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Simple and double microencapsulation of *Lactobacillus acidophilus* with chitosan using spray drying

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Abstract

The aim of this study was to evaluate the survival of *Lactobacillus acidophilus* that had been simple or double spray dried using chitosan to cause microencapsulation and which had been exposed to model gastrointestinal conditions. In addition, the study also determined the physicochemical properties of the powder containing the microencapsulated probiotic.
Chitosan-inulin or chitosan-maltodextrin (1:15 or 1:25) solutions were inoculated with 10^{12} CFU mL^{-1} of *L. acidophilus*, for simple microencapsulation. The different solutions were dried using a spray dryer with an inlet air temperature of 130 °C and a solution flux of 4.8 g min^{-1}. A two-step process was used for the double microencapsulation. In the first step, the probiotic was added to a gelatin-maltodextrin (1:25) solution and then spray dried; for the second step, the microencapsulated probiotic was added to a chitosan-inulin or chitosan-maltodextrin (1:25) solution and then it was spray dried again.
With the simple microencapsulated probiotic, a microbial reduction of 7 log cycles was obtained. With the double microencapsulated probiotic only 3 log reductions were achieved. The double microencapsulated probiotic thus demonstrated greater resistance to simulated gastrointestinal conditions. The powders produced were shown to have water activity values of 0.176 - 0.261 at 25 °C and moisture content of 0.8 – 1.0%, which are characteristic of spray dried products. The *bulk density* was significantly ($p < 0.05$) lower (300 kg m^{-3}) for simple than for double (400 kg m^{-3}) microencapsulated probiotic powders. Solubility and dispersibility of the powder microcapsules were better at lower pH values.
Double microencapsulation using a process of spray drying is therefore recommended for probiotics, thus exploiting chitosan's insolubility in water, which can be applied for the of development food products.

Keywords: Encapsulation; Probiotics; Microcapsules; Physicochemical powder properties

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Abbreviations

Ch-I:	coating of chitosan and inulin mixtures
Ch-M:	coating of chitosan and maltodextrin mixtures
Ch-I(G-M):	coating of double microencapsulation with chitosan and inulin mixtures of G-M
Ch-M(G-M):	coating of double microencapsulation with chitosan and maltodextrin mixtures of G-M
DE:	dextrose equivalent
G-M:	coating of gelatin and maltodextrin mixture for simple microencapsulation
SGF:	simulated gastric fluid
SI:	solubility index
SIF:	simulated intestinal fluid
TS:	total solids content
wb:	wet basis

1 Introduction

Functional foods that contain probiotics are continuously increasing in the global market (Amin, Thakur, Jain, et al., 2013). Probiotics especially have been added to dairy products like fermented milk, yogurt, cheese, butter, ice-cream or milk-drink beverages, and have been developed more than any other food product group (Kailasapathy, 2006; Karimi, Mortazavian, & Da Cruz, 2011; Di Criscio et al., 2010). Non-dairy food products such as fruit and vegetables drinks, soy, cereals and meat products with probiotics have been recently tested (Barboza, Marquez, Parra, Patricia Pinero, & Medina, 2012; Gawkowski & Chikindas, 2013; Granato, Branco, Nazzaro, Cruz, & Faria, 2010). It has been noted that desired levels of the survival of probiotics at suggested concentration of 10^7 CFU g^{-1} in the final food product to assure the beneficial effects in the body (FAO/WHO, 2006) are not always retained. Therefore, the evaluation of functionality of food formulated with probiotics under model gastrointestinal conditions *in vitro* is suggested (Gbassi & Vandamme, 2012). Different probiotic strains (*Lactobacillus* spp. and *Bifidobacterium* spp.) have also been studied to evaluate their resistance to acid conditions and bile salts. Of these, *Lactobacillus acidophilus* and *L. salivarius* were the most acid-tolerant and just as susceptible to bile salt exposure as *Bifidobacterium* spp. (Ding & Shah, 2007).

Several microencapsulation techniques (extrusion, emulsion, freeze-drying and spray drying) have also been proposed to enhance probiotics survival during food processing, storage and the passage through the gastrointestinal tract (Amin et al., 2013; Anal & Singh, 2007). The efficacy of each technique is dependent on the bacteria strain (its resistance to the stress factors within the microencapsulation process and further release), the wall material used (physicochemical properties and stability), the cost-effectiveness of the microencapsulated probiotic, the final application in a food product (fluid, semi-fluid or solid) and its storage temperature (freeze, refrigeration or room temperature) (Rokka & Rantamaki, 2010).

Probiotic microencapsulation through a process of spray drying has been studied from different perspectives, mainly testing different coating materials and process conditions, in the aim of ensuring probiotics viability, stability and a wider application in food products (Amin et al., 2013; Corona-Hernandez et al., 2013; Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007). The commonly used coating materials for spray drying microencapsulation are carbohydrates (dextroses, maltodextrins, starches, gum arabic, alginate, inulin, fructo-oligosaccharides and chitosan), proteins (whey protein, gelatin and skimmed milk) and lipids (vegetable oils) (Estevinho, Rocha, Santos, & Alves, 2013; Fritzen-Freire et al., 2012; Rokka & Rantamaki, 2010). Some selection criteria for the coating material used are based on the physicochemical properties, solubility, viscosity in the prepared solution, the compatibility with the core-material and the intended size and surface of the final mi-

crocapsules (Chávez & Ledeboer, 2007). Since the use of a single material does not satisfy all desired requirements, the combination of different coating materials has been suggested to be used in mixture or to create multilayers (Desai & Park, 2005).

Chitosan is a cationic polymer obtained with different degrees of deacetylation (40 – 98%) of chitin (N-acetyl-glucosamine polymer) and having a molecular weight of 50 - 2000 kDa; these parameters determine characteristics like crystallinity, biodegradability and viscosity when it is dissolved in solvent systems (Estevinho et al., 2013). The microcapsules obtained by spray drying when using chitosan are characterized by high sphericity and low water vapour sorptivity, they are therefore very stable during storage, and demonstrate controlled release characteristics caused by its low solubility at neutral pH values (Adamiec & Modrzejewska, 2005). Chitosan has been applied as coating material for spray drying microencapsulation of a wide variety of pharmaceutical products, enzymes, emulsions and vitamins (Estevinho et al., 2013). While there are many studies concerning probiotic microencapsulation with chitosan by the extrusion technique (Rokka & Rantamaki, 2010; Teoh, Mirhosseini, Mustafa, Hussin, & Manap, 2011), not much information has been found regarding probiotic spray drying microencapsulation using chitosan as coating material. Chitosan has antimicrobial activity which may necessitate the use of other coating materials as co-protectors or a two-step microencapsulation (Amin et al., 2013; Ivanovska et al., 2012; Teoh et al., 2011).

The objectives of this study were to evaluate the survival of *Lactobacillus acidophilus* that had been simple or double spray dried using chitosan for microencapsulation and which had been exposed to model gastrointestinal conditions. In addition, the study also determined the physicochemical and physical properties of microencapsulated probiotic in powder.

2 Materials and Methods

2.1 Materials

Probiotic culture

The probiotic strain used in this study was *Lactobacillus acidophilus* NRRL (B-4495), donated by the Agriculture Research Services Department of the USDA to the Food Microbiology Laboratory of Universidad de las Américas Puebla (UDLAP). This strain was cultivated in Man, Rogosa & Sharpe (MRS) agar (DB Difco, France), anaerobically at 35-37 °C. *L. acidophilus* was then adapted to 40 °C in MRS broth (DB Difco, France) to increase its thermal resistance when submitted to spray drying.

Coating agents

The coating materials used were chitosan with deacetylation degree >75% (Sigma, Mexico), maltodextrin 10 DE (Globe 19100, Mexico), agave inulin (Fructagave, Mexico) and gelatin (Gelco SA, Colombia).

2.2 Methods

Simple and double microencapsulation

Mixtures of chitosan-inulin (Ch-I) (1:15 or 1:25), chitosan-maltodextrin (Ch-M) (1:15 or 1:25) and gelatin-maltodextrin (G-M) (1:25), at 26% w/w, were dissolved with stirring in ascorbic acid solution at 1%. For simple microencapsulation these solutions were inoculated with 10^{12} CFU mL^{-1} of *L. acidophilus*. The different solutions were atomized using a spray dryer (B-290, Büchi Laboertechnik, Switzerland) with an inlet air temperature of 130 °C and a solution flux of 4.8 g min^{-1}. A two-step process was used for the double microencapsulation. In the first step, the probiotic was added to G-M (1:25) aqueous solution and then was spray dried with the same process conditions as for the simple microencapsulation; for the second step, one gram of the microencapsulated probiotic was added to 100 mL of Ch-I or Ch-M (1:25) solutions previously prepared, and

then spray dried using the same process conditions as in the first step.

L. acidophilus viability

The microencapsulated probiotic viability was determined by microbial survival count, 1 g of the microencapsulated probiotic powder was dispersed in 9 mL sterile peptone water (0.1%). Solutions were serially diluted (10^{-1} to 10^{-9}) to determine bacterial enumeration (30- 300 CFU plate^{-1}) in MRS agar (DB Difco, France). The process was carried out in triplicate. Inoculated plates were incubated at 35-37 °C under anaerobic conditions and, colonies were counted after 48 h (Hernández-Carranza, López-Malo, & Jiménez-Munguía, 2013).

Gastrointestinal model simulation

According to the USP (2002), simulated gastric fluid (SGF) (2 g NaCl, 3.2 g pepsin, 7 mL of HCl, made up to 1 L volume and adjusted to pH of 2.0) and simulated intestinal fluid (SIF) (6.8 g KH_2PO_4, 10 g pancreatin, 190 mL of NaOH 0.2N, made up to 1 L volume, and adjusted to pH of 7.0) were formulated. 1 g of the microencapsulated probiotic powder was added to 9 mL of SGF and incubated at 35 °C for 2 h, and then 1 mL of this preparation was added to 9 mL of SIF and was incubated at 35 °C for 3 h. At the beginning and after each step of the gastrointestinal model simulation, *L. acidophilus* viability was determined as described in section 2.2.2.

Physical properties of powders

The measurements of the physical properties of the powders were conducted in triplicate. Particle diameter of the powders was measured with a particle analyzer (Bluewave S3500, Microtrac, USA). *Bulk density* (ρ_b) was determined by calculation of the relation of mass and volume of the powder, specific mass of powder was weighed and poured into a cylinder (10 mL) without tapping. *Tapped density* (ρ_t) was determined by manually tapping the cylinder 250 times and recording the final volume occupied by the powder. *Particle density* (ρ_p) *or apparent density* was determined by recording the volume occupied in a cylinder of a known quantity of powder and 6 mL of petroleum ether as described by Telang and Thorat (2010). *Powder porosity* (ε) was calculated as: $\varepsilon = 1 - (\rho_b/\rho_p)$.

Phsysicochemical properties of powders

Water activity (a_w) was measured using a hygrometer (Aqua lab, Mod. 3TE, Decagon Devices Inc., USA). *Moisture content* was determined by the 925.45 AOAC method (AOAC, 2000). *Hygroscopicity* was determined by exposing 1 g of powder to 75% relative humidity, using supersaturated NaCl solution at 25 °C. The powder weight change was recorded every 2 days to constant weight until a weight difference of 0.001 g between sequential data was recorded. The measurements of a_w and moisture content of the powders were conducted in triplicate, and the moisture gain kinetic for the hygroscopicity property was carried out in duplicate.

Reconstitution properties of powders

The reconstitution properties of microencapsulated powders were determined, in triplicate, in aqueous solutions adjusted to pH 3.0, 5.0 or 7.0. *Immersion time* was determined as the time needed for 1 g of powder to disappear from the surface of 200 mL of solution, no stirring applied. For the *dispersibility* test, 10 g of powder was poured into 9 mL of the solution, then particle size distributions were measured with a particle analyzer (Bluewave S3500, Microtrac, USA), every minute, during 10 min, setting a flow of 12 mL min^{-1}; the different pH solutions were used as carrier liquid for the measurements. *Solubility* test was performed as described by Telang and Thorat (2010) with some modifications; 1.3 g of powder in 10 mL of solution was centrifuged at 1,000 rpm for 5 min and total solids content (TS) was determined in the residue, by the 925.45 AOAC method (AOAC, 2000). *Solubility index (SI)* was calculated as the solids solubilized in the solutions after centrifugation: SI = (1.3 – TS)/ 1.3.

Statistical analysis

To determine significant differences among the different treatments, analysis of variance (ANOVA) and Tukey tests were applied to the data with a confidence level of 95% ($\alpha = 0.05$), using Minitab v.16.0 software (Minitab Inc., USA).

3 Results and Discussion

3.1 Probiotic survival

Spray drying process

The use of chitosan as coating material by spray drying produced powders with good stability; however, its known antimicrobial properties were of great concern for probiotic encapsulation thus the viability of the encapsulated bacteria had to be evaluated. In the present study, simple microencapsulation using a mixture Ch-M or Ch-I was performed comparing two proportions (1:15 and 1:25). Results of *L. acidophilus* population reductions after spray drying (Table 1) demonstrated that only for the coating mixture of Ch-M, the proportion of 1:25 was significantly smaller ($p< 0.05$) than the proportion of 1:15. This result could be attributed to the increase of solids in the sprayed solution which presumably promoted a better protection against thermal damage (Avila-Reyes, Garcia-Suarez, Teresa Jimenez, San Martin-Gonzalez, & Bello-Perez, 2014; Desmond, Ross, O'Callaghan, Fitzgerald, & Stanton, 2002). Nevertheless, the log reductions of the bacteria were very high (> 7.13 log cycles). These could be due to the antimicrobial property of chitosan; cationic materials such as chitosan are mediated by electrostatic forces with negatively charged parts of bacteria cell wall, due to competition with available calcium ions, resulting in cell wall disruption (Corona-Hernandez et al., 2013). Therefore, to reduce the probiotic cell damage, a double microencapsulation was proposed, first encapsulating with G-M and then microencapsulation using a mixture of Ch-M or Ch-I, avoiding the direct contact of chitosan with the bacteria. For the double encapsulation, Ch-M(G-M) and Ch-I(G-M), *L. acidophilus* only demostrated log reductions of 2.77 and 3.03 respectively, after the spray drying process. Similar reductions were obtained by Ivanovska et al. (2012) when double microencapsulating *L. casei*, first using alginate and fructooligosaccharide solutions for spray-drying and then using chitosan and calcium chloride for complexation with the alginate and finally freeze drying, demonstred 2.67 log reductions.

Gastrointestinal model conditions

One of the main advantages of using chitosan is its ability to protect probiotics in simulated gastrointestinal fluids, nevertheless in literature this has been demonstrated only for microencapsulation by the extrusion technique with chitosan and alginate beads freeze-dried, spray-dried or not (Lee, Cha, & Park, 2004; Ivanovska et al., 2012; Teoh et al., 2011; Urbanska, Bhathena, & Prakash, 2007). In most of the cases, the comparison between non-encapsulated probiotic cells and microencapsulated with chitosan have shown that there is 5 to 6 log cycles of difference. In our study, *L. acidophilus* free cells were also exposed to SGF and after 2 h, 6.23 log cycle reductions were obtained. Probiotic population with simple encapsulation in G-M had a reduction of 5.21 log cycles while for the double encapsulation of the probiotic with Ch-M(G-M) or Ch-I(G-M), the population reduction reported was of 1.1 log cycles. The subsequent exposure of these cells to SIF for 3 h, resulted in a smaller additional log cycles reduction, demonstrating a total probiotic reduction of 1.65 and 1.84 cycles for the double encapsulation with Ch-M(G-M) or Ch-I(G-M), in contrast to 5.84 and 7.00 log cycles for the simple microencapsulated probiotic with G-M and free cells respectively (Fig. 1). The slightly higher survival levels noted when using the mixture coating of Ch-I(G-M) could be due to the high solubility of inulin at neutral pH values. Besides, prebiotics have been recommended for use as co-protectants for microencapsulation, promoting a further bacteria proliferation once these are released in the colon (Chen, Chen, Liu, Lin, & Chiu, 2005; Corcoran, Ross, Fitzgerald, & Stanton, 2004).

Table 1: Survival and log reductions of *L. acidophilus* after simple or double microencapsulation by spray drying

Microencapsulation	Coating mixture		Initial population (CFU g^{-1})	Final population (CFU g^{-1})	Log reduction
	Material	Proportion			
Simple	Ch-M	01:15	$9.90x10^{13}$	$2.50x10^{6}$	7.58 ± 0.02^{a}
		01:25	$9.90x10^{13}$	$3.70x10^{6}$	7.45 ± 0.03^{b}
	Ch-I	01:15	$7.20x10^{14}$	$5.20x10^{7}$	7.13 ± 0.02^{c}
		01:25	$5.80x10^{14}$	$3.30x10^{7}$	7.21 ± 0.04^{c}
Double	G-M	01:25	$3.45x10^{13}$	$4.30x10^{12}$	0.93 ± 0.01^{d}
	Ch-M(G-M)	01:25	$3.60x10^{12}$	$6.05x10^{9}$	2.77 ± 0.01^{e}
	Ch-I(G-M)	01:25	$3.60x10^{12}$	$3.17x10^{9}$	3.03 ± 0.03^{f}

$^{a-f}$Different letters in the same column indicate significant difference ($p < 0.05$) by Tukey test Ch: chitosan, M: maltodextrin, I: inulin, G: gelatin

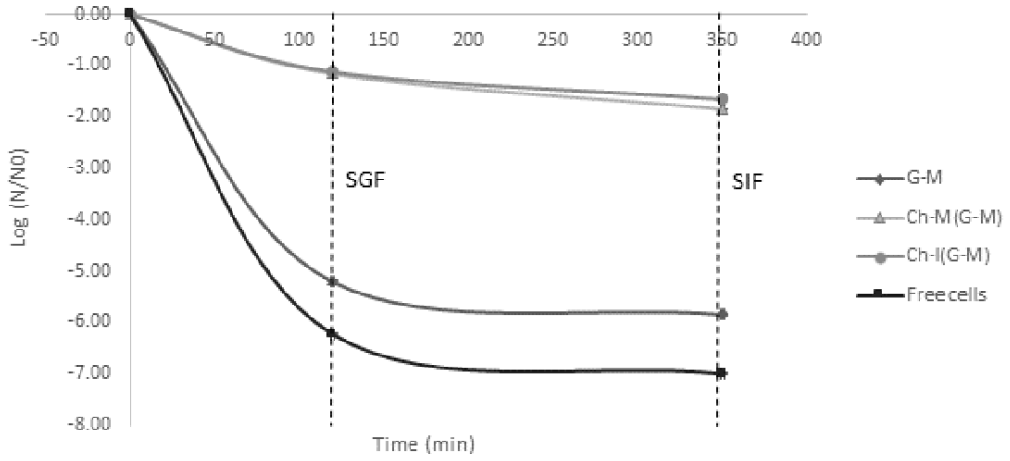

Figure 1: Survival of *L. acidophilus* exposed to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), non-microencapsulated (free cells), simple microencapsulated (G-M) and double microencapsulated (Ch-M(G-M), Ch-I(G-M)). G: gelatin, M: maltodextrin, Ch: chitosan, I: inulin

3.2 Properties of encapsulated probiotic powders

Physical properties

Spray dried microcapsules commonly have a particle diameter range of 5 to 100 μm (Fritzen-Freire et al., 2012; Hernández-Carranza et al., 2013; Rokka & Rantamaki, 2010). It has been reported that small particle sizes facilitates a greater contact surface for the nutrients availability (Avila-Reyes et al., 2014). Besides, microencapsulates with mean sizes smaller than 100 μm do not affect palatability of food products when these are incorporated (Corona-Hernandez et al., 2013). Our results correspond to the particle size reported in literature of spray drying microencapsulation of probiotics, presenting a particle median diameter average for the simple microencapsulated probiotic of 11.39 μm, while for the double microencapsulated probiotic median diameters with Ch-M(G-M) and Ch-I(G-M) were 13.94 and 21.37 μm, respectively. The double microencapsulated probiotic showed a wider particle distribution than the simple microencapsulated ones, with significantly higher *bulk density* (ρ_b) values (378 - 400 kg m^{-3}) ($p < 0.05$) and lower powder porosity ($\varepsilon = 0.80$) (Table 2). A heterogeneous particle distribution allows the rearrangement of the individual particles and consequently a more compact powder (Fuchs et al., 2006). For this reason, tapped densities (ρ_t) for the double microencapsulating probiotic powders were also significantly higher ($p < 0.05$) than the rest of the powders.

Physicochemical properties

Coating materials composition determines microcapsule stability during storage because the materials are strongly related to the final physicochemical properties of the spray dried powders (Rokka & Rantamaki, 2010). The obtained a_w values in this study are in the range of 0.144 - 0.261 (Table 3). According to the statistical analysis, the simple microcapsules with a higher proportion of the coating material (1:25) demonstrated significantly higher a_w values ($p < 0.05$). The increase of the solids content in the suspensions to be spray dried may have retained more water molecules embedded in the obtained microcapsules, therefore increasing a_w. In a previous study, Avila-Reyes et al. (2014) presented a_w values in a range of 0.200 - 0.240 when using inulin as coating material, similar to the a_w value (0.233) obtained with the double microencapsulation mixture with inulin, Ch-I(G-M) in the present study. Simple and double layered microcapsules showed a_w values below 0.300, which is characteristic of spray-dried products with good stability during storage (Ananta, Volkert, & Knorr, 2005; Fritzen-Freire et al., 2012).

With respect to the moisture content, the powders showed values ranging from 0.84 - 1.87% (wb), with no significant difference ($p > 0.05$) between the different systems studied, except for the microcapsules obtained for simple probiotic microencapsulation with G-M (Table 3). This result may be related to the good entrapment properties of gelatin and fast coat forming during spray drying (Gharsallaoui et al., 2007; Rokka & Rantamaki, 2010).

Besides moisture content and water activity (a_w), the hygroscopicity of powders helps to determine associated problems due to caking or agglomeration during storage. For this reason, moisture gain of powders was evaluated at 75 % of relative humidity (Fig. 2). It is showed that simple microcapsules with inulin in the coating mixture (Ch-I) presented the highest moisture gain (0.30 g H_2O g^{-1}), while the double microcapsules Ch-I(G-M) and Ch-M(G-M) gained 0.20 g H_2O g^{-1}. The chemical structure of inulin has a large number of available bonds for hydrogen bonding and therefore easily captures water molecules present in the environment; this is why it is usually used in mixture with other coating materials for spray drying microencapsulation (Corona-Hernandez et al., 2013).

Reconstitution properties

The reconstitution properties were determined in the microcapsules that showed a higher probiotic survival after single or double microencapsulation, in order to know the different pH conditions in which the release of the probiotics takes place easily and consider it for its further application in foodstuff.

Table 2: Bulk, tapped and particle densities of simple or double microencapsulated *L. acidophilus*

Micro-encapsulation	Coating mixture		Density (kg m^{-3})			
	Material	Proportion	ρ_b	ρ_t	ρ_p	ε
Simple	Ch-M	01:15	303.23±0.01^a	526.64±0.02^a	5004.25±0.35^a	0.94±0.00^a
		01:25	303.24±0.02^a	526.68±0.04^a	5003.75±0.35^a	0.94±0.00^a
	Ch-I	01:15	322.77±0.01^b	476.46±0.02^a	2505.63±0.53^b	0.87±0.00^b
		01:25	328.03±0.02^c	476.43±0.03^a	2501.88±0.18^c	0.86±0.00^c
	G-M	01:25	417.11±0.13^d	625.67±0.20^b	5007.25±0.35^d	0.92±0.00^d
Double	Ch-M(G-M)	01:25	400.48±0.08^d	715.14±0.15^c	2005.50±0.14^e	0.80±0.00^e
	Ch-I(G-M)	01:25	378.00±0.05^d	742.10±0.01^d	2002.60±0.00^d	0.81±0.00^f

$^{a-f}$Different letters in the same column indicate significant difference ($p < 0.05$) by Tukey test
Ch: chitosan, M: maltodextrin, I: inulin, G: gelatin
ρ_b: Bulk density, ρ_t: tapped density, ρ_p: particle density, ε: porosity

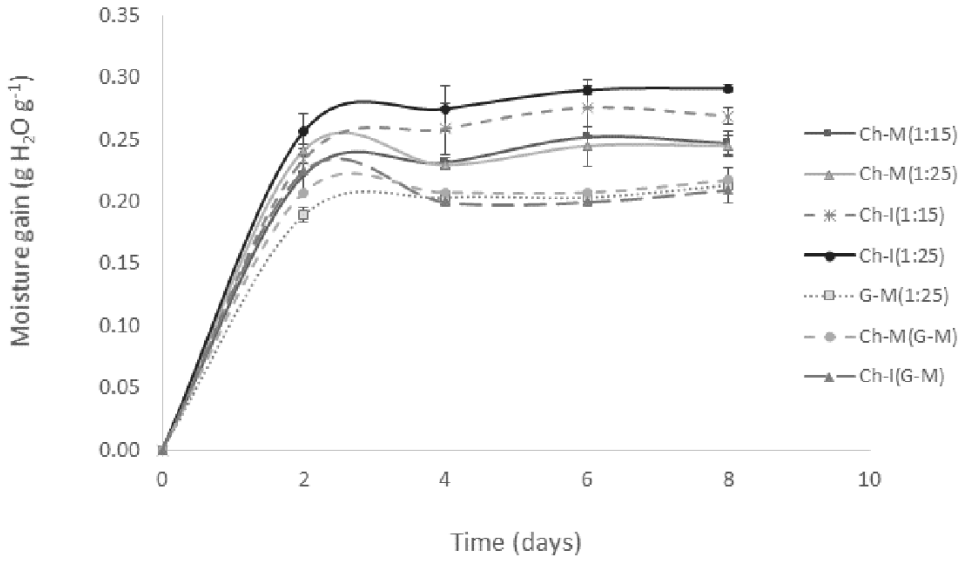

Figure 2: Moisture gain of simple microencapsulated or double microencapsulated *L. acidophilus* with different mixtures of coating materials and proportions (G: gelatin, M: maltodextrin, Ch: chitosan, I: inulin), at 75 % of relative humidity and 25 °C

Table 3: Physicochemical properties of simple or double microencapsulated *L. acidophilus*

Microencapsulation	Coating mixture		a_w	Moisture (% wb)
	Material	Proportion		
Simple	Ch-M	01:15	0.178 ± 0.002^a	0.84 ± 0.01^a
		01:25	0.255 ± 0.001^b	0.98 ± 0.01^a
	Ch-I	01:15	0.197 ± 0.002^a	1.01 ± 0.03^a
		01:25	0.261 ± 0.001^b	1.00 ± 0.14^a
	G-M	01:25	0.232 ± 0.001^b	1.87 ± 0.33^b
Double	Ch-M(G-M)	01:25	0.144 ± 0.001^c	0.98 ± 0.03^a
	Ch-I(G-M)	01:25	0.233 ± 0.001^b	1.06 ± 0.01^a

$^{a-c}$Different letters in the same column indicate significant difference ($p < 0.05$) by Tukey test
Ch: chitosan, M: maltodextrin, I: inulin, G: gelatin
a_w: water activity

According to Schubert (1987), powders poured on a liquid follows four stages: a) wettability (penetration of the liquid into the porosity of the powder by capillarity), b) sinkability (sinking of the particles below the liquid surface), c) dispersability (dispersion of the powder under stirring) and d) solubility (solution of the particles in the liquid). For instant powders, these stages are expected to occur in order of seconds. In the case of microcapsules by spray drying, these reconstitution properties may not take place in order of seconds but minutes, because the particles do not have significant particle porosity nor do they possess powder porosity as agglomerates powders may have. However, these properties give a good estimate of the powder behaviour in different media. The reconstitution properties determined in this study in three different pH values (3.0, 5.0 or 7.0) were: immersion time (related to sinkability), dispersibility and solubility. The immersion time and solubility of simple microencapsulated probiotic in G-M and double microencapsulated in Ch-M(G-M) or Ch-I(G-M) are presented in Table 4. For all the evaluated microcapsules, the immersion time was more than 5 min for the different solutions adjusted to different pH values, while the solubility test did demonstrate differences ($p < 0.05$) among the different powders and solutions. Results showed that simple microcapsules with G-M were completely solubilized in all the solutions adjusted at different pH values (SI = 1.0), while double microcapsules presented SI < 0.5, demonstrating lower SI values with higher pH values. The coating mixture containing inulin, Ch-I(G-M), resulted in a more soluble powder than the coating mixture with maltodextrin, Ch-M(G-M). This behaviour is attributed to the hydrolysis of the inulin which undergoes at pH values below 4.0.

In the dispersibility test (Fig. 3), the effect of pH was not clearly demonstrated for the simple microencapsulated probiotic (G-M), significant overlap of the particle distributions occurred in all tested times, which means that particles were solubilized rapidly. Meanwhile, with the double microencapsulated probiotic Ch-M(G-M) and Ch-I(G-M), agglomerates were formed at the beginning of the test and later showed a gradual dispersion over time, with smaller particle sizes detected. Besides, in accordance with the solubility test, the double microencapsulated probiotic with inulin, Ch-I(G-M), also showed smaller particle sizes at lower pH values than with maltodextrin in the coating mixture, Ch-M(G-M).

These reconstitution tests are of great interest since the level of effectiveness of chitosan and gelatin as coating materials in simple and double microencapsulation could be demonstrated, even if the powders were formulated with a small quantity of these materials, which are highly responsible of the slow particle solubility and dispersibility in solutions adjusted to different pH values.

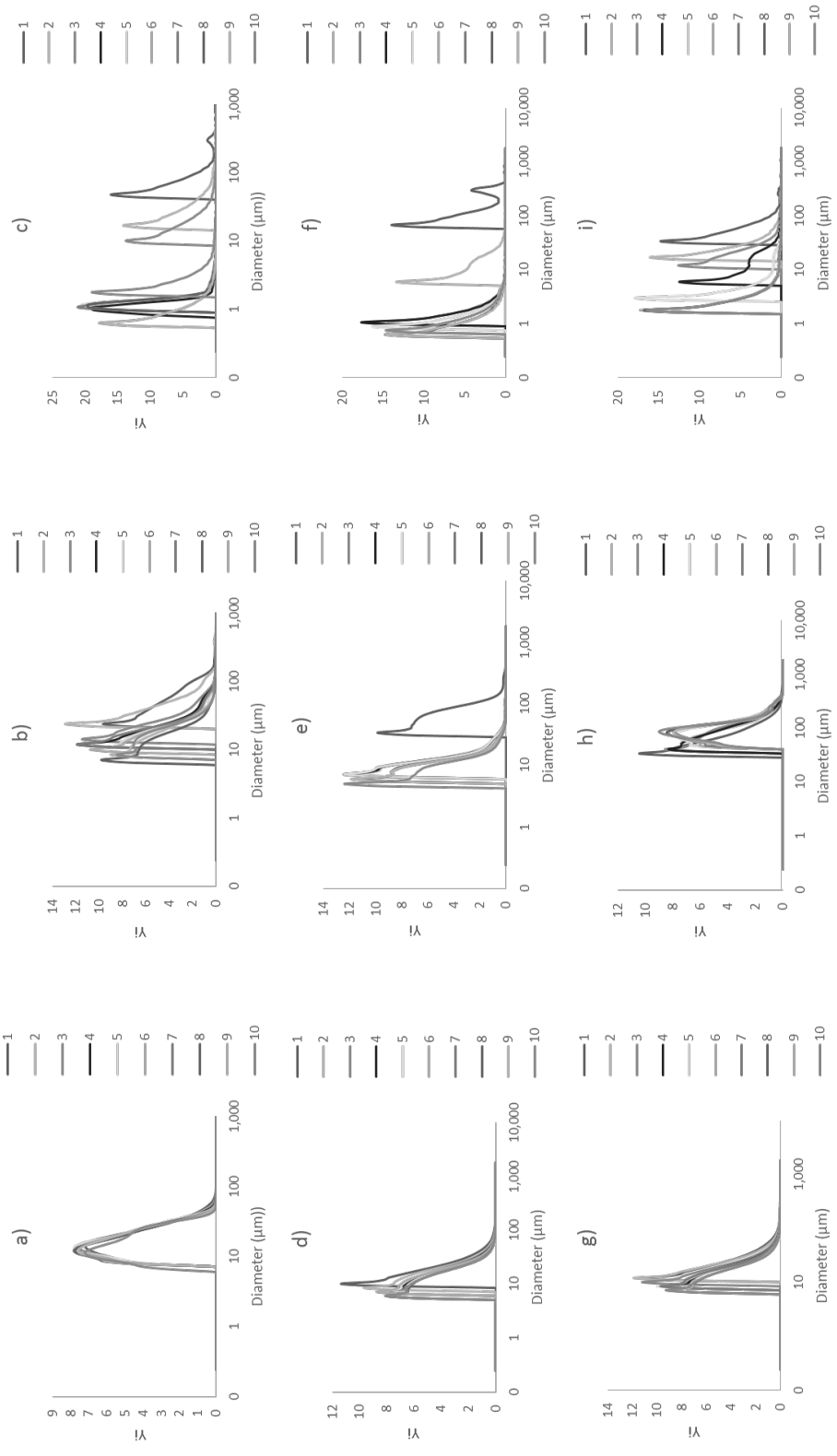

Figure 3: Particle size distribution of dispersibility test measured every minute, during 10 min, in solutions of pH 3.0 (a, b, c), pH 5.0 (d, e, f) and pH 7.0 (g, h, i) for simple microencapsulated *L. acidophilus* with G-M (a,d,g), double microencapsulated with Ch-M(G-M) (b,d,h) or Ch-I(G-M) (c,f,i). (G: gelatin, M: maltodextrin, Ch: chitosan, I: inulin).

Table 4: Reconstitution properties of simple or double microencapsulated *L. acidophilus*

Microencapsulation	Coating mixture	pH	Immersion time (min)	Solubility index (SI)
Simple	G-M	3	$> 5^a$	1.000 ± 0.000^a
		5	$> 5^a$	1.000 ± 0.000^a
		7	$> 5^a$	1.000 ± 0.000^a
Double	Ch-M(G-M)	3	$> 5^a$	0.356 ± 0.025^b
		5	$> 5^a$	0.361 ± 0.010^b
		7	$> 5^a$	0.339 ± 0.031^c
	Ch-I(G-M)	3	$> 5^a$	0.488 ± 0.047^d
		5	$> 5^a$	0.433 ± 0.011^e
		7	$> 5^a$	0.425 ± 0.002^e

$^{a-e}$Different letters in the same column indicate significant difference ($p < 0.05$) by Tukey test
Ch: chitosan, M: maltodextrin, I: inulin, G: gelatin

4 Conclusions

Double microencapsulation for probiotics by spray drying is a good alternative method for the production of insoluble powders when selecting chitosan as coating material. In this study, it was demonstrated that chitosan was effective for probiotic microencapsulation when it was exposed to gastric acid conditions when the probiotic was double encapsulated, thus maintaining its viability for further release in the colon. The use of different mixtures of coating materials (chitosan, inulin, maltodextrin and gelatin) produced stable powder microcapsules with different powder reconstitution properties, expanding the options for probiotics application in different food products.

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