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1 # *Lactobacillus rhamnosus* GG encapsulation by spray-drying: Milk proteins
2 clotting control to produce innovative matrices

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24 **Abstract**

25 A well-known probiotic strain, *L. rhamnosus* GG, was encapsulated by spray-drying in milk
26 water-insoluble matrices upon reconstitution in hot water by exploiting and controlling the
27 clotting reaction of milk proteins during the process. The feed solution, composed of probiotic
28 bacteria and milk proteins, was or not subjected to the action of chymosin, a proteolytic enzyme.
29 To optimize microencapsulation efficiency, different outlet air temperatures were tested (55, 70
30 and 85 °C). After spray-drying, small microparticles were recovered for further characterization.
31 All drying conditions led to excellent bacterial survival rates (< 0.5 log reduction) whereas only
32 the highest outlet air temperature allowed the production of microparticles with acceptable
33 moisture contents (< 7 %) to ensure storage stability. Finally, enzymatic cleavage of milk
34 proteins by chymosin before atomization led to matrices presenting innovative functionalities
35 when microparticles are reconstituted with water: rehydration or dispersion in cold (8 °C) or
36 warm (40 °C) water, respectively.

37

38 **Keywords**

39 *L. rhamnosus* GG; encapsulation; milk proteins; spray-drying, rehydration; Scanning Electron
40 Microscopy

41

42 **Abbreviations**

43 CFU: Colony Forming Units; CMP: Caseino-macro-peptide; IMCU: International Milk Clotting
44 Units; SEM: Scanning Electron Microscopy; MRS: Man, Rogosa, Sharpe broth culture.

45

46 ## 1. Introduction

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47 Consumer requests for healthy food products that prevent illnesses strongly increased at the
48 beginning of the 21st century. The interest for functional food, among which probiotic food, has
49 rapidly grown these last years (Abd El-Salam and El-Shibiny, 2015). Probiotic bacteria are
50 defined as “live microorganisms which when administered in adequate amounts confer a health
51 benefit on the host” (FAO/WHO, 2002). Probiotics have been incorporated in many food
52 products, such as dairy products that usually constitute suitable probiotic carriers (Burgain et al.,
53 2011; Granato et al., 2010). Nevertheless, after introduction in the product, a loss of viable cells
54 during preservation has often been observed (Blanchette et al., 1996; Ding and Shah, 2008; Shah
55 et al., 1995; Tripathi and Giri, 2014). For example, during refrigerated storage, five commercial
56 yogurts containing *Lactobacillus acidophilus* and *Bifidobacterium bifidum* presented a constant
57 decrease in the viable counts of these two strains (Shah et al., 1995). In dairy products, pH
58 decrease and accumulation of inhibitory substances, like lactic acid produced during
59 fermentation, were the main factors identified for the loss of probiotics viability in yogurt (Shah,
60 2000). This viability decrease was also observed in other dairy product such as cheeses (Amine et
61 al., 2014; Blanchette et al., 1996; Gobbetti et al., 1998) and in a huge number of other food
62 products, such as dark chocolate (Laličić-Petronijević et al., 2015) and fruit juices (Ding and
63 Shah, 2008; Saarela et al., 2006). During digestion, a decrease in viable probiotics in the
64 gastrointestinal transit is well documented (Burgain, Gaiani, Cailliez-Grimal, Jeandel, & Scher,
65 2013; Jantzen, Gopel, & Beermann, 2013; Pinto et al., 2015) meaning that few or no
66 microorganisms would be able to reach the intestine to exert their activity, presumably because of
67 the low gastric pH and the presence of bile salts (Charteris et al., 1998; Cook et al., 2012). For
68 example, a high loss of viable cells is observed for some strains (*L. rhamnosus* GG, *L. reuteri* and
69 *Bifidobacterium* BB-12) in simulated gastric digestion, but these bacteria remain stable under the

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70 physicochemical conditions of the small intestine (Burgain et al., 2013; Charteris et al., 1998;
71 Jantzen et al., 2013; Pinto et al., 2015).

72 To exert their probiotic activity, bacteria need to be viable when reaching the intestine.
73 Microencapsulation is undeniably one solution. This technology is used to maintain probiotic
74 viability from their processing up to their consumption and their passage through the
75 gastrointestinal tract by entrapping and protecting sensitive living cells (De Prisco and Mauriello,
76 2016). Many microencapsulation technologies are successful in encapsulating probiotic bacteria,
77 e.g., spray-drying, emulsion, coacervation, extrusion, fluid bed or gel-particle technologies
78 (Burgain, Gaiani, Linder, & Scher, 2011; Krasaekoopt, Bhandari, & Deeth, 2003; Martín, Lara-
79 Villoslada, Ruiz, & Morales, 2015).

80 Among these encapsulation methods, gelation properties of milk proteins are sometimes
81 exploited (Burgain, Gaiani, Cailliez-Grimal, et al., 2013; Heidebach, Först, & Kulozik, 2009). To
82 this end, milk-clotting enzymes, as rennet or transglutaminase, are required to form a resistant
83 matrix. Rennet is a proteolytic enzyme that is capable of hydrolyzing the κ-casein from the casein
84 micelle surface and releases the caseinomacropeptide. By releasing these hydrophilic fragments,
85 repulsive forces between caseins disappear allowing thereby the close approach of the micellar
86 caseins via calcium bond formation. Upon heat treatment, micelle aggregation occurs until
87 forming a gel (Dalgleish and Corredig, 2012). Different authors encapsulated probiotic cells (*L.*
88 *rhamnosus* GG, *B. lactis* Bb12, *L. paracasei* F19) in milk water-insoluble microparticles by
89 exploiting these enzymatically-induced gelation properties (Burgain, Gaiani, Cailliez-Grimal, et
90 al., 2013; Heidebach et al., 2009). The main drawback of these processes is the use of an
91 emulsification method that is no straight forward for the food industry.

92 The most common method used to encapsulate probiotic bacteria in food industry is spray-
93 drying. This technique presents advantages of low cost, reproducibility and rapidity and is a
94 useful way to incorporate probiotics into dairy products (Gardiner et al., 2002). Numerous studies
95 already reported the use of spray-drying to encapsulate probiotic cells, but the major
96 disadvantage of this technology is the use of high air temperatures causing a decrease in bacterial
97 survival. The ability to survive to process conditions also depends on the probiotic strain. For
98 example, spray-dried *Bifidobacterium breve* and *Lactobacillus acidophilus* presented a survival
99 rate of only 26 % and 76 %, respectively (Maciel et al., 2014; Picot and Lacroix, 2004) and for
100 *Lactobacillus reuteri*, a decrease of two log of the bacterial population was reported after spray-
101 drying. (Ananta et al., 2005) demonstrated that the reduced viability of *L. rhamnosus* GG is
102 linked to cellular membrane damage and the percentage of membrane damage increased with the
103 outlet air temperature, showing that the outlet air temperature should be carefully selected for
104 improving the encapsulation of living probiotic cells by spray-drying.

105 In the present work, enzymatically-induced gelation properties of milk proteins were for the first
106 time exploited to encapsulate *L. rhamnosus* GG by spray drying instead of an emulsification
107 technique. The clotting reaction control was able to create matrices presenting new
108 functionalities: water-insoluble upon reconstitution in hot water or water-soluble upon
109 reconstitution in cold water. Concurrently, some spray-drying parameters were tested, in
110 particular different outlet air temperatures were applied (55, 70 and 85 °C).

111

112 ## 2. Material and methods

113 ### 2.1 Material

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114 Micellar casein powder (Promilk 872 B) and whey protein powder (Promilk 752 FB) were
115 purchased from Ingredia IDI (Arras, France). Chymosin (Chymax Plus) was provided by CHR
116 Hansen (Hørsholm, Denmark).

117

118 **2.2 Preparation of proteins and chymosin solutions**

119 Micellar casein and whey protein solutions were prepared separately by rehydrating powders in
120 distilled water at 12.5 % (w/w) dry extract. Rehydration was with an overhead stirrer equipped
121 with a spiral stirrer (IKA, Staufen, Germany) at a speed of 1000 rpm for 2 h at room temperature
122 (20 °C), then overnight (4 °C). After rehydration, whey proteins were denatured by heating the
123 solution at 78 °C during 10 min (Petit et al., 2011) and the solution was cooled at 4 °C. The
124 chymosin solution was prepared by diluting ten times the initial solution (200 IMCU.mL^{-1}) in
125 distilled water.

126

127 **2.3 Preparation of bacterial suspension**

128 The strain used in this study was *L. rhamnosus* GG (ATCC 53103). The growth of
129 *L. rhamnosus* GG was performed in a laboratory-scale reactor. Bacterial stocks used for the
130 inoculation were stored at - 20 °C in MRS broth with 20 % (v/v) glycerol. A pre-culture was
131 prepared by inoculating *L. rhamnosus* GG in 200 mL of MRS broth at 37 °C for 15 h. The
132 bioreactor containing 1 L of fresh medium was inoculated with the whole pre-culture. Growth
133 was performed at 37 °C under agitation at 300 rpm and pH was adjusted at 6.8 with the addition
134 of 6 M NaOH. Cells concentration was determined by following the absorbance at 660 nm.
135 Culture was stopped at the beginning of the stationary phase and bacterial cells were harvested

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136 from the broth by gentle centrifugation (3000 *g*, 10 min). The pellet was then lyophilized during
137 72 h and stored at 4 °C before use.
138

139 **2.4 Production of microparticles by spray-drying**

140 The concentrate was prepared by mixing micellar caseins and denatured whey proteins solutions
141 in a ratio of 90/10 (v/v), respectively. Before spray-drying, the chymosin solution was added to
142 the concentrate at a final chymosin concentration of 12 IMCU.g^{-1} of proteins. The protein
143 concentrate in presence of chymosin was left for 30 min at a temperature of 8 °C to allow
144 caseinomacropeptide cleavage through the action of chymosin and to avoid casein coagulation
145 (Burgain et al., 2013). After the enzymatic cleavage step, lyophilized *L. rhamnosus* GG was
146 mixed with the concentrate at approximatively 8.0 log_{10} CFU.g^{-1} before spray-drying. After
147 incubation, the solution containing *L. rhamnosus* GG was spray-dried using a pilot-scale spray
148 drier MicraSpray 150 (Anhydro, Soeborg, Danemark). A peristaltic pump was used to deliver the
149 liquid through the bi-fluid nozzle into the spray-drying chamber with a feed flow-rate of
150 87 mL.min^{-1} and a nozzle pressure of 1 bar. In this study, different theoretical outlet air
151 temperatures were tested: 85, 70 and 55 °C. The real (measured) inlet and outlet temperatures
152 were collected in **Table S1**. The spray-dried microparticles were stored in plastic vessel at 4 °C
153 before use. Two independent productions were realized. A thermo-humidity sensor was placed at
154 the output of the cyclone to measure the relative humidity and the temperature of the humid air
155 (**Table S1**).
156

157 **2.5 Survival of *L. rhamnosus* GG after spray-drying**

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158 To evaluate the survival of *L. rhamnosus* GG during encapsulation, cell counts were determined
159 before and after spray-drying. Cell counts were obtained by determining the number of CFU in
160 1 mL concentrate before spray-drying and in 1 g spray-dried powder. For this purpose, 1 mL
161 (concentrate) or 1 g (powder) sample was introduced in 9 mL of tryptone salt broth and the
162 solution was mixed during 2 min using a vortex homogenizer. Sample was serially diluted in
163 tryptone salt broth and plated on MRS agar. After 48 h incubation at 37 °C, cell counts were
164 determined and expressed as $CFU.g^{-1}$. The concentrate density was measured to be able to
165 translate the 1 ml of concentrate in grams. For this, a known volume of concentrate was weighed.
166 The experiment was realized in triplicate for each formulation.

167

168 **2.6 Shear effect of the bi-fluid nozzle on *L. rhamnosus* GG viability and cellular**
169 **organization**

170 During spray-drying, bacterial cells were exposed to high shear stresses when passing through the
171 bi-fluid nozzle. Shear stress may impact cellular organization and viability. Here, the protein
172 concentrate previously incubate with chymosin or not and containing bacteria cells was sprayed
173 through the nozzle at 1 bar air pressure, 87 $mL.min^{-1}$ feed rate and ambient temperature (18 °C)
174 to mimic the spraying conditions of the spray-drying experiments. The solution was collected at a
175 distance of about 50 cm of the nozzle exit, thus avoiding altering the formation of droplets in the
176 spray while maximizing the recovery of atomized concentrate. Cells counts were determined
177 before and after spraying as described in section 2.5. A shear stress factor was determined as
178 follow:

179 Eq. (1) $$Shear\ stress\ factor = \frac{10^{[log_{10}CFU/mL]\ after\ spraying}}{10^{[log_{10}CFU/mL]\ before\ spraying}}$$

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180 Cellular organization before and after spraying was observed by Gram staining. For this, a drop
181 of the concentrate containing *L. rhamnosus* GG before and after spraying was fixed on a slide.
182 The fixed concentrate was gently flooded with crystal violet and let stand for 1 min. The slide
183 was rinsed with water and the same procedure was repeated with iodine solution. After
184 discoloration of the concentrate with 90 % ethanol, a new coloration with fuchsin solution for 1
185 min was performed. Once rinsed with water, the concentrate was observed by using a light-
186 microscope under oil-immersion.

187

188 **2.7 Determination of powder moisture content and particle size distribution**

189 The water content was determined by weight loss after drying 2 g powder at 103 °C for 3 h as
190 described by the International Dairy Federation standard (IDF, 2004). Three repetitions per
191 sample were carried out.

192 The particle size distribution was determined using a laser granulometer (Mastersizer 3000,
193 Malvern Instruments, UK) with Aero S dry powder dispersion unit. To obtain a correct
194 obscuration, all samples were dispersed at 1 bar air pressure, 30% feed rate and 3 mm hopper
195 length. Five measurements were conducted for each sample. The particle size distribution was
196 calculated from Mie theory. The particle size estimator was the *d*(50), which means that 50 % of
197 particles have a lower diameter.

198

199 **2.8 Wettability, dispersibility and solubility**

200 All experiments were performed in water at 20 °C. Wettability of a powder is the time necessary
201 for the whole amount of powder to be wetted by water. The dispersibility is the proportion of

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202 powder dry matter that can be dispersed in water (Niro Atomizer, 1978). These two properties
203 were determined as described by the International Dairy Federation standards (IDF, 1979) with
204 some modifications due to powder amount restrictions. Only 2.5 g powder was poured into 25 g
205 distilled water. The solubility is the ability of a powder to be dissolved in water (Niro Atomizer,
206 1978), expressed in percentage, and was determined as described by the International Dairy
207 Federation standards (IDF, 2005).

208 *Powder solubility according to reconstitution water temperature*

209 For solubility results presented in Table 3, the protocol was modified to highlight solubility
210 differences with temperature. For this purpose, the rehydration time before measuring the
211 solubility was longer (30 minutes) instead of 30 seconds. Indeed, the powder was rehydrated for
212 30 min in water at 8 °C, 15 °C, 20 °C, 30 °C and 40 °C before measuring the solubility.

213

214 **2.9 Microparticles behavior depending on water temperature**

215 *Scanning Electron Microscopy*

216 A high-resolution field-emission scanning electron microscope (SEM) type JEOL JSM-7100F
217 supplied with a hot (Schottky) electron gun (JEOL Ltd., Tokyo, Japan) and having a resolution
218 around 1 nm at 30 kV was used to investigate the microparticles in dry and rehydrated states. The
219 equipment was operated at 5 kV.

220 For dry microparticles, samples were mounted onto SEM stubs by sputtering them on a carbon
221 double-sided adhesive tape. Coating was done with iridium (Q150T Turbo-Pumped Sputter
222 Coater, ProSciTech Pty Ltd, Queensland, Australia) for 2 min in three sides (around 10 nm
223 thickness per side).

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224 For particles in hydrated conditions, the study was conducted according to (Mimouni et al., 2010)
225 with small modifications. The suspension of powder particles under rehydration was deposited on
226 a silicon chip wafer (ProSciTech, Kirwan, Australia) that has previously been coated with poly-
227 L-Lys (Sigma, Castle Hill, Australia). The suspension was kept in contact with the wafer for 5
228 min, then the wafer was drained and rinsed with 100 mM phosphate buffer at pH = 7. Chemical
229 fixation of proteins was achieved by immersing the wafer in a solution composed of 2.5 %
230 glutaraldehyde in 100 mM phosphate buffer at pH = 7 for 15 min. After that, the samples were
231 gently washed in 100 mM phosphate buffer at pH = 7 and dehydrated using the following graded
232 ethanol baths series: 50 %, 60 %, 70 %, 80 %, 90 % (once) and 100 % (three times). The elapsed
233 time per solution was 3 min (Dalgleish et al., 2004). Samples were then dried by using CO_2 in a
234 Supercritical Autosamdri-815B critical point dryer (Tousimis, Rockville, MD, USA). The silicon
235 wafer was then mounted onto SEM stubs thanks to a carbon double-sided adhesive tape. Finally,
236 samples were coated with iridium for 2 min (until reaching about 10 nm coating thickness).

237 *Turbiscan measurements*

238 Powder rehydration (0.1 %, w/w) was performed in water at 8 or 40 °C during 120 min and the
239 dispersion was poured into a glass cell. Sample stability after rehydration was followed using a
240 Turbiscan Classic (Formulaction, France). This technology used the principle of multiple light
241 scattering that consists in illuminating a liquid sample with a pulsed near infrared light source (λ
242 = 800 nm). After multiple scattering, photons emerge from the sample and are detected by two
243 detectors: a transmission detector that receives the light transmitted through the sample (in the
244 same direction as the light source) and a backscattering detector that receives the light reflected
245 by the sample (at 135 ° of the light source direction). Transmitted and backscattered are
246 informative for translucent and opaque samples, respectively. The detection head scanned the

247 entire height of the sample cell, acquiring transmission and backscattering data by 40 µm steps.
248 Sample was scanned every minute for 30 min.

249

250 **2.10 Statistical analysis**

251 All measurements presented in this paper were performed on two independent spray-drying
252 experiences. Reported data were analyzed by ANOVA using KyPlot software version 2.0 in order
253 to determine the presence of significant differences between samples. Data were then analyzed
254 using Tukey's pair-wise comparison, at 5 % level of significance, to determine what samples are
255 significantly different.

256

257 **3. Results and discussion**

258 **3.1. Identification of the best matrix formulation and process conditions**

259 *L. rhamnosus* GG was encapsulated by spray-drying using dairy matrices composed of casein and
260 denatured whey proteins being previously or not incubated with chymosin. Regarding process
261 conditions, different outlet air temperatures were tested (85, 70 and 55 °C) for each matrix
262 formulation (**Table S1**). Overall six powders were produced per batch. It appears that theoretical
263 and measured outlet temperatures were very close. A good reproducibility between the two
264 batches were observed.

265

266 **3.1.1. Survival of *L. rhamnosus* GG after spray-drying**

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267 The counts of viable cells before and after spray-drying in produced microparticles are shown in
268 **Table 1**. Bacterial cell concentrations in powders were systematically comprised between 7.8 and
269 8.9 log_{10}CFU/g (**Table 1**). To provide health benefits, a concentration of 10^6 CFU/mL in the
270 product at the time of consumption or a daily intake of 10^8 - 10^9 probiotics is often recommended
271 (Tripathi and Giri, 2014). Taking into account these reference values, the concentration level find
272 in our study was satisfying. Surprisingly, bacterial concentration in most of fresh powders (after
273 drying) was found higher than the concentration in the feed solution (before drying). These
274 results were associated with bacterial chain fragmentation outcome, which was already described
275 when encapsulating the same bacteria by extrusion, another process causing high shear stress to
276 the feed solution (Doherty et al., 2010). When spraying the concentrate, the passage through the
277 small orifices of the bi-fluid nozzle and the subsequent nebulization mechanism (leading to
278 conversion of the liquid jet into droplets) are known to apply high shear stresses to the feed
279 solution. The survival and the colony organization were investigated before and after spraying
280 (**Figure 1**). Light-microscope images on bacteria colored by Gram staining revealed significant
281 modifications in bacterial cell organization. Before spraying, rod-shaped *L. rhamnosus* GG was
282 organized in small linear chains whereas this organization was modified by spraying: individual
283 cells were recovered in sprayed solution. Because both a bacterial chain and a single isolated cell
284 lead to one colony on a petri dish, the shear stress due to spraying, which is responsible for
285 breaking bacterial chains, caused an increase in bacterial cells concentration. In concentrate not
286 incubated with the chymosin, bacterial cells concentration was of 6.8 and 7.4 log_{10}CFU/g before
287 and after spraying respectively. With previous enzymatic step in the concentrate, bacteria cells
288 concentration was of 7.3 and 7.8 log_{10}CFU/g before and after spraying, respectively. In
289 concentrate not incubated or incubated with chymosin, the shear stress was responsible for an
290 increase of 3.3 and 2.7 times more cells counted after spraying compared to cells counted before

291 spraying. Petit et al., (2015) showed that the formation of droplets by shear effect during spraying
292 in bi-fluid nozzles was essentially controlled by air pressure and liquid viscosity. Thus, the outlet
293 air temperature in the feed solution was not expected to influence the increase factor measured
294 during spraying only, and the same increase factor was considered for the calculations related to
295 spray-drying experiments. Finally, using corrected values, a log reduction of the cell counts can
296 be measured providing information of the probiotic cells ability to survive to spray-drying
297 conditions. In every cases, a decrease of less than 0.5 log was observed and confirmed the
298 excellent survival of the bacteria during encapsulation by spray-drying (**Table 1**).

299

300 **3.1.2. Powder physicochemical properties**

301 **Powder moisture content**. Powder moisture content was determined for microparticles that were
302 produced after incubation or not with the chymosin for each drying temperature. Regarding
303 powders obtained from a concentrate that was not incubated with the chymosin, moisture
304 contents of about 6.3, 7.9 and 11.6 % were observed for air outlet temperatures of 85, 70 and 55
305 °C, respectively (**Table 2**). Similar values were obtained for the formulations incubated with
306 chymosin: moisture contents were equal to circa 5.8, 8.0 and 12.0 % for outlet air temperatures of
307 85, 70 and 55 °C respectively. For a given evaporation capacity, the decrease in the outlet air
308 temperature in spray-drying is linked to an increase in its relative humidity. This results in an
309 significant increase in the powder moisture content, as its water activity tends to equilibrate with
310 air relative humidity (Schuck et al., 2012). Incubation of the protein concentrate with the
311 chymosin did not significantly influence the moisture content values as shown in **Table 2**.
312 Powder moisture content strongly influence the product stability and can also influence the
313 probiotic viability during storage which is one of the quality parameter to take into account for

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314 powders containing cells (Ying et al., 2010). A moisture content between 4 and 7 % is usually
315 recommended for a good storage (Ananta et al., 2005). This condition was only achieved for the
316 highest outlet air temperature used in the present study (i.e. 85 °C).

317 **Particle size**. Particle size and more precisely the mean diameter (d_{50}), was not significantly
318 influenced by the outlet air temperature and by the incubation of the feed solution with chymosin
319 (**Table 2**). All produced powders presented a mean size below 18 µm, well below 100 µm. This
320 particle size range is advantageous to avoid negative sensorial impact when added to food
321 (Hansen et al., 2002).

322 **Powder morphology**. SEM images of powders did not permit to evidence any significant shape
323 modification due to outlet air temperature or previous incubation with chymosin (**Figure 2**). All
324 particles were smooth and non-spherical. Sadek et al. (2014) demonstrated that particle structure
325 was governed by the composition of milk matrix. For example, whey proteins are known to form
326 smooth, spherical and open hollow powders. On the contrary, the presence of caseins in the
327 matrix is responsible of more wrinkled, non-spherical and dense powder structures (Gaiani et al.,
328 2007; Sadek et al., 2014). Here, the matrix was a 90:10 mixture of caseins and denatured whey
329 proteins. As expected, particle morphology presented in **Figure 2** was characteristic of high
330 casein content powders. No bacteria were observed on the microparticles surface, even though
331 more than one hundred microparticles were examined at elevated magnifications. The same
332 phenomenon was already observed previously (Khem et al., 2016; Liu et al., 2015). It may be
333 suggested that bacterial cells were totally embedded inside the microparticles and it will be check
334 later in the paper.

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336 **3.1.3. Importance of chymosin incubation for powder reconstitution properties**

337 Powder rehydration properties are strongly linked to the following measures: wettability,
338 dispersibility and solubility. These properties were measured for each formulation and spray-
339 drying conditions (**Table 2**).

340 Powders wettability was not found significantly affected by the drying temperature. Only the
341 powder produced without chymosin incubation at 55 °C presented a better wetting time
342 compared to powder obtained at other outlet air temperatures (**Table 2**). This powder presented a
343 high moisture content and was expected to have a stickier surface, making them prone to
344 agglomeration and thus improving their wetting time by increasing their size (Ji et al., 2016). For
345 powders coming from concentrate previously incubated with chymosin, their wettability was
346 slightly improved. The wetting time of a milk powder is known to be strongly dependent on its
347 composition (Fitzpatrick et al., 2016). For example, for a similar particle size, casein powders are
348 known to present better wetting times than whey protein powders (Gaiani et al., 2011). All
349 studied powders were not wetted in less than 5 min. The high wetting time observed here was
350 surely the consequence of the low mean particle size of all samples (d_{50} < 18 µm), as fine
351 particles present high difficulties to overcome water surface tension.

352 Powders dispersibility produced with or without chymosin incubation was not found significantly
353 affected by the drying temperature. Only powders produced after chymosin incubation at 55 °C
354 presented significant differences (**Table 2**). Indeed, the elevated moisture content may induced
355 powder agglomeration. Thus, an increase in particle size due to agglomerate can improve the
356 dispersibility (Sharma et al., 2012). Generally, powders mainly composed of whey proteins
357 present a good dispersibility, above 80 %, whereas for casein powders, the dispersibility can

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358 decreases to only 10 % (Sadek et al., 2014). In the current study, the dispersibility results were
359 characteristic of powders containing high casein content (**Table 2**).

360 From a general point of view, small difference were observed in both wettability and
361 dispersibility for all the powders. On the contrary, for solubility, strong differences are measured
362 between powders coming from concentrate previously incubated or not with chymosin. The
363 powders solubility was systematically lower when the feed solution was incubated with chymosin
364 prior to spray-drying (**Table 2**). However, the solubility was not affected by the drying
365 temperature. A recent work shown that whey protein and micellar casein powders present a
366 solubility of 100 or 55 %, respectively (Sadek et al., 2014). In the current study, the solubility
367 results for powders that were not incubated with chymosin were characteristic of high casein
368 content powders and around 80 % (**Table 2**). On the other hand, for powders obtained after the
369 chymosin incubation step, a significant lower solubility was measured around 30 % due to the
370 production of water-insoluble microparticles. The exact phenomena occurring here will be
371 detailed in the section 3.2.

372

373 **3.1.4 Selection of the best combination of matrix formulation and spray-drying temperature**
374 **for bacterial encapsulation**

375 This first part of the study permitted to identify the best matrix formulation and the best spray-
376 drying conditions to encapsulate *L. rhamnosus* GG. Since all experiments permitted a high
377 bacterial survival rate, the selection of the optimal powder was performed on the basis of
378 physicochemical properties. In our study, the moisture content allowing a suitable storage and a
379 good functional stability of the powder was only achieved for the highest outlet air temperature

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380 (*i.e.* about 6 % moisture content for powders produced at 85 °C). The main functional advantage
381 of the chymosin action before spray-drying resided in the production of powder with low
382 solubility when reconstituted in water. The low solubility may be interesting in the food industry
383 for the production of water-insoluble microparticles. For example, these structures may be able to
384 vehicle and protect probiotic bacteria in high moisture content food by avoiding the bacteria
385 dispersion in the product.

386 Consequently, the best combinations for formulation (i) and spray-drying (ii) conditions to
387 encapsulate *L. rhamnosus* GG were to use an initial incubation step with chymosin (i) followed
388 by a spray-drying at 85 °C (ii). The end of the study will be focused on this particular powder.

389

390 3.2. **In-depth characterization of optimal powder: proteins previously incubated with**
391 **chymosin and spray-dried at 85 °C**

392 SEM was used to characterize the powder behavior when reconstituted in water at 8 or 40 °C.
393 When added to cold water (8 °C), the powder was partially rehydrated, allowing probiotics
394 release in the medium (**Figures 3A** and **3B**). Probiotic bacteria visualization in the partially
395 rehydrated powder confirmed that bacteria were totally embedded inside the microparticles
396 before reconstitution in water. On the contrary, after 30 min reconstitution in warm water (40
397 °C), powder microparticles were dispersed but not rehydrated. Indeed, microparticles at 40 °C
398 were totally intact on SEM images, which allowed bacteria retention into microparticles structure
399 (**Figure 3D** and **3E**). Some of them were still visible in cracks at the microparticles surface. This
400 temperature-sensitive reconstitution behavior likely resulted from the action of chymosin in the
401 feed solution prior to the spray-drying process. Chymosin is an enzyme used for milk clotting,
402 which involves the enzymatic hydrolysis of κ-casein followed by the non-enzymatic interaction

403 between destabilized casein micelles leading to gel formation (Carlson et al., 1987). This gel
404 formation is irreversible. In the developed microparticles production process, the enzymatic
405 reaction took place before the spray-drying process but the formation of casein micelle network
406 was avoided by maintaining feed solution temperature at 4 °C, as gel formation starts to be
407 significant over 10 °C. The non-enzymatic reaction occurs only when powder was reconstituted
408 above 10 °C, explaining the discrepancy in reconstitution behavior demonstrated at 8 and 40 °C
409 (**Figure 4**). Indeed, at such high temperature, casein micelles react together by creating a compact
410 network that prevents their rehydration, leading to a suspension of microparticles entrapping
411 bacteria (**Figure 3F**). At 8 °C, repulsion forces between casein micelles kept them distant from
412 each other and the matrix structure was more porous, permitting the rehydration of microparticles
413 (**Figure 3C**). The decoupling of the enzymatic and non-enzymatic steps of the milk clotting
414 mechanism was previously developed to encapsulate probiotic bacteria, unfortunately resulting in
415 humid microparticles needing an expensive drying step to confer them a good storage stability
416 (Burgain et al., 2013; Heidebach et al., 2009). Here, the good storage stability was obtained in
417 one process step only and at a low cost by using spray-drying.

418 This tremendous influence of chymosin on the different reconstitution behavior of microparticles
419 at 8 and 40 °C was confirmed by measuring powder solubility after 30 minutes rehydration at
420 different temperature ranging from 8 to 40 °C (**Table 3**). In this part, the time of powder
421 rehydration before measuring solubility was increased to 30 minutes to accentuate solubility
422 differences between the samples. First, powders produced without the chymosin incubation step
423 were considered as control samples for the role of chymosin incubation and their solubility was
424 determined. At 8 °C, probiotics were released in the medium (**Figure 3**) but some insoluble
425 material remained in solution and was responsible for the incomplete solubility (79.6 %). Powder

426 solubility followed an increasing trend when reconstitution temperature was increased from 8 to
427 40 °C, in agreement with Schuck et al. (1994). A totally different behavior was observed for
428 microparticles produced after incubation with chymosin (**Table 3**) : at 8 °C, powder solubility
429 fell to 35.7 %, and increasing the reconstitution temperature lowered even more powder
430 solubility. The low powder solubility at 8 °C may be explained by the formation of insoluble
431 materials during spray-drying process, likely owing to the triggering of casein gel formation
432 when the temperature of the feed solution droplets increased in the course of the spray-drying
433 process (i.e. after spraying but before the droplets reached the solid state). Insoluble material can
434 be seen on SEM images obtained for microparticles reconstituted at 8 °C (**Figure 3B**).

435 The stability of reconstituted powders after addition to water at 8 and 40 °C was followed by
436 Turbiscan analysis with a view to confirm the temperature dependence of powder reconstitution
437 behavior. At 8 °C, a small increase in transmitted light at the top of the tube was observed,
438 corresponding to the thinning out of the medium. At the bottom of the tube, the sedimentation of
439 only few particles only was detectable (**Figure 5A**). At 40 °C, the same phenomena were
440 observed but in a well more marked extent (**Figure 5B**). These measurements confirmed that
441 microparticles were mostly rehydrated at 8 °C and insoluble and dispersed at 40 °C. The slight
442 sedimentation observed at 8 °C may result from the few insoluble material produced during
443 spray-drying.

444

445 **Conclusion**

446 A combination of matrix composition and process condition able to encapsulate *L. rhamnosus*
447 GG by spray-drying and presenting new temperature-dependent reconstitution behaviors was

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448 successfully developed. These new functionalities were the result of chymosin action before
449 spray-drying, which was decoupled from gel formation (**Figure 6**). This new process may be
450 interesting for industry as:

451 - Powder form provides many advantages for storage and transportation purposes (i),

452 - Irreversible production of water-insoluble microparticles when dispersed in warm water
453 presents interests for bacteria vectorization in high moisture content food products (such as milk,
454 fermented drink, juice, yogurts, etc.) (ii),

455 - Powder ability to almost fully rehydrate in cold water may be interesting for ferment
456 production, as the release of encapsulated bacteria can be easily achieved by a judicious choice of
457 reconstitution temperature (iii).

458

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466

467 **References**

ACCEPTED MANUSCRIPT

468 Abd El-Salam, M., El-Shibiny, S., 2015. Preparation and properties of milk proteins-based
469 encapsulated probiotics: a review. Dairy Sci. Technol. 95, 393–412.
470 Amine, K.M., Champagne, C.P., Raymond, Y., St-Gelais, D., Britten, M., Fustier, P., Salmieri,
471 S., Lacroix, M., 2014. Survival of microencapsulated Bifidobacterium longum in Cheddar
472 cheese during production and storage. Food Control 37, 193–199.
473 Ananta, E., Volkert, M., & Knorr, D., 2005. Cellular injuries and storage stability of spray-dried
474 Lactobacillus rhamnosus GG. International Dairy Journal, 15(4), 399–409.
475 Blanchette, L., Roy, D., Bélanger, G., Gauthier, S.F., 1996. Production of Cottage Cheese Using
476 Dressing Fermented by Bifidobaceria1. J. Dairy Sci. 79, 8–15.
477 Burgain, J., Gaiani, C., Cailliez-Grimal, C., Jeandel, C., Scher, J., 2013. Encapsulation of
478 Lactobacillus rhamnosus GG in microparticles: Influence of casein to whey protein ratio
479 on bacterial survival during digestion. Innov. Food Sci. Emerg. Technol. 19, 233–242.
480 Burgain, J., Gaiani, C., Linder, M., Scher, J., 2011. Encapsulation of probiotic living cells: From
481 laboratory scale to industrial applications. J. Food Eng. 104, 467–483.
482 Carlson, A., Hill, C.G., Olson, N.F., 1987. Kinetics of milk coagulation: I. The kinetics of kappa
483 casein hydrolysis in the presence of enzyme deactivation. Biotechnol. Bioeng. 29, 582–
484 589.
485 Charteris, W.P., Kelly, P.M., Morelli, L., Collins, J.K., 1998. Development and application of an
486 in vitro methodology to determine the transit tolerance of potentially probiotic
487 Lactobacillus and Bifidobacterium species in the upper human gastrointestinal tract. J.
488 Appl. Microbiol. 84, 759–768.
489 Cook, M.T., Tzortzis, G., Charalampopoulos, D., Khutoryanskiy, V.V., 2012.
490 Microencapsulation of probiotics for gastrointestinal delivery. J. Controlled Release 162,
491 56–67.
492 Dalgleish, D.G., Corredig, M., 2012. The structure of the casein micelle of milk and its changes
493 during processing. Annu. Rev. Food Sci. Technol. 3, 449–467.
494 Dalgleish, D.G., Spagnuolo, P.A., Douglas Goff, H., 2004. A possible structure of the casein
495 micelle based on high-resolution field-emission scanning electron microscopy. Int. Dairy
496 J. 14, 1025–1031.
497 De Prisco, A., Mauriello, G., 2016. Probiotication of foods: A focus on microencapsulation tool.
498 Trends Food Sci. Technol. 48, 27–39.
499 Ding, W.K., Shah, N.P., 2008. Survival of Free and Microencapsulated Probiotic Bacteria in
500 Orange and Apple Juices. Int. Food Res. J. 15(2), 219-232.
501 Doherty, S.B., Wang, L., Ross, R.P., Stanton, C., Fitzgerald, G.F., Brodkorb, A., 2010. Use of
502 viability staining in combination with flow cytometry for rapid viability assessment of
503 Lactobacillus rhamnosus GG in complex protein matrices. J. Microbiol. Methods 82,
504 301–310.
505 Fitzpatrick, J.J., van Lauwe, A., Coursol, M., O'Brien, A., Fitzpatrick, K.L., Ji, J., Miao, S., 2016.
506 Investigation of the rehydration behaviour of food powders by comparing the behaviour
507 of twelve powders with different properties. Powder Tech. 297, 340-348.
508 Gaiani, C., Boyanova, P., Hussain, R., Murrieta Pazos, I., Karam, M.C., Burgain, J., Scher, J.,
509 2011. Morphological descriptors and colour as a tool to better understand rehydration
510 properties of dairy powders. Int. Dairy J. 21, 462–469.
511 Gaiani, C., Schuck, P., Scher, J., Desobry, S., Banon, S., 2007. Dairy Powder Rehydration:
512 Influence of Protein State, Incorporation Mode, and Agglomeration. J. Dairy Sci. 90,
513 570–581.

514 Gardiner, G.E., Bouchier, P., O'Sullivan, E., Kelly, J., Kevin Collins, J., Fitzgerald, G., Paul
515 Ross, R., Stanton, C., 2002. A spray-dried culture for probiotic Cheddar cheese
516 manufacture. Int. Dairy J. 12, 749–756.
517 Gobbetti, M., Corsetti, A., Smacchi, E., Zocchetti, A., Angelis, M.D., 1998. Production of
518 Crescenza Cheese by Incorporation of Bifidobacteria. J. Dairy Sci. 81, 37–47.
519 Granato, D., Branco, G.F., Cruz, A.G., Faria, J. de A.F., Shah, N.P., 2010. Probiotic Dairy
520 Products as Functional Foods. Compr. Rev. Food Sci. Food Saf. 9, 455–470.
521 Hansen, L.T., Allan-Wojtas, P.M., Jin, Y.-L., Paulson, A.T., 2002. Survival of Ca-alginate
522 microencapsulated Bifidobacterium spp. in milk and simulated gastrointestinal conditions.
523 Food Microbiol. 19, 35–45.
524 Heidebach, T., Först, P., Kulozik, U., 2009. Microencapsulation of probiotic cells by means of
525 rennet-gelation of milk proteins. Food Hydrocoll. 23, 1670–1677.
526 Jantzen, M., Gopel, A., Beermann, C., 2013. Direct spray drying and microencapsulation of
527 probiotic Lactobacillus reuteri from slurry fermentation with whey. J Appl Microbiol 115,
528 1029–36.
529 Ji, J., Fitzpatrick, J., Cronin, K., Maguire, P., Zhang, H., Miao, S., 2016. Rehydration behaviours
530 of high protein dairy powders: The influence of agglomeration on wettability,
531 dispersibility and solubility. Food Hydrocoll. 58, 194–203.
532 Khem, S., Small, D.M., May, B.K., 2016. The behaviour of whey protein isolate in protecting
533 Lactobacillus plantarum. Food Chem. 190, 717–723.
534 Krasaekoopt, W., Bhandari, B., Deeth, H., 2003. Evaluation of encapsulation techniques of
535 probiotics for yoghurt. Int. Dairy J. 13, 3–13.
536 Laličić-Petronijević, J., Popov-Raljić, J., Obradović, D., Radulović, Z., Paunović, D., Petrušić,
537 M., Pezo, L., 2015. Viability of probiotic strains Lactobacillus acidophilus NCFM® and
538 Bifidobacterium lactis HN019 and their impact on sensory and rheological properties of
539 milk and dark chocolates during storage for 180 days. J. Funct. Foods 15, 541–550.
540 Liu, H., Gong, J., Chabot, D., Miller, S.S., Cui, S.W., Ma, J., Zhong, F., Wang, Q., 2015.
541 Protection of heat-sensitive probiotic bacteria during spray-drying by sodium caseinate
542 stabilized fat particles. Food Hydrocoll. 51, 459–467.
543 Maciel, G.M., Chaves, K.S., Grosso, C.R.F., Gigante, M.L., 2014. Microencapsulation of
544 Lactobacillus acidophilus La-5 by spray-drying using sweet whey and skim milk as
545 encapsulating materials. J. Dairy Sci. 97, 1991–1998.
546 Martín, M.J., Lara-Villoslada, F., Ruiz, M.A., Morales, M.E., 2015. Microencapsulation of
547 bacteria: A review of different technologies and their impact on the probiotic effects.
548 Innov. Food Sci. Emerg. Technol. 27, 15–25.
549 Mimouni, A., Deeth, H.C., Whittaker, A.K., Gidley, M.J., Bhandari, B.R., 2010. Investigation of
550 the microstructure of milk protein concentrate powders during rehydration: alterations
551 during storage. J. Dairy Sci. 93, 463–472.
552 Petit, J., Herbig, A.-L., Moreau, A., Delaplace, G., 2011. Influence of calcium on β-lactoglobulin
553 denaturation kinetics: Implications in unfolding and aggregation mechanisms. J. Dairy
554 Sci. 94, 5794–5810.
555 Petit, J., Méjean, S., Accart, P., Galet, L., Schuck, P., Le Floch-Fouéré, C., Delaplace, G., Jeantet,
556 R., 2015. A dimensional analysis approach for modelling the size of droplets formed by
557 bi-fluid atomisation. J. Food Eng. 149, 237–247.
558 Picot, A., Lacroix, C., 2004. Encapsulation of bifidobacteria in whey protein-based
559 microcapsules and survival in simulated gastrointestinal conditions and in yoghurt. Int.
560 Dairy J. 14, 505–515.

561 Pinto, S.S., Fritzen-Freire, C.B., Benedetti, S., Murakami, F.S., Petrus, J.C.C., Prudêncio, E.S.,
562 Amboni, R.D.M.C., 2015. Potential use of whey concentrate and prebiotics as carrier
563 agents to protect Bifidobacterium-BB-12 microencapsulated by spray drying. Food Res.
564 Int. 67, 400–408.
565 Saarela, M., Virkajärvi, I., Alakomi, H.-L., Sigvart-Mattila, P., Mättö, J., 2006. Stability and
566 functionality of freeze-dried probiotic Bifidobacterium cells during storage in juice and
567 milk. Int. Dairy J. 16, 1477–1482.
568 Sadek, C., Li, H., Schuck, P., Fallourd, Y., Pradeau, N., Floch-Fouéré, C.L., Jeantet, R., 2014. To
569 What Extent Do Whey and Casein Micelle Proteins Influence the Morphology and
570 Properties of the Resulting Powder? Dry. Technol. 32, 1540–1551.
571 Shah, N.P., 2000. Probiotic Bacteria: Selective Enumeration and Survival in Dairy Foods. J.
572 Dairy Sci. 83, 894–907.
573 Shah, N.P., Lankaputhra, W.E.V., Britz, M.L., Kyle, W.S.A., 1995. Survival of Lactobacillus
574 acidophilus and Bifidobacterium bifidum in commercial yoghurt during refrigerated
575 storage. Int. Dairy J. 5, 515–521.
576 Sharma, A., Jana, A.H., Chavan, R.S., 2012. Functionality of Milk Powders and Milk-Based
577 Powders for End Use Applications—A Review. Compr. Rev. Food Sci. Food Saf. 11,
578 518–528.
579 Tripathi, M.K., Giri, S.K., 2014. Probiotic functional foods: Survival of probiotics during
580 processing and storage. J. Funct. Foods 9, 225–241.
581 Schuck, P., Jeantet, R., & Dolivet, A., 2012. Analytical Methods for Food and Dairy Powders.
582 Wiley-Blackwell, (Chapter 1).
583 Ying, D.Y., Phoon, M.C., Sanguansri, L., Weerakkody, R., Burgar, I., Augustin, M.A., 2010.
584 Microencapsulated Lactobacillus rhamnosus GG Powders: Relationship of Powder
585 Physical Properties to Probiotic Survival during Storage. J. Food Sci. 75, 588–595.
586

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Table 1: *L. rhamnosus* GG concentration before and after drying with associated the Log reduction. A shear stress factor was used to correct values before drying to take into account the shear effect of the process.

Sample	Theoretical outlet air temperature (°C)	Shear stress factor	Bacteria cells concentration ($\log_{10}$ CFU/g)			Log reduction
			Before drying	Before drying with correcting factor	After drying	
without chymosin incubation	85	3.3	7.5 ± 0.2	8.1 ± 0.2	7.8 ± 0.1	0.2
	70		7.9 ± 0.2	8.4 ± 0.2	8.3 ± 0.2	0.1
	55		7.6 ± 0.1	8.1 ± 0.1	7.8 ± 0.1	0.3
with chymosin incubation	85	2.7	8.6 ± 0.6	9.0 ± 0.6	8.7 ± 0.1	0.3
	70		9.0 ± 0.8	9.4 ± 0.8	8.9 ± 0.3	0.5
	55		9.0 ± 1.1	9.4 ± 1.1	8.9 ± 0.9	0.5

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Table 2: Physicochemical properties of microparticles depending on spray-drying conditions and matrix composition (moisture content, mean particle size d_{50} and rehydration properties).

Sample	Theoretical outlet air temperature (°C)	Moisture content (%)	Mean particle size (µm)	Rehydration properties		
				Wettability (s)	Dispersibility (%)	Solubility (%)
without chymosin incubation	85	6.3 ± 0.1^{a}	17.7 ± 0.4^{a}	2048 ± 43^{a}	16.3 ± 1.2^{a}	76.6 ± 1.9^{a}
	70	7.9 ± 0.2^{b}	13.8 ± 0.4^{ab}	217 ± 25^{a}	15.1 ± 0.2^{a}	87.1 ± 1.1^{b}
	55	11.6 ± 0.1^{c}	10.6 ± 0.9^{ab}	1734 ± 284^{b}	15.1 ± 0.4^{a}	83.9 ± 1.3^{ab}
with chymosin incubation	85	5.8 ± 0.2^{a}	14.7 ± 2.8^{ab}	1661 ± 64^{b}	18.5 ± 1.3^{ab}	30.5 ± 2.5^{c}
	70	8.0 ± 0.1^{b}	13.1 ± 0.5^{ab}	1539 ± 70^{b}	18.3 ± 1.7^{ab}	31.7 ± 0.7^{c}
	55	12.0 ± 0.1^{c}	9.7 ± 1.0^{b}	1606 ± 82^{b}	22.2 ± 0.9^{b}	32.9 ± 1.6^{c}

Values followed by a different superscript letter in the same column are significantly different at $P < 0.05$.

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Table 3: Powder solubility (for formulations spray-dried at 85 °C) according to reconstitution temperature (°C).

Reconstitution temperatures (°C)	Solubility (%)	
	Microparticles without chymosin incubation	Microparticles with chymosin incubation
8	79.6 ± 1.2^{a}	35.7 ± 0.4^{a}
15	74.5 ± 1.8^{a}	31.2 ± 0.4^{a}
20	87.5 ± 0.3^{b}	26.8 ± 0.9^{ab}
30	86.3 ± 1.9^{bc}	25.6 ± 0.1^{bc}
40	93.8 ± 0.3^{bc}	14.6 ± 0.1^{d}

Values followed by a different superscript letter in the same column are significantly different at $P < 0.05$.

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Figure 1: Effect of spraying-induced shear stress on cellular organization.

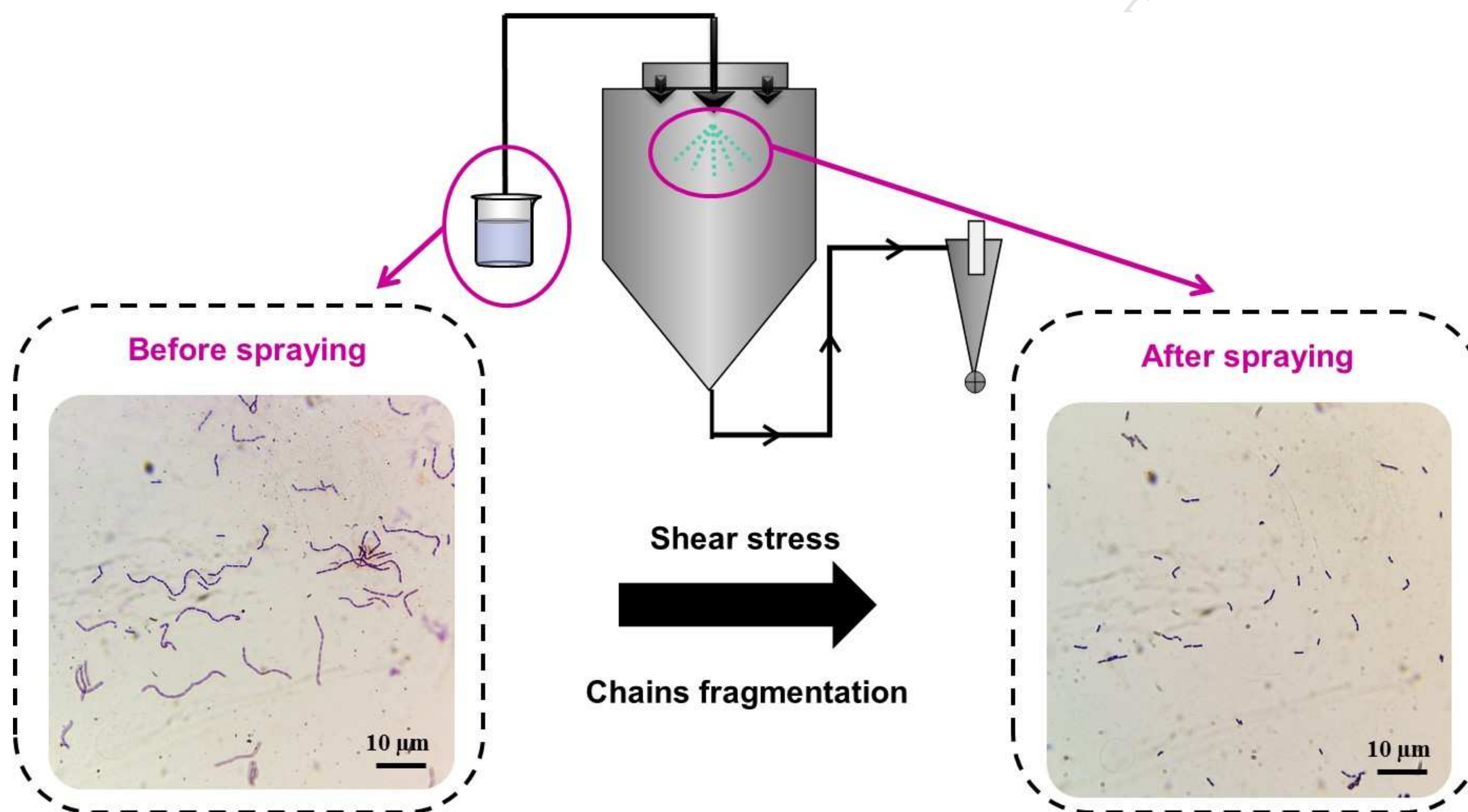

Figure 2: Scanning electron microscopy of microparticles (x 3000 magnification). *L. rhamnosus* GG was encapsulated in milk matrices without (1, 2 and 3) or with (1', 2' and 3') chymosin incubation before spray-drying. Different outlet air temperatures were used during spray-drying: 85 °C (1 and 1'), 70 °C (2 and 2') and 55 °C (3 and 3').

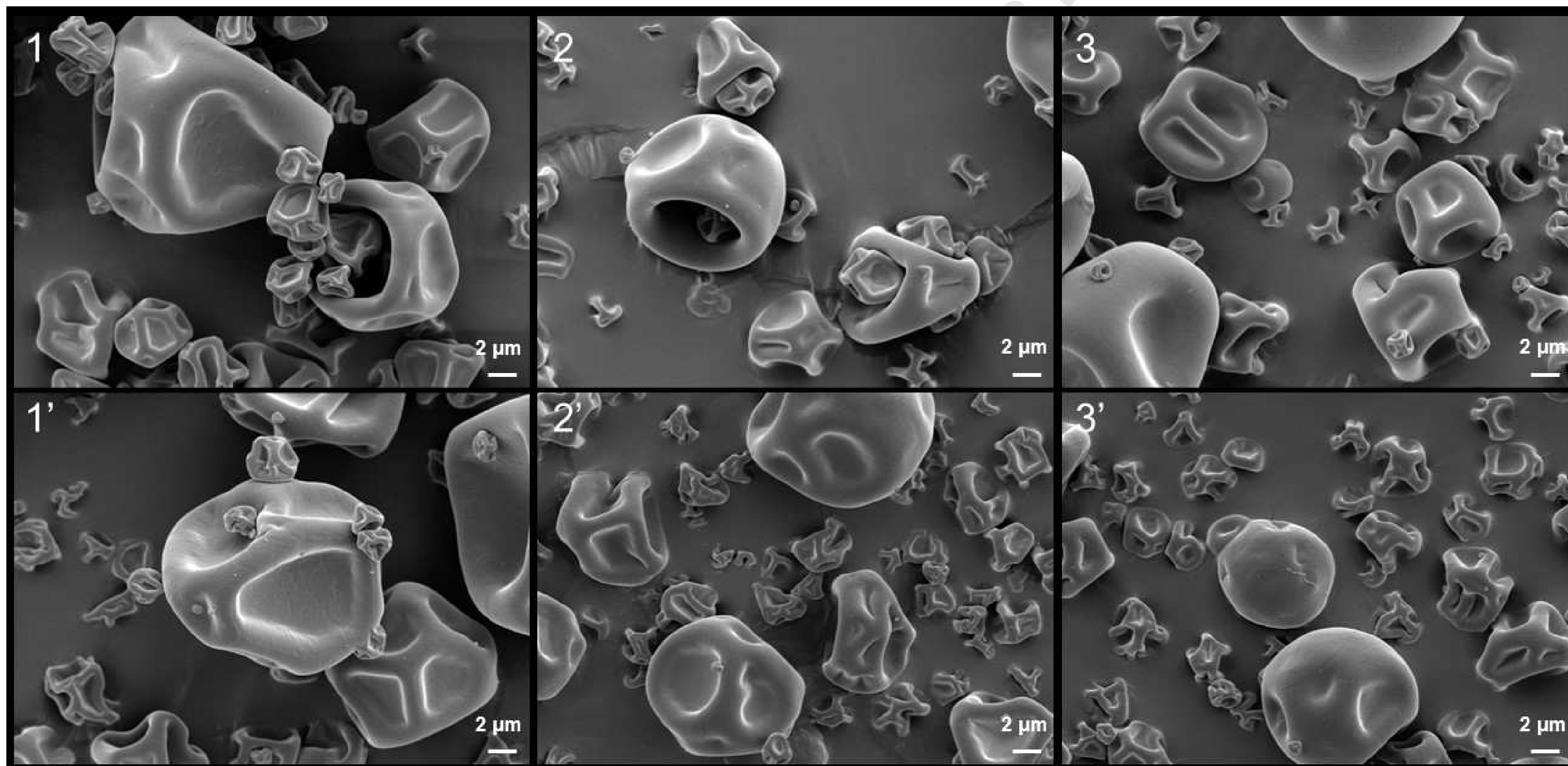

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Figure 3: Scanning electron microscopy of microparticles after reconstitution at 8 °C (A, B and C) and 40 °C (D, E and F). Microparticles were reconstituted in water during 2 h under stirring (500 rpm). Magnifications: × 2000 (A, D), × 10000 (B, E), × 50000 (C, F).

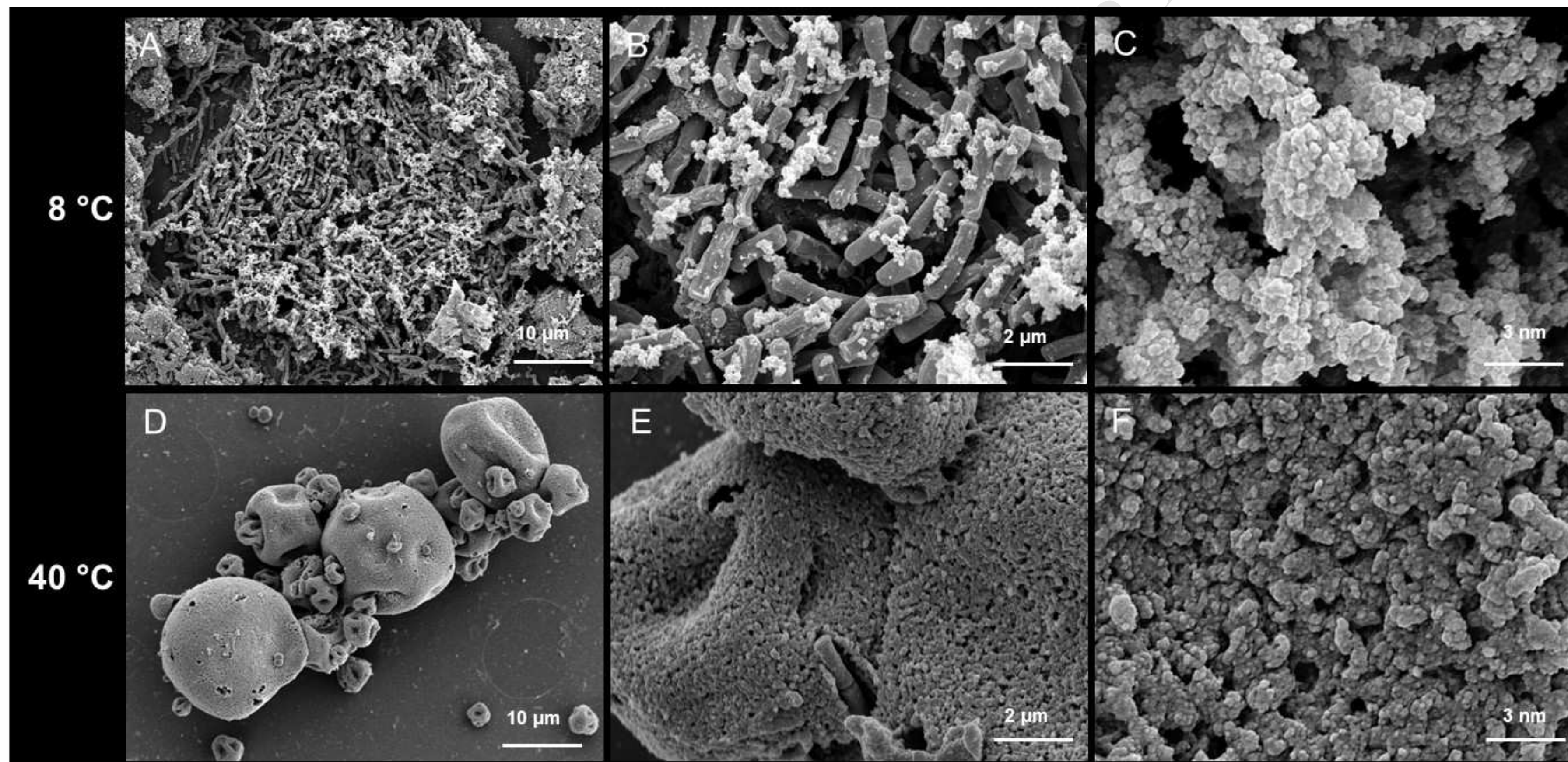

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Figure 4: Influence of chymosin action prior to spray-drying on microparticles reconstitution behavior at 8 and 40 °C. CMP: caseinomacropeptide

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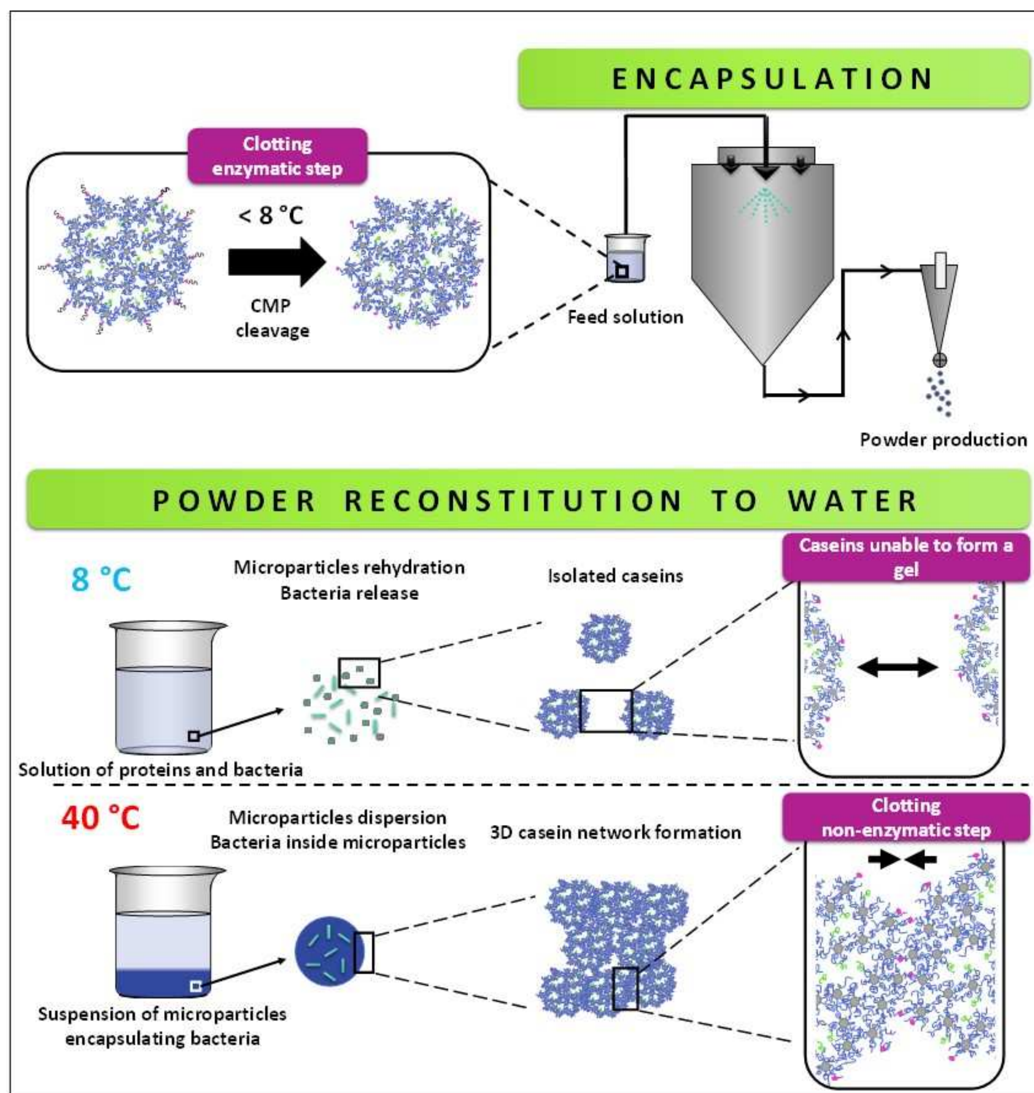

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Figure 5: Evolution of backscattered and the transmitted light after reconstitution of microparticles in water at 8 (**A**) or 40 °C (**B**). Arrows indicate the direction of evolution from the beginning to the end (20 minutes) of the measure. Backscattering and transmission were used to follow microparticles sedimentation at the bottom of the tube and clarification of the sample at the top of the tube respectively.

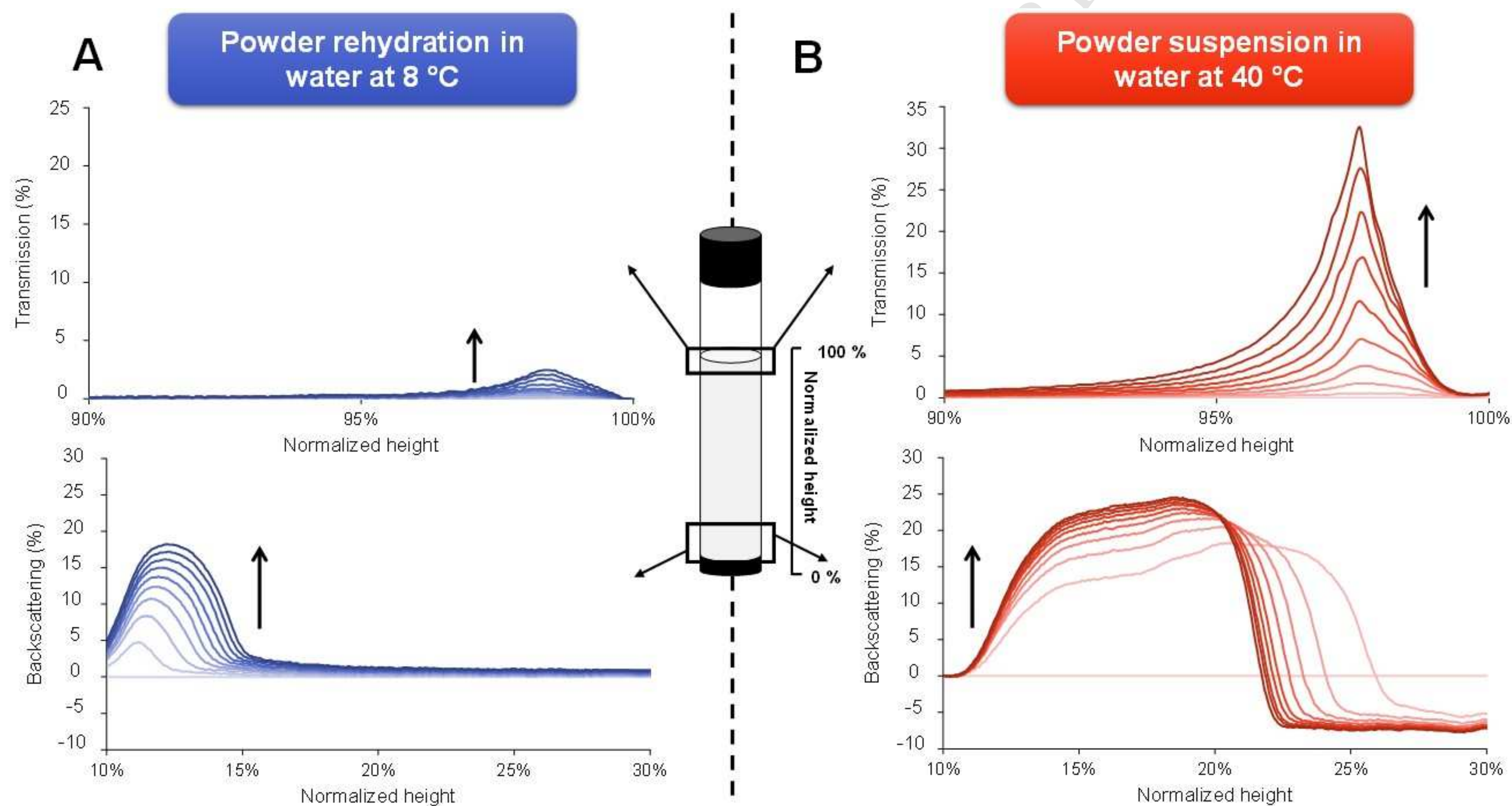
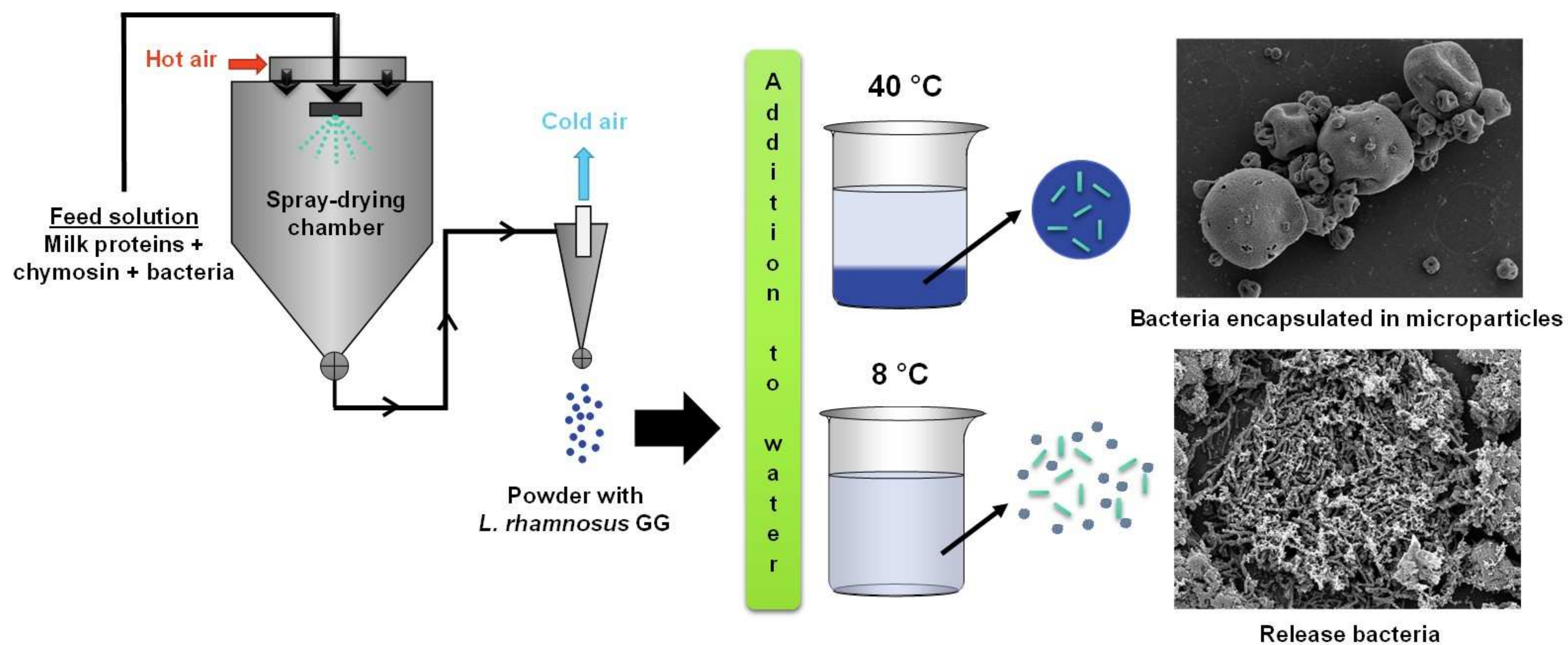


Figure 6: Production of microparticles containing LGG by spray drying: general process.



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Highlights

- Good survival of *L. rhamnosus* GG after spray-drying in milk protein matrices
- Clotting reaction control during process to produce matrices with new functionalities
- Water-insoluble matrices when microparticles are reconstituted in hot water
- Microparticles rehydration and probiotic release upon reconstitution in cold water

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