International Journal of Food Microbiology 142 (2010) 53–59

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Resistance of *Cronobacter sakazakii* in reconstituted powdered infant formula during ultrasound at controlled temperatures: A quantitative approach on microbial responses

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ARTICLE INFO

Article history:
Received 29 January 2010
Received in revised form 23 May 2010
Accepted 30 May 2010

Keywords:
Cronobacter sakazakii
Ultrasound
Predictive microbiology
Inactivation
Non-thermal technologies

ABSTRACT

Many of the documented outbreaks of *Cronobacter sakazakii* have been linked to infant formula. The aims of this work are to monitor the inactivation kinetics of *C. sakazakii* NCTC 08155 and ATCC 11467 and to determine quantitatively the effectiveness of ultrasonic treatments as an alternative to heat processing of reconstituted infant milk formula before feeding of infants at highest risk. Inactivation studies of *C. sakazakii* inoculated in reconstituted infant formula were performed at the combined conditions of temperature, i.e., 25 °C, 35 °C, 50 °C and amplitude, i.e., 24.4, 30.5, 42.7, 54.9, 61 μm and the kinetics were described by a range of inactivation models. The dependency of the specific inactivation rate with respect to the product of temperature and amplitude was described by a modified Bigelow type model. Ultrasound combined with temperature was efficient to reduce significantly the microbial levels of *C. sakazakii*. *C. sakazakii* strain NCTC 08155 was at the same range of temperature and amplitude resistance as strain ATCC 11467. Application of ultrasound is an alternative process for the production of safe reconstituted infant formula. This study contributes on the quantitative assessment of the resistance of *C. sakazakii*.

1. Introduction

Enterobacter sakazakii is a bacterium regarded as an emerging opportunistic human pathogen and is the aetiological agent in life-threatening bacterial infections in low birth-weight neonates and infants. The genus of *Cronobacter* has been recently proposed to accommodate a number of biogroups of the genetically diverse and taxonomically complex species of *Enterobacteria sakazakii* (Iversen et al., 2008). Clinical *Cronobacter* is an emerging food-borne pathogen associated with a rare but life-threatening form of neonatal meningitis, sepsis and necrotizing enterocolitis (NEC) in newborns and premature infants. The International Commission on Microbiological Specification for Foods (ICMSF 2002) classified *Cronobacter* as a severe hazard for restricted populations.

Recent reviews (Gurtler et al., 2005; Friedemann, 2007) have summarized some reports on the presence of *E. sakazakii* in a wide variety of food and food ingredients of animal and vegetable origin. *Cronobacter* spp. have also been detected in ready-to-eat foods other than infant formula like sprouts, fresh herbs/salads, spices, dried herbs confectionary samples (Baumgartner et al., 2009). Nevertheless, it appears that reconstituted infant milk formula (IMF) (or powdered infant formula (PIF) as per WHO) and powdered milk have been the most common vehicles implicated in neonatal *Cronobacter sakazakii* infections and many of its documented outbreaks have been linked to IMF (Iversen and Forsythe, 2003; Anonymous, 2007b). In a recent international survey (by 8 laboratories in 7 countries) *C. sakazakii* was isolated at 3% of follow up formulas and 12% infant foods and drinks (Chap et al., 2009).

The organism can survive for long periods of up to two years in the desiccated state, and can be recovered from a large number of powdered foods in addition to powdered infant formula (Osaili and Forsythe, 2009). *E. sakazakii* displays greater heat resistance than most other (dairy-isolated) Enterobacteriaceae (NazarowecWhite and Farber, 1997a; Dancer et al., 2009). However it was not found to be as heat resistant as *L. monocytogenes* (Nazarowec-White et al., 1999). The predominant process by which PIF (powdered infant formula) is manufactured is employing wet mix processes. Microbial contamination of the product can occur following the addition of non heat-treated ingredients (including vitamins and minerals) to the powder, and/or more probably, during post-pasteurization by contamination originating from the surrounding environment (Mullane et al., 2005). On reconstitution of the PIF, the organism may rapidly multiply. According to Iversen and Forsythe (2003) among the control measures to be taken is to ensure hygienic preparation of non-

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doi:10.1016/j.ijfoodmicro.2010.05.028

heat treated ingredients or to prevent post-pasteurization contamination. Nevertheless, heat processing of reconstituted PIF before feeding of infants at highest risk (pro-term, underweight, immunocompromised) in hospitals appears to be essential especially if commercially sterile liquid formulas are not used (Perez et al., 2007a).

Although heating is an efficient and reliable process reducing the risks associated with foodborne pathogens while diminishing the activity of several enzymes, excessive heat causes undesirable side effects in the organoleptic, nutritional and functional properties of foods (Barbosa-Canovas et al., 2005). In recent years, other preservation techniques that could eliminate microbial activity whilst significantly minimizing or completely eliminating the amount of heat required have been developed. Technologies which have been reported to inactivate *C. sakazakii* include Pulsed Electric Field (Pina-Perez et al., 2009), microwaves (Kindle et al., 1996), gamma radiation (Lee et al., 2006) and high-pressure processing (Perez et al., 2007b). Ultrasound is another technology that can be combined with heat for achieving desired microbial inactivation. Ultrasound has the potential to inactivate bacterial populations due to cavitations caused by the changes in pressure created by the ultrasonic waves and has proven successful in its application to liquid or semi-liquid foods. The bactericidal effect of ultrasound has been reported by several authors (Rodgers and Ryser, 2004 Ugarte-Romero et al., 2007). Determining the effect of ultrasound on microbial inactivation efficiency appears to be one of the research needs (Piyasena et al., 2003). To the knowledge of the authors the microbial responses of *C. sakazakii* in reconstituted PIF processed by combined ultrasound and temperature treatments have not been reported in the literature.

The EU Food Safety Authority has recommended the introduction of Performance Objectives (PO) for infant formulas (Anonymous, 2003) and has recently suggested the monitoring of Enterobacteriaceae in the processing environment and in the product to confirm the application of GMP/GHP and HACCP which would be expected to reduce the prevalence of *E. sakazakii* (Anonymous, 2007a). The aim of this work is to monitor the microbial reduction of *C. sakazakii* strains of NCTC 08155 and ATCC 11467 and to determine quantitatively the effectiveness of ultrasound treatments by the use of rigorous modelling approaches in order to define the desired safety margins of the process.

2. Materials and methods

2.1. Bacterial cultures

Two strains of *C. sakazakii* (NCTC 08155 and ATCC 11467) were used for this study and were obtained from the UCD Food Safety Culture Collection. These strains were chosen as they are sufficiently characterised in the literature and could permit preliminary evaluation of the efficacy of the studied technology. The isolates were stored in glycerol serum vials below −20 °C. To activate the cultures, 100 µl of glycerol stock was inoculated into 10 ml Tryptone Soya Broth (TSB: CM0129; Oxoid Ltd., Basingstoke, Hampshire, U.K.) vortexed and incubated for 18–24 h at 37 °C. This was followed by a second aseptic transfer and incubation in TSB to produce an active culture. The resulting culture was T-streaked on Tryptone Soya Agar (TSA: CM0131; Oxoid Ltd., Basingstoke, Hampshire, U.K.) to obtain individual colony forming units.

2.2. Preparation of cultures

A single colony from a Tryptone Soya Agar (TSA: CM0131; Oxoid Ltd., Basingstoke, Hampshire, U.K.) was aseptically inoculated into 10 ml TSB (Oxoid) which was incubated at 37 °C for 18–24 h. The culture was centrifuged at 8000 rpm for 10 min at 4 °C (Model 4K15, Sigma Laboratory Centrifuges, AGB Scientific Limited, Dublin, Ireland) and the remaining pellet was re-suspended in 9 ml of sterile Ringers solution, prepared according to instructions on container (BR0052G; Oxoid Inc, Basingstoke, Hampshire, U.K.) and centrifuged as before. The test medium, commercial powdered infant formula (Milupa Aptamil: First) was prepared and reconstituted according to the manufacturer's directions (pH 6.82). The composition of the reconstituted PIF was 1.3 g of protein, 7.3 g of carbonhydrate, 3.5 g of fat. 50 ml of reconstituted formula was dispensed into 250 ml capped Duran bottles (Schott Duran, Germany) and autoclaved at 110 °C for 10 min. Prior to the inoculation of the washed culture into the sterile reconstituted formula, a 1 ml sample was taken and spread out onto Tryptone Soya Agar plates (TSA: CM0131; Oxoid Inc, Basingstoke, Hampshire, U.K.) to assess sterility. The washed pellet was then re-suspended in the sterile PIF to give a concentration of approximately 10^9 CFU/mL which was vortexed to ensure sample uniformity. High inoculum levels were selected in order to ensure that inactivation kinetics could be described accurately by the collection of significant amount of microbial inactivation data. The inoculated PIF was transferred into a jacketed beaker for ultrasonic treatments. A control which received heat treatment only at 25 °C, 35 °C and 50 °C was also prepared to assess the impact of ultrasound induced heating. This was carried out separately for each strain.

2.3. Ultrasound treatment

A 1500 W ultrasonic processor (VC 1500, Sonics and Materials Inc., Newtown, USA) with a 19 mm diameter probe and at a frequency of 20 kHz was used for sonication. The probe was autoclaved before each experiment to avoid any environmental contamination. 50 ml reconstituted PIF was placed in a 100 ml jacketed vessel through which water at 25 ± 1.0 °C, 35 ± 1.0 °C and 50 ± 1.0 °C and a flow rate of 0.5 L/min were circulated respectively. The temperature inside the reactor was monitored by a squirrel data logger (Grant Instruments Ltd, Cambridge, UK) at each temperature. Samples of 1 ml of PIF were taken at specified treatment time points (0, 4, 8, 12, 16 and 20 min), amplitudes (24.4, 30.5, 42.7, 54.9 and 61 µm), temperatures (25 °C and 35 °C) and varied with pulse durations of 5 s on and 5 s off. At 50 °C, the treatment time points were between 0 and 8 min depending on the amplitude level. The ultrasound probe was submerged to a depth of 25 mm in the sample. All treatments were carried out in duplicate for both types NCTC 08155 and ATCC 11467.

2.4. Cell enumeration

C. sakazakii survivors were enumerated by diluting each 1 ml sample in 9 ml of sterile Ringers solution (Oxoid). The solution was vortexed and a serial decimal dilution was prepared. Duplicate 0.1 ml samples, from each dilution, were inoculated onto TSA (CM0131; Oxoid Ltd., Basingstoke, Hampshire, U.K.) using the spread plate method. The plates were dried and placed at 25 °C for 2 h to allow cell recovery. The inoculated TSA plates were then overlaid with Violet Red Bile Glucose Agar plates (VRBGA: CM485; Oxoid Ltd., Basingstoke, Hampshire, U.K) as a precaution to prevent outgrowth of *Bacillus* spp. spores that might remain from germinating. Previous researchers have found that autoclaving the formula was not always sufficient to eliminate bacillus endospores (personal communication). Preliminary results have shown that any more than 2 h did not increase recovery substantially. Once the overlay had set, the plates were incubated overnight at 37 °C for 18–24 h. The colony forming units (CFU/mL) were enumerated manually.

2.5. Mathematical modelling

2.5.1. Primary model

Initially, the inactivation models reviewed and presented by Geeraerd et al. (2005) have been evaluated for their performances on all the experimental data. Based on the results of this preliminary