Optimization of electrospraying conditions for the microencapsulation of probiotics and evaluation of their resistance during storage and *in-vitro* digestion

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ABSTRACT

Electrospraying has recently emerged as a novel microencapsulation technique with potential for the protection of probiotics. However, research efforts are still needed to minimize the viability loss observed during the processing of sensitive strains, and to maximize productivity. The aim of the present work was the optimization of the electrospraying conditions for the microencapsulation of a model probiotic microorganism, *Lactobacillus plantarum*, within a whey protein concentrate matrix. In a pre-optimization step, the convenience of encapsulating fresh culture instead of freeze-dried bacteria was established. Additionally, a surface response methodology was used to study the effect of the applied voltage, surfactant concentration, and addition of a prebiotic to the formulation on cell viability and productivity. Viability losses lower than 1 $\log_{10}$ CFU were achieved and the bacterial counts of the final products exceeded 8.5 $\log_{10}$ CFU/g. The protection ability of the developed structures during storage and *in-vitro* digestion was also evaluated.

Keywords

Electrospraying, encapsulation, *L. plantarum*, probiotic, whey protein

1 INTRODUCTION

Microencapsulation technologies constitute a plausible approach for the preservation of biologically active ingredients in food systems including probiotic bacteria (Chandramouli, Kailasapathy, Peiris, & Jones, 2004; Kailasapathy & Champagne, 2011; Krasaekoopt, Bhandari, & Deeth, 2003), not only during processing or storage, but also during gastrointestinal transit, improving the delivery of probiotic strains to the large intestine (Shori, 2015). Several methods have been reported to microencapsulate probiotic microorganisms, including spray-coating (Champagne, Raymond, & Tompkins, 2010), emulsion and/or spray-drying (Picot & Lacroix, 2003), extrusion (Doherty et al., 2012), and gel-particle technologies (Chandramouli et al., 2004). Being a well-established process that can produce large amounts of material, spray-drying is the most commonly used microencapsulation technique in the food industry (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007). However, this technique involves the use of high temperatures, which results in significant cell mortality (Salar-Behzadi et al., 2013). On the other hand, spray-coating and entrapment in gel particles generate relatively big particles (90-250 μm and >200 μm, respectively) that may affect food sensory qualities (Augustin, 2003), and the latter is considerably expensive to be scaled up in the food industry (Champagne et al., 2010; Krasaekoopt et al., 2003). Recently, electrospraying has emerged as an alternative microencapsulation technique (Bock, Dargaville, & Woodruff, 2012) which can generate very fine particulate structures in a one-step process (Chakraborty, Liao, Adler, & Leong, 2009) under mild conditions (López-Rubio & Lagaron, 2012). It basically involves the application of a high voltage electrical field to a polymer solution, dispersion or melt which is sprayed towards a charged collector, where the dry nano- or microparticles are deposited (Bhardwaj & Kundu, 2010; Bhushani & Anandharamakrishnan, 2014; Chakraborty et

al., 2009) (cf. Supplementary material). Some of the advantages of electrospraying include the possibility of working under mild ambient conditions and using food-grade solvents, achieving high encapsulation efficiencies and obtaining smaller particle sizes than in conventional mechanical atomisers (Bock et al., 2012; Chakraborty et al., 2009; Jaworek & Sobczyk, 2008). Recently, electrohydrodynamic processes were proposed for the entrapment of living bifidobacteria within ultrathin polymeric fibers (López-Rubio, Sanchez, Sanz, & Lagaron, 2009), which significantly reduced their viability loss during storage at different temperatures. Furthermore, the electrospraying technique proved to be useful for the encapsulation of *Bifidobacterium animalis* subsp. *lactis* Bb12 within edible hydrocolloids for functional foods applications, effectively prolonging bacterial survival at different relative humidities (López-Rubio, Sanchez, Wilkanowicz, Sanz, & Lagaron, 2012). Although the viability of the aforementioned commercial strain was not significantly affected by the electrohydrodynamic process, it was found that a certain viability loss occurred when trying to microencapsulate non-commercial probiotic cultures during the electrospraying processing. In addition, the different process parameters and the properties of the probiotic feed suspension have an impact on the productivity of the electrospraying technique, as the formation of stable jets from aqueous media is complicated (R. Pérez-Masiá, J. M. Lagaron, & A. López-Rubio, 2014) and often leads to dripping of the polymeric solution if conditions are not optimized. Thus, a study of the impact of different electrospraying variables on the bacterial viability loss and the process yield is needed.

Lactobacillus plantarum, a prominent species among lactic acid bacteria which has been found to colonize healthy human gastrointestinal tracts (Gbassi, Vandamme, Ennahar, & Marchioni, 2009) and to whom a number of health benefits have been attributed, such as reduction of serum cholesterol levels (Yoon et al., 2013) or

88 downregulation of proinflammatory genes (Baüerl et al., 2013), has been used as a
89 model probiotic microorganism for the present study. Regarding the encapsulation
90 matrix, a whey protein concentrate (WPC) was selected as the main wall component of
91 the capsules, as its capability for preserving the viability of probiotic microorganisms
92 had already been demonstrated in a previous work using *B. animalis* (López-Rubio et
93 al., 2012). Moreover, whey proteins are cheap by-products from the cheese industry and
94 possess functional characteristics (López-Rubio & Lagaron, 2012).
95 The aim of the present work was to study the impact of the electrospraying conditions
96 on the viability of *L. plantarum* within the obtained WPC microstructures, as well as on
97 the productivity of the process. This mathematical modelling has been attempted
98 following a Design of Experiments (DoE) methodology (Jovanović, Rakić, Ivanović, &
99 Jančić–Stojanović, 2014), applying a second-order Box-Behnken design to maximize
100 the bacterial viability and the process yield. The capability of the microencapsulation
101 structures obtained by applying the optimal electrospraying conditions to prolong the
102 survival of *L. plantarum* during storage at different relative humidities, as well as during
103 static *in-vitro* digestion, was also evaluated.
104

105 ## 2 MATERIALS AND METHODS

106 ### 2.1 Materials

107 Whey protein concentrate (WPC), under the commercial name of Lacprodan® DI-8090
108 and with a w/w composition of ~80% protein, ~9% lactose and ~8% lipids, was kindly
109 donated by ARLA (ARLA Food Ingredients, Viby, Denmark), and was used without
110 further purification. *Lactobacillus plantarum* strain CECT 748 T was obtained from the
111 Spanish Cell Culture Collection (CECT) and routinely grown in Man, Rogosa and
112 Sharpe (MRS) broth (Scharlau, Barcelona, Spain). Serial dilutions were made in 1 g/L

meat peptone solution and plate counting was performed on MRS agar, both provided by Conda Pronadisa (Spain). Surfactant Tween20®, maltodextrin (with dextrose equivalent 16.5-19.5), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, bile extract porcine and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (Spain). The commercial resistant starch Fibersol® was manufactured by ADM/Matsutani (Iowa, USA). The LIVE/DEAD BacLight Bacterial Viability Kit was purchased from Invitrogen (California, USA). All inorganic salts used for the *in-vitro* digestion tests were used as received.

2.2 Preparation of WPC dispersions

WPC dispersions were prepared by mixing the protein concentrate with distilled water or skimmed milk under magnetic stirring at room temperature to achieve a concentration of 0.3 g/mL. This concentration had been previously optimized based on previous works (López-Rubio & Lagaron, 2012; López-Rubio et al., 2012) in order to minimize dripping of the suspensions during electrospraying. Fibersol® and/or Tween20® were also added to some of the formulations in variable amounts.

2.3 Preparation of probiotic cells suspensions

Two different strategies were used to incorporate the probiotic cells within the WPC dispersions. The first one involved the use of a fresh culture of *L. plantarum*. Bacteria were grown in MRS broth for 24 h at 37ºC, reaching the growth stationary state as observed from the growth curves (results not shown) constructed using a POLARstar Omega Microplate Reader from BMG LABTECH (Ortenberg, Germany) (final cell density of 9-10 $\log_{10}$ CFU/mL). The lactobacilli were then collected by centrifugation in

50 mL tubes at 4000 rpm for 5 min using an Eppendorf Centrifuge 5804R equipped with an Eppendorf Rotor S-4-72, obtaining a pellet that was subsequently washed twice with PBS and re-suspended in the WPC dispersions. The second approach consisted in freeze-drying the cell culture after re-suspension of the twice-washed pellet in a PBS solution containing 0.1 g/mL of maltodextrin, and subsequent incorporation of the freeze-dried cells into the WPC dispersions.

2.4 Preparation of probiotic-containing capsules through electrospraying

The suspensions were processed using a Fluidnatek® LE-10 electrospinning/ electrospraying apparatus, equipped with a variable high voltage 0–30 kV power supply, purchased from BioInicia S.L. (Valencia, Spain). Probiotic-containing WPC suspensions were introduced into a sterile 5 mL plastic syringe and pumped at a steady flow-rate of 0.15 mL/h through a stainless-steel needle (2.41 mm of inner diameter). The needle was connected through a PTFE wire to the syringe, which was placed on a digitally controlled syringe pump (KD Scientific Inc., Holliston, U.S.A.). The obtained encapsulation structures were collected on a stainless-steel plate connected to the ground electrode of the power supply and placed at a distance of 10 cm with respect to the tip of the needle. The suspensions were processed during a fixed time of 4h. Applied voltage varied within the range of 10-14 kV. A schematic representation of the setup used for microencapsulation can be found in the supplementary material.

2.5 Viability of encapsulated and non-encapsulated *L. plantarum*

The viability of *L. plantarum* was evaluated by plate counting. Samples were subjected to 10-fold serial dilutions in 1 g/L meat peptone solution and plated on MRS agar. After 24-48 h incubation at 37ºC, the number of colony-forming units (CFU) was determined.

Cell viability was evaluated for the probiotics-containing WPC suspensions before processing (N_0) and in the dry electrosprayed products (N_{ES}) by resuspension of a precise amount of the powder in 1 mL of peptone solution. Tests were made in triplicate.

2.6 Morphological characterization of the capsules

Scanning electron microscopy (SEM) was conducted on a Hitachi microscope (Hitachi S-4800) at an accelerating voltage of 10 kV and a working distance of 10-12 mm. Samples were sputter-coated with a gold-palladium mixture under vacuum prior to examination.

In addition, optical microscopy images were taken using a digital microscopy system (Nikon Eclipse 90i) fitted with a 12 V, 100 W halogen lamp and equipped with a digital imaging head which integrates an epifluorescence illuminator. A digital camera head (Nikon DS-5Mc) was attached to the microscope. Nis Elements software was used for image capturing.

In order to confirm the presence of the probiotic bacteria within the WPC capsules, the cells were stained using a LIVE/DEAD kit (BacLight® viability kit, Invitrogen) prior to the electrospraying process. Because the dye used to observe live cells in green (SYTO 9®) was found to also stain WPC, it was not possible to discern live bacteria from the encapsulating matrix after the electrospraying process, and only sporadic death cells could be distinguished in red due to the propidium iodide dye. Consequently, bacteria were intentionally killed before staining by resuspension of the twice-washed bacterial pellet in 20 mL of 96% (v/v) ethanol and subsequent incubation at room temperature for 40 minutes. The ethanolic suspension was then washed and the dead pellet was resuspended in 10 mL of buffered peptone water. Propidium iodide was used as a red

187 dye according to the manufacturer protocol, before incorporating the dead bacteria to
188 the feed WPC dispersions for electrospraying.
189
190 **2.7 Optimization of electrospraying process parameters through Box-**
191 **Behnken experimental design**
192 Three key variables were selected for the optimization of the electrospraying process in
193 terms of bacterial viability loss (y_1) and product yield (y_2): the applied voltage (x_1), the
194 concentration of a prebiotic additive (Fibersol®) incorporated into the formulation (x_2),
195 and the concentration of a surfactant (Tween20®) added to the feed suspensions (x_3). A
196 Box-Behnken fractional-factorial experimental design was developed with these three
197 variables at three levels (3^3) in order to reduce the number of experimental runs. This
198 model was used to correlate the response variables, y_1 and y_2, to the independent
199 variables, x_1, x_2 and x_3, by fitting them to a polynomial second order model, whose
200 general equation is Eq. 1, where y_i are each of the predicted responses, x_i and x_j are the
201 input variables affecting the response variables, β_0 is the offset term, β_i are the linear
202 coefficients, β_{ii} are the quadratic coefficients and β_{ij} are the interaction coefficients
203 (Ismail & Nampoothiri, 2010). Table 1 summarizes the independent variables (factors)
204 used and their assayed levels (coded as +1, 0 and -1).
205
206 $$y_i = \beta_0 + \beta_i \sum x_i + \beta_{ii} \sum x_i^2 + \beta_{ij} \sum_i \sum_j x_i x_j \qquad \text{(Eq. 1)}$$
207
208 INSERT TABLE 1 ABOUT HERE
209

A small number of experimental runs (i.e. 15 runs, each made in triplicate) was necessary for the optimization process (cf. Table 3). The design included replicated central points. The bacterial viability loss (ΔN) was calculated as the difference in viability per unit mass of dry solids in the suspensions before processing and in the dry electrosprayed products, according to Eq. 2. The product yield was determined according to Eq. 3.

$$\Delta N = N_0 - N_{ES} \quad \text{(Eq. 2)}$$

$$Product\ yield\ (\%) = \frac{Mass\ of\ electrosprayed\ product\ recovered\ from\ collector}{Mass\ of\ solids\ in\ the\ processed\ suspension} \times 100 \quad \text{(Eq. 3)}$$

2.8 Survival of encapsulated *L. plantarum* under stress conditions

Selected electrosprayed capsules containing *L. plantarum* were stored at medium and high relative humidities (RH), i.e. 53 and 75%, as described in (López-Rubio et al., 2012), and their bacterial viability was tested after different time intervals. For this purpose, the powders were introduced in desiccators containing $Mg(NO_3)_2$ and NaCl saturated solutions, respectively. Similarly, freeze-dried samples of *L. plantarum* obtained from the same formulations used for the electrospraying process (by lyophilization of the feed WPC-based suspensions) were also stored at the same conditions, in order to compare the proposed microencapsulation technique with a well-established preservation method.

2.9 Survival of encapsulated *L. plantarum* during digestion

Suspensions (0.03 g/mL) of the electrosprayed capsules or their freeze-dried counterparts, respectively, in distilled water were subjected to *in-vitro* gastrointestinal digestion in order to evaluate the survival of protected *L. plantarum* during simulated consumption. Digestion was simulated according to the standardized static *in vitro* digestion protocol developed within the framework of the Infogest COST Action (Minekus et al., 2014). Simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared according to the harmonized compositions (Minekus et al., 2014). In the oral phase, the suspensions were mixed with SSF (50:50 v/v) and incubated at 37ºC for 2 min under agitation in a thermostatic bath. In the gastric phase, the oral digesta was mixed with SGF (50:50 v/v) and porcine pepsin (2000 U/mL), and incubated at 37ºC for 2 h under agitation. In the duodenal phase, the gastric digesta was mixed with SIF (50:50 v/v), porcine bile extract (10 mM) and porcine pancreatin (100 U/mL of trypsin activity), and incubated at 37ºC for 2 h under agitation. The pH was adjusted to 7, 3, and 7 in the oral, gastric and duodenal phases, respectively. Aliquots were collected after the gastric and the duodenal phases and the viability of *L. plantarum* in the digestas was assessed by plate counting.

2.10 Statistical analysis

The response surface modelling was conducted using the software Unscrambler X (version 10.1, CAMO software AS, Oslo, Norway, 2010). The statistical analysis of the Box-Behnken model was performed through analysis of variance (ANOVA). A $p < 0.05$ was considered significant. The determination coefficient (R^2), which measures the goodness of fit of the regression model, was used as an indicator of the quality of the model to predict the experimental data.

257 A statistical analysis of the rest of the experimental data was performed through
258 analysis of variance (one-way ANOVA) using OriginPro 8 (OriginLab Corp.,
259 Northampton, USA). Homogeneous sample groups were obtained by using Fisher LSD
260 test (95% significance level, $p < 0.05$).
261
262 **3 RESULTS AND DISCUSSION**
263 **3.1 Pre-optimization of the electrospraying conditions**
264 The first (preliminary) step in this process optimization was to determine whether the
265 initial state of the cells had an effect on cell viability after encapsulation and, also, to
266 determine the maximum cell load which could be incorporated within the protein
267 matrix. For this purpose, freeze-dried bacteria were added to the WPC dispersions in
268 different amounts, and compared with addition of bacterial pellets obtained from
269 different volumes of fresh culture in 5 mL of WPC dispersion. The use of a non-ionic
270 surfactant, Tween20® (a polysorbate), to improve the electrospraying process (R.
271 Pérez-Masiá, J. Lagaron, & A. López-Rubio, 2014) and limit the dripping of the
272 aqueous solutions was also considered, and its influence on the bacterial viability was
273 thus studied. Table 2 summarizes the test conditions used is this first stage. The applied
274 voltage was fixed at 10 kV in all cases, keeping the rest of the processing parameters as
275 stated in Section 2.4.
276
277 INSERT TABLE 2 ABOUT HERE
278
279 First, the initial viability (N_0) of *L. plantarum* in the WPC suspensions was compared
280 using the different formulations. It was observed that adding higher amounts of freeze-
281 dried bacteria (i.e. $5 \cdot 10^{-3}$, 10^{-2} and $1.5 \cdot 10^{-2}$ g/mL) did not yield significant differences (p

282 < 0.05) in the initial cell counts. All the formulations prepared using freeze-dried
283 bacteria exhibited an initial bacterial viability in the range of 7.23 ± 0.21 log_{10} CFU/g.
284 Similarly, no significant differences ($p < 0.05$) were found in the initial cell counts
285 when using pellets obtained from 5 mL or 10 mL of fresh culture to prepare the
286 suspensions. In these cases, the average initial viability was 9.38 ± 0.42 log_{10} CFU/g.
287 Lastly, the suspension prepared using a pellet obtained from 100 mL of fresh culture
288 had an average N_0 of 10.28 ± 0.35 log_{10} CFU/g. These results showed that significantly
289 higher initial cell counts were achieved when using fresh culture for the preparation of
290 the suspensions. It is worth mentioning that higher amounts of freeze-dried bacteria
291 could not successfully be incorporated into the WPC dispersions because the resultant
292 feed suspensions aggregated and precipitated in the syringe during the electropraying
293 process.
294
295 Figure 1 summarizes the results from viability loss (ΔN) and product yields of *L.*
296 *plantarum* after the electrospraying process of the different formulations containing
297 freeze-dried bacteria (a, c) and fresh culture (b, d). In general, greater viability losses
298 and lower productivities were observed when using freeze-dried microorganisms than
299 for fresh cultures, mainly explained by the poor bacterial dispersion within the WPC
300 suspension when using the freeze-dried form of the probiotic strain. However, the
301 suspension prepared using 100 mL of fresh culture also exhibited a high viability drop
302 and lower productivities, which could be attributed to an excess of biomass in the
303 formulation which apart from hindering proper microencapsulation of the bacteria, led
304 to extensive dripping of the solution. In general, the product yield was considerably
305 improved by the addition of Tween20® which, apart from favouring bacterial
306 dispersion, led to lower surface tensions of the suspensions (Rocío Pérez-Masiá et al.,

2014) and it did not significantly affect the viability loss of *L. plantarum* at the low concentrations used (1-5% w/w).

INSERT FIGURE 1 ABOUT HERE

In view of the results, the formulation containing a bacterial pellet obtained from 10 mL of fresh broth and 5 wt.% of Tween20® provided the greatest product recovery percentage (16.1 ± 2.1%) while experiencing one of the lowest viability losses (0.6 ± 0.1 $\log_{10}$ CFU/g). This specific formulation resulted in capsules containing over 10^9 CFU/g (cf. Figure 2), a final bacterial count in the dry product which was similar to that obtained from a 100 mL-pellet, although with much higher productivity. Thus, this formulation was chosen as the starting point for further optimization of the electrospraying process through a Box-Behnken experimental design, in order to increase the product yield while maintaining high bacterial viabilities in the final product.

INSERT FIGURE 2 ABOUT HERE

3.2 Mathematical modelling of the electrospraying process

Three key factors potentially influencing the bacterial viability and product yield in the microencapsulation of probiotics through electrospraying processes were selected for the Box-Behnken modelling: the applied voltage (x_1), the concentration of Fibersol® in the formulations (x_2), and the ratio of Tween20® in the feed suspensions (x_3). The applied voltage is known to exert an effect on the properties of electrosprayed materials (Bock et al., 2012). On one hand it must be sufficiently high to overcome the surface

tension of the suspensions, in order to efficiently produce the microcapsules. On the other hand, it was hypothesized that too strong electric fields might impose a source of stress on the probiotic strain, having an impact on its viability. Fibersol® is a commercial resistant starch recognized as GRAS by the FDA, so it was used as a prebiotic additive (Topping & Clifton, 2001) to ascertain whether the addition of the carbohydrate, apart from giving rise to a symbiotic product, could enhance bacterial viability within the capsules. Other prebiotics have been previously reported to increase the viability of probiotic bacteria upon microencapsulation through spray-drying (Fritzen-Freire et al., 2012). Lastly, the positive effect of Tween20® on the product yield was evidenced in the previous section and a more exhaustive study of its impact on bacterial viability loss should be carried out in order to optimize the feed formulation.

Hence, a Box-Behnken design was developed with these three factors at three levels in order to assess their impact on the response variables and find the optimum combination of these parameters able to yield the best results. The lower and upper levels of each factor (cf. Table 1) were fixed based on preliminary experiments carried out to determine the limits which allowed a stable electrospraying process (results not shown). A total of 15 experimental runs, each made in triplicate, were necessary to construct the design models. Table 3 summarizes the full design and the experimental values obtained for the response variables in each run.

INSERT TABLE 3 ABOUT HERE

356 The results in Table 3 were used to construct two polynomial second order models
357 (according to Eq. 1) with the aid of the software Unscrambler X, each corresponding to
358 one of the response variables y_1 and y_2. Both models were statistically analyzed using
359 analysis of variance (ANOVA) in order to check the significance of their linear,
360 quadratic and interaction terms, as well as the significance of the models themselves.
361 The quality of the models was also checked by comparing the experimental results in
362 Table 3 with the values predicted by the models. As observed in Figure 3, the model
363 obtained for y_1 did not accurately describe the experimental values. This was attributed
364 to the intrinsic variability of the results and consequent high deviations that are obtained
365 when studying microbiological systems. Due to its great lack of fit, the use of this
366 model for the prediction and optimization of the viability loss during electrospraying
367 was considered risky and thus the model was disregarded. Conversely, an acceptable
368 value was obtained for the lack of fit of the model for y_2 and, thus, this model was
369 considered adequate for the prediction and optimization of the product yield for the
370 proposed electrospraying process.
371
372 INSERT FIGURE 3 ABOUT HERE
373
374 The final model equation which correlates the product yield with the three factors, after
375 disregarding non significant terms, is expressed in Eq. 4, where x_i are the coded values
376 of the factors (from -1 to +1, cf. Table 1). Table 4 shows the results of the ANOVA
377 analysis for this model, from where it could be concluded that the linear terms
378 corresponding to the applied voltage and the ratio of Tween20®, as well as their
379 interaction, are highly significant ($p<0.01$). The linear and quadratic terms involving
380 the ratio of Fibersol® in the formulation were also statistically significant ($p<0.05$). The

offset term (β_0 = 27.9) corresponds to the predicted value of the product yield at the central point (x_1=0; x_2=0; x_3=0), and its value is not significantly different from the experimental result (31.0 ± 7.8 %). This further confirms the goodness of fitting of this model.

$$y_2(\%) = (27.9 + 12.4x_1 + 3.8x_2 + 23.8x_3 + 10.7x_3x_1 + 7.5x_2^2) \quad \text{(Eq. 4)}$$

INSERT TABLE 4 ABOUT HERE

In practice, the mathematical model in Eq. 4 can be interpreted by comparing the magnitude of its coefficients. Firstly, all of them are positive, which means that an increase in any of the three factors within the limits of the model had a favourable effect on product yield. The factors which had the greatest impact were the concentration of surfactant (β_3 = 23.8) and the applied voltage (β_1 = 12.4). Indeed, the addition of surfactants has been proposed as a useful strategy for the successful production of electrosprayed materials from biopolymeric aqueous solutions or dispersions, as they reduce their high surface tension and thus facilitate their spraying at acceptable voltages (Rocío Pérez-Masiá et al., 2014). On the other hand, increasing the applied voltage helps overcoming the surface tension of the fluid and facilitates the electrospraying process (Bock et al., 2012). In fact, both factors had a synergistic effect, as evidenced from their high interaction coefficient (β_{13} = 10.7). Figure 5 shows the interaction effect of varying these two factors on the product yield according to the model, for a constant level of x_2. The addition of the prebiotic carbohydrate also improved the product yield, although to a lesser extent (β_2 = 3.8; β_2^2 = 7.5). It is worth noting that product yields

405 over 65% were achieved for the best combinations, that is, more than three times greater
406 than the best result obtained from the pre-optimization stage, showing a considerable
407 improvement.
408
409 INSERT FIGURE 4 ABOUT HERE
410
411 Regarding the viability loss during electrospraying, although a successful mathematical
412 modelling was not achieved in terms of the three proposed factors, the results allowed
413 the extraction of some qualitative conclusions about the effects of these variables on the
414 process. While a clear tendency was not observed for variations in the applied voltage
415 or the concentration of Fibersol® in the formulation, a slight increase in the viability
416 loss during electrospraying was found in average for increasing Tween20® contents. In
417 any case, the viability loss always remained below 1 $\log_{10}$ CFU/g, thus yielding average
418 bacterial counts in the final electrosprayed materials in the range of 8.7-9.3 $\log_{10}$
419 CFU/g.
420
421 **3.3 Morphology of selected encapsulation structures**
422 Two of the electrosprayed materials obtained from section 3.2. were selected for further
423 evaluation. The first one was the powder produced in Run 8, as the conditions used for
424 these tests (x_1=1; x_2=0; x_3=1) resulted in one of the highest product yields. No
425 significant differences were found between the product yields obtained for Run 8 and 12
426 (cf. Table 3), but the former showed a slightly lower viability loss and, thus, this sample
427 was chosen. The second one was selected in order to obtain minimal viability losses.
428 However, the runs which resulted in the minimum losses had too low product yields.
429 Thus, a minimum product yield of 50% was fixed as an acceptable limit considering

that lower yields would not be industrially attractive for production. The conditions used in Run 4 (x_1=1; x_2=1; x_3=0) resulted in the electrosprayed capsules which, meeting this requirement, experienced the lowest viability loss, so the product obtained from this specific composition was chosen for further testing.

Figure 5 shows the morphology of the selected samples. The electrosprayed product obtained from Run 8 exhibits a more homogeneous structure, where individual and spherical particles could be distinguished. In contrast, the powder obtained in Run 4 presents a rather amorphous shape, with some individual capsules but also some fused structures. These differences might be attributed to the greater ratio of Tween20® used in Run 8, which facilitated the electrospraying process by reducing the surface tension of the suspensions. The location of the lactobacilli inside the electrosprayed capsules was confirmed by the optical and fluorescence microscopy images of the materials (cf. Figure 5 C and D). As stated in Section 2.6, bacteria had to be killed before staining to avoid the use of the dye SYTO 9®, which also stained the WPC matrix and precluded the identification of live cells. Although many of the particles did not contain microorganisms, the presence of dead bacteria within some of the WPC-based capsules was confirmed.

INSERT FIGURE 5 ABOUT HERE

3.4 Survival of encapsulated *L. plantarum* under stress conditions

The ability of the selected capsules to protect *L. plantarum* when subjected to stress conditions was assessed by measuring the viability of the probiotic within the materials after storage during certain time periods at different relative humidity conditions (i.e.

455 53% and 75%). The survival of the lactobacilli within the electrosprayed capsules was
456 compared to that of freeze-dried samples containing the same formulations as in Runs 4
457 and 8, respectively. All materials exhibited similar initial cell counts regardless of the
458 method used for their preservation, confirming that the viability losses observed upon
459 electrospraying are similar to those produced during freeze-drying. Figure 6 shows that
460 electrosprayed microcapsules provided enhanced protection to the bacteria compared to
461 freeze-drying for the same formulations at both storage conditions. Indeed, for the
462 freeze-dried materials, a reduction of 1 $\log_{10}$ CFU/g was observed after 1 day of storage
463 at 75% RH, and there were no cell counts after 1 week. In contrast, no viability losses
464 were found for microencapsulated bacteria after 24h and their survival was prolonged
465 for 10 days at the same conditions. Similarly, less than 1 $\log_{10}$ CFU/g reduction was
466 observed for microencapsulated bacteria after 3 weeks of storage at 53% RH while the
467 viability of freeze-dried bacteria decreased almost 3 $\log_{10}$ CFU/g in the same period.
468 Furthermore, while no cell counts were found for the freeze-dried samples after 45 days,
469 the microcapsules only experienced about 3 $\log_{10}$ CFU/g viability loss in the same time
470 period. These results are attributed to a better material organization in the compact,
471 capsular assemblies than in the porous, random structure of the freeze-dried material,
472 which resulted in the prolonged viability of the encapsulated bacteria.
473
474 INSERT FIGURE 6 ABOUT HERE
475
476 **3.5 Survival of encapsulated *L. plantarum* during digestion**
477 Viability of *L. plantarum* microcapsules and freeze-dried material was also evaluated
478 after an *in-vitro* digestion process. Table 5 shows the cell counts obtained initially and
479 after the gastric and duodenal phases of the digestion. Again, all the materials presented

similar cell counts prior to the digestion process. It was observed that the main viability loss occurred after the gastric phase, due to the acidic conditions of this stage (pH=3). However, the microencapsulation achieved a slightly better protection for the bacteria than freeze-drying, probably because the arrangement of the wall material into capsular structures delayed its dissolution, thus slightly delaying the exposure of the probiotic bacteria to the simulated gastric fluid and enhancing the protective effect of the matrix in comparison with the freeze-dried samples. However, very small differences were observed after the intestinal phase, probably because in this step the capsules were completely disrupted and could not protect the bacteria. Nevertheless, the slight differences observed between both processing techniques could also be ascribed to the high resistance of this specific strain to acidic conditions, as observed in preliminary trials which showed that *L. plantarum* viability was hardly affected by acid conditions (pH = 3.8) after 1h of exposure (data not shown).

INSERT TABLE 5 ABOUT HERE

4 CONCLUSIONS

The present work shows the convenience of using fresh culture of *L. plantarum* over freeze-dried bacteria for the preparation of the feed suspensions, as this approach led to higher initial cell counts in the WPC suspensions, lower viability losses during electrospraying and greater process productivities. Also, the addition of a surfactant, Tween20®, to the feed suspensions considerably increased the product yield. Although the model obtained for the viability loss could not explain the experimental results with statistical significance, a model for the product yield was successfully developed through a Box-Behnken experimental design. According to this model, an increase in

any of the three selected factors selected (applied voltage, surfactant concentration and addition of a prebiotic) had a favourable effect on the product yield, being the concentration of surfactant and the applied voltage the factors which had the greatest impact on the product yield, exhibiting a synergistic effect. Regarding the bacterial viability loss during electrospraying, it remained below 1 $\log_{10}$ CFU/g in all tests, so that the final electrosprayed materials had average bacterial counts in the range of 8.7-9.3 $\log_{10}$ CFU/g. Finally, while the electrosprayed microcapsules conferred *L. plantarum* similar protection against digestion as compared to a more widely-used preservation method such as freeze-drying, they proved to offer enhanced protection during storage of the food ingredient at high relative humidity. Since the ingredients used to prepare the encapsulating matrices in this study are edible and/or recognized by the FDA as GRAS, incorporation of the proposed structures into food products would be a feasible approach for the development of enhanced functional food products containing probiotics.

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622 Lactobacillus plantarum NR74 from fermented foods. *International Journal of Food*
623 *Sciences and Nutrition 64*(1), 44-52.
624
625
626

627 **Table 1.** Factors for the Box- Behnken design and their levels. Mass fractions are
628 expressed with respect to the amount of WPC
629
630
631
632

Factors		Levels		
		-1	0	+1
x_1	Voltage (kV)	10	12	14
x_2	Fibersol® (wt.%)	0	10	20
x_3	Tween20® (wt.%)	1	5	9

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Table 2. Different conditions evaluated during the pre-optimization stage

State of bacteria	Amount of bacteria	Proportion of Tween20
Freeze-dried	5 mg/mL$_{WPC\ susp.}$	-
Freeze-dried	10 mg/mL$_{WPC\ susp.}$	-
Freeze-dried	15 mg/mL$_{WPC\ susp.}$	-
Freeze-dried	5 mg/mL$_{WPC\ susp.}$	1 g/100g $_{WPC}$
Freeze-dried	5 mg/mL$_{WPC\ susp.}$	5 g/100g $_{WPC}$
Fresh culture	Pellet from 5 mL broth	-
Fresh culture	Pellet from 5 mL broth	5 g/100g $_{WPC}$
Fresh culture	Pellet from 10 mL broth	5 g/100g $_{WPC}$
Fresh culture	Pellet from 100 mL broth	5 g/100g $_{WPC}$

649

650 **Table 3.** Complete full design and results for the response variables

651

Run	x_1 (kV)	x_2 (wt.%)	x_3 (wt.%)	y_1 (log_{10} CFU/g)	y_2 (%)
1(*)	10	0	5	0.92 ± 0.16	16.8 ± 1.6
2	14	0	5	0.96 ± 0.13	54.5 ± 8.4
3	10	20	5	0.76 ± 0.15	24.6 ± 1.7
4	14	20	5	0.74 ± 0.03	56.4 ± 4.1
5	10	10	1	0.64 ± 0.09	9.3 ± 7.9
6	14	10	1	0.50 ± 0.12	3.2 ± 3.0
7	10	10	9	0.66 ± 0.12	29.6 ± 9.4
8	14	10	9	0.83 ± 0.05	65.7 ± 9.2
9	12	0	1	0.72 ± 0.15	5.0 ± 1.6
10	12	20	1	0.84 ± 0.20	7.2 ± 2.5
11	12	0	9	0.99 ± 0.09	50.6 ± 10.1
12	12	20	9	0.89 ± 0.18	68.5 ± 3.5
13-15	12	10	5	0.54 ± 0.04	31.0 ± 7.8

652 (*) From previous section

653

654

655

656 **Table 4.** ANOVA analysis for the response surface quadratic model in Eq. 4

657

	Sum of Squares	DF(*)	Mean Square	F ratio	p-value
Model	1.955	5	0.3909	55.54	4.1×10^{-15}
x_1	0.373	1	0.3725	52.92	2.4×10^{-8}
x_2	0.035	1	0.0353	5.01	3.2×10^{-2}
x_3	1.359	1	1.3585	193.01	2.4×10^{-15}
x_1x_3	0.137	1	0.1365	19.40	1.0×10^{-4}
x_2^2	0.052	1	0.0517	7.34	1.0×10^{-2}
Lack of Fit	0.129	7	0.0184	4.63	0.0018
$R^2 = 0.894$					

658 (*) DF = Degrees of freedom

659

660

661

662 **Table 5.** Viability of *L. plantarum* before digestion and after the gastric and duodenal
663 phases. Different letters (a-c) within the same column indicate significant differences
664 among the samples

Sample	Initial (UFC/g)	After Gastric Phase (UFC/g)	After Intestinal Phase (UFC/g)
Freeze-Drying (Run 4)	$(2.3 \pm 1.4) \times 10^{9\,a}$	$(1.5 \pm 0.1) \times 10^{7\,a}$	$(4.7 \pm 1.1) \times 10^{6\,a}$
Electrospraying (Run 4)	$(2.0 \pm 1.4) \times 10^{9\,a}$	$(2.7 \pm 0.5) \times 10^{7\,b}$	$(8.7 \pm 3.3) \times 10^{6\,a}$
Freeze-Dring (Run 8)	$(1.7 \pm 0.8) \times 10^{9\,a}$	$(1.4 \pm 0.4) \times 10^{7\,a}$	$(6.7 \pm 1.6) \times 10^{6\,a}$
Electrospraying (Run 8)	$(2.3 \pm 0.6) \times 10^{9\,a}$	$(7.3 \pm 0.7) \times 10^{7\,c}$	$(1.6 \pm 0.2) \times 10^{7\,b}$

665

666

667

668 **FIGURE CAPTIONS**

669

670 **Figure 1.** Viability losses obtained using a) freeze-dried bacteria and b) fresh culture,
671 and product yields using c) freeze-dried bacteria and d) fresh culture. Different letters in
672 each pair of graphs denote statistically significant differences between results ($p<0.05$).

673

674 **Figure 2.** Cell viability of the final electrosprayed products obtained from pellets of
675 fresh culture of *L. plantarum*. Different letters denote statistically significant differences
676 between results ($p<0.05$).

677

678 **Figure 3.** Predicted versus experimental values for a) viability loss and b) product yield.

679

680 **Figure 4.** Response surface for the product yield, showing the interaction of the applied
681 voltage and the ratio of Tween20® at a constant level of prebiotic concentration (level
682 0).

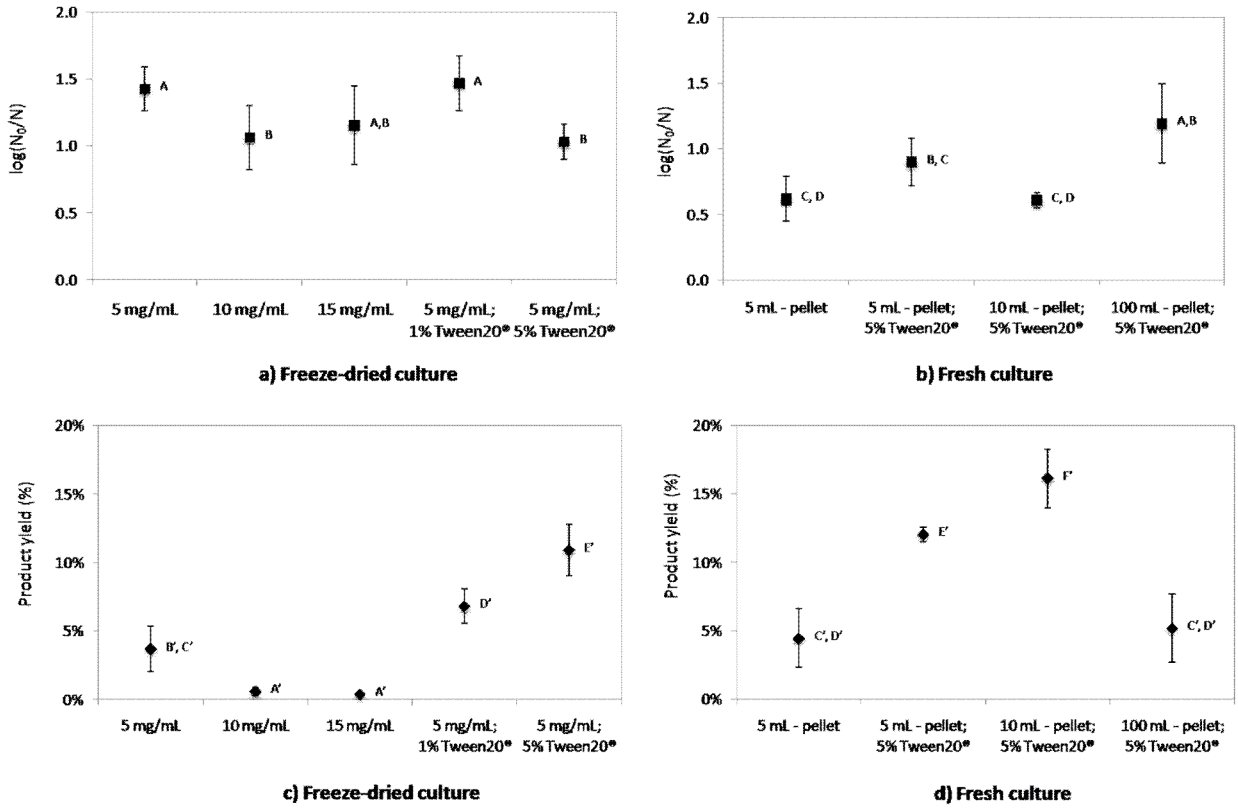
683

684 **Figure 5.** SEM images of WPC-based electrosprayed microcapsules containing *L.*
685 *plantarum*, obtained under conditions in Run 4 (A) and Run 8 (B), and optical
686 micrographs of the latter under normal light (C) and using a fluorescence source (D).
687 Scale bars in all images correspond to 20 μm.

688

689 **Figure 5.** Survival of electrosprayed microcapsules (continuous lines) vs. freeze-dried
690 materials (dotted lines) after storage at different relative humidities.

691



692

693 FIGURE 1.

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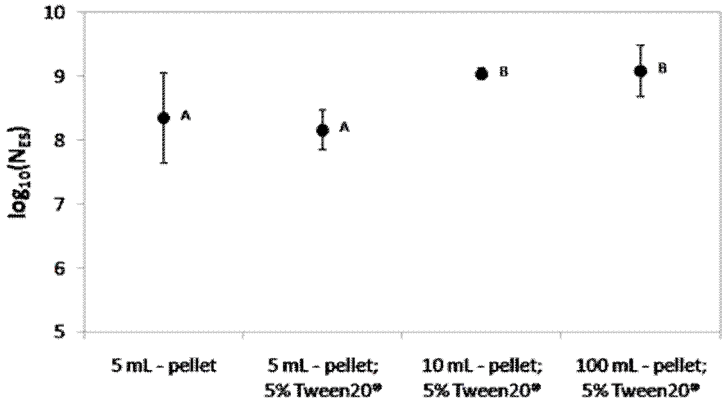

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696 FIGURE 2.

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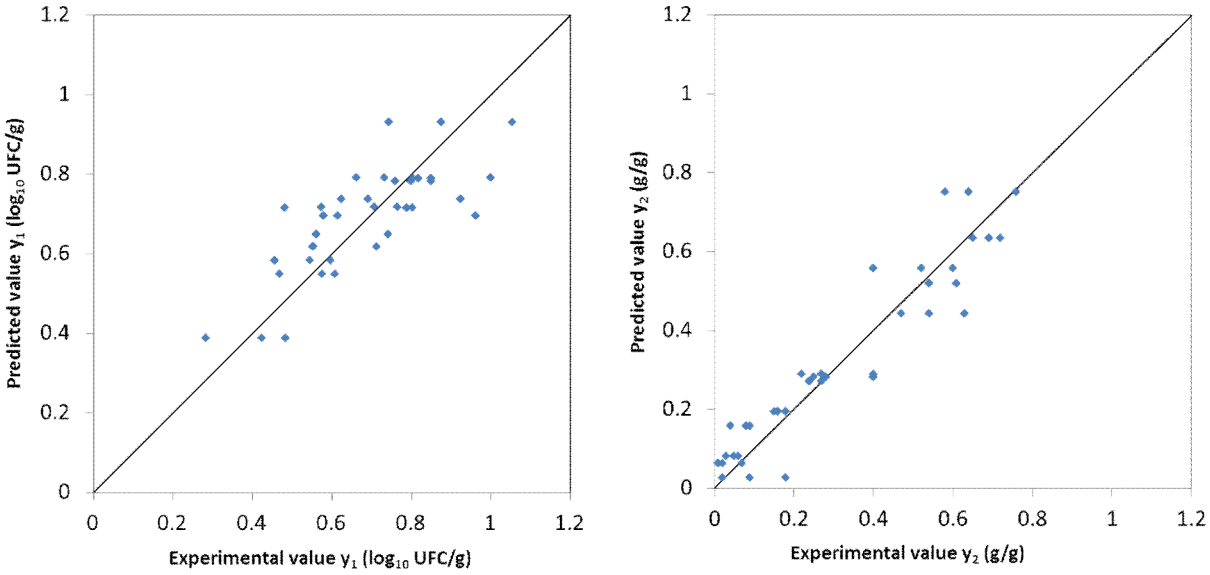

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699 FIGURE 3.

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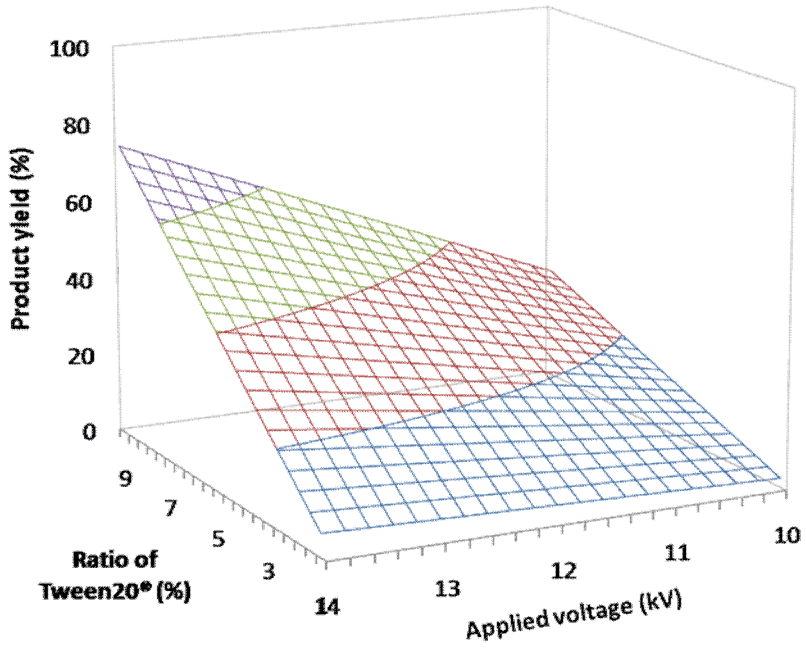

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702 FIGURE 4.

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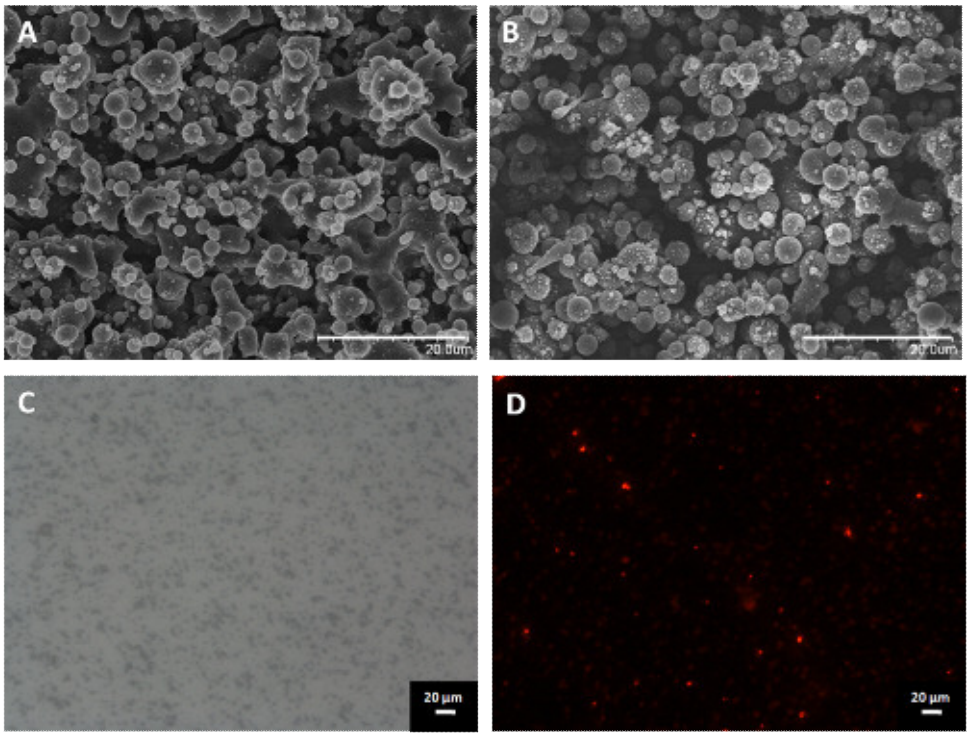
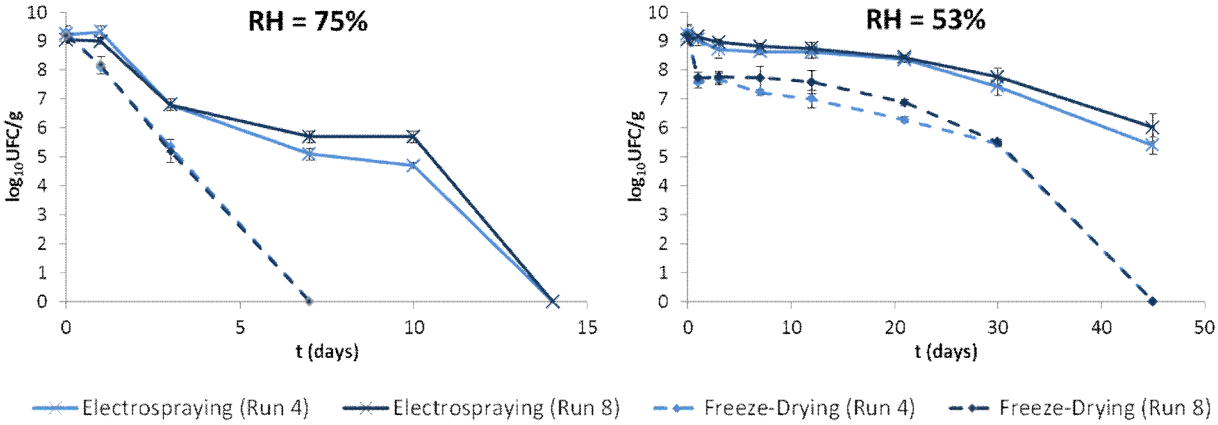


FIGURE 5.



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709 FIGURE 6.

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711 Highlights

712 • Surface response methodology was used to optimize a probiotic encapsulation process
713 • Electrospraying was used to encapsulate *L. plantarum* as a model probiotic
714 • Voltage, surfactant and prebiotic concentration were the three variables considered
715 • Low viability losses and great product yields were obtained after optimization
716 • Enhanced protection during storage at high relative humidity was also observed
717

718

Supplementary Material

Scheme 1. Simplified representation of the electrospraying setup used for the microencapsulation of *L. plantarum*