Received: 12 January 2022, Accepted: 6 March 2022

Original Research

Journal of Advanced Veterinary Research
(2022) Volume 12, Issue 2, 118-127

Molecular Characterization of *Y. enterocolitica* Isolated from Dairy Environment with Special Reference to the Antimicrobial Activity of Milk Proteins Hydrolysates

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Abstract

Yersinia species, especially *Yersinia enterocolitica*, are considered as the most prevalent milk-borne pathogens. *Y. enterocolitica* is the causative agent of yersiniosis, a zoonotic disease of growing epidemiological importance with significant consequences for public health. A total of 300 samples out of milk and milk products water and environmental samples were collected from dairy cattle rural house and local dairy shops and vendors to be investigated for presence of *Yersinia* spp. Isolates were molecularly identified and screened for virulence markers, biofilm production and antimicrobial resistance profile. Finally, the antimicrobial activity of milk proteins hydrolysates against *Y. enterocolitica* was detected. *Yersinia* spp. was recovered from 50% of the examined samples. The most prevalent species was *Y. enterocolitica* (isolated from 21.7 and 30% of milk and its products, and environmental samples, respectively). Detection of virulence genes revealed that 24% of *Y. enterocolitica* isolates harbored both *ail* and *yst* genes. *Y. enterocolitica* isolates showed high antimicrobial resistance to various antimicrobials. Also, different biofilm phenotypes were produced by these isolates. The most produced biofilm phenotype was moderate (68.9%). The parent proteins (CCP, CWP) and their pepsin hydrolysates (P-CCP and P-CWP) were potentially effective in inhibiting *Y. enterocolitica* growth and peptides P-CWP exhibited the strongest effect against *Y. enterocolitica*.

KEYWORDS
Antimicrobial biopeptides; Biofilm formation; ;Dairy environment; ; Milk & Milk products; *Yersinia enterocolitica*; Virulence and antimicrobial resistance

INTRODUCTION

Yersinia enterocolitica is a Gram-negative, non-spore former, facultative anaerobe, short rod-shaped bacterium. *Yersinia* are pathogens that able to withstand low temperature, can thrive at 4 ºC and are widely dispersed in the environment; consequently, in cold chain food items, particularly milk and cheese, they might pose a potential food safety risk (Grahek-Ogden *et al*., 2007). *Yersinia* include pathogenic and several non-pathogenic strains. There are 11 species of *Yersinia*, three of which (*Yersinia enterocolitica*, *Yersinia pestis*, and *Yersinia pseudotuberculosis*) have been proven to cause human illness (Tennant *et al*., 2005).

Yersinia enterocolitica is a psychotropic opportunistic waterborne and foodborne enteropathogen. Acute enteritis (particularly in children), enterocolitis, acute diarrhea, fever, abdominal pain, mesenteric lymphadenitis, and pseudoappendicitis are the most frequent clinical signs of *Y. enterocolitica* infections (Younis *et al*., 2019). It has a high proclivity for extraintestinal dissemination under certain host conditions, resulting in extraintestinal manifestations including a wide range of clinical symptoms (Krajinović *et al*., 2007). The majority of cases are sporadic or occur in tiny clusters, although massive outbreaks have been documented around the world in families, schools, hospitals, and in combination with community gatherings (Leclercq *et al*., 2005).

The precise infection dosage needed to produce yersiniosis in humans is more than 4 log colony forming units (CFU), reaching 7-9 log cells (Robins-Browne, 2013). *Y. enterocolitica* strains in raw milk were able to survive in the presence of many competing microorganisms and retain the virulence plasmid throughout prolonged cold storage (Larkin *et al*., 1991). Because of the several virulence factors carried on its virulence plasmid, which is solely associated with virulent strains, *Y. enterocolitica* strains

ISSN: 2090-6277/2090-6269/

have the ability to cause disease (Fàbrega and Vila, 2012). Two virulent-associated genes (the adhesion protein *ail* and the enterotoxin *yst*) included in *Y. enterocolitica*; the *ail* gene is only found in pathogenic strains and encodes a surface factor that enhances epithelial cell penetration (adhesion and invasion) and protects bacteria from complement bactericidal actions (Ahmed *et al*., 2019). The *yst* gene encodes enterotoxin, which induces liquid retention in the intestine via absorption by the intestinal villi and is typically found in diarrheagenic biotype 1A strains (Younis *et al*., 2019).

The most effective treatment strategy for *Y. enterocolitica* infections is still antibiotic therapy. However, the antibiotic resistance profiles of *Y. enterocolitica* isolates are alarming for public health because of the likelihood of human transmission (Ahmed *et al*., 2019). Antibiotic resistance is mostly caused by the acquisition of transferrable multidrug resistance genes or antibiotic misuse (Bharathy *et al*., 2014). The World Health Organization (WHO) announced in its infectious diseases report that antibiotic resistance is a severe threat to human health and that issue is expanding globally (Pandove *et al*., 2012). Ciprofloxacin, ceftriaxone, and cefotaxime have all been used effectively to treat complex infections caused by *Y. enterocolitica* (Lupi *et al*., 2013). To the best of the authors knowledge, no data on the antimicrobial susceptibilities of *Y. enterocolitica* isolates from milk, milk products, water, and farm environments in Egypt has been published.

Microbial biofilms are a serious concern in human and veterinary medicine, as well as in food safety, since it enhances bacterial resistance to various physical and chemical parameters used to optimize hygiene in the food industry (Brown and Gilbert, 1993). Biofilms are communities of microorganisms that develop permanently associated with each other, producing an extracellular polymeric substance (ESP) of carbohydrate or exopolysaccharide, attached to living or inert substrates enclosed in a polymeric matrix generated by themselves (Mah *et al*., 2001). Biofilms provide bacteria with advantages over planktonic cells as sessile cells are more resistant to environmental changes, host defenses, and antibiotic treatment. In clinical medicine, the most significant feature of biofilm formations is the increased resistance to antimicrobial agents owing to ESP protection, which promotes to multidrug resistance and treatment failure (Kunin and Steele, 1985; Nickel *et al*., 1985; Gristina *et al*., 1987; Costerton *et al*., 1993).

Recently, it has also been explored more additional health benefits of milk for human, beyond their basic nutritional values. Among its various nutrients, protein is one of the most functionally diverse nutrients, with a wide range of biologically active peptides (Guha *et al*., 2021). The antimicrobial activities of milk protein-derived biopeptides are very diverse, including those with a prebiotic effect, peptides with the ability to prevent pathogen attachment or invasion and peptides inhibiting the growth of microorganisms (Ahmed *et al*., 2020). During enzymatic hydrolysis or milk fermentation, several biopeptides with antibacterial activity have been produced from bovine milk proteins. As reviewed by Sibel Akalın (2014), these antibacterial biopeptides have been proven to exhibit a wide range of activity against gram-positive and gram-negative bacteria. With the increasing demand of evidence-based medicine, it is important to identify the bioactive peptides of milk proteins, which may be responsible for antimicrobial against *Y. enterocolitica*.

The present study aimed to investigate the prevalence of *Yersinia* species especially *Y. enterocolitica* in milk, milk products, water and dairy cow macro-environmental samples in Qena city, Egypt, in addition to detection of chromosomal virulence genes (*ail* and *yst*) in *Y. enterocolitica* isolates and the antibiotic susceptibility profiles of the isolated strains for different antimicrobial agents. Moreover, the aim of the study was to determine the ability to form biofilm by the isolated strains. The antimicrobial activity of milk proteins hydrolysates against isolated and identified *Y. enterocolitica* isolates were evaluated in vitro.

MATERIALS AND METHODS

Collection of samples

The current study was designed to target the household rearing of cattle in Qena city, Egypt, and to determine the prevalence and monitor the main source of *Yersinia* species especially *Y. enterocolitica* in cow's house environment, dairy shops and vendors. A total of 300 samples, which included milk and milk products (180 samples), water (30 samples) and environmental samples (90 samples) were collected from dairy cattle rural house and local dairy shops and vendors.

From local dairy houses, shops and vendors, a total of 60 raw milk samples (household and street vendor milk) were collected in clean sterilized 50 ml Falcon™ conical tubes. Additionally, 120 milk product samples were also collected from each of the following categories: Kareish cheese, Yoghurt, Ice cream and Rice milk (30 samples each). Water samples were collected from local water sources inside framer houses according to the recommendation of WHO (1971). Dairy manure and bedding samples were collected according to Rendos *et al*. (1975), while equipment's and working swabs were collected as described by Collins *et al*. (1991).

Isolation and identification of Yersinia species

The isolation of *Yersinia* species was adopted according to FDA (2007); Shwimmer *et al*., (2007) and Fukushima *et al*., (2011). About 25 ml/g of each sample was aseptically homogenized in 225 ml of trypticase soya broth (TSB) supplemented with *Yersinia* selective supplement (Oxoid, SR0109); mixed well and incubated at 4 ºC for up to 14 days for cold enrichment. The samples were treated with KOH solution (KOH 0.5% + saline 0.5%) to suppress the background flora grown after enrichment. Then cultures were streaked on the *Yersinia* selective agar base plate (Oxoid, CM0653) supplemented with *Yersinia* selective supplement (Oxoid, SR0109) and incubated at 30 ºC for 48-72 h.

Presumptive *Yersinia* spp. isolates (Colonies with red bull's eye-like colonies (small, with a red center and surrounded by a transparent border)) were purified on tryptic soya agar (Oxoid, CM0131) and confirmed according to Gram reaction and biochemical identification (Rahimi *et al*., 2014; Weagant *et al*., 2017). The isolated and characterized *Y. enterocolitica* strains were confirmed using Microbact Biochemical Identification *Yersinia* 12A and 12B Combined Kits S*yst*em (Oxoid) according to Chye *et al*. (2004).

Molecular identification of Y. enterocolitica

Suspected isolates were confirmed by 16S rRNA gene-based PCR-technique using the primers listed in Table 1. Moreover, The PCR-technique was applied for molecular characterization of two virulence-associated genes using two sets of primers in confirmed *Y. enterocolitica* isolates. Those genes were *ail* (attachment-invasion locus protein) and *yst* (*Yersinia* heat-stable enterotoxin). Primer sequences, amplicon size and PCR program used in this study were presented in Table 1, according to the referred authors. PCR was applied following QIA amp DNA mini kit

Table 1. PCR protocol including primer sequences, Amplicon size and amplification reactions.

Target gene		Primers sequences	Amplified segment (bp)	Primary	Amplification (35 cycles)			Final extension	Reference
				denaturation	Secondary denaturation	Annealing	Extension		
Y. enterocolitica 16S rRNA	Y1	AATACCGCATAACGTCTTCG	330	94 °C	94 °C	62 °C	72 °C	72 °C	Wannet *et al.* (2001)
	Y2	CTTCTTCTGCGAGTAACGTC		5 min	30 sec	40 sec	40 sec	10 min	
ail	ail-F	TAATGTGTACGCTGCGAG	351	94 °C	94 °C	55 °C	72 °C	72 °C	Koua *et al.* (2014)
	ail-R	GACGTCTTACTTGCACTG		5 min	30 sec	40 sec	40 sec	10 min	
yst	Pr2a	AATGCTGTCTTCATTTGGAGC	145	94 °C	94 °C	55 °C	72 °C	72 °C	
	Pr2c	ATCCCAATCACTACTGACTTC		5 min	30 sec	30 sec	30 sec	7 min	

instructions (Catalogue no.51304); Dream Taq Green PCR Master Mix (2X) (Thermo Scientific) Cat No. K1081 and agarose gel electrophoreses (Sambrook *et al*., 1989).

Antibiogram pattern of Y. enterocolitica

All *Y. enterocolitica* isolates were examined for their antibiogram pattern by disc diffusion technique as described by Bauer *et al*. (1966) against a panel of 11 antibiotics. The selection of antibiotics (11) was based on their common use in human and animals in Egypt which included Amoxycillin (AM, 20 µg), Ampicillin (AMP, 10 µg), Cephalexin (CL, 30 µg), Chloramphenicol (C, 30 µg), Ciprofloxacin (CIP, 10 µg), Erythromycin (E, 15 µg), Gentamicin (GEN, 10 µg), Penicillin (P, 10 unit), Oxacillin (OX, 1 µg), Streptomycin (S, 10 µg), Tetracycline (TE, 30 µg). All antibiotics used were obtained from Oxoid, UK. The results were interpreted as sensitive or resistant based on CLSI interpretive standards (CLSI, 2018).

Detection of biofilm formation by the microplate (MP) method

The method for assessment of biofilm formation by the MP method was based on the techniques described by Zadernowska and Chajęcka-Wierzchowska (2017). Wells of a sterile 96-well-flat-bottomed sterile poly*styr*ene microtiter plates (Nunc) were filled with 200 µl of fresh sterile broth BHI (Merck Millipore). 20 µl of overnight cultures of each *Y. enterocolitica* isolates with a cell density of 1×10^9 cells/ml were added in triplicate, onto a 96-well. Negative control wells contained broth only. The plates were covered and incubated aerobically at 30 ºC for 24 h. The bacterial suspension was aspirated, and each well was washed three times with 250 µl of PBS buffer (Sigma). After that, the biofilm was fixed with 200 µl of ethanol (99%) for 15 min and was later removed. The plates were dried at room temperature, stained with a 200 µl of cr*yst*al violet solution used for Gram staining for 5 min, washed in running water until unbound cr*yst*al violet was removed and dried at room temperature. The dye bound to the adherent cells was re-solubilized with 160 µl of 33% (v/v) glacial acetic acid per well. Absorbance was read using plate reader at 570 nm. The optical density (ODs) of each strain was obtained by the arithmetic mean of the absorbance of three wells and this value was compared with the cut-off OD (ODc) which was defined as three standard deviations above the mean OD of the negative control. The following classification was used for the determination of biofilm formation: no biofilm production (OD ≤ ODc), weak biofilm production (ODc < OD ≤ 2ODc), moderate biofilm production (2ODc < OD ≤ 4ODc) and strong biofilm production (4ODc < OD).

Antimicrobial activity of milk proteins hydrolysates against Y. enterocolitica

Microorganisms

Recovered *Y. enterocolitica* virulent strains were enriched in tryptic soy broth before cultivation on *Yersinia* selective agar base plate supplemented with *Yersinia* selective supplement for 24 h at 30 °C before assay.

Hydrolysis of cow milk proteins with pepsin enzyme

Separation of caseins and whey proteins were performed according to Ahmed *et al*. (2015); briefly centrifugation was used to remove fat from 250 ml raw milk, and the skimmed milk was then passed through three layers of gauze. The defatted milk was adjusted to pH 4.6 with 10% acetic acid and subjected to centrifugation. Cow casein proteins (CCP) and cow whey proteins (CWP) were separated and lyophilized.

In vitro pepsin digestion of CCP and CWP

The CCP and CWP solutions in Milli-Q water were adjusted to pH 3.0 with HCl. Pepsin in 1 mM HCl was added to the protein solutions at enzyme-to-substrate (E/S) ratio of 1:50 (w/w), followed by incubation for 2 h at 37 ºC with mild shaking. Heating at 85 °C for 5 min was used to inactivate pepsin followed by and immediately cooling in ice for 5 min. Centrifugation was applied to remove insoluble solids; and the obtained supernatants were adjusted to pH 7.0, to inactivate pepsin fully, followed by lyophilization (Ibrahim *et al*., 2017). The CCP and CWP, as well as their hydrolysates (P-CCP and P-CWP), were tested for antimicrobial activity against *Y. enterocolitica* as described below.

Antimicrobial assay

The liquid-broth method (Ahmed *et al*., 2020) was used to assess the bactericidal activity of milk proteins hydrolysates against *Y. enterocolitica* at different concentrations. Bacteria, grown to mid-logarithmic phase in brain heart infusion (BHI) broth, were washed and resuspended (to give 6-7 log10 CFU/ml) in 1% trypticase soya broth (TSB), pH 7.3. Aliquots of bacterial suspension (100 µl) were pipetted onto wells of 96-well microplate containing 100 µl of test protein or hydrolysates at different concentrations (0-1500 µg/ml). After incubation at 30 ºC for 24 h, absorbance was monitored from three parallel wells per sample at 620 nm. The reading was background-corrected (value in the absence of bacteria) and the results are representative of three independent experiments.

RESULTS

Bacteriological analysis of samples

A total of 180, 30 and 90 milk & milk products, water and environmental samples were screened for the presence of *Yersinia* spp. especially *Y. enterocolitica*, overall, 85 (47.2%), 9 (30%) and 56 (62.2%) samples yielded *Yersinia* spp., respectively. Of these

39 (21.7%), 5 (16.7%) and 31 (34.4%) samples were positive for *Y. enterocolitica*, respectively (Table 2). The highest occurrence percentage of *Y. enterocolitica* was detected in street vendor milk (33.3%), followed in order by household milk (26.7%), rice milk (23.3%), ice cream (16.7%) and water (16.7%). Moreover, the highest occurrence percentage of *Y. enterocolitica* in environmental samples was detected in dairy manure and bedding (43.3%), working surfaces (36.7%) and dairy equipment's (23.3%) (Table 2).

As shown in Table 2, isolates of *Yersinia* spp. were studied for morphological, biochemical properties and Microbact Biochemical Identification *Yersinia* 12A and 12B Combined Kits *Syst*em (Oxoid). These tests were recommended for the identification of *Yersinia* spp. according to Chye *et al.* (2004); Rahimi *et al.* (2014) and Weagant *et al.* (2017). The identification with classical methods based on morphological and biochemical criteria as well as Combined Microbact Kits *Syst*em showed that *Yersinia* spp. isolates were identified as *Y. enterocolitica*, *Y. pseudotuberculosis*, *Y. intermedia* and *Y. frederiksenii*. Among these isolates, it can be indicated that *Y. enterocolitica* was the most frequently occurring *Yersinia* spp. with an incidence of 25% of isolates followed by *Y. intermedia* (9.7%), *Y. frederiksenii* (8.3%) and *Y. pseudotuberculosis* (7%) (Table 2).

Incidence of Virulence Genes in isolated Y. enterocolitica

PCR assay was performed on identified *Y. enterocolitica* isolates using primers designed for virulence genes (Table 3). The *ail* and *yst* coding for attachment-invasion locus protein and *Yersinia* heat-stable enterotoxin were demonstrated in 41.0, 46.2% and 58.3, 41.6% among *Y. enterocolitica* isolates of milk, milk products and environmental samples, respectively (Table 3 and Figure 1).

A total of 75 *Y. enterocolitica* isolates were recovered. Among of them, 52 strains (69.3%) exhibited virulent profiles while 23 (30.7%) were non-virulent isolates according to screened virulent genes. Specific virulence-gene profiles turned out to be more prevalent than others in this study. Four virulence-gene profiles

Table 2. Incidence of different *Yersinia* spp. detected in the examined samples (n= 30).

Type of sample		Total No. of *Yersinia* spp.		*Y. enterocolitica*		*Y. pseudotuberculosis*		*Y. intermedia*		*Y. frederiksenii*	
		No.	%	No.	%	No.	%	No.	%	No.	%
Milk and milk products samples	Household milk	18	60	8	26.7	3	10	5	16.7	2	6.7
	Street vendor milk	20	66.7	10	33.3	3	10	4	13.3	3	10
	Kareish cheese	14	46.7	5	16.7	2	6.7	4	13.3	3	10
	Yoghurt	10	33.3	4	13.3	2	6.7	2	6.7	2	6.7
	Ice cream	11	36.7	5	16.7	1	3.3	1	3.3	4	13.3
	Rice milk	12	40	7	23.3	1	3.3	2	6.7	2	6.7
Total (180)		85	47.2	39	21.7	12	6.7	18	10	16	8.9
Dairy Cattle Macroenvironment	Water	9	30	5	16.7	1	3.3	1	3.3	2	6.7
	Dairy Equipment's	12	40	7	23.3	2	6.7	2	6.7	1	3.3
	Dairy Manure and Bedding	24	80	13	43.3	3	10	4	13.3	4	13.3
	Working surfaces	20	66.7	11	36.7	3	10	4	13.3	2	6.7
Total (120)		65	54.2	36	30	9	7.5	11	9.2	9	7.5
Overall Total (300)		150	50	75	25	21	7	29	9.7	25	8.3

Table 3. Virulence-gene (*ail* (A) and *yst* (Y)) and their distribution profile in *Y. enterocolitica* strains screened by PCR.

Type of sample		*Y. enterocolitica*	*ail*		*yst*		A+Y+		A+Y-		A-Y+		A-Y-	
		No.	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Milk and milk products samples	Household milk	8	5	62.5	4	50	2	25	3	37.5	2	25	1	12.5
	Street vendor milk	10	4	40	6	60	3	30	1	10	3	30	3	30
	Kareish cheese	5	1	20	1	20	0	0	1	20	1	20	3	60
	Yoghurt	4	0	0	2	50	0	0	0	0	2	50	2	50
	Ice cream	5	2	40	2	40	1	20	1	20	1	20	2	40
	Rice milk	7	4	57.1	3	42.9	2	28.6	2	28.6	1	14.2	2	28.6
Total (180)		39	16	41	18	46.2	8	20.5	8	20.5	10	25.6	13	33.4
Dairy Cattle Macroenvironment	Water	5	4	80	3	60	3	60	1	20	0	0	1	20
	Dairy Equipment's	7	3	42.9	4	57.1	2	28.6	1	14.3	2	28.6	2	28.6
	Dairy Manure and Bedding	13	8	61.5	5	38.5	4	30.8	4	30.8	1	7.6	4	30.8
	Working surfaces	11	6	54.5	3	27.3	1	9.1	5	45.5	2	18.2	3	27.3
Total (120)		36	21	58.3	15	41.6	10	27.8	11	30.5	5	13.8	10	27.8
Overall Total (300)		75	37	49.3	33	44	18	24	19	25.3	15	20	23	30.7

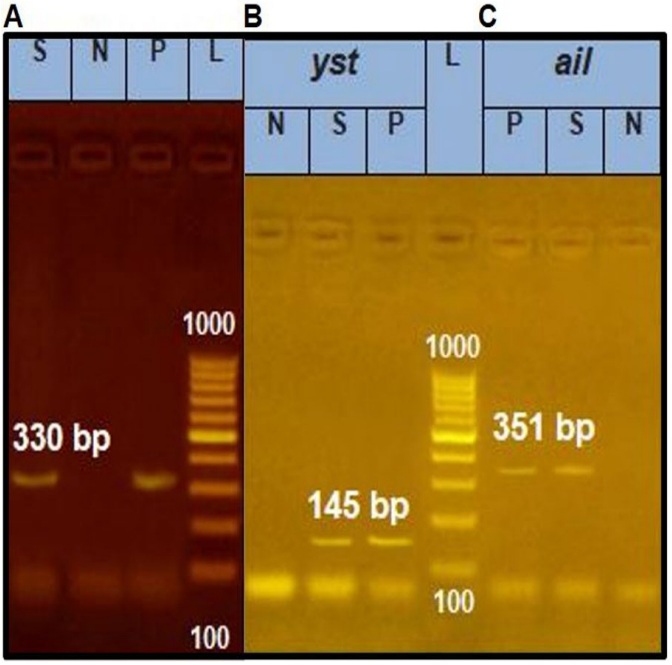

Fig. 1. Amplification of *Y. enterocolitica* 16S rRNA (A) and virulent genes (B and C) visualized on agarose gel electrophoresis. The expected molecular size of amplified DNA: 330 bp for *Y. enterocolitica* 16S rRNA (A), 145 bp for *yst* gene (B) and 351 bp for *ail* gene (C). Lane L: 100-1000 bp DNA Ladder; S.: Sample; N.: Negative control; P.: Positive control.

were detected in the obtained survey, which covered a total of 52 isolates. The most common virulence profile found among the isolates was virulent profile 2 (*ail* +/ *yst* -) (25.3%), where strains isolated from most examined samples were found, followed by virulent profile 1 (*ail* +/ *yst* +) and profile 3 (*ail* +/ *yst* +). In almost all profiles, isolated strains in milk, dairy product, and environmental samples were involved (Table 3).

Antibiogram pattern of Y. enterocolitica isolates

Antimicrobial susceptibilities for 11 antimicrobial agents commonly used in veterinary clinics and farms were assessed in 75 *Y. enterocolitica* isolates (Table 4 and Figure 2). Irrespective of the origin (milk or milk products, water sources, environmental samples) of the isolates, the explored isolates were generally resistant to Penicillin (89.3%), Oxacillin (85.3%), Erythromycin (72.0%) and Ampicillin (70.7%). They were, however, susceptible to other antimicrobials such as Tetracycline (72.0%), Ciprofloxacin (69.3%), Chloramphenicol (64.0%), Cephalexin (58.7%) and Streptomycin (53.3%) (Table 4). The multiple drug resistance (MDR) patterns are shown in Table 5. Seventy-five *Y. enterocolitica* isolates elicited 9 different patterns of antibiotic resistance to the antimicrobial agents used in this study (Table 5). The most common multidrug pattern observed in this study was MDR to 5 antibiotics (23/75, 30.67%), followed by 6 antibiotics (19/75, 25.33%), 7 antibiotics (8/75, 10.67%), and 4 antibiotics (7/75, 9.33%).

Biofilm formation activity of Y. enterocolitica isolates

From the 75 *Y. enterocolitica* isolates, 45 (60%) strains produced biofilm with variable degrees; 2.7,.41.3 and 16 % of the biofilm producing strains were characterized as strong, moderate and weak biofilm producer. These isolates were distributed as 28 (71.8%) from raw milk and dairy products and 17 (47.2%) from environmental samples. Biofilm producing *Y. enterocolitica* isolated from raw milk and dairy products formed strong, moderate and weak biofilm with incidences of 5.1, 51.3 and 15.4%, respec-

Table 4. Antibiogram resistance pattern of *Y. enterocolitica* isolates.

Samples	No. of Y. *enterocolitica*		Amoxicillin		Ampicillin		Cephalexin		Chloramphenicol		Ciprofloxacin		Erythromycin		Gentamicin		Penicillin		Oxacillin		Streptomycin		Tetracycline	
			No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Household milk	8	3	37.5	6	75	2	25	2	25	1	12.5	5	62.5	3	37.5	7	87.5	7	87.5	3	37.5	1	12.5
Street vendor milk	10	4	40	7	70	3	30	3	30	2	20	6	60	4	40	7	70	7	70	4	40	2	20
Kareish cheese	5	2	40	4	80	2	40	1	20	1	20	3	60	2	40	5	100	4	80	2	40	1	20
Yoghurt	4	2	50	3	75	2	50	1	25	1	25	2	50	1	25	4	100	3	75	2	50	1	25
Ice cream	5	2	40	3	60	2	40	2	40	2	40	3	60	2	40	5	100	4	80	2	40	1	20
Rice milk	7	3	42.9	5	71.4	3	42.9	2	28.5	2	28.5	4	57.1	2	28.5	6	85.7	6	85.7	3	42.9	2	28.6
Water	5	3	60	3	60	2	40	2	40	2	40	4	80	3	60	5	100	5	100	3	60	2	40
Dairy Equipment's	7	5	71.4	6	85.7	4	57.1	4	57.1	4	57.1	5	71.4	4	57.1	6	85.7	6	85.7	4	57.1	3	42.9
Dairy Manure and Bedding	13	7	53.8	11	84.6	6	46.2	6	46.2	5	38.5	12	92.3	8	61.5	12	92.3	12	92.3	7	53.8	4	30.8
Working surfaces	11	6	54.5	5	45.5	5	45.5	4	36.4	3	27.3	10	90.9	7	63.6	10	90.9	10	90.9	5	45.5	4	36.4
Resistance	75	37	49.3	53	70.7	31	41.3	27	36	23	30.7	54	72	36	48	67	89.3	64	85.3	35	46.7	21	28
Susceptible		38	50.7	22	29.3	44	58.7	48	64	52	69.3	21	28	39	52	8	10.7	11	14.7	40	53.3	54	72

Table 5. The antimicrobial resistance distribution pattern of *Y. enterocolitica* isolates obtained from the screened samples.

No. of antibiotic resistance	Milk and milk products samples						Dairy cattle macroenvironment				Total No. of isolates (%)
	Household milk (n.=8)	Street vendor milk (n.=10)	Kareish cheese (n.=5)	Yoghurt (n.=4)	Ice cream (n.=5)	Rice milk (n.=7)	Water (n.=5)	Dairy Equipment's (n.=7)	Dairy Manure and Bedding (n.=13)	Working surfaces (n.=11)	
3	-	1	-	-	-	-	-	-	1	1	3 (-4.00%)
4	1	1	-	1	-	1	-	1	1	1	7 (-9.33%)
5	6	6	3	-	2	2	1	-	-	3	23 (-30.67%)
6	1	2	2	3	3	4	-	-	3	1	19 (-25.33%)
7	-	-	-	-	-	-	3	2	2	1	8 (-10.67%)
8	-	-	-	-	-	-	1	3	3	2	9 (-12.00%)
9	-	-	-	-	-	-	-	1	3	2	6 (-8.00%)
Total											75 (100%)

Table 6. Biofilm production by *Y. enterocolitica* isolates

Type of sample		*Y. enterocolitica*	Total biofilm producing isolates	Strong	Moderate	Weak
		No.	No. (%)	No. (%)	No. (%)	No. (%)
Milk and milk products samples	Household milk	8	8 (100)	1 (12.5)	7 (87.5)	0
	Street vendor milk	10	9 (90)	0	5 (50)	4 (40)
	Kareish cheese	5	5 (100)	1 (20)	3 (60)	1(20)
	Yoghurt	4	1 (25)	0	1 (25)	0
	Ice cream	5	5 (100)	0	4 (80)	1 (20)
	Rice milk	7	0	0	0	0
Total (180)		39	28 (71.8)	2 (5.1)	20 (51.3)	6(15.4)
Dairy Cattle Macroenvironment	Water	5	2 (40)	0	1 (20)	1 (50)
	Dairy Equipment's	7	5 (71.4)	0	3 (42.9)	2 (40)
	Dairy Manure and Bedding	13	6 (46.2)	0	4 (66.7)	2 (30.8)
	Working surfaces	11	4 (36.4)	0	3 (75)	1 (9.1)
Total (120)		36	17 (47.2)	0	11 (30.6)	6 (16.7)
Overall Total of each biofilm phenotype		75	45 (60)	2 (2.7)	31 (41.3)	12 (16)

tively. While moderate, and weak biofilms were produced by 30.6 and 16.7%, respectively in isolates obtained from environmental samples. The distribution of biofilm phenotypes in each category of the examined samples is presented in (Table 6).

Antimicrobial activity

Cow milk proteins and its pepsin hydrolysates were evaluated for their antimicrobial activity against the isolates of recovered *Y. enterocolitica* using a bactericidal assay method measured as bacterial growth monitored at 620 nm (Figure 3). The parent proteins (CCP, CWP) and their pepsin hydrolysates (P-CCP and P-CWP) were potentially effective in inhibiting *Y. enterocolitica* growth in a dose-dependent manner and with variable potency. Their activity was expressed as bacterial growth monitored at 620 nm as a function of tested samples concentration incubated with *Y. enterocolitica* for 24 hrs. The intact proteins (CCP and CWP) exhibited little to moderate antimicrobial activity. While P-CWP and P-CCP were very effective against *Y. enterocolitica* resulting in a severe reduction in the bacterial growth (Δabs 620) at a concentration of 1.5 mg/ml of P-CWP and P-CCP, respectively (Figure 3). It can be noted that peptides P-CWP exhibited the strongest effect against *Y. enterocolitica*.

DISCUSSION

Nowadays, public health concern associated with microbial food and water safety has risen (Ahmed *et al*., 2022). Pathogenic microorganisms including *Y. enterocolitica* can spread directly to milk from widely dispersed environmental sources during and after milking, causing severe health problems (Grahek-Ogden *et al*., 2007). The current study revealed the ubiquitous distribution of *Y. enterocolitica* among the investigated dairy environment. Overall, of 21.7 and 30% among dairy and environmental samples were positive for *Y. enterocolitica*. Results from this study are comparable to a similar study that was aimed to isolate *Y. enterocolitica* from a range of farm environments samples included

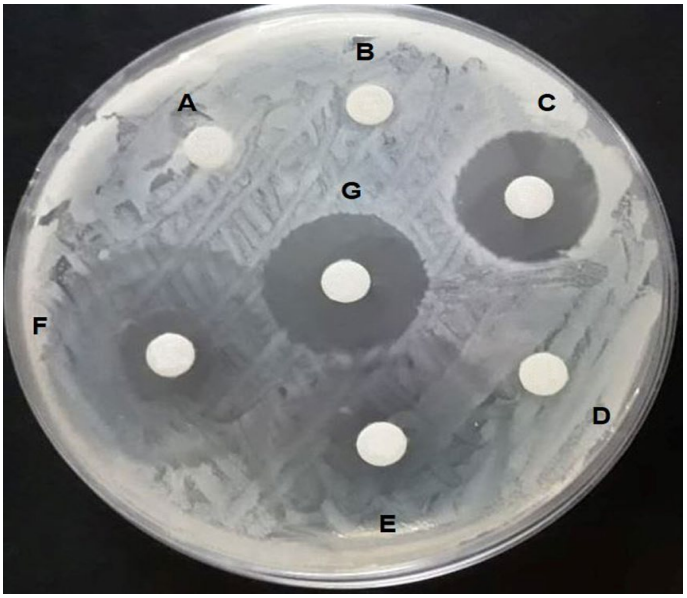

Fig. 2. Disc diffusion test; from A-G samples of disc diffusion test for Penicillin, Oxacillin, Tetracycline, Erythromycin, Chloramphenicol, Cephalexin, Ciprofloxacin.

milk, milk products, water, dairy equipments, bedding materials and working surfaces. We agreed with their conclusion that *Y. enterocolitica* was widely distributed within the dairy farm environment with multiple sources for contamination of raw milk.

The obtained results highlighted that the prevalence of *Y. enterocolitica* in raw milk samples was 30% (both household and street vendor milk samples). Comparable findings were reported by several studies performed in Turkey, India, Mexico and Egypt found that the prevalence of *Y. enterocolitica* in raw milk reached 25, 29.3, 34.9, 38.9, and 22%, respectively (Yucel and Ulusoy, 2006; Subha *et al*., 2009; Bernardino-Varo *et al*., 2013; Darwish and Allam, 2015; Ahmed *et al*., 2019). In the present study, rice milk had the highest isolation rate of *Y. enterocolitica* among dairy products, followed by kareish cheese, ice cream and yoghurt. Results from the present study agreed well with other studies in Egypt and globally in different studies in Iran and Turkey (Yucel and Ulusoy, 2006; Rahimi *et al*., 2014; Ali *et al*., 2015; Ahmed *et al*., 2019).

The high prevalence of *Y. enterocolitica* in milk and dairy products could be due to fecal contamination of milk, milking with unsanitary methods, marketing and using raw milk without pasteurization, using traditional dairy products produced in unsanitary conditions or from raw milk, the ability of this psychrotrophic microorganism in growing in raw milk and viability at refrigeration temperatures for long time (Rahimi *et al*., 2014; Ali *et al*., 2015; Ahmed *et al*., 2019). In Egypt, raw cow's milk is sold and unpasteurized milk is used in manufacturing of dairy products (International Labour Organization, 2020). These products have the potential to transmit diseases to people, particularly *Y. enterocolitica* (Bernardino-Varo *et al*., 2013). As a result of these circumstances, humans suffer from a variety of illnesses. Therefore, improving milking methods, monthly checks of milking halls for *Y. enterocolitica*, especially in animal feces, frequent fumigation of milking halls, inspecting hygiene during milking, boiling the milk, using pasteurized and even sterilized milk for dairy products, keeping dairy products in cool and dry places away from the sun, and finally preventing contamination of dairy products with extrinsic factors such as insects and rodents are all important (Rahimi *et al*., 2014).

Sahota *et al*. (2014) concluded that *Yersinia* spp. were present in 68.9% of drinking water samples collected from different water utilities of urban areas in India of which pathogenically characterized *Y. enterocolitica* was the most frequently isolated. They concluded that the detection of *Y. enterocolitica* in drinking water sources studied represent a public health concern and must be taken into account for assessing the quality of drinking water. Moreover, in a study performed in Greece, 20 *Yersinia* spp. (4%), three of them being Y enterocolitica and all the others being Y intermedia were isolated from drinking water samples (Arvanitidou *et al*., 1994). They concluded that *Yersinia* spp. with minimal or low virulence may be pathogenic in the immunocomprornised

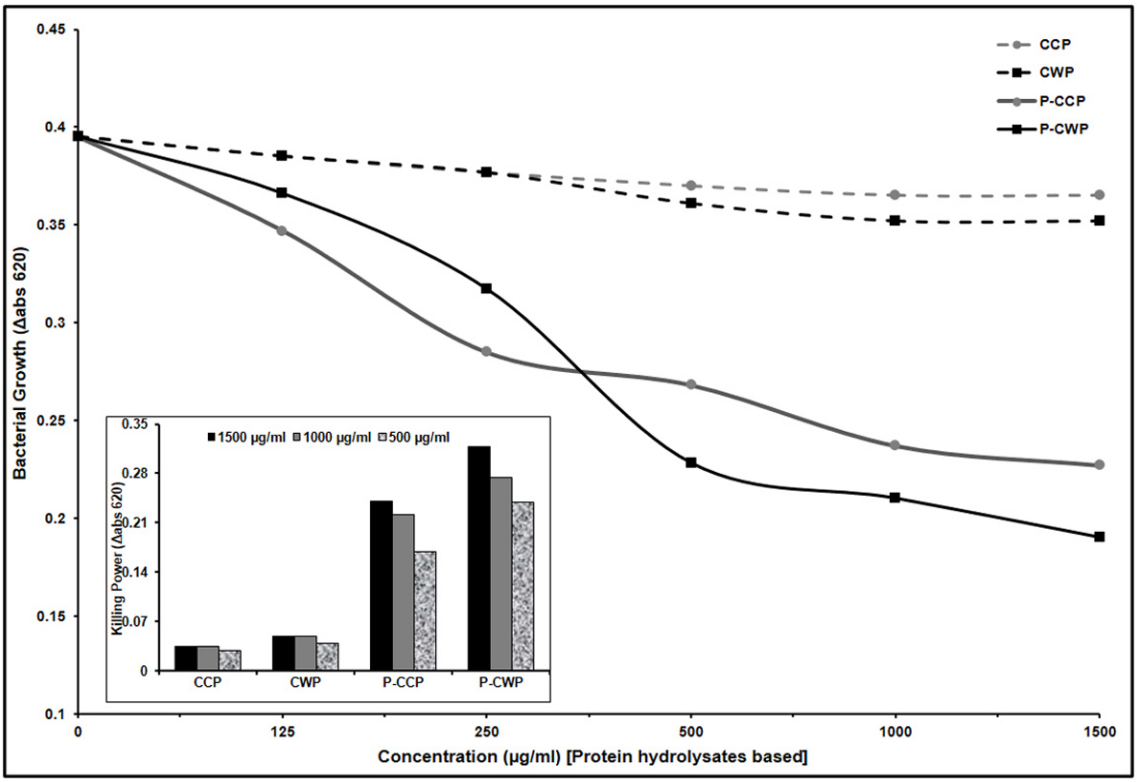

Fig. 3. Antibacterial activity of CCP and CWP and its pepsin hydrolysates against *Y. enterocolitica* at different concentrations. The data is presented as bacterial growth monitored at 620 nm. (Inset) Killing power of protein hydrolysates (1500, 500 and 2500 µg/ml) against *Y. enterocolitica* as bacterial growth monitored at 620 nm. The assays were performed in triplicate.

host. Therefore, proper sanitary survey, regular bacteriological assessment, design and implementation of water sanitation projects, regular disinfection and supervisions of water sources of all water sources for drinking should be planned and conducted to prevent occurrence of *Y. enterocolitica* in drinking water.

Additional sources of microbial contamination of cow's milk include milking equipment and working surfaces. Van Duynhoven *et al*. (2009) stated that *Y. enterocolitica* outbreaks resulted from post-pasteurization contamination, contaminated equipment and milk bottles rinsing with untreated water prior to filling milk. Most microbes have the ability to attach to surfaces and form biofilms, which are surface-associated multicellular communities. Because the self-produced matrix of extracellular polymeric material functions as a protective barrier against the effects of detergent and disinfectant solutions, bacteria embedded in biofilms often show greater resistance to antimicrobial treatments. When cleaning and disinfection procedures are insufficient and not adequately administered for a long length of time, biofilms can build in the equipment, transport line, and storage tank (Van Bokhorst-van de Veen *et al*., 2015).

Hughes (1979) concluded that *Y. enterocolitica* is capable of growth at refrigeration temperatures and if it gained entry into milk via contaminated working surfaces, dairy equipment, dust or any other pathway it could then multiply during refrigerated storage prior to pasteurization. Also, it can survive pasteurization and multiply in the refrigerated pasteurized product, especially if pasteurization temperatures are not strictly observed. *Y. enterocolitica* may gain access to the pasteurized product by post-pasteurization contamination from raw milk, inadequate cleaned surfaces and equipment (Hughes, 1979).

Also, Fukushima *et al*. (1983) and Bozcal *et al*. (2015) stated that *Y. enterocolitica* were isolated from animal feces and wastewater in Japan and Turkey. They declared that cows can be a source of *Yersinia* spp. contamination in milk, particularly *Y. enterocolitica*, which was found in bovine feces and caused a community outbreak of *Y. enterocolitica* enteritis. Pathogenic strains of *Y. enterocolitica* that are transmitted by the fecal-oral route. animal species, such as cattle are considered the principal reservoir for *Y. enterocolitica* pathogenic to humans (Bozcal *et al*., 2015).

In the present study, virulent *Y. enterocolitica* strains harboring *ail* and *yst* were detected in 49.3 and 44% among *Y. enterocolitica* isolates obtained from examined samples, respectively (Table 3). More than half the *Y. enterocolitica* isolated from dairy products (66.6%) and environmental samples (72.2%) was positive for more than one virulence-associated gene, while none of 33.4 and 27.8% from dairy products and environmental samples, respectively, harbored any virulence-associated gene (Table 3) and were likely less virulent than those with multiple virulence-associated genes. Moreover, the most common virulence profile found among the isolates was virulence profile 2 (*ail* +/ *yst* -), where strains isolated from most types of examined samples were found. The occurrence of *ail* and *yst*, observed in this study is consistent with previous studies (Jamali *et al*., 2015; Bonardi *et al*., 2018). Also, the positive rates for *ail* (41%) and *yst* (46.2%) in dairy samples were higher than those found by Ahmed *et al*. (2019) in dairy samples in Egypt, where the positive rates of *ail* and *yst* were approximately 30% and 10%, respectively. Jamali *et al*. (2015) stated that the *ail* and *yst* proteins are critical for virulence in *Y. enterocolitica*. As a result, the *ail* and *yst* genes, which code for these proteins, are utilized as pathogenicity markers for *Y. enterocolitica* isolates.

Contamination of food with antimicrobial-resistant *Y. enterocolitica* is a threat to public health (Ahmed *et al*., 2019). All isolates in the present study were tested for antimicrobial resistance against 11 antibiotics. *Y. enterocolitica* isolates showed resistance to Penicillin, Oxacillin, Erythromycin and Ampicillin with 89.3, 85.3, 72 and 70.7%, respectively; and susceptible to other antimicrobials such as Tetracycline, Ciprofloxacin, Chloramphenicol, Cephalexin and Streptomycin with 72.0, 69.3, 64.0, 58.7 and 53.3%, respectively (Table 4). The most common multidrug resistance pattern observed among *Y. enterocolitica* isolates were MDR 5, MDR 6, MDR 7 and MDR 4 antibiotics with 30.67, 25.33, 10.67 and 9.33%, respectively (Table 5). The results of antimicrobials' susceptibility tests were generally consistent with the previous studies from Egypt and other countries (Jamali *et al*., 2015; Bonardi *et al*., 2018; Ahmed *et al*., 2019). They declared that *Y. enterocolitica* strains were found to be sensitive to Tetracycline, Ciprofloxacin, Chloramphenicol, Cephalexin, Streptomycin and gentamicin. Antimicrobial drugs are used to treat microbial infections as well as stimulate animal growth. Excessive use of these drugs has recently resulted in an increase in the number of antimicrobial-resistant microorganism isolates (Jamali *et al*., 2015). The higher rates of multidrug antibiotic resistance among *Y. enterocolitica* isolates in in foods, especially in milk indicates alarming situation for designing prevention and control measures to prevent the potential transmission of the pathogen with contaminated food to the consumers.

Biofilms are well known as a source of foodborne human pathogens (Bridier *et al*., 2015). However, in case of *Y. enterocolitica* the role of biofilms and the factors that lead to biofilm formation are largely unknown. There is evidence based on studies with very limited number of strains, that *Y. enterocolitica* able to form biofilms (Izquierdo *et al*., 2002; Kim *et al*., 2008; Ioannidis *et al*., 2014; Wang *et al*., 2017; Lenchenko *et al*., 2019). Also, Itoh *et al*. (2005) and Ioannidis *et al*. (2014) indicated that biofilm formation by *Y. enterocolitica* might be an inherent feature.

It is difficult to compare the results of the current study with other studies because of the limited number of isolates in other studies and the variation in results interpretation among studies. However, the obtained results disagree with Zadernowska and Chajęcka-Wierzchowska (2017) where none of the strains showed a strong ability to produce biofilm and the majority of the Y. enterocolitica strains were non or weak biofilm producer. On the other hand, findings from the current study agree with those of Kot *et al*. (2011), who found that a high percentage of *Y. enterocolitica* strains have adhesive properties, and with Castro *et al*. (2019), who observed that absorbance levels were higher in milk samples than in feces isolates.

The ability of pathogens to form biofilms has two implications. From a clinical perspective, the ability to form is a feature that demonstrates the strain's virulence. Components of a biofilm matrix protect bacteria from immune attacks (such as phagocytosis) and antibiotics (Shi and Zhu, 2009). A slow growth rate and horizontal transfer of resistance genes are two factors that contribute to antimicrobial tolerance via metabolic dormancy or molecular persistence programmes (Del Pozo, 2017). Moreover, in terms of industrial significance, strains that can form biofilms on manufacturing surfaces can be a major concern; they are more difficult to be eliminated from those surfaces, less sensitive to disinfectants, and frequently cause cross contamination in the food industry (Shi and Zhu, 2009).

It has become increasingly difficult to protect human health from the negative consequences of pathogenic *Y. enterocolitica*. The harmful effects of chemical preservatives on human health, as well as their limited application, susceptibility, toxicity, and microbial resistance, have increased the demand for potentially effective, healthy, safer, and natural antimicrobial agents with a unique mechanism of action against pathogens especially *Y. enterocolitica*. Thus, the antimicrobial activity of cow milk proteins and their pepsin hydrolysates can provide a key aspect of *Y. enterocolitica* infection treatment that traditional antimicrobial agents cannot, and they can be used as natural preservatives to ensure healthy and safe food without the unpleasant side effects of chemical preservatives. We have shown that pepsin hydrolysates of cow milk proteins exerted strong bactericidal activity, dose-dependently, against *Y. enterocolitica*. Notably, *Y. enterocolitica* killing by P-CWP and P-CCP were observed at concentrations as low as 1.5 mg/ml. P-CWP and P-CCP were potentially effective against tested pathogens with variable potency resulting in a severe reduction in the bacterial growth (Δabs 620) (Figure 3).

Various studies evaluated the antimicrobial activities of the cow and other lactating animal milk proteins hydrolysates (P-CWP and P-CCP). Different hydrolytic enzymes viz., gastric juice, intestinal juice, enzymes of microbial, plant or animal origin are used to break large polypeptides into specific small peptides to investigate its antimicrobial activity against both Gram-positive and Gram-negative bacteria including *Y. enterocolitica* (Benkerroum, 2010; Khan *et al*., 2018; Guha *et al*., 2021). Pepsin hydrolysates of milk proteins have been shown to have antimicrobial action, with clear inhibitory effects on a variety of harmful bacteria especially *Y. enterocolitica* (Minervini *et al*., 2003; Rizzello *et al*., 2005; Silva *et al*., 2018).

CONCLUSION

The present study provided an insight into the incidence of different *Yersinia* spp. in in raw milk and its products as well as the dairy environment. Using raw milk without pasteurization, traditional dairy products produced from unpasteurized milk, are the main resources of *Y. enterocolitica*. Also, environmental samples including water represent potential hazards for public health. Therefore, application of good hygienic practices during milking, routine monitoring of the dairy environments to detect *Y. enterocolitica* especially in the animal feces, heat treatment of milk, are the best ways to prevent *Y. enterocolitica* infections. Finally, further investigation for antibiotic alternatives is fundamental for control of *Y. enterocolitica*. In this context, the parent proteins (CCP, CWP) and their pepsin hydrolysates (P-CCP and P-CWP) gave promising results in *Y. enterocolitica* growth. The current study's findings and recommendations will alert key health decision-makers to take the appropriate countermeasures.

ACKNOWLEDGMENT

The authors would like to acknowledge South Valley University, Qena, Egypt for supports.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

Ahmed, A.S., Alsayeqh, A.F., Diab, H.M., 2020. Enterotoxigenic Profiles of Virulent *Bacillus cereus* Isolated from Dairy Environments: Antimicrobials Resistant Pattern and Sporicidal Disinfectants Efficacy. Adv. Anim. Vet. Sci. 8.

Ahmed, A.S., Diab, H.M., Alkahtani, M.A., Alshehri, M.A., Saber, H., Badr, H., Dandrawy, M.K., El-Mansi, A.A., Shati, A.A., Ahmed, A.E., 2022. Molecular epidemiology of virulent *E. coli* among rural small scale dairy herds and shops: Efficacy of selected marine algal extracts and disinfectants. Int. J. Environ. Health Res. 32, 72-94.

Ahmed, A.S., El-Bassiony, T., Elmalt, L.M., Ibrahim, H.R., 2015. Identification of potent antioxidant bioactive peptides from goat milk proteins. Food Res. Int. 74, 80–88.

Ahmed, H.A., Tahoun, A.B.M.B., Abou Elez, R.M.M., Abd El-Hamid, M.I., Abd Ellatif, S.S., 2019. Prevalence of *Yersinia enterocolitica* in milk and dairy products and the effects of storage temperatures on survival and virulence gene expression. Int. Dairy J. 94, 16–21.

Ali, A.M., Attiah, S.A., Aly, S.A., 2015. Incidence of *Yersinia enterocolitica* in milk and some milk products. International Journal of Advanced Biological Research 5, 285-288.

Arvanitidou, M., Stathopoulos, G.A., Katsouyannopoulos, V.C., 1994. Isolation of *Campylobacter* and *Yersinia* spp. from Drinking Waters. J. Travel Med. 1, 156–159.

Bauer, A.W., Kirby, W.M.M., Sherris, J.C., Turck, M., 1966. Antibiotic Susceptibility Testing by a Standardized Single Disk Method. Am. J. Clin. Pathol. 45, 493–496.

Benkerroum, N., 2010. Antimicrobial peptides generated from milk proteins: a survey and prospects for application in the food industry. A review. Int. J. Dairy Technol. 63, 320–338.

Bernardino-Varo, L., Quiñones-Ramírez, E.I., Fernández, F.J., Vázquez-Salinas, C., 2013. Prevalence of *Yersinia enterocolitica* in Raw Cow's Milk Collected from Stables of Mexico City. J. Food Prot. 76, 694–698.

Bharathy, S., Swetha, S.C., Venkateswara, R., Sudhanthiramani, S., Radhika, B., 2014. Prevalence and antibiogram of *Yersinia enterocolitica* in milk and fecal swab of dairy product cows from different places of Tirupthi Region Andhra Pradesh, India. International Journal of Recent Scientific Research 6, 5469-5475.

Bonardi, S., Le Guern, A.S., Savin, C., Pupillo, G., Bolzoni, L., Cavalca, M., Pongolini, S., 2018. Detection, virulence and antimicrobial resistance of *Yersinia enterocolitica* in bulk tank milk in Italy. Int. Dairy J. 84, 46–53.

Bozcal, E., Uzel, A., Aydemir, S., Skurnik, M., 2015. Isolation of pathogenic *Yersinia enterocolitica* strains from different sources in Izmir region, Turkey. Folia Microbiol. (Praha). 60, 523–529.

Bridier, A., Sanchez-Vizuete, P., Guilbaud, M., Piard, J.-C., Naïtali, M., Briandet, R., 2015. Biofilm-associated persistence of food-borne pathogens. Food Microbiol. 45, 167–178.

Brown, M.R.W., Gilbert, P., 1993. Sensitivity of biofilms to antimicrobial agents. J. Appl. Bacteriol. 74, 87S-97S.

Castro, H., Jaakkonen, A., Hakakorpi, A., Hakkinen, M., Isidro, J., Korkeala, H., Lindström, M., Hallanvuo, S., 2019. Genomic Epidemiology and Phenotyping Reveal on-Farm Persistence and Cold Adaptation of Raw Milk Outbreak-Associated *Yersinia pseudotuberculosis*. Front. Microbiol. 10, 1049.

Chye, F.Y., Abdullah, A., Ayob, M.K., 2004. Bacteriological quality and safety of raw milk in Malaysia. Food Microbiol. 21, 535–541.

CLSI (Clinical and Laboratory Standards Institute), 2018. Performance standards for antimicrobial disk susceptibility testing. 27th ed., Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA.

Collins C.H., Lyne P.M., Gran J.M., 1991. Microbiological Methods. 6th ed., Butterworth- Heinemann: Oxford. London. pp.127-140

Costerton, J.W., Khoury, A.E., Ward, K.H., Anwar, H., 1993. Practical Measures to Control Device-Related Bacterial Infections. Int. J. Artif. Organs 16, 765–770.

Darwish, S., Allam, H., 2015. Incidence of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in Raw Milk Samples of Different Animal Species Using Conventional and Molecular Methods. Alexandria J. Vet. Sci. 44, 174.

Del Pozo, J.L., 2017. Biofilm-related disease. Expert Rev. Anti. Infect. Ther. 16, 51–65.

FDA (Food and Drug Administrator), 2007. In: *Yersinia enterocolitica*. Bacteriological Analytical Manual Online. Weagant SD, Feng P, editors. United States: Food and Drug Administration Center for Food Safety and Applied Nutrition.

Fàbrega, A., Vila, J., 2012. *Yersinia enterocolitica*: Pathogenesis, virulence and antimicrobial resistance. Enferm. Infec. Microbiol. Clin. 30, 24–32.

Fukushima, H., Saito, K., Tsubokura, M., Otsuki, K., Kawaoka, Y., 1983. Isolation of *Yersinia* spp. from bovine feces. J. Clin. Microbiol. 18, 981–982.

Fukushima, H., Shimizu, S., Inatsu, Y., 2011. *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* Detection in Foods. J. Pathog. 2011, 735308.

Grahek-Ogden, D., Schimmer, B., Cudjoe, K.S., Nygård, K., Kapperud, G., 2007. Outbreak of *Yersinia enterocolitica* serogroup O:9 infection and processed pork, Norway. Emerg. Infect. Dis. 13, 754–756.

Gristina, A.G., Hobgood, C.D., Webb, L.X., Myrvik, Q.N., 1987. Adhesive colonization of biomaterials and antibiotic resistance. Biomaterials 8, 423–426.

Guha, S., Sharma, H., Deshwal, G.K., Rao, P.S., 2021. A comprehensive review on bioactive peptides derived from milk and milk products of minor dairy species. Food Prod. Process. Nutr. 3, Article number: 2 (2021).

Hughes, D., 1979. Isolation of *Yersinia enterocolitica* from Milk and a Dairy Farm in Australia. J. Appl. Bacteriol. 46, 125–130.

Ibrahim, H.R., Ahmed, A.S., Miyata, T., 2017. Novel angiotensin-converting enzyme inhibitory peptides from caseins and whey proteins of goat milk. J. Adv. Res. 8, 63–71.

International Labour Organization, 2020. X Developing the Dairy Value Chain in Egypt's Delta Market S*yst*em Analysis. A*vail*able at: https://www.ilo.org/wcmsp5/groups/public/---africa/---ro-abidjan/---sro-cairo/documents/publication/wcms_754764.pdf

Ioannidis, A., Kyratsa, A., Ioannidou, V., Bersimis, S., Chatzipanagiotou, S., 2014. Detection of Biofilm Production of *Yersinia enterocolitica* Strains Isolated from Infected Children and Comparative Antimicrobial Susceptibility of Biofilm Versus Planktonic Forms. Mol. Diagn. Ther. 18, 309–314.

Itoh, Y., Wang, X., Hinnebusch, B.J., Preston, J.F., Romeo, T., 2005. Depolymerization of beta-1,6-N-acetyl-D-glucosamine disrupts the integrity of diverse bacterial biofilms. J. Bacteriol. 187, 382–387.

Izquierdo, L., Abitiu, N., Coderch, N., Hita, B., Merino, S., Gavin, R., Tomás, J.M., Regué, M., 2002. The inner-core lipopolysaccharide biosynthetic waaE gene: function and genetic distribution among some Entero-

bacteriaceae. The GenBank accession number for the waaE gene sequences of P. mirabilis CECT170, *Y. enterocolitica* R102 and Ent. aerogenes CECT684 reported in this paper are AY075039, AY075041 and AY075040, respectively. Microbiology 148, 3485–3496.

Jamali, H., Paydar, M., Radmehr, B., Ism*ail*, S., 2015. Prevalence, characterization, and antimicrobial resistance of *Yersinia* species and *Yersinia enterocolitica* isolated from raw milk in farm bulk tanks. J. Dairy Sci. 98, 798–803.

Khan, M.U., Pirzadeh, M., Förster, C.Y., Shityakov, S., Shariati, M.A., 2018. Role of Milk-Derived Antibacterial Peptides in Modern Food Biotechnology: Their Synthesis, Applications and Future Perspectives. Biomolecules 8, 110.

Kim, T.-J., Young, B.M., Young, G.M., 2008. Effect of flagellar mutations on *Yersinia enterocolitica* biofilm formation. Appl. Environ. Microbiol. 74, 5466–5474.

Kot, B., Piechota, M.A.Ł.G.O.R.Z.A.T.A., Zdunek, E., Borkowska, D., Binek, T., 2011. Occurrence of virulence genes and biofilm formation in *Yersinia enterocolitica* isolated from pigs. Bull. Vet. Inst. Pulawy 55, 187-191.

Koua, A., Solange, K.N.E., Germain, K.T., Mireille, D., Marcellin, D.K., 2014. Characterization of *Yersinia* spp. strains isolated from pigs in Abidjan, Cte dIvoire, West Africa. African Journal of Microbiology Research 8, 1909-1915.

Krajinović, V., Tambić Andrašević, A., Baršić, B., 2007. Tricuspidal Valve Endocarditis Due to *Yersinia enterocolitica*. Infection 35, 203–205.

Kunin, C.M., Steele, C., 1985. Culture of the surfaces of urinary catheters to sample urethral flora and study the effect of antimicrobial therapy. J. Clin. Microbiol. 21, 902–908.

Larkin, L.L., Vasadava, P.C., Marth, E.H., 1991. Incidence of *Yersinia enterocolitica* in raw milk as related to its quality. Milchwissenschaft 46, 500-502.

Leclercq, A., Martin, L., Vergnes, M.L., Ounnoughene, N., Laran, J.-F., Giraud, P., Carniel, E., 2005. Fatal *Yersinia enterocolitica* biotype 4 serovar O:3 sepsis after red blood cell transfusion. Transfusion 45, 814–818.

Lenchenko, E., Lozovoy, D., Strizhakov, A., Vatnikov, Y., Byakhova, V., Kulikov, E., Sturov, N., Kuznetsov, V., Avdotin, V., Grishin, V., 2019. Features of formation of *Yersinia enterocolitica* biofilms. Vet. world 12, 136–140.

Lupi, A., Poletti, F., Mondino, V., Canale, C., Leonardo, L., Rognoni, A., Bongo, A.S., Caimmi, P.P., Nardi, F., 2013. Subacute endocarditis caused by *Yersinia enterocolitica*: A case report. Scand. J. Infect. Dis. 45, 329–333.

Mah, T. F.C., O'Toole, G.A., 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 9, 34–39.

Minervini, F., Algaron, F., Rizzello, C.G., Fox, P.F., Monnet, V., Gobbetti, M., 2003. Angiotensin I-converting-enzyme-inhibitory and antibacterial peptides from Lactobacillus helveticus PR4 proteinase-hydrolyzed caseins of milk from six species. Appl. Environ. Microbiol. 69, 5297–5305.

Nickel, J.C., Ruseska, I., Wright, J.B., Costerton, J.W., 1985. Tobramycin resistance of Pseudomonas aeruginosa cells growing as a biofilm on urinary catheter material. Antimicrob. Agents Chemother. 27, 619–624.

Pandove, G., Sahota, P., Verma, S.K., Mahajan, V., Singh, D., Singh, J. P., 2012. Epidemiology, virulence and public health significance of *Yersinia enterocolitica* in drinking water. African J. Microbiol. Res. 6, 5905-5913.

Rahimi, E., Sepehri, S., Safarpoor Dehkordi, F., Shaygan, S., Momtaz, H., 2014. Prevalence of *Yersinia* species in traditional and commercial dairy products in isfahan province, iran. Jundishapur J. Microbiol. 7, e9249–e9249.

Rendos, J.J., Eberhart, R.J., Kesler, E.M., 1975. Microbial Populations of Teat Ends of Dairy Cows, and Bedding Materials. J. Dairy Sci. 58, 1492–1500.

Rizzello, C.G., Losito, I., Gobbetti, M., Carbonara, T., De Bari, M.D., Zambonin, P.G., 2005. Antibacterial Activities of Peptides from the Water-Soluble Extracts of Italian Cheese Varieties. J. Dairy Sci. 88, 2348–2360.

Robins-Browne, R.M., 2013. *Yersinia enterocolitica*. In Doyle, M.P., Buchanan, R.L. (Eds.), Food microbiology: Fundamentals and frontiers (4th ed.). Washington, DC, USA: ASM Press.

Sahota, P., Sharma, N., Pandove, G., 2014. Occurrence of *Yersinia enterocolitica* in drinking water in the absence of indicator organism. World Journal of Pharmaceutical Research 3, 529-542.

Sambrook, J. Fritsch, E.F., Montias, T., 1989. Molecular Biology. In: Molecular cloning. Laboratory manual, Second Edition. Cold Spring Harbor Laboratory press, USA. p. 268.

Shi, X., Zhu, X., 2009. Biofilm formation and food safety in food industries. Trends Food Sci. Technol. 20, 407–413.

Shwimmer, A., Freed, M., Blum, S., Khatib, N., Weissblit, L., Friedman, S., Elad, D., 2007. Mastitis Caused by *Yersinia pseudotuberculosis* in Israeli Dairy Cattle and Public Health Implications. Zoonoses Public Health 54, 353–357.

Sibel Akalın, A., 2014. Dairy-derived antimicrobial peptides: Action mechanisms, pharmaceutical uses and production proposals. Trends Food Sci. Technol. 36, 79–95.

Silva, C.C.G., Silva, S.P.M., Ribeiro, S.C., 2018. Application of Bacteriocins and Protective Cultures in Dairy Food Preservation. Front. Microbiol. 9, 594.

Subha, B., Ramakrishnan, D., Suganthi, V., 2009. Antimicrobial resistance pattern of selected *Yersinia enterocolitica* isolated from raw cow milk and pork samples of Namakkal District, Tamilnadu, South India. Global J. Environ. Res. 3, 169-177.

Tennant, S.M., Skinner, N.A., Joe, A., Robins-Browne, R.M., 2005. Homologues of insecticidal toxin complex genes in *Yersinia enterocolitica* biotype 1A and their contribution to virulence. Infect. Immun. 73, 6860–6867.

van Bokhorst-van de Veen, H., Minor, M., Zwietering, M., Groot, M.N., 2015. Microbial hazards in the dairy chain: A literature study (No. 1553). Wageningen UR Food & Biobased Research.

Van Duynhoven, Y.T.H.P., Isken, L.D., Borgen, K., Besselse, M., Soethoudt, K., Haitsma, O., Mulder, B., Notermans, D.W., De Jonge, R., Kock, P., Van Pelt, W., Stenvers, O., Van Steenbergen, J., 2009. A prolonged outbreak of Salmonella typhimurium infection related to an uncommon vehicle: hard cheese made from raw milk. Epidemiol. Infect. 137, 1548–1557.

Wang, H., Tay, M., Palmer, J., Flint, S., 2017. Biofilm formation of *Yersinia enterocolitica* and its persistence following treatment with different sanitation agents. Food Control 73, 433–437.

Wannet, W.J., Reessink, M., Brunings, H.A., Maas, H.M., 2001. Detection of pathogenic *Yersinia enterocolitica* by a rapid and sensitive duplex PCR assay. J. Clin. Microbiol. 39, 4483–4486.

Weagant, S.D., Feng, P., Stanfield, J.T., 2017. BAM Chapter 8, *Yersinia enterocolitica*. Bacteriological Analytical Manual (BAM) US Food and Drug Administration. Silver Spring: US Food and Drug Administration.

WHO, 1971. International standards for drinking water. Plais de Nation, 3rd Ed, Geneva.

Younis, G., Mady, M., Awad, A., 2019. *Yersinia enterocolitica*: Prevalence, virulence, and antimicrobial resistance from ret*ail* and processed meat in Egypt. Vet. world 12, 1078–1084.

Yucel, N., Ulusoy, H., 2006. A Turkey survey of hygiene indicator bacteria and *Yersinia enterocolitica* in raw milk and cheese samples. Food Control 17, 383–388.

Zadernowska, A., Chajęcka-Wierzchowska, W., 2017. Prevalence, biofilm formation and virulence markers of Salmonella sp. and *Yersinia enterocolitica* in food of animal origin in Poland. LWT 75, 552–556.