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# Preservation of viability and anti-*Listeria* activity of lactic acid bacteria, *Lactococcus lactis* and *Lactobacillus paracasei*, entrapped in gelling matrices of alginate or alginate/caseinate

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ABSTRACT

In order to control undesirable microorganisms growth in foods, the performance of alginate and alginate–caseinate (an aqueous two-phase system) matrices entrapping lactic acid bacteria (LAB) (*Lactobacillus paracasei* LAB1 and *Lactococcus lactis* LAB3) was investigated. Polymeric matrices were initially loaded with LAB cells at ~$10^{8-10}$ or ~$10^{4-6}$ CFU $mL^{-1}$, and were monitored, in liquid and gelled form (beads), for 12 days at 30 °C. In the liquid form, maximum cell density (~$10^{9}$ CFU $mL^{-1}$) was reached after 24 h whatever the matrix. Then, the LAB population decreased but remained higher in alginate–caseinate matrices: $10^{7}$ and $10^{6}$ CFU $mL^{-1}$ of LAB3 cells were enumerated after 12 days in alginate–caseinate and in alginate matrices, respectively. Anti-*Listeria* activity (assayed by agar well diffusion method) did not vary much over 12 days and was also higher for cells entrapped in alginate–caseinate matrices. When matrices were gelled, similar trends were observed: at "Day 12", LAB3 population was $10^{4-5}$ and $10^{2-3}$ CFU/bead, and, LAB1 population was $10^{5-6}$ and $10^{3-4}$ CFU/bead, in alginate–caseinate and alginate beads, respectively. Antimicrobial activity of alginate–caseinate beads containing LAB1 cells was quite constant over 12 days. The anti-*Listeria* activity of LAB cell-free supernatants incorporated in matrices with caseinate was also higher. In fact, the presence of caseinate was shown to promote both the survival of LAB cells and the release of their antimicrobial metabolites. Observation of liquid and gelled matrices by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) revealed a preferential localization of LAB cells in casein-rich microdomains which could affect favorably the efficiency of bipolymeric matrices.



## 1. Introduction

During the last decades, innovative bioactive packaging enriched with antimicrobial metabolites (mainly bacteriocins) have been intensively developed (Cao-Hoang, Chaine, Grégoire, & Waché, 2010; Ercolini et al., 2010; Guiga et al., 2010; Iseppi et al., 2011; Millette, Le Tien, Smoragiewicz, & Lacroix, 2007; Neetoo et al., 2008; Scannell et al., 2000). However, the problem generally encountered is the decrease of the film's antimicrobial activity throughout time (Concha-Meyer, Schöbitz, Brito, & Fuentes, 2011; Kristo, Koutsoumanis, & Biliaderis, 2008). In order to solve this problem, the direct incorporation of bacteriocin-producing strains into polymeric matrices was investigated (Concha-Meyer et al., 2011; Gialamas, Zinoviadou, Biliaderis, & Koutsoumanis, 2010; Iseppi et al., 2011; Sanchez-Gonzalez, Ivan, Saavedra, & Chiralt, 2013). Particularly, Gialamas et al. (2010) developed sodium caseinate films incorporating *Lactobacillus sakei* cells with interesting anti-*Listeria* activity. Application of these films to both synthetic medium (agar) and food model system (fresh beef) previously contaminated with *Listeria monocytogenes* induced a significant inhibition of the pathogen growth throughout 14 days storage at 4 °C. In this sense, this new approach to control pathogen

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growth opens the lines of research on the possibility of using polymers as a support for viable pathogen antagonists, such as lactic acid bacteria (LAB), and could lead to an alternative method of food preservation.

The antagonistic effect against undesirable microorganisms exerted by LAB is due to different mechanisms acting synergistically or not: competition for nutrients, pH lowering (resulting from the production of organics acids), production of hydrogen peroxide, gas (carbon dioxide), reuterin, diacetyl, or bacteriocins (Galvez, Abriouel, Lopez, & Ben Omar, 2007). Besides, for future applications, it is interesting to note that most of LAB are considered Generally Recognized As Safe (GRAS) by the U.S. Food and Drug Administration (FDA). Particularly, *Lactococcus lactis* is one LAB species used for food preservation because of its ability to produce bacteriocins such as nisin (the only bacteriocin with a food additive status in the European Union), lacticin, and lactococcin (Charlier, Cretenet, Even, & Le Loir, 2009). In the same way, interest in other LAB species grows increasingly as it is the case for *Lactobacillus paracasei* strains: a number of papers have been recently published on their bacteriocinogenic properties (Billah, Islam, Sabrin, Islam, & Islam, 2012; Lozo, Vukasinovic, Strahinic, & Topisirovic, 2004; Todorov & Dicks, 2007; Tolinacki et al., 2012; Vamanu & Vamanu, 2010).

Biopolymer-based delivery systems have been extensively used as biomaterials to encapsulate, protect, and release various functional food ingredients (vitamins, flavors, colors, nutraceuticals, antimicrobial agents …) (Matalanis, Jones, & McClements, 2011) or microorganisms (Bevilacqua, Sinigaglia, & Corbo, 2010). These matrices should be prepared from food-grade ingredients using economical and reliable processing operations (McClements, 2012). There is a growing interest in protein/polysaccharide mixtures which allow to obtain a variety of microstructures depending on the interactions between the two biopolymers, the nature of biopolymers, the solution composition, and the environmental conditions (Matalanis et al., 2011). In a one-phase system, the two biopolymers can exist either as individual molecules or as soluble complexes that are evenly distributed throughout the entire system. In a two-phase system, the solution separates into two distinct phases that have different biopolymer compositions (Grinberg & Tolstoguzov, 1997; McClements, 2006). When biopolymer–solvent interactions are favored at high concentrations in biopolymers, both phases contain preferably one biopolymer, the solvent being divided between both phases. This type of phase separation is called segregative phase separation and is governed by thermodynamic incompatibility (Capron, Costeux, & Djabourov, 2001; Grinberg & Tolstoguzov, 1997; McClements, 2006). With phase-separated biopolymer systems, different microstructures (spheres, fibers, teardrops) could be obtained and it is possible to fix them, for example by gelling one biopolymeric phase (Matalanis et al., 2011; McClements, 2012). These different microstructures can thus be used to encapsulate active substances or cells. Nevertheless, very few studies about the incorporation of bacterial cells in an aqueous two-phase system were published. Such systems were first used to separate biological materials under soft conditions (Pacek, Ding, Nienow, & Wedd, 2000), to characterize physico-chemical properties of cell surface (isoelectric point, charge, hydrophobicity) (Umaskoski et al., 1997) or to separate bacterial cells and their metabolites (Antov & Pericin, 2001; Antov, Pericin, & Dimic, 2001; Planas, Radström, Tjerneld, & Hahn-Hägerdal, 1996; Rito-Palomares, Negrete, Galindo, & Serrano-Carreon, 2000; Rito-Palomares et al., 2001; Schwarz-Linek et al., 2010). Moreover, aqueous two-phase systems (ATPS) were used to microencapsulate probiotic stains (Andrade, Ferreira, Cardoso, & Cardoso, 2010; Leja, Dembczyński, Bialas, & Jankowski, 2009; Millqvist-Fureby, Malmsten, & Bergenstaahl, 2000). The preservation of probiotic cultures was improved by confining bacterial cells in an ATPS in which one phase is dispersed in the other, the continuous phase. These cells were thus double protected: they were entrapped in the dispersed phase and covered by the continuous phase (Leja et al., 2009).

The present work is focused on comparing anti-*Listeria* activity of alginate and alginate–caseinate gelling matrices incorporating cells of two LAB strains, either *Lb. paracasei* LAB1 or *Lc. lactis* LAB3. Caseinate was used in combination with alginate in order to form an aqueous two-phase system. We have recently shown the enhanced antimicrobial properties of this system (Léonard, Degraeve, Gharsallaoui, Saurel, & Oulahal, 2014; Léonard et al., 2013) and a preferential localization of LAB3 cells in the caseinate-rich phase has been observed at microscopic scale in such liquid ATPS (Léonard et al., 2013). In addition, caseinate could represent a potential source of nutrients for LAB cells (Hugenholtz & Kleerebezem, 1999). In the present work, the culturability and the anti-*Listeria* activity of LAB1 and LAB3 cells entrapped either in liquid or in gelled alginate and alginate–caseinate matrices stored at 30 °C were measured for 12 days. Concerning liquid matrices, microstructures and LAB cell viability were monitored by fluorescent labeling and confocal laser scanning microscopy. The anti-*Listeria* activity of LAB cell-free supernatants, which were incorporated in liquid or gelled alginate or alginate–caseinate matrices, was assayed to assess the matrix effect on the release of antimicrobial metabolites produced by each LAB strain. Finally, observations by confocal laser scanning microscopy and scanning electron microscopy were performed to localize LAB cells and to establish if microstructure could have a role for the maintenance of culturability and antimicrobial activity.

## 2. Materials and methods

### 2.1. Bacterial strains, media, and growth conditions

*Lb. paracasei* subsp. *paracasei* LAB1 (Holdbac™ Protective Culture, Danisco, DuPont Nutrition & Health, France) and *Lc. lactis* subsp. *lactis* LAB3 (commercial strater MD089, Ezal line, Rhône Poulenc, Dangé Saint-Romain, France) (Lamboley, Lacroix, Champagne, & Vuillemard, 1997) were stored at −20 °C in "de Man Rogosa and Sharpe" broth ($MRS_b$) (Biokar Diagnostics, Beauvais, France) supplemented with 15% (v/v) glycerol. Before use, *Lb. paracasei* LAB1 and *Lc. lactis* LAB3 cells were subcultured under anaerobic conditions in $MRS_b$ at 10% (v/v) for 24 h at 30 °C, then precultured in $MRS_b$ at 10% (v/v) for 14 h at 30 °C and finally incubated at 30 °C for the requested experimental time. These two LAB strains were selected because they both produce bacteriocin-like substances: bacteriocin-like substance(s) produced by LAB1 strain are sensitive to *Aspergillus oryzae* protease, trypsin, and α-chymotrypsin or to a 1 h thermal treatment at 100 °C (data not shown) and it was previously reported that LAB3 strain produced bacteriocin-like substances (Léonard et al., 2014).

The antagonistic activity was assayed against three target strains of *Listeria* genus: *Listeria innocua* ATCC 33090, *L. innocua* LRGIA01 (isolated from a dairy) (Chadeau, Oulahal, Dubost, Favergeon, & Degraeve, 2010) and *L. monocytogenes* S162 (serotype 1/2a, isolated from a dairy) (Mariani et al., 2011). Stock cultures were maintained at −20 °C in Tryptone Soy broth (TSB) (Biokar Diagnostics, Beauvais, France) supplemented with 15% (v/v) glycerol. Before each test, the three target strains were subcultured on TSB at 10% (v/v) for 7 h at 30 °C and then precultured in TSB at 10% (v/v) for 14 h at 30 °C.