Control of *Cronobacter* in reconstituded infant formula by combined application of cathelicidin LL-37 and bacteriophages

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Summary

Cronobacter spp. is responsible for rare but fatal cases of infections in neonates and immunocompromised infants. The reconstituded powdered infant formula (PIF) is one of main sources of newborn infections. The aim of our study was to assess application of recombinant human cathelicidin LL-37, an antimicrobial peptide, alone and in combination with bacteriophages against *Cronobacter* in PIF. The minimal inhibitory concentration (*MIC*) of LL-37 determined by radial diffusion method for ten representative *Cronobacter* strains ranged from 2.56 $\mu g \cdot ml^{-1}$ to 8.83 $\mu g \cdot ml^{-1}$, and the values were below *MIC* of *E. coli* standard strain. By testing the antimicrobial activity in liquid growth medium, substantial inhibition of strains was observed at 30 $\mu g \cdot ml^{-1}$ LL-37, whereas 20 $\mu g \cdot ml^{-1}$ showed only moderate effect. However, higher peptide concentrations were necessary to inhibit *Cronobacter* in reconstituted PIF. By using 50 $\mu g \cdot ml^{-1}$ and 100 $\mu g \cdot ml^{-1}$ LL-37, numbers of cells decreased after 4-hour cultivation by approx. 70% and 96%, respectively. Finally, simultaneous application of LL-37 and bacteriophage Dev-CS-701 resulted in synergistic effect, as a 1 300-fold reduction of cell numbers in PIF was observed. Application of the two antimicrobial agents thus facilitated their reduced dosing and decreased the probability of generation of phage-resistant cells.

Keywords

Cronobacter; cathelicidin; bacteriophage; powdered infant formula

Cronobacter spp. are Gram-negative, rod shaped bacteria from the family *Enterobacteriaceae*. They were formerly known as *Enterobacter sakazakii* and as a separate genus were defined in 2007 [1]. Recently, genus *Cronobacter* has undergone a number of revisions and currently contains 7 species. *Cronobacter* spp. are opportunistic pathogens that can cause serious infections in neonates, including meningitis, necrotizing enterocolitis and sepsis with low frequency but high lethality rate [2, 3]. Infections in adults were also reported, in particular among the elderly and immunocompromised patients [4].

Whereas *Cronobacter* spp. are ubiquitous and were isolated from various foods and environments [2, 5], the main vehicle for their transmission in neonatal infections is re-hydrated powdered infant formula (PIF) [2, 6–8]. *Cronobacter* spp. were found at levels less than 1 colony forming unit (CFU) per 100 g of the infant formula responsible for infection. As milk pasteurization is the adequate treatment to devitalize the bacterium, the likely source of *Cronobacter* spp. is post-process contamination of the products. *Cronobacter* spp. are much more resistant than other *Enterobacteriaceae* to desiccation and to osmotic stress, some strains having shown also increased thermotolerance [9–10]. The high frequency of *Cronobacter* occurrence in dried foods and powdered ingredients is well-known and some powdered ingredients added to milk powder without heat treatment enhance the risk of contamination [11]. The ubiquitous occurrence of this pathogen makes its control difficult. Therefore, it is necessary to investigate alternative antimicrobial treatments against this bacterium in infant food.

Antibacterial peptides (AMP) are present on the external surfaces of potentially all living animals, where they function as the first line of defense against pathogens. LL-37 is the only hu-

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man AMP belonging to cathelicidin family. It is secreted from activated neutrophil granulocytes and possesses bactericidal and immunomodulatory activities. It is a 37 amino-acid long, cationic, α-helical, amphipathic peptide, which has a broad spectrum of activity against Gram-negative bacteria, Gram-positive bacteria, various viruses and fungi [12–14]. LL-37 found antibacterial application in clinical therapy and in food protection.

The aim of the present study was to assess the antibacterial activity of LL-37 against a collection of *Cronobacter* strains, and to compare its antimicrobial effects in laboratory growth media and in reconstituted PIF. Moreover, combined application of AMP and bacteriophages in PIF was tested.

MATERIAL AND METHODS

Bacterial strains and bacteriophages

Bacterial strains from Comenius University (Bratislava, Slovakia), Food Research Institute (Bratislava, Slovakia), Nottingham Trent University (Nottingham, United Kingdom) and Belgian Coordinated Collections of Microorganism (Gent, Belgium) were characterized previously [5, 15–16]. Luria-Bertani (LB) and tryptic soy broth (TSB) media were used for strain cultivation. Bacteriophage Dev-CS-701 was isolated from waste water and was characterized by several phenotyping and molecular methods in our laboratory (unpublished results).

Minimum inhibitory concentration of LL-37 determined by radial diffusion assay

Recombinant LL-37 was prepared in *E. coli* system and its minimum inhibitory concentration (*MIC*) was determined as described previously [17]. Briefly, bacterial strains were cultured in 3% TSB to optical density (*OD*) of 0.3 at 600 nm, and then washed three times with a solution containing 100 mmol·l^{-1} NaCl and 10 mmol·l^{-1} phosphate buffer, pH 6. Then, microbal suspension of 10^6 CFU·ml^{-1} was added to 10 ml of pre-warmed underlay gel containing 100 mmol·l^{-1} NaCl; 10 mmol·l^{-1} phosphate buffer, pH 6; 0.03% TSB; 1% agarose and 0.02% Tween 20, and the mixture was immediately poured into a Petri dish. After solidifying, wells of approx. 3 mm depth were cut in the underlay gel using a Pasteur pipette. Next, 5 μl samples with different concentrations of LL-37 were applied into wells and let to soak. The plates were incubated for 3 h at 37 °C. After this period, the underlay gel was covered with 10 ml of nutrient-rich overlay agar (6% TSB; 1% agarose) and incubated for next 18–24 h at 37 °C. The plates were photographed and the diameters of the clear zones were measured using ImageJ 1.42q freeware, a Java-based image-processing programme developed at the National Institutes of Health (Bethesda, Maryland, USA). Diameters of clear zones, after subtracting the diameter of the central well, were plotted against logarithm of the peptide concentration, and *MIC* was calculated from the graph as a peptide concentration at zero diameter.

Antimicrobial activity of LL-37 in liquid media

Antimicrobial activity of LL-37 was evaluated in 10 ml 0.1% TSB inoculated with a bacterial culture of 10^6 CFU·ml^{-1} prepared in the same way as described for *MIC* determination. LL-37 was added at 20 μg.ml^{-1} and 30 μg.ml^{-1} and the cultures were incubated for 4 h at 37 °C with shaking. Then, 100 μl samples were spread on tryptic soy agar (TSA), incubated overnight at 37 °C and the colonies were counted. The assays were performed in triplicates. For determination of LL-37 activity in milk, 20 ml of the reconstituted infant formula (0.3 g·ml^{-1}) was inoculated with 10^5 CFU·ml^{-1} *C. sakazakii* NTU 701. LL-37 was added at 50 μg.ml^{-1} and 100 μg·ml^{-1} and the cultures were incubated at 37 °C for 4 h with shaking. Bacterial counts were determined by the plate-count method in one hour intervals. Assessment of the synergistic effect of the antimicrobial peptide and bacteriophages was made in the same way with the exception that Dev-CS-701 phage was added into the infant formula at the beginning of experiment at 10^4 phage forming units (PFU)·per millilitre.

RESULTS AND DISCUSSION

Antimicrobial peptides are attractive candidates for clinical therapy and food protection as they provide selective activity against prokaryotic cells by interacting with membrane components abundant in bacterial cells but infrequent in eukaryotic cells. The compounds target the mostly conserved cell structures resulting in lack of resistance to them [18]. One of the most frequently studied AMP is human cathelicidin LL-37, which was proven to inhibit the growth of a variety of Gram-negative (*Pseudomonas*, *Salmonella*, *Escherichia*) and Gram-positive (*Staphylococcus*, *Listeria, Enterococcus*) pathogens [13]. Antibacterial activity of recombinant LL-37 against the opportunistic pathogen *Cronobacter* spp. was determined in the present study.

During our initial experiments, bactericidal activity of LL-37 was assessed by radial diffusion

Tab. 1. Minimum inhibitory concentrations of LL-37 for *Cronobacter* strains.

Strain	Source	*MIC* [μg·ml^{-1}]
C. condimendti NTU 1330	food	2.56
C. dublinensis LMG 23823	environment	6.89
C. dublinensis 088/09/P	food	7.68
C. malonaticus LMG 23826	clinical	3.14
C. muytjensii ATCC 51329	unknown	7.81
C. sakazakii ATCC 29544	clinical	5.21
C. sakazakii NTU 701	clinical	3.53
C. turicensis NTU 57	food	8.83
C. turicensis 290708/7	food	4.72
C. universalis NTU 581	water	4.24
Escherichia coli ML35P	unknown	9.23

MIC – minimum inhibitory concentrations.

method. Ten representative *Cronobacter* strains belonging to seven different species were selected covering clinical as well as food isolates and reflecting the variabilty within this genus. *E. coli* ML-35p strain was employed as a comparator. This strain was previously used to monitor permeation of both the inner and outer membranes [19]. LL-37 demonstrated consistent antimicrobial activity against all tested strains with *MIC* ranging from 2.56 μg·ml^{-1} for *C. condimendti* NTU 1330 to 8.83 μg·ml^{-1} for *C. turicensis* NTU 57 (Tab. 1). The values for all *Cronobacter* strains were below the value of 9.23 μg·ml^{-1} obtained for *E. coli* ML-35p. The *MIC* values for *Cronobacter* were in the range obtained for the majority of Gram-negative bacteria strains, 0.9–7.7 μg·ml^{-1} [13, 17].

In next experiments, antimicrobial activity of LL-37 in liquid media was studied using the same strains. LL-37 was applied at two concentrations (20 μg·ml^{-1} and 30 μg·ml^{-1}) in 0.1% TSB and reduction of bacterial counts was observed after 4 h (Fig. 1). LL-37 at 20 μg·ml^{-1} caused a moderate growth inhibition, as bacterial counts decreased by approx. 30–60% compared to the control culture. At the higher concentration of 30 μg·ml^{-1}, LL-37 caused a more pronounced effect, as bacterial counts decreased at least by seven orders of magnitude compared to the control. Actually, these values corresponded to the reduction by more than three orders of magnitude compared to the initial level at inoculation. Furthermore, no bacteria were detected in several replicates. We did not observe any direct correlation between *MIC* obtained by radial diffusion assay and the level of bacterial inhibition in TSB.

Cronobacter spp. poses risk especially for neonates and its potential vehicle is the contaminated infant formula [2, 6]. Hence, we further extended our studies to examine the effect of LL-37 in reconstituted PIF. *C. sakazakii* NTU 701 isolated from a fatal case of neonatal meningitis and belonging to virulent MLST sequence type ST4 [16] was used in these experiments. We observed that the strain grew in reconstituted PIF to densities comparable to those obtained in TSB. However,

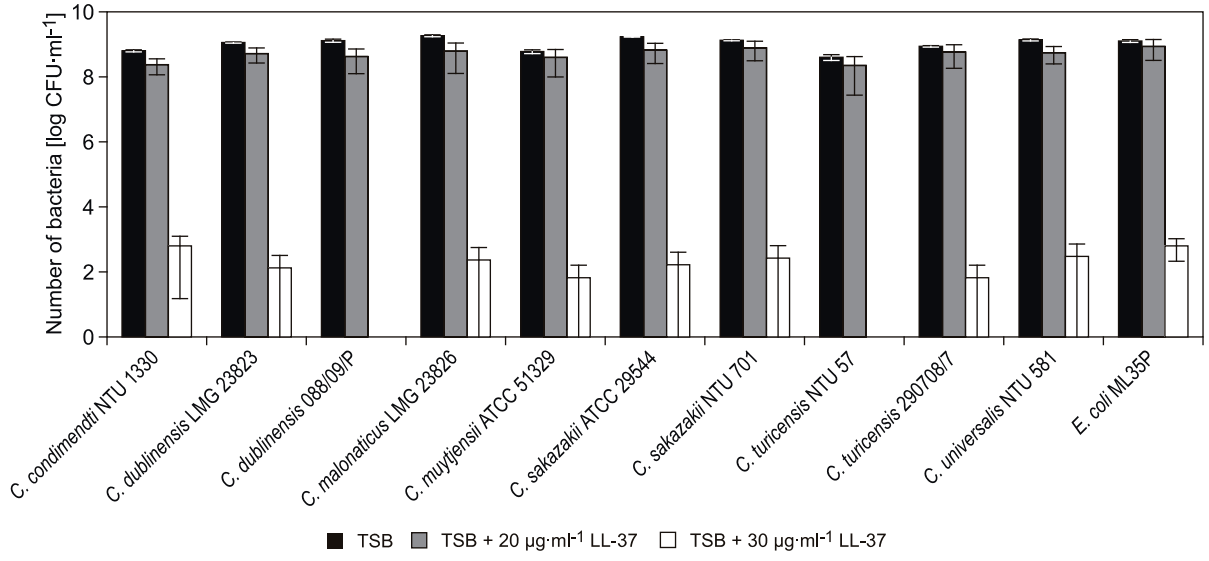

Fig. 1. Growth of *Cronobacter* and *E. coli* strains.

Strains were grown for 4 h at 37 °C in TSB, in TSB supplemented with LL-37 at 20 μg·ml^{-1} and in TSB supplemented with LL-37 at 30 μg·ml^{-1}.

higher LL-37 concentrations were necessary for *Cronobacter* reduction in PIF (Fig. 2). LL-37 at 50 $\mu g \cdot ml^{-1}$ reduced bacterial counts by approx. 70% both at 2 h and 4 h of cultivation, and 90–98% reduction was achieved with a concentration of 100 $\mu g \cdot ml^{-1}$. It can be supposed that the lower antimicrobial activity in recostituted PIF, compared the laboratory growth medium, was probably due to the interaction of LL-37 with proteins and/or certain anionic components of PIF. This explanation is based on data reported by TURNER et al. [13], who observed that *MIC* values of LL-37 in a standard culture medium were at least three-fold higher than those obtained in the medium refined by anion-exchange chromatography.

Because interaction of LL-37, which is a positively charged peptide, with negatively-charged compounds could pose a problem at application to food, we tested simultaneous application of LL-37 and bacteriophages. Bacteriophages represent a potential bio-conservation tool and application of two or more antibacterial agents possessing different modes of action is a recommended strategy in food protection [20]. Phage Dev-CS-701, which was isolated in our laboratory, was used as this phage belonging to T4-like group of phages possesses a broad-host range specificity against strains belonging to *Cronobacter* genus (unpublished results). Bacteriophage Dev-CS-701 was highly lytic on *C. sakazakii* NTU 701 grown in reconstituted PIF. We observed that bacterial counts decreased almost 50-fold in the presence of the bacteriophage after 4 h, compared to initial bacterial counts. The synergistic effect of the bacteriophage and antimicrobial peptide was also observed, as simultaneous application of Dev-CS-701 phage and LL-37 caused a 1300-fold reduction of bacterial counts (Fig. 3). Similar reduction of *Cronobacter* counts by phages was obtained in previous studies [21–23]. Simultaneous application of the bacteriophage and LL-37 in the present work facilitated the use of the bacteriophage at a lower concentration and decreased the probability of generation of phage-resistant cells.

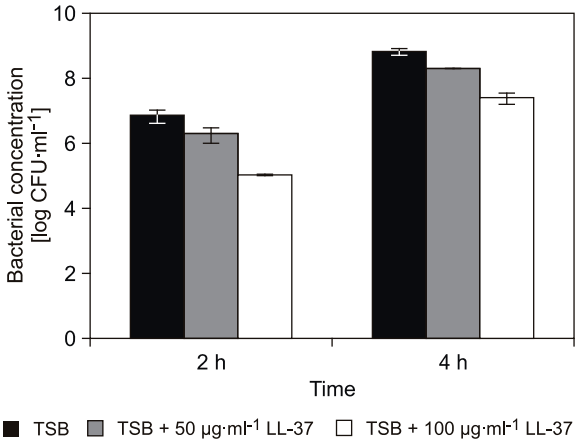

Fig. 2. Growth inhibition of *C. sakazakii* NTU 701 by LL-37 in reconstituted powdered infant formula.

Cronobacter strains were grown for 2 h and 4 h at 37 °C in TSB, in TSB supplemented with LL-37 at 50 $\mu g \cdot ml^{-1}$ and in TSB supplemented with LL-37 at 100 $\mu g \cdot ml^{-1}$.

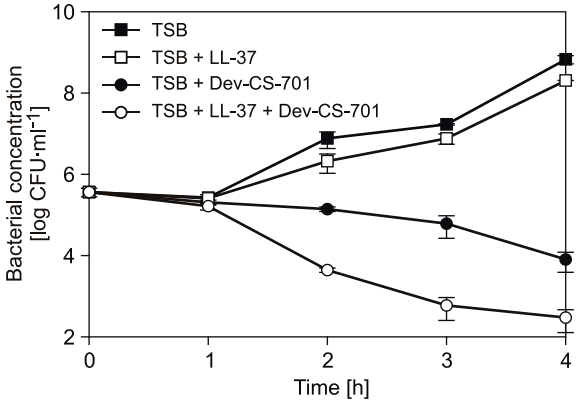

Fig. 3. Synergistic inhibitory effect of LL-37 and Dev-CS-701 phage on *C. sakazakii* NTU 701 growth in reconstituted powdered infant formula.

C. sakazakii was grown for 4 h at 37 °C in TSB, in TSB supplemented with 50 $\mu g \cdot ml^{-1}$ LL-37, in TSB with initial concentration of 10^4 PFU·ml^{-1} Dev-CS-701 phage and in TSB containing 50 $\mu g \cdot ml^{-1}$ LL-37 and 10^4 PFU·ml^{-1} Dev-CS-701 phage.

CONCLUSION

In our study we proved that cathelicidin LL-37 possesses profound antibacterial activity against different *Cronobacter* strains in tryptic soy broth, which was similar or higher than its activity against *E. coli* standard strain. By application of LL-37 in reconstituted infant formula, higher concentrations were necessary for *Cronobacter* reduction compared to TSB medium. At simultaneous application of LL-37 and bacteriophages, a synergistic antimicrobial effect was observed. We propose that combined application of antimicrobial peptides and bacteriophages has a potential to be used as a strategy for *Cronobacter* control in reconstituted infant milk formula.

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