RESEARCH ARTICLE

Viabilidad de una bacteria láctica encapsulada e incorporada en una matriz de cobertura de chocolate

Viability of encapsulated lactic bacteria added in a matrix of chocolate coverage

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Resumen

Se evaluó la viabilidad durante el almacenamiento de *Weissella confusa* incorporada en una matriz de cobertura de chocolate. La bacteria probiótica se encapsuló empleando tres materiales de pared, gel de Aloe vera, gel Aloe vera + Almidón al 10 % y gel de Aloe vera + Almidón al 15 % y células libres como control. Posteriormente se liofilizó. La bacteria probiótica encapsulada, se incorporó en una matriz de cobertura de chocolate. Los chips se empacaron y almacenaron durante 5 semanas a 4 °C, cada semana se midieron cambios en la viabilidad de la bacteria probiótica y en la actividad de agua. En la quinta semana, los chips se sometieron a condiciones simuladas de jugos intestinales. Durante el almacenamiento los chips mantuvieron su carácter probiótico (>10^6 UFC/g), sin embargo, cuando la bacteria probiótica se encapsuló en gel aloe vera, se obtuvo mayor número de bacterias probióticas vivas dentro de la matriz sólida (2,1x10^8 UFC/g). La actividad de agua varió de 0,470 a 0,810. La bacteria probiótica permaneció viva por 2 horas en medios simulados de jugos intestinales, lo cual ratifica que la matriz sólida y los medios de encapsulación seleccionados son adecuados para el desarrollo de productos sólidos probióticos ricos en grasa vegetal.

Palabras clave: probiótico, chip, encapsulación, Aloe, almidón, viabilidad celular.

Abstract

Viability during storage of *Weissella confusa* incorporated in a chocolate coating matrix was evaluated. Probiotic bacteria was encapsulated using three wall materials, Aloe vera gel, Aloe vera gel + 10 % starch and aloe vera gel + 15 % starch and free cells as control. Subsequently lyophilized. Probiotic bacteria encapsulated, was incorporated into a chocolate coating matrix. The chips were packed and stored for 5 weeks at 4 °C, were measured weekly changes in viability of the probiotic bacteria and water activity. In the fifth week, the chips were subjected to simulated conditions of intestinal juices. During storage chips remained probiotic character (>10^6 CFU/g), however, if the probiotic bacteria are encapsulated in aloe vera gel, the greater number of living probiotic bacteria was obtained within the solid matrix (2,1x10^8 CFU/g). Water activity ranged from 0.470-0,810. Probiotic bacteria remained alive for 2 hours in simulated intestinal fluid media, which confirms that the solid matrix and the selected encapsulation means are suitable for the development of solid product rich in vegetable fat probiotics.

Key words: probiotic, chip, encapsulation, Aloe, starch, cellular viability.

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Introduction

The selection of adequate food matrices to incorporate probiotics is an important factor that must be considered in the development of probiotic food (Ranadheera *et al.* 2010). It has been reported that the best matrices for probiotic bacteria are fermented dairy products (Rivera and Gallardo, 2010). However, there has been a growing demand for non-dairy probiotic products, and therefore, probiotics are being incorporated into solid and liquid matrices and being sold as tablets, capsules

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and freeze-dried (lyophilized) products (Ranadheera *et al.* 2010; Rivera and Gallardo, 2010).

The viability of the microorganism within the food matrix and its functional activity must also be taken into account in the design of these new products (Jankovic *et al.* 2010; Da Silva, 2011). The incorporation of probiotic microorganisms into a dry food matrix would have many advantages compared to liquid matrices (Ouwehand *et al.* 2004). However, for the solid probiotic matrix to have beneficial effects on human health, it is necessary to ensure that the bacteria remain alive during storage of the product and that once consumed, the probiotic microorganisms resist their passage through the gastrointestinal system (Burgain *et al.* 2011).

An alternative to increase the effectiveness of probiotic products is to incorporate them into food matrices that provide protection (Possemiers *et al.* 2010). Developments have been shown in the incorporation of probiotics into matrices based on chocolate products such as mousse, cake and bars (Aragon *et al.* 2007; Malmo *et al.* 2011; Possemiers *et al.* 2010), demonstrating that chocolate is a good vehicle for the incorporation of probiotics because of its fat and carbohydrate content. One of the chocolate substitutes is chocolate coating and one of its most common forms is the chocolate chip, which is used in the preparation of cookies, ice-cream and cold cakes, thus making it innovative to use this matrix as a vehicle.

Weisella confusa is a lactic acid bacterium that is currently considered to have probiotic potential because it presents antimicrobial activity against different pathogenic microorganisms (Nam *et al.* 2002; Serna *et al.* 2010; Ayeni *et al.* 2011; Lee *et al.* 2012). Similarly, it has been found that aloe vera is a promising wall material to encapsulate this lactic bacterium (Serna *et al.* 2012). Therefore, the objective of this work was to evaluate changes in the viability of the probiotic microorganism during storage and changes in the water activity of probiotic chips prepared with a chocolate coating and with the *Weissella confusa* lactic acid bacterium encapsulated in different wall materials. Additionally, it had the objective of establishing the resistance of the probiotic microorganism under simulated conditions of intestinal juices.

Materials and Methods

Probiotic Bacteria

A cryopreserved strain of the *Weissella confusa* lactic acid bacterium was used, which was isolated in previous studies by Serna *et al.* (2010). To obtain the necessary amount of bacteria, discontinuous fermentation was carried out using MRS broth (Scharlau, Spain) (De Man *et al.* 1960) to which 40 gL^{-1} of glucose was added as a source of carbon. The substrate was inoculated with 10% of previously inoculated broth according to the volume of the substrate. Four fermentations were carried out in a 1000 ml Erlenmeyer flask, which was ellipsoidally agitated at 100 rpm, for 6 hours at 37 °C (Incubating Orbital Shaker model 5000I, USA). After the fermentation time had passed, the bacteria were separated, centrifuging the fermented material to 4480 x g (Eppendorf AG, Germany). The supernatant was discarded and the precipitate was washed with 5 ml of NaCl at 0.9% and it was centrifuged for 5 minutes at 4480 x g. The precipitate was washed again with 10 ml of sterile distilled water, it was centrifuged for 5 minutes at 4480 x g and the supernatant was discarded. Later, the probiotic bacteria were encapsulated.

Encapsulation of Probiotic Bacteria

Aloe vera (*Aloe barbadensis* Miller) gel and commercial starch (Industrias del maíz AMISOL 2143, Colombia) were used in two concentrations, 10% and 15%, as encapsulation materials (better encapsulation results not documented). The aloe vera gel was subjected to thermal treatment at 65 °C for 15 minutes in a thermostatic bath (Julabo 13A, Germany), and it was refrigerated at 4 °C. The starch with 10% and 15% concentrations was used in combination with aloe in the proportion of 1:1. The probiotic bacteria obtained by fermentation at a concentration of 10^{11} CFU/g was mixed at a ratio of 1:4 with the encapsulation materials. AV treatment was appointed to the bacteria encapsulated in aloe vera, encapsulated in aloe vera + starch with 10% concentration (AA10%) and encapsulated in aloe vera + starch at 15% (AA15%). Later, the encapsulated bacteria were lyophilized (Eyela FDU 1100, Japan) (freezing at -20 °C, vacuum pressure of 2 Pa, temperature in the condenser at -60 °C, and drying temperature at 25 °C). As a control treatment, a non-encapsulated probiotic bacterium (*W. confusa*) was used, diluted in sterile distilled water (CL). The CL control was lyophilized in the same conditions as described above.

Preparation and Storage of Probiotic Chocolate Chip Coating

Dark chocolate coating was used (Nacional de Chocolates, Colombia) with a fat content of 37 g / 100 g . The chocolate coating was melted at 48 °C and it was tempered at 30 °C. Later, the AV, AA10, AA15 and CL treatments were added to the melted chocolate coating and they were manually standardized. The mixture was poured into PET (caliber 15) models for chips with a diameter of 6 mm. The chips solidified at a temperature of 4 °C, they were taken out of the molds and packed in polyamide bags, covered with low-density polyethylene with a permeability of 4.4 – 4.9 $g/m^2/h/atm$. The probiotic chips were refrigerated at 4 °C. The temperature of 4 °C was selected because the chips are widely used for the manufacturing of products in-

cluding refrigerated cakes and desserts, as well as ice-cream.

Determining the Viability of Probiotic Bacteria

The viability of the probiotic bacteria was measured immediately after the lyophilization process, and the chips every week during storage (5 weeks).

To establish the number of live bacteria in each treatment, 1 g of chips of each treatment (or 1 g of the control treatment) and 9 ml of peptone water at 0.1% previously cooled to 48 °C were taken in dilution flasks and they were shaken for 1 minute in a vortex mixer (Heidolph Reax control, Germany) to release the bacteria from their encapsulation material. Later, decimal dilutions were carried out using 0.1% peptone water, the samples were deeply planted in MRS agar and they were counted after 48 hours of incubation at 37 ºC. The boxes were counted, which contained 30 to 300 colonies, and the count of live cells was expressed in CFU g^{-1}.

The viability of the microorganisms was established with Equation 1:

$$Viability\ \% = \frac{N}{N_0} \qquad (Eq.\ 1)$$

Where, for encapsulation: N_0 is the number of live bacteria per gram before the encapsulation process, and N is the number of live bacteria per gram after the encapsulation process.

For lyophilization, N_0 is the number of live bacteria per gram after encapsulation and N is the number of live bacteria per gram after lyophilization.

For storage: N_0 is the count of live bacteria at the time the probiotic chips are prepared, and N: is the number of live bacteria per gram at each week of storage.

Water Activity (a_w)

The water activity of the probiotic chips was measured during storage using a water activity meter (Agualab Model Series 3 TE, USA) at a temperature of 25 °C.

Resistance of Probiotic Bacteria to Simulated Intestinal Juices

The greatest component in the chocolate chips' nutrients is vegetable fat. The process of fat absorption is mainly carried out in the small intestine where the bile and pancreatic lipase intervene, both with an alkaline pH (Cueto and Aragón, 2012). Therefore, after five weeks of storage, the different treatments were subjected to simulated environments of intestinal juices using the modified methodology of Malmo *et al.* (2011). The intestinal juices (IJ) were prepared with bile salts 4.5% (w/v) (Sharlau, Barcelona, Spain) and adjusted to pH 8.0 with sterile NaOH 0.1 mol/L. The solution was sterilized by filtration using a membrane of 0.22 µm. The probiotic chips were softened and 0.5 g of chips were added to 45 ml of the IJ solution, they were taken to ellipsoidal agitation (80 rpm) in a shaker (Incubating Orbital Shaker, VWR, Radnor, Pennsylvania, USA) for 2 hours at 37 °C. The viability of the probiotic bacteria was measured when chips were mixed with the IJ and at 2 hours. The viability was measured in the same way as described above.

Experimental Design

To evaluate the effect of the encapsulation materials on the viability of the probiotic and the product's water activity during the probiotic chips' storage, a one-factor design was used with repeated measures. As a factor, encapsulation material with three levels (AV, AA10% and AA15%) was used for the probiotic bacteria. The response variables were the water activity and the viability percentage of the probiotic. The response variables were measured at 6 times (0, 1, 2, 3, 4 and 5 weeks of storage). A Tukey test was carried out for differences between the means with a significance level of $p < 0.05$ and the data were analyzed using the SAS software, version 9.3. Additionally, the viability of the probiotic was measured after lyophilization in the three treatments, AV, AA10% and AA15%, and after each treatment, it was subjected to two hours of intestinal juices.

Results and Discussion

Viability of the Probiotic Bacteria after Lyophilization in the AV, AA10% and AA15% Treatments

The AV treatment presented the greatest percentage of live probiotic bacteria after the lyophilization process (Table 1). These results coincide with those reported by Abadias *et al.* (2001), who assessed the viability of *Candida sake* when covering it with different protective materials during lyophilization, obtaining a viability of 0.2% when a 1% concentration of glucose and fructose was used and a viability of 1% when a 1% solution of galactose was used.

An explanation for the drastic reduction in viability during lyophilization can be attributed to the formation of ice crystals in the freezing process, which damage the cell membrane of the bacteria (Homayouni *et al.* 2008). Added to this is the low solubility of starch and the possible nucleation and separation of phases (crystallization) of the two aloe vera sugars (Pehkonen *et al.* 2007), because the crystallization of sugar deactivates the interaction between the sugar and the cell structures; an interaction which provides additional protection to the cells (Crowe *et al.* 1998 cited by Foerst *et al.* 2011).

Table 1. Viability percentage of the probiotic (*W. confusa*) after the lyophilization process and initial count of the probiotic once it has been incorporated into the chocolate.

Treatment	Viability %	Cells in Chocolate (CFU/g)
CL	0.084 ± 0.010	2.8x109 ± 4.24x107
AV	1.184 ± 0.110	4.2x108 ± 1.41x106
AA10	0.183 ± 0.065	2.8x107 ± 4.24x105
AA15	0.005 ± 0.001	1.0x106 ± 4.24x104

Viability of Probiotic Bacteria in Chocolate Coated Chips during Storage

Significant differences (p < 0.0001) arose between treatments in the first week (Figure 1). The CL treatment presented the greatest viability, followed by the AV, AA15 and AA10 treatments.

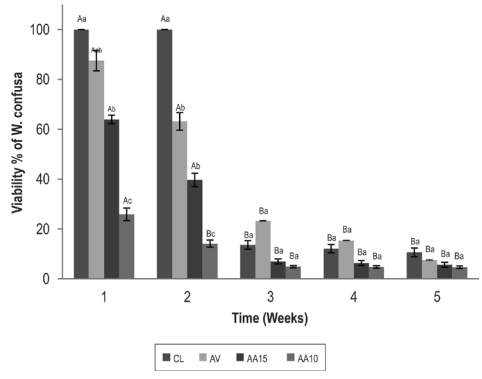

Figure 1. Viability percentage of *Weissella confusa* in chocolate chips stored for 5 weeks.

The uppercase letters indicate significant differences (p < 0.0001) for the same treatment during the assessed period. The lowercase letters indicate significant differences (p < 0.0001) between different treatments in the same week.

The behavior of CL in the first and second weeks of storage indicates that the chocolate coating is a food matrix that alone provides initial protection to the cells (Nebesny *et al.* 2007; Aragon *et al.* 2007). However, at the third week of storage, CL presented a drastic drop in viability. This behavior could be explained by the fat present in the chocolate coating (37 g / 100 g) providing protection to the probiotic bacteria, preventing the cells from being exposed to water (present in the chocolate coating and in the space at the top of the packaging) and preventing stress factors such as those produced by the H+ ions (Lahtinen *et al.* 2007). However, it is possible that as the storage time passes, the layers of fat in the matrix form cracks through which water can directly migrate (Weinbreck *et al.* 2010). Other causes attributable to the decrease in the probiotic's viability may be the reactions to the deterioration of the chocolate coating, such as the oxidation of phytosterols, which may generate several kinds of hydroxyl, epoxy and keto and derivatives of triol (Botelho *et al.* 2014), which have antimicrobial activity (Brudzynski, 2006). Additionally, chocolate substitutes, such as the chocolate coating, contain polyphenols which come from cacao and have antimicrobial effects (Possemiers *et al.* 2010), with the consequent loss of viability.

Figure 2 presents the cell count during 5 weeks of storage. For all the treatments, the final cell concentration was within the range in which food is considered to be probiotic (10^6 – 10^7 CFU/g) (Krasaekoopt *et al.* 2003), recommended concentrations for the consumption of probiotic foods (FAO/WHO, 2001). The products that contain probiotics must be kept refrigerated to maintain high viability. The expiry period for these products, whether for a juice or bar, is shorter than for products without probiotics (Christopher *et al.* 2009).

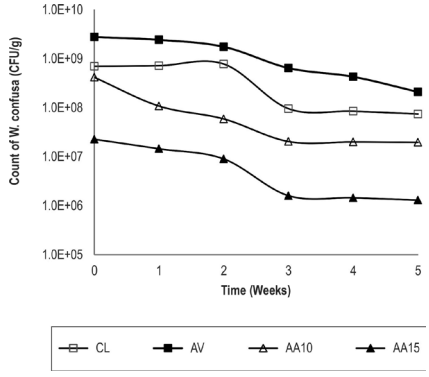

Figure 2. Count of *W. confusa* in the different treatments assessed during five weeks of storage at 4 °C.

The chips were packed in packaging with high permeability to water vapor (4.4 - 4.9 g/m^2/h/atm), and therefore, they presented a substantial increase in a_w during the five weeks of storage (Figure 3) with an average of 0.6 to 0.8. This may be explained by a phenomenon that occurs in chocolate called crystallization (also known as "Bloom"), where water is released and water activity could increase (Vercet, 2003). This increase in water activity could affect the texture of the chip. Katz and Labuza (1981) show that an increase in water activity from 0.4 to 0.45 could induce a loss of hardness in products with a low humidity content, as well as the proliferation of mold or yeast, which directly influences the deterioration of the food (Beuchat, 2002).

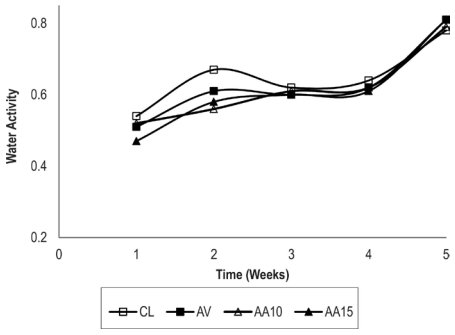

Figure 3. Water activity of chocolate coated chips during storage at 4 °C in polyamide packaging with permeability of 4.4 – 4 g/m^2/h/atm.

One of the important criteria for the selection of a probiotic is its capacity to survive stress conditions caused by a low or high pH (Cook *et al.* 2012); like in the case of the gastrointestinal tract. The number of probiotic microorganisms that managed to survive in the chocolate chips after five weeks of storage and after being subject to 2 hours in a simulated system of intestinal juices (pH 8.0) are presented in Table 2. All of the treatments showed tolerance to the bile salts with reductions equal to or lower than 1 logarithmic unit. This same behavior was presented in the results of Lee *et al.* (2012), who showed a reduction of 1 logarithmic unit ($5.6x10^{10}$ to $7.2x10^{9}$ CFU/g) when evaluating the same microorganism (*W. confusa)* in the same conditions of this study. The concentration used in this study (0.3% bile salts) is considered to be a critical concentration used for the selection of resistant strains (Gilliland *et al.* 1984). It is known that fats are hydrophobic molecules that need the activity of bile salts for their absorption, because the bile salts present a hydrophobic region that interacts with the fat globules, and a hydrophilic region which is orientated toward the watery environment, allowing enzymes, such as pancreatic lipase, to hydrolyze the fat in fatty acids for their later absorption in the small intestine, thus permitting the reduction of serum cholesterol (Manzano *et al.* 2012). The bile salts as well as the lipase have an alkaline pH, and therefore, with the results, it can be confirmed that the chocolate matrix as well as the *W. confusa* probiotic are suitable for the design of solid probiotic products because they resist storage and stay alive during their passage through the small intestine. Resistance to bile salts is one of the criteria for a product to be considered a probiotic.

Table 2. Count of *W. confusa* in chocolate covered chips subject to a simulated system of intestinal juices.

Time	Treatment (CFU/g)			
	CL	AV	AA10	AA15
t=0 min.	7.45x107	2.10x108	1.96x107	1.30x106
t=120 min.	8.15x106	1.34x108	4.90x106	1.28x106

Conclusions

The chocolate coating and *W. confusa* lactic acid bacterium are ideal for the development of probiotic products in solid matrices. The chocolate covered chips maintained their probiotic nature for at least five weeks, because more than 10^6 microorganisms per gram of product remained alive. The water activity increased for all the treatments, which suggests using packaging with lower transmission of water vapor and storing in conditions with relatively low humidity. The incorporation of encapsulated and free *W. confusa* into a chocolate coating matrix presents resistance to the intestinal environment with the AV and AA15 treatments presenting the lowest loss of viability.

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Bibliographic References

Abadias, M., Benabarre, A., Teixido, N., Usall, J., y Vinas, I. (2001). Effect of freeze drying and protectants on viability of the biocontrol yeast Candida sake. *International Journal of Food Microbiology*, 65(3), 173–182.

Aragon-Alegro, L.C., Alarcon Alegro, J.H., Cardarelli, R.H., Chih Chiu, M. y Isay Saad, S.M. (2007). Potentially probiotic and synbiotic chocolate mousse. *LWT - Food Science and Technology*, 40(4), 669–675.

Ayeni, F.A., Sánchez, B., Adeniyi, B.A., De Los Reyes-Gavilán, C.G., Margolles, A. y Ruas-Madiedo, P. (2011). Evaluation of the functional potential of *Weissella* and *Lactobacillus* isolates obtained from Nigerian traditional fermented foods and cow's intestine. *International Journal of Food Microbiology*, 147(2), 97–104.

Beuchat, L.R. (2002). Water activity and microbial stability. Fundamentals of water activity. IFT Continuing Education Committee. *Anaheim*, CA. 14-15.

Botelho, P.B., Galasso, M., Dias, V., Mandrioli, M., Lobato, L.P., Rodriguez-Estrada y M.T., Castro, I.A. (2014). Oxidative stability of functional phytosterol-enriched dark chocolate. *LWT - Food Science and Technology*, 55(2), 444–451.

Brinques, G.B., y Ayub, M.A.Z. (2011). Effect of microencapsulation on survival of *Lactobacillus plantarum* in simulated gastrointestinal conditions, refrigeration, and yogurt. *Journal of Food Engineering,* 103(2), 123–128.

Brudzynski, K. (2007). Effect of hydrogen peroxide on antibacterial activities materials and methods of Canadian Honeys". *Canadian Journal of Microbiology*, 52(12), 1228–1237.

Burgain, J, Gaiani, C., Linder, M. y Scher, J. (2011). Encapsulation of probiotic living cells: From laboratory scale to industrial applications. *Journal of Food Engineering*, 104(24), 467–483.

Christopher M, Reddy V. y Venkateswarlu K. (2009). Viability during storage of two *Bifidobacterium bifidum* strains in set and stirred flavoured yoghurts containing whey protein concentrates. *Indian Journal of Natural Products and Resources*, 8(1), 25-31.

Cueto, C., y Aragón, S. (2012). Evaluación del potencial probiótico de bacterias ácido lácticas para reducir el colesterol *in vitro*. *Scientia Agropecuaria*, 3(1), 45–50.

Cook, M.T., Tzortzis, G., Charalampopoulos, D., y Khutoryanskiy, V.V. (2012). Microencapsulation of probiotics for gastrointestinal delivery. *Journal of Controlled Release: Official Journal of the Controlled Release Society*, 162(1), 56–67.

Da Silva S. M. (2011). Incorporation of probiotics in cereal bars: Technological viability and stability. [MSc. Tesis social Communication]. Braga (Portugal): Universidade Católica Portuguesa. Escuela Superior de Biotecnología. 4 p.

De Man J.C., Rogosa M. y Sharpe, M. E. A. (1960). A medium for the cultivation of *Lactobacilli*. *Journal of Applied Bacteriology*, 23(1), 130-135. DOI: 10.1111/j.1365-2672.1960.tb00188.x.

FAO/WHO. Food and agriculture organization/ World Health Organization. (2001). Probiotics in food Health and nutritional properties and guidelines for evaluation. p, 1–34. Recuperado de: ftp://ftp.fao.org/docrep/fao/009/a0512e/a0512e00.pdf.

Foerst, P., Kulozik, U., Schmitt, M., Bauer, S., y Santivarangkna, C. (2012). Storage stability of vacuum-dried probiotic bacterium *Lactobacillus paracasei* F19. *Food and Bioproducts Processing*, 90(2), 295–300.

Gilliland, S.E, Staley, T.E y Bush, I.J. (1984). Importance of bile tolerance of *Lactobacillus acidophilus* used as a dietary adjunct. *Journal of Dairy Science*, 67(12), 3045-3051. DOI: http://dx.doi.org/10.3168/jds.S0022-0302(84)81670-7.

Homayouni, A., Azizi, A., Ehsani, M.R., Yarmand, M.S., y Razavi, S.H. (2008). Effect of microencapsulation and resistant starch on the probiotic survival and sensory properties of synbiotic ice cream. *Food Chemistry*, 111(1), 50–55.

Jankovic, I.; Sybesma, W.; Phothirath, P.; Ananta, E., y Mercenier, A. 2010. Application of probiotics in food products – Challenges and new approaches. *Current Opinion in Biotechnology,* 21(2), 175-181.

Katz, E.E. y Labuza T.P. (1981). Effect of water activity on the sensory crispness and mechanical deformation of snack food products. *Journal Food Science*, 46(2), 403–409. DOI: 10.1111/j.1365-2621.1981.tb04871.x.

Krasaekoopt, W., Bhandari, B., y Deeth, H. (2003). Evaluation of encapsulation techniques of probiotics for yoghurt. *International Dairy Journal*, 13(1), 3–13.

Lahtinen, S.J., Ouwehand, C., Salminen, S.J., Forssell, P., y Myllärinen, P. (2007). Effect of starch- and lipid-based encapsulation on the culturability of two *Bifidobacterium longum* strains. *Letters in Applied Microbiology*, 44(5), 500–505.

Lee, K.W., Park, J.Y., Jeong, H.R., Heo, H.J., Han, N.S., y Kim, J.H. (2012). Probiotic properties of *Weissella* strains isolated from human faeces. *Anaerobe*, 18(1), 96 – 102.

Malmo, C., Storia, A., y Mauriello, G. (2011). Microencapsulation of *Lactobacillus reuteri* DSM 17938 Cells Coated in Alginate Beads with Chitosan by Spray Drying to Use as a Probiotic Cell in a Chocolate Soufflé. *Food and Bioprocess Technology*, 6(3), 795–805.

Manzano C., Estupiñan D.G. y Poveda E. (2012). Efectos clínicos de los probióticos: Que dice la evidencia científica. *Revista Chilena de Nutrición*, 39(1), 98–110.

Nam, H., Ha, M., Bae, O. y Lee, Y. (2002). Effect of *Weissella confusa* Strain PL9001 on the Adherence and Growth of *Helicobacter pylori*. *Applied and Environmental Microcrobiology,* 68(9), 4642-4645.

Nebesny, E., Żyżelewicz, D., Motyl, I., y Libudzisz, Z. (2007). Dark chocolates supplemented with *Lactobacillus* strains. *European Food Research and Technology,* 225(1), 33–42.

Ouwehand, A.C., Kurvinen, T., y Rissanen, P. (2004). Use of a probiotic *Bifidobacterium* in a dry food matrix, an in vivo study. *International journal of food microbiology*, 95(1), 103–106.

Pehkonen, K.S., Roos, Y.H., Miao, S., Ross, R.P., y Stanton, C. (2008). State transitions and physicochemical aspects of cryoprotection and stabilization in freeze-drying of *Lactobacillus rhamnosus* GG (LGG). *Journal Applied Microbiology*, 104(6),1732–1743.

Possemiers, S., Marzorati, M., Verstraete, W. y Van De Wiele, T. (2010). Bacteria and chocolate: a successful combination for probiotic delivery. *International journal of food microbiology*, 141(1-2), 97–103.

Ranadheera, R. D. C. S.; Baines, S.K., Adams, M. C. (2010). Importance of food in probiotic efficacy. *Food Research International*, 43(1), 1-7.

Rivera-Espinoza, Y. y Gallardo-Navarro, Y. (2010). Non-dairy probiotic products. *Food Microbiology*, 27(1), 1-11.

Serna L., Valencia, L.J. y Campos, R. (2010). Cinética de fermentación y acción probiótica de *Weissella confusa* contra *Staphylococcus aureus* y *Streptococcus agalactiae*. *Revista de la Facultad de Ingeniería de la Universidad de Antioquia*, 55, 53-63.

Serna-Cock, L., Vallejo-Castillo, V.E. y García-Gonzalez, E. (2012). Gel de Aloe vera (*Aloe barbadensis* Miller) en la encapsulación de una bacteria ácido láctica. *Vitae*, 19(1), S168-S170.

Vercet, A. 2003. Browning of white chocolate during storage. *Food Chemistry*, 81(3), 371–377.

Weinbreck, F., Bodnár, I. y Marco M.L. (2010). Can encapsulation lengthen the shelf-life of probiotic bacteria in dry products? *International Journal of Food Microbiology*, 136(3), 364–367.