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# Encapsulation of *Lactobacillus rhamnosus* GG in microparticles: Influence of casein to whey protein ratio on bacterial survival during digestion

J. Burgain, C. Gaiani *, C. Cailliez-Grimal, C. Jeandel, J. Scher

*Université de Lorraine, LIBio (Laboratoire d'Ingénierie des Biomolécules), 2 avenue de la Forêt de Haye, TSA 40602, 54518 Vandoeuvre lès Nancy, France*

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ABSTRACT

Encapsulation of *Lactobacillus rhamnosus* GG in various microparticles made of only milk proteins (casein, native whey and/or denatured whey proteins) was done. The microparticles obtained were rather similar in shape (mostly round) and size (around 60 μm) whatever the formulation but the obtained gel presented different elasticity (varying between 61 and 96 Pa). An original equipment involving a granulo-morphometer coupled to a thermostated reactor was developed and validated to visualize in situ the microparticles during digestion. Although the initial particles were similar, their disintegration in simulated gastric media was totally different and characterized by two stages. An initial decrease in particle size more or less quick depending on the protein composition was followed by a stable phase characterized by the particle size and shape retention. At the end of gastric digestion, a significant amount of intact particles was still noticeable for each formulation. Nevertheless, the formulation containing a mix of casein and denatured whey presented the best bacterial survival (99%) and encapsulation rate (97%) in comparison with formulations containing either only casein or casein and native whey or casein in mixture with native and denatured whey proteins.

*Industrial relevance:* This paper is part of a global project entitled "Structured dairy matrices to enhance probiotic efficiency". The entire project will provide milk structured matrices allowing the stabilization and the vectorization of *Lactobacillus rhamnosus* GG (LGG). This project will consist of four main axes: milk constituent's interactions with LGG, stabilization process implementation, and structural and functional characterization of the matrices obtained. The scientific objective is to propose models connecting process parameters, matrix structure (from an atomic, molecular to a macro scale) and their functionality. This implies the in-depth study of interactions between milk components and probiotic strain. For this purpose, the use of genetically modified strain of LGG will allow the identification of biomolecules interacting with milk matrices. The industrial aims are to optimize and control the processes to suit the needs of industrial criterions: encapsulation rate, gastric resistance, intestinal release, storage in the final food….



## 1. Introduction

Modern consumers are increasingly buying into the concept that probiotic yogurt and other fermented milk products improve digestion, boost immunity and provide other health benefits long claimed by food companies (Burgain, Gaiani, Linder, & Scher, 2011). However, regulatory authorities, particularly in Europe, have not supported probiotic health claims on the grounds that the provided data were not sufficient. This year, the European Food Safety Authority (EFSA) has rejected almost all health claims put forward by the probiotic industry. Several reasons are invoked to justify the refusal: microbes have not been sufficiently characterized, the claimed effect was not considered beneficial or because human studies to support the claims were not provided (Schmidt, 2013). The design of biopolymer based microparticles to encapsulate, protect and release a specific bioactive component believed to benefit human health is now gaining interest (Matalanis, Jones, & McClements, 2011). In most cases, the encapsulated substance needs to be released at a specific site in the body. Thus, developing a model identifying the physico-chemical mechanism leading to the release could be a useful tool to predict microparticles' future (Matalanis et al., 2011).

In the past few years, the use of bioactive ingredients derived from the dairy sector has gained interest. Dairy ingredients are widely used in the food industry because of their technological properties. In fact, they are recognized for their surface-active and colloid stabilizing characteristics (Horne, 2009). Moreover, their health benefits are increasingly promoted (Mackie & Macierzanka, 2010). Milk proteins can be used as a carrier for health-promoting delivery system. For example,

*Abbreviations:* Bdi, Breadth of the distribution; CFU, Colony forming units; EQPC, Diameter of a circle of equal projection area; EFSA, European Food Safety Authority; ER, Encapsulation rate; IMCU, International Milk Clotting Units; LGG, *Lactobacillus rhamnosus* GG; SR, Survival rate.

* Corresponding author at: LIBio (Laboratoire d'Ingénierie des Biomolécules), Université de Lorraine, 2 avenue de la Forêt de Haye, B.P. 172, 54505 Vandoeuvre Lès Nancy Cedex, France. Tel.: +33 3 83 59 58 77; fax: +33 3 83 59 58 04.
*E-mail address:* claire.gaiani@univ-lorraine.fr (C. Gaiani).

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nanoparticles are used to solubilize and protect hydrophobic nutraceuticals. In this case, the natural digestibility of caseins is exploited (Livney, 2010). Milk contains two major protein groups: casein and soluble proteins. These proteins differ in their physico-chemical properties and more particularly in their amino acid composition. Caseins and whey proteins make up respectively around 80% and 20% of the proteins in milk. Due to their emulsifying properties and amino-acid composition, caseins play an important role in human nutrition. These characteristics explain their wide use as additives in food. Casein micelles are composed of $\alpha_{s1}$-, $\alpha_{s2}$-, β- and κ-casein proteins. Rennet coagulation of milk results from κ-casein proteolysis by chymosin enzyme. In fact, by releasing hydrophilic fragments, repulsive forces disappear allowing thereby micelle aggregation via calcium bond formation (Horne, 2009). Gelation properties of milk proteins can be exploited for probiotic encapsulation. Many health promoting claims attributed to probiotic bacteria are dependent on the cells being both viable and sufficiently numerous in the intestinal tract (Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2012). Nevertheless, the passage through the stomach of most bacteria results in an important loss of viability which lowers the efficacy of the administered supplement. Formulation of probiotics into microcapsules is an interesting method to reduce cell death during the gastrointestinal passage (Burgain et al., 2011). The use of milk protein based microparticles was poorly exploited whereas it was demonstrated that dairy matrices such as cheeses can protect bacterial cells (Stanton et al., 1998) thanks to the good buffering capacity of milk proteins for example (Livney, 2010).

Rennet gelation of milk proteins was used to develop a microencapsulation technology for the protection of two strains: *Lactobacillus paracasei* ssp. *paracasei* F19 and *Bifidobacterium lactis* Bb12 (Heidebach, Först, & Kulozik, 2009a). The obtained microparticles were spherical and water insoluble due to the use of an emulsification process. This technique allows a high encapsulation rate and a good microparticle resistance in simulated gastric conditions leading hence to an important survival rate of bacteria. By using this technique, the authors claimed that probiotic cells can resist to adverse conditions encountered in the stomach. Another enzyme (transglutaminase) was also tested to produce microparticles where milk protein gelation was exploited (Heidebach, Först, & Kulozik, 2009b). The heat induced gelation of whey proteins can be used to produce microparticles containing probiotic bacteria. In this way, *Bifidobacterium* Bb-12 was microencapsulated by spray drying with whey proteins and the entrapped cells presented better survival during simulated gastric digestion (De Castro-Cislaghi, Silva, Fritzen-Freire, Lorenz, & Sant'Anna, 2012). Another example is the microencapsulation of *Lactobacillus rhamnosus* GG (LGG) with gelled whey protein isolates. The produced matrix was able to protect the cells during in vitro stomach incubation (Doherty et al., 2011) and also during ex-vivo digestion (Doherty et al., 2012). Finally, all of these trials for microencapsulation with dairy proteins led to an increase in bacterial survival during digestion.

In this context, the first objective of this study was to compare the resistance of four matrices (without bacteria) constituted by only milk proteins in simulated gastric environment. For this purpose, an original equipment allowing the in situ determination of particle size and shape during digestion was developed. Then, the same matrices were used to encapsulate LGG. Survival rate during gastric digestion was followed during the 2 h. The selection of the best formulation should be done by the confrontation of results: particle size, shape and Bdi, encapsulation and gastric resistance rates.

## 2. Materials and methods

### 2.1. Material used

Micellar casein powder (Promilk 872B) was obtained from Ingredia IDI (Arras, France). Whey protein isolate powders (Prolacta 90) were purchased from Lactalis Ingredients (Bourgbarré, France). The chemical characterization of the powders has been already studied (Gaiani et al., 2011). Micellar casein and whey proteins present respectively a protein content around 87% and 90% (w/w).

The rennet preparation (Naturen™) was provided by CHR Hansen (Arpajon, France) and presented an activity of 140 IMCU $mL^{-1}$. The rennet solution was prepared before being used by diluting 3 g of rennet preparation in 12 g of distilled water (28 IMCU $mL^{-1}$). The sunflower oil was purchased from a local store.

LGG (ATCC 53103) was used throughout this study. Bacteria were first subcultured at 37 °C in MRS medium. This preculture was then used to inoculate 500 mL of MRS broth which was incubated overnight at 37 °C until an early stationary phase. Cells were centrifugated (15 min., 3500 *g*, room temperature), washed with physiologic water (pH 7.4) and finally harvested by centrifugation (15 min., 3500 *g*, room temperature). The pellet was then frozen and placed on the shelves of a freeze dryer (Christ alpha 1–2, freeze-dryer, Osterode, Germany). The microbial powder obtained after freeze drying has a content of $10^{11}$ CFU/g.

### 2.2. Microparticulation procedure

#### 2.2.1. Preparation of the carrier material

All the solutions were prepared by adding 12.5 g of protein powder into 100 g of distilled water (Fig. 1). The rehydration was done by stirring for 2 h at room temperature and then overnight at 4 °C. The denatured whey proteins were obtained by heating the native whey solution at 78 °C for 10 min then cooling it to room temperature. Four formulations were used to produce variable microparticles with different milk protein composition (micellar casein and whey proteins in a native or denatured state). The amount of each solution used to prepare the initial mix of protein solution is detailed in Fig. 1.

The four formulations were tested for the microencapsulation of LGG. The strain was added after mixing the protein solution and before enzymatic incubation (Fig. 1). 0.03 g of freeze dried LGG was added to 15 g of protein mixture.

#### 2.2.2. Microparticle production

Microparticles were produced by using an emulsification method (Heidebach et al., 2009a) and the procedure is detailed in Fig. 1. The encapsulation procedure was completed in a double-walled, temperature-controlled reactor, made of stainless steel. An amount of 15 g of protein mixture was added into the reactor held at 9 °C. Then, 445 μL of diluted rennet preparation was added and mixed with the solution. The mixture was left for 30 min at 9 °C allowing the rennet enzyme to cut the κ-casein. This step was followed by an emulsification. For this purpose, 150 g of cooled sunflower oil was added and stirred with the mixture at 500 RPM (Rotation Per Minute) for 5 min. Afterward, the temperature was raised to 40 °C for 15 min and under mechanical agitation.

Microparticles were removed from the reactor and separated by centrifugation (500 *g*, 1 min). The harvested particles were washed with distilled water. Afterwards, the microparticles were stripped from the container and shaken for 12 s. Another centrifugation following the same conditions was then performed. Finally, the microparticles were removed, re-suspended in distilled water and stored at 4 °C under stirring until their use the next day.

#### 2.2.3. Encapsulation rate (ER)

The enumeration of LGG living cells was done by serial dilutions. The samples (0.1 mL) were plated in duplicate on MRS agar. Colony forming units (CFU) were determined after incubation (48 h at 37 °C). To enumerate the entrapped probiotic cells, the microparticles were mechanically broken with a spatula.

The ratio between the number of bacteria added in the protein mixture and the number of bacteria in the final particles was done. The initial number of bacteria introduced in the protein mixture was obtained by determining the CFU in 1 g of solution. The result