INTRODUCTION

Milk and milk products are excellent media for the multiplication of many of the pathogenic and non-pathogenic bacteria. Improper or unhygienic conditions during the product preparation and handling of milk and milk products in the various unorganized sectors of Agra region, may therefore pose a considerable risk to the people of all age groups. They may cause an outbreak of undesirable microorganisms such as Cronobacter sakazakii (formally known as Enterbacter sakazakii).

Enterobacter sakazakii is an emerging and an opportunistic pathogen. The organism is related to outbreaks of necrotizing enterocolitis, bacteraemia (Muytjens et al., 1983) and a rare form of infant meningitis. Enterobacter sakazakii has been ranked as “severe hazard for restricted population” by International Commission on Microbiological Specification for Food. It was ranked because of its resistant nature to certain antibiotics. Better understanding of the Enterobacter sakazakii pathogenesis is needed to aid in the development of new preventive strategies.

Enterobacter sakazakii is named in honor of the Japanese microbiologist Riichi Sakazakii. The organism was initially referred to as “yellow pigmented” Enterobacter cloacae till 1980 but it was reclassified as Enterobacter sakazakii (Farmer et al., 1980) on the basis of difference in the DNA-DNA hybridization, biochemical reaction, antibiotic susceptibility and production of yellow pigmented colonies. In DNA-DNA hybridization studies, the DNA of a type strain of Cronobacter sakazakii was compared with all other strains previously known as Enterobacter cloacae. Enterobacter sakazakii was assigned to the genus Enterobacter as it was 51% similar to E. cloacae and was also phenotypically closer to E. cloacae.

Using the polyphasic approach based on the extensive geno- and phenotypic evaluations the taxonomy of Enterobacter sakazakii was updated. This resulted in the description of five novel species: Cronobacter sakazakii, C.malonaticus, C.turicensis, C. muytjensii and C. dublinensis.

Scientific Classification

Kingdom: Bacteria
Phylum: Proteobacteria
Class: Gamma Proteobacteria
Order: Enterobacteriales
Family: Enteriobacteriaceae
Genus: Cronobacter
Species: sakazakii

Cronobacteria is a gram negative straight rod having dimensions of 3 µm in length and 1 µm in width. The cells are motile by peritrichous flagella and do not forms spores.
Enterobacter sakazaki is an uncommon bacteria but also fatal and an invasive pathogen which may cause nervous system and blood stream infections. The natural habitat and reservoir of Enterobacter sakazakii remain unknown. It can be found in surface water, soil, mud, grain, domestic animals, rotting wood, bird dung cattle and raw cow’s milk (Muytjens and Kollee, 1990), lettuce (Soriano et al., 2002), tomatoes. It was also found in infant milk formula (Himelright et al., 2001) fermented bread (Gassem et al., 1999) and thermal mineral water streams (Mosso et al., 1994). Enterobacter sakazakii has also been isolated from factory production lines including powdered infant formula factories and household as well as from a wide range of clinical samples including cerebrospinal fluid, bone marrow, sputum, urine and faeces. In addition to food and clinical samples, Cronobacter spp. was isolated from various insect’s intestinal tracts such as the Mexican fruit fly Anastrepha ludens and the stable fly Stomoxys calcitrans. (Kuzina et al., 2001).

Onset of Cronobacter infections is characterized by signs and symptoms typical of infections caused by other gram negative organisms. Common symptoms include sepsis (bacteria in blood), poor feeding response, irritability, jaundice, grunting respirations and instability of body temperature. Cronobacter forms biofilms and thus resist disinfectants. They are able to attach to and form biofilms on silicon, latex, polycarbonate, stainless steel, glass and polyvinyl chloride (Lehner et al., 2005, Iversen et al.,2004). In food industries such as brewing, dairy processing, fresh produce poultry processing and red meat processing such biofilms pose major problems.

In the year 2007 the bacteria caused a multiple brain abscess case in Japan and a sepsis case in 2009 (Teramato et al., 2009). However, their source and routes of infection remain unexplained. Enterobacteria represent a dominant portion of the ‘recontaminant flora’ of dairy products made from pasteurized products. Cronobacter sakazakii causes infection in infants, immuno-compromised, pre mature, having low birth weight of 2.5 kg or less are more susceptible to be affected by Cronobacter infections (Lai, 2001).

Cronobacter is fairly resistant to osmotic, heat and dry stress, which explains the survival and presence in desiccated infant powder (Nazarowes-White and Farber, 1997). Cronobacter sakazakii can also withstand a wide range of pH (Iversen et al; 2004a). The optimum temperature for the growth of Cronobacter sakazakii is 39°C, it can even grow at the temperature less than 4°C, which enable the bacteria to replicate during refrigeration (Iverson et al 2005).

Cronobacter sakazakii carries endotoxin on its surface, however, other virulence factors also may be crucial to pathogenicity(Townsend et al., 2007). Cronobacter sakazakii shows clustered adhesion (Mange et al., 2006). Cronobacter sakazakii adhere instantaneously to host surfaces, and then proliferate rapidly until a sufficient concentration is attained .Fimbriae are not associated with adhesion of Cronobacter sakazakii to epithelial cells (Mange et al., 2006). Viscous capsular material is produced by Cronobacter sakazakii which allows the organism to form a biofilm on feeding
equipments and contact surfaces (Iverson et al., 2004). This biofilm may provide protection for *Cronobacter sakazakii*, allowing it to survive osmotic, thermal, and tensile stressors. The adhesive capacities have been shown by *Cronobacter sakazakii* to a number of *in vitro* cell lines, including endothelial and transformed epithelial lines (Mange et al., 2006). Similar to many other gram negative bacteria *Cronobacter sakazakii* expresses the outer membrane protein A that shows a high degree of homology with *omp A* genes of other gram negative bacteria. (Nair et al., 2006). The organism also induces microtubule condensation at the sites of entry in endothelial cells. *Cronobacter sakazakii* invades moderately into human intestinal epithelial (INT 407) cells, and for which OmpA expression is required (Nair et al., 2006).

*Cronobacter* enhances epithelial cell injury by inducing apoptosis in rat model of necrotizing enterocolitis. (Hunter et al., 2008). It appears that low level of *Cronobacter sakazakii* in powder infant formula (PIF) can lead to infection in neonates (Clark et al., 1990). The high levels of *Cronobacter* are necessary to cause illness in animal models. (Pagatto et al., 2003). The mishandling of PIF during the preparation, storage, and/or feeding can lead to growth of *Cronobacter* to potentially high levels.

*Cronobacter sakazakii* infection has been treated with antibiotics like ampicillin and gentamycin. As pointed out by (Lai et al., 2001) increasing resistance of *Cronobacter sakazakii* to antibiotics prompt physicians to consider carbapenems in concert with a second agent such as an aminoglycoside. Use of gamma radiations (Lee et al., 2006) and *Cronobacter*-targeted bacteriophage therapy tends to reduce bacterial growth (Kim et al., 2007).

Because of the ubiquitous nature of *Cronobacter sakazakii* and the mystery surrounding its pathogenesis, preventive measures by parents, infant formula manufacturers, and health care providers will be important in the prevention of *Cronobacter* related infections. Breast milk feeding, should be encouraged and warning should be given on powdered infant formula packages that they may be contaminated with *Cronobacter* and abstinence from the practice of rewarming of reconstituted formula. In adults, reconstituted dairy products should be avoided in immunosuppressed populations. Appropriate barrier precautions should be observed in ICU settings (both adults and neonatal), where spread of infection may be more prevalent.
Review of Literature

A new species of “Enterobacteriaceae” was isolated from clinical samples such as sputum, wounds and uninoculated blood culture bottle (Farmer, 1980). Later eight cases of neonatal meningitis and sepsis were reported in Netherland due to Enterobacter sakazakii (Muytjens et al., 1983).

Pitout et al., (1997) reported increasing antibiotic resistance of Cronobacter infections. The phenotypic and genotypic typing methods were done to characterize food and clinical isolates of Cronobacter sakazakii. (Nazarowee-White and Farber 1999). Lai (2001) studied that Cronobacter sakazakii infections can occur in neonates, infants ,children and also among adults. The natural antibiotic susceptibility of Enterobacter amnigenus, Enterobacter carcerogenus, and strains were found to be naturally sensitive or intermediate to a wide range of antibiotics.(Stock and Wiedemann, 2002). Later Bureewer et al., (2003) analyzed desiccated and heat tolerance activity of Enterobacter sakazakii and concluded that the high tolerance of desiccation provides a competitive advantage for Cronobacter sakazakii in dry environment, as found in milk powder factories and increase the risk of post pasteurization contamination of finished products.Later Iversen et al.,(2004 a) studied the growth profile, thermo-tolerance and biofilm formation of Cronobacter sakazakii grown in infant formula milk. The small percentage of Cronobacter sakazakii cells can survive for extended periods in dehydrated powdered infant formula. (Endelson et al., 2005. Subsequently Iversen et al., (2005) compared the methods for the isolation of Cronobacter sakazakii. 16 S rRNA gene based analysis of Cronobacter sakazakii has been done from different sources and PCR assay was developed for its identification (Lehner et al.,2004). A real time PCR assay targeting the dnaG gene was developed (Seo et al., 2005).

Subsequently in 2006 Dadhich published a report on “How Safe are Infant Formulas?” and suggested that there is a association between Cronobacter sakazakii infections and use of commercial powdered infant formulas and clinicians should be aware that powdered formulas are not sterile products and might contain opportunistic bacterial pathogens.Later Mullane et al.,(2006) detected Cronobacter sakazakii in dried infant milk formula by developing simple methods, combining charged separation and growth on selective agar which can reliably detect 1 to 5 CFU(colony forming unit) of Cronobacter in 500g of Infant Milk Formula in less than 24h. Later Iversen and Forsythe (2007) compared media for the isolation of Cronobacter sakazakii. (Hunter et al., 2008), studied the origin detection and pathogenesis of Cronobacter. The inhibitory activities of some natural antimicrobial compounds alone or in combination with nisin aagainst Cronobacter sakazakii were determined by Lee and Jin (2008). The pathogenesis of Cronobacter sakazakii was studied by Pagatto et al.,(2008). Further the virulence of Cronobacter sakazakii was studied by Townsend et al., (2008). They isolated four distinguishable pulsotypes of Cronobacter sakazakii during the outbreak and the deaths were attributed to one pulsotype. The inactivation of Cronobacter sakazakii by water-soluble muscadine seeds was
studied by Kim et al., (2009). He concluded that muscadine seed extract contains antimicrobial inhibitors which can be used against *Cronobacter sakazakii* infections. The inhibitory effect of organic acids against *Cronobacter sakazakii* has been observed in laboratory media and liquid foods (Young Back et al., 2009). *Cronobacter* spp. were isolated from infant food, herbs and environmental samples and they were identified and confirmed using biochemical, chromogenic assays, PCR and 16S rRNA sequencing. (Jaradat et al., 2009). The sequencing of *Cronobacter sakazakii* strain BAA-894 and comparative genomic hybridization analysis of *Cronobacter* species was reported by Kucerova et al., (2010). Oonaka et al., (2010) analyzed powdered infant formula (PIF) as the source of infantile infections and isolated *Cronobacter sakazakii* from PIF samples and also performed drug sensitivity tests of *Cronobacter*. Later Bennour et al., (2010) combined the genotypic methods (pulsed field gel electrophoresis, 16S rRNA gene sequencing and automated ribotyping) with traditional phenotypic biochemical methods to characterize a collection of *Cronobacter* isolates from various origins.
PLAN OF WORK AND METHODOLOGY

SAMPLING AND ISOLATION OF PLANKTONIC AND SESSILE FORMS OF *Cronobacter sakazakii*.

- Milk samples of cow, buffalo and goat will be collected from different locations of Agra city.
- Aseptic method will be followed for collection of samples.
- In order to obtain pure culture, streak plate and pour plate method will be adopted using selective media.
- Maintenance of suspected colonies in selective broth
- Setting up of bacterial culture on selective broth
- Identification and biochemical characterization of sessile and planktonic forms of *Cronobacter sakazakii* from milk and milk products.

MOLECULAR CHARACTERIZATION

- Genotyping of the isolates from milk and milk products as well as from the biofilms using molecular techniques like 16s rRNA gene sequencing techniques (Bennour et al., 2010).

REMEDIAL MEASURES

- Control/Inhibition of planktonic and sessile form of the bacteria through the action of various
  1. Antibiotics by disk diffusion method (NCCLS, 1997)
  2. Natural products by well diffusion methods (Saxena and Gomber, 2006).
  3. Probiotic Control
OBJECTIVES

1. Sampling of cow, goat and buffalo milk and milk products collected from different sites of Agra region.
2. Isolation and characterization of planktonic form of *Cronobacter sakazakii*.
3. Isolation and characterization of *Cronobacter sakazakii* from biofilms.
4. Comparison of planktonic and sessile forms of *Cronobacter sakazakii* by molecular analysis.
5. Antibiotic sensitivity of the various isolates obtained and remedial measures for the control of planktonic and sessile form of *Cronobacter sakazakii* through the use of various molecules like natural products and probiotic bacteria.
PLAN OF WORK

North

West

AGRA REGION

East

South

Sampling

Milk and Milk Product

Isolation

*Cronobacter sakazakii*

PLANKTONIC FORM

SESSILE FORM

Biochemical and Molecular Characterization

Antibiotic Sensitivity and other Control Measures for Confirmed Isolates