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Prevalence of *Yersinia enterocolitica* in milk and dairy products and the effects of storage temperatures on survival and virulence gene expression

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ABSTRACT

Yersinia enterocolitica was isolated from raw milk and dairy products from 10% of examined samples. The highest isolation rate was 22%, from raw milk, followed by 12%, 4% and 2% from fermented milk (Rayeb), pasteurised milk and ripened salted cheese, respectively. The virulence-associated genes *ail* and *yst* were detected in 30% and 10% of the isolates, respectively, while these genes were present simultaneously in 10% of the isolates. All the isolates showed susceptibility to gentamicin, ciprofloxacin and chloramphenicol, while only two of the isolates exhibited multidrug resistance. Storage of inoculated pasteurised milk at refrigeration (4 °C), freezing (−20 °C) and room (25 °C) temperatures revealed significant differences in *Y. enterocolitica* counts and relative expression of the two virulence genes. The isolation of potentially pathogenic *Y. enterocolitica* isolates from retail dairy products indicates risk to consumers; screening of prevalence, pathogenicity potential and antibiotic resistance is essential to implement control measures.

1. Introduction

Yersinia enterocolitica are Gram-negative, psychrotrophic enterobacteria of the family *Enterobacteriaceae*. These bacteria are considered the third most common foodborne zoonotic pathogens, following *Campylobacter* and *Salmonella* species (EFSA, 2015). *Y. enterocolitica* are classified into six biotypes and more than 57 O serogroups, of which biotypes 1B, 2, 3, and 4 and serotype O:8 are the most pathogenic (Fàbrega & Vila, 2012). More than two-thirds of human yersiniosis infections manifest as acute diarrhoea lasting for 1–3 weeks, and individuals below five years of age are the most widely affected (Bernardino-Varo, Quiñones-Ramírez, Fernández, & Vázquez-Salinas, 2013). Moreover, extra-intestinal and post-infectious manifestations, including reactive arthritis and erythema nodosum, have been reported (Huovinen et al., 2010). This organism is ubiquitous and has been reported to contaminate food, soil and water sources (Fredriksson-Ahomaa & Korkeala, 2003).

The exact infection dose required to cause yersiniosis in humans is not known; however, the dose exceeds 4 log colony forming units (cfu), reaching 7–9 log cells (Bhunia, 2008; Robins-Browne, 2013). The foods most commonly implicated as causes of *Y. enterocolitica* infection in humans are those of animal origin (Rahimi, Sepehri, Safarpoor Dehkordi, Shaygan, & Momtaz, 2014). Milk is a highly nutritious food for human consumption; however, milk serves as a vehicle for different foodborne pathogens, including *Y. enterocolitica* (Bernardino-Varo et al., 2013).

Two important chromosomal virulence markers of *Y. enterocolitica* are the *ail* and *yst* genes, encoding the adhesion protein Ail and the enterotoxin Yst, respectively (Revell & Miller, 2001). The Ail protein protects the bacteria from complement bactericidal actions and plays a role in invasion and adhesion in vivo; the *ail* gene is present in only pathogenic strains (Thoerner et al., 2003). The Yst protein causes the accumulation of liquids in the intestine via absorption by the intestinal villi (Revell & Miller, 2001). This protein is produced by both pathogenic and non-pathogenic strains.

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Contamination of food with antimicrobial-resistant bacteria is of concern due to the possibility of transmission to humans. The emergence of these bacteria results from the misuse or overuse of antibiotics for treatment of humans and in veterinary therapy or as growth promoters in animal production (Özdemir & Arslan, 2015).

The ability of *Y. enterocolitica* to grow at low temperatures in food and in products with long shelf lives has led to increased research interest in this organism (Kowalik & Lobacz, 2015). Storage of food under refrigeration for prolonged periods creates a favourable environment for bacterial growth. The optimal temperature for *Y. enterocolitica* growth is 22–29 °C; however, as psychrotrophs, these bacteria may survive and multiply even at 0 °C. The ability of *Y. enterocolitica* to grow at and below refrigeration temperature (4 °C) is potentially hazardous for consumers (Jamali, Paydar, Radmehr, & Ismail, 2015; Yucel & Ulusoy, 2006). Pathogen survival and expression of virulence genes are promoted by changes in temperature either during storage or after entry into the host (Yehualaeshet et al., 2013).

This study aimed to determine the prevalence of potentially virulent *Y. enterocolitica* in milk and dairy products. The effects of storage temperatures on the survival of *Y. enterocolitica* in pasteurised milk and on virulence gene expression were also investigated.

2. Materials and methods

2.1. Sample collection

Raw, pasteurised and fermented milk (commercial Rayeb; a stirred yoghurt made using ABT probiotic mixed culture of *Lactobacillus acidophilus* La-5 + *Bifidobacterium animalis* Bb-12 + *Streptococcus thermophilus*, [ABT-4; Chr.Hansen]) and ripened salted hard cheese samples (50, each) were randomly purchased from outlets and examined during the period from September 2017 to May 2018. The samples were collected from small dairy outlets and vendors in Sharkia Governorate, Egypt. The study was approved by the Committee of Animal Welfare and Research Ethics, Faculty of Veterinary Medicine, Zagazig University, Egypt.

2.2. Bacteriological examination

Samples (10 mL of milk, 10 g of cheese) were each homogenised with 90 mL of trypticase soya broth (TSB, Oxoid CM 0129B) and incubated at 25 °C for 24 h. Sub-culturing on selective CIN agar plates (Yersinia selective agar base; Oxoid, CM 0653B) was performed according to the method recommended by the Food and Drug Administration (FDA, 2001). Suspected colonies of typical "bull's eye" appearance on the CIN agar plates were streaked onto tryptone soya agar (Oxoid, CM 0131B) plates for purification. The isolates were then examined via Gram staining, utilisation of citrate, Kligler's iron agar reaction and urease activity testing (FDA, 2001).

2.3. Molecular identification of Yersinia enterocolitica

Bacterial DNA was extracted using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's guidelines. Suspected isolates were subjected to confirmation by PCR-based amplification of the 16S rRNA gene (Neubauer, Hensel, Aleksic, & Meyer, 2000). Isolates identified as *Y. enterocolitica* were further subjected to molecular characterisation of the *ail* (Thoerner et al., 2003) and *yst* (Ibrahim, Liesack, Griffiths, & Robins-Browne, 1997) virulence-associated genes.

A reaction mixture with no added DNA was run in the PCR as a negative control, and a positive control DNA from *Y. enterocolitica* strain (NCTC 11174) kindly provided from Biotechnology Unit, Reference Lab for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Dokki, Giza, Egypt, was also run in the reaction.

2.4. Biotyping of Y. enterocolitica

Confirmed *Y. enterocolitica* isolates were biotyped using previously described biochemical tests (Wauters, 1981). The tests included lipase, esculin/salicin (24 h), indole, xylose, trehalose, pyrazinamidase, β-D-glucosidase and Voges-Proskauer tests. The *Y. enterocolitica* strain (NCTC 11174) was used as a control.

2.5. Antimicrobial susceptibility testing

The antibiotic susceptibility of *Y. enterocolitica* isolates was determined with the Kirby-Bauer disc diffusion method as described by the National Committee for Clinical Laboratory Standards (NCCLS), and the zones of inhibition were measured and interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2016). The 10 antibiotic discs (Oxoid) used were ampicillin (AMP, 10 μg), amoxicillin/clavulanic acid (AMC, 20/10 μg), nalidixic acid (NA, 30 μg), ciprofloxacin (CIP, 5 μg), chloramphenicol (C, 30 μg), gentamicin (CN, 10 μg), trimethoprim-sulphamethoxazole (SXT, 1.25/23.75 μg), kanamycin (K, 30 μg), streptomycin (S, 10 μg) and tetracycline (TE, 30 μg). *Escherichia coli* ATCC 25922 was used as a quality control organism. All the isolates were examined by PCR for the presence of resistance genes encoding for resistance to β-lactams (*bla*TEM, *bla*SHV) (Colom et al., 2003), gentamicin (*aad*B, *aad*C) (Frana, Carlson, & Griffith, 2001; Lynne, Rhodes-Clark, Bliven, Zhao, & Foley, 2008), streptomycin (*aad*A1, *aad*A2) (Randall, Cooles, Osborn, Piddock, & Woodward, 2004; Walker, Lindsay, Woodward, Ward, & Threlfall, 2001), chloramphenicol (*flo*R) (Doublet et al., 2003), tetracyclin (*tet*A(A), *tet*A(B)) (Randall et al., 2004), fluoroquinolones (*qnr*, *aac(6_)-Ib-cr*) (Lunn, Fabrega, Sanchez-Cespedes, & Vila, 2010; Robicsek et al., 2006) and sulphonamides (*sul*1) (Ibekwe, Murinda, & Graves, 2011).

2.6. Survival of Y. enterocolitica in pasteurised milk and virulence gene expression at different storage temperatures

2.6.1. Preparation of a Y. enterocolitica isolate

Y. enterocolitica isolate (isolated from pasteurised milk during our study and was positive for both the *ail* and *yst* virulence-associated genes and showed multidrug resistance [isolate code: PM22]) was used in the experiment. The isolate was maintained in tubes containing sterile brain heart infusion (BHI) broth and incubated at 28 °C for 18 h. The turbidity of the cultures was adjusted to match a McFarland standard no. 0.5 tube (1.5×10^8 cfu mL^{-1}). The cell count was confirmed by the surface plating method on selective CIN agar plates (Thatcher & Clark, 1968).

2.6.2. Inoculation of milk samples

Pasteurised milk samples were purchased from a retail outlet and subjected to bacteriological examination to ensure that the samples were not contaminated with *Y. enterocolitica* before starting the experiment. One hundred microlitres of the adjusted bacterial broth was used to inoculate 900 μL of pasteurised milk to obtain a bacterial concentration of 1.5×10^7 cfu mL^{-1}. Three groups of inoculated milk samples were prepared for storage at room

(25 °C), refrigeration (4 °C) and freezing (−20 °C) temperatures. Each group contained 20 samples as control groups of non-inoculated pasteurised milk samples.

2.6.3. Sampling and counting

Counting of *Y. enterocolitica* was determined as cfu mL^{-1} on CIN agar at day 0, day 2, day 4, week 1 and week 2. The count was carried out in triplicate using the surface plating method (Thatcher & Clark, 1968). *Y. enterocolitica* colonies were verified by amplification of the 16S rRNA gene by PCR. Bacterial counts were measured in triplicate and are expressed as the mean values and standard deviations. A two-way analysis of variance (ANOVA) and pairwise comparison were used to determine the significant differences between the bacterial counts at different storage temperatures and storage times using SPSS ver. 21 (IBM Corp., Chicago, IL, USA). Values with $P \leq 0.05$ were considered significantly different.

2.6.4. Expression of virulence genes

At each sampling time, two volumes (1 mL) of RNAprotect Bacteria reagent (Qiagen, Germany, GmbH) was added to one volume (0.5 mL) of the harvested bacterial culture and processed as described in the manufacturer's guidelines to protect RNA from degradation. RNA was extracted from each sample using the QIAamp RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Expression of *ail*- and *yst*-associated genes was performed using SYBR Green I-based real-time PCR with the specific primers for each gene (Ibrahim et al., 1997; Thoerner et al., 2003), and the 16S rRNA gene was used as a housekeeping gene (Neubauer et al., 2000).

The primers were utilised in a 25-μL reaction containing 12.5 μL of 2 × QuantiTect SYBR Green PCR master mix (Qiagen), 0.25 μL of RevertAid reverse transcriptase (200 U mL^{-1}) (ThermoFisher), 0.5 μL of each primer (20 pM concentration), 8.25 μL of water, and 3 μL of RNA template. The reaction was performed in a Stratagene MX3005P real-time PCR machine. The reaction conditions included reverse transcription at 50 °C for 30 min, primary denaturation at 94 °C for 15 min and 40 cycles of denaturation (94 °C for 15 s), annealing (62 °C/30 s for 16S rRNA, 50 °C/30 s for *ail*, 55 °C/30 s for *yst*) and extension at 72 °C for 30 s. The melting curve was performed after all cycles of amplification and fluorescence reading was performed at every degree between 55 °C and 95 °C to ensure that only one PCR product was amplified.

Amplification curves and cycle threshold (Ct) values were determined by Stratagene MX3005P software. To estimate the variations in RNA gene expression of the different samples, the Ct of each sample was compared with that of the positive control group by the comparative Ct method, ΔΔCt, according to Yuan, Reed, Chen, and Stewart (2006) using the following ratio: $(2^{-\Delta\Delta ct})$.where:

$$\Delta\Delta Ct = \Delta Ct\ reference - Ct\ target. \quad (1)$$

$$\Delta Ct\ target = Ct\ control - Ct\ treatment\ and\ \Delta Ct\ reference = Ct\ control - Ct\ treatment \quad (2)$$

The fold change values of the expression of the two genes in the samples at each storage temperature were compared using two-way ANOVA and pairwise comparison; SPSS ver. 21 (IBM Corp., Chicago, IL, USA) was used for the analyses. Values with $P \leq 0.05$ were considered significantly different.

3. Results

Y. enterocolitica was recovered from 20 (10%) of the examined samples. The isolation rates from raw milk, fermented milk (Rayeb), pasteurised milk and ripened salted cheese were 22%, 12%, 4% and 2%, respectively (Table 1).

Virulence-associated genes were detected in 10 (50%) of the isolates. The two genes (*ail* and *yst*) were amplified in 6 (30%) and 2 (10%) of the isolates, respectively, and were present simultaneously in 2 (10%) isolates from the pasteurised milk samples (Table 1).

Biotyping of the 20 confirmed *Y. enterocolitica* isolates revealed that 10 (50%) were of biotype 4, and all of the isolates tested positive for the *ail* and/or *yst* gene. The remaining 10 (50%) *Y. enterocolitica* isolates were of biotype 1A, and none of the isolates harboured any of the two investigated virulence genes.

The results of the antibiotic sensitivity test revealed that the examined isolates were highly resistant to ampicillin (85%), while, 100% susceptibility was observed to gentamicin, ciprofloxacin and chloramphenicol (Table 2). Only the two isolates from pasteurised

Table 1
Proportion of *Y. enterocolitica* isolated from milk and dairy products and the distribution of the *ail* (A) and *yst* (Y) virulence-associated genes in the examined isolates.

Sample	Number examined	*Y. enterocolitica*	A + Y−	A−Y+	A + Y+	A−Y−
Raw milk	50	11 (22%)	4	1	0	6
Pasteurised milk	50	2 (4%)	0	0	2	0
Fermented milk	50	6 (12%)	1	1	0	4
Ripened salted cheese	50	1 (2%)	1	0	0	0
Total	200	20 (10%)	6 (30%)	2 (10%)	2 (10%)	10 (50%)

Table 2
Results of antibiotic susceptibility tests of *Y. enterocolitica* isolates.

Antibiotic class	Antibiotic	Susceptible	Intermediate	Resistant
Penicillin	Ampicillin	2 (10%)	1 (5%)	17 (85%)
	Amoxicillin-clavulanic acid	15 (75%)	5 (25%)	0
Aminoglycosides	Gentamicin	20 (100%)	0	0
	Kanamycin	17 (85%)	1 (5%)	2 (10%)
	Streptomycin	18 (90%)	0	2 (10%)
Quinolones	Nalidixic acid	13 (65%)	4 (20%)	3 (15%)
	Ciprofloxacin	20 (100%)	0	0
Sulphonamides	Trimethoprim-sulphamethoxazole	15 (75%)	4 (20%)	1 (5%)
Phenolics	Chloramphenicol	20 (100%)	0	0
Tetracyclines	Tetracycline	17 (85%)	2 (10%)	1 (5%)

Table 3
Y. enterocolitica counts in pasteurised milk stored at −20 °C, 4 °C and 25 °C.[a]

Storage time (days)	Bacterial count (log_{10} cfu mL^{-1})		
	−20 °C	4 °C	25 °C
Day 0	6.48 ± 0.03^{a}	6.48 ± 0.03^{a}	6.48 ± 0.03^{a}
Day 2	5.47 ± 0.01^{b}	6.51 ± 0.18^{a}	7.93 ± 0.001^{c}
Day 4	5.28 ± 0.01^{d}	6.73 ± 0.03^{e}	8.44 ± 0.01^{f}
Day 7	5.16 ± 0.01^{g}	6.98 ± 0.01^{h}	8.09 ± 0.05^{f}
Day 14	0	6.99 ± 0.01^{h}	8.42 ± 0.03^{i}

[a] Values are means ± SD; means with different superscript letters are significantly different ($P \leq 0.05$).

milk showed multidrug resistance (MDR) to four or five drugs of different groups. The *bla*TEM gene was identified in 85% of the isolates, while, none of the other investigated genes were detected.

The effects of different storage temperatures were evaluated on the survival of an MDR *Y. enterocolitica* isolate recovered from pasteurised milk during our study, the isolate harboured both the *ail* and *yst* virulence-associated genes as determined by PCR. Expression of the two genes in pasteurised milk samples stored at different temperatures was also investigated (Tables 3 and 4). All control samples at −20 °C, 4 °C and 25 °C tested negative for *Y. enterocolitica* growth during the experiment. Table 3 shows the means of the cfu in the examined samples at the three storage temperatures. At −20 °C, there was a significant ($P < 0.05$) decrease in the *Y. enterocolitica* count from 6.48 ± 0.03 to 5.16 ± 0.01 log_{10} cfu mL^{-1} after one week of storage, while no colonies were observed after two weeks of storage.

A significant increase was observed at 4 °C after 4 days (6.73 ± 0.03 log_{10} cfu mL^{-1}), one week (6.98 ± 0.01 log_{10} cfu mL^{-1}) and two weeks (6.99 ± 0.01 log_{10} cfu mL^{-1}) of storage. However, at 25 °C, a significant increase in the bacterial count was observed, reaching 8.42 ± 0.03 log_{10} cfu mL^{-1}, which was a 1.94-fold increase compared to the value at day zero ($P < 0.05$).

The relative expression of the *ail* and *yst* genes in *Y. enterocolitica* inoculated in pasteurised milk and stored At −20 °C, revealed significant down-regulation in the relative expression levels of the *ail* gene (0.53-fold change) and *yst* gene (0.45-fold change) after one week of storage, while no viable bacteria were detected after two weeks (Table 4). Refrigeration (storage at 4 °C) also significantly suppressed the expression of the two genes, reaching 0.34- and 0.24-fold changes in *ail* and *yst* expression, respectively. Significant up-regulation of the relative expression of the two genes was observed in all the examined samples, reaching 3.17- and 2.35-fold changes for the *ail* and *yst* genes, respectively, after two weeks of storage at 25 °C.

4. Discussion

In our study, 10% of the samples were contaminated with *Y. enterocolitica*, and the isolation rates from raw, fermented and pasteurised milk samples were 22%, 12% and 4%, respectively, while only 2% of the cheese samples were contaminated. Different studies have reported the contamination of raw milk with *Y. enterocolitica*, for instance, 25% (Yucel & Ulusoy, 2006), 3.3% (Özdemir & Arslan, 2015) in Turkey, 34.9% in Mexico (Bernardino-Varo et al., 2013), 3.1% in Italy (Bonardi et al., 2018), 7.7% in Finland (Ruusunen et al., 2013) and 10.6% in India (Subha, Ramakrishnan, & Suganthi, 2009). In Iran, different studies have reported the isolation of *Y. enterocolitica* from raw milk samples with comparable percentages: 2.3% (Hanifian & Khani, 2012; Soltan-Dallal, Tabarraie, & MoezArdalan, 2004) and 5.8% (Jamali et al., 2015).

Raw milk was reported to be contaminated with *Y. enterocolitica* in a number of studies from Egypt, with isolation rates of 10% (Ali, Attiah, & Aly, 2015) and 19.4% (Abd El Aal & Atta, 2009; Darwish, Asfour, & Allam, 2015). In dairy farms, faecal contamination of milk is expected to occur during milking. Storage and transport of contaminated milk under refrigeration may favour the growth of this psychotropic microorganism (Bonardi et al., 2018). Although the consumption of raw milk is not frequent, the manufacturing of homemade dairy products such as cheese from raw milk is common in Egypt, posing a risk to consumers. In Turkey, 35.7% (Yucel & Ulusoy, 2006) and 2.5% (Özdemir & Arslan, 2015) of cheese samples were positive, while in Egypt, 4% (Abd El Aal & Atta, 2009) and 6.7% (Ali et al., 2015) were contaminated. However, none of the investigated cheese samples were positive for *Y. enterocolitica* in Poland (Zadernowska & Chajęcka-Wierzchowska, 2017).

The isolation of *Y. enterocolitica* from pasteurised milk samples in our study (4%) was inconsistent with the results of another study in Egypt, which failed to isolate the bacterium from pasteurised milk (Abd El Aal & Atta, 2009). However, an outbreak of yersiniosis due to the consumption of milk and dairy products was reported in the USA in 2011, with 22 cases occurring due to the consumption of pasteurised milk (Longenberger et al., 2014). Other outbreaks have also been reported (Schiemann, 1987; Tacket et al., 1984). Previous reports have shown that this organism is unable to survive the pasteurisation process; however, the isolation of the pathogen from pasteurised milk could be due to failure of pasteurisation or contamination post-pasteurisation (Bernardino-Varo et al., 2013). The variation in *Y. enterocolitica* incidence among different studies could be attributable to several factors, including geographic region, season, hygienic practices and isolation methods (Bernardino-Varo et al., 2013).

Of the confirmed *Y. enterocolitica* isolates, 10% harboured virulence-associated genes (*ail* and/or *yst*). All these isolates were of biotype 4, whereas the other 10 (50%) *Y. enterocolitica* isolates were of biotype 1A and were negative for the examined genes. In

Table 4
Relative expression of the *ail* and *yst* genes in pasteurised milk stored at −20 °C, 4 °C and 25 °C.[a]

Storage time (days)	*ail*			*yst*		
	−20 °C	4 °C	25 °C	− 20 °C	4 °C	25 °C
Day 0	1^{a}	1^{a}	1^{a}	1^{a}	1^{a}	1^{a}
Day 2	0.96 ± 0.01^{a}	0.72 ± 0.01^{b}	2.59 ± 0.03^{f}	0.85 ± 0.01^{b}	0.72 ± 0.01^{f}	2.26 ± 0.04^{g}
Day 4	0.73 ± 0.02^{b}	0.503 ± 0.01^{c}	2.75 ± 0.01^{g}	0.77 ± 0.002^{c}	0.54 ± 0.01^{h}	2.33 ± 0.03^{i}
Day 7	0.53 ± 0.01^{c}	0.34 ± 0.01^{d}	3.18 ± 0.08^{h}	0.45 ± 0.003^{d}	0.24 ± 0.004^{j}	2.34 ± 0.02^{i}
Day 14	0	0.22 ± 0.01^{e}	3.17 ± 0.09^{h}	0	0.17 ± 0.003^{e}	2.35 ± 0.02^{i}

[a] Values are means ± SD; means with different superscript letters are significantly different ($P \leq 0.05$).

Mexico, biotypes 1A, 2, 3 and 4 were identified in raw milk samples (Bernardino-Varo et al., 2013), while in Iran, *Y. enterocolitica* belonging to the 1A, 1B and 4 biotypes were detected in raw cows' milk (Jamali et al., 2015). Another study, from Italy, reported the isolation of biotypes 2 and 1A from raw milk, and all the biotype 2 strains were positive for the *ail* gene, while only one was positive for the *yst* gene, and none of the 1A isolates harboured any of the two genes (Bonardi et al., 2018).

Biotype 1A is classified as non-pathogenic due to the absence of the pYV plasmid and major chromosomal virulence genes (Bhagat & Virdi, 2011). However, other studies have suggested that some strains of biotype 1A may cause gastroenteritis with symptoms similar to those produced by pathogenic biotypes (Söderqvist, Boqvist, Wauters, Vågsholm, & Thisted-Lambertz, 2012). This finding was attributed to the ability of biotype 1A to invade epithelial cells and to the resistance of this biotype to phagocytosis (Grant, Bennett-Wood, & Robins-Browne, 1999). Other studies have reported that biotype 1A was implicated in nosocomial (Ratnam, Mercer, Picco, Parsons, & Butler, 1982) and foodborne outbreaks (Greenwood & Hooper, 1990).

Contamination of food with antimicrobial-resistant bacteria is a threat to public health. The increased misuse or overuse of antimicrobials in animal production, veterinary medicine, and human therapy has resulted in increased resistance (Özdemir & Arslan, 2015). In our study, 85% of the isolates were resistant to ampicillin, while 85–100% were sensitive to different antibiotics, including gentamicin, ciprofloxacin, chloramphenicol, streptomycin, kanamycin and tetracycline. Comparable results were previously reported by other studies (Bonardi et al., 2018; Jamali et al., 2015; Soltan-Dallal et al., 2004). Only *bla*TEM gene was identified in the examined isolates exhibiting phenotypic resistance to ampicillin. In accordance, other studies reported the intrinsic resistance of *Y. enterocolitica* to ampicillin (Fàbrega & Vila, 2012; Frazão, Andrade, Darini, & Falcão, 2017). The observed low frequency of the multiple resistance phenotypes in our study is comparable with the previously reported rare MDR of *Y. enterocolitica* (Fàbrega & Vila, 2012; Frazão et al., 2017). The sensitivity of our isolates to ciprofloxacin is of concern because this drug is recommended for the treatment of *Y. enterocolitica* infection (Bonardi et al., 2018).

Y. enterocolitica is a psychrophilic organism that has the ability to grow at temperatures as low as 0 °C. According to the World Health Organisation, 25% of foodborne infections occur due to cross-contamination as a result of insufficient production and hygienic storage practices (Zadernowska & Chajęcka-Wierzchowska, 2017). In our study, there was a significant increase in the count of *Y. enterocolitica* isolates stored at refrigeration (4 °C) and ambient (25 °C) temperatures after one and two weeks of storage; however, a significant decrease was observed at freezing temperature (−20 °C). Accordingly, *Y. enterocolitica* growth increased at 4 °C and 24 °C by 1.8 and 3.4 logs, respectively, after storage for one week (Yehualaeshet et al., 2013).

The growth of *Y. enterocolitica* at refrigeration temperature (4 °C) has been previously reported in refrigerated water (Harvey, Greenwood, Pickett, & Mah, 1976; Highsmith, Feeley, Skaliy, Wells, & Wood, 1977; Karapinar & Gönül, 1991). Another study reported the ability of *Y. enterocolitica* to grow and survive in cheese stored at a wide range of temperatures from 3 to 15 °C, indicating the psychrotrophic nature of this organism (Kowalik & Lobacz, 2015). There was a significant increase in *Y. enterocolitica* growth in cheese stored at 8, 14 and 25 °C (AbdollahI & Hanifian, 2016). *Y. enterocolitica* can also multiply in milk at temperatures close to 0 °C (Bari, Hossain, Isshiki, & Ukuku, 2011). It has been reported that the *Y. enterocolitica* count increased by one order of magnitude after 20 days of storage of reconstituted powdered milk at 4 °C (Divya & Varadaraj, 2013). Another study reported that *Y. enterocolitica* was able to survive and grow in pasteurised milk at 8 °C and 24 °C (Bursová, Necidová, Haruštiaková, & Janštová, 2017). The authors reported 4 log increases after 7 days at 8 °C, while at room temperature, the counts increased sharply within two days.

Gene expression is temperature dependent; therefore, temperature plays an important role in pathogenesis in humans (Wunschel et al., 2005; Yehualaeshet et al., 2013). Virulence factors of *Yersinia* species are regulated and active at room temperature or host temperature (Herbst et al., 2009). Pathogenic bacteria, including *Yersinia* species, evolved sophisticated systems to sense the temperature of their environment (Straley & Perry, 1995). Our results revealed that *ail* and *yst* gene expression was significantly up-regulated by storage at ambient temperature; however, significant down-regulation of the two genes was observed at refrigeration and freezing temperatures. Consistent with our study, as the storage temperature increased from 4 °C to 24 °C, 8-fold and 2-fold increases in the expression of 38 genes and 237 genes, respectively, were observed (Yehualaeshet et al., 2013). Heat-stable enterotoxin was produced by toxigenic *Y. enterocolitica* in milk stored at 25 °C but not in milk stored at 4 °C (Bari et al., 2011; Francis, Spaulding, & Lovett, 1980). Moreover, strains that grew well in milk at 4 °C did not produce significant amounts of toxins (Olsvik & Kapperud, 1982). Our results revealed that temperature acts as a signal that results in up-regulation of the *ail* and *yst* genes. This finding is consistent with the results of a study on virulence gene expression in *Y. enterocolitica* and *Listeria monocytogenes* during storage (Rantsiou, Mataragas, Alessandria, & Cocolin, 2012; Yehualaeshet et al., 2013). However, further studies are required to understand the effects of temperature, and the underlying mechanism, on the expression of different genes in *Y. enterocolitica*.

5. Conclusions

Our results revealed that *Y. enterocolitica* biotype 4 is frequently isolated from retail milk and dairy products in the study area. The presence of the *ail* and *yst* genes indicates the potential risk posed by these bacteria. This study highlights the potential of *Y. enterocolitica* to survive significantly at different storage temperatures with different gene expression patterns, which could be important for the safety and processing of milk and dairy products. Monitoring of the survival and growth of *Y. enterocolitica* in milk and dairy products under storage conditions is recommended.

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