NIT)

Table 5: Distribution of multidrug-resistance profiles among *E. faecium and B. subtilis* isolates of cheese origin

Drug resistance profile	No of resistant antimicrobials	No. (%) of isolates	
		B. subtilis (n=8)	*E. faecium* (n=33)
GHL; CLI; DOX	3	0 (0)	2 (6)
SHL; CLI; TET	3	0 (0)	2 (6)
FEP; OXA; CHL	3	1 (12.5)	0 (0)
AMP; CLI; AMC; VAN	4	1 (12.5)	0 (0)
Total		2(25)	4 (12)

Number of resistant antimicrobials means group number not individual agent

Of note, Table-5 shows that 25% of *B. subtilis* isolates were identified as MDR, which were isolated from Ras cheese samples, with most common antibiotic resistance patterns AMP/CLI/AMC/VAN (12.5%) and FEP/OXA/CHL (12.5%). In addition, 12% of the *E. feacium* isolated from Ras cheese were identified as MDR isolates.

Discussion

The present study reveals the microbial load, distribution of species, and antimicrobial resistance profiles among bacillus and enterococcus isolates recovered from raw, UHT and pasteurized milk, Ras cheese, and dairy desserts samples collected at El-Sharkia Governorate, Egypt, for one year.

All analyzed samples in the present study had Bacillus counts of not more than 4 log CFU/g (Table 1). Spoilage or even diseases can occur at low levels of *B. cereus* contamination ($\leq 10^3$ CFU/g) (2).

Although differences in isolation percentages were noticeable, *B. cereus* was the predominant species recovered from both raw milk (75%) and UHT milk (72.7%) samples (Figure 1). These results were less than that recorded by Gundogan (16) who reported that *B. cereus* prevalence in raw milk was 90%. The presence of high levels of *B. cereus* in examined raw milk samples may be due to environmental contamination and/or adaptation of bacillus in the mammary gland producing mastitis. *B. cereus* can persist as subclinical form imitating a great safety problem as the secreted milk pass for human consumption or enter further processing (17). The present study highlights the great necessity for applying controlled hygienic measures in small-scale points during milking and handling of raw milk. On the other side, *B. cereus*, *B. mycoides*, and *B. thurigiensis* were detected in UHT milk samples as 72%, 18%, and 9%, respectively. Aouadhi et al. (18) documented different findings, where *B. cereus* was 2.5% in UHT milk marketed in Tunisia. Spoilage of UHT milk is allied to contamination with spores from the soil, animal feeding, milking equipment, and packaging material (19). Sporulating bacteria in the UHT milk chain is of great significance to the dairy industry, since they produce proteolytic and heat-resistant lipolytic enzymes that cause off-flavors, sweet coagulation, and bitter flavor in milk (20).The present study stated that *B. cereus* wasn't detected in Ras cheese. Consistent with a previous result, the occurrence of potentially toxigenic *B. cereus* in hard rennet-coagulated cheeses has been recorded by Hammad et al. (21) at a low percentage (10%). Low *B. cereus* loads in artisanal manufactured Egyptian Ras cheese, may be ascribed to their inactivation during the ripening period by salt, drop in pH, and antimicrobial agents secreted from competitor microorganisms (22).

B. subtilis was the predominant species recovered from both Ras cheese (100%) and dairy desserts samples (100%) (Figure 1). Moschonas et al. (23) also stated that *B. subtilis* was the principal spoilage microbe in neutral-pH dairy desserts. Their high prevalence in dairy desserts samples may be due to the adding starch during production, which is a good medium for bacillus organisms or using inadequate thermal treatment during the production process (24).

Enterococcus species are abundant and have a great resistance to adverse environmental conditions increasing their spreading through the food chain. Their existence is an indicator of fecal contamination (8).

E. faecium was the predominant species recovered from both pasteurized milk and Ras cheese samples (Figure 2). In accordance, *E. faecium* was the major isolated species (53.4%) from dairy products as reported by Chajęcka-Wierzchowska et al. (25), while Fracalanzza et al. (26) found that *E. faecium* represented only 0.8% of enterococcus isolates from pasteurized milk. The relatively high heat resistance of enterococcus may be the cause of their existence in pasteurized milk. Finally, it is supposed that heat-resistant aerobic and/or spore-forming bacteria downgrade food quality.

B. subtilis isolated from Ras cheese samples and dairy desserts showed predominant resistance to FEP, OXA, AMP, CHL, CLI, AMC, and VAN, which were investigated in Table-3. Luna et al. (27) found that several *Bacillus* species showed resistance to CLI and ERY. The introduction of bacillus strains in food of humans and animals as probiotics is increasing, regardless of the restricted data about their resistance to standard antibiotics besides their role in the transmission of antibiotic resistance genes (27).

In the present study, *E. faecium* were resistant to HLG, HLS, CLI, DOX, and TET that were isolated from Ras cheese samples, while those isolates from pasteurized milk samples showed resistance to CLI only (Table 4). Fracalanzza et al. (26) found that enterococci isolated from pasteurized milk samples were resistant to streptomycin, and tetracycline. Chingwaru et al. (28) recorded high levels of resistance to tetracycline by *E. faecium* isolated from milk samples, the same as our results but in Ras chees isolates. Malek et al. (6) reported that *E. faecium* isolates of Ras cheese samples were sensitive to AMP, NIT, TET, and VAN, the same as *E. faecium* isolated from Ras cheese herein. The high-level aminoglycosides resistance (HLAR) was noted only in *E. faecium* isolated from Ras cheese samples representing about 52% in this study. Teuber and Miele (29) recognized HLAR in 80% of *Enterococcus* species isolated from cheese. Though, resistance to high-level gentamicin indicates elimination of the synergistic bactericidal effect of combined exposure to a cell wall-active agent and gentamicin.

Multidrug-resistant enterococcus populations are commonly introduced into human intestinal microflora by the consumption of food. These enterococcal resistances to multiple antimicrobial groups may be transferred by conjugation to other bacteria of the same or different genus (9). As well as their susceptibility to acquire new traits continues to create therapeutic problems and becomes dangerous for public health (30).In this work, MDR trait was particularly detected among *E. faecium* and *B. subtilis* recovered from Ras cheese (Table 5).

Similarly, MDR enterococci strains were identified as 13.75% with 23 different phenotypes from dairy products studied by Chajęcka-Wierzchowska et al. (25).

Conclusion

This study revealed that the tested dairy products were contaminated with some thermoduric bacteria, which will consequently affect the commercial shelf life of these products. Furthermore, the health-related hazards which are associated with these microorganisms cannot be neglected, especially with the presence of MDR strains.

Acknowledgments

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