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Evaluation of *in vivo* Encapsulated Biopolymers of Moringa Leaves (*Moringa oleifera*) in Ruminal Overrun

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ABSTRACT

An experiment was conducted to evaluate encapsulating biopolymers that allowed the ruminal overrun of Moringa (Moringa oleífera) with high protein value for which the following treatments were designed in two test phases: alginate (2.0, 2.5, 3.0%) and alginate (2.0, 2.5, 3.0%) + Chitosan (0.75%) as the capturing matrix of the foliar material, under in vitro conditions and with a nested factorial design. In the next phase, those matrices that maintained greater integrity in the first phase were evaluated in vivo. To do this, three male sheep of the Damara breed with a live weight of 14 to 16 kg of weight and an age of 4 months were used, which was performed by abdominal laparotomy a fistula in the rumen, with a completely random design. Significant differences were found (p <0.05) between the treatments, concluding that the alginate polymer matrices elaborated at 2.5% and alginate (3.0%) + chitosan (0.75%) maintain their integrity after 12 h of the retention time under conditions of rumen in vitro and in vivo.

Keywords: *Alginate, Chitosan, Ionic gelling, Sheep, Legumes*

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INTRODUCTION

Livestock farming in Mexico is one of the most dynamic productive activities in rural areas (SAGARPA 2012), placing Chiapas in tenth place as a national producer of animal protein. However, it faces various problems, among which the variability of the quantity and quality of forage throughout the year stands out, negatively impacting the productive and reproductive parameters of livestock, where the main factors are the low content of protein and soluble carbohydrates, including the high concentration of fiber and low digestibility (Ku-Vera *et al*., 2014). These factors affect the metabolic activity of the microorganisms present in the rumen as there is low availability of nutrients in the diet, limiting the rumen fermentation process, directly modifying the production of components such as volatile fatty acids, peptides, amino acids, and biodegradable microbial protein (Rosales & Pinzón, 2005; Gutiérrez *et al*., 2015). Therefore, coating techniques are proposed to protect physically, chemically, and biologically, such as lipid encapsulating materials, phenols, gums, and carbohydrates (Benchaar *et al*., 2008).

Chitosan is one of the polymers mostly used for capture matrices since it is a linear polysaccharide formed by units of 2-deoxy-N-acetyl-D-glucosamine and 2-deoxy-D-glucosamine linked by ß-(1) glycosidic bonds. →4), which is obtained from the deacetylation of chitin, is hydrophilic and can form a swellable matrix by ionic cross-linking between the positively charged amino groups (Agnihotri *et al*., 2004; Prashanth & Tharanathan, 2007; Tokárová *et al*., 2013). Alginate is a linear polysaccharide that comes from brown algae and is used in the encapsulation of microorganisms, and active components, and in the formation of artificial seeds. It owes its polyanionic character to the carboxyl groups that appear along the chain, which allows it to establish ionic interactions with other polymers, in addition to this Rayment *et al*. (2009) reported promoting the stability of the bioactive against gastric conditions, in this way, both biological materials serve as polymeric matrices in the protection of food (Hernández *et al*., 2005).

Moringa oleifera (MO) is a perennial tree that is available in tropical and subtropical regions around the world, and it is considered a potential forage tree. It is propagated through sexual and asexual means and it requires a low demand for soil nutrients and water, besides has acceptability and palatability for ruminants, and there are no problems in fermentation and the tree leaf provides protein of plant origin (García *et al*., 2008; Adepapo *et al*., 2009). Therefore, this study aimed to evaluate the integrity of the polymeric matrices for moringa (*Moringa oleifera*) leaf material in the ruminal bypass of sheep.

MATERIALS AND METHODS

The work was carried out in the research laboratory of *Instituto de Biociencias* at *UNACH*. The sample of biological material was collected at the agroecological ranch "AYOL" (14º49'45" N, 92º17'47" W) located in Tapachula, Chiapas and the experimental animals were kept at the "Red Brangus" livestock

ranch (14º55'55.62'' N, 92º24'27.4 W") located in Mazatán, Chiapas.

The experiment was established in two phases:
Phase I: *In vitro evaluation* of the capture matrices.
To carry out the *in vitro evaluation,* the moringa leaves were dehydrated in a drying oven at 60°C for 24 h, subsequently, they were ground to obtain 80-micron particles (MF 10 basic IKA WERKE) and subsequently the determination of crude protein (total nitrogen) by the Kjeldahl method (Standard NMX-F-068-S-1980).
Low-viscosity chitosan (SIGMA Life-Science) and pure sodium alginate (MEYER CHEMICALS ®) were used. The matrices were formed by the ionic gelation method with the two encapsulating materials alginate (2.0, 2.5 and 3.0%) and the mixture of alginate (2.0, 2.5 and 3.0%) with chitosan (0.75%), and 20% moringa flour *(Moringa oleifera* L.*)*, during and after the formation of the matrices they were kept in $CaCl_2.2H_2O$ [1.16%] solution for 30 minutes under constant stirring (300 RPM) at room temperature for stabilization, then they were washed with tap water. Matrices with a diameter range (D) of 7 to 9 mm were selected, which were measured with a digital vernier (*Stainless hardened*). The initial breaking force (initial BF) was determined through a Texturometer (TA1 AMETEK), and commercial gelatin capsules (PHARMAKOOS No. 00) were used as a positive control. They were filled with 1.0 g of moringa flour. Five matrices with similar diameters per treatment were deposited in 50 mL Erlenmeyer flasks containing 20 mL of phosphate buffer solution [0.1M] (KH_2PO_4 and Na_2HPO_4) at pH values 6.0, 6.5, and 7.0 for each treatment. The flasks were sealed and placed in an incubator (BJPX-BANGOR BIOBASE) at 40ºC with constant shaking of 150 RPM and were removed from the incubator at 4, 6, 8, 10, and 12 hours, and then the matrices of each were recovered. In one of the treatments, they were washed with tap water and the variables Diameter (D) and Breaking Strength (BF) were measured.
Five polymer matrices from the alginate (A) and alginate+chitosan (A+Q) treatments were placed in Erlenmeyer flasks (50 mL), with 20 mL of rumen fluid (pH 6.28) obtained from sheep with the help of a gastroesophageal cannula. , which were kept in incubation at 40°C, at 150 RPM and removed from the incubator at 4, 6, 8, 10, and 12 hours later, to later recover the matrices of each of the treatments, they were washed with a water tap and the variables D and BF were measured.
A 2x3x3x4 nested factorial experimental design was used where the study variables were: type and concentration of the encapsulating material, retention time, and pH values. Each of them with five repetitions per treatment, a total of 72 treatments were carried out. Having as response variables, the diameter (D) and breaking force (BF) before and after the tests.

Phase II: Treatments that preserved physical characteristics were evaluated in *in vivo* tests.
Three male sheep of the Damara breed with a live weight between 14 to 16 kg and age of 4 months were used, in which a fistula in the rumen was performed by abdominal laparotomy, which was fixed to the left lateral wall. The sheep were housed in pens where they had permanent water and a grazing feeding system with meadows of colocho or Swazirland grass (*Digitaria swazilandensis*).
After seven days of recovery, a cloth bag with a pore size of 80 microns was introduced into the fistula of each sheep, the two treatments selected according to the statistical analysis previously carried out, arranged in a completely randomized design. They remained for 10 hours and then were removed and taken to the laboratory to measure the corresponding variables. The variables of the polymeric matrices were measured [Diameter (D), Breaking Strength (BF), and weight (P)] before starting the treatments and after being subjected to *in vivo tests.*

Data analysis
The data from phase I was subjected to an analysis of variance (ANOVA) to determine if there were significant differences between the means of the treatments and subjected to a Tukey test for multiple comparisons of means with the INFOSTAT [MR statistical software], to select the treatment that keeps the matrix intact and preserves the moringa flour *(Moringa oleifera)*.
The data from phase II were subjected to an analysis of variance (ANOVA) to determine if there were significant differences between the means of the treatments, with a Tukey test for multiple comparison of means and a multivariate analysis of variance with the test of Lawley-Hotelling distribution to identify whether changes in the independent variables had significant effects on the dependent variables with the INFOSTAT [MR statistical software].

RESULTS AND DISCUSSION

The protein content of the moringa leaf material was 16.78% ± 3.05, which indicates the protein value of this forage plant. In the results of the *in vitro tests* with the alginate matrices, an increase in the final D was recorded as the retention time elapsed; however, it was found that the initial and final D of the alginate capture matrices are not significantly different in the retention times and pH values evaluated ($p<0.05$). On the other hand, the gelatin capsules (positive control) were solubilized within the first 4 h of the retention time for the different pH values.
In the initial BF, significant differences were found between the concentrations of the matrices associated with retention times 4, 6, and 8 hours different from the remaining times ($p<0.05$). Likewise, the tendency to decrease was observed with respect to retention times and pH values, observing a greater effect of degradation of the matrix at pH 7.0; the effect of pH at values of 6.5 and 7.0 causes greater damage to the resistance of the matrices concerning those evaluated at pH 6.0. When the means of all treatments of the final BF were compared, it was found that the 2.5% alginate concentration, after the longest retention time at pH 6.5, required the highest BF ($p<0.05$). The dBF presented significant differences between the treatments with respect to the pH value of 7.0, reflecting the damage caused by the resistance of the matrices in the alginate concentrations of 2.0 and 3.0% at the different retention times ($p<0.05$). The initial and final D presented significant differences ($p<0.05$) between the treatments with respect to the concentration of A+Q at 3.0%, which presented the highest values of D with respect to the other concentrations at the different retention times.
The initial BF showed significant differences with respect to the 2.0% A+Q concentration at a pH of 6.5 at the different retention times, since they were the matrices with the lowest BF in

contrast to the other concentrations of A+Q ($p<0.05$). In the concentration of A+Q at 2.0% at pH 6.0, the final BF values presented a reducing trend where only in the first two hours (4 and 6) the final values were higher than the initial ones, which resulted in a difference in the breaking force of negative value. Significant differences ($p<0.05$) were found in the final BF with respect to the concentrations of A+Q at 2.0 and 2.5% at pH 6.5, because the resistance of the matrices was reduced 6 hours after the retention time, on the other hand, the concentration of A+Q at 3.0% were those matrices that best preserved their structure. The dBF has a similar behavior to the initial BF. Significant differences ($p<0.05$) were found with respect to the concentration of 2.5% A+Q at pH 6.0 during the 12 h incubation time, in which matrices less resistant to the other concentrations were obtained. The treatments evaluated at pH 7.0 were solubilized in the first 4 h of incubation for effect **(Figure 1)**.

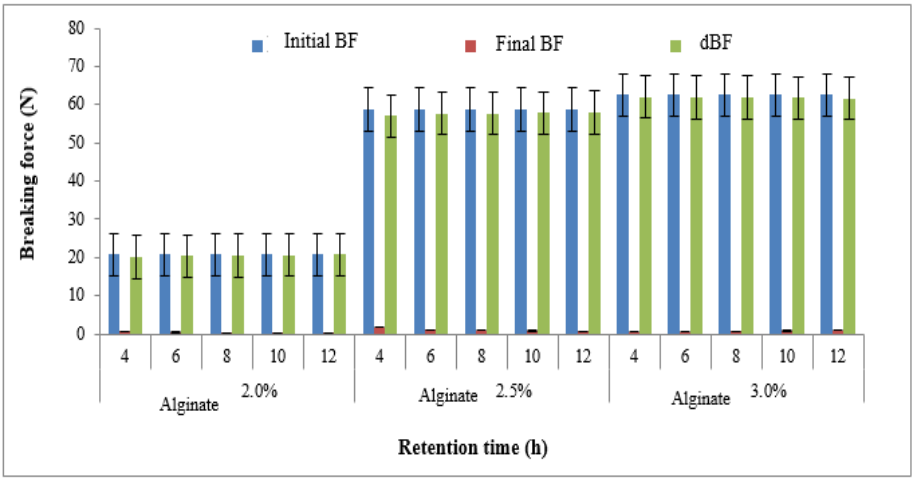


Figure 1. Resistance of the alginate matrix in different concentrations, incubated for 12 hours with ruminal fluid (pH 6.28).

The results obtained demonstrated that as the retention time elapsed, the resistance of the matrices changed since the firmness was reduced according to the exposure time. Lupo *et al.* (2012) mentioned that prolonged exposure to heat treatments degrades the polymer, however, the incubation time was 12 h at 40°C, and even so, the treatments made with A maintained their structure firm compared to those made with A+. Q. In contrast to the pH range of the experiment (6.0, 6.5, 7.0), it was the values of 6.0 and 7.0 that directly affected the structure of the matrices in the treatments; on the other hand, Sankalia *et al.* (2005) described that alginate capsules increase their disintegration capacity under neutral and alkaline pH conditions (5.5 – 7.0), in this same work they mention that after one hour of *in vitro incubation* in simulated intestinal solution at pH 6.8, the capsule disintegrates completely; arguing this fact as a consequence of an increase in the affinity between the calcium ion and the sodium phosphate buffer, causing weakness in the cross-linking of the polymer generating the total disintegration of the alginate matrix. Pawar and Edgar (2012) attribute this structural weakening of the alginate matrix to the repulsion between the carboxylic groups (COO^-) due to the effect of neutral and alkaline pH, generating as a consequence a high absorption of water, a phenomenon known as swelling with a consequent increase of capsule size, as happened in the *in vitro test* with the matrices in the different treatments in which a minimal increase in the diameter size was obtained, even though said matrices were wet before starting the experiment, negatively influencing the strength of the matrix, causing erosion in the polymer matrix (Bhattacharya *et al.*, 2014; Yang *et al.*, 2015).

The results of the alginate (A) and alginate mixed with chitosan (A+Q) matrices exposed in ruminal fluid at pH 6.28, recorded that the initial BF (20.88 N) was always the same at all retention times. It was also observed that the initial BF values in the 2.5 and 3.0% matrices were 58.74N and 62.55N, respectively. The statistical analysis showed significant differences ($p<0.05$) with respect to the 2.0% alginate concentration at all retention times, because they presented lower initial resistance in contrast to the other concentrations. The final BF value in the 2.0% and 3.0% alginate concentrations was less than 0.8 N, where the trend was to be lower, which can be interpreted as a change in consistency relative to the elapsed retention time. On the contrary, with what was observed in the 2.5% alginate matrix where the BF value was >1.0 N with a tendency to be lower, they presented differences ($p<0.05$). The dBF values have a behavior similar to that observed in the initial BF. In general, 2.5 and 3.0% alginate matrices are stronger than the 2.0% concentration. Furthermore, between 2.5 and 3.0%, based on the final BF value, the 2.5% alginate matrices had greater resistance for all retention times ($p<0.05$) **(Figure 2)**.

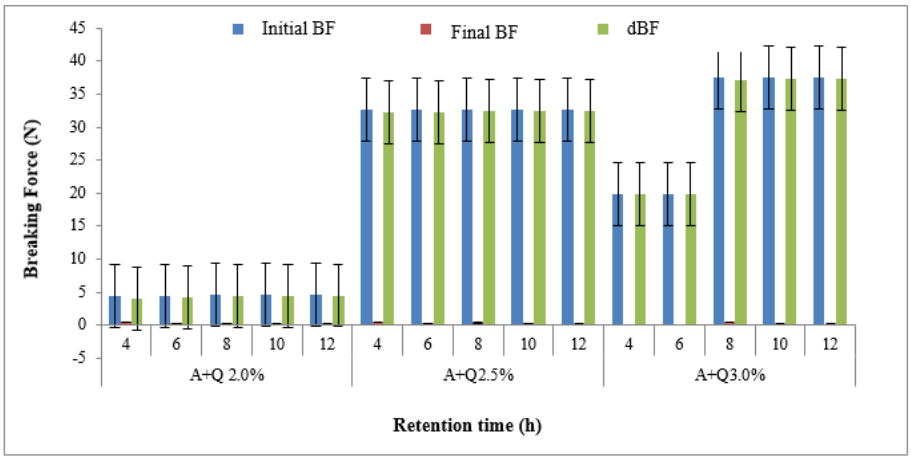


Figura 2. Resistance of A+Q matrices [0.75% (1:2)], incubated for 12 hours with ruminal fluid (pH 6.28).

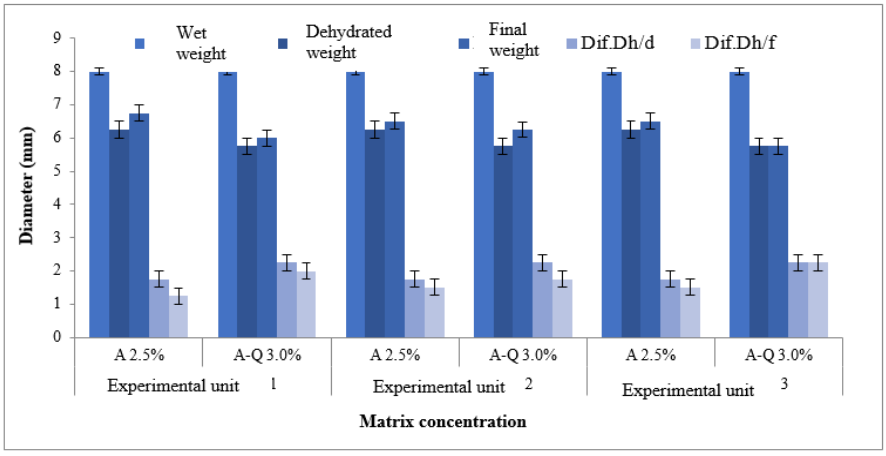
In the initial BF value, there were significant differences ($p<0.05$) between the treatments with respect to the concentration of A+Q at 2.0%, because they presented the lowest resistance in contrast to the other concentrations at the different retention times. On the other hand, the final BF in the three concentrations at all retention times was less than 0.5N, with a decreasing trend; the analysis revealed significant differences ($p<0.05$) with respect to the concentration of A+Q at 2.5 %, since at retention times 4 and 6 hours the matrices had been solubilized, the concentration of A+Q at 3.0% presented better resistance of the matrices at the different retention times. Regarding the dBF values, the values presented a similar behavior to that observed with the initial BF values in the three concentrations at all retention times, presenting significant differences ($p<0.05$).

Phase II: *in vivo tests.*

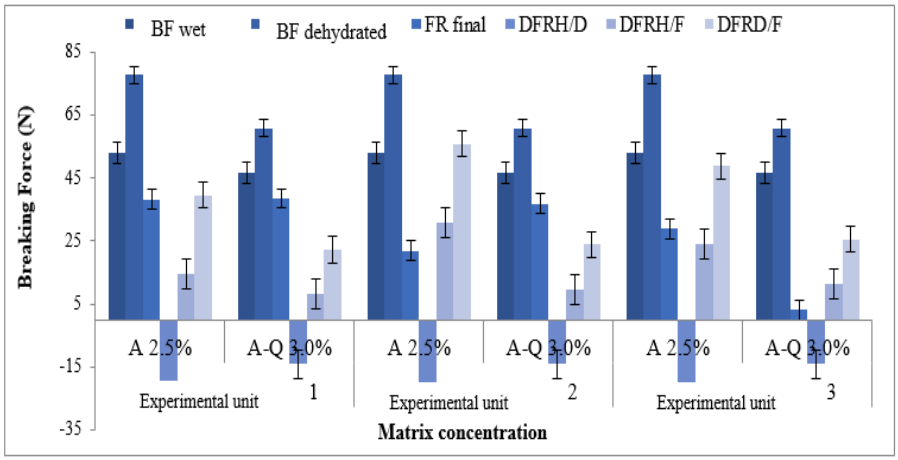
The variation in the diameter value of the matrices at different concentrations was observed during a retention time of 10 h in the three experimental units. After selecting the same matrix diameter value in both concentrations (A 2.5% and A+Q 3.0%) and subjecting them to dehydration, a significant variation ($p<0.05$) was found between the initial diameter and the diameter of the dehydrated matrices. (D.Dh/d). The above was more noticeable at the concentration of 3.0% A+Q. After their exposure in the experimental unit, a rehydration of these matrices was observed resulting in an increase in the diameter value in most of the experimental units, except in the third experimental unit, at the concentration of 3.0%, the diameter value was not modified and remained constant. The percentage of humidity of the A 2.5% and A+Q 3.0% matrices was: 72.92 and 60.04%, respectively **(Figure 3)**.

Regarding the weight of the matrices, a difference was observed associated with the percentage of alginate for each experimental unit in the wet weight and dry weight conditions. The difference between these two variables was significant ($p<0.05$) and likewise between the dry weight values with the rehydrated weight. The difference observed between experimental units was significant ($p<0.05$), supported mostly by the weight values of experimental unit 3, at a concentration of 3% alginate.

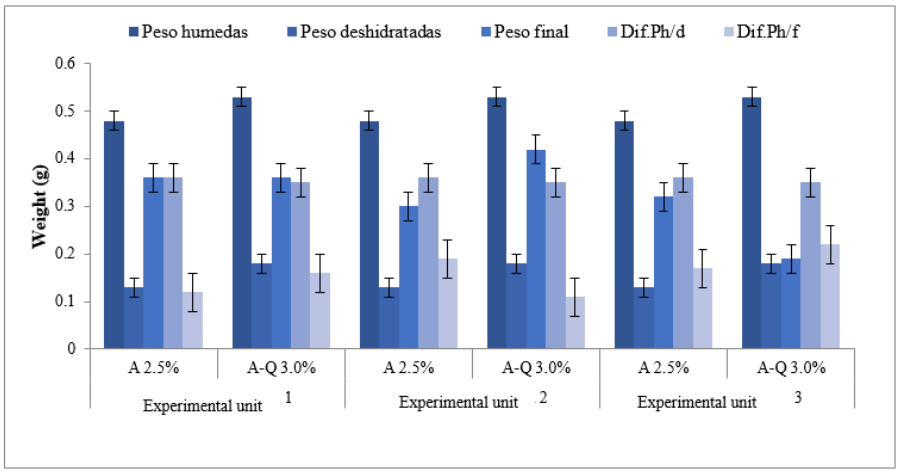
In the rupture force of the matrices, a difference was observed inversely associated with the percentage of alginate, where the higher the concentration of alginate, the lower the value of rupture force, both in wet and dehydrated conditions in all experimental units. The difference between the breaking force values in wet and dehydrated conditions was significant ($p<0.05$) for each experimental unit and between them.



a)



b)



c)

Figure 3. Change of the variables (D, FR, and P) in the matrices, before and after being evaluated in the *in vivo* test. [Dif.Ph/d (Difference in the weight (g) of the wet matrices minus the weight (g) of the dehydrated ones), Dif.Ph/f (Difference in the weight of the wet matrices minus the weight of the dehydrated ones)].

Despite the damage caused by the abiotic conditions of the *In vitro* incubation after the 12 hours elapsed retention time in the treatments with alginate encapsulating material, the matrices were recovered, although their physical appearance was damaged and their resistance had been reduced in contrast to Gombotz and Fong (1998), who subjected alginate capsules to alkaline pH (7.4) and verified that the capsules actively released all the contents for 2 hours. These authors described that the release under these conditions can occur through two routes by the formation of pores and/or by progressive degradation of the polymer. Hernández (2015) also described that when the alginate capsules are subjected to alkaline pH, there is an increase in the size of the pores of the matrix, generating swelling, degradation of the polymer, and disintegration of the capsules in a short time. The matrices most susceptible to changes in the polymeric membrane were those produced in alginate concentrations of 2.0 and 3.0%, in contrast to what was mentioned by Tello *et al.* (2015), who used alginate solution at 2.0% in gastrointestinal conditions in a range of pH 3.0 – 7.0 at 37°C, for 2 and 5 hours, respectively, the alginate matrices were recovered after the incubation times and they still retained their spherical shape, as well as Chen *et al.* (2012) who mention that alginate at a 2.0% concentration with $CaCl_2$ at 5.0% w/v has proven to be sufficient for the development of spherical and resistant capsules. Chitosan is hydrophilic and can form a swellable matrix by ionic cross-linking between positively charged amino groups and polymers or negatively charged ions, in addition to this, it can be influenced by the addition of a porogen (polysaccharides) causing greater hydration of the matrix, same effect. which could be related to the matrices prepared in different concentrations of A+Q at pH 7.0 which were solubilized in the first 4 hours of the retention time, on the contrary, Chávarri *et al.* (2010) produced 2.0% alginate capsules mixed with 0.4% chitosan exposed to gastrointestinal conditions in incubation at 37°C for 120 min and concluded that said complex reduces the porosity of the alginate beads and decreases the leakage of the encapsulated probiotic and In addition, it is stable in wide pH ranges. In the tests with ruminal fluid, all treatments were affected, even the matrices made that contained chitosan, which due to its antimicrobial characteristic would be expected to have minimal degradation with respect to the alginate matrices (A), however, Hejazi and Amiji (2003) suggest that the microbiota produces chitosan degradation.

Soliman *et al.* (2013) and Badarinath *et al*. (2010) evaluated the effects of the concentration of the polysaccharide (alginate), the concentration of the cationic solution ($CaCl_2$), and cross-linking time on the encapsulation efficiency. The authors indicate that the encapsulation efficiency increases by increasing the concentration of cationic solution.

CONCLUSION

The polymer matrices of A (2.5%) and A (3.0%) + Q (0.75%) were those that protected the Moringa leaf material under *in vitro conditions*. The dehydrated A (2.5%) and A (3.0%) + Q (0.75%) polymer matrices maintained integrity after 10 h retention time under *in vivo* conditions. The dehydrated A (3.0%) + Q (0.75%) matrices capture a greater amount of *Moringa oleifera* flour in contrast to those made only with Alginate.

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CONFLICT OF INTEREST: None

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ETHICS STATEMENT: In this study, there were animals involved and it was approved by "Asociación Ganadera de Tapachula", and the reference number is 9626953024 the first author is a certified veterinarian.

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