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Effects of wall materials and lyophilization on the viability of *Weissella confusa*

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The effects of wall materials and encapsulation by lyophilization on the viability of *Weissella confusa* were evaluated. Aloe vera gel, sodium casein at 5 and 15% p/v, sodium alginate at 2% p/v, buffer phosphate, and a mixture (Aloe vera gel, sodium casein, and sodium alginate) as wall materials, were used. Bacteria without encapsulation (*W. confusa*) as control were used. Encapsulated bacteria were freeze dried for 48 h, in order to determine their viability in the freezing and sublimation-drying stages. Results indicate that bacteria without encapsulation, showed greater loss of viability in the sublimation-drying stage. All the wall materials evaluated, may be used for encapsulation of bacteria, because, at the end of the freeze-drying process, the encapsulated bacteria showed higher viability percentages than non-encapsulated bacteria, with significant statistical difference ($p<0.05$). The protective effect of wall materials was higher in the sublimation-drying stage, compared to freezing stage.

Key word: Aloe, *Weissella*, probiotic, encapsulation.

INTRODUCTION

Probiotic lactic acid bacteria (LAB) are useful for the dairy and nutraceutical industry, due to their applications to human and animal health (Reddy et al., 2009). Several studies have demonstrated the probiotic potential of *Weissella confusa*, its antimicrobial activity against pathogenic microorganisms, including *Helicobacter pylori* (Nam et al., 2002), *Staphylococcus aureus*, *Streptococcus agalactiae*, *Escherichia coli, Klebsiella pneumonic* (Serna-Cock et al., 2012), and its ability to adhere itself to the vaginal and intestinal epitheliums (Ayeni et al., 2011; Lee et al., 2012). In the food probiotics, processing is important; the concentration of probiotic bacteria (WHO / FAO, 2006), and the techniques are used to maintain their viability (Carvalho et al., 2004).

Encapsulation techniques have been developed and successfully used in the preservation and protection of probiotic LAB. In encapsulation, the material used to trap the substance or microorganism to be encapsulated is called encapsulation material, cover membrane, shell, vehicle, wall material, or external phase matrix (Serna-Cock and Vallejo-Castillo, 2013). Encapsulation of LAB reduces damage caused by external factors such as storage conditions (time, temperature, moisture, oxygen)

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(Burgain et al., 2011), and also, the encapsulation of LAB, decreases the degradation of bacteria in the human gastrointestinal tract, where the pH is less than 2.00 (Kailasapathy, 2006). Lyophilization (freeze drying), is an encapsulation technique consisting in the elimination of water of a product, by means of sublimation of free water in the solid stage, (previous freezing of the product), followed by vacuum pressure application (Abdelwahed et al., 2006).

Sublimation occurs when vapor pressure and ice surface temperature are below water triple point (Song et al., 2005). Lyophilization is one of the best methods to conserve the properties of biological products (Shui et al., 2006). Lyiophilization-encapsulated probiotics are more stable along storing, especially at low temperatures and inert atmospheres (nitrogen or vacuum) (Manojlović et al., 2010).

Khoramnia et al. (2011) used skimmed milk, sucrose, and lactose as wall materials in the lyophilization-encapsulation of *Lactobacillus reuteri*; these authors report viabilities of 96.4 and 73.8%, for 6-month storage at 4 and 30°C, respectively. Carvalho et al. (2003) evaluated the effect of sorbitol and monosodic glutamate in a skimmed milk solution at 11% on the viability of stored *Lactobacillus bulgaricus, Lactobacillus plantarum, Lactobacillus rhamnosus, Enterococcus durans,* and *Enterococcus faecalis,* using encapsulation by lyophilization.

The findings show that sorbitol and monosodic glutamate maintain the viability of the strains along prolonged storage, with no significant differences between the viability of encapsulated and free cells. Chan et al. (2011), obtained cell viabilities of 5%, using liofilization and sodium alginate (2%) and sodium caseinate (10%) as wall materials, for stabilizing the viability of encapsulated cells. *Lactobacillus paracasei ssp. paracasei F19 (Lactobacillus F19)* and *Bifidobacterium lactis Bb12* in sodium caseinate (15% w/w) were microencapsulated, and retained the cell viability in 16 and 43%, respectively (Heidebach et al. (2010). Sodium caseinate offer suitable physical and functional properties for microencapsulation, due to its amphiphilic character and emulsifying characteristics (Hogan et al., 2001). Studies show improving viability when different types of wall materials as polysaccharides and proteins were included. Hence, cell viability during the lyophization encapsulation process is affected by the type of strains, the parameters of the lyophilization process, the physiological cell state, and the use of cryoprotectors (Abadias et al., 2001). Thus, is necessary to carry out specific encapsulation studies for each type of strain.

The main causes leading to cell viability loss during lyophilization are, ice formation and high osmolarity, (resulting from high internal solutes concentration) which causes cell membrane damage, macromolecular denaturelization, and water loss (Huang et al., 2006). The choice of a cryoprotector is important to maintaining the viability of LAB during the dehydration and storage stages (Carvalho et al., 2004). The most commonly used cryoprotectors include skimmed milk, glycerol, manitol, sorbitol, trealhose, sucrose, maltose, fructose, and proteins (Abadias et al., 2001; Carvalho et al., 2004; Gbassi et al., 2009; Huang et al., 2006). However, the most important selection criterion of an encapsulation material is its functionality in respect to the probiotic (Nedovic et al., 2011). At present, there is a high interest in Aloe vera for the food industry, thus being used as functional nutrient in drinks and ice cream (Martínez-Romero et al., 2006). In spite of its functionality, Aloe vera has not been used as wall material in encapsulation.

A mixture between carbohydrates and proteins can improve the effectiveness of encapsulated probiotics. Therefore, the aim of this present study was to evaluate the effects of wall materials and the lyophilization on the viability of *W. confusa*. The wall materials were Aloe vera gel, sodium casein at 5 and 15% p/v, sodium alginate at 2% p/v, buffer phosphate pH 7.5, and a mixture of pure Aloe vera, casein at 10% and alginate at 2% p/v. During the lyophilization process, the freezing and sublimation-drying stages were evaluated.

MATERIALS AND METHODS

Microorganism culture conditions

A cryoconserved strain, biochemically identified as *Weissella confuse*, isolated by Serna et al. (2010) was used. *W. confusa* strain, was replicated for three generations using MRS commercial substrate (De Man et al., 1960; Scharlau, Spain) (24 h at 37 ± 0.2ºC). For its growing, batch fermentation was used, following the methodology of Serna et al. (2010). After fermentation, *W. confuse* was separated from its metabolites, using centrifugation for 30 min at 5000 rpm (Eppendor Centrifuge 5804R, Germany). The bacteria were washed using 1 mL of NaCl at 0.9% and then centrifuged for 5 min at 5000 rpm. Finally, the supernatant was discarded (Picot and Lacroix, 2004).

Wall materials (encapsulation materials)

Sodium casein of 92.7% (alanate 180, Fonterra, New Zealandd), Sodium alginate (Sigma-Aldrich Co. USA), buffer phosphate, and Aloe vera (Aloe barbadensis Miller), were used as wall materials. In addition, a mixture of these materials was used. The phosphate buffer was selected, because this compound is used for the release of encapsulated cells. Aloe vera was obtained from an experimental plantation at Universidad Nacional de Colombia-Palmira, located 1,100 m above sea level.

Sodium casein was used in aqueous solutions at 5% p/v (C5) and 15% p/v (C15). The solutions were shaken for 12 h (Heidebach et al., 2010). Sodium alginate was used at 2% p/v (AG) (Kailasapathy, 2006). The buffer phosphate (BP) solution was used at pH 7.5. In order to produce Aloe vera gel, acibar (a yellow color liquid) was extracted by cutting the base of the leaf and leaving it drain for 1 h (Miranda et al., 2010). Then, the crystals from leaf epidermis were isolated and processed in a juice extractor (Black & Decker JE2200B, USA), under aseptic conditions. The frozen Aloe

Table 1. Viability percentage of *Weissella confusa* in different wall materials, during freezing and sublimation-drying.

Time (h)	AG	A	BP	C5	C15	FC	MZ
0	100.00 ± 0.00^{a}						
12	41.93 ± 2.03^{Ab}	97.57 ± 1.98^{Da}	92.71 ± 2.38^{Da}	90.33 ± 1.03C^{Dab}	90.14 ± 4.47^{CDa}	79.84 ± 0.12^{Bb}	83.11 ± 1.39^{BCb}
24	41.52 ± 2.46^{Ab}	96.58 ± 1.93^{Da}	91.00 ± 4.20^{CDa}	82.79 ± 6.47^{BCb}	88.65 ± 2.90^{CDa}	71.77 ± 0.16^{Bc}	81.85 ± 0.81^{BCb}
36	41.18 ± 1.24^{Ab}	88.10 ± 0.58^{Bb}	47.20 ± 4.57^{Ab}	80.67 ± 2.77^{Bb}	85.91 ± 6.39^{Ba}	45.16 ± 0.31^{Ad}	79.74 ± 0.84^{Bb}
48	40.87 ± 1.23^{CDb}	81.70 ± 0.50^{Fc}	46.77 ± 4.57^{Db}	36.51 ± 1.29^{BCc}	31.37 ± 0.43^{Bb}	0.02 ± 0.00^{Ae}	69.19 ± 0.96^{Ec}

Mean ± SD. FC = bacteria without encapsulation, C5 sodium casein at 5% p/v, C15 = casein at 15% p/v, AG = sodium alginate, A = Aloe vera, BP = buffer phosphate, MZ = mixture of Aloe vera, sodium casein at 10% and sodium alginate at 2% p/v.

vera gel (A) was stored at 5°C for 12 h and used undiluted. Additionally, a mixture (MZ) of Aloe vera, sodium casein at 10%, and sodium alginate at 2% p/v. was used (this mixture was evaluated in previous experiments).

Encapsulation process by freeze-drying

Bacteria without encapsulation (FC) in concentration of 10,344 ± 0,038 Log_{10}UFC g^{-1} were separately mixed with the corresponding wall materials, using a 1:4 ratio between the bacteria and the wall material (Brinques and Ayub, 2011). Encapsulated and non-encapsulated bacteria were freeze-dried (frozen at - 20°C, vacuum pressure 0.120 mbar and condensing temperature -50°C) using (Labconco, England). During the freezing process, cell count was made at 0, 12 and 24 h, and during the sublimation-drying process, cell count was made at 24, 26 and 48 h. The cell count made at 24 h in freezing process, corresponded to initial conditions of the sublimation-drying stage. FC treatment corresponded to treatment control. FC was freezing-dried to the same conditions described above (Doherty et al., 2010; Kailasapathy, 2006) (Doherty et al., 2010; Kailasapathy, 2006).

Quantification of living bacteria

For liberation of bacteria, the encapsulated bacteria were dissolved (1:10, v/v) in buffer phosphate (pH 7.5), and were centrifuged for 2 min at 5000 rpm (Eppendorf Centrifuge-5804R, Germany). Quantification of cell viability was done using spread-plate (agar MRS, 48 h, and 37 ± 0, 2°C). Afterward, plates containing 30-300 colonies were enumerated, expressing the counting in $UFCg^{-1}$ (Doherty et al., 2010, 2011).

Viability of the probiotic strain

Viability was assessed after freezing and sublimation-drying, in accordance with Doherty et al. (2010), using equation 1:

$$\% \text{ Viability} = (100 \times N/N_o) \quad (1)$$

Where N, is the number of viable cells after freezing in UFC g^{-1} and N_o, number of viable cells before freezing in en UFC g^{-1}.

During the sublimation-drying process, the percentage of viable cells was calculated using Equation 2:

$$\% \text{ Viability} = (100 \times N_t^*/N_o^*) \quad (2)$$

Where, N_t^* is the number of viable encapsulated cells in UFC g^{-1}, each time, along sublimation-drying and N_o^* is the number of viable encapsulated cells before freezing in $UFCg^{-1}$ (Semyonov et al., 2010).

Statistical analysis

A univariate design with 7 levels, FC, C5, C15, AG, A, BP and MZ was used. The response variable was the percentage of viability. Response variable was evaluated during the freezing and drying-sublimation processes, at the time t0 = 0 h, t1= 12 h, t2 = 24 h, t3 = 36 h and t4 =48 h. Results were presented as mean ± standard deviation (SD) of three replicates. The results were analyzed using SPSS 15.0 for Windows (SPSS Inc, Chicago IL, USA). The comparison between averages was made using Tukey, with a probability of $p< 0.05$.

RESULTS AND DISCUSSION

Table 1 shows the means of percentages of viability of *W.confusa* in different wall materials, during freezing and sublimation-drying times.

Different letters in the superscripts in the same column or row indicate significant differences, according to Tukey's comparison ($p <0.05$). Capital letters indicate significant differences ($p <0.05$) between different treatments (row). Lowercase letters indicate significant differences ($p <0.05$) for the same treatment during the time evaluated (column).

Percent viability of FC, presented statistically significant decrease during the freezing and sublimation-drying (Table 1). At the end of the freezing process (24 h), the viability of FC decreased to 28.23%, however its viability percentage was statistically equal to treatments C5 and MZ (Table 1). This indicates that C5 and MZ have no effect cell cryoprotectant in the freezing step. At the end of the process of sublimation-drying (48 h), FC had the lowest percentage of viability compared to all treatments, with statistical significance of $p<0.05$ (Table 1).

Figure 1 shows that after 36 h of process, the cell concentration, measured as $Log_{10}CFUg^{-1}$, decreased significantly reaching 6,722 ± 0.033 $Log_{10}CFUg^{-1}$ after 48 h (In Figure 1, axis Y was divided for including information of FC treatment). For all treatments, the behavior of the cell concentration during the sublimation drying process was similar to the behavior of % viability, since this percentage was calculated from the cell concentration.

This may be caused by cell stress produced by the

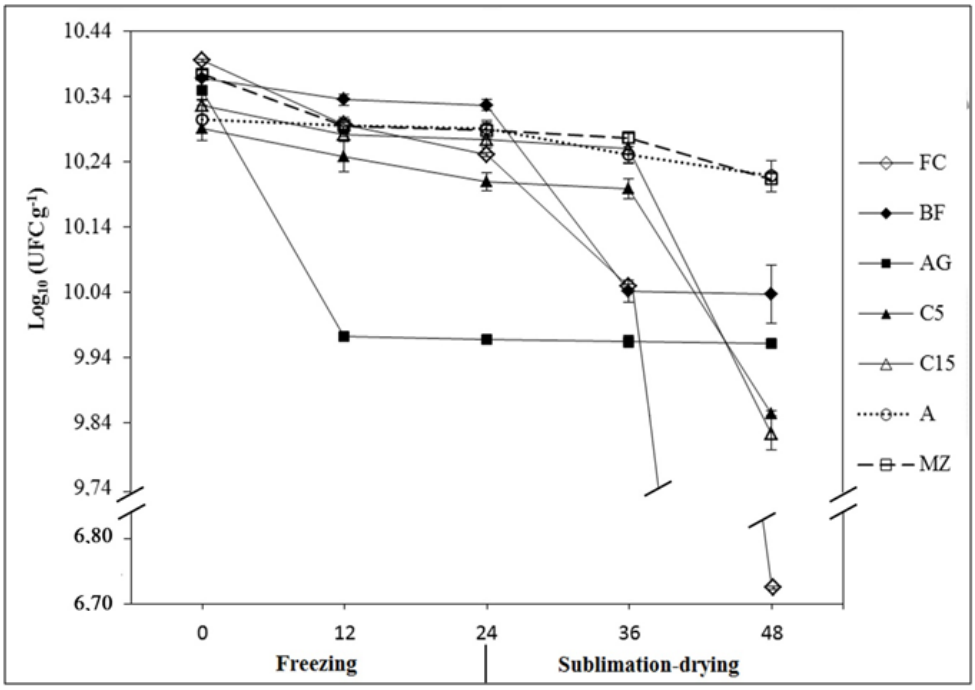

Figure 1. Cell count of *Weissella confusa* for different encapsulation materials during the freezing-sublimation stage of lyophilization. FC = bacteria without encapsulation; C5, sodium casein at 5% p/v; C15 = casein at 15% p/v; AG = sodium alginate; A = Aloe vera; BP = buffer phosphate; MZ = mixture of Aloe vera, sodium casein at 10% and sodium alginate at 2 % p/v.

formation of ice crystals during freezing and during the sublimation-drying stage in lyophilization (Otero et al., 2007). When bacteria are lyophilized, some membrane regions may be negatively affected, mainly because of water crystals in the freezing stage, as well as changes in membrane permeability and protein denaturalization (De Giulio et al., 2005; Yang et al., 2012).

At the end of sublimation-drying process (Table 1), the encapsulated bacteria with wall material A, showed the highest percentage of viability (81.70 ± 0.50%) ($p < 0.05$), however, from 36 h of processing, there showed a significant decrease in the viability percentage ($p < 0.05$), which shows that there is an adverse effect on the stage of sublimation-drying (Table 1). Referring to the cell concentration, at the end of the sublimation-drying process, 10.217±0.019 $\log_{10}$CFUg^{-1} was obtained, this being the highest value for all treatments. At the end of the freezing process, the encapsulated bacteria with the wall material A, showed no statistically significant differences with BP and C15 treatments (Table 1). Kanmani et al. (2011), used glucose and galactose at a 35% concentration as protecting substances for the probiotic *Enterococcus faecium* MC13, obtaining viabilities of 85.6 and 84.7%, after encapsulation by lyophilization, respectively. Zayed and Roos (2004) also evaluated a mixture of sucrose and trehalose for lyophilization encapsulation of *Lactobacillus salivarius*, obtaining 80% viability of the strain. The viability percentage obtained with Aloe vera gel as cryoprotector, is due to its high content of polysaccharides, such as mannose, glucose, and galactose (Chang et al., 2011). The use of sugar-based cryoprotectors (mono and disaccharides) produces high rates of viability (Chávez and Ledeboer, 2007; Huang et al., 2006; Khoramnia et al., 2011; Semyonov et al., 2010). This is attributed to the protection that exerted the sugars on the functionality of cell proteins. The sugars create a glassy matrix during the lyophilization stage, which presents high viscosity and low mobility. Additionally, the increase in cell viability is attributed to the fixation of solutes to cell proteins, due to the fact that the solutes behave as a substitute for water, when the zones of protein hydration are altered as a result of drying (Carvalho et al., 2004). Aloe vera has high glass transition temperature (Tg=70°C in lyophilization) (Nindo et al., 2010), and this gives additional protection to the cells, compared to other polysaccharides such as sucrose. The drying temperature in this study was 25°C, when a biological product is stored below the glass transition temperature, the chemical reactions such as oxidation of free radicals is slowed and the cellular damage is limited (Fu and Chen, 2011). The main bioactive compound of Aloe vera is acemannan, a water-soluble carbohydrate, and consists of glucose and manose monomers linked by glucosíde β–(1,4) bonds

(Femenia et al., 2003; Reynolds and Dweck, 1999). Aloe vera pulp contains 93% in dry base of polysaccharide, of which 62.9% is mannose, 13.1% is glucose and 1.5% is galactose (Ni et al., 2004). Carvalho et al. (2004), evaluated the influence of the addition of different sugars on the survival of *L. bulgaricus,* during freeze-drying, the study showed that the presence of mannose produced higher rate of survival of the bacteria, after freeze-drying. Abadias et al. (2001), evaluated during freeze-drying, the viability of *Candida sake*, when it was coated with different protective materials. They obtained viabilities of 0.2% when concentration of glucose and fructose of 1% were used, and viabilities of 1% when galactose solution to 1% was used. The acemannan plays an important role in the healing of wounds, due to inhibiting bacterial growth and promoting macrophage activity (Djeraba and Quere, 2000), therefore, Aloe vera provides functional advantages, compared with other wall materials.

The MZ treatment viability decreased, in the first 12 h of processing and at the end of the sublimation-drying process. The viability showed statistically different values, compared to the other treatments. These findings are in accordance with those reported by Nanasombat and Sriwong (2007), they used mixtures of skimmed milk, lactose, sucrose, and trehalosa in different combinations, for the lyophilization-encapsulation of *Lactococcus lactis* 13IS3 and Lactobacillus sakei 13IS4, and they obtained viabilities of 61 and 75% for *L. lactis* and 64 and 74% for *Lactobacillus sakei*. The viability percentages of a mixture of materials were used and higher compared with not mixed materials.

Collagen, trehalosa, L-cisteín and glycerol in the encapsulation by lyophilization of Bifidobacterium longum BIOMA 5920, were used. Viabilities of 83% using mixtures of wall materials were obtained, and viabilities of 53.22% using no-mixture wall materials were obtained (Yang et al., 2012). Gbassi et al. (2009), used lyophilization, and sodium alginate (20 g/L) in combination with whey protein (10 g/L), to encapsulate *L. plantarum* 299v, *L. plantarum* 800 and *L. plantarum* CIP A159 strains. The researchers concluded that the combination of polysaccharides and proteins is a feasible alternative, since it improves cell viability. Furthermore, they assessed the viability of the strains in gastric and intestinal simulated juices; the results showed that encapsulated strains had higher viability in gastric juices than no-encapsulated cells. In intestinal juice only encapsulated bacteria maintained viability.

In buffer treatment, viability did not differ significantly with AG treatment. The buffer is used to release the cells from the capsules, and subsequently obtaining a cell count (Doherty et al., 2010, 2011). AG treatment, presented at 12 h of processing, the largest decrease in the percentage of viability. Values were statistically different compared to the other treatments (Table 1). After 12 h of the process, the percentage viability was statistically unchanged until the end of sublimation-drying process. This behavior was similar to that found in Chan et al. (2011); they indicated that the sodium alginate do not protects cells during lyophilization, due to physical properties of sodium alginate cause cellular stress.

Between all encapsulation materials, C5 and C15 treatments had the lowest percentage of viability at the end of sublimation-drying process (cell concentration of 9.854 ± 0.003 and 9.823 ± 0.003 $Log_{10}CFUg^{-1}$, respectively) (Figure 1). In these two treatments after 36 h, the greatest decrease in the percentage of viability was observed. C5 treatment did not differ significantly with treatments AG and C15 to 48 h of processing. These findings are similar to those reported by Heidebach et al. (2010); they used lyophilization with sodium casein at 15% to encapsulate *Bifidobacterium* Bb12 and *Lactobacillus* F19, obtaining viabilities of 40 and 30%, respectively.

The low viability percentages found in this study can be attributed to the ability of bacteria to survive in different ways under the same adverse or comfort conditions (Carvalho et al., 2004; Meng et al., 2008). Therefore, for each strain there should be an evaluation of different encapsulation materials in order to find the most convenient strain (Carvalho et al., 2003; Otero et al., 2007). Further research is necessary to test new wall materials and determine other variables such as viscosity, molecular weight, gelification, composition, *Tg*, and other properties that can be useful in technical applications and materials optimization, in order to enhance viability.

Conclusions

Using wall materials, the life of the bacteria was protected in higher percentage in the sublimation-drying stage. Bacteria without-encapsulation showed higher viability decrease in the sublimation-drying stage (24-48 h).

All wall materials evaluated in this study, have potential in the encapsulation of lactic acid bacteria, due to, cell counts at the end of the process, are found within the ranges accepted by several conuntries, for probiotics foods (at least 7 to 9 Log 10 probiotic cultures per serving of product). Thus, *W. confusa* encapsulated can be used in the formulation of probiotics.

Aloe vera gel is a promising material for the encapsulation of active compounds because it improves the functionality of the material to be encapsulated. In this study, Aloe vera was the only one wall material that maintained the viability of *W. confusa* above 80%. Likewise, buffer phosphate was found to be a cheap material that could be used as a complement in the formulation of wall materials.

Encapsulation of *W. confusa* expands the application horizons of this lactic acid bacteria to the food industry, including foods with probiotic effects, as application in

milk fermented, desserts, ice cream, and powdered starter culture (for fermentation process).

Conflict of Interests

The author(s) have not declared any conflict of interests.

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