Influence of pH and Storage Period on the Antibacterial Susceptibility of Enterotoxigenic *Bacillus cereus* in Pasteurized Cow Milk during Low Temperature Storage

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Abstract: Food preservation processes are usually applied with the aim of slowing down or preventing spoilage and pathogenic bacteria in food materials. This involves the application of one or more environmental stresses (or hurdles) in the form of extremes of temperature, pH, and the manipulation of other optimal growth conditions.Environmental stress that may result from the use of hurdles, has however been shown to play a role in the emergence of antimicrobial resistance. This study was conducted with the aim of studying the combined effects of pH, low storage temperature and storage period on the antibacterial susceptibility of enterotoxigenic *Bacillus cereus* isolates from pasteurized cow-milk to selected antibacterial agents. Mcfarland standard 1 (about 8.5 log units) of a confirmed *B. cereus* isolate from raw milk was inoculated into sterile pasteurized cow-milk samples at varying pH levels (6.3.6.4,6.7,6.8), and stored at refrigeration temperature (4-10^{0}C) for a period of 72 h. Isolates were assayed for the *B. cereus* diarrhoel enterotoxin haemolysin BL (HBL) production using B. cereus enterotoxin reversed passive agglutination (BCET-RPLA) toxin detection kits (OXOID) and then subjected to antibacterial susceptibility tests using the Kirby-Bauer method against 10 antimicrobial agents (OXOID). Antibacterial agents included Ampicillin (10µg), Cephalothin (30µg), Amoxicillin-clavulanic acid(20/10µg), Cefpodoxime (10µg) Ceftriaxone (30µg), Erythromycin (30µg), Amikacin (30g), Tetracycline (30µg)Ciprofloxacin (5µg) and Trimetoprim Sulfamethoxazole (1.25/23.75µg). Results revealed resistance of all isolates to Ampicillin, Amoxicillin-clavulanic acid, Cefpodoxime, Ceftiaxone and Trimetoprim Sulfamethoxazole. All isolates were sensitive to Erythromycin, and Ciprofloxacin.Varying patterns were observed for Cephalothin, where only isolates from milk at pH 6.8 stored for 72 h and which exhibited suppressed toxigenic ability, were sensitive. Similarly, all isolates showed susceptibility to Tetracycline except for isolates at pH 6.3 which showed less sensitivity (intermediate response) to the antibacterial agent over a 48 h period. Findings suggest pH and storage conditions of foods could induce varying degrees of susceptibility to antibacterial agents in some enterotoxigenic *B. cereus* isolates.
Keywords: *B. cereus*, Haemolysin BL, low temperature storage, pH, antibacterial susceptibility

INTRODUCTION

The world today is faced with a major challenge of an increasing number of pathogens being resistant to antimicrobial agents. With antibiotic resistance, bacteria change in the way they would ordinarily respond to medicines used to prevent or treat infections. Global health agencies are funding researches to tackle this challenge (Ebinesh and Kailash, 2016). This challenge has also elicited a large number of researches by individuals, aimed at understanding the mechanisms fueling the changes in microbial susceptibility to conventional antimicrobial agents (Russell, 2004; Lister *et al*., 2009; Stickland *et al*., 2010; Hosseini *et al*., 2014; Shafaati *et al*., 2016).

Bacillus cereus, the type species of the *Bacillus cereus* group, (Senesi and Ghelardi, 2010), is a Gram positive, rod shaped bacterium capable of facultative aerobic metabolism. The organism is widely distributed in the environment, mainly in the soil. *B. cereus* causes two types of foodborne illness-emetic (vomiting) and diarrheal syndromes. The emetic syndrome is an intoxication caused by ingestion of a pre-formed toxin called cereulide in food during growth of the organism. This syndrome has a short incubation period and recovery time. The symptoms of nausea, vomiting and abdominal cramping occur within 1-5 hours of ingestion, with recovery usually within 6-24 hours (Schoeni and Wong 2005; Senesi and Ghelardi 2010).

The diarrheal syndrome is caused by enterotoxins produced by *B. cereus* inside the host following ingestion of food contaminated with vegetative cells and also spores (Wilcks *et al*., 2006).The diarrheal syndrome is mainly caused by three pore-forming enterotoxins; cytolysin K (CytK), non-hemolytic enterotoxin (NHE), and hemolysin BL (HBL) (Stenfors-Arnesen *et al*., 2008). Haemolysin BL (HBL) is believed to be the major diarrhoeal toxin of *B. cereus* (Burgess and Horwood, 2006)**.** Within an incubation period of 8-16 hours, the diarrheal illness usually lasts for 12-14 hours, although it can continue for several days. Symptoms are usually mild with abdominal cramps, watery diarrhea and nausea (Granum, 2007). The mild nature and short duration of symptoms associated with *B. cereus* infections are thought to be reasons why the illness is underreported, consequently, the prevalence of *B. cereus* infections is underestimated in many countries (Mols, 2009). To buttress this fact, it was observed that the organism was the cause of most incidents with an identified agent in the Netherlands in 2007 (Doorduyn *et al.*, 2008); It was the causative agent of foodborne illnesses in that country at levels of up to 41%between 2004 and 2007. Food products that are of potential risks for *B. cereus* infections include pasta, milk and milk products (Wijnands *et al*., 2006). The organism has a remarkable ability to survive adverse conditions due to its ability to form endospores and also display adaptive stress responses (Mols and Abee, 2011). Spores are able to survive many food processing operations and have been isolated from pasteurized milk and milk products like ice cream and infant formula milk (Mohammed, 2016). A number of preservative factors are applied in food preservation that act as hurdles against microorganisms. These preservative factors act by inhibiting their growth, as such serve as essential control measures for particular foodborne pathogens (Pujol *et al*., 2012).

Studies have however shown environmental stress that may result from the use of hurdles, plays a role in the emergence of antimicrobial resistance (Ebinesh, 2017). Environmental stresses are external factors that have adverse effects on the physiological welfare of bacterial cells, leading to reduction in growth rate, inhibition and/or death, at individual cell or population levels (McMahon et al., 2007). Exposure of bacterial cells to environmental stress results in changes to their genomic, phenotypic and physiological nature to enable them survive (Boor, 2006). In order to cope with these environmental dynamics, microorganisms have developed stress adaptation strategies that lead to the possibility that bacteria overcome harsher conditions for a variety of stresses (Desriac *et al.*, 2013).

Case reports of *B. cereus* infections depict a broad picture of sensitivity to antibiotics like Ciprofloxacin, Tetracycline, Vancomycin, Chloramphenicol, Amoxicillin-clavulanic acid (Turnball*et al.* 2004; Luna *et al*., 2007) and resistance to Penicillin, Ampicillin, Cephalosporins and trimethoprim (Turnball *et al*., 2004). Varied responses also exist where resistance of *B. cereus* toErythromycin and Clindamycin has been documented, whereas many preceding case reports of *B. cereus* show the organism as susceptible to these antibiotics (Luna *et al*., 2007).

While it is essential to employ hurdles in food preservation to meet rising consumer demands for safe and nutritious foods, it is also necessary to ensure these hurdles do not increase resistance in microorganisms or spores that become exposed to these preservative measures following post-processing contamination or inadequate heat processing. There is therefore an urgent need to carry out studies to better understand the relationship between specific hurdles in food matrices and antimicrobial susceptibility. This study is thus an attempt at studying the combined effects of pH, low storage temperature and storage period on the antibacterial susceptibility of enterotoxigenic *Bacillus cereus* isolates from pasteurized cow-milk to selected antibacterial agents.

MATERIALS AND METHODS

Growth of *B. cereus* Isolates at Varying pH Levels at Low Storage Temperature

Retail raw milk samples purchased at milking points from Fulani herdsmen in some parts of Kaduna town, Nigeria, were subjected to 10-fold serial dilutions in sterile 0.1% peptone water by aseptically diluting 1ml of the homogenate through a series of five 9ml sterile dilution blanks in test tubes. One millilitre of dilutions 10^{-1}, 10^{-3}, and 10^{-5} of serially diluted raw milk samples were pour plated on sterile plates of Mannitol Egg-yolk Polymyxin Agar (MYP) (OXOID, UK), a highly selective *Bacillus cereus* medium developed by Holbrook and Anderson, (1980). MYP plates of the food samples were incubated at 37°C for 24-48 h (until the appearance of distinct colonies). Flat, white granular colonies that appeared surrounded by zones of clearance on pink or red backgrounds on the MYP plates were counted and recorded as presumptive *Bacillus cereus*(MFLP-42, Http:// www.hc-sc.gc.ca. retrieved 27/7/2007). Colonies were streaked on nutrient agar slants, incubated at 37°C for 24 to 48 h, and stored at 4°C until required for characterization.

B. cereus isolates from raw cow milk were characterized using a Microgen® Bacillus ID identification kit (Microgen Bioproducts, U.K.). A confirmed isolate was then inoculated into pasteurized fresh cow milk obtained from the dairy processing unit of National Animal production and Research Institute (NAPRI) in Shika, Kaduna State. This was then tyndallized at 72^0C for 15 min (HTST) in a Grant JB series (Grant Instruments, U.K.) water bath for three days. Following tyndallisation, 0.1 m1 of the sample was then spread on sterile nutrient agar plate after the three-day heat treatment, and incubated for 24 h at 37^0C to test for sterility. One hundred and fifty millitres (150ml) (enough quantity that allowed for retrieval of samples for subsequent analysis) of pasteurized milk was then dispensed into four sterile 250m1 conical flasks. The milk in each of these conical flasks had its pH adjusted to 6.3, 6.4, 6.7 and 6.8 respectively, using 0.1Meach of lactic acid and sodium hydroxide.Lactic acid was used to lower and sodium hydroxide to increase pH levels as required.The pH in each case was monitored with a JENWAY 3150 series (JENWAY, U.K.) pH meter. These pH levels were selected to include the minimum and maximum levels recorded for retail samples analyzed for presence of *B. cereus,* as well as one point below, and above, these levels. Twenty milliliter (20ml) aliquots of milk sample from each conical flask were then dispensed into each of 3 sterile glass bottles. These bottles were each inoculated with 1 m1 suspension of an approximate cell count of 3 x 10^8 cfu/m1 (McFarland standard 1) *B. cereus* isolate and refrigerated (4-10^0C) for 72 h.

Determination of Production of Haemolysin BL (HBL) Enterotoxin by *B. cereus* From Pasteurized Cow Milk at varying pH Levels During Low Temperature Storage.

B. cereus isolates from milk samples were subjected to assay for production of haemolysin BL (HBL) diarrhoeal enterotoxin (Rowan *et al*., 2001) at 24 h intervals over the 72h storage period using *B.cereus* enterotoxin reversed passive agglutination(BCET-RPLA) toxin detection kits (OXOID, U.K.) according to manufacturer's instructions. One loopful of *B. cereus* isolates was inoculated into Brain heart infusion (BHI) broth (OXOID, U.K.), and incubated at 37^0C for 18 h. One milliliter (1m1) of each culture fluid was then dispensed into a sterile Eppendorf centrifuge tube, and centrifuged at 300rpm for 20 min at 40^oC using a TGL-16G series refrigerated centrifuge (Ningbo biocotek, China). Culture filtrates were retained and assayed for enterotoxin content in V-well microtitre plates (TITERTEK, U.S.A).

Antibacterial Susceptibility of HBL Enterotoxin Producing *B. cereus* Isolates

HBL enterotoxin producing *B. cereus* isolates were subjected to antimicrobial susceptibility tests by the Kirby-Bauer disc diffusion method (Agwa *et al*., 2012). Isolates were tested for their sensitivity to ten antimicrobial agents using filter paper discs impregnated with specified

concentrations of the antimicrobial agents (OXOID, U.K.). Antibacterial agents included Ampicillin (10μg), Cephalothin (30μg), Amoxicillin-clavulanic acid (20/10μg), Cefpodoxime (10μg) Ceftriaxone (30μg), Erythromycin (30μg), Amikacin (30g), Tetracycline (30μg)Ciprofloxacin (5μg) and Trimetoprim Sulfamethoxazole (1.25/23.75μg). These were selected to include some commonly used antibacterial agents from different target groups.

Plates were observed for zones of growth inhibition following the incubation period and the diameters of the zones were measured to the nearest millimeter for each of 11 HBL enterotoxin producing isolates. The responses of test isolates to the antimicrobial agents were then interpreted according to the clinical and Laboratory Standards Institute methodology (CLSI M100-S17, 2011) as either resistant, intermediate or sensitive.

RESULTS

Determination of Production of HBL Enterotoxin by *B. cereus* From Pasteurized Cow Milk at Varying pH Levels During Low Temperature Storage

Toxin titer values of≥>64 ng/m1 were recorded only for samples at pH 6.3 at 24 h of storage and at pH 6.7 at 48 h of storage (Table 1). Values ranged from ≥2 to 32 ng/m1 for samples at pH 6.4 ,6.7 and 6.8 at 24h; samples at pH 6.3, 6.4, and 6.8 at 48h and at samples at pH 6.3, 6.4, 6.7 ,6.8 at 72 h of low temperature storage (4-10^{o}C).Samples at pH 6.8 at 48 h of storage had the lowest toxin titre value of ≥2 ng/m1. There appeared to be a positive correlation between toxin titre and counts for isolates over the 72 h storage period with values ranging from 0.40 at 24 h to 0.7 at 72 h.

Table 1: Combined Effects of pH and Storage Time on HBL Enterotoxin Production by *B. cereus* Isolates(Log10cfu/ml)from pasteurized cow milk during refrigeration

Temperature(^{0}C)	pH	Storage Time (h)	Counts ±**SE**	Toxin Titre (ng/ml)*	Correlation coefficient (r)	P**
4-10	6.3	24	8.5±0.3	≥ 64	0.40	0.11
	6.4		8.2±0.5	4		
	6.7		8.0±0.5	32		
	6.8		7.7±0.2	32		
	6.3	48	6.6±0.3	4	0.70	
	6.4		8.4±0.5	8		
	6.7		8.7±0.1	≥ 64		
	6.8		7.2±0.2	<2		
	6.3	72	7.7±0.4	16		
	6.4		7.9±0.4	2	0.71	
	6.7		8.4±0.5	2		
	6.8		8.0±0.3	8		

Key: cfu/m1: colony forming units per milliliter of triplicate samples (initial inoculum levels of 8.5log10cfu/m1)

*Sensitivity at 2ng/ml, **Level of significance at p<0.05 for toxin titres at all storage periods.

Antibacterial Susceptibility of HBL enterotoxin Producing *B. cereus* Isolates

Results revealed resistance of all isolates to Ampicillin, Amoxicillin-clavulanic acid, Cefpodoxime, Ceftriaxone and Trimethoprim-sulfamethoxazole (Table 2). All isolates were also resistant to Cephalothin, except for the isolate from milk at pH 6.8 following storage for 72 h which was sensitive. All isolates were sensitive to Erythromycin, and Ciprofloxacin.

Similarly, all isolates showed susceptibility to Tetracycline except for isolates at pH 6.3 which showed less sensitivity (intermediate response) to the antibacterial agent over a 48 h period.

Table 2: Antimicrobial Susceptibility of HBL Enterotoxin Producing *B. cereus* Isolates From Pasteurized Milk during Refrigeration Storage at Varying pH Levels

Food Sample	Diameter of zone of inhibition (mm) to antibiotic (potency in µg)									
	AMC (30)	AK (30)	SXT (25)	E (30)	CPD (10)	KF (30)	TE (30)	CIP (5)	CRO (30)	AMP (10)
pH 6.7 at 24 h	11(R)	27(S)	0(R)	34(S)	0(R)	8(R)	24(S)	29(S)	8(R)	0(R)
pH 6.7 at 48 h	8(R)	28(S)	0(R)	29(S)	0(R)	11(R)	21(S)	32(S)	7(R)	0(R)
pH 6.7 at 72 h	15(R)	27(S)	0(R)	29(S)	0(R)	9(R)	26(S)	29(S)	8(R)	0(R)
pH 6.8 at 24 h	0(R)	27(S)	0(R)	34(S)	0(R)	0(R)	27(S)	31(S)	7(R)	0(R)
pH 6.8 at 48 h	*	*	*	*	*	*	*	*	*	*
pH 6.8 at 72 h	18(R)	28(S)	0(R)	25(S)	0(R)	22(S)	24(S)	29(S)	7(R)	0(R)
pH 6.3 at 24 h	14(R)	30(S)	0(R)	34(S)	0(R)	14(R)	14(I)	38(S)	0(R)	0(R)
pH 6.3 at 48 h	13(R)	24(S)	0(R)	32(S)	0(R)	11(R)	13(I)	31(S)	7(R)	0(R)
pH 6.3 at 72 h	11(R)	25(S)	0(R)	29(S)	0(R)	10(R)	16(S)	28(S)	7(R)	0(R)
pH 6.4 at 24 h	12(R)	28(S)	0(R)	34(S)	0(R)	9(R)	24(S)	28(S)	14(R)	0(R)
pH 6.4 at 48 h	8(R)	28(S)	0(R)	31(S)	0(R)	0(R)	16(S)	31(S)	7(R)	0(R)
pH 6.4 at 72 h	11(R)	25(S)	0(R)	32(S)	0(R*)	8(R)	23(S)	29(S)	10(R)	0(R)

Key: R: resistant, I: intermediate, S: susceptible, AMC: amoxicillin-clavulanic acid, AK: amikacin, SXT: trimethoprim-sulfamethoxazole, E: erythromycin, CPD: cefpodoxime, KF: cephalothin, TE: tetracycline, CIP: ciprofloxacin, CRO: ceftriaxone, AMP: ampicillin, refrigeration temperature 4-10^{0}C, *: non-enterotoxin producing isolate.

DISCUSSION

B. cereus isolates grown in refrigerated pasteurized milk, showed HBL toxin titres that correlated positively with counts. There was a general pattern of toxin production gradually increasing for isolates, under conditions of slow increases in cell density. These observations infer a positive impact of cell count on HBL production by *B. cereus* from milk during storage at refrigeration temperatures (4-10^{0}C). A transcriptional regulator, the phospholipase C regulator (PlcR), controls most known virulence factors in *B. cereus* (Gohar *et al.*, 2002). Its transcription is auto induced by a PlcR associated protein (PapR), an auto inducer peptide that accumulates inside the bacteria when high density cells are reached (Slamti *et al.,* 2002). This could also explain the interesting observation made of toxin titre levels of <2ng/m1 for isolates from milk at pH 6.8 after 48 h refrigeration, being associated with a sustained decreased in cell density during the storage period.

The results of sensitivity tests on HBL enterotoxin producing *B. cereus* isolates revealed resistance of all isolates to Ampicillin, an extended spectrum penicillin, as well as to Amoxicillin-clavulanic acid, a penicillin plus β-lactamase inhibitor combination. This agrees with the findings of Dikbas (2010), who also documented 100% resistance of *B. cereus* isolates from different food sources to penicillins. Similar findings have also been reported by Whong and Kwaga, (2006); Merens *et al.* (2008); Banerjee *et al.* (2011) and Owusu-kwarteng *et al.*, (2017). *Bacillus cereus* has been documented as a producer of a potent broad spectrum β-lactamase, which negatively affects the activity of penicillins and cephalosporins (Andrews and Wise, 2002).

Results of this study showed all isolates being resistant to Cefpodoxime a broad spectrum (third generation) cephalosporin.

Whong *et al*. (2006) also documented resistance of 56.7% of food isolates to Cefotaxime, another broad-spectrum cephalosporin. Isolates in this study were also resistant (96%) to ceftriaxone, another broad-spectrum cephalosporin.

Findings of this study also revealed all isolates being resistant to Cephalothin, a narrow spectrum (first generation) cephalosporin, except for the isolate from pasteurized milk at pH 6.8 stored at refrigeration temperature for 72 h, which appeared sensitive. Interestingly, isolates from this particular milk sample tested negative for the presence of the diarrhoeal enterotoxin HBL following 48 h of refrigerated storage. This may infer that the combined effects of pH and temperature not only suppressed the ability of the isolates to express the HBL diarrhoeal enterotoxin at 48 h, but may also have suppressed the ability of surviving isolates to resist the effects of this particular antibacterial agent at 72 h. It has been reported that virulence and resistance are closely related such that genetic elements may carry genes associated with both virulence and resistance (Soto, 2009).

Broad spectrum cephalosporins have been shown to be less active than the narrow spectrum agents against Gram positive cocci (Yao and Moellering, 2007). This may explain the sensitivity of these *B. cereus* milk isolates to Cephalothin (a narrow spectrum agent), and their resistance to Ceftriaxone and Cefpodoxime (broad spectrum agents). Sensitivity of all isolates in this study to Amikacin, an aminoglycoside, as well as to Ciprofloxacin a fluoroquinolone agrees with the findings of Whong *et al*. (2006); Dikbas (2010); Banerjee *et al*. (2011) and Hafiz *et al*. (2012).

A pattern of sustained intermediate response to Tetracycline over a 48h storage period was exhibited by *B. cereus* isolates from pasteurized milk at pH 6.3. Counts in this sample appeared to decrease during the initial 48 h of storage, which was then followed by an increase in cell count at 72h. It is noteworthy that in contrast to other test isolates from milk samples at higher pH levels, only isolates from this particular milk sample (pasteurized milk at pH 6.3) exhibited a sustained intermediate response to tetracycline during storage for 48h. In a related study, McMahon *et al*. (2007) also documented increased antibiotic resistance in food pathogens when incubated under sublethal stress conditions. Resistance to Trimethoprim-sulfamethoxazole (agent which target folate biosynthesis in bacteria) observed for all isolates in this study is consistent with findings from other researches (Turnball *et al*., 2004; Luna *et al.,* 2007).

CONCLUSION

Antibacterial sensitivity tests in this study suggest that decreased resistance to Cephalothin, a first generation cephalosporin, could be induced in *B. cereus* isolates following periods of suppressed HBL production in milk isolates. Findings also suggest that suppressed *B. cereus* growth in refrigerated milk at pH levels as low as 6.3 could be associated with increased resistance to Tetracycline. In addition, results also reaffirm the findings of previous researchers who documented sustained responses to antibacterial agents by bacteria even after removal of stress factors.

RECOMMENDATIONS

More research needs be carried out to understand the underlying mechanisms regulating virulence and antimicrobial susceptibility of food borne pathogens. This could reveal simple effective measures that would aid in the control of foodborne illnesses.

REFERENCES

Agwa, O.K., Uzoigwe, C.I., and Wokoma, E.C. (2012). Incidence and antibiotic sensitivity of Bacillus cereus isolated from ready to eat foods sold in some markets in portharcourt, rivers state, Nigeria. Asian *Journal of Microbiology Biotechnology and Environmental Science,* 14 (1): 13-18.

Andrews, J.M. and Wise, R. (2002). *Susceptibility testing of Bacillus species. Journal of antimicrobial Chemotherapy,* 49:1039-1046.

Banerjee, M., Nair, G.B. and Ramamurthy, T. (2011). Phenotyhpic & Genetic characterization of Bacillus cereus isolated from acute diarrhoeal patients *Indian Journal of Medical Research,* 133(1):88-95.

Boor, K.J. (2006). Bacterial stress response: What doesn't kill them makes them stronger. *PLOS Biology* 4:23.

Burgess, G. and Horwood, P. (2006). Development of improved molecular detection methods for *Bacillus cereus* toxins. A report for the Rural Industrial Research and Development Corporation, Australia.

CLSI (Clinical and Laboratory Standards Institute) (2011). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-First Informational Supplement. CLSI document M100-S21 (ISBN 1-56238-742-1). Clinical and Laboratory Standard Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087 U.S.A, pp 70-75

Desriac, N., Broussolle, V., Postolec, F., Mathot, A., Sohier, D., Coroller, L., and Leguerinel, I. (2013). Bacillus cereus cell response upon exposure to acid environment: toward the identification of potential biomarkers. *Front Microbiology,* 4:284. Published online October 2, 2013.

Dikbas, N. (2010). Determination of antibiotic susceptibility and fatty acid methy1 ester profiles of *Bacillus cereus* strains isolated from different food sources in Turkey. *African Journal of Biotechnology,* 9(11):1641-1647.

Doorduyn, Y., de Boer, E., and van Pelt, W. (2008). *Registrative voedselinfecties en vergiftigingenbij de Inspectievoor de Gezondheidszorg en de Voedsel en Waren Autoriteit, 2007. In: RIVM. In : Mols,M*. (2009). *Bacillus cereus* acid stress responses. PhD thesis, Wageningen University. P 18.

Ebinesh, A. (2017). Bacterial stress response and cross resistance to antibiotics in the light of natural selection. *Journal of infectious Diseases and Immune Therapies*1:1-2

Ebinesh, A. andKailash, T.V. (2016). Looking into antibiotics failure: a deemed threat, indeed not! *International Journal of Applied Research Studis*5: 1247 Fenselfeld, G. (2014). A brief history of epigenetics. Cold Spring Harbour Perspectives in Biology**6**(1). http://www.ncbi.nlm.nih.gov/pubmed. Retrieved 20/04/2018.

Gohar. M., Okstad, O.A., Gilois, N., Sanchis, V., Kolsto. A.B. and Lereclus, D. (2002). Two-dimensional electrophoresis analysis of the extracellular *proteome* of *Bacillus cereus* reveals the importance of the Plc Pregulon. Proteomics, 2(6): 784-791.

Hafiz, Y., Iqbal, A., Ahmad, M. and Ali, A. (2012). Antibiograms of B. cereus isolated from street vended foods in Srinigar area of Kashmir valley. *Indian Journal of Field Veterinarians,* 8(1):34-37.

Holbrook, R. and Anderson, J.M. (1980). An improved selective and diagnostic medium for the isolation and enumeration of *Bacillus cereus* in foods. *Canadian Journal of Microbiology,* 26**:**753-759

Hoseini, A.S.M., Motamedifar, M., Hadi, N. and Sedigh, E.S.H. (2014). Analysis of Virulence Genes Among

Methicillin Resistant *Staphylococcus aureus* (MRSA) Strains. Jundishapur Journal of Microbiology, 7(6):e10741. doi:10.5812/jjm.10741

Luna, V.A., King, D.S. Gulledge, J. Cannons, A.C., Amuse, P.T., and Cattani, J. (2007). Susceptibility *of Bacillus anhracis, Bacilus cereus, Bacillus mycoides, Bacillus pseudomycoides and Bacillus thuringiensis* to 24 antimicrobials using sensitive automated microbroth dilution and test agar gradient diffusion methods. *Journal of Antimicrobial Chemotheraphy;* 60(3):555-567.

McMahon, M.S.,Xu, J., Moore, J.E., Blair, I.S. and McDowell, D.A. (2007). Environmental Stress and Antibiotic Resistance in Food-Related Pathogens. *Applied and Environmental Microbiology,* 73(1):211-217.

MFLP-42 (Laboratory Procedures For The Microbiological Analysis Of Foods) (2003). Isolation and Enumeration of *Bacillus cereus* in Foods. Http:// www.hc-sc.gc.ca. Retrieved 27/7/2007.

Mols, M. (2009). *Bacillus cereus* acid stress responses. PhD thesis, Wageningen University, Netherlands. P18.

Mols, M., and Abee, T. (2011) *Bacillus cereus* responses to acid stress. *Environmental Microbiology* 13:2835-2843

Mohammed, A. (2016). Occurrence of Bacillus cereus in raw milk and some dairy products in Egypt. Japanese *Journal of veterinary research.* 66:595-102

Owusu-kwarteng, J., wuni, A., akabanda, f., tano-debrah, k. and Jespersen, I. (2017). Prevalence, virulence factor genes and antibiotic resistance of *B. cereus* sensulato isolated from dairy farms and traditional dairy products. *Bio. Med. Central Microbiology* 2017, 17:65 Doi: 10.1186/s12866-017-0975-9

Pujol, L., Kan-Kin-YU D., Le Marc, Y., Johnston, M., Rama-Heizard, F., Guillou, S and Membre, J. (2012). Establishing Equivalence for Microbial-Growth-Inhibitory Effects ("Iso-Hurdle Rules") by Analzying Disparate *Listeria monocytogenes* Data with a Gamma-Type Predictive Model. *Applied and Environmental Microbiology,* 78(4):1069-1080.

Rowan, N.J., Deans, J.0., Anderson, C.0., Gemmell, C.0., Hunter, I.S. and Chaithong, T. (2001). Putative virulence factor expression by clinical and food isolates of *Bacillus* spp. after growth in reconstituted infant milk formulae. *Applied and Environmental Microbiology,* **67:**3873-3881.

Russell, A.D. (2004). Bacterial adaption and resistance to antiseptics, disinfectants and preservatives is not a new phenomenon. *Journal of Hospital Infections,* 57(2):97-104.

Schoeni, J., and Wong, A.C.L. (2005). Bacillus cereus food poisoning and its toxins *Journal of Food Protection,* 68:636-48.

Senesi, S. and Ghelardi, E. (2010). Production, Secretion and Biological Activity of *Bacillus cereus* Enterotoxins. Toxins, 2:1690-1703.

Shafaati, M., Boroumand, M., Nowroozi, J., Amiri, P. and Kazemian, H. (2016). Correlation Between qacE and qacEDeltal Efflux Pump Genes, Antibiotic and Disinfectant Resistant Among Clinical Isolates of *E. Coli. Recent Patents on Antinfective Drug Discovery* 11(2):189-95.

Slamti, L and Lereclus, D. (2002). A cell-cell signaling pepide activates the PlcR virulence regulon in bacteria of the *Bacillus cereus* group. EMBO Journal, 21: 4550-4550.

Soto, S.M. (2009). relationship between virulence and antimicrobial resistance in bacteria. Reviews in medial microbiology, 20:84-90

Stenfors-Arnesen, L.P., Fagerlund, A., and Granum, P.E. (2008). From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiology Review,*32:579-606.

Stickland, H.G., Davenport, P.W., Lilley, K.S., Griffin, J.L. and Welch, M. (2010). Mutation ofnfxB causes global changes in the physiology and metabolism of *Pseudomonas aeruginosa. Journal of Proteome Research,* 9:2957-67.

Turnball, P.C.B., Sirranni, C., Samaan, M.N., Sutton, F.N, Reyes, A.E. andPeruski Jr., L.F. (2004). MICs of selected antibiotics for *Bacillus anthracis, Bacillus cereus, Bacillus thuringiensis, and Bacillus* mycoides from a range of clinical and environmental sources as determined by the Etest Journal of Clinical Microbiology, 42:3626-3634.

Wijnands, L., Dufrenne, J., Zwietering, M. H., and Leusden, F. (2006). Spores from mesophilic *Bacillus cereus* strains germinate better and grow faster in simulated gastro-intestinal conditions than spores from psychrotrophic strains. *International Journal of Food Microbiology*, 112(2):120-128.

Wilcks, A., Hansen, B., Hendriksen, N.B. and Licht, T. R. (2006). Fate and effect of ingested Bacillus cereus spores and vegetative cells in the intestinal tract of human flor-associated rates. *FEMS Immunology and Medical* Microbiology 46:70-77

Whong, C.M.Z., Kwaga, J.K.P., Umoh, V.J. and Ameh, J.B. (2006). Densities of *Bacillus cereus* in some Nigerian foods and food ingredients. *Nigerian Journal of Microbiology*, **20**(2):960-970.

Whong, C.M.Z. and Kwaga, J.K.P. (2007). Antibiograms of Bacillus cereus isolates from some *Nigerian foods Nigerian food Journal,* 25(1):178-183.

Yao, J.D.C., and Moellering JR, R.C. (2007). Antibacterial agents. In: Patrick, R. Murray, Ellen Jo Baron, James H. Jorgensen, Marie Louise Landry, and Michael Pfaller (eds). *Manual of clinical microbiology.* (9th edition). ASM press, Washington DC, USA, p 1080.