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Carrier systems for bacteriophages to supplement food systems: Encapsulation and controlled release to modulate the human gut microbiota

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ABSTRACT

The use of phages in food systems to modulate the human gut microbiota is currently gaining increasing interest. However, several studies have reported low viability of phages under gastrointestinal conditions. Furthermore, processed foods nowadays contain only few phage particles. Hence, this study aimed at encapsulating phages to increase their survival under gastrointestinal conditions allowing the release of phages in active form in the intestine.

Lactococcus phage P008 was entrapped in different matrix materials, using three different encapsulation techniques (i.e., emulsion or extrusion method). Results showed that non-encapsulated phages were sensitive to acid conditions at pH 2.0. However, the investigated capsules which contained milk proteins provided good protection of phages at pH 2.0: a phage reduction of only 0.5 log-units was observed after incubation for 120 min at 37 °C. The release of phages from capsules after 2 h incubation in simulated intestinal fluid (pH 6.8) was also determined. Depending on the encapsulation technique, phages were completely released from the capsules. Micrographs confirmed these observations and showed a dissolution of the capsule matrix due to swelling and permeation of pancreatic enzyme. The study indicates that encapsulated phages may be targeted via food formulation to the intestine to modulate the human gut microbiota.

1. Introduction

Bacteriophages and their occurrence in the fermentation industries have been described in detail in various studies (Atamer et al., 2009; Barrangou, Yoon, Breidt, Fleming, & Klaenhammer, 2002; Brüssow, 2001; Deasy, Mahony, Neve, Heller, & Van Sinderen, 2011; Marco, Moineau, & Quiberoni, 2012; Müller-Merbach, Rauscher, & Hinrichs, 2005; Samtlebe et al., 2015). However, in recent years it has been widely recognized that bacteriophages are not only a significant problem in the production of fermented products, (i.e., cheese, yoghurt), but that they may also be associated with beneficial use. Several opportunities and application fields in the food industry for the use of phages are receiving increasing attention (Kutateladze & Adamia, 2010; Lu & Koeris, 2011; Tsonos et al., 2014; Wittebole, De Roock, & Opal, 2014). In food production and processing, phage may be relevant to improve food safety and quality, and have been proposed either for detecting pathogenic bacteria or as biocontrol agents (García, Martínez, Obeso, & Rodríguez, 2008; Gill, 2010; Hagens & Loessner, 2007; Hudson, Billington, Carey-Smith, & Greening, 2005).

Various studies have suggested an interrelation between gut microbiome, human health and diseases (Clemente, Ursell, Parfrey, & Knight, 2012; Dalmasso, Hill, & Ross, 2014; Dave, Higgins, Middha, & Rioux, 2012; Mills et al., 2013; Reyes et al., 2010). The bacterial composition of the human intestinal microbiota was

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found to be highly specific for individuals, and the complex composition of the bacterial populations was shown to be not consistent, but rather subject to many fluctuations resulting from changes in human diet, intervention with antibiotics, health status or age (Lepage et al., 2013; Power, O'Toole, Stanton, Ross, & Fitzgerald, 2014). In recent studies, numerous phage populations have been characterized from human gut samples (Dalmasso et al., 2014; Hoyles et al., 2014; Lepage et al., 2013; Waller et al., 2014). In this regard, bacteriophages have been discussed to control, shape and modulate the microbiota associated with the human gastrointestinal tract. Waller et al. (2014) have shown that phage abundance, diversity and variation are related to the human host and thus to a personalized feature. As phage are (i) ubiquitous, (ii) natural predators of bacteria and (iii) represent the most abundant biological entity on earth with an estimated population of about 10^{31}, they have a substantial potential for use in food formulations to alter the composition of the gut microbiota via the diet. Müller-Merbach, Peter, Weidendorfer, and Hinrichs (2007) showed that phages have limited diffusion characteristics in viscous media and hence are not able to inactivate bacterial populations in the gut completely. Furthermore, phages are generally highly host strain specific. Thus, they may be selected as to not infect the healthy intestinal microbiota (Abeles & Pride, 2014; Dalmasso et al., 2014; García et al., 2008; Hendrix, 2003; Mills et al., 2013). Nevertheless, the application of phage in food formulations faces numerous challenges, i.e., limited host ranges, bacterial resistances to phage, manufacturing issues and delivery systems (Hagens & Loessner, 2010; Hudson et al., 2005; Tang et al., 2013; Tsonos et al., 2014).

In a former study, the effects of acid and alkaline solutions on the activity of *Lactococcus lactis* phages were investigated. Although phages survived at pH values of 3–10, they were completely inactivated at pH 2 after san incubation time of 10 min (Atamer & Hinrichs, 2010). Other studies confirmed these results and showed that the acidic conditions of the gastrointestinal tract are critical and need to be carefully analyzed (Ma et al., 2008; Smith, Huggins, & Shaw, 1987).

In an increasing number of publications, encapsulation of bioactive compounds such as proteins, antibodies and probiotics within different matrices was suggested to protect them from the chemical conditions of the gastrointestinal tract (Heidebach, Först, & Kulozik, 2009; Picot & Lacroix, 2004; Schell & Beermann, 2014). However, only few methodologies have so far been applied to encapsulate bacteriophages. No data on the encapsulation of phages in enzymatically induced milk gels are currently available. Moreover, to the best of our knowledge, previous studies analyzed either the effect of acidic gastric fluid, or of intestinal fluid on survival of phages, but not their combined effects. (Ma et al., 2012; Tang et al., 2013; Tang, Huang, Sabour, Chambers, & Wang, 2015; Vonasek, Le, & Nitin, 2014). The survival and release of phages after exposure to first the gastric fluid and subsequent to the intestinal fluid, has not yet been determined.

In this study, it was hypothesized that phage can be encapsulated in such a way, that they resist the passage through the gastrointestinal tract and that they are thus released in active form in the intestine. Different methods for encapsulation of infective phage particles used in previous related studies (Heidebach et al., 2009; Ma et al., 2012; Picot & Lacroix, 2004) were therefore tested and optimized. A commonly studied dairy phage, *L. lactis* phage P008 (Loof, Lembke, & Teuber, 1983) was used as model phage for the experiments. Tolerance of both free and encapsulated phages to simulated human stomach conditions, and release of encapsulated phages in simulated intestinal fluid was determined in *in-vitro* experiments.

2. Material and methods

2.1. Bacteriophage and host strain

The well described *L. lactis* bacteriophage P008, which belongs to the 936 phage species (Braun, Hertwig, Neve, Geis, & Teuber, 1989) was propagated as described before (Samtlebe et al., 2015) and concentrated by cesium chloride density gradient centrifugation essentially done according to Sambrook and Russel (2001) (final phage titer of 5×10^{12} pfu mL^{-1}). Its mesophilic host *L. lactis* subsp. *lactis* biovar. *diacetylactis* F7/2 was grown at 30 °C in M17-broth (Merck Millipore, Schwalbach, Germany) (Terzaghi & Sandine, 1975) supplemented with 0.5% (w/v) lactose and 10 mM $CaCl_2$. Titers of phage P008 in suspensions were determined as plaque-forming units (pfu) per mL using the double agar layer method (plaque assay) of Adams (1959).

2.2. Preparation of encapsulated phages

Encapsulated phages were prepared using the three different methods shown in Fig. 1. Phages were either encapsulated using an emulsion method with enzymatically gelled milk protein (1A), or by extrusion with alginate (1B) or alginate mixed with whey protein (1C), respectively.

2.2.1. Encapsulation in enzymatically gelled milk protein

The method of Heidebach et al. (2009) was modified to prepare enzymatically gelled milk protein capsules (Fig. 1A). Skim milk powder (VWR International, Leuven, Belgium) was suspended in distilled water at a concentration of 35% (w/w) overnight at 5 °C. The milk-protein-suspension (30 g) was spiked with 100 μL of P008 phage stock. The initial phage titer (N_0) in the milk-protein-phage-mixture was determined after gently stirring for 10 min. The mixture was incubated with 60 μL rennet (200 IMCU (international milk clotting units) mL^{-1}) at 5 °C for 60 min. Then, 180 μL $CaCl_2$ (100 mM) was added and the mixture was stirred for another 5 min. Fifteen grams of milk-protein-phage-mixture were emulsified in 200 mL sunflower oil (5 °C), while mixing with a magnetic stir bar for 5 min. After this, gelation was induced by increasing the temperature to 40 °C and stirring was continued for another 15 min. The resulting microcapsules were separated from the oil by low-speed centrifugation (500× g, 1 min, 20 °C). Subsequent to removing the supernatant, the sedimented microcapsules were washed with distilled water, centrifuged again as described before and finally stored at 5 °C. The amount of encapsulated phages (N_0*) per gram wet weight was determined by destroying the microcapsules mechanically. For this, 1 g glass beads (⌀ 1 mm) and 1 g capsule-sediment were added to a 2 mL reaction tube. The microcapsules were disrupted in a mixer mill (Retsch mill MM 2000, Retsch GmbH, Haan, Germany) at 30 Hz for 10 min at 10 °C.

2.2.2. Encapsulation in alginate

Calcium-alginate capsules were prepared using an extrusion technique adapted from Ma et al. (2012) (Fig. 1B). Thirty grams of a 1.6% alginate-solution (A2033, Sigma–Aldrich Chemie GmbH, Steinheim, Germany) were prepared with distilled water and spiked with 100 μL of the P008 phage stock. The initial phage titer (N_0) was determined. Capsules were made by extruding the alginate-phage-mixture through a 0.55 mm nozzle into a 100 mM $CaCl_2$ solution at 20 °C. The capsules were kept for 30 min in the $CaCl_2$ solution for complete hardening. Subsequently, they were washed with distilled water, filtered and stored as sediment at 5 °C. The amount of phages (N_0*) in the capsules per gram wet weight was determined by dissolving them (1 g) for 15 min in a solution (9 mL) composed of 50 mM sodium citrate, 200 mM sodium