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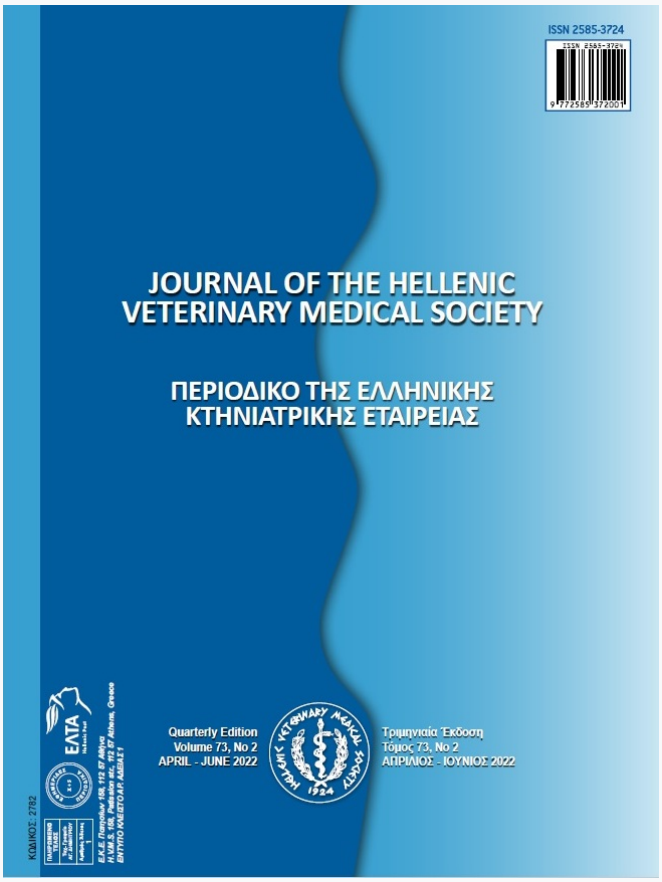

Biofilm formation, siderophore production, virulence-associated genes and genetic diversity of Yersinia enterocolitica from food

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Biofilm formation, siderophore production, virulence-associated genes and genetic diversity of *Yersinia enterocolitica* isolated from foods

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ABSTRACT: *Yersinia enterocolitica* is a foodborne pathogen widely found in nature and foods. The ability of this organism to multiply at refrigeration temperatures in food products with a prolonged shelf-life renders it a potential health risk for consumers. *Y. enterocolitica* isolates from foods were investigated for biofilm formation and siderophore production at different temperatures (12, 25 and 37 °C) using the microtiter plate and CAS agar plate methods, respectively. The isolates were also evaluated with PCR for the presence of virulence genes and rep-PCR for genetic diversity. Most of the isolates showed high capability to form biofilm at all temperatures after 24 and 48 h of incubation. The results at 12 and 25 °C ($P < 0.05$) as well as at 25 and 37 °C ($P < 0.05$) showed a statistically significant difference in quantification of biofilm formation after 24 h; however, no difference was observed between findings at 12 and 37 °C ($P > 0.05$). There was a significant difference at 12 and 37 °C ($P < 0.05$) as well as at 25 and 37 °C ($P < 0.05$) after 48 h of incubation; conversely, no significant difference was observed between 12 and 25 °C ($P > 0.05$). Strong biofilm-producing isolates at 12 °C were more prevalent at 48 h than at 24 h. Siderophore production was observed in 83.3% and 100% of the isolates at 25 and 37 ºC, respectively. However, no isolate produced siderophores at 12 °C. Genes *myfA*, *fepA*, *fepD*, *fes*, *hreP*, *tccC* and *ymoA* were detected in 16.7%, 33.3%, 100%, 100%, 44.4%, 5.6%, and 33.3% of the isolates, respectively. The rep-PCR revealed a high level of genetic differentiation among the isolates. The results indicate that foods of animal origin may act as a potential vehicle for dissemination in the community of *Y. enterocolitica* strains producing biofilm and siderophore and carrying virulence-associated genes.

Keywords: *Yersinia enterocolitica*; biofilms; virulence markers; rep-PCR

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INTRODUCTION

Yersinia enterocolitica is isolated from foods, water, sewage and animals. It can grow within the range of temperatures between 0 and 44 °C, optimally at 25-29 °C (Bhunia, 2008). As a psychrophilic organism, *Y. enterocolitica* can survive and multiply at refrigeration temperatures in foods with a prolonged shelf-life (Bhunia, 2008; Ye *et al.*, 2016). This ability makes it difficult to eliminate from food, and ingestion of food contaminated with this bacterium represents health risk for the consumers (Bhunia, 2008; Bonardi *et al.*, 2018). *Y. enterocolitica* is an important foodborne pathogen causing a variety of gastrointestinal infections, septicemia and other acute extra-intestinal infections in humans (Bottone, 2015).

Y. enterocolitica has the ability to adhere on biotic or abiotic surfaces and form biofilm that contributes to its survival in the environment and pathogenesis (Kim *et al.*, 2008; Kot *et al.*, 2011). Biofilm constitutes an alternative lifestyle that allows the bacteria to survive and propagate in harsh environmental conditions (Van Houdt and Michiels, 2010; Zadernowska and Chajecka-Wierzchowska, 2017). Biofilm formation on food surfaces and food contact equipment may serve as a significant source of contamination, leading to food spoilage and transmission of foodborne pathogens. Removal of a formed biofilm is very difficult due to its enhanced tolerance to physical and chemical treatments (Van Houdt and Michiels, 2010). The biofilm forming capacities of bacteria are influenced by several factors such as the material of attachment surface, temperature, growth medium and other environmental conditions (Van Houdt and Michiels, 2010; Singal *et al.*, 2019). In a study, all hydrophilic and hydrophobic *Y. enterocolitica* isolates adhered to polystyrene in varying degrees. Cell-surface hydrophobicity of *Y. enterocolitica* has been shown to be insignificant in its adhesion and subsequent biofilm formation (Kot *et al.*, 2011). Biofilm production in the isolates of *Y. enterocolitica* was also affected by temperature (Kim *et al.*, 2008) and culture medium (Singhal *et al.*, 2019). Previous studies have shown variations in the biofilm forming capability of *Y. enterocolitica* strains under conditions related to food environments (Wang *et al.*, 2017; Zadernowska and Chajecka-Wierzchowska, 2017).

Siderophores, which are low molecular weight and high-affinity iron chelating compounds, are secreted by *Y. enterocolitica* under iron deficient conditions and regarded as virulence factor (Bhagat and Virdi, 2011; Bottone, 2015). The siderophore-iron complex is transported by specific membrane receptors on the bacterial surface into the cell and internalized. After internalization of the ferric-siderophore complex, iron is released inside the cell (Bhunia, 2008). Siderophores have been demonstrated to be secreted by mouse-lethal strains of *Y. enterocolitica* (serotypes O:8, O:13, O:20, and O:40), but not in mouse non-lethal strains (serotypes O:3, O:5,27, and O:9) (Bottone, 2015).

Several factors contribute to the virulence of *Y. enterocolitica* to establish infection in the host tissue (Bhunia, 2008). The *myfA* (mucoid *Yersinia* factor) gene as a genetically stable virulence marker, encodes fibrillar surface antigen. It has an important role at the beginning of infection and in binding to distinct sites in the intestinal epithelial cells (Bhagat and Virdi, 2011; Bottone, 2015). Genes *fepA* (enterochelin receptor protein), *fepD* (enterochelin ABC transporter) and *fes* (enterochelin esterase) are involved in iron utilization; *hreP*, which encodes a subtilisin/kexin-like protease; *tccC*, which encodes the insecticidal toxin protein; *ymoA*, which encodes the *Yersinia* modulator protein (Grant *et al.*, 1998; Schubert *et al.*, 1999; Bhagat and Virdi, 2007).

Various molecular typing methods including multilocus sequence typing (MLST), whole genome sequencing (WGS), multiple-locus variable number tandem-repeat analysis (MLVA), PCR-ribotyping, pulsed-field gel electrophoresis (PFGE), repetitive element palindromic PCR (rep-PCR), enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) and random amplified polymorphic DNA (RAPD), whose information content and discriminatory power vary broadly, have been applied to characterize the microorganisms (Versalovic *et al.*,1991; Bhagat and Virdi, 2011; Petsios *et al.*, 2016). Among other valuable methods, rep-PCR which is quite reproducible with an increased accuracy has been used successfully to reveal the genetic diversity of *Y. enterocolitica* strains (Sachdeva and Virdi, 2004; Shanmugapriya *et al.*, 2014).

Y. enterocolitica is considered as substantial in terms of food hygiene because of its ability to grow and form biofilm at refrigeration temperatures along the food production chain which allows its persistence for long periods on food surfaces and its virulence potential (Bhunia, 2008; Petsios *et al.*, 2016; Bonardi *et al.*, 2018). Therefore, the objective of this study was

to investigate the biofilm formation and siderophore production ability under different temperatures as well as the presence of virulence genes (*myfA*, *fepA*, *fepD*, *fes*, *hreP*, *tccC* and *ymoA*) and the genetic diversity among the *Y. enterocolitica* isolates from meat and milk products.

MATERIALS AND METHODS

Bacterial isolates

Eighteen isolates of *Y. enterocolitica* recovered from chicken meat (10 isolates), ground beef (3 isolates), open white cheese (3 isolates) and raw milk (2 isolates), and identified by phenotypic and genotypic methods, were used in this study. The samples of raw cow's milk from public bazaars, open (unpackaged) white cheese made from pasteurized cow's milk from supermarkets, chicken meat containing breast and leg parts and ground beef from supermarkets, butchers and delicatessen shops were obtained. All isolates belonging to biotype 1A were activated from glycerol stock cultures (−20 °C) in Brain Heart Infusion broth (BHI) (Merck, Darmstadt, Germany) and grown overnight at 28 °C.

Detection of biofilm formation

Biofilm formation ability of *Y. enterocolitica* isolates was tested by the microtiter plate method according to Stepanovic *et al.* (2000) with minor modifications. The isolates were grown overnight in Tryptic Soy Broth (TSB) (Merck) at 28 °C. The bacterial suspension (adjusted to 0.5 Mc Farland scale) was transferred into the wells of three microtiter plates and incubated at 12, 25 and 37 ºC for 24 and 48 h. Wells with sterile TSB were used as negative control. After incubation, each well was washed three times with 250 µL of sterile phosphate-buffered saline and fixed with 200 µL of 99% methanol (Merck) for 15 minutes. Then the plates were air dried and stained with 200 µL of 0.1% crystal violet solution for 15 minutes. After removing crystal violet, the wells were washed with sterile distilled water and air-dried. The dye bound to the adherent bacteria was resolubilized with 160 µL of 33% (v/v) glacial acetic acid (Merck) per well. Absorbance at 570 nm was measured with a Multiskan Ascent plate reader (Thermo Electron Corporation, Finland). The isolates were classified as strong, moderate, weak and no biofilm producer according to the results of biofilm formation.

Detection of siderophore production

Siderophore production was investigated using chrome azurol S (CAS) blue plates according to the method of Schwyn and Neilands (1986) with some modifications recommended by Fiss and Brooks (1991). The CAS agar was prepared for 1L as follows: 60.5 mg CAS was dissolved in 50 mL water and mixed with 10 mL iron (III) solution (1 mM $FeCl_3.6H_2O$, 10 mM HCl). Then, this solution was slowly added to 72.9 mg hexadecyl-trimethyl-ammonium-bromide (HDTMA) dissolved in 40 mL water. The dark blue solution was autoclaved. Later, the basic medium of the CAS indicator solution was made. This indicator solution contains KH_2PO_4, 0.3 g/L; NaCl, 0.5 g/L; NH_4Cl, 1.0 g/L; PIPES (piperazine-*N, N'*-bis [2-ethanesulfonic acid]), 0.1 M, pH 6.8; L- asparagine, 5.0 g/L; and agar, 15 g/L. After the basic medium was autoclaved, the sterile CAS indicator solution was added slowly, followed with 20 mL of sterile glycerol. The overnight cultures of the *Y. enterocolitica* isolates were subcultured on the CAS agar plates. These plates were incubated at 12, 25 and 37 °C for 5 days. The appearance of orange-yellow zone around the colonies indicated siderophore production by the isolates. *Aeromonas hydrophila* ATCC 7966 and *Pseudomonas aeruginosa* ATCC 9027 were the control strains for siderophore production in this study.

Detection of virulence-associated genes

Genomic DNA of the *Y. enterocolitica* isolates was extracted from culture kept in BHI broth (Merck) at 28 °C for 18 h using the cetyltrimethylammonium bromide (CTAB) method according to Ausubel *et al.* (1991). All PCR reactions were carried out in a T100 Thermal Cycler (Bio-Rad Lab Inc., Hercules, USA). Each *Y. enterocolitica* isolate was tested for the presence of virulence genes *myfA*, *fepA*, *fepD*, *fes*, *hreP*, *tccC* and *ymoA* by PCR as previously described (Grant *et al.*, 1998; Schubert *et al.*, 1999; Kot and Trafny, 2004; Bhagat and Virdi, 2007). The products were resolved in 1% (w/v) agarose gel, analyzed by electrophoresis and visualized with UV transillumination (DNR Minilumi Bio-imaging Systems, Israel). DNA molecular marker-100 bp Plus DNA Ladder (Vivantis) was included as standard. *Y. enterocolitica* ATCC 23715 was used as a positive control.

Detection of genetic diversity by rep-PCR

The rep-PCR fingerprinting analysis was carried out with primers rep 1R-Dt and rep 2-Dt as described by Versalovic *et al.* (1991). The PCR reaction mixture (total volume 50 µL) contained 0.4 µM each primer (Biomers, Ulm, Germany), 4 µL DNA template (50

ng/μL), 5 μL 10X PCR buffer (Vivantis, USA), 4 mM $MgCl_2$ (Vivantis), 0.2 mM dNTP (Thermo Fisher Scientific, USA), 2 U Taq DNA polymerase (Vivantis) and 31.6 μL of PCR-grade water (AppliChem, Germany). The cycling conditions were as follows: initial denaturation at 94 °C for 3 min, 35 cycles at 94 °C for 2 min, 50 °C for 1 min and 72 °C for 5 min; with the final extension at 72 °C for 7 min. Amplification products were analyzed by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide (0.5 μg/mL) and photographed on a UV transilluminator (DNR Minilumi Bio-imaging Systems, Israel). The molecular weight of fragments was determined by comparison with a 100 bp Plus DNA Ladder (Vivantis) and the data matrix was generated from rep-PCR band patterns by scoring the presence and absence of bands as 1 and 0, respectively. The data matrix was generated from rep-PCR band patterns by scoring the presence of band as 1 and absence as 0. Cluster analysis was performed with the NTSYS-pc (version 2.10) software using the Dice similarity coefficient (1% position tolerance) on the basis of unweighted pair group method using arithmetic averages (UPGMA).

Statistical analysis

The results of biofilm formation at different temperatures were compared using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. All analyses were performed using the SigmaPlot 12.3 (Systat Software Inc., USA). A P value of < 0.05 was accepted to be significant.

RESULTS

Biofilm formation

The majority of the isolates produced biofilm at the three different temperatures (12, 25 and 37 °C) (Tables 1 and 2). The results of biofilm formation af-

Table 1. Biofilm formation, siderophore production and virulence gene profiles of *Y. enterocolitica* isolates

		Biofilm formation *						Siderophore production			Virulence-associated genes						
		24 h			48 h												
Isolate	Origin	12 °C	25 °C	37 °C	12 °C	25 °C	37 °C	12 °C	25 °C	37 °C	*myfA*	*fepA*	*fepD*	*fes*	*hreP*	*tccC*	*ymoA*
T1	Chicken meat	0.33 ± 0.10 (S)	0.67±0.01 (M)	0.73±0.03 (S)	0.80±0.31 (S)	0.64±0.28 (S)	0.34±0.06 (M)	-	+	+	-	+	+	+	+	-	+
T2	Chicken meat	0.15±0.01 (M)	1.25±0.07 (S)	0.25±0.04 (M)	0.45±0.015 (S)	0.77±0.18 (S)	0.20±0.08 (M)	-	+	+	-	+	+	+	+	-	+
T3	Chicken meat	0.11± 0.02 (M)	0.56±0.06 (M)	0.34±0.04 (S)	0.33±0.03 (S)	0.55±0.15 (S)	0.18±0.01 (M)	-	+	+	-	+	+	+	+	-	+
T4	Chicken meat	0.11± 0.01 (M)	0.22±0.13 (M)	0.06±0.02 (N)	0.16±0.03 (M)	0.27±0.04 (M)	0.05±0.01 (N)	-	+	+	-	-	+	+	-	-	-
T5	Chicken meat	0.06±0.00 (N)	0.18±0.15 (M)	0.09±0.04 (W)	0.06±0.01 (N)	0.56±0.29 (S)	0.07±0.01 (N)	-	+	+	+	+	+	+	-	-	+
T6	Chicken meat	0.10±0.04 (M)	0.30±0.04 (M)	0.08±0.01 (N)	0.13±0.01 (W)	0.50±0.13 (S)	0.07±0.01 (N)	-	+	+	-	+	+	+	+	-	+
T7	Chicken meat	1.14±0.24 (S)	1.49±0.04 (S)	0.08±0.00 (N)	1.57±0.10 (S)	1.07±0.42 (S)	0.14±0.10 (M)	-	+	+	-	-	+	+	-	-	-
T8	Chicken meat	0.68±0.36 (S)	1.10±0.03 (S)	0.39±012 (S)	0.81±0.05 (S)	1.07±0.14 (S)	0.35±0.02 (S)	-	+	+	-	-	+	+	-	-	-
T9	Chicken meat	0.07±0.01 (W)	0.22±0.11 (M)	0.28±0.02 (M)	0.13±0.03 (M)	0.97±0.37 (S)	0.15±0.05 (M)	-	+	+	+	+	+	+	+	+	+
T10	Chicken meat	0.74±0.12 (S)	0.81±0.03 (S)	0.39±0.16 (S)	1.86±0.29 (S)	0.66±0.07 (S)	0.29±0.06 (M)	-	-	+	+	-	+	+	+	-	-
K1	Ground beef	0.42±0.06 (S)	0.64±0.29 (M)	0.40±0.14 (S)	1.15±0.41 (S)	0.62±0.18 (S)	0.28±0.07 (M)	-	+	+	-	-	+	+	-	-	-
K2	Ground beef	0.32±0.06 (S)	0.40±0.04 (M)	0.13±0.03 (W)	1.31±0.63 (S)	0.64±0.07 (S)	0.09±0.03 (W)	-	+	+	-	-	+	+	-	-	-
K3	Ground beef	0.35±0.18 (S)	1.41±0.15 (S)	0.58±0.02 (S)	1.71±0.40 (S)	1.06±0.26 (S)	0.35±0.13 (S)	-	-	+	-	-	+	+	-	-	-
M1	Open white cheese	0.15±0.03 (M)	0.21±0.05 (M)	0.09±0.01 (W)	1.16±0.15 (S)	0.09±0.03 (W)	0.08±0.00 (N)	-	+	+	-	-	+	+	+	-	-
M2	Open white cheese	0.19±0.04 (M)	0.82±0.11 (S)	0.17±0.03 (M)	0.77±0.21 (S)	0.78±0.16 (S)	0.35±0.07 (S)	-	+	+	-	-	+	+	+	-	-
M3	Open white cheese	0.55±0.07 (S)	1.05±0.12 (S)	0.10±0.01 (W)	1.33±0.28 (S)	0.68±0.29 (S)	0.18±0.04 (M)	-	+	+	-	-	+	+	-	-	-
S1	Raw milk	0.25±0.07 (S)	0.58±0.27 (S)	0.06±0.01 (N)	0.64±0.15 (S)	0.58±0.15 (S)	0.08±0.02 (N)	-	+	+	-	-	+	+	-	-	-
S2	Raw milk	0.06±0.00 (N)	0.14±0.04 (W)	0.20±0.10 (S)	0.26±0.06 (S)	0.73±0.19 (S)	0.22±0.24 (M)	-	+	+	-	-	+	+	-	-	-

*Mean optical density values ± standard deviation; S, M, W and N in parentheses indicates strong, moderate, weak and no biofilm producer, respectively.

Table 2. Biofilm formation of *Y. enterocolitica* isolates at 12 °C, 25 °C and 37 °C after 24 and 48 h

Time	Temperature	No. (%) of biofilm producing isolates	Biofilm forming ability							
			Strong		Moderate		Weak		No biofilm	
			No. (%)	OD*	No. (%)	OD	No. (%)	OD	No. (%)	OD
24 h	12 °C	16 (88.9)	9 (50)	0.530 ± 0.141	6 (33.3)	0.135 ± 0.027	1 (5.6)	0.073 ± 0.007	2 (11.1)	0.056 ± 0.003
	25 °C	18 (100)	8 (44.4)	1.062 ± 0.137	9 (50)	0.378 ± 0.097	1 (5.6)	0.143 ± 0.038	0 (0)	-
	37 °C	14 (77.8)	7 (38.9)	0.431 ± 0.088	3 (16.7)	0.234 ± 0.031	4 (22.2)	0.103 ± 0.023	4 (22.2)	0.072 ± 0.008
48 h	12 °C	17 (94.4)	14 (77.8)	1.010 ± 0.220	2 (11.1)	0.147 ± 0.027	1 (5.6)	0.127 ± 0.014	1 (5.6)	0.060 ± 0.011
	25 °C	18 (100)	16 (88.9)	0.740 ± 0.208	1 (5.6)	0.273 ± 0.040	1 (5.6)	0.093 ± 0.026	0 (0)	-
	37 °C	13 (72.2)	3 (16.7)	0.350 ± 0.074	9 (50)	0.219 ± 0.077	1 (5.6)	0.091 ± 0.035	5 (27.8)	0.073 ± 0.010

*Optical density, values are expressed as mean ± standard deviation

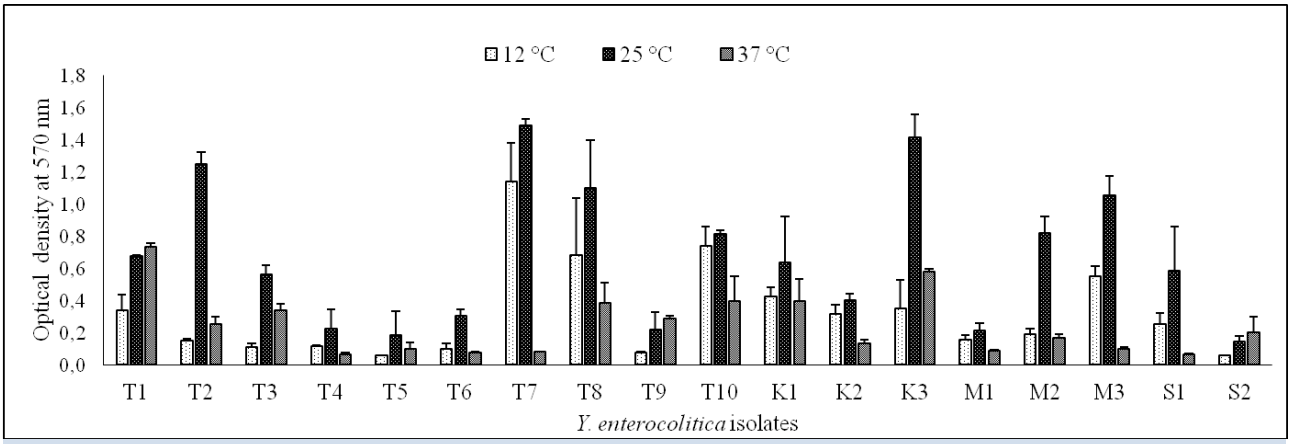

Figure 1. Biofilm formation of *Y. enterocolitica* isolates from meat and milk products at 12, 25 and 37 °C after 24 h

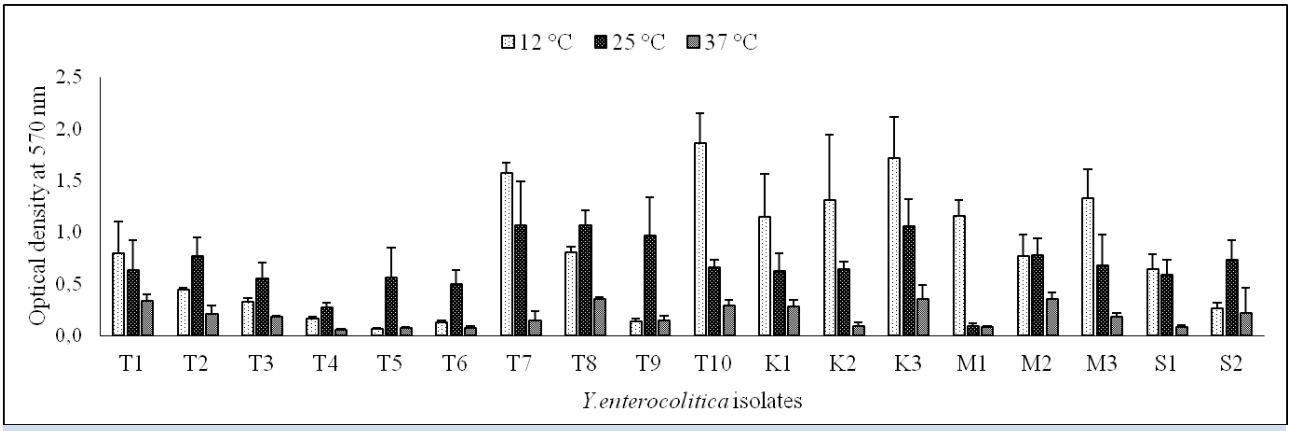

Figure 2. Biofilm formation of *Y. enterocolitica* isolates from meat and milk products at 12, 25 and 37 °C after 48 h

ter 24 and 48 h of incubation at 12, 25 and 37 °C are shown in Figures 1 and 2, respectively. The results at 12 and 25 °C ($P < 0.05$) as well as at 25 and 37 °C ($P < 0.05$) displayed a statistically significant difference in quantification of biofilm formation after 24 h; however, no difference was observed between 12 and 37 °C ($P > 0.05$). There was a significant difference at 12 and 37 °C ($P < 0.05$) as well as at 25 and 37 °C ($P < 0.05$) after 48 h of incubation; conversely, no significant difference was observed between findings at 12 and 25 °C ($P > 0.05$). After 24 h biofilm formation was detected in 16 (88.9%), 18 (100%) and 14 (77.8%) of the isolates at 12, 25 and 37 °C, respectively. In addition, after 48 h of incubation, 17 (94.4%), 18 (100%) and 13 (72.2%) of the isolates formed biofilm at 12, 25 and 37 °C, respectively. In particular, *Y. enterocolitica* isolates produced the strongest biofilms at 25 °C after 24 h and 48 h of incubation.

Siderophore production

Overall, 88.9% and 100% of the isolates produced siderophores at 25 and 37 °C respectively, in Chrome Azurol S (CAS) agar medium. Two meat isolates were negative for siderophore production at 25 °C.

However, none of the isolates were positive for siderophore production at 12 °C (Table 1).

Virulence-associated genes

The virulence gene profiles of the *Y. enterocolitica* isolates are shown in Table 1. The presence of virulence-associated genes *myfA*, *fepA*, *fepD*, *fes*, *hreP*, *tccC* and *ymoA* was detected in 16.7%, 33.3%, 100%, 100%, 44.4%, 5.6%, and 33.3% of the isolates, respectively. All isolates were found to carry at least two or more virulence genes. Only one isolate from chicken meat was positive for all tested virulence genes. At least four or more virulence genes were present in seven (38.9%) isolates. Nine (50%) isolates were found to be positive for the same two genes, namely *fepD* and *fes*.

Genetic diversity

The genetic diversity among the 18 *Y. enterocolitica* isolates from various foods was evaluated using the rep-PCR technique. Dendrogram generated from rep-PCR fingerprinting of these isolates is shown in Figure 3. The rep-PCR analysis revealed the presence of 16 different genotypes. Isolates T1 and T5 from chicken meat as well as isolates K1 and K3 from ground beef exhibited 80% similarity. The remaining 14 (77.8%) isolates were singletons. The discrimination index of rep-PCR in this study was found to be 0.987. No clear correlation betwen the origin of the isolates and rep-PCR fingerprint profiles was observed.

DISCUSSION

Y. enterocolitica as a significant food-borne pathogen has the capacity to grow and survive in a wide range of temperatures, including refrigeration temperatures. This makes it a major concern to food safety (Bhunia, 2008). *Y. enterocolitica* infections occur as a result of consumption of contaminated food with this pathogen harboring virulence markers (Bhunia, 2008; Bottone, 2015).

The ability to form biofilm which contributes to virulence of the strain is believed to be an important property of bacterial pathogens (Bhunia, 2008; Zadernowska and Chajecka-Wierzchowska, 2017). In this study, all *Y. enterocolitica* biotype 1A isolates from various foods produced biofilms after 24 and 48 h of incubation at 25 °C. Similarly, a recent study in India reported that 100% of *Y. enterocolitica* biotype 1A strains isolated from clinical samples, wastewater, pigs and pork formed biofilms at 28 °C after 24 and 48 h of incubation (Singhal *et al.*, 2019). The optimum growth temperature of *Y. enterocolitica* is 25-29 °C (Bhunia, 2008). Our results indicated high ability to form biofilm at 25 °C, which is consistent with a previous study (Kim *et al.*, 2008). After 24 h -incubation at 37 °C, 77.8% of the isolates produced biofilm and 38.9% of these isolates were strong biofilm producers (Table 2). However, a study conducted by Zadernowska and Chajecka-Wierzchowska (2017) indicated that 57.1% of the *Y. enterocolitica* strains from poultry and minced meat were biofilm producers after 24 h of incubation at 36 °C but none of them had a strong biofilm forming ability. After overnight incubation at 37 °C, Kot *et al.* (2011) reported that 18.2% of the isolates from pigs adhered strongly. This percentage is lower than our results (38.9%). Over 88% of the *Y. enterocolitica* isolates from food were biofilm producers at 12 °C after 24 and 48 h of incubation (Table 2). This finding may present a threat to the food processing industry, because this temperature is closely related to processing and storage practices in food industry (Mercier, 2017; Ndraha *et al.*, 2018). Previous studies demonstrated the biofilm-forming capacity might help *Y. enterocolitica* to persist in food and food-processing environment (Wang *et al.*, 2017; Zadernowska and Chajecka-Wierzchowska, 2017). Biofilm-forming pathogens in food environments pose a risk to food safety and human health (Van Houdt and Michiels, 2010).

Many bacteria including *Y. enterocolitica* produce several iron-chelating substances known as siderophores which are essential for environmental iron uptake (Schubert *et al.*, 1999; Bhunia, 2008). Siderophore plays an important role in *Y. enterocolitica* virulence. Siderophore production by *Y. enterocolitica* on the CAS agar was reported in many previous studies (Brem *et al.*, 2001; Rakin *et al.*, 2012). In a study, the influence of temperature on siderophore -producing ocean isolates was investigated and the isolates showed varying siderophore production at different temperatures (Sinha *et al.*, 2019). In another study, bacterial species including *Enterobacter*, *Erwinia*, *Citrobacter*, *Bacillus*, *Micrococcus* and *Microbacterium* were isolated from environments and screened for siderophore production at 28 and 37 °C on the CAS agar plates. Siderophore production at these temperatures was found to be variable (Chaudhary *et al.*, 2017). In this study, siderophore production was detected in 16 (88.9%) and all (100%) of the isolates at 25 and 37 °C, respectively. However, siderophore production was not detected in any of the isolates at 12 °C on the CAS agar plate.

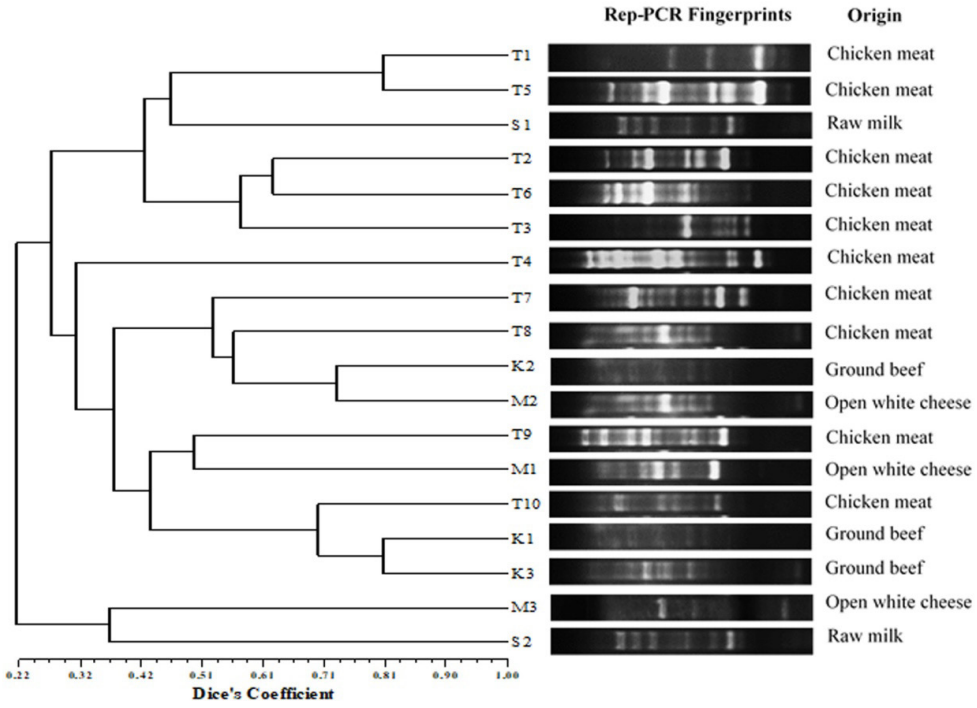

Figure 3. Rep-PCR based genotyping of the 18 *Y. enterocolitica* isolates from meat and milk products

In the present study, the prevalence of the *myfA* gene (16.7%), related to fimbriae production, was the same (16.7%) with previous findings of a study in Italy concerning *Y. enterocolitica* strains isolated from raw milk (Bonardi *et al.*, 2018). In contrast, a study done in India found that prevalence of the *myfA* gene was higher (60%) in nonclinical strains of *Y. enterocolitica* compared with clinical strains (35%) (Bhagat and Virdi, 2007). All *Y. enterocolitica* isolates from pork production chain in Brazil were positive for *myfA* (Martins *et al.*, 2018). The genes *fepA* and *fepD* encode the siderophore transport proteins and *fes* functions in iron release into the bacterial cytoplasm (Bhagat and Virdi, 2011). We found that the prevalence of *fepA*, *fepD* and *fes* genes in the *Y. enterocolitica* isolates was 33.3%, 100%, 100%, respectively. The result for both the *fepD* and *fes* genes (100%) was higher in this study than that (79%) observed earlier by Campioni and Falcao (2014). However, the result for the *fepA* (33.3%) was quite lower than that observed in that study (72%). Furthermore, previous studies demonstrated the presence of the *fepD* and *fes* genes in all isolates of *Y. enterocolitica* biotype 1A (Bhagat and Virdi, 2007; Ye *et al.*, 2016), similar to our results. In contrast to our results, Martins *et al.* (2018) indicated that *Y. enterocolitica* isolates belonged to biotype 1A did not carry the *fepA*, *fepD* and *fes* genes. The *hreP* and *ymoA* genes were detected in 44.4% and 33.3% of the isolates, respectively, in this study. Previous reports demonstrated that the *hreP* gene was detected in the majority of the *Y. enterocolitica* isolates (>70%) (Bhagat and Virdi, 2007; Campioni and Falcao, 2014) and the *ymoA* gene was found in all *Y. enterocolitica* isolates (100%) (Bhagat and Virdi, 2007; Martins *et al.*, 2018). The *tccC* gene, which encodes insecticidal toxins, was found in 5.6% of the *Y. enterocolitica* isolates in this study, which is lower than the prevalence reported for nonclinical isolates by Campioni and Falcao (2014) (21%). However, Bhagat and Virdi (2007) showed that none of the nonclinical isolates of *Y. enterocolitica* carried the *tccC* gene. Previous studies in clinical strains reported greater carriage frequency of *tccC* gene (Tennant *et al.*, 2005; Bhagat and Virdi, 2007).

Genetic variability of the *Y. enterocolitica* biotype 1A isolates from various foods was analyzed using the rep-PCR fingerprinting (Figure 3). The index of discrimination was found to be 0.987, indicating a high level of genetic diversity among these isolates. Wojciech *et al.* (2004) observed that rep-PCR was more discriminative for *Y. enterocolitica* isolates than ERIC-PCR and ITS profiling. In epidemiological studies, rep-PCR has been considered as a more suitable method for the typing of *Y. enterocolitica* due to its discriminatory power (Wojciech *et al.*, 2004; Shanmugapriya *et al.*, 2014). The current study did not reveal an explicit relationship between rep-PCR

genotypes and the origin of the isolates, which is similar to a previous study in India (Sachdeva and Virdi, 2004).

CONCLUSIONS

This study highlighted the biofilm-formation ability and siderophore production at 12, 25 and 37 °C as well as the virulence determinants and genetic relationship of the *Y. enterocolitica* biotype 1A isolates from various foods. All isolates were positive for biofilm and siderophore production at different temperatures. In particular, *Y. enterocolitica* isolates produced high levels of biofilm at 12 and 25 °C. The ability of this pathogen to form biofilm and persist for long time at these temperatures used during food processing and storage may increase the risk of contamination, resulting in consumers' health hazard. Furthermore, all isolates carried at least two or more virulence genes. The rep-PCR analysis revealed a high genetic diversity among these isolates. Consequently, the results of this study indicated that the presence of *Y. enterocolitica* strains with possessing putative virulence characteristics pose a health risk to consumers.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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