Food Control 102 (2019) 94–103

Contents lists available at ScienceDirect

Food Control

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

Biocontrol potential of phage Deep-Blue against psychrotolerant *Bacillus weihenstephanensis*

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ARTICLE INFO

Keywords:
Bacillus cereus
Bacillus weihenstephanensis
Bacteriophage
Biocontrol
Biofilm
Deep-blue
Myoviridae
Psychrotolerance

ABSTRACT

Some strains of the *Bacillus cereus* group can be implicated in two types of foodborne intoxication syndromes, namely diarrheal and emetic. The emetic syndrome results from the ingestion of the cereulide toxin produced by particular strains of *B. cereus* and *Bacillus weihenstephanensis*. It represents a major concern since it can lead, in severe cases, to the death of the intoxicated patients. This study aimed to characterize the virulent phage Deep-Blue and investigate its biocontrol potential against psychrotolerant *B. weihenstephanensis*. This myovirus was isolated from an agricultural soil and is stable for at least 6 months at 4 °C. It is specific to members of the *B. cereus* group and shows a lytic activity against five of twelve *B. weihenstephanensis*. Its lytic cycle lasts less than 1 h with a final phage progeny of ca. 300 virions. Regarding its bactericidal potential, Deep-Blue was able to reduce *B. weihenstephanensis* contamination under laboratory conditions (LB broth) and in milk, and to control bacterial growth in food matrices such as milk rice. Finally, the application of Deep-Blue as preventive treatment was shown to efficiently control the development of *B. weihenstephanensis* biofilm.

1. Introduction

Bacillus cereus sensu lato (*s.l.*) is a group of Gram-positive, rod-shaped and ubiquitous spore-forming bacteria that includes strains associated with foodborne intoxications. In Europe, this pathogen is involved in more than 1,000 intoxications each year and represented 6.8% of all foodborne outbreaks reported in 2015 (EFSA & ECDC, 2016). Some strains of *B. cereus* may provoke two types of infection. First, the diarrheal syndrome is caused by heat-labile enterotoxins (for which NHE (Non Hemolytic Enterotoxins), HBL (Hemolysin BL) and CytK (Cytotoxin K) are potential candidates) produced in the small intestine of the patient (Forghani, Kim, & Oh, 2014; Stenfors Arnesen, Fagerlund, & Granum, 2008). Between eight to 16 h after the ingestion of contaminated food, symptoms including diarrhoea and abdominal pain, appear. This syndrome resembles, and is therefore often confused with the one caused by *Clostridium perfringens* foodborne infection (Park et al., 2009). The second type of intoxication, the emetic syndrome, is caused by the ingestion of the cereulide toxin, a 1.2 kDa heat and acid stable cyclic dodecadepsipeptide ([D-*O*-Leu-D-Ala-L-*O*-Val-L-Val]$_3$) (Agata et al., 1994; Hoton et al., 2009). This toxin is produced by certain strains of *B. cereus sensu stricto* (*s.s.*) (Delbrassinne et al., 2012), but also by some strains of the psychrotolerant *Bacillus weihenstephanensis* (Thorsen et al., 2006), at temperatures as low as 8 °C (Guérin et al., 2017). It should be noted that although *B. weihenstephanensis* was originally referring to psychrotolerant isolates of *B. cereus s.l.* (Lechner et al., 1998), it was recently proposed to consider this species as a later heterotypic synonym of *Bacillus mycoides* (Liu, Lai, & Shao, 2018).

The emetic toxin, preformed in food, causes nausea and vomiting, from 30 min to 6 h after consumption of the contaminated food. In this case, symptoms are similar to those of *Staphylococcus aureus* food poisoning (Granum & Lund, 1997). Both syndromes are usually mild and self-recovering, but in some severe cases, cereulide can lead to the patient death due to irreversible liver damage, especially for vulnerable individuals such as children or elderly people (Delbrassinne, Botteldoorn, Andjelkovic, Dierick, & Denayer, 2014; Dierick et al., 2005; Mahler et al., 1997; Naranjo et al., 2011).

Because of their ubiquity, sporulation capacity and the extreme resistance of cereulide (pH, heat, proteases), conventional methods (*e.g.* pasteurization) cannot provide an efficient sanitation of *B. cereus s.l.* strains. Moreover, some emetic strains are psychrotolerant (*e.g.* emetic *B. weihenstephanensis*) and are consequently able to multiply in the consumer's fridge. Therefore, there is a real need to develop alternative approaches to control the growth of emetic strains in foodstuffs, in particular those able to develop under refrigeration conditions. In this context, bacteriophages seem to be a good alternative.

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https://doi.org/10.1016/j.foodcont.2019.03.014
Received 15 January 2019; Received in revised form 25 February 2019; Accepted 14 March 2019
Available online 19 March 2019
0956-7135/

Bacteriophages, or phages, are natural killers of bacteria. Since their discovery, one hundred years ago (d'Hérelle, 1917), they have been used as therapeutic agents in phage therapy (Stent, 2000). Compared to other bactericidal agents (*e.g.* antibiotics and/or bacteriocins), phages offer several advantages for pathogen control in foodstuffs (Kazi & Annapure, 2016). They are naturally present in food and are innocuous to humans, animals and plants (Billington, Hudson, & D'Sa, 2014; Hosseinidoust, Olsson, & Tufenkji, 2014), and thanks to their specificity, they show no side effects against commensal microbiota or beneficial bacteria. They also have an excellent capacity of adaptation: when resistant bacteria arise, phages are prone to co-evolve counter strategies against bacterial variants (d'Hérelle, 1917; Labrie, Samson, & Moineau, 2010; Hargreaves, Kropinski, & Clokie, 2014). Nevertheless, important characteristics are essential for phage application as food biopreservative: only strictly lytic phages are good candidates and long-term stability, under various conditions, are also expected. Finally, if phages are used in cocktails of different phage-types, the emergence of bacterial resistance can be hampered (Chan, Abedon, & Loc-Carrillo, 2013).

So far, the Food and Drug Administration (FDA, 2015) has recognized several phage preparations as GRAS (Generally Recognized As Safe) to be used in food. ListShield™ (Intralytix Inc.) was the first phage preparation active against *Listeria monocytogenes* approved in 2006. The SalmoFresh™ of Intralytix Inc., the FO1a-S16 *Salmonella* phage preparation of Micreos B.V., the SalmoPro® and a *Salmonella* phage preparation (Phagelux) both active against *Salmonella enterica*, Listex™ P100 (Micreos B.V.) active on *L. monocytogenes* and EcoShield™, PhageGuard E™, Secure Shield E1 (FINK TEC GmbH) as well as Shigashield™ displaying a lytic activity on *Escherichia coli* and *Shigella* spp. respectively, also received GRAS status (FDA, 2015). Although it has recently been reported that some phages could partially control bacteria of the *B. cereus* group in foodstuffs (Bandara, Jo, Ryu, & Kim, 2012; Kong & Ryu, 2015; Lee, Billington, Hudson, & Heinemann, 2011; Shin, Bandara, Shin, Ryu, & Kim, 2011), no study focusing on the control of *B. weihenstephanensis* and its emetic strains has been published yet. So far, only two phages infecting *B. weihenstephanensis* have been reported: the virulent podovirus MG-B1 (Redondo, Kupczok, Stift, & Bollback, 2013) and more recently the lytic phage Deep-Blue (Hock, Gillis, & Mahillon, 2016). In this work, Deep-Blue was characterized and its biocontrol potential against *B. weihenstephanensis* investigated, within the ultimate goal of developing new phage cocktails to control this psychrotolerant foodborne pathogen.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Details on the *B. cereus s.l.* strains used in this work are given in Table 1. All bacteria were grown overnight (O/N) in Lysogeny Broth (LB) (5 g L^{-1} NaCl, 5 g L^{-1} yeast extract, 10 g L^{-1} Tryptone) or LB-agar plates (1.5% agar) at 30 °C for the *Bacillus* spp. strains and at 37 °C for *Staphylococcus* spp., *Listeria* spp., *Pseudomonas aeruginosa*, *E. coli* and *S. enterica* strains.

2.2. Bacteriophage isolation and propagation

Phage Deep-Blue was isolated from an agricultural soil collected in Gembloux (Belgium) (Hock et al., 2016). The collected sample was diluted ten times with modified LB broth (LB broth supplemented with 0.3% of $CaCl_2$ and 0.5% of $MgSO_4$) and incubated for 1 h at room temperature. After centrifugation (2,000 *g* for 5 min), the supernatant was filtered using 0.45 µm pore size filter (Sartorius Stedim). The filtrate was then mixed with a combination of O/N cultures of three emetic *B. weihenstephanensis* strains: MC67 (Thorsen et al., 2006), BtB2-4 (Castiaux et al., 2014; Hoton et al., 2009) and LH002 (Hock, Gillis, & Mahillon, 2018) in modified LB broth. In order to increase the concentration of the phages present in the sample, the filtrate was incubated at 30 °C O/N with shaking (120 rpm). After centrifugation (2,000 *g* for 10 min), the supernatant was filter-sterilized as described above. Individual phage purifications were then performed as follows: 500 µL of the filtrates containing the phage mixture were incubated 30 min at room temperature with an equal volume of an O/N culture of a target strain. The mixture was then added to a molten modified LB soft agar (0.4%), poured on a LB agar plate and incubated O/N at 30 °C. A single phage plaque was picked up with a sterile pipette tip and eluted in modified LB broth. These plaque isolation and elution steps were repeated at least three times to purify single phage displaying constant plaque morphology.

Once the phage purification was achieved, high titre stocks (10^8 to 10^9 PFU mL^{-1}) were prepared as follows. First, a phage suspension was obtained by vortexing a plug of agar corresponding to a single phage plaque forming unit (PFU) in modified LB broth. The resulting phage suspension was added to 10 mL of modified LB broth inoculated with 1% of an O/N host culture. After 5 h at 30 °C, 120 rpm, the culture was centrifuged (2,000 *g* for 5 min) and the supernatant was filtrated. In a second step, 50 µL of this filtrate were added to 10 mL of modified LB broth contaminated with 100 µL of a concentrate culture of the host bacterium. To obtain this high bacterial concentration, 10 mL O/N culture of the host bacterium were previously centrifuged (4,000 *g* for 10 min) and resuspended in 0.5 mL of modified LB broth. After 5 h at 30 °C, 120 rpm, the culture was centrifuged and the supernatant was filtrated as indicated above. Finally, phages were purified by precipitation with PEG (PolyEthylene Glycol) 8000 (30%, 0.5 M NaCl) during 30 min at room temperature. After 10 min centrifugation at 15,000 *g*, the phage pellet was resuspended in sterile water. This purification step was repeated twice.

2.3. Morphological analysis by transmission electron microscopy

The phage morphology was determined using transmission electron microscopy (Mica Technology Platform, UCL). A 20-µL drop of purified phage sample was loaded and absorbed for 15 min on a carbon-coated copper grid, followed by negative staining with 2% potassium phosphotungstate. Phage size was calculated by means of at least three measurements.

2.4. Host spectrum

Deep-Blue host range was assessed by spot-on-plate assays on ten *B. cereus*, five *B. thuringiensis* and twelve *B. weihenstephanensis*, including six emetic strains (Table 1). Thirty-four *Bacillus* spp. (including ten *Bacillus subtilis*), three strains of *Staphylococcus* spp., two *Listeria* spp., two *P. aeruginosa*, three *E. coli* and three *S. enterica* were also tested (the main features of these bacterial strains can be obtained from the authors upon request). Ten-µL drop of isolated phages (*ca.* 10^9 PFU mL^{-1}) and 10-fold dilutions were spotted on a 0.6% modified LB soft agar overlay containing 200 µL of an O/N bacterial culture. The plates were incubated O/N at 30 °C or 37 °C according to the optimal growth temperature of each bacterium.

2.5. Temperature and pH sensitivity

To examine their thermo-sensitivity, phage suspensions (1 mL) were incubated at different temperatures (4, 30, 40, 50, 60, 70 and 90 °C) for 1 h in the presence of SM buffer (50 mM Tris, 100 mM NaCl, 8 mM $MgSO_4$, 0.01% gelatine, pH 7.5). The phage survival was estimated by the number of remaining phages at the tested temperature using the protocol described above for phage isolation and propagation (section 2.2), with *B. weihenstephanensis* BtB2-4 as bacterial host. To evaluate the pH-sensitivity, phages were diluted 10-times in appropriate buffers (citrate buffer for pH 4–5, phosphate buffer for pH 6–8, Tris-HCl buffer for pH 9, carbonate buffer for pH10-11 and KCl-NaOH buffer for pH 12) and the phage survival was assessed after 1 h incubation at room

Table 1
Strains used in this work.

Bacillus species	Strain	Features/origin	References
cereus s.s.	AH621	Environmental	Helgason et al., (1998)
	ATCC 14579	Reference strain	Ivanova et al., (2003)
	MSX-A1	Environmental	Van der Auwera, Feldgarden, Kolter, and Mahillon (2013)
	TIAC71	Foodborne	Castiaux, Liu, Delbrassinne, and Mahillon (2015)
	TIAC139	Foodborne	Castiaux et al. (2015)
	TIAC299	Foodborne	Castiaux et al. (2015)
	TIAC468	Foodborne	Castiaux et al. (2015)
	VD21	Environmental	Van der Auwera et al. (2013)
	VD45	Environmental	Van der Auwera et al. (2013)
	VD78	Environmental	Van der Auwera et al. (2013)
thuringiensis	DBT242	Environmental	N. B. Hendriksen, Aarhus University, Denmark
	DBT684	Environmental	N. B. Hendriksen, Aarhus University, Denmark
	T50 001	sv. *navarrensis*	Iriarte et al., (2000)
	4Q7	sv. *israelensis*, acrystalliferous	Jeong, Park, and Choi (2014)
	BMG1.7	Environmental, sv. *kurstaki*	Van der Auwera et al. (2013)
weihenstephanensis	BtB2-4	Emetic	Hoton et al., 2009; Castiaux et al., 2014
	Cer057	Emetic	Hoton et al., 2009; Castiaux et al., 2014
	Cer074	Emetic	Hoton et al., 2009; Castiaux et al., 2014
	LH002	Emetic	Hock et al. (2018)
	MC67	Emetic	Thorsen et al., (2006)
	MC118	Emetic	Thorsen et al., (2006)
	Bw 2-1	Non-emetic	This work
	KBAB4	Non-emetic	Lapidus et al., (2008)
	Si0170	Non-emetic	This work
	Si0239	Non-emetic	This work
	WSBC10202	Non-emetic	Lechner et al., (1998)
	WSBC10204	Non-emetic, reference strain	Lechner et al., (1998)

temperature. Finally, to assess long-term stability, phage suspensions were mixed with an equal volume of water and stored at 4 and 20 °C. The phage concentration was monitored by the spot-on-plate method.

2.6. Phage adsorption assay and one-step growth curve

To evaluate the phage adsorption rate, an 18-h bacterial culture of *B. weihenstephanensis* BtB2-4 was diluted 4-fold in modified LB broth to reach an OD_{600} between 0.1 and 0.2 (*ca.* 10^7 CFU mL^{-1}). After 5 min at 30 °C, with an agitation of 120 rpm, phage suspension, diluted to reach a PFU/CFU ratio of 0.1, was added. A sample was taken at 2-min intervals during 20 min, diluted 200 times and placed on ice to reduce additional adsorption of phages to host cells. At the end of the adsorption experiment, all samples were immediately centrifuged (10,000 *g* for 10 min at 4 °C) and filtrated to remove bacterial cells and to determine the number of unbound free phage particles by the spot-on-plate method. A linear regression of free phage proportion (phage concentration divided by the initial phage concentration), in Log value, in function of time was performed using JMP Pro 12 (SAS Institute, Inc.). The adsorption rate constant (k) was calculated by dividing the negative of adsorption line slope by the concentration of bacterial cells. The times required to achieve 50 and 90% adsorption were determined according to the formula:

$$t = 2.3 \times \text{Log}\ (P_0/P)/(B \times k),$$

where k is the adsorption rate constant, B is the concentration of bacterial cells (CFU mL^{-1}), t is the time interval (min) during which the phage titre (PFU mL^{-1}) falls from P_0 (original) to P (final) (Clokie & Kropinski, 2009).

Life cycle parameters were assessed by building a one-step growth curve. An 18-h bacterial culture of *B. weihenstephanensis* BtB2-4 was mixed in warm LB broth with phages at the PFU/CFU ratio of 0.1, and an adsorption of 50% of phages was allowed using the time determined above. The mixture was centrifuged (15,000 *g* for 2 min) and the supernatant was discarded to remove the residual phages. The cell pellet was resuspended in warm modified LB broth and the culture was further incubated at 30 °C with shaking (120 rpm). Two sets of samples were collected at 10-min intervals. One was immediately centrifuged (13,000 *g* for 2 min) and filter-sterilized whereas the other was treated with 5% of chloroform to release intracellular phages before centrifugation and filtration. Phage titres were determined for each sample. The one-step growth curve was drawn by plotting at each time point the number of PFU divided by the number of infective centres (*i.e.* bacteria on which a phage is adsorbed).

2.7. Bacterial challenge test in liquid culture

Bacterial inocula were prepared using a standardized challenge test used for proficiency testing (Requasud, Belgium; M. Abdel Massih, pers. comm.). Fresh colonies of *B. weihenstephanensis* BtB2-4 strain were re-suspended in Ringer solution (Oxoid, UK) to reach two McFarland units (densitometer Den-1, Grant Instruments, Cambridge, UK), which corresponds to a bacterial concentration of *ca.* 2×10^7 CFU mL^{-1}. Using 96-well polystyrene microtiter plates, the efficiency of different Deep-Blue PFU/CFU ratio (10^{-3} to 10^3) was tested in a final volume of 200 µL. Each well contained 160 µL of modified LB broth, 20 µL of bacterial suspension (10^4 CFU mL^{-1}) and 20 µL of phage suspension in different concentrations. A low concentration of the bacterial host allowed mimicking conditions likely to be found in non-artificially contaminated food matrices. The bacterial growth kinetics was monitored every hour, during 24 h at 30 °C using a Multiskan™ FC Microplate spectrophotometer (Thermo Fisher Scientific, Watham, USA) with an absorbance of 595 nm. Each condition was performed in quadruplicate.

Deep-Blue efficiency was also tested in 20 mL of modified LB broth using a PFU/CFU ratio of 1 and 1,000 at temperatures of 7, 20 and 30 °C. Bacterial and phage concentrations were monitored after 24 and 48 h of incubation (5 and 7 days for the incubation at 7 °C). Each condition was performed in triplicate.

2.8. Biocontrol in food matrices

B. weihenstephanensis BtB2-4 and phage suspensions were prepared as described in the previous section. Deep-Blue biocontrol efficiency (PFU/CFU ratios of 1 and 1,000) was evaluated in 20 mL of semi-skimmed UHT milk (Delhaize, Belgium) and 50 g of milk rice

(Everyday, Colruyt, Belgium). Homogenisation of solid food matrices (milk rice) was performed with a sterilized spoon. Foods were incubated at 20 °C (and also 30 °C for milk) and 7 °C during 48 h and 7 days, respectively. To monitor the bacterial concentration, 10 g of solid food were diluted in 90 mL of Ringer solution and homogenized with a stomacher (Stomacher^R 400 circulator, Seward, UK) for 30 s at 230 rpm, whereas liquid food matrices were directly spread without further processing. The bacterial concentrations were estimated by counting CFU on decimal serial dilutions spread on MYP agar (Biokar, France). The number of indigenous *B. cereus s.l.* microflora potentially present in the food was assessed using a non-inoculated food sample.

The same experiments were reproduced with a mix of three *B. weihenstephanensis* strains. Spontaneous mutants of strains BtB2-4, Cer074 and Cer057 (Castiaux et al., 2014; Hoton et al., 2009), resistant to nalidixic acid (Nal^R), rifampicin (Rif^R) and streptomycin (Sm^R), respectively, were used to be easily distinguished from each other. Milk and rice were contaminated with the bacteria as described before. Rice was cooked in water in an autoclave (121 °C, 15 min) and homogenisation of bacteria and phages in the matrices was done in a blender (Microtron MB 550, Kinematica Ag, Switzerland) with an equal amount of rice and cooking juice (*i.e.* both 25 g). Phage treatments were performed during 48 h at 20 °C and extended for 7 days at 7 °C with a PFU/CFU of 1,000.

2.9. Assessment of phage activity on biofilms

Bacterial biofilms were obtained using the Biostream flow cell device (Vanzieleghem et al., 2013, 2016). Glass slides (available biofilm surface of 320 mm^2) were inserted inside the flow cells and sterile PBS was injected to force out the air. The bacterial suspensions (*ca.* 10^5 CFU mL^{-1}) were pumped (0.5 mL per min) through the flow cells for 20 min. LB broth diluted twice was injected during 24 h to allow the biofilm to develop. The efficacy of phage treatments was evaluated using either a preventive or a curative approach. For the former, a phage suspension (10^7 PFU mL^{-1}) was flowed along the glass slide for 20 min before bacterial injection. The latter treatment consisted in the addition of phages (10^7 PFU mL^{-1}) for 4 h on a 20 h biofilm. The experiment was performed at 30 °C, in triplicate. To evaluate the bacterial concentration in the biofilm, the glass slides were vortexed for 2 min in 40 mL of Ringer solution and spread on LB agar. Deep-Blue efficiency was evaluated on biofilms containing BtB2-4 (Nal^R), Cer074 (Rif^R) and Cer057 (Sm^R) in LB or milk. Concentration of each strain was estimated by plating on LB containing the appropriate antibiotic (added at the following concentrations: Nal, 15 μg mL^{-1}, Rif, 50 μg mL^{-1} and Sm, 100 μg mL^{-1}).

2.10. Statistical analysis

Statistical analyses of results were done using JMP PRO 12.2.0 software and Student test with a confidence level of 95%.

3. Results

3.1. Characteristics and morphology of phage Deep-Blue

Phage Deep-Blue (Fig. 1) is a lytic phage isolated and purified from an agricultural soil (Hock et al., 2016) using the emetic *B. weihenstephanensis* strain BtB2-4 (Hoton et al., 2009) as host. Phage preparations reaching *ca.* 10^8 PFU mL^{-1} were routinely obtained after PEG precipitation. As shown in Fig. 1, Deep-Blue possesses an icosahedral head of about 48 nm in diameter and a contractile tail of 129 nm in length, suggesting that it belongs to the family *Myoviridae* (order *Caudovirales*). Its genomic sequence has been recently analysed (Hock et al., 2016).

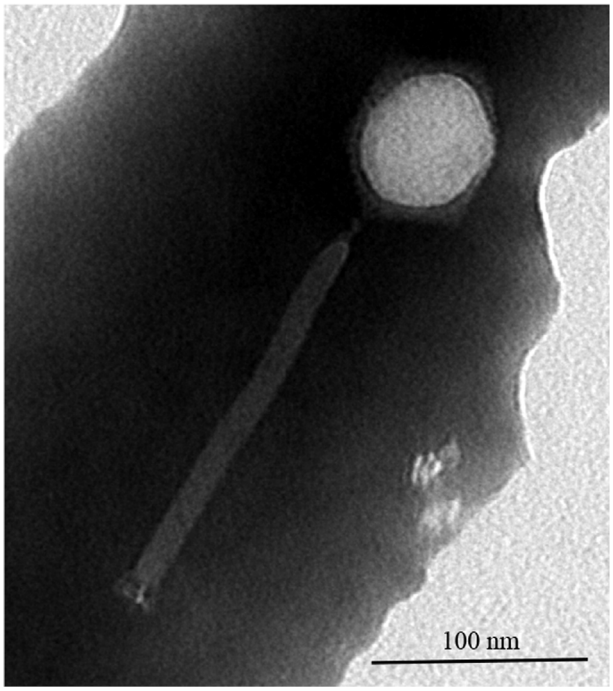

Fig. 1. Transmission electron microscopy image of phage Deep-Blue, negatively stained with 2% potassium phosphotungstate.

3.2. Deep-Blue host spectrum and stability

The host spectrum of Deep-Blue was evaluated using spot assays on twelve strains of the *B. weihenstephanensis*, including six emetic, ten strains of *B. cereus s.s.* and five strains of *B. thuringiensis* (Table 1). It was also tested on 34 *Bacillus* spp. and thirteen strains of *Staphylococcus* spp., *Listeria* spp., *P. aeruginosa*, *E. coli* and *S. enterica*. Among the twelve *B. weihenstephanensis* tested, five were sensitive to Deep-Blue, including three emetic (BtB2-4, CER057 and CER074) and two non-emetic strains (Si0239 and WSBC10202). Two strains of the ten *B. cereus s.s.* (TIAC139 and VD21) and two of the five *B. thuringiensis* strains (DBT242 and T50 001) were also sensitive to the Deep-Blue phage preparations. However, none of the other bacterial species were sensitive to this phage.

The thermal and pH stabilities of Deep-Blue were evaluated after 1-h incubation at different temperatures and pH buffers. Deep-Blue virion suspensions remained stable after 1 h at 40 °C, but lost more than five-Log PFU mL^{-1} at 50 °C (Fig. 2A). They were also stable between pH 5 and 10 for 1 h at room temperature (Fig. 2B). Concerning the storage conditions, Deep-Blue stability was evaluated at 4 and 20 °C. After six months, the phage concentration decreased by less than one-Log CFU mL^{-1} at 4 °C, whereas the reduction observed at 20 °C during the same period exceeded three Log (Fig. 2C).

3.3. Phage life cycle

Growth parameters of Deep-Blue were assessed by a phage adsorption and a one-step growth curve assays using *B. weihenstephanensis* BtB2-4. One half of Deep-Blue particles were attached to BtB2-4 after 7 min of incubation at 30 °C. However, 23 min were required to reach 90% of adsorption (data not shown). The adsorption rate constant (k) was estimated around 10^{-8} mL min^{-1}. The one-step growth kinetics (Fig. 3) also indicated that Deep-Blue has an eclipse period of 40 min, a latent period time of 50 min (*i.e.* delay between phage adsorption to a bacterium and subsequent phage-progeny release (Clokie & Kropinski, 2009)), and a burst size of ca. 300 virions/infective centre, when infecting *B. weihenstephanensis* BtB2-4 under the tested conditions.

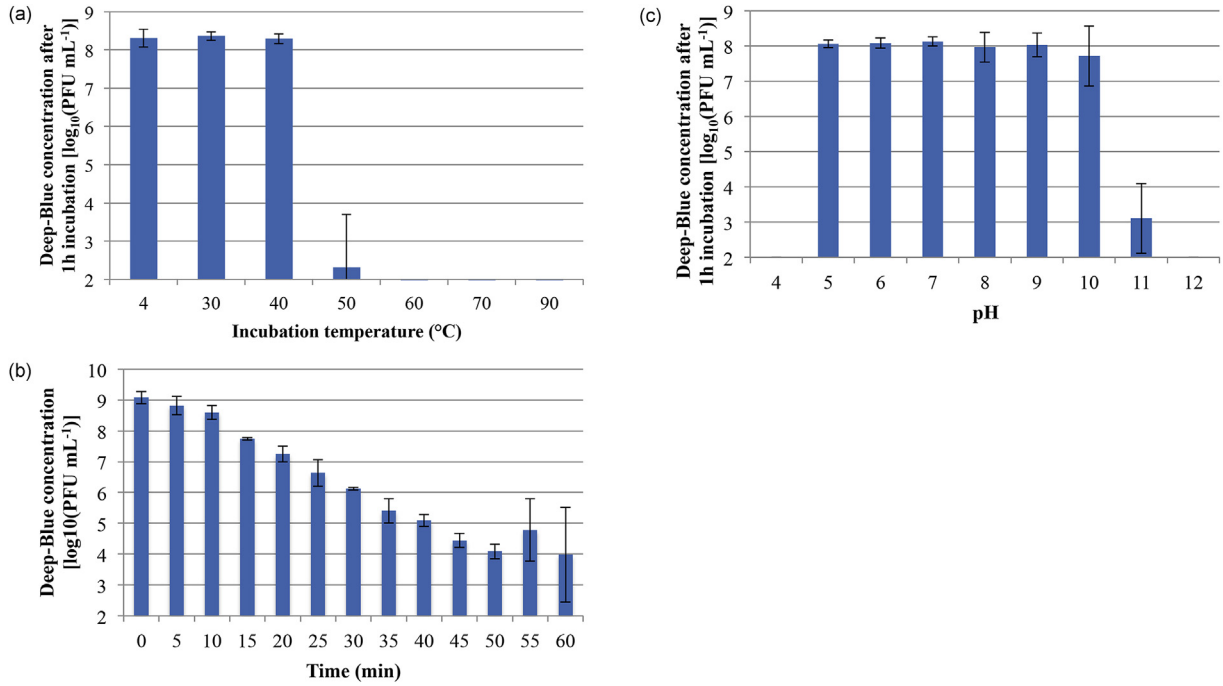

Fig. 2. Thermal-, pH- and time-sensitivity of phage Deep-Blue suspensions. **(A)**: Thermo-sensitivity after 1 h incubation at 4, 30, 40, 50, 60, 70 and 90 °C. **(B)**: Deep-Blue pH-sensitivity after 1 h incubation in appropriate buffered pH. **(C):** Stability when stored at different temperatures. Means with standard deviations were calculated from experiments done in triplicate. The limit of detection (LOD) was 2 Log PFU mL^{-1}. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. Deep-Blue biocontrol in artificial media

The ability of Deep-Blue to inhibit the growth of emetic *B. weihenstephanensis* BtB2-4 was assessed in a 96-well microtiter plate. Bacteria (in modified LB broth) were mixed with Deep-Blue at a PFU/CFU ratio ranging from 0.001 to 1,000. In the absence of phages, the growth of BtB2-4 followed a classical bacterial growth curve (Fig. 4). With the addition of phages at PFU/CFU ratio between 0.001 and 1, the exponential phase was affected and its duration reduced. The higher the phage concentration, the quicker bacterial lysis was observed. For PFU/CFU ratios above 10, no bacterial growth could be recorded by spectrophotometric measurements.

In order to better quantify the ability of Deep-Blue to control BtB2-4 growth, the same assay was performed with PFU/CFU ratios of 1 and 1,000 using the colony counting method to assess the bacterial concentration. Three incubation temperatures were tested: 30 °C which corresponds to the optimal growth temperature for BtB2-4, 20 °C to represent storage at room temperature and 7 °C to reflect *B. weihenstephanensis* capacity to grow at refrigeration temperature. After seven days at 7 °C, no statistical difference was observed when BtB2-4 was treated with Deep-Blue at the PFU/CFU ratio of 1, as compared to a non-treated BtB2-4 culture (Fig. 5A). On the other hand, the addition of Deep-Blue at the PFU/CFU ratio of 1,000 prevented the growth of BtB2-4 and produced a 2.2 and 3.9 Log reduction after five and seven days, respectively. In addition, the final bacterial concentration was 0.4 Log lower than the initial contamination.

At room temperature, the addition of phage at a PFU/CFU ratio of 1,000 prevented the bacterial growth (difference of 5.3 Log with the control after 48 h) (Fig. 5B). Moreover, the final bacterial concentration was 0.4 Log lower than the initial contamination. On the other hand, with a PFU/CFU ratio of 1, the bacterial count was reduced by 3.8 Log but the bacterial growth resumed after 48 h. Finally, the same observations were done at 30 °C after 48 h, with 4.8 and 0.7 Log reductions compared to the control and to the initial contamination, respectively, at a PFU/CFU ratio of 1,000 (Fig. 5C). However, only one Log reduction in the bacterial count was observed with phages at a

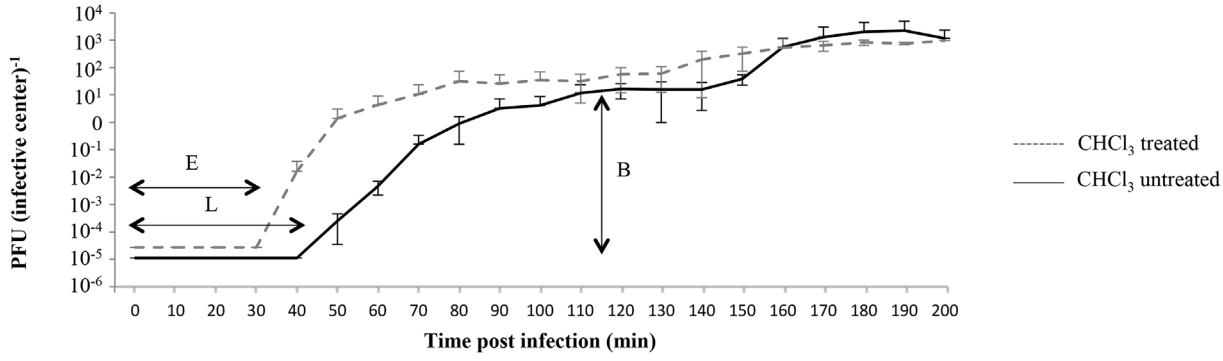

Fig. 3. One-step growth curve of phage Deep-Blue on *B. weihenstephanensis* BtB2-4 with a PFU/CFU ratio of 0.1. Every 10 min, the number of free phages was estimated and divided by the number of host cells (*i.e.* infective centres). The solid curve represents free phages in the medium (PFU/infective centre) whereas the dot curve represents the free phages in the medium after cell lysis by chloroform. E, eclipse period; L, latent period; B, burst size. Means with standard deviations were calculated from experiments done in triplicate.

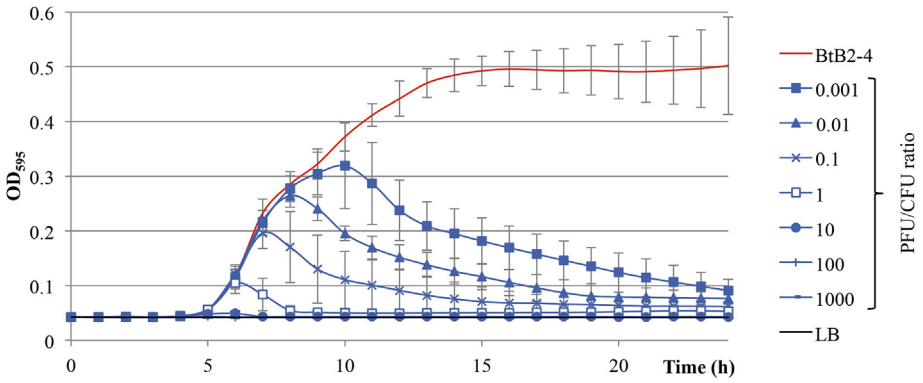

Fig. 4. Inhibition of emetic *B. weihenstephanensis* BtB2-4 by phage Deep-Blue in LB medium. The bacterial concentration was estimated with the Optical Density at 595 nm (OD_{595}). Tests were performed with different PFU/CFU ratios. Means with standard deviations were calculated from quadruplicate assays. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

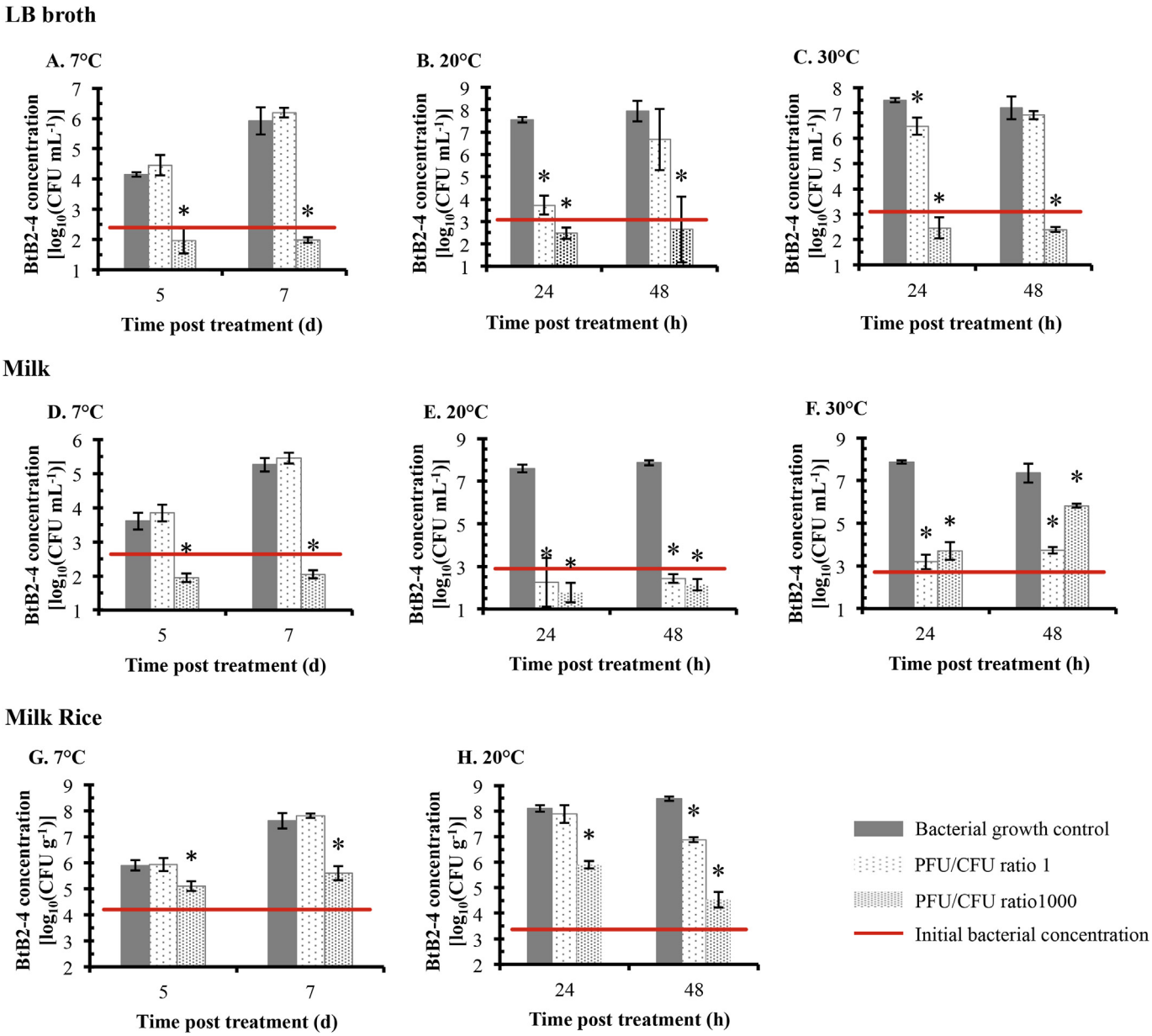

Fig. 5. Deep-Blue phage efficiency to inhibit *B. weihenstephanensis* BtB2-4 growth in LB broth **(A–C)**, milk **(D–F)** and milk rice **(G–H)** at 7 °C **(A, D, G)**, 20 °C **(B, E, H)** and 30 °C **(C, F)**. The time scale is expressed in days for incubation at 7 °C, and in hours for 20 °C and 30 °C incubations. The bacterial concentration is given in logarithmic scale, with a LOD of 1 Log CFU mL^{-1}. The red line represents the initial BtB2-4 concentration. Stars indicate statistical differences (Student test, *p*-value of 0.05) in comparison with the bacterial growth control. Means with standard deviations were calculated from triplicate assays. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

PFU/CFU ratio of 1 after 24 h.

3.5. Deep-Blue biocontrol efficiency in food

To assess the biocontrol ability of Deep-Blue in food matrices, milk and milk rice were artificially contaminated with BtB2-4 and treated with Deep-Blue at PFU/CFU ratios of 1 and 1,000. When phages were added at low concentration, no bactericidal effect was observed in milk after 1 week at fridge temperature (Fig. 5D). However, a 3.2 and 0.6 Log reduction of bacterial counts compared to the bacterial growth control and the initial contamination, respectively, were obtained after 7 days at 7 °C with a PFU/CFU ratio of 1,000.

At 20 °C, the addition of phages was efficient even at low concentration (Fig. 5E). Indeed, the phage addition at a PFU/CFU ratio of 1 and 1,000 produced 5.4 and 5.7 Log reduction of bacterial counts, respectively, compared to the bacterial growth controls. In addition, the bacterial contamination was lower (0.5 and 0.8 Log) than the initial level. A smaller decrease (3.6 and 1.5 Log reduction in comparison with the control for PFU/CFU ratio of 1 and 1,000, respectively) was obtained at 30 °C compared to room temperature (Fig. 5F). In milk rice, only the phage treatment at a PFU/CFU ratio of 1,000 was efficient and reduced by 2 Log the bacterial concentration in comparison with the bacterial growth control after 7 days at 7 °C (Fig. 5G). At room temperature, 1.6 and 4 Log reductions of bacterial concentration were observed after 48 h with a PFU/CFU ratio of 1 and 1,000, respectively (Fig. 5H). In both cases, the final bacterial concentration was higher than the initial contamination. Therefore, a high phage concentration was able to control *B. weihenstephanensis* BtB2-4 in milk rice but could not reduce the original level of bacterial contamination.

When food matrices (milk and rice) were simultaneously contaminated by three different emetic strains of *B. weihenstephanensis* (displaying distinct antibiotic-resistances), a 48 h Deep-Blue treatment with a PFU/CFU ratio of 1,000 prevented the multiplication of all three strains (Fig. 6). In milk, differences ranging from 5.2 to 6.1 Log CFU mL^{-1} were observed between the bacterial counts with and without phages after an incubation at 20 °C for 48 h. After one week at 7 °C, the same trend was observed with reductions between 3.8 and 5.1 Log CFU mL^{-1}. In both cases, the emetic *B. weihenstephanensis* concentration after the phage treatment was under the initial contamination level (Fig. 6A and B). In rice, the bacterial growth was lower at 7 °C than at room temperature but a 2.1–3.5 Log reduction was observed in the presence of phages compared to the bacterial growth control. At 20 °C, reductions ranging from 3.5 to 4.7 Log were observed, depending on the strains. However, these reductions were not sufficient to decrease the bacterial counts below the initial contamination for all the tested strains (Fig. 6C and D).

3.6. Deep-Blue prevention and elimination of B. weihenstephanensis biofilm

B. weihenstephanensis BtB2-4 biofilms were formed in flow cells after a bacterial suspension was first added in a continuous flow for 20 min, followed by LB broth for 24 h to allow adherent bacteria to form biofilm on the glass surface.

The biofilms were much less dense when phages were applied in preventive treatment (Fig. S1). Indeed, the application of Deep-Blue before the biofilm development induced 4.7 Log reduction of the bacterial counts inside the single strain biofilm whereas the addition of Deep-Blue on a mature biofilm caused a 2.1 Log reduction (Fig. 7). Similarly, in the three-strain biofilm, the bacterial count reductions were higher in preventive treatment (between 2.7 and 4 Log reduction as compared to the 0.8 to 2 Log reduction in curative application) (Fig. 8). When milk was used rather than LB, the reductions of bacterial counts were lower but the preventive treatment (prophylaxis) remained the most efficient phage application.

4. Discussion

In the context of foodborne diseases, some pathogens are resistant to current sanitation methods. This is particularly true for members of the *B. cereus* group, which are spore-forming bacteria able to survive in extremely harsh environments. Moreover, the cereulide toxin produced by the emetic strains is resistant to most preservation techniques used

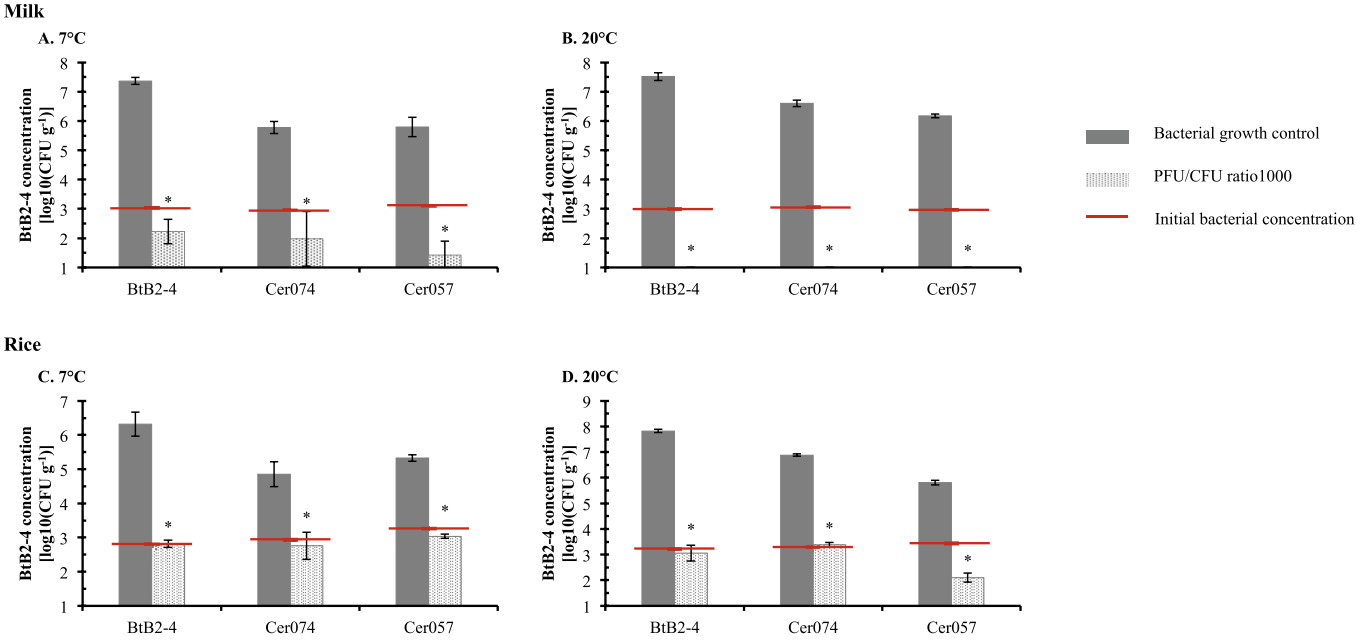

Fig. 6. Deep-Blue phage efficiency to inhibit *B. weihenstephanensis* BtB2-4, Cer074 and Cer057 growth in milk **(A, B)** and rice **(C, D)** after 7 days of incubation at 7 °C **(A, C)** and 48 h of incubation at 20 °C **(B, D)**. The bacterial concentration is given in logarithmic scale, with a LOD of 1 Log CFU mL^{-1}. The red line represents the initial bacterial concentration. Stars indicate statistical differences (Student test, *p*-value of 0.05) in comparison with the bacterial growth control. Means with standard deviations were calculated from triplicate assays. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

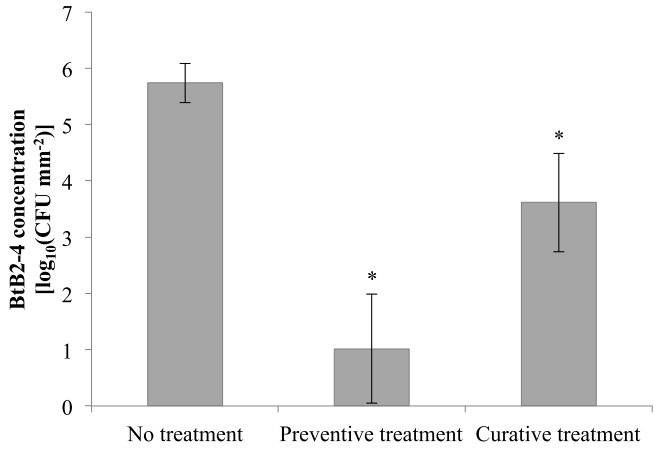

Fig. 7. Evaluation of *B. weihenstephanensis* BtB2-4 concentration in a 24-h biofilm in LB broth without phage treatment, and with preventive or curative Deep-Blue phage treatments. The bacterial concentration is expressed in logarithmic scale per surface unit, with a LOD of 1 Log CFU $(mm^2)^{-1}$. Stars indicate statistical differences (Student test, *p*-value of 0.05) in comparison with the biofilm control without phages. Means with standard deviations were calculated from triplicate assays.

in food industries. The application of phages as biocontrol agents of foodborne bacterial pathogens is a well-established strategy in the cases of *L. monocytogenes*, *S. enterica* and *E. coli* (FDA, 2015). In this work, phage Deep-Blue, isolated from soil, was characterized and its activity as biocontrol agent was assessed in biofilm and food matrices artificially contaminated by psychrotolerant *B. weihenstephanensis*.

In order to be a good candidate for biocontrol or therapy, a phage must fulfil certain conditions. First, the phage must not enhance bacterial virulence and therefore must be strictly lytic. According to its morphology observed by TEM and the ICTV classification, Deep-Blue is a member of Bastille-like group phage in the subfamily *Spounavirinae* (Barylski, Nowicki, & Goździcka-Józefiak, 2014; Gillis & Mahillon, 2014; Asare et al., 2015) of the *Myoviridae*. Moreover, the genome synteny with phages JBP901, Bcp1 and vB_BceM_Bc431v3 (GenBank Acc. # KJ676859, KJ451625, and JX094431, respectively), as well as the lack of bacterial pathogenicity genes and lysogenic determinants (prophage integration-mediating enzymes or prophage maintenance enzymes) (Hock et al., 2016) lend to support the virulent nature of Deep-Blue.

Second, the host spectrum of the candidate phage must correspond to the target. In this case, Deep-Blue infected five (out of twelve) *B. weihenstephanensis* strains and is apparently limited to the *B. cereus* group (it is also active on 2/10 and 2/5 of the *B. cereus s.s.* and *B. thuringiensis*, respectively). Consequently, it could be combined with one or several other phages in order to lyse all *B. weihenstephanensis* emetic strains. Conversely, Deep-Blue specific host spectrum suggests that it will most likely not interfere with food microflora or gut microbiota.

Third, the phage stability during food processing and storage is essential. Deep-Blue lytic activity was stable during 1 h at 40 °C and pH from 5 to 10, allowing appropriate phage biocontrol application to a large range of foodstuffs. In comparison, the *Bacillus Myoviridae* BPS10c and BPS13 phages were shown to resist at 50 °C during 1 h and for 24 h between pH 5 and 8.8 (Shin, Lee, Park, Heu, & Ryu, 2014). Also, FDA approved phage-based products guarantee an effective application between 2 °C and 42 °C (ListShield™ and SalmoFresh™, Intralytix). Fridge temperature (4 °C) was also appropriate to maintain an optimal lytic activity of the phage preparation during time.

Pathogenic bacteria must also be efficiently controlled by the phage under all conditions encountered during the entire shelf life of the food product. Deep-Blue efficiency to control *B. weihenstephanensis* BtB2-4 was therefore assessed in both artificial media and food matrices. In LB medium, Deep-Blue was able to reduce the bacterial growth, and the treatment was more effective when a higher phage concentration was applied. Regarding food matrices, the efficiency of Deep-Blue was evaluated in milk, milk rice and rice because psychrotrophic strains, such as *B. weihenstephanensis*, are regularly responsible for spoilage in the dairy industry (Owusu-Kwarteng, Wuni, Akabanda, Tano-Debrah, & Jespersen, 2017), the potential production of cereulide in milk is high (Ehling-Schulz, Fricker, & Scherer, 2004) and rice is commonly involved in emetic *B. cereus* intoxications (Delbrassinne et al., 2012). The addition of elevated concentrations of phage was necessary to control the bacterial development in milk stored under fridge conditions during one week. At room temperature, Deep-Blue was able to reduce the bacterial load below the initial artificial contamination whereas it slowed down the bacterial growth at 30 °C. The rate of bacterial cell divisions at 30 °C was probably faster than the phage-mediated bacterial cell killing. In rice, mixed with its cooking juice, Deep-Blue allowed to maintain the level of *B. weihenstephanensis* closed to the initial contamination, regardless of the host strains. In milk rice, Deep-Blue was only able to partially limit *B. weihenstephanensis* growth but the final bacterial concentration was higher than its initial level. This decrease in efficiency was probably due to the semi-solid nature of the matrix, which could have hampered the phage diffusion and consequently the phage-bacterium encounter (Hagens & Loessner, 2010). Moreover, phage biocontrol efficiency in food matrices (such as milk or milk rice) also depends on other factors including unfavourable pH (Leverentz et al., 2003), compounds that adversely affect the phage (Guenther, Huwyler, Richard, & Loessner, 2009) or interactions with other bacteria and/or molecules that prevent phage binding to host cell (Fister et al., 2016). In order to increase Deep-Blue efficiency in solid food, the likelihood of phages/bacteria interactions could be boosted

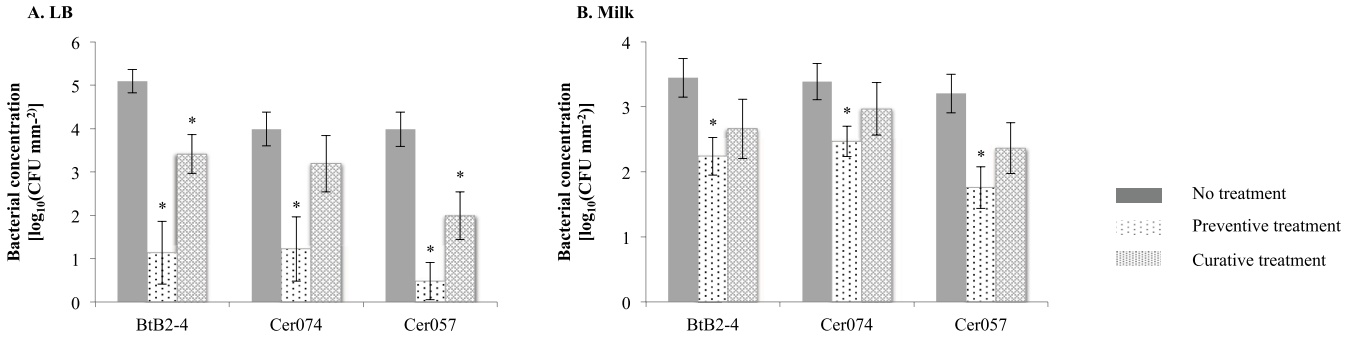

Fig. 8. Deep-Blue efficiency on biofilms containing BtB2-4 (Nal^R), Cer074 (Rif^R) and Cer057 (Sm^R) in LB (**A**) or milk (**B**). Estimation of *B. weihenstephanensis* concentrations was made in 24-h multi-strain biofilms without phage treatment, and with preventive or curative Deep-Blue phage treatments. The bacterial concentration is expressed in logarithmic scale per surface unit, with a LOD of 1 Log CFU $(mm^2)^{-1}$. Stars indicate statistical differences (Student test, *p*-value of 0.05) in comparison with the biofilm control without phages. Means with standard deviations were calculated from triplicate assays.

with the application of higher phage concentrations and/or by thoroughly mixing the phage suspensions with the food matrix.

By comparison, other myoviruses, such as FWLBc1 and FWLBc2, were able to reduce by more than 6 Log CFU mL^{-1} the *B. cereus* concentration in mashed potatoes stored for 24 h at 25 °C (Lee et al., 2011), whereas the siphovirus PBC1 lowered the bacterial contamination in rice to an undetectable level (Kong & Ryu, 2015). These efficacies are similar to those of Deep-Blue in milk but a lower efficiency was observed in our case for milk rice. However, solid food matrices used in previous studies (Kong & Ryu, 2015; Lee et al., 2011) were diluted at least five times in water, which would allow a better phage diffusion and accessibility to bacteria, but not actually mimicking the marketed food product.

Comparatively to FDA approved phages, ListShield™ allows a reduction of *Listeria* spp. ranging from 2 to 4.6 Log CFU g^{-1} in melons stored at 10 °C during one week (Leverentz et al., 2003), SalmoFresh™ decreases the population of *Salmonella* by 1.3 Log CFU g^{-1} after 7 days in turkey breast cutlets stored at 4 °C (Sharma, Dhakal, & Nannapaneni, 2015). With the addition of Deep-Blue in milk and milk rice, a decrease of 0.4 and 2 Log CFU g^{-1} was observed after 7 days at 7 °C. Unlike antibiotics which eliminate the target bacteria, the aim behind the use of phages is to control the target bacterial population and therefore prevent, or considerably limit, the appearance of resistant bacteria.

Concerning its application as biosanitizer to control bacterial biofilm, Deep-Blue was most efficient against *B. weihenstephanensis* BtB2-4 biofilms on glass as preventive treatment, rather than the curative approach. Phage infection of planktonic cells is easier than biofilm forming cells, which are incorporated in a complex matrix (Pires, Melo, Boas, Sillankorva, & Azeredo, 2017). A 24 h application of Listex™ on 2-day biofilms developed on stainless steel allowed a *L. monocytogenes* reduction of 5.4 Log CFU $(cm^2)^{-1}$ in static conditions (Soni & Nannapaneni, 2010). These results are similar to those observed for Deep-Blue in preventive treatments.

5. Conclusions

Taken together, Deep-Blue is a novel potential biocontrol candidate against *B. weihenstephanensis* and its emetic pathotypes, thanks to its efficient control, even at low temperatures, of both bacterial growth in food matrices and biofilm development. The inclusion of Deep-Blue in a cocktail of phages displaying distinct and complementary host ranges should limit the emergence of bacterial resistance and help to better control the entire spectra of *B. weihenstephanensis* strains.

Further developments are still necessary before an industrial application of Deep-Blue in food matrices. First, an in-depth study of Deep-Blue stability on target food matrices should be performed. Second, the method of phage application still needs to be optimised, including the possible combinations of Deep-Blue with other bactericidal or bacteriostatic compounds (e.g. bacteriocins). Finally, the inclusion of Deep-Blue in multi-phage cocktails is also to be developed.

Conflicts of interest

The authors declare no conflict of interest.

Funding sources

This work was supported by the FRIA (*Fonds pour la formation à la Recherche dans l'Industrie et dans l'Agriculture*) through a grant to L. Hock, the FNRS (National Fund for Scientific Research) through grants to A. Gillis and J. Mahillon, the Université Catholique de Louvain (UCLouvain) and the Research Department of the Communauté française de Belgique (Concerted Research Action, ARC12-17/046).

Acknowledgments

We are grateful to P. Lipnik and D. Magnin for their technical assistance with transmission electron microscopy. We also thank C. Nannan for her help in the biofilm experiments. We also express our appreciation to S. Hiel and S. Pötgens for their contribution to the initial phage characterization and to O. Henriet for his critical reading of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2019.03.014.

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