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Effect of microencapsulation in whey protein and water-soluble chitosan derivative on the viability of the probiotic *Kluyveromyces marxianus* VM004 during storage and in simulated gastrointestinal conditions

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ABSTRACT

The aim of this study was to evaluate the effect of the microencapsulation (by spray drying) of the whey-native probiotic yeast *Kluyveromyces marxianus* VM004 in matrices of whey protein concentrate (WPC) and water-soluble chitosan (WSCh) on the viability of the yeast during drying and storage and in simulated gastrointestinal conditions. The optimized outlet drying temperature was 68 °C. This temperature allowed obtaining an encapsulation efficiency of 91% for a suspension of 10% (w/v) WPC. Yeasts microencapsulated in WSCh showed a significantly improved tolerance to simulated gastrointestinal conditions in comparison to free yeasts and yeasts microencapsulated in WPC. Besides, the solids content showed a significant influence on the probiotic viability during storage, with a suspension with 30% (w/v) solids (29:1 WPC:WSCh) showing 95% of viability after passing through gastrointestinal conditions. The results allow expanding the development of mixtures of encapsulating materials to improve the probiotic aptitude of a food ingredient powder.

1. Introduction

The microencapsulation of probiotics, as bioactive agents, has become an alternative to preserve the viable microorganisms not only during storage and digestion, but also during food processing (Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2012). The method mostly used to develop microcapsules is spray drying, because it comprises the lowest production costs at the industrial level to develop active ingredients (Bernucci et al., 2017).

In the microencapsulation of probiotics, the wall materials have an important role in microorganism protection (Eckert et al., 2017; Huang et al., 2017a). Wall materials may consist of low-molecular-weight carbohydrates, proteins or polysaccharides. Low-molecular-weight carbohydrates may protect cells by replacing the water of the membrane, and thus decreasing its transition temperature (Morgan, Herman, White, & Vesey, 2006). Proteins can protect cells from damage by stabilization of membrane components. In particular, whey proteins have varied functional properties such as emulsification, gelation and foaming stability (Kareb & Aïder, 2018). Polysaccharides considered as dietary fiber (chitosan, alginate, hydroxypropyl methylcellulose) can protect microorganisms against gastric conditions and allow their release in the colon with appropriate counts (Cook et al., 2012; Díaz-Vergara et al., 2017; Yonekura, Sun, Soukoulis, & Fisk, 2014).

Matrices that involve mixes of whey proteins and polysaccharides as encapsulating materials have been shown to enhance the qualities of each polymer separately. Chitosan (Ch), for example, reduces the initial counts of cells in the suspensions to be dried due to its natural antimicrobial capacity, but, during storage, as well as under simulated digestive conditions, it increases their survival rates (Yonekura et al., 2014).

The yeast genus *Kluyveromyces*, mainly isolated from whey, can grow in a wide variety of substrates and temperatures. In particular, numerous studies have described the probiotic properties of *Kluyveromyces marxianus* and *Kluyveromyces lactis* (Díaz-Vergara et al., 2017; Pedersen Lindegaard, Owusu-kwarteng, Thorsen, & Jespersen, 2012; Rodicio & Heinisch, 2013; Romanin et al., 2010). Díaz-Vergara

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et al. (2017) studied the encapsulation of *Kluyveromyces marxianus* in a Ch-glucose derivative and found an appropriate probiotic count inside the capsules. Arslan, Erbas, Tontul, and Topuz (2015) microencapsulated another yeast genus (*Saccharomyces cerevisiae* var. *boulardii*) with six different wall materials (gelatin, whey protein concentrate (WPC), modified starch, maltodextrin, pea protein isolate and gum Arabic), and observed the highest product yield with WPC, and the best resistance to gastric conditions with gum Arabic, followed by gelatin and pea protein.

The water-soluble chitosan (WSCh) previously obtained by us (Vanden Braber et al., 2017), which has a molecular weight of 98 kDa and 63% degree of deacetylation, has been shown to have preserved antioxidant activity, gastrointestinal resistance and ability as wall material for microencapsulation, which indicates that it is a functional coating material (Vanden Braber et al., 2018a).

Based on the above, the aim of this study was to evaluate the effect of the microencapsulation (by spray drying) of the whey-native probiotic yeast *K. marxianus* VM004 in matrices of WPC and WSCh on the yeast viability under storage and digestion conditions. To this end, we first optimized the outlet temperature to obtain not only high encapsulation efficiency, but also a powder with better characteristics for yeast preservation, and then evaluated the cytotoxic effect of both wall materials against the normal epithelial cells of the rat ileum (IEC-18). Then, we obtained three formulations of microencapsulated *K. marxianus* in WPC:WSCh mixtures and evaluated the efficiency, morphology, and tolerance of microcapsules under simulated gastrointestinal conditions and viability during storage.

2. Materials and methods

2.1. Reagents

Ch (molecular weight of 583 kDa and 78% deacetylated), glucosamine hydrochloride (GAHC), 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide (MTT), trypsin, chymotrypsin and pepsin were obtained from Sigma-Aldrich (MO, USA). Dulbecco's Modified Eagle Medium (DMEM), trypsin–EDTA (0.05%) and GlutaMAX™-I Supplement were purchased from Gibco (Invitrogen, USA). Fetal bovine serum (FBS) was from Natocor (Argentina). Dimethyl sulfoxide (DMSO) was from Sintorgan (Argentina). Glacial acetic acid (CH_3COOH), potassium phosphate monobasic (KH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), sodium chloride (NaCl), sodium hydroxide (NaOH), hydrochloride acid (HCl) and potassium chloride (KCl) were from Cicarelli (Argentina). Yeast extract, peptone, dextrose, agar-agar and ox bile extract were from Britania (Argentina). IEC − 18 cells were provided by the American Type Culture Collection (USA). Finally, WPC (35% (w/w)) was kindly donated by Molfino Hnos. S.A. (Córdoba, Argentina), and whey-native *K. marxianus* VM004 was previously isolated and characterized in our laboratory (Díaz-Vergara et al., 2017).

2.2. Methods

2.2.1. Preparation of WSCh derivative by Maillard reaction

WSCh was obtained by Maillard reaction between Ch and GAHC, as described in Vanden Braber et al. (2017).

2.2.2. Evaluation of the cytotoxicity of WSCh on IEC-18 cells

IEC-18 cells were used to evaluate the cytotoxicity of WSCh and WPC (as control compound) by the MTT method. Cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin-100 μg/mL streptomycin and 1/100 CTS™ GlutaMAX™-I Supplement in an incubator with 5% CO_2 and humidified atmosphere at 37 °C. For the assays, cells were plated in 96-well plates (5×10^4 cells/well) and allowed to attach for 24 h. After different treatments of cells with WSCh and WPC, the medium was replaced with DMEM without FBS containing 0.5 mg/mL of MTT. After incubation for 4 h, the MTT solution was discarded and the blue crystals were solubilized with DMSO. Absorbance at 570 nm, which was directly proportional to the production of formazan and represents the number of viable cells, was detected by a microplate spectrophotometer reader Multiskan GO (ThermoFisher Scientific). IEC-18 cells were exposed to different concentrations of WSCh (0.06–0.5 mg/mL) and WPC (0.1–1 mg/mL) for 24 h. WSCh was solubilized in acetic acid (≤0.03% in the culture medium). Control cells were tested in parallel and subjected to the same changes in medium with 0.03% (v/v) acetic acid. WPC was directly dissolved in DMEM. The cytotoxic effect was calculated as the percentage of cell viability values with respect to the control cells. Data represent the mean ± standard deviation (SD) of three wells per treatment and are representative of three experiments.

2.2.3. Preparation of K. marxianus VM004 culture

After *K. marxianus* VM004 had grown on yeast extract, peptone and dextrose (YPD) agar at 37 °C for 24 h, one colony was transferred to YPD broth and incubated at 37 °C at 150 rpm for 24 h. Cells were harvested by centrifugation (1540 RCF, 4 °C, 10 min) and re-suspended in fresh sterile YPD broth to a final concentration of 10^9 colony-forming units (CFU)/mL.

2.2.4. Spray-drying microencapsulation of K. marxianus VM004

The microencapsulation process was separated into two stages. First, the outlet temperature was optimized to achieve the highest cell viability and an appropriate yield at the end of the drying process, and microencapsulated yeasts (McY) were obtained by spray-drying in a BÜCHI Mini B-290 Spray Dryer under the conditions shown in Table 1. Three working conditions (A, B, C) were tested, adjusting the outlet temperature in a range from 55 to 80 °C by varying the sample feed flow rate. All mixtures were homogenized at 18000 rpm for 1 min using a PRO250 homogenizer (Pro Scientific, USA). Condition B was chosen as optimal.

Second, three formulations (from now on called F1, F2, F3) of *K. marxianus* VM004 microencapsulated in mixtures of WPC and WSCh were obtained under the parameters of condition B. The proportions (% (w/v)) of the wall materials (WPC:WSCh) in F1, F2 and F3 were as follows: 10 (9:1 in weight), 20 (19:1 in weight) and 30 (29:1 in weight), respectively. *K. marxianus* VM004 was also microencapsulated in 1% (w/v) WSCh alone.

In all cases, 1 mL of free yeast suspension per gram of wall material was added, so that the same proportion of yeast (10^9 CFU/g) was obtained in each dry powder sample. Empty microcapsules were obtained for all cases. McY were stored at 25 and 8 °C.

2.2.5. Microencapsulation efficiency and yield

The yield of the microencapsulation process (*MY*) was calculated according to Eq. (1):

$$MY\,(\%) = \left[\frac{m_{McY}}{m_T}\right] \times 100 \quad (1)$$

where m_{McY} and m_T are the mass of solids obtained before and after the spray-drying encapsulation.

Under sterile conditions, 0.1 g of McY was suspended in 1 mL of

Table 1
Spray-drying parameters.

Parameter	Condition		
	A	B	C
Inlet temperature (°C)	120	120	120
Outlet temperature (°C)	55	68	80
Liquid flow (mL/min)	14	8	6
Compressed spray air flow (m^3/h)	1.05	1.05	1.05
Aspiration rate (%)	100	100	100

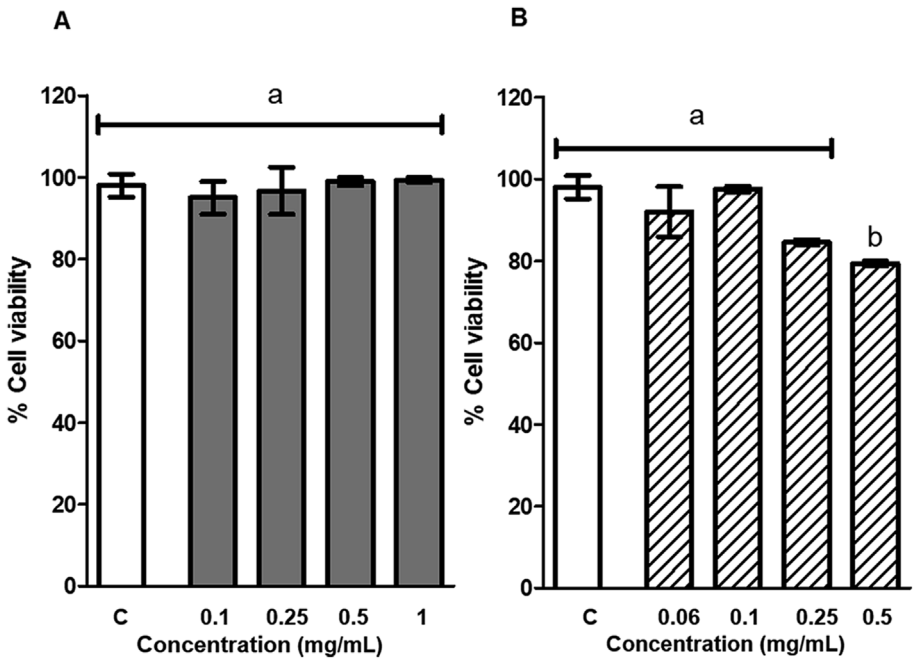

Fig. 1. Cell viability of IEC-18 cells treated with WPC (0.1–1 mg/mL) (A) and WSCh (0.06–0.5 mg/mL) (B) for 24 h. The white bar corresponds to the control. The values are presented as the mean ± SD (n = 3). ($p < 0.05$ vs the control group).

0.1 mol/L phosphate buffer saline (PBS) at pH 7.4 and serial dilutions of the respective initial suspensions were seeded by the microdroplet technique in YPD agar plates. Plates were incubated at 37 °C for 24 h.

Microencapsulation efficiency (*ME*) was calculated with Eq. (2):

$$ME\,(\%) = \left[\log N \Big/ \log N_0 \right] \times 100 \quad (2)$$

where N is the number of viable cells (CFU/g) in McY powder and N_0 is the number of viable cells (CFU/g of dry material) in the initial suspensions.

Viability tests of McY were evaluated at 20, 40, 70, and 90 days.

2.2.6. Tolerance to gastric and intestinal conditions

McY powders and free yeast were compared for their tolerance to *in vitro* gastric and intestinal conditions according to Kühle, Skovgaard, & Jespersen (2005). For this, 1 g of McY or 1 mL of free yeast was suspended in 9 mL of a simulated gastric solution containing 5 g/L NaCl and 3 g/L pepsin at pH 2. At 120 min of incubation, the samples were centrifuged for 5 min at 6160 RCF, the supernatant was discarded and the pellet was resuspended in 9 mL of a simulated intestinal solution containing 5 g/L NaCl, 4.5% (w/v) ox bile, 0.1% (w/v) trypsin and 0.1% (w/v) chymotrypsin at pH 7.4 for 4 h. The pH values of the solutions were adjusted with 1 mol/L HCl or NaOH. Cultures were incubated at 37 °C under agitation at 150 rpm.

At certain times, 100 μL of each sample was serially diluted in PBS and seeded by the microdroplet technique in YPD agar plates. Plates were incubated at 37 °C for 24 h before the count.

2.2.7. Morphology and size of microcapsules

The morphology and size distribution of the spray-dried powders were evaluated by scanning electronic microscopy (SEM) with a ZEISS SUPRA 55VP Scanning Electron Microscope (ZEISS, Germany). Empty microcapsules and McY dried powders were placed in stubs containing a double-faced adhesive metallic tape and coated with gold in a JFC-1100 ion sputter (JEOL, USA). The size distribution of spray-dried powders was evaluated with the ImageJ 2014 software (Rasban, National Institute of Health, USA).

At the same time, empty microcapsules and McY powders were pretreated with Karnofsky fixative solution (phosphate buffer pH 7.2, glutaraldehyde 1.7% and paraformaldehyde 2.7%) for 3 h at room temperature to dissolve the microcapsules and observe the yeast cells released from the wall material. Then, samples were dehydrated by transferring them to vials containing graded water–ethanol series (30, 50, 70, 90 and 100%) and finally acetone. The critical drying point was performed in a DCP-1 critical point dryer (Denton Vacuum, USA). Dehydrated samples were fixed in stubs containing a double-faced adhesive metallic tape, coated with gold and evaluated by SEM.

2.2.8. Moisture content and water activity

The moisture content (MC) of the powders was evaluated according to the AOAC. Official Methods of Analysis (2005) method. Briefly, 1 g of McY was dried at 105 °C until its constant weight. MC was expressed as a percentage of the initial weight.

The water activity (a_w) of McY was measured using an AquaLab water activity meter (AquaLab Series 3, Decagon Devices, INC., USA).

2.3. Statistical analysis

All the experiments were performed in triplicate and data are presented as mean value ± SD. Data on the tolerance to gastric and intestinal conditions were processed and analyzed with Orig-inPro 8.5® of OriginLab Software Corporation. Statistical differences between groups were determined by one-way analysis of variance (ANOVA) followed by Tukey's post-test, using the InfoStat software version 2017 (Facultad de Ciencias Agrarias, Universidad Nacional de Córdoba, Argentina). Values of $p < 0.01$ and $p < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Cytotoxicity of wall materials

The cytotoxicity of WSCh and WPC on IEC-18 cells was estimated by the MTT method, after incubation for 24 h. Cells treated with WSCh showed no statistically significant differences with control cells, and viability was conserved until a concentration of 0.3 mg/mL. At a concentration of 0.5 mg/mL WSCh decreased cell viability to 79% respect

Table 2
Characteristics of spray-dried powders. Microencapsulation process yield (MY), microencapsulation efficiency (ME), water activity (a_w), moisture content (MC).

Condition	*MY(%)*	*ME(%)*	a_w	MC (% w/w)
A	39 ± 2^{a}	94 ± 1^{b}	0.34 ± 0.02^{b}	8.43 ± 0.34^{b}
B	53 ± 3^{b}	$91 \pm 2b^{**}$	0.33 ± 0.02^{b}	6.50 ± 0.21^{a}
C	77 ± 3^{c}	75 ± 2^{a}	0.26 ± 0.01^{a}	6.37 ± 0.39^{a}
Formulation				
F1	50 ± 3^{a}	$87 \pm 0.5^{b*}$	0.30 ± 0.02^{b}	6.46 ± 0.06^{b}
F2	65 ± 3^{b}	82 ± 1^{a}	0.24 ± 0.01^{a}	5.75 ± 0.21^{a}
F3	66 ± 3^{b}	82 ± 1^{a}	0.23 ± 0.01^{a}	5.77 ± 0.09^{a}

Means with different letters in the same column are significantly different ($p < 0.01$). Means with different symbols in the same column but different experiments are significantly different ($p < 0.01$).

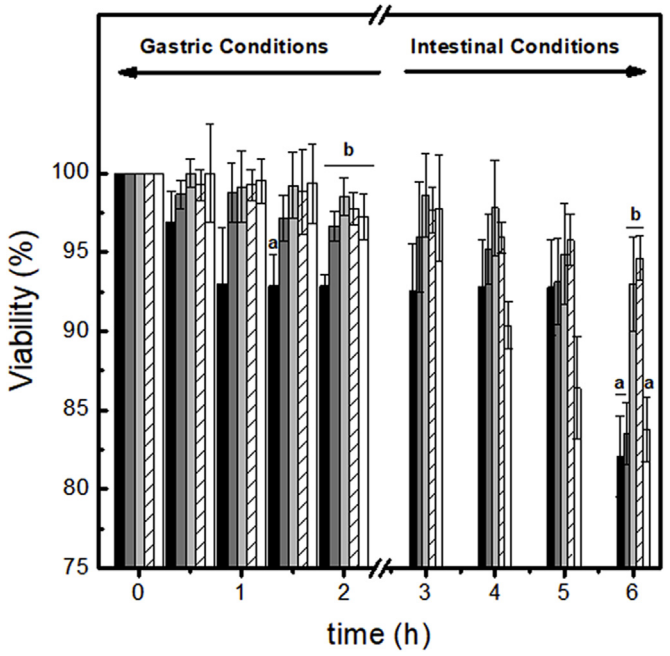

Fig. 2. Effect of gastrointestinal solution on the viability of free *Kluyveromyces marxianus* VM004 and *Kluyveromyces marxianus* VM004 microencapsulated in WPC. Black bars: condition A, dark gray bars: condition B, light gray bar: condition C, bars with diagonal stripes: WSCh, and white bars: free yeast suspension. Different letters indicate significant differences ($p < 0.01$).

to control cells, exhibiting a concentration-dependent effect. On the other hand, cells treated with WPC showed no statistically significant difference with control cells (Fig. 1).

The concentration-dependent cytotoxic effect of Ch and its water-soluble derivatives has been previously demonstrated (Arata Badano et al., 2019).

A compound can be considered as not toxic, weakly toxic or toxic, when cell viability is > 70%, between 50% and 70% or < 50%, respectively (Abdillahi, Verschaeve, Finnie, & Van Staden, 2012). According to this, neither WPC nor WSCh presented cytotoxicity in the concentration range evaluated.

3.2. Optimization of the drying outlet temperature

The principal parameter that influences the viability of probiotic powders is the outlet temperature (Huang et al., 2017a). Thus, to evaluate how the outlet temperature affects the viability of McY (Table 2), we tested the three working conditions mentioned above (A, B, C) (Table 1). The inlet temperature was set at 120 °C, and the sample feed flow was adjusted to obtain outlet temperatures in a range from 55 to 80 °C.

The results present in Table 2 show that the powders obtained from condition A presented the highest MC ($p < 0.01$), those obtained from condition C presented the lowest a_w ($p < 0.01$), and those obtained from condition B showed a *ME* value without statistical difference with that from condition A, but a statistical higher *MY* and a lower MC (condition C).

The viability of microencapsulated probiotics is known to be considerably affected by the residual water content (Liao et al., 2017). Thus, the recommended MC value for food powders is 4% (Ananta, Volkert, & Knorr, 2005). Arslan et al. (2015) and Loyeau et al. (2018) obtained microcapsules of probiotics in different wall materials by spray drying with MC between 7 and 10% (w/w). So, condition B was more appropriate to microencapsulate *K. marxianus* VM004 by spray drying, with 91% of *ME*, 53% of *MY* and a viable initial count of 8.38 log CFU/g. Condition C presented the lowest MC but also the lowest viable count per gram of powder ($p < 0.01$).

The results of tolerance to gastric and intestinal conditions (Fig. 2) for condition B did not exhibit greater tolerance than that of free yeast ($p > 0.01$); in contrast, the tolerance for condition C was significantly greater than that of free yeast ($p < 0.01$). Likewise, the initial count of the powder from condition C was significantly lower ($p < 0.01$) than that of the powder from condition B. Thus, at the end of the passage by the simulated intestinal solution of condition B, we obtained a powder with higher viable probiotic counts.

Under condition B, 10% (w/v) WPC protected the yeast during the

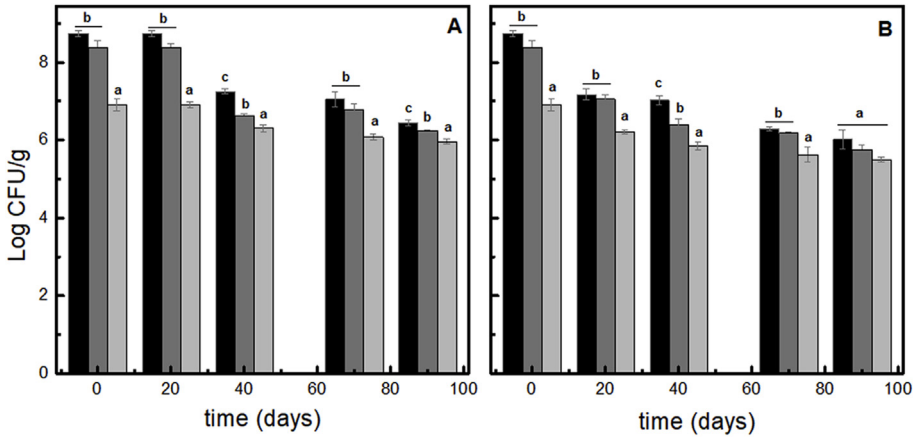

Fig. 3. Viability of *Kluyveromyces marxianus* VM004 microencapsulated in WPC 10% (w/v) during storage at 8 °C (A) and 25 °C (B). Black bars: condition A, dark gray bars: condition B, light gray bars: condition C. Different letters indicate significant differences ($p < 0.01$).

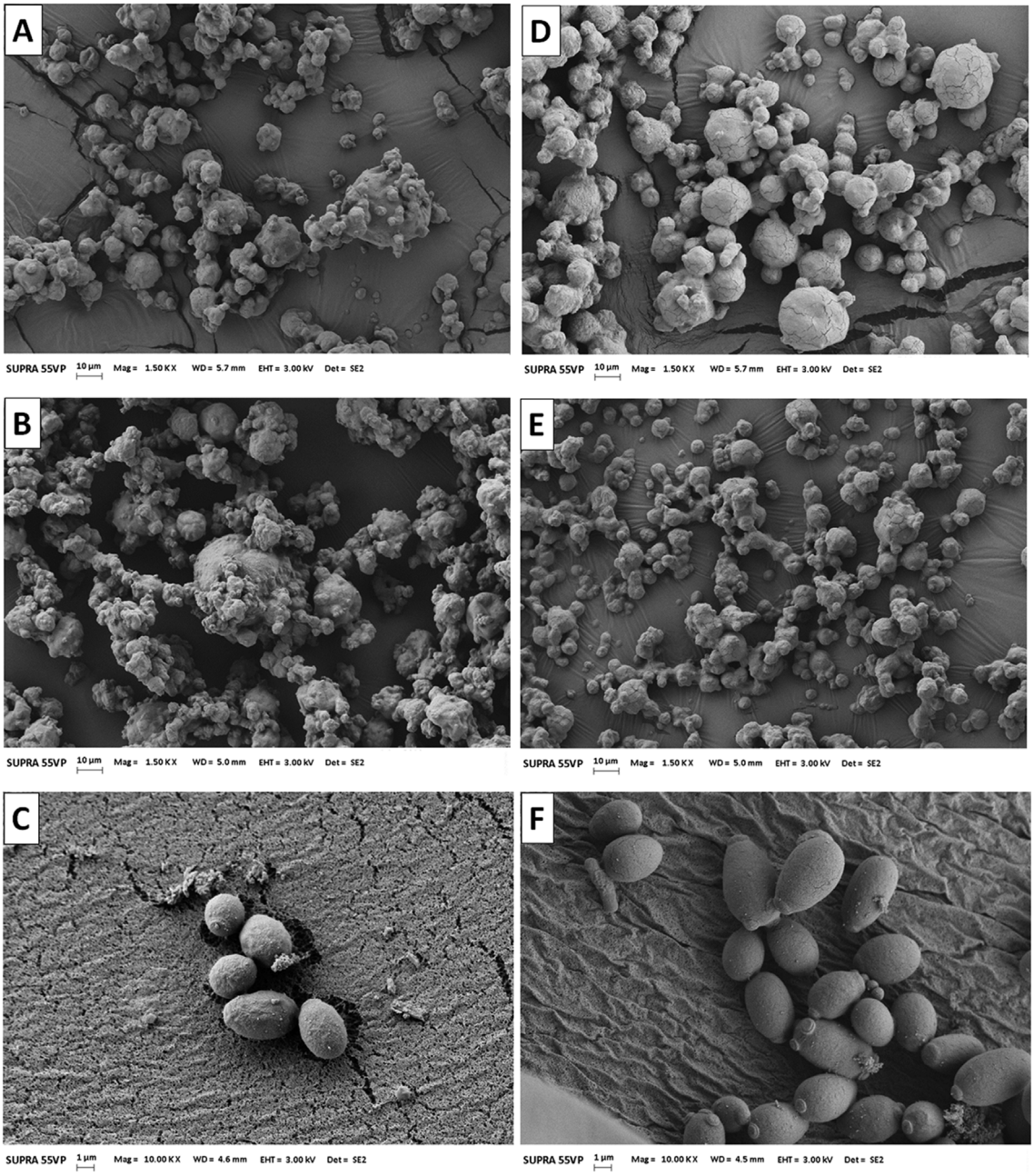

Fig. 4. SEM images. Empty microcapsules of 10% (w/v) WPC (A), *Kluyveromyces marxianus* VM004 microencapsulated in 10% (w/v) WPC (B), *Kluyveromyces marxianus* VM004 from dissolved microcapsules of 10% (w/v) WPC (C), empty microcapsules of WPC:WSCh (29:1) (D), *Kluyveromyces marxianus* VM004 microencapsulated in WPC:WSCh (29:1) (E). *Kluyveromyces marxianus* VM004 from dissolved microcapsules of WPC:WSCh (29:1) (F). All images come from samples obtained under condition B.

drying process, with 1 Log less than the initial suspension, and allowed obtaining a probiotic powder able to maintain a count of 10^7 CFU/g for 20 days at 25 °C (Fig. 3B). Arslan et al. (2015) observed the same decrease in the count of *Saccharomyces cerevisiae* var. *boulardii* microencapsulated in 20% (w/v) WPC after drying, but our powders of *K. marxianus* VM004 showed greater tolerance to our simulated gastric conditions. This may be explained by the fact that, as *K. marxianus* VM004 is isolated from whey, it shows tolerance to acidic pH (Díaz-Vergara et al., 2017; Huang et al., 2017b). Fig. 4 shows the SEM microphotographs of WPC empty microcapsules (A), WPC McY (B) and yeasts from dissolved WPC McY (C) obtained under condition B. The particles showed a spherical shape with irregularity and surface roughness (Fig. 4A and B). Particles were of various sizes, agglomerated, with cracks or surface fissures, but without microcapsule rupture, which confirms good structural integrity. The absence of free surface yeast indicates good encapsulating performance of the powders (Fritzen-Freire et al., 2012). Fig. 4C allows observing bud scar formation, which would indicate that the microorganism remained viable after cell division; the average McY size was 10 ± 3 µm (n = 40), while the released yeast presented an average size of 4 ± 1 µm (n = 40).

3.3. Microcapsules with WSCh as wall material

After the drying process, the count of *K. marxianus* VM004 in the powders obtained with WSCh as wall material was reduced 3 Log; however, the count then increased throughout the 90 days of storage ($p < 0.05$) (Table S1 of supplementary material). The presence of Ch

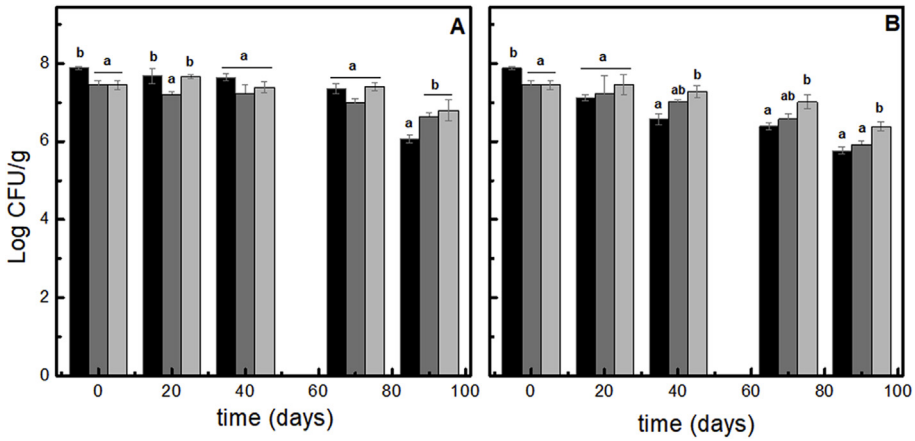

Fig. 5. Viability of *Kluyveromyces marxianus* VM004 microencapsulated in WPC:WSCh at different proportions during storage at 8 °C (A) and 25 °C (B). Black bars: formulation F1, dark gray bars: formulation F2, light gray bars: formulation F3. Different letters indicate significant differences ($p < 0.01$).

Table 3
Inactivation rate of *Kluyveromyces marxianus* VM004 microencapsulated in WPC:WSCh storage for 90 days.

Formulation	Inactivation rate (Log CFU/day)	
	8 °C	25 °C
F1	0.016 ± 0.001[b]	0.023 ± 0.003[b]
F2	0.007 ± 0.003[a]	0.017 ± 0.002[a]
F3	0.007 ± 0.001[a]	0.012 ± 0.002[a]

Means with different letters in the same column are significantly different ($p < 0.05$).

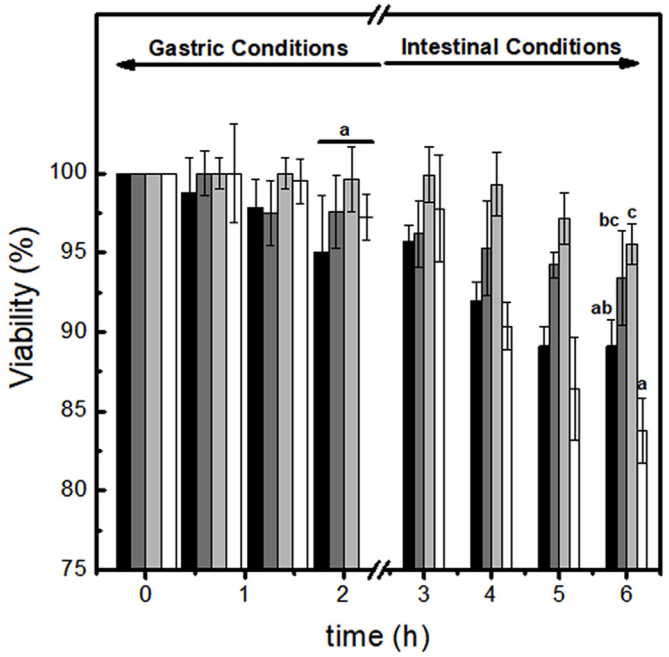

Fig. 6. Effect of gastrointestinal solution on the viability of free *Kluyveromyces marxianus* VM004 and *Kluyveromyces marxianus* VM004 microencapsulated in WPC:WSCh. Black bars: formulation F1, dark gray bars: formulation F2, light gray bars: formulation F3, and white bars: free yeast suspension. Different letters indicate significant differences ($p < 0.01$).

could give the microcapsule a lower permeability to oxygen and water vapor, a lower molecular mobility of water in the powder, improving the stability of the microorganism and allowing the budding process to complete (Fig. S1 of supplementary material), resulting in a slight increase in viable cell count during storage (Hoobin et al., 2013; Moayyedi et al., 2018; Perez-Gago & Krochta, 2001). The low concentration of solids in the WSCh suspension to dry was not enough to preserve the probiotic during the drying process. This result is similar to that obtained by Huang et al. (2017a). The antimicrobial properties of Ch and its WSCh derivative could influence the initial count of yeasts in the suspension to be dried (Vanden Braber et al., 2017). Nevertheless, in this work, we observed that 1% (w/v) WSCh not only increased the tolerance to gastric and intestinal conditions of yeast, but also kept the viability of the microorganism during storage (Fig. 2).

In a study on *Saccharomyces cerevisiae* dried by fluidized bed drying, Garre, Raginel, Palacios, Julien, and Matallana (2010) observed changes in the gene expression related to redox balance systems. In our previous works, we evaluated the antioxidant activity of Ch and its WSCh derivative and found that WSCh preserved the antioxidant activity of Ch and contributed to the activity of microencapsulated flavonoids (Vanden Braber et al., 2017, 2018a, 2018b). The oxidation of membrane lipids and their products can cause damage to the probiotic cell, with loss of viability throughout the storage time (Teixeira, Castro, & Kirby, 1995). Yonekura et al. (2014) demonstrated that Ch acts as a barrier preserving the viability of *Lactobacillus acidophilus* NCIMB 701748. Cook, Tzortzis, Charalampopoulos, and Khutoryanskiy (2011) and (Li, Chen, Sun, Park, & Cha, 2011) explained that Ch could form a gel or denser coating on the drop surface during the spray-drying process that protects the probiotic microorganism from external conditions which could improve the viability through the storage. Ch is a cationic polysaccharide which presents mucoadhesive properties and resistance to digestion (Cook, Bull, Methven, Parker, & Khutoryanskiy, 2017), whereas WSCh has been shown to protect flavonoids from simulated gastrointestinal conditions and to reach the colon to release the antioxidant in an *in vivo* model (Vanden Braber et al., 2018a, 2018b).

3.4. Microcapsules with WPC:WSCh formulations as wall material

For microcapsules of WPC:WSCh (9:1) in formulation (F1), obtained under drying condition B, the *ME* was statistically lower ($p < 0.01$) than that of microcapsules of 10% (w/v) WPC from condition B, with a difference of 0.49 Log (Table 2). However, at the end of the storage period at 25 °C, the probiotic count of both powders showed no significant differences, which explains a better preservation of *K. marxianus* VM004 in the presence of WSCh (Fig. 5B). Also, the powder of F1 showed an improved tolerance to intestinal conditions ($p < 0.05$), with viabilities of 90 and 83% for F1 and condition B, respectively (Figs. 2 and 4). This improved tolerance to gastrointestinal conditions could be explained by the Ch buffer effect and its ability to inhibit pancreatic enzymes (Cook et al., 2012; Tsujita, Takaichi, Takaku,

Sawai, & Yoshida, 2007).

The *MY* was statistically higher (around 15%) for F2 and F3 than for F1 ($p < 0.01$), not so the *ME*, which was 5% lower (Table 2). Fig. 5 shows the viability of *K. marxianus* VM004 microencapsulated in WPC:WSCh during storage at 8 and 25 °C. The lower MC and a_w ($p < 0.01$) for F2 and F3 than for F1 (Table 2) could explain that the F2 and F3 formulations presented a 7 Log count until 40 days of storage at 25 °C. Only the F3 formulation kept a viable count during the 70 days of storage.

The inactivation rates (Table 3), which were calculated as the linear slope of the variation of Log (CFU/g) at 90 days of storage (Fig. S2 of supplementary material), showed that, at 8 and 25 °C, F2 and F3 preserved the yeast with slower inactivation than F1. Spray drying is more energy-efficient when the solids content is high. While a low solids content could preserve the osmotic balance and improve cell viability in the powder obtained (Huang et al., 2016)., Huang et al. (2016) showed that adaptation to hyper osmolality stress is dependent on exposure time. So, the difference presented for the yeast viability of F1 (after spray-drying) is attributed to the osmotic balance in the suspension to be dried because of the solid concentration was lower than F2 and F3, but the physical properties of F1 could not contribute to the preservation of the probiotic in time through the storage time. A high solids content (20–30%) ensures better microencapsulation of the probiotic agent and greater viability during storage due to low molecular mobility (Chávez & Ledeboer, 2007; Poddar et al., 2014). In the present study, F1 showed a *ME* of 87%, which could be explained as a result of lower osmotic stress in the suspension to be dried, whereas the other characteristics (MC, a_w, *MY* and viability during storage) support working with at least 20% of total solids.

The evaluation of the tolerance to simulated gastrointestinal conditions showed that the solids content significantly affected the final yeast count in intestinal conditions (Fig. 6). Due to their buffer capacity, whey proteins can protect the probiotic microorganism from digestive stress (Huang et al., 2017b). At the same time, if the WPC amount is greater, digestive hydrolysis is delayed and viability increases (Hébrard et al., 2010). If the protein concentration is between 22 and 30% (w/w), a sol-gel transition may occur and a skin is progressively thickened with the consequent formation of a vacuole (Sadek et al., 2013). At higher protein concentration, the walls of the vacuole or microcapsule will be more resistant (Sadek et al., 2013). In the present study, the yeast from F3 showed the highest ($p < 0.01$) tolerance.

Fig. 4 shows the SEM micrographs of WPC:WSCh empty microcapsules (D), WPC:WSCh McY (E) and yeast from WPC:WSCh McY (F) obtained from F3, under condition B. The particles shown in Fig. 4D and E have a shape and appearance similar to those of the particles obtained for WPC. Fig. 4F allows observing bud scar formation, which would indicate that the microorganism remained viable after cell division; the average McY size was 12 ± 3 μm (n = 40).

4. Conclusions

In the present work, microcapsules loaded with probiotic *K. marxianus* VM004 were obtained by spray drying by using WPC and WSCh as wall materials. At the concentrations evaluated, neither WPC nor WSCh showed cytotoxicity effect against IEC-18 cells. The outlet drying temperature was optimized; this showed that a temperature of 68 °C allowed obtaining a powder with an appropriate MC for storage and a probiotic count of 8.38 log CFU/g. Then, the evaluation of different mixtures of WPC:WSCh showed that WSCh in formulations significantly improved the tolerance to simulated conditions of gastrointestinal digestion in comparison to the free yeast and the WPC microcapsule (condition B). Finally, it was determined that the solids concentration in the suspension to be dried enhances the general probiotic count of McY (F2 and F3) during simulated gastrointestinal conditions and storage.

Our results allow us to conclude that matrices of WPC:WSCh constitute an excellent alternative to develop probiotic microcapsules as a bioactive ingredient of functional foods.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2019.108844.

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