International Journal of Food Microbiology 136 (2009) 221–226

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Influence of desiccation on the sensitivity of *Cronobacter* spp. to lactoferrin or nisin in broth and powdered infant formula

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ARTICLE INFO

Keywords:
Lactoferrin
Nisin
Cronobacter spp.
Infant milk formula
Bactericidal action
Natural antimicrobial

ABSTRACT

Although outbreaks caused by *Cronobacter* spp. (*Enterobacter sakazakii*) are rare, infections by this organism have a case-fatality rate which may reach 80%. Powdered infant milk formula (PIMF) is considered a major source for human infection with *Cronobacter* spp. The organism has the capability to survive in dry environments for long periods (~2 years). Current interest in the use of natural antimicrobials including lactoferrin (LF) and nisin has developed because of the desire for preservative-free food products. The objective of the present study was to evaluate the antimicrobial activity of bovine LF or nisin against undesiccated and desiccated *Cronobacter* spp. cells in 0.2% peptone water (PW) and reconstituted PIMF at different temperatures. In 0.2% PW, 2.5 mg/ml LF was able to inactivate 4 $\log_{10}$ CFU/ml of undesiccated cells of *Cronobacter* spp. in 4 h at 37 °C but at lower temperatures, higher concentrations of LF as well as longer exposure were needed to achieve the same effect as at 37 °C. Similarly, the effect of nisin against undesiccated cells of *Cronobacter* spp. was concentration and temperature dependent in 0.2% PW. It was found that 1500 IU/ml caused a 4 $\log_{10}$ CFU/ml reduction of undesiccated cells of *Cronobacter* spp. at 21 °C and 37 °C. Desiccated *Cronobacter* spp. cells in 0.2% PW were more sensitive to LF action than were undesiccated cells. A 4 $\log_{10}$ CFU/ml reduction was obtained with 2.5 mg/ml LF after 1 h at 21 and 37 °C or 8 h at 10 °C. In contrast, desiccated cells of *Cronobacter* spp. were more resistant to nisin. Furthermore, neither LF nor nisin had detectable antimicrobial activity against desiccated or undesiccated *Cronobacter* spp. in reconstituted PIFM. Heating at 55 °C for 5 min with nisin in reconstituted PIFM did not enhance the antimicrobial activity of nisin. Unexpectedly, nisin appeared to protect *Cronobacter* spp. from the damaging effects of heat treatment. The reduced antimicrobial activity of LF and nisin in reconstituted PIMF was potentially explained by the higher concentration of Ca^{2+}, Mg^{2+} and Fe^{3+} in the latter.

1. Introduction

Cronobacter (*Enterobacter sakazakii*) is an emerging foodborne pathogen that has been associated with meningitis (Burdette and Santos, 2000), sepsis (Simmons et al., 1989), bacteremia (Noriega et al., 1990), and necrotizing enterocolitis (Van Acker et al., 2001) in preterm neonates and immunocompromised adults (Lai, 2001). Recently, Iversen et al. (2008) reclassified *E. sakazakii* as a new genus, *Cronobacter*, comprising five spp.; *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii* and *C. dublinensis*. Although illness outbreaks caused by this pathogen are rare, *C. sakazakii* infections are usually accompanied by a high case-fatality rate that may reach 80% (Lehner and Stephan, 2004; Kim and Beuchat, 2005). Powdered infant milk formula (PIMF) has been associated primarily with most of the outbreaks caused by *Cronobacter* spp. This bacterium is usually inactivated during pasteurization of PIFM (Nazarowec-White and Farber, 1997a). Therefore, the presence of *Cronobacter* spp. in PIMF is mainly due to post-processing environmental contamination, the addition of contaminated ingredients during powder production (Nazarowec-White and Farber, 1997b) or is due to colonization by *C. sakazakii* of utensils such as bottles, brushes and spoons used in PIMF preparation. *C. sakazakii* has been shown to have a remarkable capacity to survive in dry environments for periods of ≤2 years (Caubilla-Barron and Forsythe, 2007). This characteristic represents a competitive advantage, facilitating its prevalence in dry environments such as PIFM (Edelson-Mammel et al., 2005). *Cronobacter* spp. is capable of accumulating compatible solutes such as trehalose that protect the organism against osmotic stress by stabilizing membrane phospholipids and proteins (Breeuwer et al., 2003). Elimination of *Cronobacter* spp. at PIMF reconstitution represents a final opportunity to prevent illness.

Heat treatment during reconstitution of PIMF at ≥70 °C before feeding has been recommended by FAO and WHO (2004). However,

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doi:10.1016/j.ijfoodmicro.2009.08.008

this treatment could negatively affect its organoleptic quality and nutritive value. It should be possible to reduce the heat treatment required to reduce the numbers of *Cronobacter* cells in line with the WHO recommendations if the treatment were combined with a secondary antimicrobial. Recently, interest has arisen in the possible use of natural antimicrobials such as lactoferrin and nisin because of the demand for preservative-free food products (Payne et al., 1990; Paulsson et al., 1993; Al-Holy et al., 2004a).

Lactoferrin (LF), the main iron-binding glycoprotein present in the milk of mammals, inhibits the growth of many bacteria, fungi and parasites (Farnaud and Evans, 2003). The LF concentration in human milk is >2 mg/ml, and it is present at levels ranging from 0.02 to 0.20 mg/ml in bovine milk (Shimazaki, 2000). LF also has antioxidant, antiviral, anti-inflammatory, immune-modulating as well as anti-cancer activities, and can promote the growth of probiotic bacteria such as *Bifidobacterium* (Aguila and Brock, 2001; Al-Nabulsi and Holley, 2007). LF has been shown to be bacteriostatic due to its ability to bind iron and limit its availability in the growth environment (Orsi, 2004). Additionally, binding of LF to the surface of Gram-negative bacteria initiates bactericidal effects by releasing lipopolysaccharide (LPS) from the membrane (Ellison et al., 1988).

Nisin is a heat-stable bacteriocin produced by certain strains of *Lactococcus lactis*. The Joint Expert Committee on Food Additives, the U. S. Food and Drug Administration, and the European Union recognize nisin to be safe for food use (Budu-Amoako et al., 1999). Nisin is primarily active against Gram-positive bacteria including *Listeria*, *Clostridium*, *Bacillus* and *Staphylococcus* spp. (Ray and Daeschel, 1994). It is generally accepted that the underlying mode of action of nisin is through disruption of membrane function which is caused by pore formation in the membrane. This is followed by leakage of intracellular material (Winkowski et al., 1996; Ueckert et al., 1998). In contrast, Gram-negative bacteria are resistant to nisin unless treated to allow nisin access to the inner cell membrane by altering permeability of the outer cell membrane. Sublethal heat, freezing, chelating agents, high pressure, organic acids and their derivatives, as well as lysozyme have been used successfully with nisin against these organisms. Branen and Davidson (2004) reported that nisin in combination with EDTA synergistically inhibited enterohemorrhagic *Escherichia coli* strains in Tryptic Soy Broth (TSB). EDTA may enhance nisin activity by releasing lipopolysaccharides from the outer membrane and subsequently increasing its permeability. Recently, Lee and Jin (2008) reported the synergistic interaction of diacetyl with nisin in inhibiting the growth of *Cronobacter* spp in TSB. Furthermore, growth inhibition of *Salmonella enteritidis* PT4 and *S. enteritidis* PT7 by nisin was enhanced with heating at 55 °C for 10 min in nutrient broth (Boziaris et al., 2001).

The objective of the present study was to evaluate the antimicrobial activity of bovine LF or nisin on *Cronobacter* spp. in peptone water and reconstituted PIMF (RIMF) at different temperatures. Additionally, the combined effect of nisin and mild heat treatment on the growth of *Cronobacter* spp. in reconstituted PIMF was investigated.

2. Materials and methods

2.1. Preparation of culture

Five different *Cronobacter* strains were used in the present study, including *C. muytjensii* ATCC 51329 (formally known as *E. sakazakii*), and four food isolates obtained from the Department of Nutrition and Food Technology at Jordan University of Science and Technology (Shaker et al., 2007); IMF1 and IMF2 (infant milk formula isolates), IFF1 (infant food formula isolate), and CW1 (crushed wheat isolate). All *Cronobacter* isolates were kept individually at −40 °C in Brain Heart Infusion (BHI, Difco, Becton Dickinson, Sparks, MD, USA) containing 20% glycerol. They were maintained at 4 °C on Tryptic Soy Agar (TSA, Difco) slants. Three culture transfers were performed to resuscitate each culture and these were then transferred individually to BHI broth prior to the experiment and grown for 24 h at 37 °C (to reach the stationary phase). One ml of each culture was pooled in a 10 ml test tube and mixed to obtain an approximately equal number of cells of each strain in the mixed culture. The mixed (cocktail) culture was diluted using the test medium (peptone or reconstituted milk powder) to give a final concentration of about 4 $\log_{10}$ CFU/ml.

2.2. Preparation of antimicrobials

Bovine lactoferrin (Bioferrin 1000) was obtained from Glanbia Nutritionals (Glanbia Ingredients Inc., Monroe, WI, USA). Stock solutions of LF were prepared in distilled water to give final concentrations of 2.5, 5.0, 10.0, 15.0, 60.0 and 75.0 mg/ml in the test media, sterilized using 0.22 µm syringe filters (Fisher Scientific, Fair Lawn, NJ, USA), and then added to heat-sterilized growth media. The iron saturation of LF was 4 mg/100 g as determined by Glanbia which is less than in its normal physiological state (15–20% saturated) (Steijns and van Hooijdonk, 2000).

A stock solution of 10^4 IU/ml nisin (Danesco, Denmark) was prepared by dissolving 0.769 g of nisin in 50 ml of 0.02N HCl and boiled for 8 min. The stock solution was diluted to give final concentrations of 0, 500, 1000, 1500, 2000 IU/ml in the test medium. The stock solution was kept at −18 °C between use.

2.3. Preparation of desiccated cells of Cronobacter spp.

Desiccated cells of the *Cronobacter* spp. cocktail were prepared as described by Osaili et al. (2007). One ml of freshly prepared *Cronobacter* spp. cocktail was dispensed in 50 µl portions on the bottom of a sterile petri dish. The plate was stored without a lid in a 40 °C incubator to dry over dehydrated silica gel. After drying (<2 h), the plate was covered and kept at 21 °C for 4d. Preliminary study showed that the drying and storage times used in the current study decreased the initial number of cells by 1 log and ≤1 log/g, respectively.

Thereafter, 2 ml of 0.2% (w/v) peptone water was added to the plate to collect desiccated cells, mixed with 8 ml 0.2% peptone water, and serial decimal dilutions in peptone water were used to yield 4 $\log_{10}$ CFU/ml of *Cronobacter* spp. For reconstituted PIMF, the five isolates of *Cronobacter* spp. were individually desiccated in dry infant milk formula as described by Osaili et al. (2007) with minor modifications. Approximately 0.5 ml of each freshly grown culture was sprayed on 100 g of dry PIMF and spread evenly on the bottom of a sterile 50 cm diameter stainless steel mixing bowl. Spraying of the sample was performed using a chromatography reagent sprayer (model 422530-005, Kontes Glass Company, Vineland, N.J.) with nitrogen gas as carrier at 2 lb/in^2. The formula was mixed with a sterile spatula between applications of each of the inoculum cultures and then was sieved using a sterile 0.5 mm diameter pore size sieve. After incubation at room temperature for one month, an equal weight of each inoculated PIMF was mixed and then 9 g was dissolved in 60 ml sterile distilled water to yield an initial level of *Cronobacter* spp. of 4 $\log_{10}$ CFU/ml.

2.4. Test media

Peptone water (0.2% w/v, Difco) and PIMF were used to assess the antimicrobial activity of LF or nisin. Commercial PIMF cans containing 450 g powder (56.6% carbohydrate, 11.4% protein, and 25.4% fat) were obtained from a local processor. The formula was screened and found to be free of *Cronobacter* spp. according to the method described by Iversen et al. (2004). In addition, no viable organisms were recovered following plating of PIMF on violet red bile glucose agar (VRBGA, Difco). A total of 9 g of PIMF was transferred to sterile150 ml capacity flasks and reconstituted, based on the manufacturer's recommendation, at a ratio of 1:7.6, with 60 ml sterile water.