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In vitro synbiotic activity of *Lactobacillus plantarum* encapsulated with mixtures of Aloe vera mucilage, agave fructans and food additives as wall materials

Actividad sinbiótica *in vitro* de *Lactobacillus plantarum* encapsulado con mezclas de mucílago de Aloe vera, fructanos de agave y aditivos alimenticios como materiales de pared

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Abstract

In this work, *Lactobacillus plantarum* was encapsulated in matrices made from the prebiotics gum Arabic (GA), whey protein concentrate (WPC), Aloe vera mucilage (AVM) and agave fructans with a high degree of polymerization (AFHDP). The synbiotic behavior was assessed by rheological mucoadhesion, simulation of gastrointestinal digestion, growth inhibition of pathogens, antibiotic activity of extracellular metabolites and viability of the probiotic during storage at room and refrigeration temperatures. Rheological studies demonstrated that the biopolymer mixture interacted with intestinal adhesion proteins (mucin) via hydrogen bonding, forming weak gels. During the simulated gastrointestinal digestion, the viability of the microorganism released from mixtures with GA increases after two hours, which evidence microorganism release from the capsule. The extracellular metabolites extract proved to be significantly more effective in the growth inhibition of pathogens and the storage viability reduction is lower than 0.3 log CFU/g after 70 days. These results show for the first time, that *L. plantarum*, when microencapsulated in a suitable prebiotic matrix, displays improved synbiotic activity, guaranteeing that the probiotic will produce potential health benefits and could be used in the formulation of functional foods and products.

Keywords: Synbiotic, microencapsulation, rheological mucoadhesion, antibiotic activity.

Resumen

En este trabajo, se encapsuló ha *Lactobacillus plantarum* en materiales de pared prebióticos (GA, WPC, AVM, y AFHDP). El comportamiento sinbiótico in vitro se evaluó mediante las propiedades de mucoadhesión reológica, simulación de digestión gastrointestinal, inhibición del crecimiento de patógenos, actividad antibiótica de metabolitos extracelulares y viabilidad del probiótico durante el almacenamiento y refrigeración. Los estudios reológicos demostraron que la mezcla de biopolímeros podría interactuar con las proteínas de adhesión intestinal mediante puentes de hidrogeno formando geles débiles. Durante la simulación gastrointestinal, la viabilidad del microorganismo liberado de las mezclas con GA incrementa después de 2 horas, y es indicio de liberación. El extracto de metabolitos extracelulares demostró ser significativamente eficaz en la inhibición del crecimiento de patógenos y la reducción de la viabilidad durante el almacenamiento es inferior a 0.3 log UFC/g después de 70 días. Estos resultados podrían demostrar por primera vez, que *L. plantarum* encapsulado en una matriz prebiótica exhibe una actividad sinbiótica mejorada, garantizando que el probiótico produzca beneficios para la salud y podría utilizarse en la formulación de alimentos y productos funcionales.

Palabras clave: Sinbiótico, microencapsulación, mucoadhesión-reológica, actividad antibiótica.

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1 Introduction

Gut microbiota (GM) has a relevant role in the overall wellness of the immune and gastrointestinal systems. A disequilibrium between pathogens and beneficial microorganisms, known as dysbiosis, is associated with diseases such as inflammatory intestinal disease, diarrhea, colon cancer, colitis, obesity, cardiac and renal diseases (Pandey *et al*., 2015). A few studies concluded that the principal factors that induce the GM dysbiosis are lifestyle (nutrition, stress, physical activity), chronic diseases or even drugs (antibiotics, cancer treatment). Thus, finding adequate strategies to reverse it become necessary (Drago *et al*., 2019; Kalinkovich and Livshits, 2019; Wieland *et al*., 2015). Curative or preventive treatments with synbiotic products represent a novel strategy, which according to the International Scientific Association for Probiotics and Prebiotics (ISAPP) are: "products of mixtures comprising live microorganisms (probiotic) and substrate(s) (prebiotic), selectively utilized by host microorganisms that confers a health benefit on the host" (Swanson *et al*., 2020; Hadi *et al*., 2019; Sengupta *et al*., 2019; Talebi *et al*., 2020). This integration provides greater survivability of the beneficial bacteria at the passage through the gastrointestinal tract, and increasing the colonization rate and growth in the colon (Uraipan *et al*., 2014). Moreover, most lactic acid bacteria can ferment fructo-oligosaccharides, galacto-oligosaccharides and hetero-biopolymers with mannose, xylose, rhamnose, arabinose among another monosaccharide (Zhang *et al*., 2010).

In recent years, has been confirmed the prebiotic effect (García-Gamboa *et al*., 2020; Balderas-Hernández *et al*., 2020), and functional efficiency of native agave fructans and mainly of fractions enriched with a high degree of polymerization as encapsulant material (Ortiz-Basurto *et al*., 2017; Aldrete-Herrera *et al*., 2019; Cancino-Castillo *et al*., 2020; Juarez-Trujillo *et al*., 2021). The property of biopolymers to encapsulate bioactive compounds and microorganisms depends on the structural conformation and random coil entanglement that predominate in fructans with a high degree of polymerization (AFHDP) and other biopolymers such as mucilaginous extracts and gums (Ceja-Medina *et al*., 2020a; Macias-Cortes *et al*., 2020; Medina-Torres *et al*., 2016; 2019). Technologies such as spray drying for the microencapsulation of bioactive compounds and most recently microorganisms, are considered a novelty with excellent results for their relative simplicity, low cost and high efficiency (González-Quijano *et al*., 2019; Estrada-Villegas *et al*., 2019; Trujillo-Cárdenas *et al*., 2018). In a previous work (Ceja-Medina *et al*., 2020a), reported that mixtures of prebiotic biopolymers from Aloe vera and Agave fructans can be used to encapsulate *Lactobacillus plantarum* by spray drying, obtaining microcapsules with a bacterial count higher than 6 log UFC/g after release. These microcapsules in powder form could be considered as a synbiotic functional food. However, the characterization of its probiotic properties must be conducted at least by *in vitro* trials, to ensure that the microorganism is metabolically active in conditions in which the microorganism will produce a probiotic effect (González-Figueredo *et al*., 2020; Melgar-Lalanne *et al*., 2019).

In the present work, microcapsules of *L. plantarum* with novel mixtures of Aloe vera mucilage and AFHDP as wall materials were studied *in vitro* tests with the intention to determinate their possible synbiotic activity. Properties were evaluated by rheological mucoadhesion, release on gastrointestinal simulation, pathogen growth inhibition, antibiotic activity of extracellular metabolites and viability loss during storage.

2 Materials and methods

2.1 Materials

Aloe vera plants growing in a greenhouse located within the facilities of Instituto Tecnológico de Tepic, Tepic, Nayarit, México, were cultivated with constant irrigation and fertilizer application. From these plants, leaves of 70-90 cm of length, 9-10 cm of width and 6-8 cm thick, were selected for their bright intense green color and no signs of spots or disease. Characterization of pH, degrees Brix, total solid content and moisture was performed with the methodology described by Medina-Torres *et al*., (2019). Agave fructans of high degree of polymerization (AFHDP) in powder form, were obtained by membrane ultrafiltration in a Pellicon 2 system fitted with a 5 kDa MWCO membrane (Millipore, Massachusetts) and stabilized in a rotary disk spray dryer as described by Ceja-Medina *et al*., (2020b). Gum Arabic (GA) from A. senegal was purchased from Sigma-Aldrich (CAS: 9000-01-5 MO,

USA). Whey protein concentrate (WPC) Lacprodan SP-8011 with a 80% w/w protein concentration, was purchased from Arla Foods Ingredients (Sønderhøj, Denmark).

These four biopolymers were used as wall materials, because have demonstrated that their hetero-polysaccharide composition and structural arrangement, remains unaltered after stressful heat and mechanical process, such as spray drying (Ceja-Medina *et al*., 2020a). However, other technological functionalities such as emulsifiers and water-soluble biopolymers were also considered.

2.2 Biomass

Biomass proliferation started by a lyophilized strain of *Lactobacillus plantarum* 115 isolated from feces of healthy infants and donated by CIATEJ (Jalisco, Mexico). The strain was first dispersed (1% w/v) in a 50 mL flask with MRS broth (De Man, Rogosa and Sharpe, NutriSelect^{TM} Sigma-Aldrich, USA), and incubated at 37 °C during 18 h in semi anaerobic conditions. A second propagation consisted of 3 L of MRS broth inoculated with 50 mL of the first propagation; this broth volume was incubated at the same conditions by 24 h, which is the time lapse at which *L. plantarum* reaches early stationary phase. Cell recovery was carried out by centrifugation at 10000 rpm by 10 minutes, after removing the supernatant, cells were washed two times with a solution of sodium chloride 1% (w/v). All glassware used on this methodology was sterilized in autoclave at 121 °C, 1.5 bar and 15 minutes.

2.3 Preparation of wall material mixtures and microencapsulation by spray draying process

Wall material preparation and microencapsulation was carried out as described by Ceja-Medina *et al*., (2020a), firstly by forming a suspension of the microorganism in the biopolymer solution then by spray drying. Aqueous mixtures of AVM, AFHDP powder, AG powder and WPC powder were prepared considering a final total solid concentration of 10% (w/v). The final concentration was the sum of solids from each biopolymer and is indicated in Table 1. For mixture integration, each biopolymer was added by separate and mixed with a Braun mixer (MQ725 Braun GmbH, Germany) at 500 rpm for 10 minutes at room temperature, after this, a cell suspension of 1.74×10^8 UFC/g was added and homogenized at 500 rpm for 5 minutes. Final ratio of wall material (prebiotic biopolymers) and cells (*L. plantarum*) was 5:1 wet basis. At this step, the suspensions were spray dried as reported by Ceja-Medina *et al*., (2020a) in a LPG-5 rotary disc spray dryer (CIMA Industries, China) at inlet temperature: 150 °C, flow rate: 1.5 L/min and atomizer speed: 27,500 rpm. The outlet temperature was 78 to 90ºC; similar conditions were employed in previous studies (Medina *et al*., 2013; 2016; 2019). The microcapsule powders showed a moisture content of 7.95 ± 1.85% (dry basis) and were collected with a in tri-laminated bags and sealed hermetically for shelf life trials at room and refrigeration temperature (4 °C).

2.4 Rheological mucoadhesion of microcapsules in simulated gastrointestinal pH

The microcapsules were dispersed in aqueous solutions with pH values similar to gastrointestinal conditions. For gastric condition, a buffer of hydrochloric acid at pH 1.8±0.1 was used to rehydrate the microcapsules formed with different wall material mixtures (Table 1), to form a suspension of 2.5% (w/v). For simulated intestinal conditions a solution with phosphate buffer at pH of 6.5±0.1 adjusted with sodium hydroxide was used (Mansuri *et al*., 2016) to form a suspension of 2.5% (w/v) of microcapsules. Simulation of mucoadhesion test started with the addition of mucin from porcine stomach (M1778, type II, <1.5% of bound sialic acid, Sigma-Aldrich, USA) to complement the suspension of microcapsules in each condition (2.5% w/v gastric and 2.5% w/v intestinal buffer) up to 5% (w/v) and form the systems mucin+microcapsules (well material + *L. plantarum*). Rheological measurements of the suspensions were carried out in a controlled-stress hybrid rheometer DHR-1 (TA Instruments, USA) fitted with a Peltier system for temperature control and all measurements were done at 37 °C.

Table 1. Total solid composition (percentage w/w) of biopolymer mixture used as wall materials.

Mixture	AVM	AFHDP	GA	WPC
M1	80	20	-	-
M2	60	20	20	-
M3	60	20	-	20

AVM, Aloe Vera Mucilage; AFHDP, Agave fructans of High Degree of Polymerization; GA, Gum Arabic; WPC, Whey Protein Concentrate.

A concentric cylinder geometry (21.96 and 20.38 mm of outer and inner cylinder diameters, respectively, 59.50 mm height, and 500 μm gap) was used. Viscoelastic properties (G', storage and G'', loss moduli) under small amplitude linear oscillatory flow ($\gamma < 5\%$) in the frequency range from 0.1 to 300 rad/s were determined by separate for mucin, mixtures of biopolymers, microcapsules, and the combination of mucin+microcapsules. The rheological synergism ($\Delta G'$) was used to assess the difference on the magnitude of the elastic modulus (G'), which will be used as a measure for establishing the degree of interaction between mucin and microcapsules (Garcia-Guzman *et al*., 2019). Therefore, rheological synergism was calculated as follows:

$$\Delta G' = G'_{system} - (G'_{mucin} + G'_{microcapsules}) \quad (1)$$

2.5 *Lactobacillus plantarum release under in vitro gastric and intestinal conditions*

Simulation of gastric and intestinal digestion was carried out as described by Rajam and Anandharamakrishnan, (2015) with some modifications. A simulation of gastric fluid prepared with 0.9% (w/v) NaCl (Sodium Chloride, Bioreagent, Sigma-Aldrich, USA) and porcine gastric pepsin 0.3% (w/v) (P7125 ≥400 u/mg, Sigma-Aldrich, USA) adjusted to pH 1.8±0.1 with HCl 2N (hydrochloric acid, ACS reagent 37%, Sigma-Aldrich, USA) was used to disperse the microcapsules at concentration of 2.5% (w/v). For the intestinal simulation, fluid prepared with 0.9% (w/v) of sodium chloride, 1% (w/v) of porcine pancreatin (P3292 Sigma-Aldrich, USA) and 0.3% (w/v) of bile salts (Bile salts, 48305, Sigma-Aldrich, USA) was adjusted to pH 6.5±0.1 with sodium hydroxide, was used to disperse the microcapsules at concentration of 2.5% (w/v). *In vitro* digestions were incubated at 37 °C and 110 rpm in a benchtop orbital shaker (MaxQ 4000, Thermo Scientific, USA) for 4 hours each, aliquots were taken every 30 minutes to inactivate the enzymes and carry out a microorganism count by serial dilutions and plate pouring in MRS agar incubated at 37 °C for 24 h.

2.6 *Growth inhibition*

Microcapsules obtained from each mixture were dissolved at concentration of 5% (w/v) in a flask with 50 mL MRS broth and incubated for 24 h, 37 °C and 110 rpm. This broth culture was then stored at refrigeration temperature (4 °C) until it uses on the test.

Growth inhibition test was performed as described by Arrizon *et al*., (2014) where two pathogens known to cause gastrointestinal diseases: *Staphylococcus aureus* ATCC 6538 (gram positive), *Escherichia coli* O157:H7 (gram negative) and a phytopathogen fungus *Aspergillus niger* isolated from jackfruit were tested. From a suspension of 1×10^6 CFU/mL of *S. aureus* and *E. coli*, 500 μL was poured in sterile Petri dishes with nutritive agar, homogenized and solidified under sterile air conditions. After solidification, the petri dishes were marked in three sections and a well of 5 mm in diameter was made in each one, and then inoculated with 50 μL of 1×10^8 CFU/mL of free probiotic cells released from each microcapsule mixture. Petri dishes were incubated for 24 h at 37 °C and then the inhibition halo around each hole was measured. The phytopathogen was sub-cultured every 10 days in PDA agar (Patato Dextrose Agar, NutriSelect Plus, Sigma-Aldrich, USA) and 26 °C. For the inhibition test, 1000 μL of 1×10^8 CFU/mL of free cell suspension from each microcapsule were poured in petri dishes with PDA agar; after solidification, *A. niger* was inoculated in the center of the petri dish and incubated at 26 °C for five days, after which the diameter of the fungi was measured.

2.7 *Antibiotic activity of extracellular metabolites*

Probiotic extracellular metabolites (PEM) were separated from the broth culture obtained in the previous section by centrifugation at 10000 rpm for 10 minutes, the supernatant was filtered through a 0.22 μm membrane and then stored at 4 °C until its use. The antibiotic activity of PEM was tested as described by Arrizon *et al*., (2014) in the previous section, with the modification of 50 μL inoculation of PEM instead of free cells in each well to test antibiotic activity against *S. aureus*, *E. coli* and *A. niger* using streptomycin (1 mg/mL) (Streptomycin sulfate salt, S6501, Sigma-Aldrich, USA) as control.

2.7.1 *Survival of microencapsulated L. plantarum during storage*

Viability of *L. plantarum* microcapsules was determined for 60 days at room and refrigeration temperatures (4 °C). To evaluate cell viability, *L. plantarum* was released and enumerated on MRS agar, the plot of the logarithmic value of the cell viability

($\log N_t/N_i$) versus time (t, day) was fitted to a first order kinetics model as described by the following equation:

$$\log \frac{N_t}{N_i} = k_T t \tag{2}$$

where, N_t is the number of viable bacteria after a period in CFU/g, N_i is the number of viable cells at the beginning of storage in CFU/g and t is the storage time in days. k_T represent the specific rate of viability loss per day.

3 Results and discussion

3.1 Rheological mucoadhesion of microcapsules in simulated gastrointestinal pH

Mucoadhesion is a phenomenon related to the physical bonding of inorganic or biological molecules to a mucus layer (Fuongfuchat *et al.*, 1996). Figure 1 shows the results of oscillatory flow tests at small amplitude (γ <5%), were the microcapsules of the three mixtures were dispersed at gastric (1.8±0.1) and intestinal (6.5±0.1) pH, respectively, and mixed with porcine mucin at 37 °C to form a mucoadhesive system. The results allowed to identify a possible interaction between the mucoadhesive protein mucin and the microcapsules by a rheological synergic parameter. The magnitude of G' and G'' moduli reveal that the individual mucin and microcapsules dispersions, and the combined mucin-microcapsule dispersion, presented a predominant viscous behavior were $G'' > G'$ in the lineal viscoelastic region at low frequencies (0.1 - 10 rad/s). Ceja-Medina *et al.*, (2020a) reported similar behavior in the studies of resuspended microcapsules made from AVM and AFHDP. This behavior is desirable and expected from biopolymers because it allows the component integration into the dispersed matrix, and indicates that the dispersion will behave like a liquid and flow achieving a complete liberation of the probiotic cells by shear stress, and the monomodal particle size distribution (Ceja-Medina *et al.*, 2020a; Villegas-Santiago *et al.*, 2019). Specifically, at pH 1.8±0.1 (Figure 1: A, C, E) mucin and AVM have a negative net charge (García-Guzmán *et al.*, 2019), so the carboxyl groups of acemannan (major biopolymer of AVM) and mucin, protonates and allows the molecules to unfold and stretch most of their chains into a new bar-like structure. This new conformation increases the interaction of said groups with others such as acetyl, hydroxyl and amino (Minjares-Fuentes *et al.*, 2017). Figure 1C shows that the combined mucin-microcapsules systems exhibited higher values for both viscoelastic moduli as compared to the individual components. This is probably due to the net charges of the D-glucoronic acid residues in GA, which promote hydrogen bonding and an increase in the magnitude of the van der Waals forces, which increases the mucoadhesive effect (Hagesaether and Sande, 2007). At pH 6.5±0.1, mucin is also negatively charged and hydrogen bonding interactions cause the viscoelastic moduli in the system to be higher than the values exhibited by mucin or microcapsules alone. It is remarkable that the changes at pH 6.5 are more drastic (Figure 1 B, D, F), and the results for the microcapsules of AVM-AFHDP-GA show inflexions in the curve, where the viscoelastic response is not dependent on the frequency, meaning a higher number of interactions and a higher strength of these, which evidence a more stable system, attributed to the ionization and -OH groups in the fructose of AFHDP, galactose, rhamnose and arabinose of GA. In theory, this result indicates a mucoadhesion phenomenon due to the interactions between these molecules, which is relevant since the microcapsules are expected to adhere, then dissolve and release the probiotic cells at intestinal conditions (specifically at the colon) where the microorganism will generate a beneficial effect in the host.

Table 2 shows the values of $\Delta G'$ for the three wall material mixtures, at two frequencies, selected from a region where the viscoelastic properties are frequency dependent. The analysis shows an increase of the synergism when the frequency increase.

Table 2. pH effect and frequency effect on the rheological synergism between M1 (AVM-AFHDP), M2 (AVM-AFHDP-GA) and M3 (AVM-AFHDP-WPC) with intestinal protein mucin.

pH	Frequency (rad/s)	$\Delta G'$ M1	$\Delta G'$ M2	$\Delta G'$ M3
1.8	0.15	-1.84	0.018	-0.016
	3	-12.7	0.347	-1.61
6.5	0.15	-1.46	0.025	-0.0174
	3	-17.35	0.672	-0.89

AVM, Aloe Vera Mucilage; AFHDP, Agave Fructans of High Degree of Polymerization; GA, Gum Arabic; WPC, Whey Protein Concentrate.

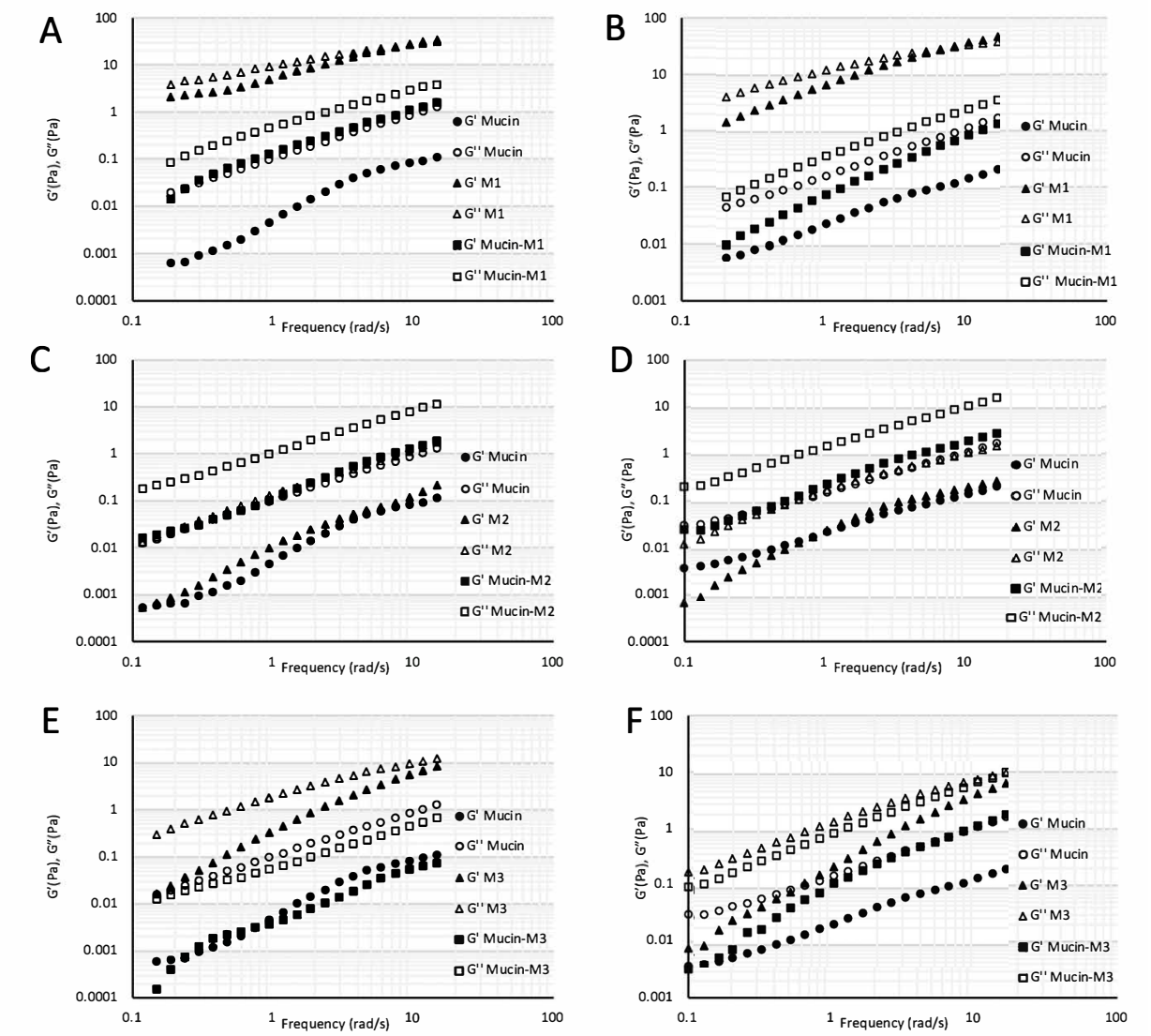

Fig. 1. Viscoelastic curves of mucin, microcapsules and microcapsule-mucin systems at simulated gastric pH 1.8 (A, C, E) and simulated intestinal pH 6.5 (B, D, F). M1; AVM-AFHDP (80-20%), M2; AVM-AFHDP-GA (60-20-20%), M3; AVM-AFHDP-WPC (60-20-20%).

At intestinal conditions pH.(6.5) M2 shows positive values of $\Delta G'$ meaning that the magnitude of the elastic modulus in the system is higher than the sum of the individual components (mucin + microcapsules), which is an evidence that the mucoadhesion occurs and is in agreement with that observed in other polymer systems (García-Guzmán *et al*., 2019; Rueda *et al*., 2015; Vernon-Carter *et al*., 2000). In the case of M1 and M3, the negative sign of the synergism, can be explained as a result of a less extended configuration of the biopolymer system that occurs at high frequency, which results in a decreased viscoelastic behavior, and weaker mucoadhesion. However, this phenomenon occurs in a greater extent in intestinal pH conditions (6.5). To our knowledge, no previous reports exist on the determination of rheological synergism of mucine-microcapsulate (AVM and AFHDP with probiotic) dispersion, but further characterization is necessary since mucoadhesion is a very complex phenomenon. The rheological studies show that all systems behave predominantly as a liquid since the viscous modulus (G'') had a higher magnitude than the elastic one.

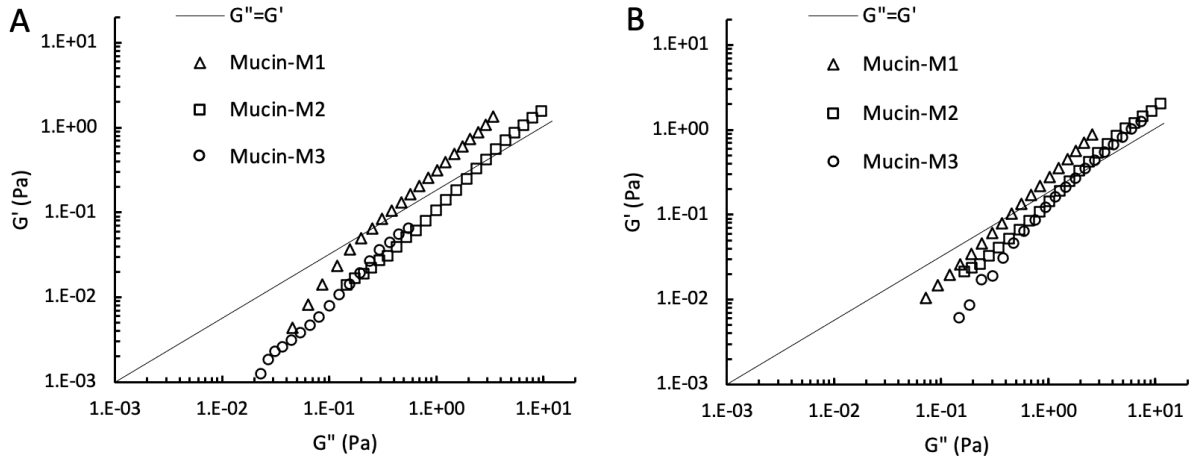

Fig. 2. Han's plot of systems microcapsule-mucin at pH 1.8 (A) and pH 6.5 (B). M1; AVM-AFHDP (80-20%), M2; AVM-AFHDP-GA (60-20-20%), M3; AVM-AFHDP-WPC (60-20-20%).

However, this only happens at low frequencies (long times) and a transition to solid-like behavior is observed at high frequencies, as shown in the change of $\Delta G'$, where a gel-like behavior and mucoadhesion is expected. For this reason, the Han plot can help to elucidate whether predictions of mucoadhesion in these systems are feasible. The plots are constructed with the elastic (G') and viscous (G'') moduli and a diagonal line was $G'=G''$, determines the transition from a viscous ($G'<G''$) behavior to an elastic one ($G'>G''$) (Medina-Torres *et al*., 2020; Hägerström and Edsman, 2003). The analysis shows that the pH (Figure 2, A and B) slightly changes the magnitude in which a transition from viscous to elastic is observed, for example, at pH 6.5, all samples cross the diagonal at low values, therefore at high frequencies the mucoadhesion effect between microcapsules and mucin is possible.

3.2 Survival of probiotic cells in simulated gastric and intestinal conditions

The survival of *L. plantarum* cells during the simulation of the digestive condition evidence the microcapsules release as a function of time. Initial CFU/g for Mixtures 1, 2 and 3 was 2×10^7, 9.1×10^7 and 2.7×10^7, respectively. A suspension of non-microencapsulated cells was also tested with a CFU/mL of 4.67×10^8. The test demonstrated that the free cells are highly susceptible to the gastric and intestinal fluid, which presented a total loss in the first hour (Figure 3 A, B). However, the encapsulation treatments with the different mixtures, increased the protection and significantly prolonged the survival (Figure 3A), which demonstrates the need of an encapsulating material when transiting through the stomach. Microcapsules of M1 (AVM and AFHDP), had the lower release in relation with the other samples, measured in relative survival (number of times plus or minus the initial count). The small increment in relative survival could mean that the release of the probiotic cells is not sustained and the solubilization of the wall material is not appropriate, which ultimately results in higher protection but not in an adequate release. The microcapsules of M2 (AVM, AFHDP, and GA) had a superior cell release reaching a relative viability three times higher than the initial release in the first two hours. The significantly higher relative viability could be the result of the appropriate solubilization of the wall material since the interaction of biopolymers in M2 has been reported as "*random coil webs*" that are easily unfolded at acid pH due the partially acetylated carboxyl groups (Ceja-Medina *et al*., 2020a) and the hydrolysis of fructans, galactose and arabinose (Aldrete-Herrera *et al*., 2019). This prompt to suggest that microcapsules of M2 could be an adequate and convenient delivery system for probiotic cells since they guarantee protection during the passage through the stomach after two hours of digestion at pH 1.8. Microcapsules from M3 (AVM, AFHDP, and WPC) started with a midpoint release at one hour, where relative viability between M1 and M2 was observed.

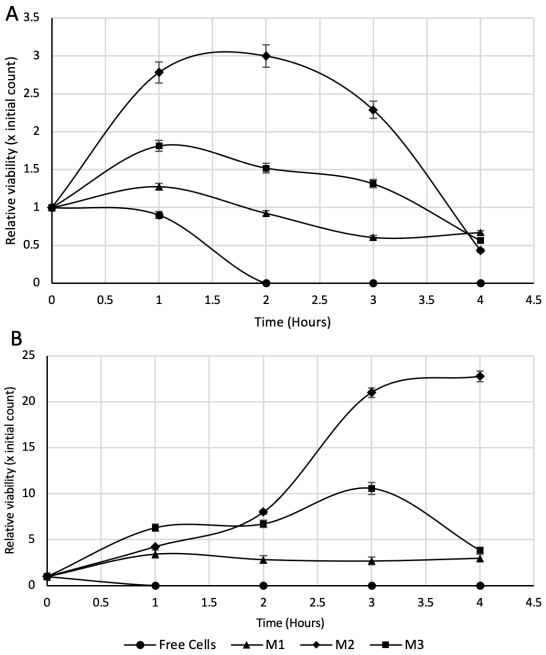

Fig. 3. Release profiles of *L. plantarum* microcapsules at *in vitro* conditions A) gastric, B) intestinal. M1: AVM-AFHDP, M2: AVM-AFHDP-GA, M3: AVM-AFHDP-WPC.

The fluctuation of the cell count during the simulation can be explained by the unfolding and hydrolysis of proteins in the WPC, resulting in irregular solubilization of the wall and not a convenient release. Some authors (Ledezma-Oblea *et al*., 2015; Andrade-Velasques *et al*., 2020) suggest that the protection of wall materials where proteins are present is relatively inferior in gastric simulations because of the action of pepsin, enzyme that hydrolyzes most of proteins in the stomach. Additionally, the microcapsules release behavior changed drastically in intestinal conditions (pH 6.5, pancreatin and bile salts) (Figure 3B). The three microcapsules mixtures showed a relative viability five times higher than the initial on the first hour. M1 not showed an increase in the count by the end of the trail, and could be an indicative that the biopolymer matrix is not solubilized completely and therefore the probiotic cells are not released. The predominant protein nature in M3 is evident as the released is observed by increments, this could be due to the conformation changes of the protein as it goes from a gastric pH (1.8) to an intestinal one (6.5), and by the end of the digestion, the solubilization of the wall material is not complete, explaining the reduction in relative viability. However, the behavior of M2 (AVM, AFHDP and GA), where an exponential release is observed starting at the second hour is noteworthy. This suggest that the solubilization of the wall material was adequate since it could protect the probiotic cells for the first two hours of intestinal transit and then completely release it by the time the microcapsules reach the colon, where the probiotic effect is expected. The higher and constant increase in the relative viability for M2, could also be result of the change in the metabolism of the probiotic, since *L. plantarum* has a facultative ability for the consumption of different carbon sources (e.g., cellulose, fructose, mannose and arabinose) after a short conditioning time (Zielińska and Fabiszewska, 2018). For this, a synbiotic effect could be expected since the microorganism does not have another source of nutrients in the simulation test, and its growth could be stimulated by the presence of the prebiotic, i.e. agave fructans, acemannan and units of arabinose and mannose (Kalita *et al*., 2018).

3.3 Growth inhibition and antibiotic activity against pathogenic bacteria

Two methods were tested to measure the growth inhibition and antibiotic activity of *L. plantarum* released from the microcapsules against pathogenic bacteria; one was the direct contact with probiotic cells (PC) and the other, the effect of probiotic's extracellular metabolites (PEM), this according to the methodology described by Arrizon *et al*., (2014). There was a significant difference ($p>0.05$) between the two methods (Table 3) and the application of PEM was more efficient in both growth inhibition and antibiotic activity test. PC and PEM from M2 microcapsules (AVM-AFHDP-AG) showed remarkable results and are in concordance with the release profiles. This confirms the protection and preservation of metabolic and growth functions of the probiotic by the wall material after the process of spray drying (Ceja-Medina *et al*., 2020a; Valero-Cases and Frutos, 2015). The superior effect of PEM over PC is explained by the different types of mechanisms and interactions that occur during the tests such as the interaction of epithelial cells of the intestine, and the induction of mucin that avoids the permeability of not only pathogens but also toxins (Guidone *et al*., 2014). Moreover, the results of mucoadhesion are important as they explain the interaction of the microcapsules with mucous proteins (Haghshenas *et al*., 2016). The mechanisms that do not require direct contact probably explain the higher efficiency of PEM.

Table 3. Growth inhibition halo (mm) of pathogen microorganisms.

Mixtures	Pathogen					
	E. coli		*S. aureus*		*A. niger*	
	PC	**PEM**	**PC**	**PEM**	**PC**	**PEM**
M1	7.23 ± 0.62^{Aa}	8.70 ± 0.08^{Ba}	6.53 ± 0.09^{Aa}	7.07 ± 0.04^{Ba}	46.82 ± 0.9^{Aa}	41.03 ± 1.2^{Ba}
M2	9.20 ± 0.45^{Ac}	11.06 ± 0.21^{Bc}	11.0 ± 0.57^{Ac}	13.20 ± 0.24^{Bc}	28.50 ± 1.76^{Ab}	24.97 ± 1.96^{Bb}
M3	8.53 ± 0.25^{Ab}	10.37 ± 0.26^{Bb}	9.94 ± 0.65^{Ab}	11.17 ± 0.85^{Ab}	36.93 ± 1.1^{Ac}	30.40 ± 0.67^{Bc}

PC: probiotic cells, PEM: Probiotic extracellular metabolites. Different uppercase letters in the same row indicate significant difference (p>0.05) between PC and PEM for each pathogen (*E. coli, S. aureus or A. niger*). Different lowercase letters in the same column indicate significant difference (p>0.05) between microcapsules (M1, M2 and M3) for PC or PEM. Values are the mean (n=3) ± standard deviation.

These mechanisms are the production of short chain fatty acids (butyric, lactic, acetic), production of inhibition peptides (lantibiotics, bacteriolicin, bacteriocins) and also production of H_2O_2 (Silva-Jara *et al*., 2019; Berbegal *et al*., 2016) that generally function as antibiotic and fungicidal agents.

3.4 Survival of L. plantarum microcapsules during storage

Viability loss during storage is mainly caused by membrane lipid oxidation, thus, factors such as aw, moisture, temperature and presence of oxygen are crucial for reactions that degrade the membrane (Rajam and Anandharamakrishnan, 2015). Initial CFU/g for mixtures 1, 2 and 3 was 2×10^7, 9.1×10^7 and 2.7×10^7, respectively. A suspension of non-microencapsulated cells was also tested with a CFU/mL of 4.67×10^8. Figure 4 shows the plot of $\log N_t/N_i$ versus time (t) for the microcapsules obtained from the studied mixtures. In all cases, the rate of viability loss followed a first order kinetics with R2 values ranging from 0.995 to 0.963. As expected, the free cells lost almost all viability at both room and refrigerated temperatures (Figure 4A), and the major loss of viability among mixtures was from microcapsules of M1 (AVM-AFHDP) at -0.5 log from initial count.

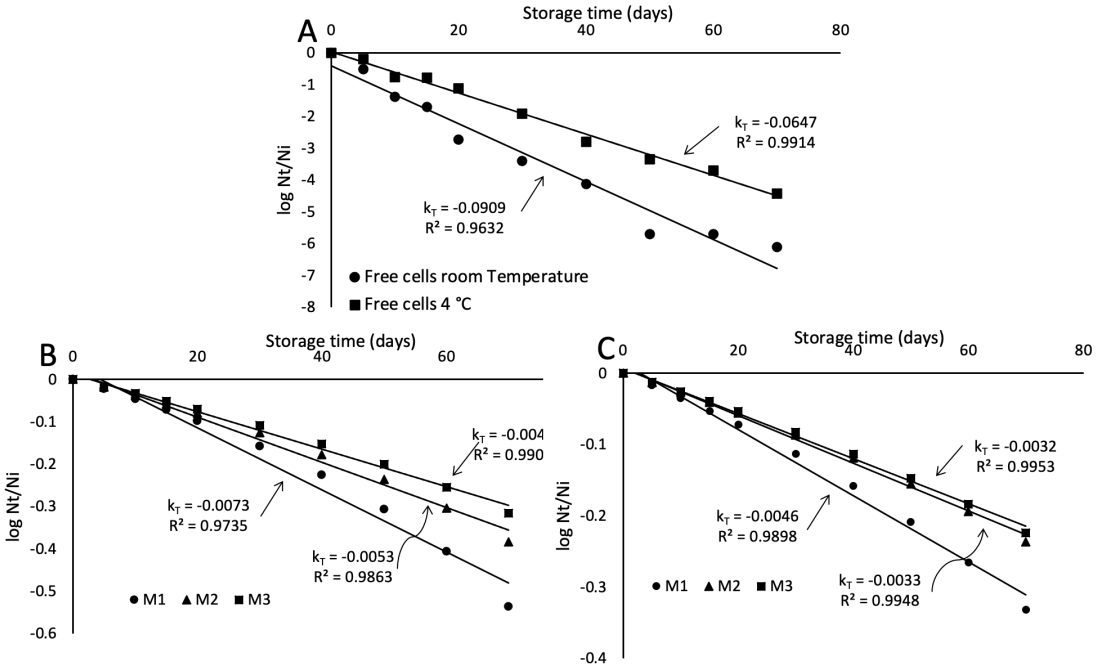

Fig. 4. Storage stability of A) Free cells, B) microcapsules at room temperature (25 ºC) and C) microcapsules at refrigeration temperature (4 °C).

Mixtures added with GA (M2) and WPC (M3) did not show significant differences on viability lost. However, temperature significantly affected the rate at which the survival is reduced with k_T values of 0.0053 for room temperature and 0.0032 at 4 °C (Figure 4 B, C). This behavior could be due to the low values of aw and moisture (less than 0.3 and 7.38%, respectively) obtained during the spray drying and subsequent microencapsulation by the mixtures, as reported in previous work (Ceja-Medina *et al*., 2020a). Overall, after a test of 70 days, the microcapsules still have a high cell count required to exert a probiotic effect, even considering the loss after digestion.

Conclusions

The study of probiotic properties of newly proposed synbiotic matrices that contain microorganisms and functional biopolymers is of utmost importance since this will define their application. We observed by means of *in vitro* rheological mucoadhesion, the release profiles under simulated gastric conditions, antibiotic, and growth inhibition test, which evidence that microcapsules of *Lactobacillus plantarum* with prebiotic biopolymers as wall materials, are capable of interact with intestinal proteins of adhesion that allow the fixation of microcapsules to the intestinal wall. Further studies may include evaluate the release profiles on real digestive tract conditions.

The mixtures of agave fructans of high degree of polymerization, biopolymers of Aloe vera mucilage and gum Arabic, guarantee the protection during the transit through the gastrointestinal tract and permit the release of the viable quantity of microorganisms required to produce a probiotic effect (*in vitro* tests). This is indicative that the microorganism growth is stimulated by the presence of the prebiotics. The antibiotic test demonstrated that the released probiotic cells preserve their metabolism and produce extracellular metabolites that are capable of inhibiting pathogens both gram positive and negative.

These results contribute to the development of new synbiotic products that benefit human health.

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