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Effect of high pressure homogenization on lactic acid bacteria phages and probiotic bacteria phages

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ABSTRACT

To investigate the effect of high pressure homogenization on virus inactivation, phages specific for *Lactobacillus delbrueckii*, *Lactobacillus helveticus*, *Streptococcus thermophilus*, *Lactococcus lactis*, *Lactobacillus paracasei* and *Lactobacillus plantarum* were studied. The influence of pressure, number of passes, suspension medium and phage concentration were studied at 25 °C. Reductions in viability were proportional to pressure and number of passes, though the inactivation extent was phage-dependent. At 100 MPa, some bacteriophages were completely inactivated (6 $\log_{10}$ reduction) after 3 or 5 passes, while others remained infective after 8 passes. For all phages, treatment at 60 MPa was insufficient for complete inactivation, even after 8 passes. No clear influence of suspending medium was observed. Inactivation seems to depend on phage concentration; the higher the initial load, the bigger the reduction achieved. Although these results showed that several phages studied are resistant to high-pressure homogenization, this strategy could be combined with others to control their presence in raw milk.

1. Introduction

High pressure processing is one of the emerging preservation technologies designed to produce safe food, maintaining its nutritional and sensory qualities (Lado and Yousef, 2002). Furthermore, it offers opportunities for developing new foods differentiated by sensory and structural characteristics and with different functional properties compared with traditional products (Fonberg-Broczek et al., 2005; Guerzoni et al., 1999; Kheadr et al., 2002). The development of technologies as alternatives to thermal treatment has received considerable attention in response to consumer demands for more "fresh" and "natural" food products. These so-called "non-thermal" technologies have the ability to inactivate microorganisms at room or near room temperature, avoiding the deleterious effect that heat has on flavour, colour and nutrient value of foods (Ross et al., 2003).

Among these, great attention has focussed on technologies based on the application of high pressure, namely high hydrostatic pressure and high pressure homogenization. Although they probably share some action mechanisms, the dynamics of the two processes do not coincide. In fact, during high pressure homogenization the fluid is forced through a narrow gap, after which it is subjected to an ultra-rapid depression. In particular, when the local pressure in a liquid is reduced without temperature change, gas-filled bubbles (or cavities) nucleate and grow within the body of liquid. The collapse of such cavities could transmit several localized forces to surfaces or particles, including the microbial cell (Middelberg, 1995). Moreover, in high-hydrostatic pressure treatment, the exposure time is in the order of minutes or more, while in high pressure homogenization it is only in the order of a second or less (Wuytack et al., 2002). Furthermore, for each technology the efficacy of inactivation depends on several parameters (Diels and Michiels, 2006; Dilek Avsaroglu et al., 2006; Grove et al., 2006; Kheadr et al., 2002; Lanciotti et al., 2004a; Müller-Merbach et al., 2005; Thiebaud et al., 2003).

High pressure homogenization (HPH) is a unit process used in the food, chemical, cosmetic and pharmaceutical industry, primarily to prepare or stabilize emulsions and suspensions. Research into new applications has been stimulated by the development over the last few years of homogenizing equipment that operates at increasingly higher pressures (Diels et al., 2005; López-Pedemonte et al., 2006). Some investigations were carried out about potential applications of HPH in the processing of liquid milk (Hayes et al., 2005; Pereda et al., 2007; Thiebaud et al., 2003)

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showing modifications of physicochemical properties of milk, the activity of its enzymes, and the processes of lipolysis and proteolysis. Other studies have evaluated the effects of HPH on cheese-making properties of milk (Burns et al., 2008; Lanciotti et al., 2004a; Vannini et al., 2008; Zamora et al., 2007) and on the production of yoghurt, probiotic fermented milk and cheese (Burns et al., 2008; Lanciotti et al., 2004b, Patrignani et al., 2007). The inactivation of several microorganisms by HPH has also been assayed (Fonberg-Broczek et al., 2005; López-Pedemonte et al., 2006; Vachon et al., 2002; Wuytack et al., 2002).

Currently, very little is known about the effect of HPH on viruses (Diels and Michiels, 2006; Moroni et al., 2002), particularly bacteriophages. Moroni et al. (2002) used a laboratory device for evaluating the influence of media, pressure, number of passes and initial viral concentration, on the viability of three different species of lactococcal phages. Up to now, this is the only report on bacteriophage inactivation by HPH. Other authors have reported the comparison of viral and phage inactivation by hydrostatic high pressure and a particular treatment that they called hydrodynamic pressure processing (Sharma et al., 2008).

Bacteriophages are one of the principal causes of failed fermentations on dairy processes (Heap and Harnett, 2002) and raw milk is one of the major sources of phages in dairy plants (Neve, 1996). Because HPH seems to be one of the most promising technological alternatives to pasteurization, which is not always useful for inactivation of dairy phages (Capra et al., 2004; Moineau, 1999; Suárez et al., 2007), the principal aim of the present study was to investigate the effect of the application of HPH on the viability of bacteriophages of lactic acid- and probiotic bacteria.

2. Materials and methods

2.1. Bacterial strains, bacteriophages and culture conditions

Table 1 lists the host strains, bacteriophages and culture conditions used in this study. Strains were maintained as frozen stocks at −80 °C in the corresponding medium supplemented with 15% (v/v) glycerol, and routinely reactivated overnight at the appropriated temperature and medium. Phage stocks were prepared as described by Neviani et al. (1992) in broth, supplemented with 10 mM $CaCl_2$, and stored at 4 °C (broth), and at −80 °C (broth with 15%, v/v, glycerol).

2.2. Plaque assay titration

Phage were enumerated [in plaque forming units (pfu) mL^{-1}] by the double-layer plate titration method (Svensson and Christiansson, 1991), using MRS (Biokar, Beauvais, France) for lactobacilli, or Elliker (Biokar, Beauvais, France) for lactococci, supplemented with agar (1.2% w/v), 10 mM $CaCl_2$ and 100 mM glycine (Lillehaug, 1997). Plates were incubated at the corresponding temperature for each phage host system (Table 1), under microaerophilic conditions.

2.3. High-pressure homogenization treatments

2.3.1. Experimental procedures

A continuous high-pressure homogenizer PANDA (Niro Soavi, Parma, Italy) was used for all homogenization treatments. The machine was supplied with a homogenizing PS type valve with a flow rate of 10 L h^{-1}. The valve assembly includes a ball-type impact head made of ceramics, a stainless steel large inner diameter impact ring and a tungsten carbide passage head. The temperature increase was about 0.25 °C MPa^{-1}.

2.3.2. Suspension media, phage type, applied pressure and number of passes

MRS broth (used for lactobacilli phages) or Elliker broth (used for lactococci phages) and reconstituted skim milk (RSM) were inoculated with one of the eight bacteriophages listed in Table 1, with an initial load of 10^6 pfu mL^{-1}. These suspensions were subjected to HPH treatment for up to 8 successive passes at 100 MPa. Viable phage particles were counted at the beginning of the assay and after 1, 3, 5 and 8 passes. At these points of sampling, temperatures of the phage suspension before passing through the homogenizer (inlet temperature) and after its pass (outlet) were also measured. The same experimental design, performed in model (broth) and skim milk, was also tested at 60 MPa for 8 passes, but only three phages (MLC-A, B1 and 13.2) were tested.

2.3.3. Effect of initial phage load and applied pressure

To evaluate the effect of inoculum level on sensitivity of phages to HPH treatment, MLC-A phage was inoculated in RSM at levels ranging between 10^2 and 10^6 pfu mL^{-1} and then subjected to a single-pass treatment at 100 and 60 MPa.

Table 1
Phages, sensitive strains and culture conditions used in this study.

Phage	Phage classification (Ackermann, 2001)	Origin/date of isolation[a]	Sensitive strain[b]	Reference	Culture conditions (broth[c], incubation temperature)
Bym	*Siphoviridae* (morphotype B1)	Yogurt production (Argentina, 1997)	*Lactobacillus delbrueckii* subsp. *bulgaricus* YSDV	Quiberoni et al. (2004)	MRS, 42 °C
832-B1	*Myoviridae*	CNRZ	*Lactobacillus helveticus* CNRZ 892		MRS, 45 °C
QP4	*Siphoviridae* (morphotype B1)	Cheese production (Argentina, 1999)	*Lactococcus lactis* Ll 15.1	Suárez et al. (2008)	Elliker, 30 °C
QF12	*Siphoviridae* (morphotype B1)	Industrial plant of Fymbo cheese (Argentina, 1998)	*Lactococcus lactis* Ll Mo12	Suárez et al. (2008)	Elliker, 30 °C
13.2	*Siphoviridae* (morphotype B1)	Large-scale-production cheese plant (Argentina, 2000)	*Streptococcus thermophilus* 13.2	Quiberoni et al. (2006)	Elliker and RSM, 42 °C
B1			*Lactobacillus plantarum* ATCC 8014		MRS, 34 °C
MLC-A	*Siphoviridae* (morphotype B1)	Probiotic dairy product (Argentina, 2003)	*Lactobacillus paracasei* A	Capra et al. (2006)	MRS, 37 °C[d]
MLC-A8	*Siphoviridae* (morphotype B1)	Probiotic dairy product (Argentina, 2005)	*Lactobacillus paracasei* A	Capra, 2007	MRS, 37 °C[d]

[a] CNRZ: Centre National de la Recherche Zootechnique.
[b] ATCC: American Type Culture Collection, Manassas, VA.
[c] MRS: de Man, Rogosa, and Sharpe broth (Biokar, Beauvais, France). RSM: reconstituted (10%) sterile skim powder (Oxoid, Basingstoke, UK). Elliker broth (Biokar, Beauvais, France).
[d] Although *Lb. paracasei* cultures were incubated at 37 °C, plates for phage enumerations were incubated at 34 °C.

2.3.4. Effect of phage type, applied pressure and number of passes at a phage concentration typical of raw milk

To assess the effect of different pressures at a certain phage concentration, typical of those found in raw milk (10^2–10^3 pfu mL^{-1}), milk suspensions of phages MLC-A (of *Lactobacillus paracasei*), B1 (of *Lb. plantarum*) and 13.2 (of *Streptococcus thermophilus*) inoculated in RSM were tested for their sensitivity to two different pressures (60 and 100 MPa). Phage enumerations were performed immediately after HPH treatment, as previously reported (Svensson and Christiansson, 1991).

2.4. Statistical analysis

Experiments were replicated three times. All data were analysed using the one-way analysis of variance (ANOVA) procedure of SPSS. Differences among means were detected by Duncan's multiple range test (Lizasoain and Joaristi, 1995).

3. Results

3.1. Influence of suspension medium, phage type, applied pressure and number of passes

Different behaviours were observed within the eight phages studied (Fig. 1) when their suspensions (10^6 pfu mL^{-1}) were subjected to HPH treatment at 100 MPa for 1, 3, 5 or 8 passes. It was noteworthy that there were differences between phages MLC-A and MLC-A8, both lytic for the same strain of *Lb. paracasei*, the latter being more sensitive than the former, in both broth and milk. Among all phages tested, φ CNRZ 832-B1 (of *Lb. helveticus*) was the most sensitive, showing a reduction of almost 2.5 $\log_{10}$ after one pass at 100 MPa, and being completely inactivated in MRS broth after three passes (Fig. 1A). In general, the greater the number of passes, the higher the rate of inactivation reached. However, even after a treatment of eight passes at 100 MPa, 50% of the phages studied remained viable at a concentration of at least 10 pfu mL^{-1}, in broth and 37.5% of them remained viable at a concentration over 60 pfu mL^{-1}, in RSM. No clear influence of suspension medium was evident (Fig. 1A and B). While several phages had similar responses in broth and RSM, others (φ CNRZ 832-B1, QP4 and MLC-A) seemed to be protected by RSM or by broth (QF12 and 13.2).

In both media, phages MLC-A (of *Lb. paracasei*) and B1 (of *Lb. plantarum*) were the most resistant, retaining after 8 passes at 100 MPa concentrations of viable phage particles of 1.6×10^2 and 7.7×10^2 pfu mL^{-1}, respectively. The inlet temperature for the first pass and the outlet temperature after 8 passes (at 100 MPa for each pass) were 21–24 °C and 60–64 °C, respectively, The measured temperatures for the other passes (third and fifth) were comprised in this range and a progressive increase was recorded as the number of passes increased.

For studies at 60 MPa, only three phages were tested (Fig. 2). In this case, it was not possible once again to demonstrate a protective effect of either media (broth or RSM). The probiotic bacteria phages, MLC-A and B1, showed again a higher resistance to HPH than the *S. thermophilus* phage 13.2. Titres of MLC-A and B1 remained between 3.8 and 5.3 $\log_{10}$ cycles after 8 passes, and phage 13.2 counts decreased up to 10^2 pfu mL^{-1}. Thus, treatment at 60 MPa was showed to be inadequate for the complete inactivation of 10^6 pfu mL^{-1}, even for a phage that exhibited an intermediate resistance against HPH treatment, and such a treatment, needed a high number of passes.

3.2. Influence of initial phage load and applied pressure

To study the influence of initial phage concentration, phage MLC-A suspended in RSM was tested using different initial phage concentrations ranging between 10^2 and 10^6 pfu mL^{-1} for one-pass treatments at 60 and 100 MPa (Fig. 3A and B). After one pass at 60 MPa (Fig. 3A), almost no differences were obtained for initial loads of 10^2, 10^3 and 10^4 pfu mL^{-1}. For the other two concentrations tested (10^5 and 10^6 pfu mL^{-1}), a decrease in viable phage concentration was observed, but reductions were only around 0.5 $\log_{10}$.

As expected, better results were obtained when the treatment was performed at 100 MPa (Fig. 3B). Similarly, for treatments at 60 MPa at higher initial loads (10^5 and 10^6 pfu mL^{-1}), significant reductions (more than 1.6 $\log_{10}$ cycles) occurred, while for the low concentrations (10^2, 10^3 and 10^4 pfu mL^{-1}), the inactivation was <1.0 $\log_{10}$.

In any case, neither treatment at 60 nor at 100 MPa was able to completely inactivate viable MLC-A phage particles, even for low initial phage loads, e.g., 10^2 pfu mL^{-1}.

3.3. Influence of phage type, applied pressure and number of passes at a phage concentration typical of raw milk

Among the three phages tested, MLC-A, B1 y 13.2 (specific for *Lb. paracasei*, *Lb. plantarum* and *S. thermophilus*, respectively), those lytic for the probiotic species (MLC-A and B1) were more resistant

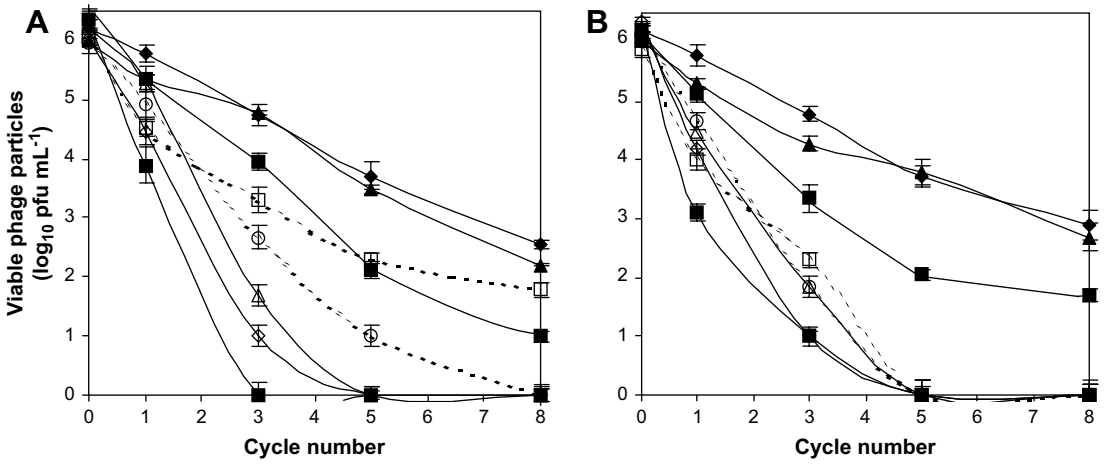

Fig. 1. Effect of high pressure homogenization at 100 MPa on the viability of lactic acid- and probiotic bacteria phages in (A) model system (MRS broth for lactobacilli phages and Elliker broth for streptococci- and lactococci phages) and (B) in reconstituted skim milk (RSM). Phages MLC-A8 (△), MLC-A (▲), Bym (○), 13.2 (○, dotted line), CNRZ 832-B1 (■), QP4 (■, dotted line), QF12 (□, dotted line) and B1 (◆). Values are the mean of data from three determinations.

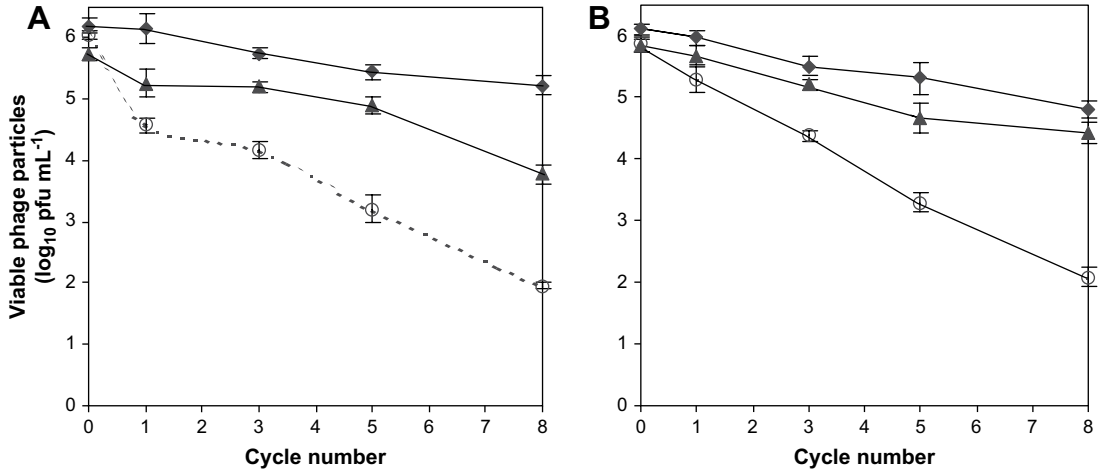

Fig. 2. Effect of high pressure homogenization at 60 MPa on the viability of phages 13.2 (○, dotted line), B1 (◆) and MLC-A (▲) in (A) model system (MRS broth for lactobacilli- and Elliker broth for the streptococci-phage) and (B) reconstituted skim milk (RSM). Values are the mean of data from three determinations.

than phage 13.2 (Figs. 1 and 4). HPH at 60 MPa was not sufficient for the complete inactivation of these three phages suspended in milk with initial concentrations of approximately 10^2 pfu mL^{-1}, even for the less resistant phage 13.2 (Fig. 4). In fact, viable counts for MLC-A and B1 remained practically unchanged at this pressure and their titres remained between 10 and 100 pfu mL^{-1} at 100 MPa (3 passes). In contrast, the phage 13.2 was completely inactivated (2.3 $\log_{10}$ reduction) when it was treated at 100 MPa even for one pass.

4. Discussion

The use of novel technological alternatives to heat treatment for inactivation of phages or to minimize the threat of their infections in the dairy industry is of interest, since most bacteriophages are able to survive conventional pasteurization. Temperatures reached after the pressure treatments used in this work were too low to inactivate completely the phages tested (Binetti and Reinheimer, 2000; Capra et al., 2004, 2006; Quiberoni et al., 1999, 2003; Suárez and Reinheimer, 2002). The few studies on the effects of HPH on viral inactivation indicate that this technology may also be promising for this purpose; therefore, this study regarding the effects of HPH on the viability of lactic acid- and probiotic bacteria phages may be of potentially high significance.

The inactivation of phages specific for *Lb. bulgaricus*, *Lb. helveticus*, *S. thermophilus*, *Lactococcus lactis*, *Lb. paracasei* and *Lb. plantarum* was dependent on the number of passes, phage type and pressure applied, though the last factor was only tested for phages 13.2 (of *S. thermophilus*), B1 (of *Lb. plantarum*) and MLC-A (of *Lb. paracasei*). In fact, at 100 MPa, some bacteriophages were completely inactivated (6 $\log_{10}$ reduction) after 3 or 5 passes, while others remained infective after 8 passes. Diels and Michiels (2006) obtained more than 1 log-cycle inactivation of Hepatitis A virus using HPH at 300 MPa and 5 passes through the homogenizing valve. Also, Moroni et al. (2002) obtained a 5 log-cycle reduction in lactococcal phages at 200 MPa after 5 passes and found that a phage with a prolate capsid (c2) was more sensitive than two others with isometric capsids.

The effect of HPH on phages could be due to alteration of their protein structure (Grove et al., 2006). In fact, it has been reported that HPH could induce a structural rearrangement of proteins, e.g., by increasing the exposure of their hydrophobic regions (Guerzoni et al., 1999). The capsid, which protects the nucleic acid, and tail structures, which are essential for the binding of phages to the bacterial cell, are both proteins and thus could be the putative targets for high pressure treatments (Guan et al., 2006; Kingsley et al., 2002; Moroni et al., 2002). Furthermore, the sophisticated structure of the tail (base plate, fibres) may increase the pressure sensitivity of the phage (Guan et al., 2006).

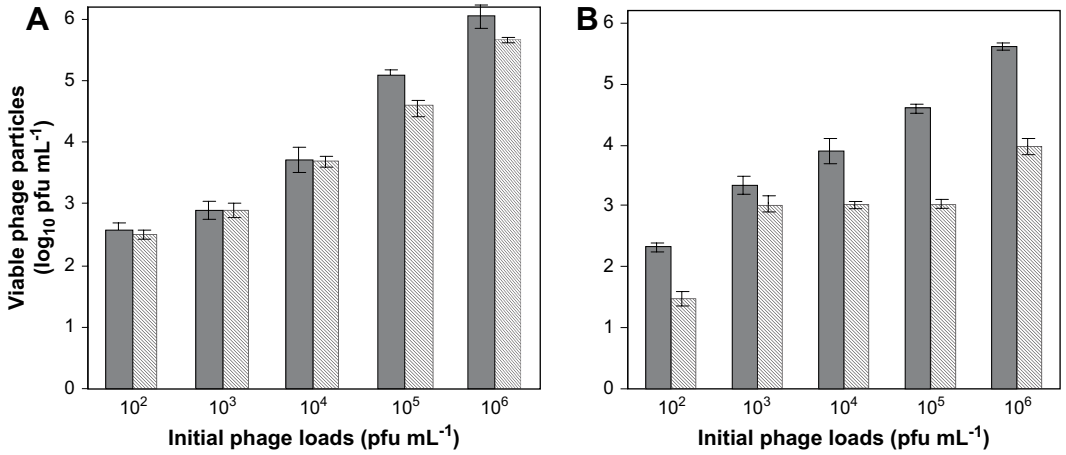

Fig. 3. Influence of initial phage concentration (milk suspension inoculated with phage MLC-A) on inactivation following high pressure homogenization at (A) 60 MPa and (B) 100 MPa; untreated samples (dark grey bars), treated samples (light grey bars). Values are the mean of data from three determinations.

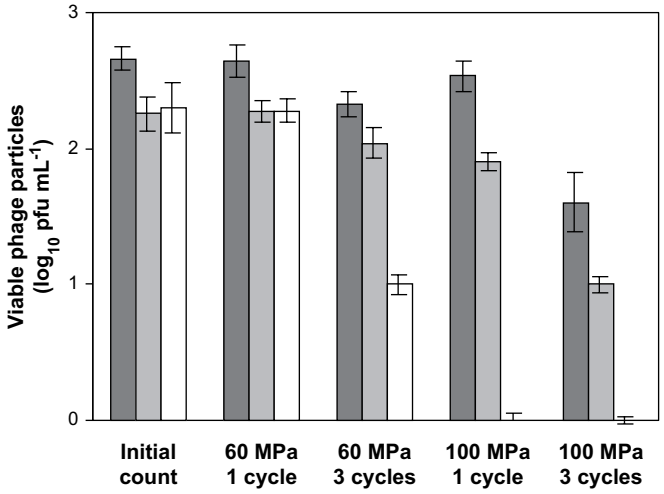

Fig. 4. Effect of different high pressure homogenization treatments on the viability of phages MLC-A (dark grey bars), B1 (light grey bars) and 13.2 (white bars) suspended in reconstituted skim milk (RSM) at approximately 10^2 pfu mL^{-1}.

Regarding phage strains, lactococcal phages QP4 and QF12 demonstrated an intermediate resistance compared to all the other phages assayed in this research. Moreover, these phages were more resistant than c2 (Moroni et al., 2002) because, after a treatment at 200 MPa for one pass, almost 10^2 c2 phage particles per mL remained viable, while the phage titres obtained for the study cited were higher even after HPH at 100 MPa for 5 passes. Also that additive effects, obtained when subsequent passes were applied, need to be considered. In addition, results show that two related phages (MLC-A and MLC-A8), lytic for the same probiotic strain, showed remarkably different behaviour when treated with HPH. Similarly, Kingsley et al. (2007) also observed that different viruses and even different strains of viruses could differ in susceptibility to high hydrostatic pressure. Furthermore, Guan et al. (2006) indicated that a similarity in viral morphological structure did not guarantee comparable inactivation behaviour by using hydrostatic pressure, which may result from the different stability of capsid proteins to pressure.

Concerning the suspending medium, no clear influence can be concluded, either for RSM or broth, on inactivation of phage by HPH. In contrast, Chen et al. (2004) reported differences in the inactivation kinetics of phage λcI 857 in milk and in SM buffer. Also, Moroni et al. (2002), using a continuous HPH system, found that the treatment was generally less effective in milk and whey permeate than in phosfate buffer solution (PBS), showing that milk and whey permeate components may provide protection. Analogously, Diels et al. (2005) stated that the variation in the inactivation of *Escherichia coli* by HPH was influenced by media (buffer and milks with different fat contents), though this could be explained only by viscosity differences between them.

The results obtained, for a resistant phage (MLC-A) indicated that the efficacy of the treatment was also dependent on the initial virus concentration. At both pressures, the highest rates of inactivation were obtained for initial loads of 10^5–10^6 pfu mL^{-1} (the highest concentrations tested). In contrast, Moroni et al. (2002), who tested initial loads of 10^4–10^9 pfu mL^{-1}, reported that the treatment became less effective with greater initial load.

It is worth noting that, even for phage concentrations in raw milk lower than the critical threshold of 10^4 pfu mL^{-1} (Moineau and Lévesque, 2005), HPH at 60 or 100 MPa for one pass was not enough to completely inactivate MLC-A phage particles. In fact, phages MLC-A and B1 were not inactivated even after treatment at 100 MPa for 3 passes.

The results of this work revealed that the phages tested are quite resistant to the pressures previously applied to milk for the production of yogurt and Crescenza cheese (60 and 100 MPa, respectively, for a single pass) (Burns et al., 2008). Consequently, in such conditions, HPH treatment cannot be used as the sole barrier against phage presence in raw milk. The application of more severe HPH treatments is required for complete inactivation of some of the studied phages. Pereda et al. (2007) found that HPH at 300 MPa at 30 °C could be a good option to perform milk homogenization and pasteurization at the same time, although further research is necessary to evaluate the effect of this treatment on the sensory and nutritional aspects of milk as. Alternatively, it cannot be excluded that the application of more passes at 100 MPa can produce milk having technological properties suitable for cheese-making. On the other hand, some authors (Lado and Yousef, 2002; Ross et al., 2003) mentioned the possibility of combining non-thermal with conventional preservation processes (such as addition of organic acids or natural antimicrobial compounds, diverse temperature treatments) or with other non-thermal process to enhance microbial inactivation. Diels and Michiels (2006) reported that the addition of some compounds (such as lytic enzymes, Sodium Dodecyl Sulphate (SDS), thylenediaminetetraacetic acid (EDTA), ions) before homogenization might increase microbial sensitivity to HPH. For phages, any substance causing instabilization of protein capsid should be tested for enabling use of reduced HPH treatment intensities.

5. Conclusions

Lactic acid bacteria phages and probiotic bacteria phages have a high resistance against HPH treatments (≤100 MPa); thus, the treatments tested demonstrated a limited efficiency for inactivation of phage in raw milk. Probably, the use of higher pressures (≥100 MPa) will increase efficiency of phage inactivation; however, such research should be accompanied by determination of their effect on physicochemical and biochemical raw milk characteristics.

Acknowledgments

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