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Effect of different stress conditions on antibiotic susceptibility of coagulase positive thermo tolerant *Staphylococcus aureus*

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ABSTRACT

The control of microorganisms is one of the most important aspects of food preservation and its destruction ensures food safety. Prevalence of antibiotic resistance of thermo tolerant *Staphylococcus aureus* isolated from a variety of foods has increased. *Staphylococcus aureus* survive under a wide range of environmental stresses. The objective of this study was to evaluate effect of osmotic stress (2, 4, 8, 12% NaCl), pH (6, 4, 2) as well as cold (4^{O}C) stress on susceptibility of two laboratory isolates of *Staph. aureus* towards 9 different antibiotics. The susceptibility of antibiotics was tested against unstressed (control), stressed or post-stressed *Staph. aureus* isolates (an ATCC strain and a dairy) using the discs diffusion method. Unstressed *Staph. aureus* were sensitive to all tested antibiotics. In general, when *Staph. aureus* exposed to salt, pH and cold stress, their antibiotic resistance increased as salt concentration increased to 8 or 12%, pH reduced to pH 4 or 2, and as temperature decreased to 4^{O}C. Results showed that the dairy isolate and ATCC reference strain, both were resistant with Amoxycillin in stressed or post-stressed condition. The bacteria developed resistance in cold and pH stress with Streptomycin and osmotic pressure stress (4% to 12% NaCl) with Gentamicin in both isolates under stressed or post-stressed condition. The resistance against Gentamicin and Getifloxacin was revealed in acid stress condition. The both the isolates developed resistance against all four antibiotics under different stress conditions suggests that increased use of antibiotics as bacteriostatic agent, rather than bactericidal purpose in food processing and preservation systems may stimulate antibiotic resistance responses in *Staphylococcus aureus.*

***Keyword**: Staphylococcus aureus; Antibiotic susceptibility; Osmotic pressure; Acid stress; Cold stress; Stress adaptation*;

1. INTRODUCTION

Staphylococcus aureus is major human pathogen involved in food-related diseases and a common cause of community-associated infection (Fridkin et al., 2005; Normanno et al., 2007). This organism proliferates in food and releases one or more heat-stable enterotoxins, causing food-borne illnesses (Balaban et al., 2000). *S. aureus* is also common cause of infections in hospitalized patients and has been a major concern over a century (Ekrami et al., 2011). The spectrum of diseases caused by this organism is ranging from superficial infections to deep-seated and systemic infections such as pneumonia, endocarditis, osteomyelitis, and sepsis (Bertini et al., 2006). The treatment of staphylococcal infections has become extremely challenging due to the propensity of the organism to rapidly evolve into antibiotic-resistant strains. Antibiotic resistance is an emerging problem worldwide (Lietzau et al., 2004).

Thermo tolerant *Staph. Aureus is* a major concern in food safety during food processing and preservation, as the bacteria may encounter variety of stressful conditions viz., chemical (acids, ethanol, alkalies, chlorine and salts) or physical (heat, radiation and pressure) stress (Yousef and Courtney, 2003). Although there are some published data on its heat resistance (Baird-Parker 2000; Kennedy et al., 2005), there is very less information on the influence of environmental factors affecting heat resistance of this microorganism. When bacteria are exposed to mild forms of such stress, opportunity is provided for them to improve their ability to adapt and become resistant to subsequent more extreme exposures through physiological adjustment, enabling reproduction (Depardieu et al., 2007). Furthermore, the adaptive responses to these stresses may enhance resistance to others such as exposure to antibiotics and lead to "cross-protection" (Doyle et al., 2006; McMahon et al., 2007; Kumar et al., 2017).

It is believed that bacterial cells sense the actions of antibiotics as just another form of environmental stress (McDowell, 2004) and that sub-lethal dose of antibiotics induce stress hardening (McMahon et al., 2007; Blackburn et al., 1994) and cross-protection (Hastings et al., 2004). Conversely

environmental stress can induce resistance to antibiotics by a range of means. Many environmental stresses, including detergents, organic solvents, dyes, food components, preservatives, and antibiotics, induce the *mar* (multiple antibiotic resistance) operon, that regulates expression of large number of genes, including those coding for at least one broad-specificity efflux pump (*arcAB* efflux pump), which are more strongly expressed under conditions of environmental stress (Ma et al., 1995; Alekshun et al., 1997; Rickard et al., 2004). This suggests a direct linkage between environmental stresses, such as those occurring in foods and the domestic environment (Alekshun et al., 1999), efflux pump expression, and the development of antibiotic resistance (ABR) (Greenway et al., 1999). Other mechanisms associated with the development or expression of ABR include expression of specialized bacterial proteins, transfer of plasmids, or generation of genetic variability through mutation. Bishop et al. (2000) indicated that the expression of bacterial lipocalin, a protein implicated in the adaptation of bacterial cells to environmental stress and the dissemination of ABR genes, is regulated by high osmolarity and starvation. The bacterial lipocalin gene (*blc*) is carried on multiple antibiotic resistance plasmids in some genes in *Enterobacteriaceae*, rendering them capable of transfer between and within bacterial species (Livermore, 2003). As well as encouraging plasmid transfer, environmental stress can modulate plasmid numbers, enhancing resistance due to higher plasmid copy numbers. Such increases induce a range of protective responses, including expression of bactericidal exoproteins to suppress the growth of competing bacteria, and mutagenesis, including the induction of the SOS response (McDowell, 2004; Velkov, 1999).

Under nonstress conditions, DNA repair systems operate very efficiently to sustain population homogeneity with strict genetic conservation and fidelity. Rates of expression of classical mutations are low, only occurring when random genetic errors evade the DNA repair systems (Foster, 2000). Some members of heterogeneous population, which possess the new genes or gene combinations, survive in the adverse conditions. This process has been described as adaptive or directed mutation. The mechanisms underlying adaptive/directed mutation include stress-induced errors during DNA synthesis, suppression of normal DNA repair checking and repair mechanisms, transient hyper-mutability, gene amplification, and stress-induced recombination processes (McDowell, 2004; Kumar et al., 2014).

Environmental stresses such as oxidative stress, extreme pH, anaerobiosis, heat shock, osmotic shock, salts, osmotic pressure, alkaline, acidic conditions and starvation are reported to induce genomic reorganization or mutation (Jolivet-Gougeon et al., 2000) in bacterial cells, and such mechanisms of adaptive mutation have been associated with the development of antibiotic resistance (Foster, 2000; ; Kumar et al., 2018).

Keeping in view the literature reviewed, stress and antimicrobial drug resistance has been studied extensively in Gram-negative bacteria viz., *Escherichia coli* and *Salmonella typhimurium*; but very less is known about such stress factors with regard to *Staph. aureus, and hence* this work was planned to determine if environmental stresses typically used in food preservation influence the development or expression of antibiotic resistance in food-related pathogens.

2. MATERIALS AND METHODS

Experimental bacterial and standard culture

The bacterial cultures used in this experiment were isolated from dairy product, in which 23 ice cream sample were collected from different places of Navsari districts of south Gujarat with aseptic precautions and processed under standard bacteriological techniques and screened for the presence of coagulase positive *Staph. aureus* (Cowan & Steel, 1975; Public health England, 2001and Quinn et al, 1994) and molecular approaches were followed with genomic DNA extraction as per manufacturer's instruction (mericon^{TM} DNA Bacteria Plus Kit) and targeted to the species specific gene *16S rRNA* by simplex PCR. The specific primer pairs used in this study were synthesized from Eurofins and detection of genes as protocol outlined by Maes et al (2002) as mentioned in (Table: 1). The recovered 5 coagulase positive *Staph. aureus* andstandard control ATCC 25923 *Staph. aureus* and antibiotic discs quality control pure culture were maintained on Nutrient Agar (HiMedia) slants at 4^{o}C under standard bacteriological procedures.

Determination of Thermo tolerance

The pure working cultures prepared by plating all 5 recovered coagulase positive *Staph. aureus* from the sample on Mannitol Salt Agar (MSA, HiMedia), incubated at 37^{o}C for 24 h, were challenged for thermo tolerance selection criteria with high temperature short time pasteurization (HTST) as per standard US protocol for pasteurization of milk, 71.7 °C $\pm$ 0.5^{o}C (161 °F $\pm$ 0.83 °F) for 15 seconds in serological water bath and there after cooled immediately at 37^{o}C for 15 min in another serological water bath. The cultures were diluted after thermal challenge in Mueller Hinton Broth (MHB, HiMedia) to give a final concentration of 0.5 McFarland turbidity standards (HiMedia), approximately 1.5 x 10^{8} CFU/ml. For the selection of thermo tolerant coagulase positive *Staph. aureus*, 0.5 ml standardized culture was inoculated with spread plate technique on MSA, (HiMedia), incubated at 37^{o}C for 24 – 48 h and upon completion of incubation, the plates were observed to recover survival isolates for experimental studies.

Selection and quality control of antibiotic discs

The 9 antibiotics (Table 2), chosen according to their mode of action. Those that inhibit cell wall synthesis included Cephalothin and Amoxycillin, Those that inhibit protein synthesis included

Tetracycline, Gentamicin, Amikacine, Chloramphenicol and Streptomycin. Those that inhibit nucleic acid synthesis included Trimethoprime and Getifloxacin were tested for discs quality with ATCC 25923 *Staphylococcus aureus* as per guidelines from NCCL (2014).

Determination of antimicrobial susceptibility

The one thermo tolerant coagulase positive *Staph. aureus* isolate along with control (ATCC 25923 *Staph. aureus)* were used for the experiment to determine antimicrobial discs susceptibility assay from among the culture inoculate from stock Nutrient Agar (HiMedia) slants maintained at 4^{o}C. The experimental isolates were prepared for working culture by growth in 5 ml BHI broth (HiMedia) incubated at 37^{o}C for 24 h. For tests, 100 µl was transferred to 5 ml BHI and incubated at 37^{o}C for 20 h. The cultures were diluted in MH broth (HiMedia) to have final concentration 0.5 McFarland turbidity standards (HiMedia).

Preparation for working culture

The fresh both unstressed cultures from stock were inoculated in 5 ml BHI broth, incubated at 37^{o}C for 24 h and subsequently they were used for experimental stress tests.

Preparation of stressed cultures

Preliminary tests showed that the mild stresses to be used in the current study decrease the initial number of cells by approximately $\leq$ 1.0 $\log_{10}$ CFU/ml except for osmotic stress at 12% NaCl, where the initial number of cells was decreased by approximately 2 x $\log_{10}$ CFU/ml. The number of survivors was determined by plating on MSA before and after exposure to stress.

Osmotic stress

Four different levels of NaCl were used; 2%, 4%, 8%, and 12% (wt/ vol) in MH broth. The NaCl-supplemented suspensions were inoculated with coagulase positive *Staph. aureus* and incubated at 37^{o}C for 24 h. *Staph. aureus* cells were harvested by centrifugationat 4000g for 20 min and the pellet was resuspended and diluted to give a final concentration of 0.5 McFarland turbidity standards, in the reaction mixtures for antibiotic disc challenge.

Acid stress

Three different levels of pH i.e. 6.0, 4.0 and 2.0 were used in the current study. The acid-stressed cells were prepared (Francois et al., 2006; Osaili et al., 2008). The 5 ml of overnight grown culture of *Staph. aureus* in BHI broth was harvested by centrifugation (4000 g) for 20 min and the pellet was resuspended with 5 ml 0.1M potassium dihydrogen phosphate buffer adjusted to pH 6.0, 4.0 and 2.0 and then held at room temperature for 30 min. The culture was diluted to give a final concentration of approximately 1.5 x 10^{8} CFU/ml in the reaction mixtures for antibiotic disc challenge.

Cold stress

Cold-stressed cultures were prepared as described by Al-Nabulsi et al., (2011). The 1 ml of freshly prepared *Staph. aureus* cell suspension was added to 9 ml of sterile 0.1 M potassium dihydrogen phosphate buffer (pH 6.8) in 15 ml screw capped test tubes, mixed thoroughly for 1 min and stored 24 h at 4^{O}C. The culture was diluted to give final concentration of 0.5 McFarland turbidity standards in the reaction mixtures for antibiotic disc challenge.

Preparation of post-stressed cultures

To examine the effect of post-stress conditions on the susceptibility of *Staph. aureus* against tested antibiotics, cells stressed by osmotic, acid, and cold exposure were inoculated into BHI broth and incubated at 37^{O}C for 24 h. Then the cells were harvested by centrifugation at 4000 g for 20 min, the pellets were resuspended and diluted to give final concentration of 0.5 McFarland turbidity standards in the reaction mixtures for antibiotic disc challenge.

Interpretation of results

The break - point to determine antibiotic discs susceptibility criteria of the antibiotics disc against *Staph. aureus* was determined using reference values recommended as per guidelines for antimicrobial susceptibility testing for commonly occurring pathogens (CLSI, 2017) Wayne from HiMedia. (Table 2).

3. RESULTS

Isolation and molecular confirmation

Out of 23 ice cream samples processed, 5 (21.73 %) samples yielded the growth of coagulase positive *Staph. aureus*, identified based on cultural (Fig. 1 and (Fig. 2)) and biochemical characteristics. The all 5 conformed isolates was subjected for simplex PCR technique for detection of *16S rRNA*, revealed that all isolates were carried*16S rRNA* gene with amplicon size of 750 bp (Fig. 3).

The thermo tolerant isolates

The cultures after final dilution to give a final concentration of 0.5 McFarland turbidity standards (HiMedia) were challenged for thermo tolerance and recovered one isolate with abundant number of colonies grown (approximately 242). The maximum numbers of survived isolates were selected for further experimental studies.

The susceptibility of unstressed isolates to antibiotics

All unstressed *Staph. aureus* (thermo tolerance and ATCC 25923) isolates were sensitive to the tested antibiotics (Table 3). However, some variation in the susceptibility of unstressed isolates was noted, for example, the dairy isolate was more susceptible to Amikacine, Amoxycillin, Cephalothin, Chloramphenicol, Gentamicin, Getifloxacin, Streptomycin and Tetracycline than the ATCC strain. The latter was the most susceptible to Trimethoprime.

The susceptibility of stressed and post-stressed cells to antibiotics

Effect of osmotic stress to antibiotic susceptibility

Both stressed or post-stressed *Staph. aureus* isolates at NaCl concentrations 4% to 8% showed decrease susceptibility to the tested antibiotics were as at 12% revealed increased susceptibility to the Amikacine, Cephalothin, Chloramphenicol, Getifloxacin, Streptomycin, Trimethoprime and Tetracycline. Osmotic stress at 4%, 8%, and 12% (wt/ vol) NaCl for 24 h reduced the susceptibility of both isolates to the extent that susceptible isolates became resistant with Amoxycillin and Gentamicin, were as at 2% NaCl concentrations showed higher susceptibility. The development of antibiotic resistance by dairy isolates during NaCl exposure was markedly greater than that shown by the ATCC reference strain. Further, this resistance increased or remained constant after removal of the stress (Table 4).

Effect of acid stress to antibiotic susceptibility

It was observed that acid stress at pH 2.0 to 6.0 for 30 min tended variegated resistance of *Staph. aureus* isolates with osmotic stress. When *Staph. aureus* was exposed to pH 2.0 to 6.0, both isolates were found to be resistant to Amoxycillin, however, the ATCC strain and the dairy isolate became resistant to Gentamicin and Getifloxacin at pH 2.0, and Streptomycin showed resistance at pH 2.0 to 4.0. While both isolates showed moderate change in susceptibility to the Amikacine, Cephalothin, Chloramphenicol, Getifloxacin, Trimethoprime and Tetracycline between pH 2.0 to 6.0 under stressed or post-stressed conditions. The antibiotic resistance of post-acid-stressed *Staph. aureus* isolates increased or remained the same as the acid-stressed cells (Table 5).

Effect of cold stress to antibiotic susceptibility

Cold stress (4^{o}C for 24 h) consistently increased the resistance of *Staph. aureus* toward some tested antibiotics, although there were differences in the extent of the increase which were both, strain and antibiotic dependent. The dairy strain and ATCC strain tended to show enhanced resistance under cold stressed or post-stressed to the development of antibiotic resistance against Amoxycillin and Streptomycin. Cold stressed or post-stressed also increased the antibiotic resistance of *Staph. aureus* isolates even when the stress was removed (Table 6).

Fig. 1. Mannitol salt agar plate showing gowned colonies of *S. aureus.*

Fig. 2. Baird-Parker agar plate showing gray to jet-black colonies of *S. aureus*.

Table 1. Primers used in the study**.**

Sl. No	Gene/ Primer	Sequence of Primer (5'-3')	Product size (bp)
1.	16S rRNA	*16S rRNA* - F AACTCTGTTATTAGGGAAGAACA	750
		16S rRNA - R CCACCTTCCTCCGGTTTGTCACC	

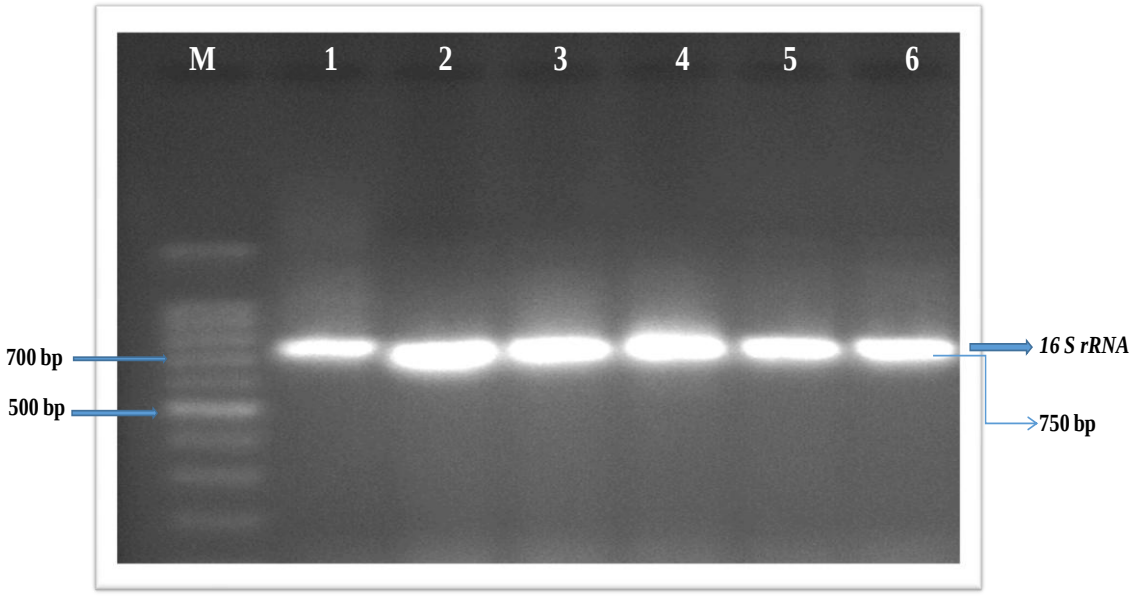

Fig. 3: Agarose gel showing amplification product of *16S rRNA* (750 bp), gene of coagulase positive *S. aureus*, M: 100 bp DNA marker, Lane 1 - 5: Sample positive and Lane 6: Standard control ATCC 25923 *S. aureus*

Table 2. Shows disc content and breakpoints used to determine antibiotic susceptibility criteria of the antimicrobial agent (HiMedia).

Sl. No.	Antimicrobial Agent	Symbol	Disc potency (µg)	Zone diameter interpretive standard (mm)			Control zone diameter limits (mm)
				Resistance	Intermediate	Susceptible	ATCC-25923, *S. aurus*
1.	Amikacine	AN	30	≤ 14	15-16	≥ 17	20-26
2.	Amoxycillin	AmX	30	≤ 19	-	≥ 20	28-36
3.	Cephalothin	CEP	30	≤ 14	15-17	≥ 18	29-37
4.	Chloramphenicol	C	30	≤ 12	13-17	≥ 18	19-26
5.	Gentamicin	GEN	10	≤ 12	13-14	≥ 15	19-27
6.	Getifloxacin	GAT	10	≤ 19	20-22	≥ 23	27-33
7.	Streptomycin	S	10	≤ 11	12-14	≥ 15	14-22
8.	Trimethoprime	TR	30	≤ 10	11-15	≥ 16	19-26
9.	Tetracycline	TE	30	≤ 14	15 – 18	≥ 19	24-30

Table 3. Changes in susceptibility of antibiotics against unstressed *Staphylococcu s aureus* strains as quality control of discs used**.**

Isolates	Susceptibility of antibiotics against unstressed *Staphylococcus aureus* strains								
	Amikacine	Amoxy cillin	Cephalot hin	Chloramphenicol	Gentamicin	Getifloxacin	Streptomycin	Trimethoprime	Tetracycline
ATCC-25923	22	28	31	32	23	30	21	30	34
Dairy (NVP-117)	40	37	36	37	27	36	31	22	37

Table 4. Changes in susceptibility of antibiotics against osmotically-stressed or post-osmotically-stressed *Staphylococcus aureus* strains**.**

Isolates	Salt %	Susceptibility of antibiotics against osmotically-stressed or post-osmotically-stressed *Staphylococcus aureus* strains																	
		Amikacine		Amoxycillin		Cephalothin		Chloramphenicol		Gentamicin		Getifloxacin		Streptomycin		Trimethoprime		Tetracycline	
		Stress	Post	Stress	Post	Stress	Post	Stress	Post	Stress	Post	Stress	Post	Stress	Post	Stress	Post	Stress	Post
ATCC-25923	2%	23	23	20	20	31	31	31	31	21	21	30	30	23	23	27	27	35	35
	4%	20	20	15	15	25	25	24	24	12	12	21	21	16	16	26	26	32	32
	8%	19	19	15	15	27	27	25	25	11	11	21	21	16	16	25	25	32	32
	12%	23	23	14	14	29	29	29	29	12	12	25	25	19	19	27	27	32	32
Dairy	2%	25	25	30	30	31	31	31	31	27	27	29	29	26	26	23	23	32	32
	4%	24	24	15	15	30	30	31	31	12	12	28	28	17	17	24	24	31	31
	8%	23	23	15	15	29	29	24	24	11	11	29	29	16	16	24	24	30	30
	12%	23	23	14	14	28	28	25	25	12	12	26	26	16	16	24	24	31	31

Table 5. Changes in susceptibility of antibiotics against acid-stressed or post-acid-stressed *Staphylococcus aureus* strains.

Isolates	pH	Susceptibility of antibiotics against acid-stressed or post-acid-stressed *Staphylococcus aureus* strains																	
		Amikacine		Amoxycillin		Cephalothin		Chloramphenicol		Gentamicin		Getifloxacin		Streptomycin		Trimethoprime		Tetracycline	
		Stress	Post	Stress	Post	Stress	Post	Stress	Post	Stress	Post	Stress	Post	Stress	Post	Stress	Post	Stress	Post
ATCC-25923	6	22	22	19	19	28	28	27	27	18	18	22	22	20	20	30	30	31	31
	4	20	20	17	17	29	29	27	27	22	22	25	25	11	11	30	30	31	31
	2	23	23	18	18	29	29	28	27	12	12	19	19	11	11	27	27	30	30
Dairy	6	22	22	19	19	28	28	28	27	18	18	22	22	20	20	30	30	31	31
	4	20	20	17	17	29	29	27	27	22	22	25	25	11	11	30	30	31	31
	2	23	23	18	18	29	29	27	27	12	12	19	19	11	11	26	26	29	29

Table 6. Changes in susceptibility of antibiotics against cold-stressed or post-cold -stressed *Staphylococcus aureus* strains.

Isolates	Temp ^{o}C	Susceptibility of antibiotics against cold-stressed or post-cold -stressed *Staphylococcus aureus* strains																	
		Amikacine		Amoxycillin		Cephalothin		Chloramphenicol		Gentamicin		Getifloxacin		Streptomycin		Trimethoprime		Tetracycline	
		Stress	Post	Stress	Post	Stress	Post	Stress	Post	Stress	Post	Stress	Post	Stress	Post	Stress	Post	Stress	Post
ATCC-25923	37	25	24	20	20	28	28	30	30	19	19	25	25	23	23	29	29	32	32
	4	27	27	18	18	31	32	31	32	21	21	29	29	11	11	30	31	35	36
Dairy	37	25	23	20	20	28	28	29	30	19	19	25	24	23	23	28	29	32	32
	4	27	26	17	17	32	32	31	32	21	21	29	29	11	11	31	32	36	35

4. DISCUSSION

Experimental thermo tolerant isolates obtained in this study under HTST pasteurization of milk (US protocol, 71.7 °C ± 0.5°C; 161 °F ± 0.83 °F, for 15 seconds, and cooling) in serological water bath has demonstrated the occurrence of heat resistance strains of *Staph. aureus* tested. While the heat-resistant strain showed a similar heat resistance and survival when compared with other pathogenic Gram positive bacteria such as *Listeria monocytogenes* (Lund et al., 2000; Hassani et al., 2005). *Staph. aureus* showed higher heat resistance under exposure to sublethal temperatures that might act as a heat shock. Several studies with other bacterial species demonstrated that thermal heating up lag phases may act as heat shock inducing a thermo tolerance enhancement (Thomson et al., 1979; Tsuchido et al., 1982; Mackey and Derrick, 1987; Hansen and Knochel, 1996; Manas et al., 2003; Hassani et al., 2005). The capacity of *Staph. aureus* strains of increasing their thermo tolerance after heat shock was checked. The induced thermo tolerance in the heat-resistant strain was only based on a higher capacity of heat damage repair as the survival of the heat-shocked cells obtained after recovery in selective conditions was similar to that obtained with non heat-shocked cells. Both the increase in intrinsic heat resistance and in capacity of heat damage repair are two general mechanisms of bacterial heat resistance increase under the influence of a heat shock (Pagan et al., 1997; Mackey, 2000).

Historically, most *Staph. aureus* strains isolated from clinical, food and environmental samples have been found susceptible to antibiotics against Gram-positive bacteria (Zhang et al., 2007; White et al., 2002). In the current study, the two *Staph. aureus* isolates examined were initially sensitive toward the antibiotics tested, but when they were exposed to osmotic, acid or cold stress, their antibiotic resistance changed. If this represents a broader phenomenon, it may in part explain the emergence of antibiotic resistance among *Staph. aureus* isolates in food products. Environmental stress like osmotic, acid and cold shock induced mutation in bacterial cells has been reported to be associated with the development of antibiotic resistance (Foster, 2000; Jolivet-Gougeon et al., 2000; Al-Nabulsi et al., 2015). The organism is able to adapt to elevated osmolarity by accumulating compatible solutes or osmolytes (Ko and Smith, 1999). In the present study, both stressed or post-stressed *Staph. aureus* isolates showed decrease susceptibility to the tested antibiotic at NaCl concentrations 4% to 8% , where as at 12% increase susceptibility to the Amikacine, Cephalothin, Chloramphenicol, Getifloxacin, Streptomycin, Trimethoprime and Tetracycline. Osmotic stress at 4%, 8%, and 12% (wt/ vol) NaCl for 24 h reduced the susceptibility of both isolates to the extent that susceptible isolates became resistant with Amoxycillin and Gentamicin, where as at 0% or 2% NaCl concentrations towards susceptibility to the tested antibiotic discs. These results were similar to those of Alonso-Hernando *et al*., (2009) who found that the resistance of *L. monocytogenes* to various antibiotics increased after exposure to acidified sodium chlorite. In contrast, Faezi-Ghasemi and Kazemi, (2015) reported that *L. monocytogenes* cells osmotically-stressed at 7% NaCl had decreased resistance to selected antibiotics including tetracycline, rifampicin, gentamycin, penicillin, ampicillin, trimethoprim-sulfamethoxazole and chloramphenicol. However, in that work *L. monocytogenes* PTCC 1297 (serotype 4a) was exposed to 7% NaCl for only 1 h before antibiotic challenge compared to the 24 h used in the present work. In further support of the present observations, McMahon et al., (2007) found that > 4.5% NaCl increased the antibiotic resistance of *E. coli*, *Salmonella Typhimurium* and *S. aureus*. In addition, Ganjian et al., (2012) found that NaCl concentrations up to 35% increased the antibiotic resistance of S. aureus cells. It is also important to note that the exposure of NaCl stress can lead to cross-protection against other stresses such as heat and acid (Skandamis et al., 2008).

In the presence of mild concentrations of weak acid preservatives, organisms have been shown to adapt by regulation of outer cell membrane protein synthesis (Beales, 2004), and by making changes in their cell membrane fatty acid composition to alter membrane permeability and fluidity (Diakogiannis *et al*., 2013). In the present study, It was recorded that acid stress at pH 2.0 to 6.0 for 30 min tended to increase the resistance of *Staph. aureus* isolates to antibiotics due to osmotic stress. When *Staph. aureus*was exposed to pH 2.0 to 6.0, both isolates were resistant to Amoxycillin, however, the ATCC strain and the dairy isolate became resistant to Gentamicin and Getifloxacin at pH 2.0, where as streptomycin developed resistance at pH 2.0 to 4.0. While both isolate became moderately susceptible to the Amikacine, Cephalothin, Chloramphenicol, Getifloxacin, Trimethoprime and Tetracycline between pH 2.0 to 6.0 in both stressed or post-stressed. Similarly, Alonso-Hernando et al., (2009) found that *L. monocytogenes* cells exposed to citric acid were more resistant to antibiotics than unexposed cells. Al-Nabulsi et al., (2011) found that acid-stressed Cronobacter sakazakii cells were more resistant toward tetracycline, tilmicosin, florfenicol, amoxicillin, ampicillin, vancomycin and enrofloxacin. McMahon et al., (2007) also found that *E. coli*, *Salmonella Typhimurium* and *S. aureus* stressed at pH 4.0 to 5.0 were more resistant to antibiotics than unstressed cells. Results from a number of studies showed that exposure of *L. monocytogenes* to acid stress increased its resistance to osmotic, ethanol, and oxidative stresses (Lou and Yousef, 1997; Phan-Thanh et al., 2000; Faleiro et al., 2003). In contrast with the present findings, Faezi-Ghasemi and Kazemi, (2015) reported that *L. monocytogenes* exposed to pH 5.0 was more susceptible to tetracycline, rifampicin, gentamycin, penicillin, ampicillin, trimethoprim sulfamethoxazole and chloramphenicol. Since the lengths of acid stress exposure in the two studies were similar (≤1 h), differences may have occurred because the *L. monocytogenes* cells used by Faezi-Ghasemi and Kazemi, (2015) were in the exponential rather than the stationary phase as used in the present study.

Storage temperature is important parameters regulating the activities of microorganisms in food systems. *Staphylococcus aureus* is a psychrotrophic microorganism which has the capacity

to grow at temperatures as low as 4°C. In the current study, Cold stress (4°C for 24 h) consistently increased the resistance of *Staph. aureus* toward tested antibiotics, although there were differences in the extent of the increases which were both strain and antibiotic dependent. The dairy strain and ATCC strain tended to show enhanced resistance under cold stressed or post-stressed towards decrease susceptibility to the development of antibiotic resistance against Amoxycillin and streptomycin. In other work with *C. sakazakii*, it was found that exposure of cells to 4°C for 24 h increased bacterial resistance to kanamycin, neomycin, tetracycline, florfenicol, ampicillin, amoxicillin, vancomycin and enrofloxacin (Al-Nabulsi et al., 2011). However, McMahon et al., (2007) found that cold stress had the opposite effect; it reduced the antibiotic resistance of *E. coli*, *S. Typhimurium* and *S. aureus*.

The increase in antibiotic resistance observed in the present work may be related to one or more of the following mechanisms: reduction of cell wall antibiotic binding sites, amplification of genes responsible for efflux pump synthesis and operation, and induction of stress shock proteins (McMahon et al., 2007). Bacteria can respond to adverse environmental challenges involving osmotic, acid and cold stress by down-regulation of penicillin binding proteins in the cell wall, can enhance their ability to reduce cytoplasmic concentrations of antibiotics by enhanced efflux pump operation and can improve survival by synthesis of chaperone proteins to maintain protein functionality during stress or antibiotic challenge (Bremer and Kramer, 2000; Rahmati-Bahram and Magee, 1997).

It was observed in the present work that *Staph. aureus* continued to show antibiotic resistance after removal of each of the three types of stress. Similar results were reported by McMahon et al., (2007) who found that NaCl or acid stressed *E. coli* and *S. aureus* were more resistant (≥4 times) to the antibiotics tested than controls. On the other hand, post- NaCl-stressed or post-acid-stressed *S. Typhimurium* cells were similarly or less resistant than unstressed ones.

5. CONCLUSIONS

This study demonstrates that *Staph. aureus* isolated from dairy food has thermal tolerance similar to that reported from *S. aureus* isolated elsewhere. Study showed that some common food preservation processes (i.e., those which employ low pH, cold or high NaCl to prevent or reduce pathogen growth rate) can lead to the development resistance to a range of currently used antibiotics.

Increase in antibiotic resistance observed were maintained for at least a day after the stress was removed. It was significant that cold (4°C), acid (pH 4 or 2) and osmotically (4%, 8% or 12% NaCl) stressed isolates were resistant to Amoxycillin whereas in cold and pH stresses developed resistance with streptomycin. The effect against Gentamicin and Getifloxacin were showed resistance in acid stressed or post-stressed among tested antibiotics. Both the isolates developed resistance against all 4 antibiotics under different stress suggested that, the increased use of bacteriostatic (sub-lethal) stress, rather than bactericidal treatments in food processing and preservation systems may stimulate antibiotic resistance responses in *Staph aureus*, if present and contributing to increase development and dissemination of antibiotic resistance among important food-related pathogens.

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