Evaluation of an Antimicrobial Ingredient Prepared from a Lactobacillus acidophilus Casein Fermentate against Enterobacter sakazakii

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ABSTRACT

Previously two antimicrobial peptides, IKHQGLPQE (casein A) and VLNENLLR (casein B), were identified following the fermentation of sodium caseinate with the proteolytic strain Lactobacillus acidophilus DPC 6026. This study evaluated the ability of these peptides to kill Enterobacter sakazakii ATCC 12868 spiked in reconstituted infant formula. The survival of E. sakazakii populations in reconstituted infant formula containing a sodium caseinate fermentate was compared with survival in formula containing positive (monocaprylin) and negative controls. The L. acidophilus DPC 6026 sodium caseinate fermentate reduced pathogen numbers by >4 log CFU/ml at 37°C, comparing favorably with the activity of monocaprylin. Additionally, E. sakazakii NCTC 8155 was inoculated into pasteurized, reconstituted infant formula (6 log CFU/ml) followed by the addition of increasing concentrations of the fermentate (0.21 to 6.7% [wt/vol]). At a concentration of 0.21% (wt/vol), pathogen viability was maintained over 4 h at 6.0 log CFU/ml. In contrast, pathogen numbers increased approximately 100-fold in the control formula in the same time frame. At higher final fermentate concentrations (≥3.33% [wt/vol]), numbers were reduced to 0 log CFU/ml over 60 min. The spectrum of activity of the fermentate against other foodborne pathogens was also determined and shown to be effective against Escherichia coli O157:H7 and Listeria innocua. Results indicate the potential of this fermentate as a built-in protection mechanism against E. sakazakii strains in reconstituted infant formula.

Enterobacter sakazakii has been associated with sporadic cases of sepsis, meningitis, cerebritis, and necrotizing enterocolitis (7). Little information, however, is available concerning its natural environmental habitat, taxonomy, and virulence factors (18). Diseases caused by E. sakazakii infection have been reported for almost every age group. However, according to the Food and Agricultural Organization of the United Nations and the World Health Organization (WHO) (32) the bacterium has been implicated most frequently as the causative agent of illness in children from 3 days to 4 years of age, and at least 76 cases of E. sakazakii infections and 19 E. sakazakii–related deaths in children have been reported (9, 13). Among infants, those at greatest risk of E. sakazakii infection include preterm infants (born at <37 weeks of gestation) and low-birthweight infants (<2,500 g). Mortality rates of 20 to 50% have been reported for patients who contract E. sakazakii–related diseases, and survivors often suffer severe neurological disorders such as hydrocephalus, quadriplegia, and retarded neural development (17).

A number of reports have implicated powdered infant formula as the source and vehicle of E. sakazakii infection (1, 3, 9, 30). In this respect, it is important to emphasize that dried powdered infant formula is a nonsterile product, unlike sterile commercially available liquid feeds. While generally the levels of E. sakazakii in dry powdered infant formula are very low (0.36 to 66 CFU/100 g (20)), the reconstituted formula provides an ideal medium for growth and multiplication, and when present, E. sakazakii can multiply during preparation, cooling, storage, and holding of the bottles (18). At room temperature (21°C) the microorganism has a doubling time of approximately 75 min in reconstituted infant formula (14). E. sakazakii is considered an opportunistic pathogen and possesses many characteristics that enable it to survive in infant formula and behave as a pathogen (10, 16, 28). Additionally, a study that evaluated the thermal resistance of pooled E. sakazakii isolates in reconstituted infant formula reported that the microorganism was among the most thermotolerant members of the family Enterobacteriaceae (23). The study reported that at 68°C, E. sakazakii was found to be more thermotolerant than Listeria monocytogenes (23, 24). A further study evaluated the thermostolerance of E. sakazakii type strain NCTC 11467 and concluded that its D-value at 58°C was 2.6 min (15).

E. sakazakii can grow at temperatures as low as 5.5°C, i.e., at abused refrigeration temperature (22), and exhibits substantial resistance to acid pH (5, 9). Furthermore, E. sakazakii is unusual in its ability to survive osmotic stress and desiccation, enabling the strain to survive the low water activity (a_w, 0.2) environment of dried infant formula. Breeuwer et al. (2) reported that the osmotic stress resistance of

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E. sakazakii at an a₉₀ of 0.934 and 25°C was greater than that of Escherichia coli. Salmonella Senftenberg, Salmonella Typhimurium, and Salmonella Enteritidis. Moreover, some strains of E. sakazakii produce capsules that contribute to macrophage invasion and may protect the microorganism in desiccated environments and during gastrointestinal transit. It has been reported that the minimum lethal dose of E. sakazakii in neonates was far higher than the numbers normally detected in contaminated infant formula; however, levels of >10⁵ CFU/ml could occur if reconstituted infant formula was held at an incorrect temperature over time due to the short lag time and generation time of the organism in reconstituted infant formula (22).

Increased antibiotic resistance among Enterobacter species has resulted in expanding interest in the use of alternate natural antimicrobials such as antimicrobial peptides in the treatment of bacterial infections. Antimicrobial peptides constitute a primitive immune defense mechanism, are generally less than 10 kDa in mass, are hydrophobic, and are membrane active (27). They can be generated through fermentation and proteolytic digestion of several proteins, including the milk proteins casein and whey (8). Additionally, antimicrobial peptides derived from milk proteins could provide a built-in safeguard against E. sakazakii contamination in powdered infant formula.

The aim of this study was to evaluate the efficacy of a filtered (≤3-kDa) L. acidophilus DPC 6026 sodium caseinate fermentate containing the peptides IKHQGLPQE (casein A) and VLLENLLR (casein B) (12) in protecting infant formula against the pathogen E. sakazakii at 37°C and the abused refrigeration temperature of 6°C.

### MATERIALS AND METHODS

**Bacterial strains and culture conditions.** E. sakazakii NCTC 8155, originally isolated by Thornley from dried milk (29), and E. sakazakii ATCC 12868 were obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen AB24 3RY, Scotland, and propagated in brain heart infusion (BHI) broth at 37°C with agitation. Test strains used to determine the spectrum of inhibition are listed in Table 1. Lactobacillus acidophilus DPC 6026 was originally isolated from the porcine small intestine and stocked in the culture collection of Teagasc, Moorepark Food Research Centre, Co. Cork, Ireland (11). This strain was propagated in deMan Rogosa Sharpe (MRS) broth (Oxoid Ltd, Basingstoke, UK) anaerobically using Anaerocult A gas packs, in accordance with the manufacturer’s instructions (Merck, Darmstadt, Germany), for 24 h at 37°C. Standard cultures were prepared by inoculation of 10 ml of MRS broth with 10 µl of a frozen stock (−80°C) and then incubated at 37°C for 16 to 24 h. E. sakazakii strains were then subcultured in 10 ml of BHI broth for 16 to 24 h at 37°C prior to inoculation into the fermentation vessels.

**Fermentation of sodium caseinate with L. acidophilus DPC 6026.** Three separate 5-liter fermentations were performed, using sodium caseinate as the substrate (2.5% [wt/vol]). Each of these was inoculated (1% [vol/vol]) with an overnight (17 h) L. acidophilus DPC 6026 culture and incubated at 37°C for 24 h (agitated at 100 rpm) at a constant pH of 7, maintained via addition of 0.1 M NaOH. The fermentates were then heated to 80°C for 15 min in a water bath to inactivate the cultures and were subsequently filtered through a size-exclusion 3-kDa spiral cartridge filter (Millipore Ltd., Hertfordshire, UK). The resultant permeate containing peptides of ≤3kDa was then freeze-dried. Following freeze-drying, a yield of 14.54 ± 0.7 g resulted from the 5-liter fermentate, representing a yield of 12%, and this was stored at −20°C.

Aliquots of the freeze-dried powder were redissolved in distilled high-pressure liquid chromatography (HPLC) grade water and filtered through a 0.45-µm-pore-size filter (Millipore), and 30 mg/ml was loaded onto a column for reverse-phase (RP) HPLC separation of peptide fractions. Peptides were then separated from sodium caseinate hydrolysates by using an RP-HPLC system containing a narrow-bore column (5 by 250 mm; Nucleosil C18, Varian Chromatography Systems, Walnut Creek, CA) and a UV detector operating at 214 nm. The mobile phase was a binary mix-
ture of acetonitrile and HPLC grade water (99.9% [vol/vol]) containing trifluoroacetic acid (0.1% [vol/vol]). The content of acetonitrile in the mobile phase was increased linearly from 0 to 100% for 72 min at a flow rate of 1 ml/min. Peptides were detected using a detector operating at a wavelength of 214 nm. Solvents were removed from the collected fractions by evaporation using a CentriVap console (Labconco Corporation, Kansas City, MO). The fractions were redissolved in 1 ml of distilled water, prior to subsequent analysis for antimicrobial activity against the indicator strain E. sakazakii ATCC 12868 by use of a well diffusion assay as described previously (11), to verify the presence of the peptides in the 3-kDa permeate filtrate. The peptide molecular masses in the collected fractions were analyzed by mass spectrometry as previously described (4).

**Determination of antibacterial activity in infant formula.**
The filtered fermentate was reconstituted (0.5 g/ml of double-distilled water) (50% [wt/vol]). The lipid 1-caprylyl-1-glycerol monocaprylin (a monoglyceride ester of caprylic acid) was purchased from Sigma Chemicals (Dublin, Ireland) and used as a positive control. The lipid was dissolved in 100% ethanol to give a final concentration of 50 mM (1%) monocaprylin as previously described (21). Monocaprylin was added to the formula after addition of the E. sakazakii ATCC 12868 or NCTC 8155 culture to the desired level.

A commercially available brand of infant milk composed of reduced-minerals whey, vegetable oils, skim milk powder, lactose, emulsifiers, calcium chloride, potassium bicarbonate, sodium citrate, and vitamins A, C, D, E, B<sub>6</sub>, B<sub>12</sub>, K, biotin, and pantothenic acid was purchased. This formula was then prepared according to the manufacturer’s recommendations as follows: 25.5 g of infant formula was reconstituted in 180 ml of sterile, distilled water and pasteurized at 63°C for 30 min in a water bath and subsequently cooled to room temperature. Following pasteurization, 6-ml volumes of the reconstituted infant formula were dispensed into 30-ml polypropylene tubes. The sodium caseinate L. acidophilus DPC 6026 filtrate (50% [wt/vol] stock) was added to the infant formula at a concentration of 6.66% (wt/vol) after addition of E. sakazakii ATCC 12868.

E. sakazakii ATCC 12868 was cultured in triplicate in 10 ml of sterile BHI in 30-ml screw-cap tubes at 37°C for 20 h with agitation (150 rpm). Following incubation, the culture was sedimented by centrifugation at 8,000 × g for 10 min, washed twice in phosphate-buffered saline (PBS 7.2), and resuspended in 2 ml of the same. Cell numbers of the culture were determined by plating 100 µl of serially diluted culture on triplicate BHI agar plates with aerobic incubation at 37°C for 240 min. E. sakazakii NCTC 8155 was also separately added to the pasteurized milk samples to give an inoculation level of approximately 10.5 log CFU/ml. The inoculated samples were then incubated at 6°C (abused refrigeration temperature), and the test fermentate (6.66 [wt/vol]) and positive and negative controls were added 30 min after inoculating the pathogen to allow time for the pathogen to establish itself in the infant formula.

E. sakazakii NCTC 8155 was also separately added to the pasteurized milk samples (prepared as described above) to give an inoculation level of approximately 10.5 log CFU/ml. The inoculated samples were then incubated at 37°C. The test fermentate (6.66, 3.33, 0.83, and 0.208% [wt/vol]) and positive and negative controls were added after 30 min, and the surviving populations of the pathogen were then counted at 0, 40, 70, 160, and 360 min for samples by plating 1:10 PBS serial dilutions on duplicate BHI agar plates. E. sakazakii colonies were identified on BHI plates based on their morphological appearance, i.e., large, round, smooth, slightly raised, glossy, and yellowish brown colonies. Duplicate samples were assayed for each treatment and control at each concentration at each specified sampling time, and the study was replicated three times. Analysis of the scientific data was carried out using the basic statistical software Excel.

**Determination of spectrum of antibacterial activity.** A well diffusion assay was used to detect antibacterial activity of the 3-kDa fermentate at concentrations within the range of 3.2 to 50% (wt/vol) (12). Briefly, this assay was performed in either BHI or Luria-Bertani agar (200 ml) seeded individually with 0.5 ml of an overnight culture of the indicator strains E. coli DPC 6053, L. innocua DPC 3306, E. sakazakii 5920 (ATCC 12868), or E. sakazakii 8272 (NCTC 8155) and other strains listed in Table 1. Wells, 4.6 mm in diameter, were cut into agar plates, and 30 µl of the 3-kDa L. acidophilus DPC 6026 sodium caseinate fermentate (3.2 to 50% [wt/vol]) was placed in each well. Plates were stored at 4°C for 4 h to permit radial diffusion of the fermentate fraction, incubated at 37°C aerobically for 24 h, and examined for zones of inhibition. The sensitivity of a strain to the peptides was scored according to the diameter of the zone of inhibition surrounding the well. The experiments were performed in triplicate, and mean zone sizes were calculated. The antimicrobial peptide cecropin P1 (Sigma Aldrich Chemie, Steinheim, Germany) and unfermented casein at concentrations ranging from 3.2 to 50% (wt/vol) were used as reference positive and negative controls, respectively.

**RESULTS**

The aim of this study was to confirm that an L. acidophilus DPC 6026 fermentation of sodium caseinate (pH 7), which was previously shown to produce the antimicrobial peptides IKHQGLPQE (casein A) and VLLENLRR (casein A) (11), contained these antibacterial peptides following upscaling of the fermentation process from laboratory scale (300 ml) to 5-liter scale. Antimicrobial peptides are generally <3 kDa, and as IKHQGLPQE and VLLENLRR had previously calculated molecular masses of 1,049.177 and 970.119, respectively, the fermentation was filtered through a 3-kDa spiral cartridge filter to concentrate these peptides. RP-HPLC was used to separate the 3-kDa permeate into 72 fractions, which were subsequently analyzed for antimicrobial activity against the indicator strain E. sakazakii NCTC 8155. The RP-HPLC chromatogram generated from the 3-kDa fermentate and the antibacterial effect of the 3-kDa L. acidophilus DPC6026 sodium caseinate fermentate on E. sakazakii NCTC 8155 are shown in Figure 1. Fractions 45 and 54, containing VLLENLRR and IKHQGLPQE, respectively (mass spectrometry data not shown), both produced zones of inhibition approximately 1.5 cm in diameter when tested for antimicrobial activity against E. sakazakii NCTC 8155, as shown in the inset of Figure 1 as reported previously (11).

The survival of E. sakazakii ATCC 12868 in rehydrated infant formula following addition of the L. acidophilus DPC 6026 ultrafiltered sodium caseinate fermentate was additionally assessed. The effect of the 3-kDa L. acidophilus DPC 6026 sodium caseinate fermentate filtrate (final volume in infant formula, 6.66% [wt/vol]) on E. sakazakii ATCC 12868 in reconstituted infant formula at 37°C is shown in Figure 2A. The mean population of E. sakazakii
ATCC 12868 in the treatment and control samples at 0 h was approximately 7.5 log CFU/ml. Following 30 min of incubation, the *L. acidophilus* DPC 6026 3-kDa sodium caseinate fermentate was added to the test sample. In the presence of 6.66% (wt/vol) of the sodium caseinate 3-kDa fermentate filtrate, *E. sakazakii* numbers were reduced to approximately 4.5 log CFU/ml within 120 min. In contrast, *E. sakazakii* ATCC12868 numbers grew to approximately 8.4 log CFU/ml in the negative control during the same period of time. This killing effect compared very favorably with that obtained with 50 mM monocaprylin, which also caused a reduction in *E. sakazakii* ATCC 12868 numbers from approximately 7.5 to 3.8 log CFU/ml after 120 min. Furthermore, the *L. acidophilus* DPC 6026 3-kDa fermentate filtrate reduced *E. sakazakii* ATCC 12868 numbers to 0 CFU/ml after 270 min, in contrast with the positive control, in which *E. sakazakii* numbers remained between 8 and 9 log CFU/ml. This result also compares favorably with that obtained with 50 mM monocaprylin, which reduced *E. sakazakii* numbers to 2.0 log CFU/ml after 270 min.

The survival of *E. sakazakii* NCTC 8155 in rehydrated infant formula following addition of the 3-kDa sodium caseinate fermentate was also assessed at 6°C (Fig. 2B). The mean population of *E. sakazakii* NCTC 8155 in the treatment and control samples at 0 h was approximately 10.5 log CFU/ml. Following 30-min incubation, the *L. acidophilus* DPC 6026 3-kDa sodium caseinate fermentate was added to the test sample. In the presence of 6.66% (wt/vol) of the sodium caseinate 3-kDa fermentate filtrate, *E. sakazakii* numbers were reduced to undetectable levels (no colonies) within 60 min. In contrast, *E. sakazakii* NCTC 8155 numbers remained between 10.4 and 11 log CFU/ml in the negative control during the same period of time. Monocaprylin, used at a concentration of 50 mM, caused a reduction in *E. sakazakii* NCTC 8155 numbers from approximately 10.5 to 4.6 log CFU/ml after 360 min.

The antimicrobial effects of different concentrations of the *L. acidophilus* DPC6026 3-kDa sodium caseinate fermentate filtrate on *E. sakazakii* NCTC 8155 were also assessed (Fig. 3). At 37°C, a final concentration of 6.66% (wt/vol) of the fermentate filtrate reduced *E. sakazakii* NCTC 8155 numbers to an undetectable level after 60 min and cell numbers were retained at 0 CFU/ml after 360 min. Similarly, a final concentration of 3.33% (wt/vol) of the fermentate filtrate reduced *E. sakazakii* NCTC 8155 numbers to 0 CFU/ml after 60 min, and this was maintained after sampling at 360 min. At the lower final concentration
of 0.83% (wt/vol), _E. sakazakii_ was reduced from 6 log CFU/ml to 5.5 log CFU/ml (an approximate 0.5-log CFU/ml reduction) after incubation for 60 min. At a final concentration of 0.208% (wt/vol), the 3-kDa fermentate filtrate did not kill _E. sakazakii_ NCTC 8155 after 360 min. At a final concentration of 0.83% (wt/vol), _E. sakazakii_ NCTC 8155 in reconstituted infant formula at 37°C was maintained at 5.5 log CFU/ml, whereas _E. sakazakii_ NCTC 8155 incubated with sodium caseinate _L. acidophilus_ DPC 6026 3-kDa powder (6.66% [wt/vol], final concentration in infant formula), _E. sakazakii_ NCTC 8155 incubated with sodium caseinate _L. acidophilus_ DPC 6026 3-kDa powder (0.83% [wt/vol]), and _E. sakazakii_ NCTC 8155 incubated with sodium caseinate _L. acidophilus_ DPC6026 3-kDa powder (6.66% [wt/vol], final concentration in infant formula), _E. sakazakii_ NCTC 8155 incubated with sodium caseinate _L. acidophilus_ DPC6026 3-kDa powder (3.33% [wt/vol], final concentration in infant formula), and _E. sakazakii_ NCTC 8155 incubated with sodium caseinate _L. acidophilus_ DPC6026 3-kDa powder (3.33% [wt/vol], final concentration in infant formula), with viability of the strain maintained at 6 log CFU/ml, which still compared favorably with numbers obtained with the negative control.

In addition, the spectrum of inhibition of the _L. acidophilus_ DPC 6026 sodium caseinate fermentate was assessed against some frequently isolated infant formula organisms such as _Enterobacter cloacae_, _Pantoea agglomerans_, and others (Table 1). At concentrations between 50 and 6.25% (wt/vol), the fermentate was active against all 11 strains shown in Table 1, with the exception of the farmyard bovine mastitis isolate _Staphylococcus aureus_ DPC 5246. However, at a concentration of 3.2% (wt/vol), the fermentate was not active against either the gram-negative strains _P. agglomerans_, _E. sakazakii_ DSM 4485, _E. coli_ JM 109, _E. coli_ O157:H7 DPC 6054, _E. coli_ O157:H7 DPC 6055, or the gram-positive strains _S. aureus_ DPC 5246 and _Listeria innocua_ DPC 3306.

### DISCUSSION

Although the WHO recommends breast-feeding, in many developing countries the use of powdered infant formula is increasing due to the proportion of subpopulations consisting of human immunodeficiency virus-infected mothers and low-birth-weight infants (32). Indeed, the WHO recommends that infants of human immunodeficiency virus-positive mothers should be fed powdered infant formula where feasible (31). Conversely, in Western societies, exclusive breast-feeding for the first 6 months following birth is recommended by the WHO (31); however, in situations where mothers have difficulty lactating and where banked human milk is not always available, preterm formulas have been specifically designed and recommended for very low birth weight infants during hospitalization (26). The natural habitat of _E. sakazakii_ is currently unknown, but epidemiological studies have implicated dried infant formula as a source and vehicle of transmission (18, 21, 22, 30). Additionally, _E. sakazakii_ infection of the newborn is most probably effected through ingestion of contaminated infant formula and not through vertical transmission from the mother during birth (19). Due to the increasing antibiotic resistance of _Enterobacter_ strains and European Food Safety Authority recommendations of very low levels of _E. sakazakii_ in infant milk formula (6), there is expanding interest in the development of novel antimicrobials for addition to infant milk formula. Since it is impractical to produce sterile dried infant formula in many cases, extreme care needs to be taken to prevent formula contamination and subsequent infant infection (25). In this respect, antimicrobial peptides which can kill or inhibit _E. sakazakii_ may act as a built-in protection mechanism against such pathogens. The antimicrobial peptides IKHQGLPQE and VLNENLLR corresponding to α1-CN f (30–37) and α1-CN f (21–29), respectively, were previously isolated from a small-scale (300 ml), pH-controlled (pH 7) sodium caseinate fermentation using the proteolytic strain _L. acidophilus_ DPC 6026 (11). The present study demonstrated that casein A and casein B were present following upscaling of the fermentation process to 5 liters and 3-kDa membrane filtration. Spiral cartridge 3-kDa membrane filtration was used, as bioactive peptides are usually between 3 and 30 amino acids in size, corresponding to peptides of ≤3-kDa. The 3-kDa membrane filtered chromatogram and the antimicrobial activity of fractions 45 and 54 against _E. sakazakii_ NCTC 8155 are shown in Figure 1. Zones of inhibition of 1.5 mm in diameter were produced by both fractions.

In addition, the fermentate was shown to rapidly inactivate _E. sakazakii_ ATCC 12868 concentrated populations (8 log CFU/ml) in infant formula at 37°C, reducing populations to 4 log CFU/ml after 120 min (Fig. 2A). Additionally, the fermentate inactivated _E. sakazakii_ NCTC 8155 concentrated populations (10.5 log CFU/ml) in infant formula at 6°C, reducing pathogen numbers to undetectable levels after 60 min. These results compare favorably with the positive control used in this study, monocaprylin, used
at a concentration of 50 mM, which reduced the *E. sakazakii* ATCC 12868 population to 2.0 log CFU/ml in the positive control samples incubated at 37°C and the *E. sakazakii* NCTC 8155 numbers to 6.7 log CFU/ml in the positive control samples incubated at 6°C after 60 min. Previously, monocaprylin was documented as reducing *E. sakazakii* pathogenic strains at 25 and 50 mM concentrations by 4.0 and 5.0 log CFU/ml when samples were incubated at 4 and 23°C, respectively (21). Not surprisingly, the antimicrobial effect of the fermentate was shown to increase with increased fermentate concentration (Fig. 3). At the lower final concentration of 0.21% (wt/vol), *E. sakazakii* NCTC 8155 growth was maintained over 4 h at 6.0 log CFU/ml, but this concentration still caused an approximate 2-log CFU/ml reduction compared with the negative control (unfermented 6.66% [wt/vol] sodium caseinate fermentate). Using a final concentration of 0.83% (wt/vol) of the 3-kDa fermented powder resulted in an approximate 2.5-log CFU/ml reduction in the growth of *E. sakazakii* NCTC 8155 compared with the negative control. In addition to *E. sakazakii*, *P. agglomerans* (formerly known as *E. sakazakii*) and *E. cloacae* are also common infant milk powder contaminants and were the most frequently isolated organisms from one-half of the 141 infant milk formulae examined by Muytjens et al. from 35 different countries (20). *L. acidophilus* DPC 6026 3-kDa sodium caseinate fermentate produced zones of inhibition against all strains tested with the exception of the bovine mastitis and methicillin-resistant isolate *S. aureus* DPC 5246.

This study has indicated that the *L. acidophilus* DPC 6026 sodium caseinate fermentate reduced populations of *E. sakazakii* NCTC 8155 in infant formula stored at 37°C. Additionally, as shown from well diffusion assays, this fermentate displayed a broad spectrum of activity against both gram-positive and gram-negative infant formula pathogens such as *L. innocua* and *P. agglomerans* after addition of 30 μl of stock concentrations within the 3.2 to 50% (wt/vol) range. Consequently, the inclusion of this sodium caseinate fermentate as an antimicrobial ingredient in powdered infant formula may be a feasible approach to prevent *E. sakazakii* contamination pending sensory analysis.

The possibility of using a sodium caseinate ultrafiltered fermentate containing the antimicrobial peptides casecin A and casecin B as a built-in mechanism of protection against infant formula pathogenic strains such as *E. sakazakii* and *P. agglomerans* may provide a useful approach for enhancing the safety of milk powders. Such a fermentate could safeguard infant formula during manufacture, preparation, and storage processes when used in combination with currently used hazard analysis and critical control point procedures.

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