Food Microbiology 63 (2017) 28–34

Contents lists available at ScienceDirect

Food Microbiology

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

The effect of bacteriophages on the acidification of a vegetable juice medium by microencapsulated *Lactobacillus plantarum*

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ARTICLE INFO

Article history:
Received 8 August 2016
Received in revised form
18 October 2016
Accepted 28 October 2016
Available online 28 October 2016

Keywords:
Fermented vegetables
Lactic acid bacteria
Microencapsulation
Alginate
Bacteriophage

ABSTRACT

Starter cultures are increasingly being used for the production of sauerkraut, kimchi and other fermented vegetables. The goal of this study was to determine whether the microencapsulation of a bacterial culture can prevent phage infection during vegetable fermentation. *Lactobacillus plantarum* HER1325 was microencapsulated in alginate beads. Some beads were used without further processing, while others were freeze-dried prior to testing. Fresh beads (diameter of 2 mm) and dried cultures of the lactobacilli (particle size of 53–1000 μm) were added to a vegetable juice medium (VJM) at 1×10^7 CFU/mL. The virulent phage HER325 was added at an initial titer of 1×10^4 PFU/mL. In the absence of phages, the pH of the vegetable juice dropped to 4.2 after 40 h of fermentation at 19 °C. In the presence of phage HER325, acidification by both the non-microencapsulated and microencapsulated starter cultures stopped after 24 h. In all assays, the alginate particles dissolved during the 40 h of VJM fermentation. When 15 g/L of calcium chloride was added to the VJM, the alginate beads did not dissolve and significant phage protection was observed. The results suggest that phage-protected microencapsulated starter cultures can be used for vegetable fermentation if means are taken to prevent them from dissolving during acidification.

1. Introduction

Lactic fermentation of vegetables can be carried out by a variety of bacteria, but the main genera are *Lactobacillus* and *Leuconostoc* (Jung et al., 2013). It is well documented that bacterial food fermentation can be impaired by virulent bacteriophages (phages) (Moineau and Lévesque, 2005). For example, several phages active against *Lactobacillus* (for a review, see Villion and Moineau, 2009) and *Leuconostoc* (for a review, see Kot et al., 2014), have been isolated. It is therefore not surprising that various studies have reported the presence of phages in sauerkraut (Barrangou et al., 2002; Lu et al., 2003a; Yoon et al., 2002; Zhang et al., 2015), fermented cucumbers (Lu et al., 2003b, 2010; Yoon et al., 2007), and kimchi (Jung et al., 2013; Yoon et al., 2001).

As a result, phages are likely to contribute to the microbial ecology of sauerkraut fermentation. When a starter-free fermentation based on the wild microbiota of the vegetable is carried out, the microbial diversity allows fermentation to proceed in the presence of phages. Microbial selection also occurs, including the emergence of phage-resistant strains. However, such microbial adaptation takes time and could be responsible for some of the variability observed in this type of fermentation (Barrangou et al., 2002).

There is growing interest in avoiding the variability linked to spontaneous fermentation caused by indigenous vegetable microbiota. To standardize fermentation processes, starter cultures containing lactic acid bacteria have been developed for sauerkraut (Mudgal et al., 2006; Gardner et al., 2001) and kimchi (Moon et al., 2013). Furthermore, with the development of low-sodium sauerkraut, the selective effect of salt favoring the lactic acid bacterial population is reduced, and inoculation with a starter culture

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http://dx.doi.org/10.1016/j.fm.2016.10.036
0740-0020/

becomes desirable (Yoon et al., 2002; Mudgal et al., 2006). However, if the same lactic starter culture is repeatedly inoculated, it is expected that specific virulent phages could emerge and adversely affect the vegetable fermentation process. This would result in killing the added starter cultures (Samson and Moineau, 2013), as has been observed in the dairy industry (Pujato et al., 2015; Johnson and Lucey, 2006), although the stability of phages in plant-based matrices and environments is not well documented. Moreover, phages of *Leuconostoc* and *Lactobacillus* have mostly specific host ranges (Barrangou et al., 2002; Kleppen et al., 2011; Johnson and Lucey, 2006; Marcó et al., 2012), suggesting that industrial acidification problems linked to phage infection could be addressed by inoculating with carefully selected strains, by using a mixture of strains and/or by rotating the cultures (Moineau and Lévesque, 2005). As with fermented dairy products, these approaches will likely generate variability in the acidification and flavour profiles.

It has been shown that encapsulating lactic acid bacteria in alginate gels protects them against phages (Steenson et al., 1987) and that milk can successfully be fermented by alginate-microencapsulated (ME) lactococci in the presence of phages (Champagne et al., 1992). What is not known, however, is whether this strategy can also be used to ferment vegetables. Alginate gel particles do not affect the sensory properties of dairy foods if the particle size is below 70 μm (Hansen et al., 2002; Sheu et al., 1993), but they are more acceptable in foods with a heterogeneous structure, such as dry sausages (Muthukumarasamy and Holley, 2006). This could be the case with sauerkraut or kimchi, but it is unknown to what extent the particle size of ME starters will influence the acidification rate and, potentially, consumer acceptance of fermented vegetables.

The goal of this study was to determine whether a *Lactobacillus plantarum* culture microencapsulated in alginate particles of varying sizes can successfully complete lactic acid fermentation in a vegetable-based medium in the presence or absence of lytic phages.

2. Materials and methods

2.1. Production of Lactobacillus plantarum reference cultures

Lactobacillus plantarum HER 1325 was obtained from the Félix d'Hérelle Reference Center for Bacterial Viruses (www.phage.ulaval.ca). The strain was plated on MRS (deMan-Rogosa-Sharpe) culture medium (Difco, Becton Dickinson, Franklin Lakes, NJ, USA) at an inoculation rate of 1% from a milk-glycerol stock culture stored in vials and frozen at −80 °C. The stock culture was prepared by mixing the following at a ratio of 1:2.5:2.5: *Lactobacillus* culture, 20% (w/w) reconstituted skim milk, and 20% (w/v) glycerol. The skim milk and glycerol were heated beforehand at 115 °C for 10 min. For autoclaving, a temperature probe was placed in a control bottle that was same size and held the same volume of liquid as the microbiological medium. The heating times reported are therefore the actual holding times for the liquids at the temperature indicated. One thawed vial was used to inoculate 100 mL of MRS medium, and the resulting suspension was incubated at 30 °C for 14 h. At the end of the incubation period, the pH was 4.4 ± 0.2, and the viable count was $1.9 \pm 0.5 \times 10^9$ CFU/mL.

2.2. Immobilization of a fresh culture of Lb. plantarum in alginate (fresh alginate beads)

A sterile alginate solution (20 g/L; Sobalg FD 126, Grindsted) was mixed with an equal volume a fresh MRS-grown *Lb. plantarum* culture prepared as described above. The resulting suspension was added dropwise, using a syringe and a 20-gauge needle, to a stirred solution (with a magnetic stirring bar) of 11 g/L calcium chloride dihydrate (VWR, Montreal, QC, Canada) to which 1 g/L peptone (BD, Franklin Lakes, NJ, USA) was added, and the pH was adjusted to 7.0. The ratio of bacterial suspension to alginate:$CaCl_2$ solution was 1:5. The beads were left without agitation in the $CaCl_2$–peptone solution at 4 °C for 30 min and then collected in a sterile sieve.

2.3. Production of Lb. plantarum biomass (starters)

The MRS for the fermentations was prepared as recommended by the manufacturer. After sterilization and just prior to inoculation, 10 mL of a 500 g/L $CaCl_2$ solution was added. This 2.5 g/L supplementation was used to maintain firmness of the beads during fermentation. Two biomass production processes were used: 1) traditional free-cell fermentation followed by centrifugation to concentrate the *Lb. plantarum* culture; and 2) addition of cell-containing alginate particles to the growth medium followed by an incubation period. For the alginate-based process, the fermentation medium (MRS) was inoculated with the ME cells at a rate of 9% (200 g of cell-containing beads added to 2 L of medium). For free-cell fermentation, to ensure inoculation with the same volume and quantity of cells, 100 mL of fresh MRS culture was mixed with 100 mL of 1 g/L peptone, and this 200 mL cell suspension was placed in the fermentor. This resulted in similar "total cells" inoculated in the fermentor (Table 1), but the CFUs per mL of the free cell system is lower than in the alginate system, because the free cells distribute themselves throughout the growth medium, while the cells in the beads remain concentrated in the alginate gel.

The fermentations were carried out in BioFlo® 3000 systems (New Brunswick Scientific, Enfield, CT, USA) at 30 °C for 15 h, and the pH was maintained at 6.0 by the addition of 5 M KOH and 5 M NH_4OH in a 5:1 proportion. The medium was agitated at 60 rpm, but agitation was increased to 100 rpm during neutralization by the addition of alkali. At the end of fermentation, the concentrated suspension of ME cells was obtained by collecting 200 g of alginate beads using a sterile sieve. For the concentrated suspension of free cells, 2.2 L of the fermentation medium was centrifuged (Avanti® J-20 XPI, Beckman Coulter, Mississauga, ON, Canada) at 5000*g* for 15 min. The cell pellet was then mixed with 200 mL of sterile 1 g/L peptone.

2.4. Freeze-drying

The freeze-drying medium was prepared (w/w) with the following final concentrations: 200 g/L skim milk powder (Crino low-heat skim milk powder, Agropur, Granby, QC, Canada), 50 g/L sucrose, and 10 g/L casitone hydrolysate (Difco). The medium was heat-treated at 60 °C for 30 min and stored at 4 °C for a maximum of 24 h. Next, the concentrated free cells or beads were mixed with the freeze-drying medium (1:1) supplemented with 1.75 g/L ascorbic acid (Sigma, St. Louis, MI, USA). Each mixture was agitated at 25 °C for 30 min and then stored at −40 °C before being freeze-dried (Caltech FTS Systems, Stone Ridge, NY, USA). The freeze-drying conditions were as follows: 4 h at −40 °C under atmospheric pressure, 16 h at 0 °C under 100 mTorr vacuum, 16 h at 20 °C under 100 mTorr vacuum, and finally 24 h at 20 °C under 10 mTorr vacuum. After freeze-drying, the free-cell culture contained 7.5×10^{10} CFU/g, and the immobilized-cell culture contained 6.7×10^{10} CFU/g. The two dried cakes thus obtained were then ground and sieved (Canadian Standard Sieve Series, W.S. Tyler, St. Catharines, ON, Canada) to retain powders with particle sizes between 53 and 90 μm (small particle size) and between 500 and 1000 μm (large particle size).

Taken together, the following *Lb. plantarum* culture powders were obtained: free-cell powder with a small particle size (FPSm),