International Journal of Food Microbiology 253 (2017) 1–11

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Investigating the biocontrol and anti-biofilm potential of a three phage cocktail against *Cronobacter sakazakii* in different brands of infant formula

Lorraine Endersen[a], Colin Buttimer[a], Eoghan Nevin[a], Aidan Coffey[a], Horst Neve[b], Hugo Oliveira[c], Rob Lavigne[d], Jim O'Mahony[a,*]

[a] Department of Biological Sciences, Cork Institute of Technology, Cork, Ireland
[b] Department of Microbiology and Biotechnology, Max Rubner-Institute, Federal Research Institute of Nutrition and Food, 1, Kiel, Germany
[c] CEB-Centre of Biological Engineering, LIBRO – Laboratório de Investigação em Biofilmes Rosário Oliveira, University of Minho, 4710-057 Braga, Portugal
[d] Laboratory of Gene Technology, KU-Leuven, Leuven, Belgium

ARTICLE INFO

Keywords:
Bacteriophages
Biocontrol
Biofilm
Cronobacter sakazakii
Infant formula

ABSTRACT

In recent years, the microbiological safety of powdered infant formula has gained increasing attention due to the identification of contaminating *C. sakazakii* and its epidemiological link with life-threatening neonatal infections. Current intervention strategies have fallen short of ensuring the production of infant formula that is free from *C. sakazakii*. In this study, we describe the isolation and characterisation of three bacteriophages (phages) and their application as a phage cocktail to inhibit the growth of *C. sakazakii* in different brands of infant formula, while also assessing the phages ability to prevent biofilm formation. All three phages, isolated from slurry, possess a relatively broad host range, verified by their ability to infect across genera and species. When all three phages were combined and used as part of a phage cocktail, 73% coverage was obtained across all *Cronobacter* strains tested. Optimum thermo-tolerance and pH stability were determined between 4 °C–37 °C, and pH 6–8, respectively, well within the normal range of application of infant formula. Genome sequencing and analysis revealed all the phages to be free from lysogenic properties, a trait which renders each favourable for phage therapy applications. As such, the combined-phage preparation (3×10^8 pfu/mL) was found to possess a strong bactericidal effect on *C. sakazakii*/*C. sakazakii* LUX cells ($\leq 10^4$ cfu/mL), resulting in a significant reduction in cell numbers, to below the limit of detection (< 10 cfu/mL). This was observed following a 20 h challenge in different brands of infant formula, where samples in the absence of the phage cocktail reached concentrations of $\sim 10^9$ cfu/mL. The phage cocktail also demonstrated promise in preventing the establishment of biofilm, as biofilm formation could not be detected for up to 48 h post treatment. These results highlight the potential application of this phage preparation for biocontrol of *C. sakazakii* contamination in reconstituted infant formula and also as a preventative agent against biofilm formation.

1. Introduction

Cronobacter spp. (formally *Enterobacter sakazakii*) consists of a diverse group of Gram-negative, facultatively anaerobic, motile bacilli belonging to the *Enterobacteriaceae* family (Iversen et al., 2007). The genus comprises seven species: *Cronobacter sakazakii, Cronobacter malonaticus, Cronobacter turicensis, Cronobacter universalis, Cronobacter muytjensi, Cronobacter dublinensis* and *Cronobacter condiment* (Brady et al., 2013; Joseph et al., 2012). In recent years, *C. sakazakii* has gained significant attention as an emerging food-borne pathogen due to the associated link between infectious disease and the consumption of contaminated foods, in particular, reconstituted infant milk formula. While *C. sakazakii* is responsible for causing severe clinical infections in immunocompromised individuals of all ages, it is pre-term, low-birth weight infants who are most at risk (FAO/WHO 2008; Healy et al., 2010). Clinical symptoms of infection in infants include meningitis, bacteraemia and severe forms of necrotising enterocolitis, with case fatality rates ranging between 40 and 80% (Friedemann, 2009). These high mortality rates and the fact that many survivors are very often left with chronic neurological and developmental disorders, highlights the damaging effect this organism has on infant health (Forsythe, 2005; Lai, 2001). Accordingly, The International Commission for Microbiological Specifications for Foods has ranked *C. sakazakii* as a "Severe hazard for restricted populations, life threatening or substantial chronic sequelae

* Corresponding author at: Department of Biological Sciences, Cork Institute of Technology, Cork, Ireland.
E-mail addresses: lorraine.endersen@mycit.ie (L. Endersen), colin.buttimer@mycit.ie (C. Buttimer), eoghan.nevin@mycit.ie (E. Nevin), aidan.coffey@cit.ie (A. Coffey), horst.neve@mri.bund.de (H. Neve), hugooliveira@deb.uminho.pt (H. Oliveira), rob.lavigne@kuleuven.be (R. Lavigne), jim.omahony@cit.ie (J. O'Mahony).

http://dx.doi.org/10.1016/j.ijfoodmicro.2017.04.009
Received 6 January 2017; Received in revised form 13 April 2017; Accepted 18 April 2017
Available online 21 April 2017

of long duration", placing the organism in the same category as *Clostridium botulinum*, *Cryptosporidium parvum* and *Listeria monocytogenes* (types A and B) (ICMSF, 2002).

C. sakazakii is ubiquitous in nature with many studies indicating that plant material is its primary niche (in particular vegetables, fruits, cereals, wheat, rice, herbs and spices). However, other more pertinent sources also found to harbour this pathogen include powdered infant formula (PIF) and milk powder manufacturing environments (Friedemann, 2007; Kandhai et al., 2004).

C. sakazakii possess physiological traits which affords its ability to survive in such environments and thus permit PIF to serve as a prime vehicle for transmission to the immunocompromised infant. These traits include, (1) resistance to desiccation and osmotic stress (Breeuwer et al., 2003), (2) an extended temperature growth range (Iversen and Forsythe, 2004), (3) thermo-tolerance compared to other *Enterobacteriaceae* found in PIF (Nazarowec-White and Farber, 1997a, 1997b), and (4) the ability to form biofilms on a range of different materials including polycarbonate which is often used to make babies bottles (Iversen and Forsythe, 2004).

PIF is not manufactured as a sterile preparation and hence can become contaminated with *C. sakazakii* during production. Although the organism is effectively inactivated during pasteurisation (Nazarowec-White and Farber, 1997a, 1997b), contamination is likely to occur from the addition of non-sterile ingredients during manufacture. Indeed, it has been suggested that PIF ingredients originating from plant material, which have not been heat treated are a potential source of *C. sakazakii* contamination (Healy et al., 2010). Other possible sources of contamination include the use of non-sterile equipment during processing or reconstitution, and from temperature abuse of the reconstituted formula itself (Al-Nabulsi et al., 2009).

Capsular polysaccharides on the outer surface of *C. sakazakii* cells play a central role in biofilm formation, giving the organism the ability to attach and colonise a variety of surfaces including stainless steel, glass, latex, polycarbonate, silicon and polyvinyl chloride (PVC) (Iversen et al., 2004). The bacterial cells embedded in a matrix of exopolymeric substances, are physiologically distinct from their planktonic counterparts. These cells demonstrate changes in growth rate and gene transcription and often exhibit a significantly higher tolerance to antibiotics ($\leq$1000 times higher) and other sanitising agents. The presence of persister cells, the reduced metabolic activity present in the inner layers of the biofilm and the decreased penetration of antibiotics through the exopolymeric matrix, all contribute to this increased resistance (Donlan and Costerton, 2002; Keren et al., 2004). As a result, complete elimination is very often compromised and re-infection commonly occurs.

Consequently, the microbiological safety of PIF is under continuous scrutiny as a result of contaminating *C. sakazakii* and its epidemiological link with life-threatening neonatal infections (Forsythe, 2005; Himelright, 2002; Hunter and Bean, 2013). The destructive economic impact the pathogen has on healthcare systems and PIF production facilities due to contaminated product recalls is also apparent (Chenu and Cox, 2009). Indeed, there is a growing requirement for the development of new and effective mechanisms to further prevent *C. sakazakii* contamination in both food, and the food processing environment.

Bacteriophages (phages) and their derivatives are well recognised for their antibacterial properties, demonstrating promise as natural, safe and effective alternatives for the prevention, treatment and/or eradication of foodborne pathogens in a range of different foods and food processing environments. These include, decontamination of livestock, sanitation of contact surfaces and equipment, in addition to biocontrol of raw meats, fresh foods and vegetables (Endersen et al., 2014; Goodridge and Bisha, 2011), cheese (Carlton et al., 2005), ready-to-eat (RTE) foods (Bigot et al., 2011), skim milk (Ellis et al., 1973; Endersen et al., 2013), and reconstituted infant formula (Kim et al., 2007), all of which demonstrate their applicability for use at each stage of the food production process. ListShield™, approved for use in the US in 2006 as a processing aid to control *Listeria monocytogenes* in meat and poultry products, marked the arrival of the first phage-based product to the commercial marketplace in the Western world (Bren, 2006). Following this significant development, several phage related products have been approved for use in the US, including preparations active against the prominent foodborne pathogens, *E. coli* O157:H7, *Salmonella*, and additional preparations against L. *monocytogenes* (Endersen et al., 2014; Goodridge and Bisha, 2011). The pioneering anti-listeria phage-based preparation is now registered in Europe as an organic food additive and has also been approved for use as a food processing aid by the Food Standards Australia & New Zealand, highlighting the fact that phage-based preparations are continuing to gain global acceptance as safe and effective alternatives for the biocontrol of harmful foodborne pathogens (Fsanz, 2012; Hodgson, 2013).

Here we report the isolation and characterisation of three phages against the opportunistic, foodborne, infant formula pathogen, *C. sakazakii*. In addition, we demonstrate the efficacy of the phages, used as part of a phage cocktail, at inhibiting the growth of *C. sakazakii* in four different brands of reconstituted infant milk formula while also assessing the phages ability to prevent biofilm formation.

2. Materials and methods

2.1. Bacterial strains and growth media

The following strains were used in this study: *C. sakazakii* ATCC BAA 894, *C. sakazakii* ATCC BAA 894 LUX, *C. sakazakii* DPC 6258, *C. muytjensi* ATCC 51329, *C. sakazakii* ATCC 29004, and *C. sakazakii* DPC 8155. These were sourced from the Dairy Products Research Centre, DPC, Moorepark, Fermoy, Co. Cork, Ireland. Strains were stored at − 80 °C and routinely grown on Luria-Bertani (LB) agar, in LB broth and in some cases supplemented with 1% or 2.5% D-(+)-glucose at 37 °C. HiChrome™ *Cronobacter* spp. Agar, Modified (14,763, Sigma Aldrich) was used for selective growth of *C. sakazakii* when necessary. Selective growth of *C. sakazakii* ATCC BAA 894 LUX was achieved by the addition of 500 μg/mL erythromycin to LB broth/agar.

2.2. Isolation of phages

Phages were isolated from slurry, sourced from a cattle farmer in Clonakilty, West Cork, Ireland, using methods described previously (Carlson, 2005). Briefly, each sample (5 mL) was added to equal volumes of LB broth (Sigma Aldrich, UK), supplemented with 10 mM $CaCl_2$ (Sigma Aldrich) and inoculated with a mid-log phase *C. sakazakii* ATCC BAA 894 culture. Samples were incubated overnight at 37 °C with shaking. Samples were centrifuged at 4000*g* for 15 min to pellet cells and debris. The supernatant was filtered through a 0.45 μM filter and the filtrate was re-enriched with mid-log phage *C. sakazakii* culture two more times. The supernatant obtained from the final enrichment step was filter-sterilised and tested for the presence of viable infective phages by adding 100 μL of the filtrate to 100 μL of early log phase *C. sakazakii* cells in a 5 mL LB 0.4% w/v overlay agar tube tempered to 50 °C and subsequently poured onto the surface of a LB 1.5% w/v agar plate. Plates were incubated at 37 °C overnight and then examined for phage plaques.

2.3. Phage purification and amplification

Presumptive phages were purified by successive single plaque isolation and were routinely propagated on *C. sakazakii* ATCC BAA 894 as previously described (O'Flaherty et al., 2005). Briefly, a single isolated plaque was aseptically picked from a lawn of *C. sakazakii* ATCC BAA 894 on LB agar using a sterile capillary tube and added to 100 μL of early-log phase culture. The sample was incubated at 37 °C overnight. The resulting lysate was centrifuged, filter sterilised and serially