Bacteriophage application for control of *Cronobacter* in liquid media and in biofilms

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Summary
Cronobacter spp. are responsible for rare but fatal cases of infection in neonates and immunocompromised persons. These bacteria are ubiquitous in the environment and were also isolated from various foods, the powdered infant formula being the main vehicle for its transmission in neonatal infections. In the present study, effects of four bacteriophages against *Cronobacter* in liquid media and in biofilm were assessed. Ten selected *Cronobacter* isolates belonging to four species showed variable inhibition by phages in minimal medium with maltose and in powdered infant formula. Bacteriophages significantly decreased growth and biofilm production of the bacterial cultures in maltose minimal medium, the highest effect being observed in the phage cocktail samples. However, the efficiency of bacteriophages for biofilm removal in powdered infant formula was limited only to three strains. Our results will facilitate development of new strategies for *Cronobacter* control in food and food processing factories.

Keywords
Cronobacter; bacteriophage; powdered infant formula; pathogen

The *Cronobacter* genus belongs to the Enterobacteriaceae family and currently contains seven species [1–3]. Members of the genus are opportunistic pathogens that can cause serious infections in neonates, including meningitis, necrotizing enterocolitis and sepsis with low frequency but at a high lethality rate [4, 5]. *Cronobacter* spp. can infect also adults, in particular the elderly and immunocompromised patients, but infections have generally milder manifestation compared to the disease in newborns [6, 7].

Cronobacter spp. are bacteria ubiquitous in the environment and were isolated also from various foods with predominance of dry products of plant origin (e.g. dry fruits, cereals, herbs and spices) [5, 8]. The powdered infant formula is the main vehicle for its transmission in neonatal infections [5, 9, 10]. Survival in dry food is mediated by the high resistance to desiccation and osmotic stress and, in the case of certain strains, also by increased thermotolerance [10, 11]. Biofilm, a community-grown bacterial population, is another form facilitating *Cronobacter* survival in the environment. Biofilm cells are attached on biotic or abiotic surfaces and enclosed by extracellular polymeric substance [12–14]. Bacteria grown in biofilm are protected against many stress conditions such as water or nutrient shortages, exposure to toxic compounds, phage infection or detachment by flow shear. Bacterial biofilms can be a continuous source of food contamination in production facilities [15]. Ability to grow in biofilm is strain-dependent and is regulated by environmental factors, e.g. nutrients, temperature or type of surface [16].

Increased stress resistance of *Cronobacter*, compared to other enterobacteria, makes control of this pathogen difficult. Therefore, it is necessary to investigate alternative treatments against this bacterium. Bacteriophages, viruses infecting bacteria, are recognized as natural, safe and effective alternatives for the prevention and eradication of foodborne pathogens in foods and food processing environments [17, 18]. However, before application, it is necessary to obtain sufficient information on biocontrol bacteriophages to guarantee their safety and reliability [19]. Several *Cronobacter*-specific bacteriophages were already described [20] and the efficiency of bacteriophage application for

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powdered infant formula decontamination [21–24] and prevention of biofilm formation was described [25].

The aim of the present study was to assess anti-biofilm activity of four bacteriophages in a collection of *Cronobacter* strains and to compare their antimicrobial effects in laboratory growth media and in reconstituted powdered infant formula. The results would support development of new strategies for *Cronobacter* control in food.

MATERIALS AND METHODS

Bacterial strains and bacteriophages

Bacterial strains originating from Comenius University (Bratislava, Slovakia), Food Research Institute (Bratislava, Slovakia), Nottingham Trent University (Nottingham, United Kingdom) and Belgian Coordinated Collections of Microorganisms (Gent, Belgium) were characterized previously [8, 26]. Luria-Bertani (LB) medium (10 g tryptone, 5 g yeast extract and 5 g NaCl in 1 000 ml, pH adjusted to 7.0 by NaOH) was used for culturing the strains at 37 °C by shaking. Bacteriophages Pet-CM3-4, Dev-CS701, Dev-CD23 and Dev-CT57 were isolated from waste water by standard procedures and purified by CsCl gradient. They were characterized by several phenotypic and molecular methods in our laboratory (unpublished results) according to Kajsík et al. [21, 22].

Phage host specificity

The host range and the efficiency of plating (EOP) was tested by plaque assay. A volume of 200 μl of overnight-grown bacterial culture was mixed with 5 ml of top agar (2 g tryptone, 2 g NaCl and 5 g agar in 1 000 ml) and poured on LB agar plate. A volume of 10 μl of the appropriate bacteriophage suspension (10^2–10^{10} plaque forming units (PFU) per millilitre) was spotted onto the plate and incubated overnight at 37 °C followed by plaque counting.

Determination of biofilm production

Biofilm-forming ability was assayed in microtiter plates using crystal violet staining [27]. Three different media were tested: LB medium, Agrobacterium minimal medium (AB medium; 2 g $(NH4)_2SO_4$, 6 g Na_2HPO_4, 3 g KH_2PO_4 in 1 000 ml supplemented with 2 mmol·l^{-1} $MgCl_2$, 0.1 mmol·l^{-1} $CaCl_2$ and 3 μmol·l^{-1} $FeCl_3.6H_2O$) with 1% saccharide (glucose, lactose or maltose) and reconstituted powdered infant formula, i.e. Sunar complex 1 (Hero, Praha, Czech Republic) reconstituted in sterile distilled water according to producer's recommendation. A volume of 190 μl of the medium was inoculated with 10 μl of overnight-grown bacterial culture of optical density (*OD*) 0.2 (absorbance at 600 nm). Uninoculated medium was used as control. Microtiter plates were cultured stationary for 24 h at 37 °C. Bacterial growth was then determined as turbidity at 600 nm.

Then, medium was discarded, microplate wells were washed three times with 150 μl phosphate-buffered saline (8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4.2H_2O$, 0.22 g KH_2PO_4, pH 7.4 in 1 l) and fixed with 150 μl methanol for 20 min at room temperature followed by drying at 37 °C. The wells were stained with 150 μl of 1% aqueous crystal violet (Sigma Aldrich, St. Louis, Missouri, USA) for 15 min. Plates were rinsed by submersion in tap water with gentle agitation until no further crystal violet was released. Plates were dried, 150 μl of 96% ethanol was added and absorbance was measured at 600 nm on varioSCAN spectrophotometer (Scanlab, Munich, Germany). Bacterial strains were classified as strong ($OD > 4\times ODc$), medium ($4\times ODc > OD > 2\times ODc$), weak ($2\times ODc > OD > 1\times ODc$) and no ($OD < 1\times ODc$) biofilm producers. Optical density cut off value (*ODc*) was defined as average *OD* of three negative controls plus three standard deviations.

Influence of bacteriophages on bacterial growth and biofilm production was measured in microtiter plates by mixing 190 μl medium, 10 μl overnight-grown bacterial culture ($OD = 0.2$) and 10 μl bacteriophage suspension (10^9 PFU·ml^{-1}). Equal volumes of the four bacteriophage suspensions were mixed to form the bacteriophage cocktail. All measurements were done in triplicate and repeated three times.

RESULTS AND DISCUSSION

Bacterial communities grown in biofilm represent important hazard to food safety and public health. Application of bacteriophages seems to be a simple and efficient method for biofilm eradication [17]. In the present study, we tested effects of four *Cronobacter*-specific bacteriophages for this purpose.

It was shown previously that *Cronobacter* strains are able to produce biofilm at various growth conditions and on various surfaces, e.g. on silicon, latex, polycarbonate, stainless steel, glass or and polyvinyl chloride surfaces, and that the biofilm production is strain-dependent [12, 14, 16]. In a pilot experiment, we tested influence of me-

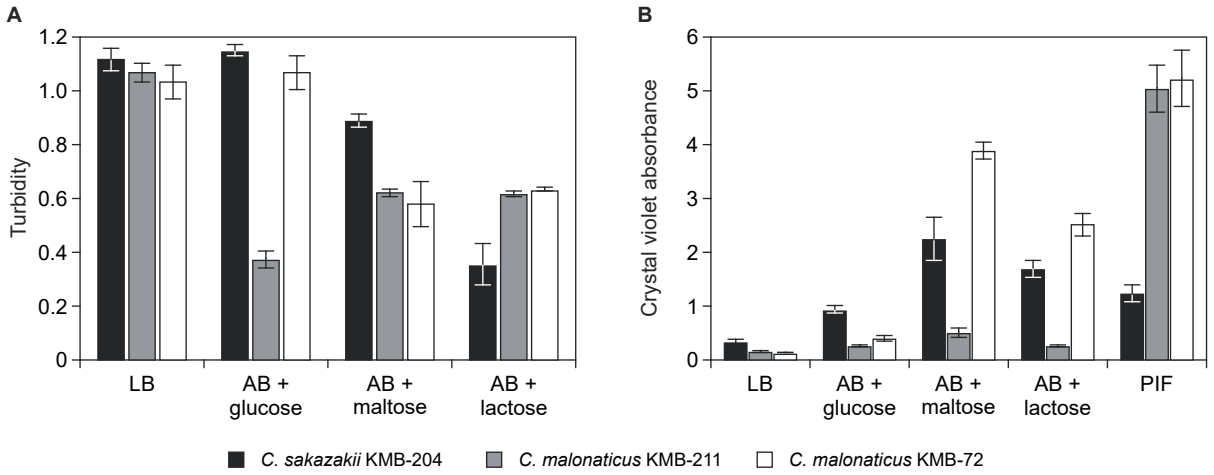

Fig. 1. Growth and biofilm formation of *Cronobacter* strains in various media.

A – growth, B – biofilm formation.
Strains were cultured for 24 h at 37 °C. Turbidity and crystal violet absorbance were measured at 600 nm.
LB – Luria-Bertani medium, AB+glucose – Agrobacterium minimal medium with 1% glucose, AB+maltose – Agrobacterium minimal medium with 1% maltose, AB+lactose – Agrobacterium minimal medium with 1% lactose, PIF – reconstituted powdered infant formula.

dium composition on biofilm growth of three representative *Cronobacter* strains. We observed that the strains were able to grow in all tested media, but the highest cell density after overnight cultivation was observed in LB medium. In AB medium, different growth was observed for individual strains and was dependent on the carbon source (Fig. 1A). However, reversed medium preferences were observed in biofilm detection, as AB medium but not LB broth supported biofilm growth of strains (Fig. 1B). Two from three tested strains produced strong biofilms in AB medium, and biofilm formation depended on saccharide, increasing from glucose to lactose and maltose. All three strains also formed strong biofilms in reconstituted infant formula, but two strains (*C. malonaticus* KMB-211 and *C. malonaticus* KMB-72) produced stronger biofilm in reconstituted infant formula comparing to AB medium with lactose, and the third strain (*C. sakazakii* KMB-204) created less biofilm in these conditions (Fig. 1B). Similar effects were observed also in other studies [16, 28].

Tab. 1. Host specificity of bacteriophages used in the study.

Strain	Serotype / Sequence type	Origin	Efficiency of lysis / Formation of single plaques			
			Pet-CM3-4	Dev-CS701	Dev-CD23	Dev-CT57
C. sakazakii ATCC 29544	O:1/8	clinical	++/+	++/+	–	–
C. sakazakii KMB-203	O:1/8	patient	++/–	++/+	–	–
C. sakazakii NTU 701	O:2/4	clinical	++/+	++/+	+/+	+/+
C. sakazakii KMB-204	O:2/513	patient	+/–	++/+	+/+	+/+
C. sakazakii KMB-544	O:2/513	patient	+/+	++/+	+/+	+/+
C. malonaticus LMG 23826	O:2/7	clinical	++/+	–	+/–	+/–
C. malonaticus KMB-211	O:2/7	patient	+/+	–	–	–
C. malonaticus KMB-72	O:3/462	food	++/+	+/+	++/+	++/+
C. dublinensis LMG 23823	O:1/106	environm	+/+	+/+	++/+	++/+
C. turicensis KMB-86	O:1 /5	food	+/+	++/+	++/+	++/+

Strains were characterized by PCR-serotyping and multilocus sequence typing in a previous study [26]. Patient strains were isolated from throat swabs of hospitalized patients [26].
Efficiency of lysis: (++) – lysis comparable to a propagating strain; (+) – lysis reduced by more than 2 orders of magnitude compared to a propagating strain; (–) – no lysis observed.
Formation of single plaques: (+) – plaques observed; (–) – no plaques observed.

According to these results, we selected AB medium with maltose and reconstituted powdered infant formula for further experiments.

Biofilm production and anti-biofilm effect of bacteriophages were tested by using ten selected *Cronobacter* isolates belonging to four species. Four *Cronobacter*-specific bacteriophages isolated in our laboratory were selected for application as anti-biofilm agents. All bacteriophages possessed broad host specificity against *Cronobacter* strains in spot tests and each strain was infected by at least one phage (Tab. 1). Besides application of single phages, a cocktail composed of all four phages was also tested.

Likewise in the preliminary experiments, we observed variable ability of *Cronobacter* strains to grow in AB medium with maltose resulting in two-fold difference in turbidity between the slowest- and fastest-growing strains (Fig. 2A). Bacteriophages decreased the growth of bacterial cultures, the highest effect being observed in cocktail samples. In nine from ten tested strains, the bacteriophage cocktail reduced the overnight culture to half and, in six of these strains, turbidity was comparable with that of negative control. Bacteriophages Pet-CM3-4 and Dev-CS701 inhibited six and five strains, being more efficient compared to Dev-CD23 and Dev-CT57, which inhibited growth of only two strains (Fig. 2A).

Great variability in biofilm production between *Cronobacter* strains was also observed. Six strains produced strong biofilm in AB medium with maltose. In all these strains, significant restriction of biofilm formation by the bacteriophage cocktail was observed, no biofilm was detected in four strains (*C. malonaticus* LMG 23826, *C. malonaticus* KMB-211, *C. malonaticus* KMB-72 and *C. dublinensis* KMB-86) and in two strains (*C. sakazakii* KMB-204 and *C. turicensis* KMB-86) only weak biofilm was created by the bacteriophage cocktail application (Fig. 2B). Effects of bacteriophages on biofilm production was com-

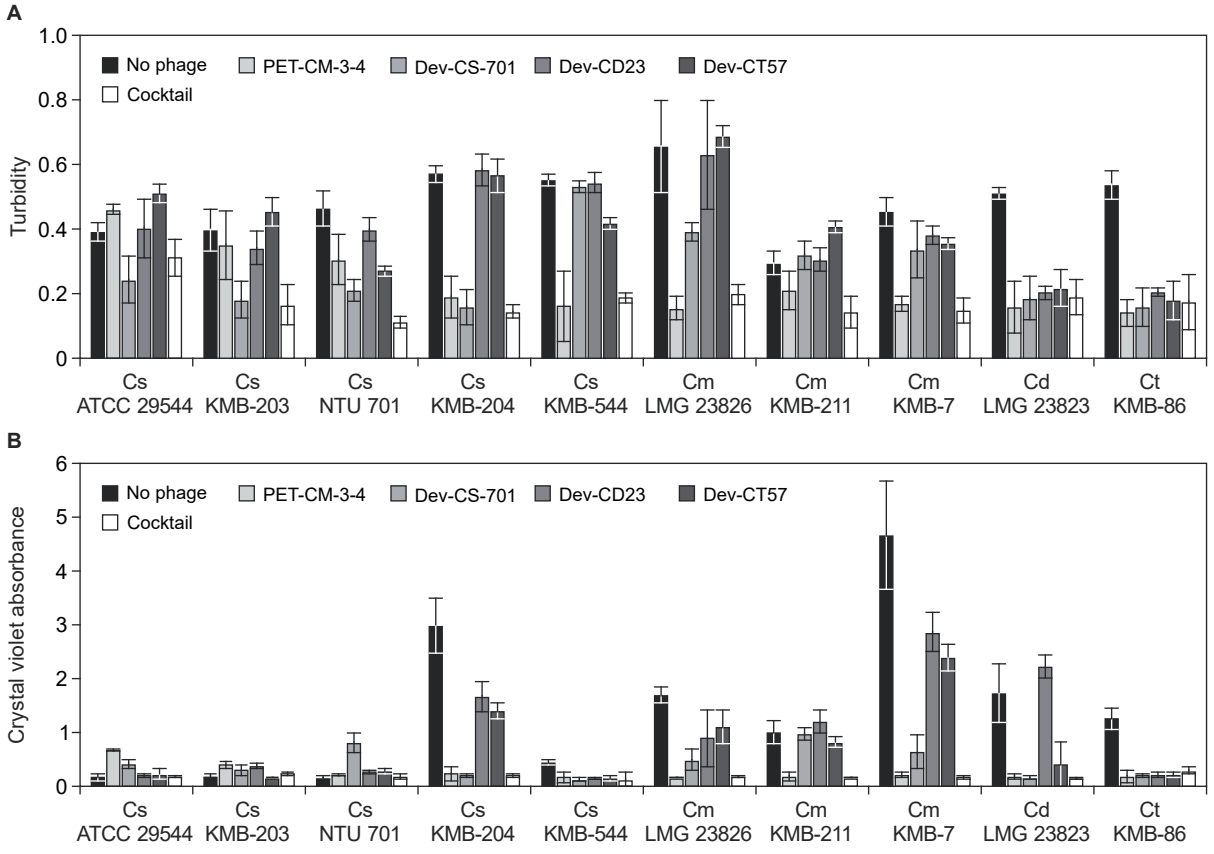

Fig. 2. Influence of bacteriophages on *Cronobacter* growth and biofilm formation.

A – *Cronobacter* growth, B – *Cronobacter* biofilm formation.
Bacteria were cultured in Agrobacterium minimal medium with 1% maltose for 24 h at 37 °C. Turbidity and crystal violet absorbance were measured at 600 nm.
Species abbreviations: Cs – *C. sakazakii*, Cm – *C. malonaticus*, Cd – *C. dublinensis*, Ct – *C. turicensis*.

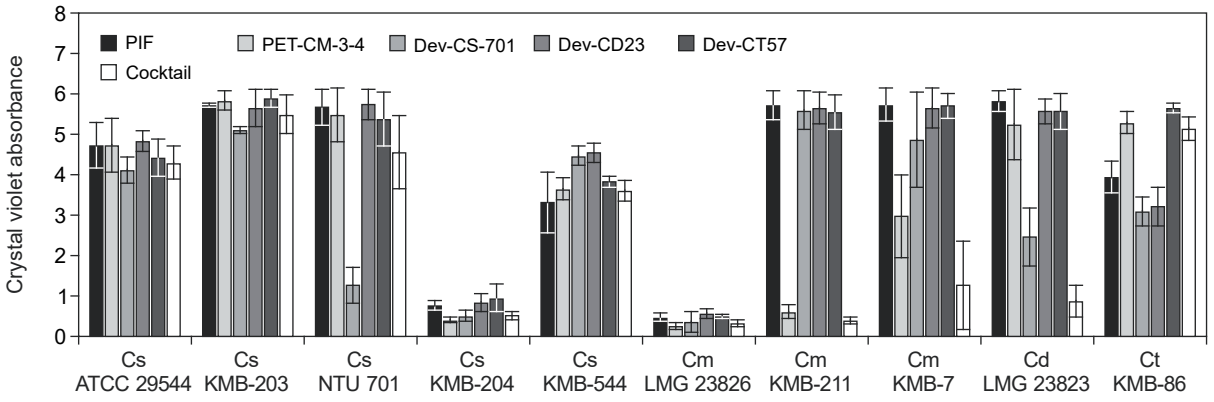

Fig. 3. Influence of bacteriophages on *Cronobacter* biofilm formation in reconstituted infant formula.

Bacteria were cultured for 24 h at 37 °C. Crystal violet absorbance was measured at 600 nm.
PIF – reconstituted powdered infant formula. Species abbreviations: Cs – *C. sakazakii*, Cm – *C. malonaticus*, Cd – *C. dublinensis*, Ct – *C. turicensis*.

parable with their effects on growth in liquid cultures, Pet-CM3-4 phage being the most efficient. However, three *Cronobacter* strains that originally did not form biofilm unexpectedly increased its production in some samples containing single phage (Fig. 2B).

In next experiments, the influence of bacteriophages on biofilm production by *Cronobacter* strains in reconstituted infant formula was determined. Besides two exceptions, *Cronobacter* strains developed strong biofilm in reconstituted infant formula and the efficiency of bacteriophages for biofilm removal in reconstituted infant formula was lower compared to that in AB medium with maltose (Fig. 3). The bacteriophage cocktail significantly decreased the biofilm amount only in three strains and, in one strain (*C. sakazakii* NTU 701), the single bacteriophage Dev-CS701 but not the whole cocktail had anti-biofilm activity (Fig. 3).

Differences in anti-biofilm activity of bacteriophages in maltose medium and in reconstituted infant formula could be partially explained by the fact that the strains that were non-responsive in reconstituted infant formula (*C. sakazakii* ATCC 29544, *C. sakazakii* KMB-203 and *C. sakazakii* NTU 701) did not produce biofilm in maltose medium and *C. sakazakii* KMB-204, which responded well in maltose medium, did not form biofilm in reconstituted infant formula. In *C. sakazakii* KMB-544 and *C. turicensis* KMB-86, which formed biofilm in both media but responded well to bacteriophages only in maltose medium, we propose that the stronger biofilm in reconstituted infant formula could pose a barrier preventing the bacteriophage attack.

In the study of ENDERSEN et al. [25], a cocktail of three bacteriophages was used for devitalization and growth inhibition of *C. sakazakii* in four different brands of infant formula. The authors demonstrated efficiency of the method on one representative strain. Similarly in other studies, bacteriophages inhibited *Cronobacter* growth at a range of different incubating temperatures when using high-titre single bacteriophage suspensions [21, 22, 24]. However, only a very limited number of strains was used in these experiments, which cannot be taken as sufficient from the point of view of industrial application, where the effectiveness against the vast majority of strains is a requirement. Hence the phage application was tested on ten selected *Cronobacter* strains in the present study. The strains used in our study included both well characterized collection strains as well as clinical and food isolates. We observed high efficiency of the bacteriophage cocktail to suppress bacterial growth in maltose AB medium, as nine strains responded to cocktail application (Fig. 2A). This result is similar to ZUBER et al. [23] where a cocktail of five bacteriophages prevented the growth of 35 out of 40 strains in artificially contaminated powdered infant formula.

Biofilms are a significant source of repeated contamination in hospitals, food production facilities and other places, contributing to the spread of pathogens. Similarly to other studies [13, 14, 16, 25], we confirmed high biofilm production in some *Cronobacter* strains (Fig. 1) and therefore biofilm prevention by the bacteriophage activities is required. ENDERSEN et. al. [25] proved that their three-bacteriophage cocktail was very effective in preventing formation of biofilm of two *C. saka-*

zakii strains. In our study, we determined a good anti-biofilm activity of the phage cocktail against ten *Cronobacter* strains in maltose AB medium, but its anti-biofilm effect in reconstituted infant formula was quite limited. Therefore, we propose that new broad-host bacteriophages are included into the bacteriophage cocktail in our next experiments to increase its efficiency against majority *Cronobacter* strains. Alternatively, combination of bacteriophages with other antimicrobial agents could be used [22].

CONCLUSIONS

The present study indicated that the use of bacteriophages can significantly decrease *Cronobacter* growth and biofilm production. However, the effect of bacteriophages was strain-dependent and was more pronounced in maltose AB medium than in reconstituted infant formula. The study contributed to development of new strategies for *Cronobacter* control in food and food processing factories.

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