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Full Length Research Paper

Effect of oxidative and temperature stress on viability and toxin production of environmental isolates of *Escherichia coli*

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The effect of oxidative stress on expression of virulence factors and the effect of low temperature stress on toxin production among *Escherichia coli* serotypes isolated from wastewater and river water samples were determined. Oxidative stress due to chemicals, salts, alcohol or low temperature stress exerted various degrees of lethality to the bacterial cells with bacterial strains losing their potential to express virulence factors with time. The cell kill index (CKI) increases as temperature stress (-5, -18 and -28°C) increases with time. However, the rate of loss of expression of virulence factors or viability was slower in isolates from wastewater and abattoir compared to those from river water. Contamination of food or drinking water sources with these strains should be prevented to avoid human infection with disease conditions such as diarrhea, urinary tract infections and gastroenteritis.

Key words: Antimicrobial resistance, beta-lactamase, haemolysin, hydrophobicity, serum resistance, verotoxins.

INTRODUCTION

Contamination and proliferation of bacterial pathogens in food and water are of great concern for food and water safety and public health. Many environmental factors such as temperature, pH, moisture content, antimicrobial agents and water activity affect the growth of bacteria in nature (FDA, 2001). The food industry has a long history of manipulating these factors to control food-borne pathogens during food processing. Among these factors, temperature control is one of the most effective to reduce or minimize populations of *Escherichia coli* in foods (Yuk and Marshall, 2003). In addition, many chemical agents including salts have also been used for preservation purposes. Several studies have reported increasing cases of disease outbreaks due to *E. coli* (Aksoy et al., 2007) and many other food-borne pathogens especially in the developing world (Yuk and Marshall, 2003; WHO, 2005; Doughari et al., 2010). Pathogenecity by these food pathogens is possible due to the possession of virulence factors. Common virulence factors include surface hydrophobicity, colonization factor, capsular polysaccharides, siderophores, serum resistance and resistance to phagocytosis, haemolysins and enterotoxins (Raksha et al., 2003; Hedge et al., 2009; Jalalpour and Ebadi, 2012). The virulence factors of *E. coli* are multiple and usually complex, affecting pathogenicity in combination with one another (Hedge et al., 2009).

For successful infection, bacterial pathogens must overcome the host innate immunity (Davies et al., 2011). Phagocytic leukocytes, especially neutrophils, play a critical role in innate immune responses against bacteria, fungi and other pathogens (Witko-Sarsat et al., 2000). Neutrophil-mediated bacterial killing can involve both oxygen-independent and oxygen-dependent processes (Mydel, et al., 2006). While oxygen-independent bacterial killing involve the use of bactericidal peptides, proteins

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and protease fibres to trap, entangle and efficiently kill invading bacteria, oxygen-dependent processes involved exertion of oxidative stress (OS) by reactive oxygen species (ROS). ROS such as phagocytic cells (neutrophils and macrophages) and superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-), generated as by-products of endogenous metabolism (Soutourina et al., 2010) destroy the invading bacterial pathogens via oxidative burst. The mechanism by which bacteria overcome these factors to successfully establish infection is said to be complex and poorly understood (Khanduja et al., 1998; Hedge et al., 2009). To develop effective virulent-factor-targeted control measures, it is important to understand the response of pathogenic bacteria to these stress factors. This study investigated the effect of oxidative stress exerted by various physicochemical parameters (crystal violet, bile salt, and sodium chloride) and effect of low temperature stress (freeze-thaw) on the virulence factors and toxin production among environmental isolates of *E. coli.* Attempts were also made to investigate the association of antibiotic resistance to stress resistance among isolates resistant to more than three classes of antibiotics.

MATERIALS AND METHODS

Source of bacterial strains and blood sample

Nineteen stock cultures of *E. coli* serotypes: *E. coli* RWW1i O103:H2; *E. coli* RWW1ii O86; *E. coli* RWW1iii O145:H2; *E. coli* PSW1iii O111: NM; *E. coli* PSW2ii O96:H9; *E. coli* PSW1iv O111:NM (from treatment plant wastewater); *E. coli* PRE1i O4; *E. coli* FSE1ii O145:H2; *E. coli* FSE1iii O86; *E. coli* PST1v O96:H9; *E. coli* PST2i O124; *E. coli* PRE1vi O111:NM; *E. coli* PST1iii O113; and *E. coli* PST1iv O4 (from abattoir wastewater) and *E. coli* RBU2i O113; *E. coli* RBD1iii O86; *E. coli* RBI2iii O96:H9; and *E. coli* PRK2ii O86 (from river) were used for this study. The strains were previously isolated from the various water sources, characterized using standard methods to be positive for verotoxins, cell surface hydrophobicity, serum resistance and haemolysin production and maintained as stock cultures in the Microbiology Laboratory, Department of Biotechnology, Faculty of Applied Sciences Cape Peninsula University of Technology, Cape Town, South Africa (Doughari et al., 2012). The non pathogenic strain *E. coli* ATCC 25922 was used as control. All the bacteria were subcultured into tryptic soy slants (TAS) and incubated at 37°C for 18 h before use. Sheep blood erythrocyte was purchased fresh from the National Research Council (NRC) Cape Town, South Africa. The blood was used without further treatment as a source of serum complement.

Antimicrobial susceptibility testing

The bacteria were tested for susceptibility against ampicillin (10 µg), cefuroxime (30 µg), cephalexin (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), aztreonam (30 µg), nalidixic acid (30 µg), amikacin (30 µg), tetracycline (30 µg), gentamicin (10 µg), ofloxacin (5 µg) and ciprofloxacin (5 µg) (Oxoid UK) (WHO, 2002) using the disc diffusion method (Aksoy et al., 2007). Antibiotic discs were placed on Molten Mueller-Hinton agar (MHA) plates, earlier pre-seeded with the test bacteria (0.5 McFarland turbidity standard), using a sterile swab stick and the plates were incubated at 37°C for 18 h, after which antimicrobial activity was determined by measurement of zone diameters of inhibition (mm) against each bacterial strain.

Effect of stress on bacterial viability and virulence

Effect of oxidative stress on surface hydrophobicity of bacterial cells

To screen isolates for the effect of oxidative stress on bacterial viability, bacterial strains from TAS were subcultured twice in chemically defined medium (CDM) of Snyder and Koch as described by Hedge et al. (2009) and each time incubated at 37°C for 24 h. After the final subculture, the cells were washed thrice in sterile physiological saline (0.85% w/v NaCl) and finally suspended in saline to get a solution of OD_{600} 0.1 (equivalent to 0.5 McFarland turbidity standard or 10^{-8} cells/ml).

To determine the effect of oxidative stress exerted by H_2O_2, CDM (10 ml) containing three different molar concentrations of H_2O_2 (0.1, 0.2 and 0.3 M) were prepared and dispensed aseptically in three sets of 100 ml Erlenmeyer flasks. The bacterial suspensions were inoculated into each of the flasks to obtain a cell inoculum of approximately 10^{-6} cells/ml, and a fourth flask containing *E. coli* ATCC 25922 suspended in CDM with no H_2O_2 was used as control. The flasks were then incubated at 37°C in a rotary water bath at 160 rpm for 24 h, centrifuged at 3000 rpm for 10 min and the supernatants discarded and packed cells retained. The packed cells were washed with sterile phosphate buffered saline (PBS, 10 ml) and then suspended in PBS to get a density of 0.3 at OD_{600} (OD Initial). From this bacterial suspension, 3 ml was withdrawn and mixed with 0.3 ml *p*-xylene and vortexed for 1 min, then left for 30 min at ambient conditions and the final OD_{600} (OD Fl) determined. Degree of retention of hydrophobicity was determined by calculating the percent hydrophobicity index (HI).

$$HI = \frac{OD\ I - OD\ F}{OD\ I} x\ 100$$

Effect of oxidative stress on bacterial haemolysin production

This was carried out using the quantitative α-haemolysin assay. The H_2O_2 treated (0.3 M) bacterial suspension earlier grown in the Erlenmeyer flasks (10 ml), was transferred into sets of tubes and centrifuged at 3000 rpm for 10 min. The supernatant was collected and diluted in 0.8% calcium chloride solution (10 ml), 1 ml withdrawn and mixed with 1% (v/v) sheep erythrocyte suspension and incubated at 37°C for 1 h. After the incubation, 2 ml of 0.8% NaCl saline was added to each tube exhibiting partial haemolysis and the bacterial suspensions centrifuged at 1500 rpm for 10 min to pellet the unlysed erythrocytes. The supernatant fluid was separated and the OD_{540} determined. Fifty percent (50%) haemolysis standard prepared by mixing 1 ml of 1% (v/v) sheep erythrocyte suspension and 3 ml of diluent was used as control. Inverse of the dilution which caused 50% lysis was recorded as HU 50 (50% haemolysis units) (Hedge et al., 2009). A tube containing non-H_2O_2 treated bacterial suspension (*E. coli* ATCC 25922) suspended in CDM was used as control.

$$HU\ 50 = \frac{ODs - ODt}{ODs} x\ 100$$

where OD_s = 50% haemolysis standard, OD_{ex} is the final haemolysis measured, HU 50 = 50% haemeolysis rate.

Effect of oxidative stress on bacterial serum resistance

This was carried out using the quantitative serum bactericidal assay (Hughes et al., 1982). H_2O_2 treated (0.3 M) bacterial suspension (0.5 ml) was mixed with 1.5 ml of fresh undiluted serum and incubated at 37°C. Cell viability was determined turbidiometrically at 600 nm after 3, 6 and 18 h. Serum resistance index (SRI) of bacteria was calculated using the formula:

$$SRI = \frac{ODI - ODF}{ODI} x\,100$$

where SRI is the serum resistance index, ODI is the initial turbidiometric reading, and ODF is the final turbidiometric reading. A tube containing non-H_2O_2 treated bacterial suspension (*E. coli* ATCC 25922) suspended in CDM was used as control.

Effect of temperature stress

The effect of low temperature treatment on viability of the bacterial isolates was determined as described in literature (Chou and Cheng, 2000). A 1 ml aliquot of bacterial suspension (initial density 10^8 CFU/ml) grown at 37°C was inoculated into 9 ml modified tryptone soy broth (mTSB) in two different sets of test tubes and thoroughly mixed. The tubes were then stored at -5, -18, and -28°C for a period of 21 days. After every 5 days of storage, the tubes were removed from the freezers and the contents thawed under running tap water for 5 min and then returned to the freezer. After the 21 days storage, the cultures were removed and 2 to 3 loopfuls inoculated into modified trypticase soy broth (mTSB), incubated for 18 h at 37°C and the viable cell index (VCI) determined by taking the OD_{600} values first at 0 min (OD Initial) then at 10 min interval for 1 h. Viable cell counts on the scale of 100% was calculated using the formula:

$$VCI = \frac{OD\,I - OD\,F}{OD\,I} x\,100$$

To confirm that viable bacteria were in a culturable state, viable counts were made by making serial dilutions of 2 to 3 loopfuls of bacterial culture in 10 ml mTSB and surface spread inoculating onto NA plates, incubating at 37°C and determining percentage survival after 18 h. Bacterial suspension containing non-H_2O_2 treated and non-freeze-thawed bacterial (*E. coli* ATCC 25922) suspension in CDM was used as control.

Effect of ionic salt concentrations and other chemicals on cell viability and verotoxin production

Zero point two milliliters (0.2 ml) of 0.3 M H_2O_2 stressed bacterial culture suspension was inoculated into 10 ml of solution containing either 0.03% crystal violet, 0.3% bile salt, 4% NaCl, 8% ethanol and incubated at ambient conditions for 1 h (Chou and Cheng, 2000). Bacterial culture not subjected to H_2O_2 stress (*E. coli* 25922), or alcohol and salts was used as control. After incubation, each of the samples was serially diluted in Butterfield's phosphate diluents and 0.1 ml surface plated on tryptone soy agar (TSA) and incubated at 37°C at 10 (initial) and 30 min (final) after which, the percent killed cells was determined. Percent kill index (CKI, %) after incubation was calculated as follows:

$$CKI\,\% = \frac{Initial\ Population - Final\ Population}{Initial\ Population} x100$$

For effect of H_2O_2 (0.3 M) stress and temperature freeze thawing on verotoxin production, the Duoperth kit (Merck, SA) antibody-based rapid slide agglutination assay was employed according to the manufacturer's instructions. The H_2O_2 stressed or freeze thawed bacterial isolates (-5, -18, and -28°C) were first precultured in 1 ml casaminacid yeast extract (CAYE) broth (20 g of casamino acid, 6 g of yeast extract, 2.5 g of NaCl, 8.71 g of KH_2PO_4, and 1 ml of trace salt solution - 0.5% $MgSO_4$, 0.5% $MnCl_2$ and 0.5% $FeCl_3$ dissolved in 0.0005 M H_2SO_4) accordingly and incubated at 37°C with rotation at 100 rpm for 24 h. After incubation, 10 µl of the pre-cultured broth (approximately 1×10^7 cells/ml) was inoculated into fresh CAYE broth and further incubated for 16 h with rotation at 100 rpm at 37°C. The culture was centrifuged at 5000 x *g* for 5 min to separate the supernatant and cell pellets. The cell pellets were then washed three times with phosphate buffered saline (PBS, 5 ml) and then suspended in 0. 25 ml 0.01 M Tris-HCl (pH 7.5). To the pellets, 0.5 ml distilled water containing 50 µg/ml polymyxin B was added and the suspension incubated at 37°C for 30 min. Two hundred microliter (200 µl) of the culture suspension was then transferred onto the test device using a sterile Pasteur pipette and the result read after 10 min. The appearance of red bands on the VTX 1 and/or VTX 2 positions on the cartridge denoted the presence of either one of or both verotoxins.

Statistical analysis

The Student *t*-test of SIGMAPLOT 8.0 statistical software package was used to determine the effect of oxidative stress on viability of bacteria at $p \leq 0.05$ and also to calculate the standard deviation and standard error of mean of values obtained.

RESULTS

Table 1 shows the effect of various concentrations of crystal violet, bile salt, sodium chloride and ethanol on virulence and effect of freeze thawing on toxin production of *E. coli* isolates obtained from wastewater and river water sources. Results showed that 4% NaCl exhibited the highest lethal effect against *E. coli* PRK2ii 086 (initial cell population - ICP/ml 8000) and *E. coli* RBU2i O113 (ICP/ml 3840) with cell kill index (CKI) values of 52.48 (final cell population - ICP/ml 6600) and 45.83% (FCP/ml 3200) respectively (Table 1), followed by 8% ethanol and 0.3% bile salt with the highest effects against *E. coli* RBD1iii O86 and *E. coli* PRK2ii O86 (CKI values of 40.00 and 38.16% respectively). The highest CKI values of 25.20 (crystal violet), 38.16 (bile salt), 40.00 (ethanol) and 52.48% (NaCl) was recorded for *E. coli* PRE1i O4 (from abattoir wastewater), *E. coli* PRK2ii O86 (from river water), *E. coli* RBD1iii O86 (River water), and *E. coli* PRK2iiO86 (river water). *E. coli* isolates from river water samples recorded the highest CKI values (4.25 to 52.48) followed by those from abattoir (CKI values, 2.2 to 32.83%) and wastewater (CKI values, 1.34 to 22.86%). The CKI values of *E. coli* ATCC 25922 (control) ranged between 1.00 and 5.67% with ethanol recording the lowest (1.00). Among all the 19 isolates, the wastewater

Table 1. Cell kill index (CKI) (%) of *Escherichia coli* serotypes isolated from wastewater and river water samples.

Escherichia coli serotypes	Crystal violet (0.03%)			Bile salt (0.3%)			NaCl (4%)			Ethanol (8%)		
	ICP/100 ml (10 min)	FCP/100 ml (30 min)	CKI	ICP/100 ml (10 min)	FCP/100 ml (30 min)	CKI	ICP/100 ml (10 min)	FCP/100 ml (30 min)	CKI	ICP/100 ml (10 min)	FCP/100 ml (30 min)	CKI
***W*aste water isolates**												
E. coli RWW1i O103:H2	6700	6610	1.34	4000	3780	5.50	4000	3840	4.00	5200	3840	26.15
E. coli RWW1ii O86	8000	7660	4.25	4000	4000	0.00	7000	7340	2.13	6500	6300	3.17
E. coli RWW1iii O145:H2	4400	4300	2.27	3940	3720	5.58	4840	6200	11.30	5000	4400	12.00
E. coli PSW1iii O111: NM	4000	3600	10.00	3800	2100	17.89	4000	3600	10.00	4000	3200	20.00
E. coli PSW2ii O96 : H9	3500	3200	8.57	3300	3120	5.45	3500	2700	22.86	4400	4320	1.82
E. coli PSW1iv O111:NM	4600	3850	6.40	3000	2920	4.36	3200	3000	3.78	4800	4100	2.68
E. coli RBI2iii O96:H9	3600	3100	4.76	3700	3120	6.72	3000	2630	4.54	4600	4000	3.03
E. coli PRE1i O4	5000	3740	25.20	6000	4030	32.83	5700	4020	29.47	5000	4100	18.00
E. coli PRE1vi O111:NM	4700	4400	9.26	5620	4800	18.47	5300	4780	11.68	4800	4620	19.76
E. coli FSE1ii O145: H2	4200	4200	0.00	4000	3750	6.25	4000	3700	7.50	6000	5670	5.50
E. coli FSE1iii O86	6500	5960	8.31	6000	5600	6.67	6500	6040	7.10	4000	3800	5.00
E. coli PST1iii O113	4350	4000	1.87	4000	3700	5.82	4700	4620	4.86	3800	3700	4.67
E. coli PST1iv O4	4800	4550	4.82	4440	4000	10.56	5020	4800	6.80	4100	3780	6.72
E. coli PST1v O96: H9	4500	4400	2.20	3000	2820	6.00	4000	3800	5.00	5200	4580	11.92
E. coli PST2i O124	4600	4600	0.00	4400	4000	9.10	6000	5300	11.67	6000	5200	13.33
River water isolates												
E. coli RBU2i O113	3840	3200	16.67	4000	3900	2.50	2400	1300	45.83	3000	2300	23.33
E. coli RBD1iii O86	3400	3200	5.88	4600	3240	29.57	3400	2600	23.23	4500	2700	40.00
E. coli PRK2ii O86	8000	6600	17.50	7600	4700	38.16	7500	2300	52.48	6500	4340	33.23
Control												
E. coli ATCC 25922	3000	2900	3.30	3000	2830	5.67	3000	2950	1.67	3000	2870	1.00

ICP, Initial cell population at 3 h incubation; FCP, final cell population at 18 h incubation.

isolates *E. coli* RWW1ii O86 from wastewater treatment plant and *E. coli* PST2i O124 from abattoir wastewater recorded the lowest CKI values of 0.00 each for bile salt and crystal violet respectively.

Results of antimicrobial susceptibility profile and the effect of temperature stress on verotoxin production (Table 2) shows that, while the majority of the isolates were resistant to more than 3 antibiotics, *E. coli* PSW1iv O111:NM, *E. coli* PRE1vi O111:NM were only resistant to one antibiotic each (amoxicillin and impenim respectively) and *E. coli* RBI2iii O96:H9, *E. coli* PST1iii O113 and *E. coli* PST1iv O4 were not resistant to any one antibiotic. The control strain was resistant to tetracycline and gentamicin. Results also showed that while *E. coli* RBU2i O113 (from river water), *E. coli* PRK2ii O86 (from river water), *E. coli* PST1v

Table 2. Effect of H_2O_2 stress and temperature freeze-thawing son verotoxin production among multi-drug resistant environmental *E. coli* serotypes.

E. coli serotypes	Antibiotic resistance pattern	0.3 M H_2O_2		Temperature (°C) stress treatment/verotoxin status							
				37*		-5		-18		-28	
		Vtx1	Vtx2	Vtx1	Vtx2	Vtx1	Vtx2	Vtx1	Vtx2	Vtx1	Vtx2
Wastewater isolates											
E. coli RWW1i O103:H2	SXT, OFX, AMP, CN, AK	+	+	+	-	+	-	+	-	+	-
E. coli RWW1ii O86	ATM, AK, CL	+	+	+	+	+	+	+	+	+	+
E. coli RWW1iii O145:H2	TE, CN, CXM, AMP	+	+	+	+	+	+	+	+		+
E. coli PSW1iii O111: NM	OFX, NA, AMP, TE	+	+	+	+	+	+	+	+	+	+
E. coli PSW2ii O96: H9	AMP, CL, CRO, IPM	+	+	+	+	+	+	+	+	+	+
E. coli PSW1iv O111:NM	AML	+	+	+	+	+	-	-	-		-
E. coli RBI2iii O96:H9	-	-	-	-	+	+	-	-	-		-
E. coli PRE1i O4	AMP, CN, OFX, NA, TE	-	+	-	+	+	+	-	+		+
E. coli PRE1vi O111:NM	IPM	+	+	+	+	-					-
E. coli FSE1ii O145: H2	CFM, CL, AMP, NA	+	+	+	+	-	+	-	+		-
E. coli FSE1iii O86	OFX, TE, AK	-	+	-	+	-	+	-	+		+
E. coli PST1iii O113	-	+	+	+	+	-	-	-	-		-
E. coli PST1iv O4	-	-	-	-	+	-	+	-	-		-
E. coli PST1v O96: H9	CL, FM, CRO	-	+	-	+	-	+	-	+		+
E. coli PST2i O124	AMP, NA, OFX, TE, SXT	+	+	+	+	+	+	+	+	+	+
River water isolates											
E. coli RBU2i O113	CL, CFM, IPM	-	+	-	+	-	+	-	+	-	+
E. coli RBD1iii O86	AML, CRO, AK	+	+	+	+	+	+	+	+	+	+
E. coli PRK2ii O86	AK, AMP, CN	+	+	-	+	-	+	-	+	-	+
Control											
E. coli ATCC 25922	TE, CN	+	+	-	-	-	-	-	-	-	-

SXT, Sulphomethaxazole/Trimethoprim; AMP, Ampicillin; ATM, aztreonam; TE, Tetracycline; AK-Amikacin; OFX, Ofloxacin; CAZ, Ceftazidim; CL, Cephalexin; CRO, Ceftriazone; CXM, Cefuroxime; AML, Amoxycillin; CN, Gentamicin; CFM, Cefixime; CIP, Ciprofloxacin; NA, Nalidixic acid; IPM, impenim; +, positive; -, negative; *, non treated.

E. coli PST1v O96: H9 (from abattoir wastewater), *E. coli* PRE1i O4 (from river water) lost the ability to produce verotoxin type 1 (expressed by *vtx 1* genes), only *E. coli* RWW1i O103:H2 (from wastewater) lost ability to produce verotoxin type 2 (expressed by *vtx* 2) at all the temperatures tested. At -28°C however, *E. coli* FSE1ii O145: H2 (from abattoir wastewater) completely lost the ability to produce any of the verotoxins. However, H_2O_2 (0.3 M) stress however, did not show any significant effect on verotoxin production among the bacterial isolates at the tested concentration.

The hydrophobicity index values (HI) for various *E. coli* isolates from wastewater and river water samples are shown in Figure 1. Results showed

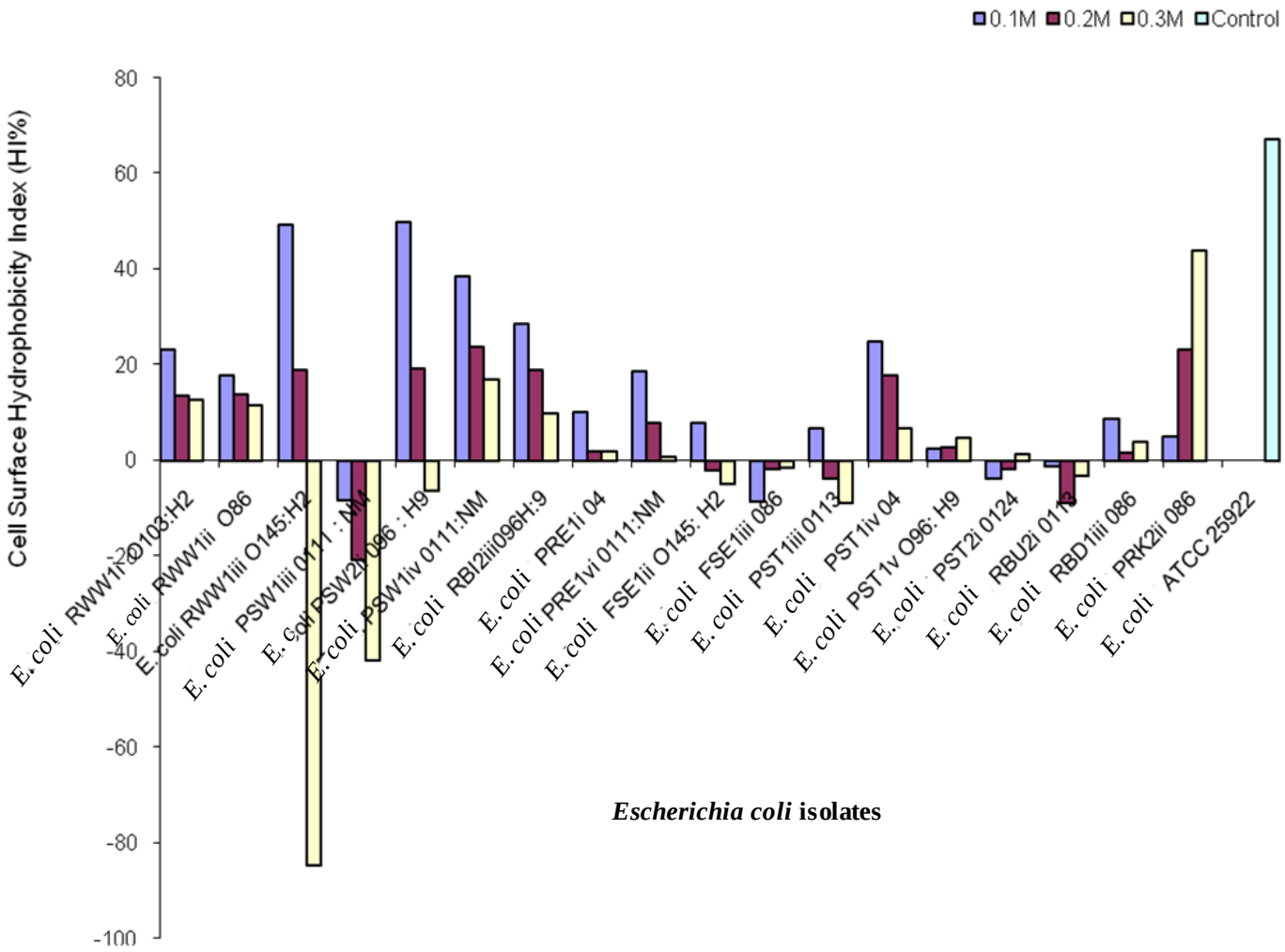

Figure 1. Effect of various concentrations of H_2O_2 on cell surface hydrophobicity (HI) values for temperature stressed *E. coli* serotypes isolated from wastewater and river water sources.

that oxidative stress exerted by H_2O_2 had significant effect on surface hydrophobicity of *E. coli.* The HI values indicating loss of surface hydrophobicity were in the order 23.12, 49.442, 49.917 and 43.967% at 0.1 M H_2O_2 for the *E. coli* serotypes RWW1i O103:H2, RWW1iii O145:H2 and PSW2ii O96:9 (all from treatment plant wastewater), and *E. coli* PRK2ii O86 (from abattoir wastewater) respectively. Results also showed that while *E. coli* PSW1iii O111:NM, *E. coli* PSW2ii O96:H9, *E. coli* FSE1ii O145:H2, *E. coli* PST1iii O113 lost their HI at 0.3 M H_2O_2, strains *E. coli* FSE1iii O86, *E. coli* PST2i O124 (both from abattoir wastewater) and *E. coli* RBU2i (from river water) completely lost their hydrophobicity at all the tested concentrations. Isolates from wastewater sources (RWW, PSW, FSE and PST) retained their HI more than isolates from river water (RBU and PRK). *E. coli* ATCC 25922 (control) which was not subjected to treatment with H_2O_2 retained its hydrophobicity.

Figure 2 shows the result of effect of oxidative stress on haemolysin production among the *E. coli* serotypes. Out of the 19 isolates, 12(63.16%) retained their potential for haemolysin production after being subjected to stress conditions with HU values ranging between 49.6 to 74.04%. *E. coli* ATCC 25922 (control) also was positive (HU 61.336%) for haemeolysin.

For bacterial serum resistance, results showed that while the erythrocytes were lethal to most of the isolates with increase in time of exposure, isolates from wastewater samples were less susceptible compared to those from river and the control (Figure 3). For wastewater isolates, *E. coli* PST1v O96:H9 (from abattoir) was the most resistant with only a very insignificant reduction in the SRI values with time (1.72, 1.69, 1.62 and 1.51% at 0, 3, 6 and 12 h respectively). Isolates from river water showed the least SRI values (1.02, 0.38, 0.05 and -.61% at 0, 3, 6 and 18 h respectively) compared to all other isolates, including the control.

Figure 4a to c shows results of viability of *E. coli*. serotypes under different low temperature conditions (-5,

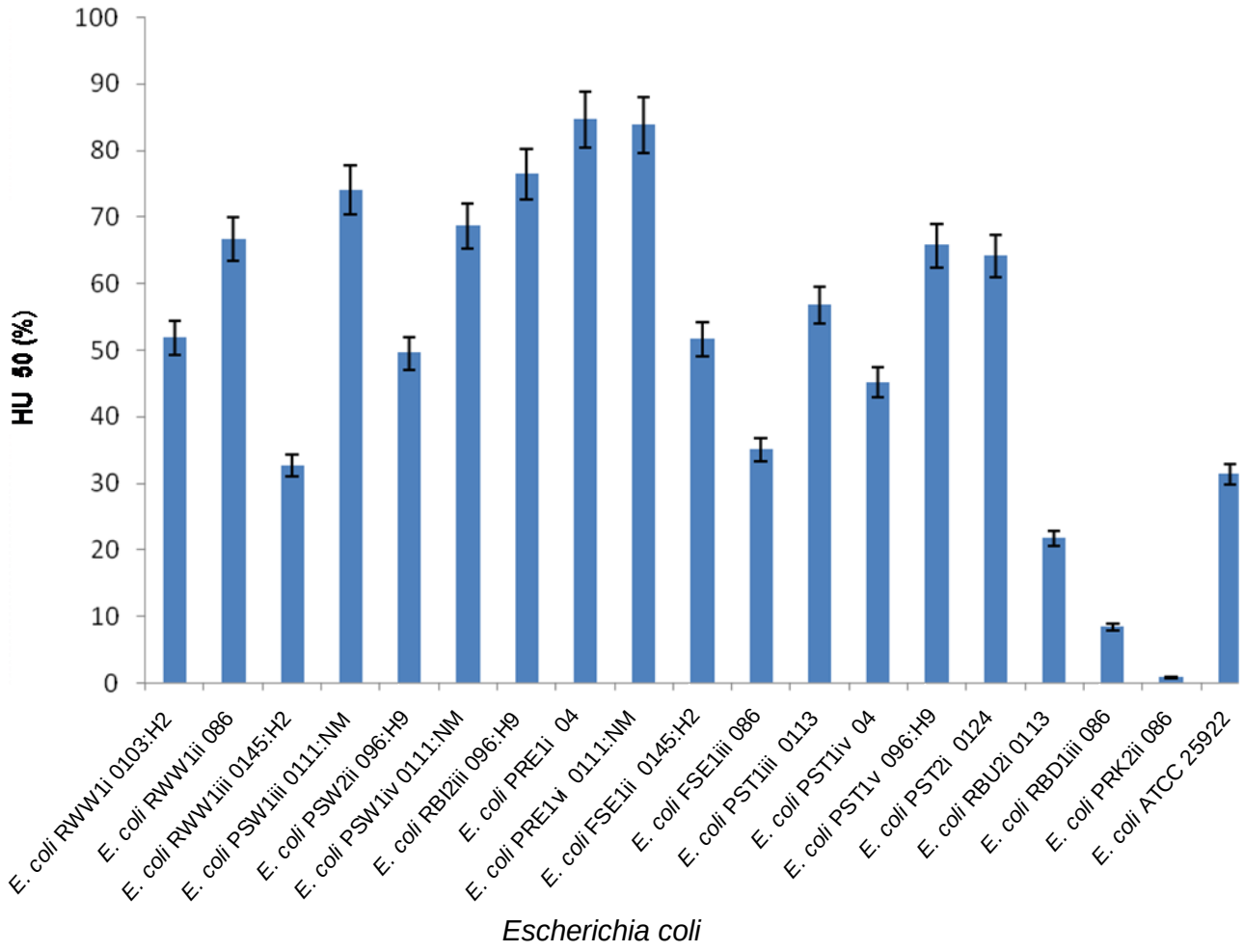

Figure 2. Effect of oxidative stress on haemolysin production of temperature-stressed *Escherichia coli* serotypes isolated from wastewater and river water sources.

-18, and -28°C). Generally, results showed that while the growth of temperature-stressed cells begins to decline after incubation for 6 h, those of non-freeze-thawed cells only declined between incubation for 10 to 12 h. For instance, at -5°C, the viable cell indexes of *E. coli* RWW1i O103:H2 (from treatment plant wastewater) increased from 0.36% at 0 h to 0.56% after 8 h incubation, and this value decreased to 0.03% after 12 h of incubation at 37°C. For *E. coli* ATCC 25922 (control), the VCI increased from 0.343 to 0.87% from 0 to 6 h of incubation, and after 12 h the VCI had increased to 0.905% (Figure 4a). A similar trend was observed for all the isolates at the various temperature stress conditions (-5, -18, and -28°C). The VCI values for isolates from river water samples were generally higher than those from wastewater samples and at 12 h incubation, about 50% of all the temperature stressed cells at -28°C recorded negative VCI values but at this temperature (-28°C) however, the control bacteria significantly ($P \leq 0.05$) recorded the highest VCI value (Figure 4c).

DISCUSSION

After exposure to various concentrations of chemicals and sodium chloride, the majority of the isolates retained their surface hydrophobicity and haemolysin production potential. Most isolates from the abattoir wastewater lost their ability to produce verotoxins but those of water treatment plant and rivers still produced the toxins and the majority of the isolates from wastewater retained high serum resistance. A higher CKI value is an indication of high susceptibility (and vise versa). Resistance among wastewater samples could be as a result of adaptation to a mixture of chemicals, salts and antibiotics of varying concentrations in the wastewaters. Exposure of isolates to harsh conditions provided by higher concentrations of these chemicals might have induced the cells to develop resistance.

The study showed that loss of verotoxin expression occurred more in the non-antibiotic resistant isolates or those that showed resistance to only one antibiotic.

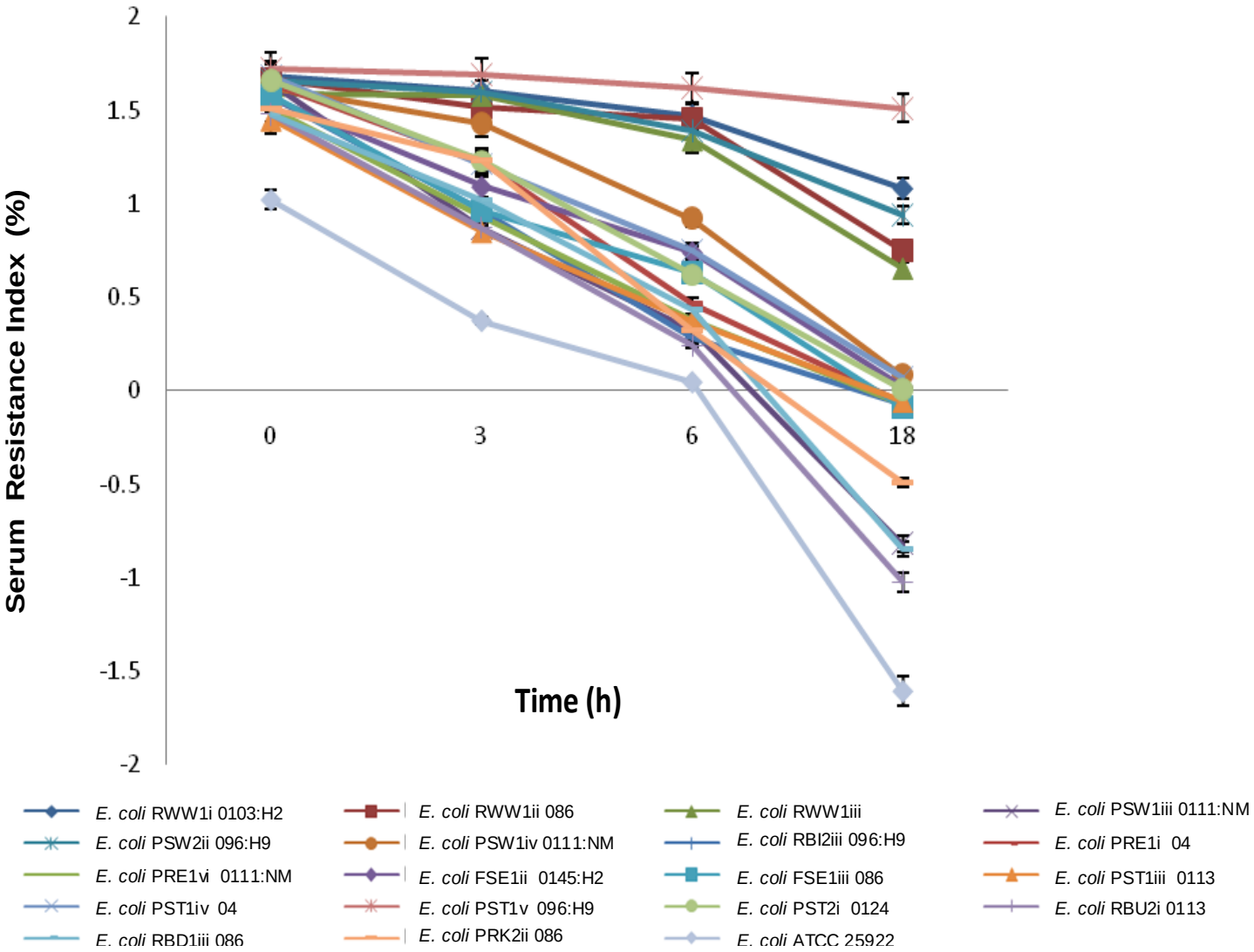

Figure 3. Effect of oxidative stress on serum resistance of H_2O_2 (0.3 M) stressed *Escherichia coli* serotypes isolated from wastewater and river water sources.

Though verotoxin and antibiotic resistance genes aregenetically mediated and are often carried within bacterial nuclei, previous studies has not established any association between antibiotic resistance and verotoxin production in bacteria (Aksoy et al., 2007). However, many of the isolates lost the ability to express the verotoxin 1 gene (*vtx* 1) compared to the verotoxin 2 (*vtx* 2) genes. Both genes are responsible for expression of toxin production in bacteria (European Food Safety Authority, 2007). No difference in the chemical nature of *vtx* 1 and *vtx* 2 genes has been reported and therefore reasons for the difference observed in this study are not immediately discernible. It will be interesting to determine the responses of these genes individually to various physiochemical parameters. At lower temperatures, isolates from abattoir wastewater completely lost the ability to express the verotoxin genes. A possible explanation is the fact that the freeze-thawing provided by the low temperature stress might have compromised the integrity of the various bacterial cell walls and membranes resulting in the alteration of the protoplasmic content, conformational changes and structural damages, consequently affecting their ability to withstand stress. According to Yuk and Marshall (2003), low-temperature bacterial growth decreases heat resistance of cells due to increase in membrane unsaturated fatty acids, which increases membrane fluidity consequently interrupting the selective permeability function of the cell membrane (Yuk and Marshall, 2003).

The population of temperature-stressed *E. coli* serotypes in this study was affected by various chemicals and salts with the effect increasing with time of exposure. Susceptibility was as a result of increased permeability in the cell wall of the bacteria caused by the freeze thawing

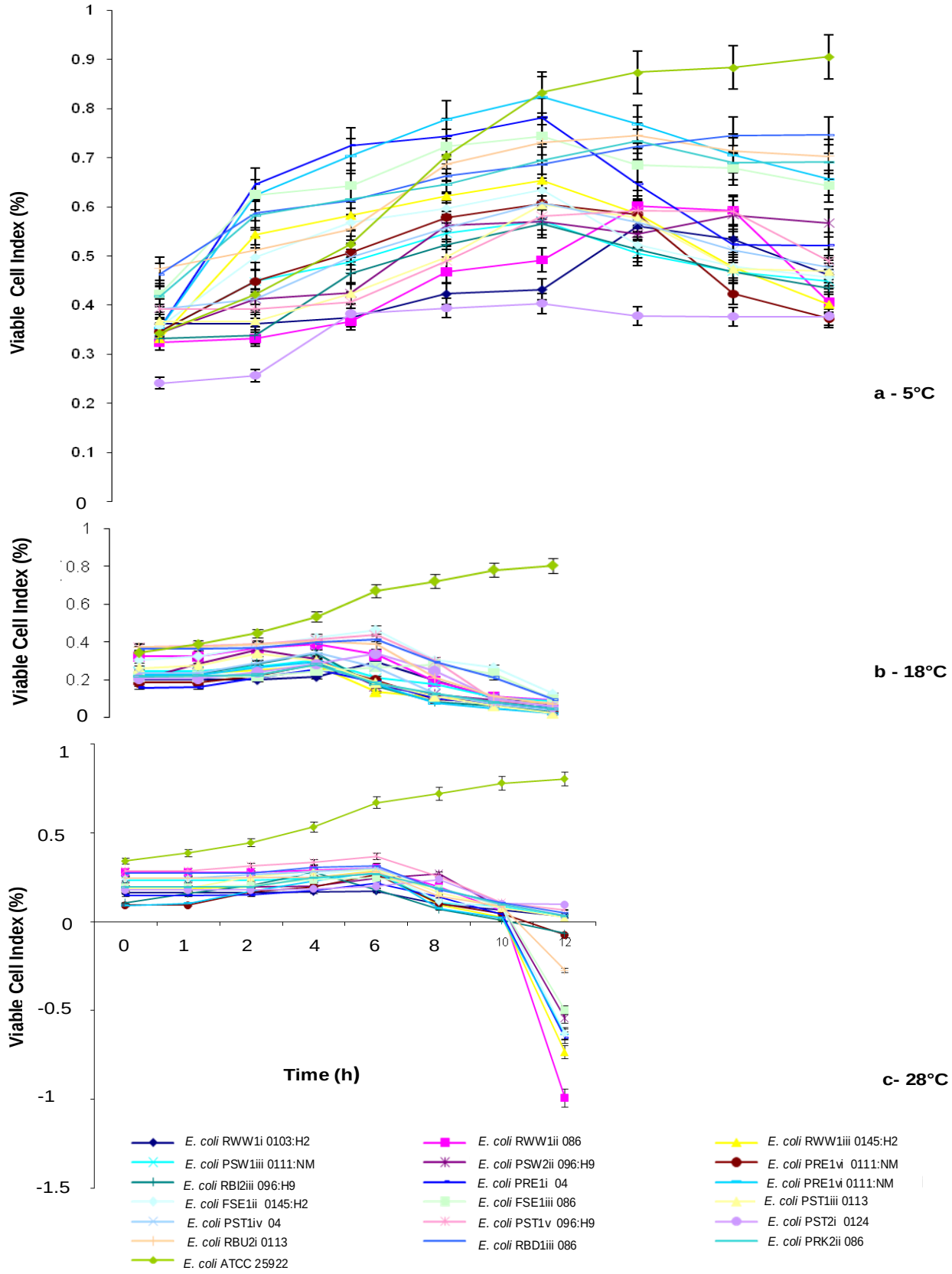

Figure 4. Effect of temperature stress (**a** -5°C, **b**-18°C, **c** -28°C) on viability of *Escherichia coli* serotypes isolated from wastewater and river water sources.

process. The increased susceptibility of *Listeria monocytogenes* to salt after freezing has earlier been reported (Golden et al., 1988). There was a slight difference in cell viability between the test and control cultures in this study, with the control showing a slightly higher ($p \leq 0.05$) viable cell index (VCI). This is an indication that their cell wall was less permeable since they were not subjected to temperature stress prior to exposure and hence have a reduced absorption rate of the salts or chemicals tested. The various *E. coli* strains responded differently in terms of production of virulent factors and cell viability to the salts of chemicals tested. This is possibly due to differences in physiological adaptability or resistance amongst the bacterial cells. The wastewater samples showed higher serum resistance compared to isolates from water samples. The exposure of this isolates to different environmental conditions might have induced the test bacteria to develop some degree of resistance to these chemicals.

Sodium chloride is one of the most important food adjuncts used for food preservation. During the handling of food and food ingredients from the farm to table, foodborne bacteria are exposed to different suboptimal physical and chemical environments. This can enable foodborne pathogens and spoilage bacteria, as well as beneficial bacteria, to develop characteristics that are different from those of normal cells. Most foodborne pathogens (especially the enteric pathogens) and spoilage bacteria (especially Gram-negative) are susceptible to low pH and die off rapidly in high-acid foods (pH 4.5) during storage. If they are first acid-adapted, they become relatively resistant to lower pH and other treatments at minimal levels and survive in food. Acid-adapted pathogenic strains surviving low pH and low heat treatment have recently been associated with outbreak of food borne diseases from the consumption of fruit juices, fermented sausages, and acidified foods containing viable *Salmonella, E. coli* O157:H7, and *L. monocytogenes* (Ray and Bhunia, 2008). Crystal violet and bile salt are commonly used in the selective inhibition of bacteria in selective bacteriological media such as MacConkey Sorbitol agar, while ethanol is used as a common food preservative as well as disinfectant. Resistance of *E. coli* to these chemicals will further pose a challenge to their laboratory cultivation, which will necessitate the search for alternative culture media. Resistance of *E. coli* to disinfectants has been a source of concern in healthcare settings (Guimarães et al., 2000).

Results showed differences in the rate of loss of hydrophobicity among the various serotypes. For instance, *E. coli* O86 from river water (*E. coli* PRK2ii O86) showed higher HI than those from wastewater (*E. coli* RWW1ii O86). While surface hydrophobicity is one of the virulence factors which contributes to the adherence of microorganisms to host tissue (Wojnicz and Jankowski, 2007; Hedge et al., 2009), previous studies have shown correlation between this factor and bacterial adherence to epithelia (Jahnn et al., 1981; Hedge et al., 2009). Strains retaining their surface hydrophobicity in this study might likely adhere more to epithelial cells if faced with similar concentrations of salt.

All the 3 isolates from river water samples lost their ability to produce haemolysins, while serum resistance was highest among the wastewater isolates compared to those from river water and the control isolates. *E. coli* haemolysins are proteins that cause *in vitro* lysis of erythrocytes from several species of animals (Kŏnig et al., 1986). Serum resistance also confers Gram-negative bacteria with the ability to resist the lytic effects of serum and to invade and survive in the human bloodstream. While bacterial haemolysins are found complexed with lipopolysaccharides, serum resistance in *E. coli* is imparted by capsular polysaccharides (CPS) and membrane proteins (La Regione and Woodward, 2002). The relative contributions of these polysaccharides and proteins to the virulence factors remains poorly understood (Cross et al., 1986). The higher serum resistance rate observed among the wastewater samples might not be unconnected with previous exposure to blood cells from the abattoir or treatment plant. The wastewater samples contain mixed wastes from both animal and human excreta, hospital and industrial environments where blood cells and antibiotics are part of the mixtures. For isolates from wastewater, prior exposure to animal blood (abattoir wastewater) and harsh pH, antibiotics and high salt concentrations (wastewater treatment plant) might have conferred them with adaptive potentials and consequently development of resistance mechanisms against erythrocytes. For instance, it has been reported that exposure of cells for an extended period to mild acidic environment (e.g. pH 5.0 to 5.8) enables them to develop resistance to subsequent exposure to pH ~ 2.5 (acid resistance of acid adaptation) and a brief exposure of cells to mild acidic environment enables them to survive subsequent exposure to pH 2.4 to 4.0 developing what is termed acid tolerance or acid tolerance response (ATR) (Ray and Bhunia, 2008). Absence of blood and less concentration of antibiotics in the river water might have posed a less challenging survival task to the bacteria.

However, a casual glance at the results showed that the antibiotic resistant (resistant to more than 3 antibiotics) isolates were slightly more tolerant to the various chemicals and salts. It is therefore possible that antibiotic resistance confers the bacteria with the advantage to withstand other chemical agents. Cooke *et al.* (2010) recently found equal distribution of virulence factors between susceptible and multidrug resistant (MDR) nosocomial and community blood stream *E. coli* isolates, but whether the degree of multidrug resistance is proportional to virulence is yet to be established.

In general, as the temperature of frozen storage increased, the percentage of surviving cells decreased.

This could be due to injury to the cells during storage, especially because the cells were freeze-thawed intermittently. The conformational changes to the bacterial cell wall as a result of the abrupt change in temperature during the freeze-thawing process limits the supply of essential nutrients such as iron, while oxidative stress results from increased levels of superoxide anion and H_2O_2 which leads to oxidative bursts, suboptimal pH conditions, osmotic stress, swelling and bursting of cell in hypotonic environments or in plasmolysis and dehydration in hypertonic conditions (Hedge et al., 2009). Although Shen et al. (2010) did not study the effect of low temperature stress; incubation of *Vibrio parahaemolyticus* at -18°C for 15 to 30 days inactivated the bacteria. The expression of virulent genes in bacteria however, is highly regulated and responds differently to environmental stimuli, such as temperature, pH and nutrient availability (Clarkem et al., 2003; Hedge et al., 2008). This explains the various responses exhibited by the various E. coli serotypes to the different stress conditions in this study. Furthermore, results showed that the CKI values of cells stored at -5°C were higher than those of -18 and -28°C. This is an indication that *E. coli* are less susceptible at -5°C. Even the control isolate which was not multidrug resistant responded differently with each virulent factor under different oxidative stress conditions.

Conclusion

In this study, retention of virulence by some strains of the *E. coli* serotypes, even after subjection to oxidative stress, is a cause for concern. This therefore calls for more proactive control and prevention measures as well as more investigation into virulence potentials associated with water and foodborne bacterial agents.

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