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Resistance of biopolymer capsules to sheep-ruminal fluid

Resistencia de cápsulas biopoliméricas al líquido ruminal ovino

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Abstract

The resistance of polymeric capsules in sheep ruminal fluid was evaluated for possible application as a vehicle of bioactive agents to be released in the stage post-ruminal. Capsules were made by ionic gelation, using sodium alginate as wall material in combination with biopolymers and chlorophyll as a bioactive agent. Chlorophyll release and digestibility of biomaterials were evaluated *in vitro* conditions using sheep-ruminal fluid and digestibility *in situ* in a sheep with a fistula. The highest resistance at the rumen digestibility (46.60 ± 3% *in vitro* and 46.30 ± 3% *in situ*) was observed in deposit-type capsules, being the alginate-guar gum formulation the most resistant to the release of the bioactive. It was evidenced that it is possible to use deposit-type capsules, formulated with biopolymers (alginate-xanthan gum and alginate-guar gum) to serve as a possible vehicle for bioactive agents and promoting the post-ruminal release in an ovine model for improvement in the quality of meat for the human consumption.
Keywords: Encapsulation, alginate, sheep, *in situ* digestibility, *in vitro* digestibility.

Resumen

Se evaluó la resistencia de las cápsulas poliméricas en el fluido ruminal de oveja para su posible aplicación como vehículo de agentes bioactivos que se liberarán en la etapa post-ruminal. Las cápsulas se hicieron por gelificación iónica, utilizando alginato de sodio como material de pared en combinación con biopolímeros y clorofila como agente bioactivo. La liberación de clorofila y la digestibilidad de los biomateriales se evaluaron en condiciones *in vitro* utilizando líquido ovino-ruminal y digestibilidad *in situ* en una oveja con una fístula. La digestibilidad ruminal más alta (46.60 ± 3% *in vitro* y 46.30 ± 3% *in situ*) y la liberación más baja fue la formulación de alginato-goma guar en cápsulas de depósito. Se evidenció que es posible usar cápsulas de depósito, formuladas con biopolímeros (alginato-goma xantana y alginato-goma guar) para servir como un posible vehículo para agentes bioactivos y promover la liberación post-ruminal en un modelo ovino para mejorar en la calidad de la carne para consumo humano.
Palabras clave: Encapsulación, alginato, ovino, digestibilidad *in situ*, digestibilidad *in vitro*.

1 Introduction

Ruminants are one of the most numerous and important animal groups for humanity because of their role in food. There is currently a growing interest in developing products that can be easily incorporated in the ruminant's diet to improve their growing up or mitigate the environmental damage associated with these animals (Cobellis *et al*., 2016).

The encapsulation of bioactive compounds by ionic gelation represents an easy, fast and viable option for the release of bioactive agents in exact sites of the digestive system. In this encapsulation process, an irreversible gel is formed with a specific ion that interacts linking the polymer chains (Elnashar *et al*., 2015).

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The application of encapsulates in the ruminant diet has two approaches, a) the release of bioactive components within the rumen (Cherdthong and Wanapat, 2010); b) the release and absorption in the intestine, and protection of bioactive agents from the polycavitary stomach (Elnashar *et al*., 2015; Spanghero *et al*., 2009).

The use of biopolymers such as alginate, collagen, and gums are preferred due to their easy obtaining and wide use in foods. Sodium alginate is one of the main hydrocolloids in the food industry, is obtained from marine algae and structurally is a linear polyionic polysaccharide formed by D-mannuronic and L-guluronic acids, and is capable of instantly forming irreversible gels in the presence of calcium ion, a quality that allows rapid capsule formation in a spray system on a solution rich in calcium ions (Bhosale *et al*., 2014; Elnashar *et al*., 2015).

Biopolymers are ideal as wall-materials for the encapsulation of bioactive agents, however, it is important to evaluate the resistance in the ruminal fluid and any change due to the type of capsule formed by these biopolymers, for application in the feeding of ruminants (Gutiérrez-Rebolledo *et al*., 2018). The correct protection of bioactive compounds with functional activity, as well as their controlled release, are a technological strategy to favour the meat quality for human consumption, free of toxic compounds. Thus, this work analyses the resistance of polymeric capsules in sheep ruminal fluid for possible application as a vehicle of bioactive agents.

2 Materials and methods

2.1 Materials

Sodium alginate of low viscosity food grade (Cas No. 9005-38-3, A854), guar-gum (G440), Arabic-gum (CAS-9000-01-5), xanthan-gum (CAS-11138-66-2) and hydrolysed pork collagen (Farmacia París SA de CV, Mexico) all food grade were used as wall materials. As a bioactive agent, an extract of peppermint (Mentha arvensis and M. spicata) (Cas No. 1406-65-1, Naturalhealth, Mexico) with a concentration of 14 μg/mL of total chlorophyll was used. The viscosity of the formulations was determined using a viscometer, Discovery Hybrid Rheometer (HR-3, TA Instruments, USA) and following the method proposed by Zamudio-Flores *et al*. (2015) rheological measurements were performed in stress control for one minute, at room temperature (25 °C) and 60 °C.

Table 1. Formulations and viscosity of wall materials dispersions previous ionic-gelation and the operating conditions of the encapsulation equipment.

Biopolymers dispersions:	Viscosity (Pa.s)		Pressure (Pa)	Frequency (Hz)	Charge (V)	Temperature (°C)
Matrix-type capsules	25°C	60°C				
Alginate	1.60	-	55000	1500	800	25
Alginate-collagen	1.67	-	66100	1500	1200	25
Alginate-Arabic gum	1.69	-	66800	1500	600	25
Alginate-xanthan gum	4.32	1.18	83000	1500	600	65
Alginate-guar gum	33.59	12.64	83000	1500	500	65
Deposit-type capsules						
Alginate	7.19	3.76	85000	1500	500	25
Alginate-collagen	7.87	5.17	85000	1500	500	25
Alginate-Arabic gum	7.47	3.73	96000	1300	500	25
Alginate-xanthan gum	10.47	5.78	98000	1300	500	65
Alginate-guar gum	290.50	155.44	110000	1300	500	65

2.2 *Preparation of capsules*

The capsules were formed using an encapsulation equipment (B-390, Büchi, Switzerland). The operating conditions (frequency, pressure, load, and temperature) used to manufacture all the capsules are shown in Table 1. The formulations used included sodium alginate (2%) in combination with other biopolymers: 1.5% Arabic-gum, 1% xanthan gum, 1.5% guar-gum or 2% hydrolysed collagen.

The Matrix-type capsules (M-capsules) were manufactured using a concentric nozzle (1000 μm aperture). A wall material dispersion was mixed with the chlorophyll, in a 1:10 ratio; the mixture was pumped to the nozzle under the conditions mentioned in Table 1. The Deposit-type capsules (D-capsules) were manufactured with an internal concentric nozzle of 450 μm (core nozzle) and an external nozzle of 900 μm (shell nozzle). The wall material dispersions were pumped to the external nozzle (shell nozzle) until reaching a stable drop size, the chlorophyll was subsequently pumped into the internal nozzle (core nozzle), maintaining the operating conditions (Table 1). In both cases, the sprinkled drops were collected in a solution of calcium chloride (0.5 mol/L) (Elnashar et al, 2015), kept in gelation during 30 min maintaining constant stirring at low revolutions. Subsequently, the solution was drained and the capsules were dried with air in a fixed bed at 25 °C, in the dark for 48 h, and storage in glass jars with airtight seal until further analysis.

2.3 *Digital image analysis*

Capsules were analysed using a stereoscopic microscope (SMZ1500, Nikon®, USA) coupled to a camera (Nikon Digital Sight DS-2Mv, TV Lens 0.55X, DS, Japan). The images were processed using the ImageJ program (1.50i, Java1.6.0_24 (64-bit), National Institutes of Health, USA). Morphometric parameters (area, Feret diameter, circularity, radius aspect, roundness, and sphericity) were calculated (Quintanilla-Carvajal *et al*., 2011) . An average of 50 micrographs was used to establish the pixel-millimetre reference scale using the measuring bar provided by the microscope software and using a boiling glass bead of known size (Jones *et al*., 2012). Statistical analysis of morphometric parameters was carried out with a coefficient of variation between particles (≤ 10%) for each of the parameters.

2.4 *Chlorophyll quantification*

1g of encapsulates was previously rehydrated in 3 mL of distilled water heated (50 °C)/1h and extracted using 3 mL of a mixture of acetone water (1:1). To quantify the chlorophyll, 100 μL were placed in 10 mL of acetone and the absorbency (Abs) was measured at 663 nm and 645 nm with a visible UV spectrophotometer (Genesys 10S UV-Vis, Thermo Fisher Scientific Inc., USA), using equations 1 and 2 to estimate chlorophyll a, chlorophyll b, and total chlorophyll (Barros *et al*., 2010; Plazola-Jacinto *et al*., 2019).

$$Chlorophyll\ a = 0.999(Abs_{663nm}) - 0.0989(Abs_{645nm}) \quad (1)$$

$$Chlorophyll\ b = -0.328(Abs_{663nm}) + 1.77(Abs_{645nm}) \quad (2)$$

2.5 *Biomaterial stability test and ovine ruminal fluid release*

2 g (dry basis) of capsules were placed in 5 mL distilled water incubating in a closed bottle in a 39 °C water-bath. The chlorophyll concentration in the medium was measured since 1 until 48 h of incubation (Kostewicz *et al*., 2014). Subsequently, an *in vitro* fermentation model was used, following the method proposed by Del Razo-Rodriguez *et al*. (2013). 2 g of capsules were placed in 5 mL of ruminal fluid. Incubation of the capsules in water were carried out in a sealed tube containing CO_2 (24 h, 39°C). The estimated time for the capsules to remain in the rumen, the solubility of the materials and the release of chlorophyll in the medium were measured at 24 h of fermentation (Almaraz-Buendia *et al*., 2018). Previously, the ruminal fluid was obtained from a fistula sheep (Suffolk x Rambouillet, 6 years old), prior to the first food intake. This liquid was filtered three times with sterile gauze and kept at a constant temperature (39°C) until use. Sheep was maintained in accordance with the regulations established by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) of Autonomous University of Hidalgo State (UAEH), Mexico. The medical-veterinary care before and after the surgery was performed according to regulations (NOM-062-ZOO, 1999).

2.6 *Ruminal fermentation and determination of in vitro gas production*

Biomaterials of all capsules were evaluated by emulating the digestive conditions of a grazing animal, with an *in vitro* fermentation test with oat hay (0.5 g) as substrate. The amount of capsules was determined from a dose of 250 μg/kg body weight; a mix of ruminal fluid and wall material was used as a white control to eliminate the effect caused by residual forage and bacterial growth. The volume of gas produced at 0,1,2,3,4,5,7,9,12 and 24 h was performed, with which the accumulated gas kinetics was calculated, considering: maximum volume of gas produced (V) in mL/g dry matter; gas production rate (S) in h^{-1} and delay time or Lag phase (L) in h. The time was established based on the average time of the sheep feed rate, due to the particle size it is expected not to exceed that time in the rumen. The *in vitro* fermentation and the kinetics of gas production were performed following the methodology reported by Almaraz-Buendia *et al*. (2018) with adaptations for the work material.

2.7 *Ruminal fermentation in situ*

Samples of capsules were placed inside nylon filter bags (F57, Ankon Technology, USA) with 25 μm pore, and these in turn in a nylon mesh container bag (pore of 150 μm, capacity of 2 L and length of 70 cm). Additionally, three 3 cm diameter glass spheres were placed to give weight and the bag was introduced into the rumen of the sheep through the fistula. The fermentation process was carried out for 24 hours; the bags once recovered were washed with distilled water and dried in an oven at 60°C, until reached constant weight, and the calculations were performed (Danesh-Mesgaran and Stern, 2005). The animal was fed with oat hay and water ad libitum, in a metabolic cage and was monitored throughout the experiments (Almaraz-Buendia *et al*., 2018; Del Razo-Rodriguez *et al*., 2013).

2.8 *Statistical analysis*

The experimental design was randomized using the SAS® MIXED procedure (SAS-Institute-Inc., 2012) taking into consideration in the statistical model the treatment and time as a fixed effect. The least-squares means were obtained with the SAS® LSMEANS function and the comparison of means was performed for variables with significant treatment effect (Almaraz-Buendia *et al*., 2018; Campos-Montiel *et al*., 2008). Gas production kinetics variables were calculated using the statistical model of of Schofield and Pell (1995) Va = V / (1 + e2-4S (tL)) with the SAS® NLIN procedure SAS-Institute-Inc (2012).

3 Results and discussion

3.1 *Morphometric parameters determined by digital image analysis*

To achieve a homogeneous release of the bioactive component, it is desirable that the capsules acquire a spherical shape. In M-capsules, chlorophyll was distributed in the matrix structure formed by the wall-materials (Figure 1). In the case of D-capsules, the chlorophyll was located forming a wet centre (core) inside the capsule and the wall-material (shell) surrounding the core.

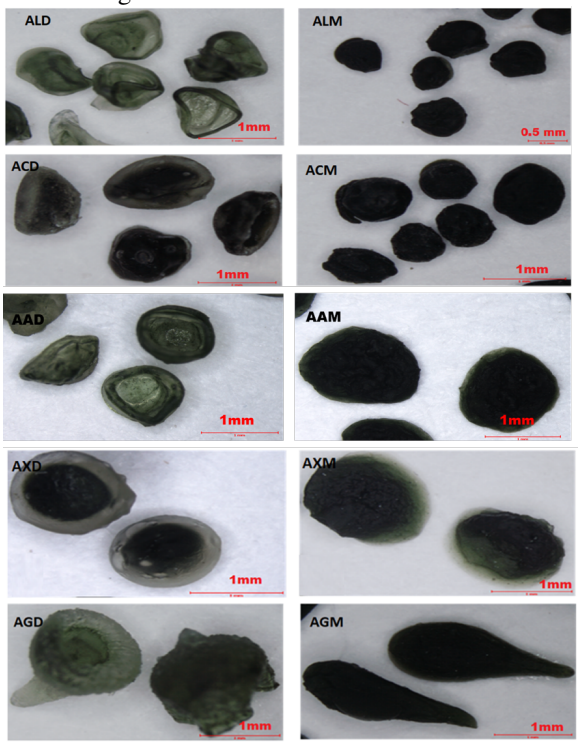

Fig. 1. On the left are the deposit type capsules and on the right the matrix type capsules. The images were captured by stereoscopic microscopy; the first two letters indicate the wall material and the third one the capsule type: (AL) sodium alginate, (AC) alginate and collagen, (AA) alginate and Arabic gum, (AX) alginate and xanthan gum, (AG) alginate and guar gum; matrix-type capsules (M), deposit-type capsules (D).

Table 2. Water activity and morphometric characterization of the capsules.

Type of Capsule	Water activity (25°C)	Number of capsules analysed (CV*≤10%)	Circularity (mm)	Aspect ratio (AR) (mm)	Roundness (mm)	Sphericity factor (SF)	Feret Diameter (FD) (mm)
ALD	0.3260	249	0.74 ± 0.08^{aA}	1.5 ± 0.27^{aA}	0.69 ± 0.12^{aA}	0.19 ± 0.07^{aA}	1.16 ± 0.12^{aA}
ACD	0.3533	182	0.80 ± 0.08^{aA}	1.26 ± 0.17^{aA}	0.81 ± 0.10^{aA}	0.12 ± 0.06^{aA}	1.27 ± 0.01^{aA}
AAD	0.2770	201	0.77 ± 0.08^{aA}	1.36 ± 0.28^{aA}	0.76 ± 0.13^{aA}	0.15 ± 0.08^{aA}	1.40 ± 0.15^{aA}
AXD	0.2968	231	0.80 ± 0.07^{aA}	1.24 ± 0.18^{aA}	0.82 ± 0.11^{aA}	0.11 ± 0.06^{aA}	1.18 ± 0.10^{aA}
AGD	0.2569	157	0.65 ± 0.06^{aA}	1.31 ± 0.21^{aA}	0.78 ± 0.10^{aA}	0.16 ± 0.06^{aA}	2.10 ± 0.19^{bA}
ALM	0.3578	482	0.84 ± 0.04^{aA}	1.18 ± 0.13^{aA}	0.85 ± 0.08^{aA}	0.09 ± 0.04^{aA}	0.75 ± 0.06^{aB}
ACM	0.3521	229	0.82 ± 0.07^{aA}	1.24 ± 0.21^{aA}	0.82 ± 0.10^{aA}	0.11 ± 0.06^{aA}	1.06 ± 0.1^{bB}
AAM	0.2860	134	0.80 ± 0.06^{aA}	1.29 ± 0.17^{aA}	0.79 ± 0.10^{aA}	0.13 ± 0.06^{aA}	1.45 ± 0.13^{cA}
AXM	0.1894	109	0.74 ± 0.09^{aA}	1.32 ± 0.25^{aA}	0.78 ± 0.12^{aA}	0.13 ± 0.07^{aA}	2.21 ± 0.20^{dB}
AGM	0.2582	221	0.55 ± 0.06^{bA}	2.42 ± 0.48^{bB}	0.43 ± 0.09^{bB}	0.37 ± 0.08^{bB}	2.47 ± 0.25^{dA}

Nomenclature of each sample: the first two letters indicate the wall material and the third letter the type of capsule: sodium alginate (AL), alginate and collagen (AC), alginate and Arabic gum (AA), alginate and xanthan gum (AX), alginate and guar gum (AG); matrix-type capsules (M), deposit-type capsules (D). The letters identified the differences ($P<0.05$) using the least squares analysis. Lower letters show differences between formulations per group of particles, capital letters show the contrast between particles. (*) CV≤10%: coefficient of variation less or equal to 10 percent.

The average size of capsules after being dehydrated, measured as Feret's diameter was 2.21 to 1.06 mm. The dehydrated M-capsules had a granulated appearance similar to sea salt. The dried D-capsules had a flake form since they collapse on one of their sides (alginate and alginate-collagen). In formulations with gums, the appearance of capsules was similar to a bell or flower button (Figure 1). This appearance may be due to the thickeners and elastic characteristics of the gums, in addition to the interweaving of the polysaccharide chains derived from the mixture (Bhosale *et al*., 2014; Bokkhim *et al*., 2016).

The shape descriptors indicate that a circularity value of 1.0 corresponds to a perfect circle while values close to 0.0 are elongated shapes; the aspect ratio is suitable for elongated or elliptical particles, while roundness is preferred for spherical particles (Ferreira and Rasband, 2012). The morphometric descriptors are shown in Table 2. The sodium alginate capsules showed the smallest size, while the ones with the largest diameter were those made with alginate and guar gum, observing that with this mixture the capsules increase their average size due to the elongation of major-axis, so the use of this mixture does not allow obtaining spherical capsules, since the structural strength of the matrix is not enough to maintain the shape. During ionic-gelation and drying these capsules collapse acquiring an elliptic form. The M-capsules performed only with alginate were the most spherical and smaller. Capsules that tend to sphericity are preferred because they exhibit homogeneous release, it some cases it could imply that the enzymatic actions are similar on all capsules sites (Phaechamud and Darunkaisorn, 2016; Plazola-Jacinto *et al*., 2019).

All capsules are smaller or similar to some seeds used in the estimation of the digestive passage speed of sheep, reason why all the capsules have an adequate size (Danesh-Mesgaran and Stern, 2005). Small particle sizes allow not only the rumination phenomenon, and also reduce rumination fermentation time by easily passing between forage leaves and stems from the upper stratum of the ruminal content and through the omasal-reticulum orifice, continuing to the intestine(Chan *et al*., 2010).

3.2 Stability of capsules to rehydration in ruminal fluid and gas production

The production of gas derived from the fermentation started with the ruminal microorganisms allowed to know the behaviour of this microbial population. The normal pH of the rumen ranges from 7.0 to 7.3 and this was acidified as the fermentation time

increases, it is known that the biopolymers used as wall material are more stable in pH acids (Mudgil and Barak, 2013). The fermentation solution had an initial pH of 7.2 and this was reduced after fermentation with substrate to a range between 6.82-6.91; this suggests that the implementation of these biopolymers at this concentration does not represent a factor that influences subclinical acidosis whose values of pH ranges between 6.5-5.7 (Fanning *et al*., 2018). However, 3 formulations (ALM, AAM, AGM) showed differences in pH reduction with respect to the type of capsule (Table 3), these results suggest that the M-capsules degrade more than the D-capsules and of these capsules the ones made with alginate and alginate-gum Arabic are highlighted, which show statistical differences in the reduction of pH with respect to oat hay (Fanning *et al*., 2018). No significant differences were observed between biopolymers with the same capsule conformation, this results in the pH change suggest that the M-capsules degrade better by the effect of the microenvironment of the rumen and that the capsules with better resistance would be those formulated with alginate-guar gum and alginate-xanthan gum, followed by the alginate-collagen formulations (Table 3).

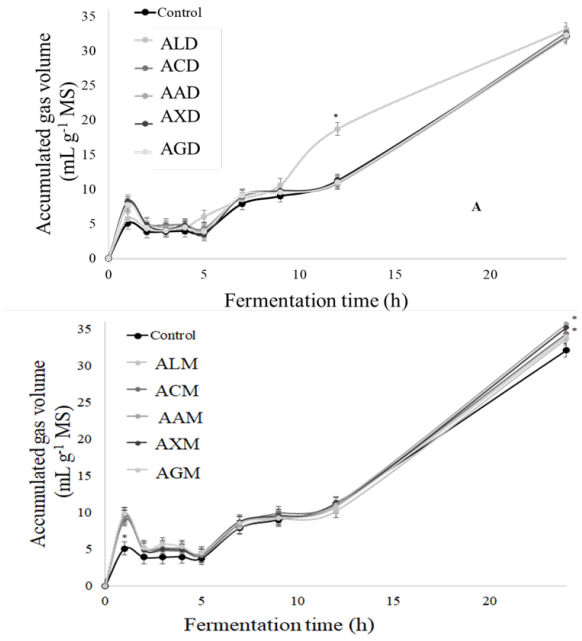

Fig. 2. Accumulated gas volume generated *in vitro* fermentation of oat hay for 24 h with capsules. (A) deposit-type capsules, (B) matrix-type capsules (*significant difference between wall material per type of capsules ($P \leq 0.05$)).

Table 3. pH, gas production rate and maximum gas volume during *in vitro* fermentation with oat hay.

Capsule	Dose (g)	Final pH	Lag phase (h)	Gas production rate (S) (mL/h)	Maximum gas volume (V) (mL)
ALD	0.016	6.90±0.03^{aA}	2.14±0.79^{aA}	0.07±0.1^{aA}	205.1±87^{aA}
ACD	0.016	6.87±0.03^{aA}	1.08±0.79^{aA}	0.06±0.1^{aA}	190.1±87^{aA}
AAD	0.016	6.90±0.03^{aA}	1.09±0.79^{aA}	0.06±0.1^{aA}	189.5±87^{aA}
AXD	0.016	6.90±0.03^{aA}	1.04±0.79^{aA}	0.06±0.1^{aA}	192.6±87^{aA}
AGD	0.016	6.91±0.03^{aA}	1.17±0.79^{aA}	0.06±0.1^{aA}	190.1±87^{aA}
ALM	0.05	6.81±0.03*^{aB}	0.48±0.79^{aB}	0.059±0.1^{aA}	186.2±87^{aA}
ACM	0.05	6.85±0.03^{aA}	0.75±0.79^{aA}	0.062±0.1^{aA}	191.5±87^{aA}
AAM	0.05	6.82±0.03*^{aB}	0.70±0.79^{aA}	0.060±0.1^{aA}	191.2±87^{aA}
AXM	0.05	6.84±0.03^{aA}	0.32±0.79*^{aA}	0.059±0.1^{aA}	196.1±87^{aA}
AGM	0.05	6.84±0.03^{aB}	0.43±0.79*^{aA}	0.060±0.1^{aA}	191.2±87^{aA}
OAT HAY	0.5	6.91±0.03*	2.02±0.79*	0.065±0.1	185.83±87

Significant difference between capsules and wall materials ($P \leq 0.05$). Lower letters show differences between formulations per group of particles, capital letters show the contrast between particles. *Significant differences with the forage control ($P \leq 0.05$).

The *in vitro* test incubation of capsules were similar between treatments with respect to fodder in the gas production test, suggesting that the fermentation of the biopolymers by the ruminal microbiota does not affect the normal digestibility of the forage thus avoiding metabolic problems (Campos-Montiel *et al*., 2008; Fanning *et al*., 2018). The AGM capsules (Lag = 0.43 ± 0.79 h) and AXM (Lag = 0.32 ± 0.79 h) showed significant differences ($p<0.05$) in the Lag phase with respect to oat hay (Lag = 2.02 h) (Figure 2). Similarly, significant differences ($p <0.05$) were also observed in the Lag phase depending on the type of capsule when the only alginate was used as wall material, the ALD encapsulates had the longest Lag time (L = 2.1427 h) (Table 3).

M-capsules showed major gas production than D-capsules, under controlled incubation, within the first hours of the incubation stage (Table 3 and Figure 2). When observing the behaviour of M-capsules in the production of gas, during the first 5 hours there are no differences between formulations (Figure 2), which suggests that the incorporation of chlorophyll in the polymer matrix of the formulations makes them susceptible to ruminal fluid (da S. Gulão *et al*., 2014; Erben *et al*., 2019). Zamudio-Flores *et al*. (2015) reported when other substances were incorporated in the substrate matrix, the digestibility was affected.

Regarding the rate of gas production (S) no significant differences were shown between any treatments (Table 3), this may suggest an enzymatic and bacterial preference directed to oat hay rather than that of wall materials; however, it is possible to appreciate differences in digestibility derived from the type of capsule. This idea is reinforced by observing the apparent digestibility values of the capsules in the ruminal fluid solubility test without oats hay, where all the formulations presented significant differences ($p <0.05$) between type of capsule (Table 4) (Carlson *et al*., 2016).

Table 4. Digestibility of polymeric capsules in fermentations *in vitro* and *in situ*.

	Fermentation *in vitro*		*Fermentation In Situ**
Capsule	With substrate (%)	Without substrate (%)	(%)
ALD	$36.85\pm3.6^{\underline{a}aA}$	28.96 ± 4.2^{aA}	52.1 ± 3.2^{aA}
ACD	$35.67\pm3.6^{\underline{a}aA}$	53.45 ± 4.2^{bA}	56.2 ± 3.2^{aA}
AAD	$39.98\pm3.6^{\underline{a}abA}$	44.82 ± 4.2^{bA}	51.2 ± 3.2^{aA}
AXD	$40.23\pm3.6^{\underline{a}abA}$	38.81 ± 4.2^{aA}	43.8 ± 3.2^{bA}
AGD	$46.60\pm3.6^{\underline{a}bA}$	35.16 ± 4.2^{aA}	46.3 ± 3.2^{abA}
ALM	$32.52\pm3.6^{\underline{a}aA}$	70.51 ± 4.2^{aB}	45.7 ± 3.2^{aB}
ACM	$35.51\pm3.6^{\underline{a}aA}$	74.53 ± 4.2^{aB}	52.3 ± 3.2^{aA}
AAM	$40.79\pm3.6^{\underline{a}aA}$	61.92 ± 4.2^{bB}	64.3 ± 3.2^{cB}
AXM	$40.95\pm3.6^{\underline{a}aA}$	67.63 ± 4.2^{aB}	59.3 ± 3.2^{bB}
AGM	$36.33\pm3.6^{\underline{a}aB}$	60.66 ± 4.2^{bB}	56.2 ± 3.2^{abB}
OAT HAY	$40.17\pm3.6^{\underline{a}}$	-	-

*Sheep fed *ad libitum*. Significant difference between capsules and wall materials ($P \leq 0.05$). Lower letters show differences between formulations per group of particles, capital letters show the contrast between particles, underlined letters show the relationship with the control.

The total volume of gas produced in all treatments does not show significant differences with respect to the control ($p < 0.05$). This data suggests the biopolymers used will not generate changes in the production of volatile compounds in the rumen (Fanning *et al*., 2018) and, suggests its functionality as a carrier of substances sensitive to ruminal digestibility through it (Elnashar *et al*., 2015). However, although there are no significant differences with respect to the total volume of gas produced according to the model, the behaviour in the gas production of the capsules suggests that the formulations AAM (34.5 mL gas) and AXM (33.6 mL gas) they could produce more gas if the kinetics continued, since these formulations show significant differences ($P \leq 0.05$) at the end of the kinetics (Figure 2). It is known that gum Arabic can be digested by ruminants (Westendorf and Wohlt, 2002), however, the AAD formulation has the lowest gas volume (189.5 ± 87 mL gas) of this type of capsule; on the other hand the formulations AXD and AXM showed similar production of gas, however the results observed in Figure 2, suggest that the AXM formulation could be further degraded at a longer time (BeMiller, 2019; Bhosale *et al*., 2014), these results suggest that rumen fermentation resistance is dependent on the type of particle and the arrangement of polysaccharide chains during their formation, since the simplest formulation (ALD) showed the highest volume of gas (205.1 ± 87 mL gas). It is inferred that the potential of the biopolymers as carriers of bioactive compounds depends on the distribution of the components in the capsule. The M-capsules showed a greater production of gas than the D-capsules during the first hour, but less quantity at 12 h and the final volume at 24 h, it shows a greater production of gas of M-capsules showing that the type of capsule plays an important role in the polysaccharide digestibility. The incidence of pores in substrate improves fermentation and increases methane production (Sanchez-Herrera *et al*., 2018). Gómez-Guerrero *et al*. (2019) observed that gas production can be self-limiting, depending on the bacteria's ability to ferment the substrate and the kind of substrate used.

3.3 Ruminal fermentation in vitro and in situ

The results of the digestibility of biopolymers capsules due *in vitro* fermentation, did not show significant differences between treatments with respect to oat hay (40.17 ± 3.6%) (Table 4). Therefore, the incorporation of the biopolymers does not affect the normal digestibility of the fodder consumed (Almaraz-Buendia *et al*., 2018; Spanghero *et al*., 2009), However, differences were observed significant ($P \leq 0.05$), among the formulations of the D-capsules; AGD (46.60 ± 3.6%) showed significant differences with respect to ALD (36.85 ± 3.6%) and ACD (35.67 ± 3.6), the gums showed an average digestibility of 40%. The results obtained during fermentation *in situ*, show that AGD capsules maintained their digestibility percentage (46.3 ± 3.2%) (Table 4), these results, together with those obtained from the oats hay solubility test, suggest that of the AGD (35.16 ± 4.2%), it is the second formulation with greater resistance to the ruminal microenvironment. However, the results of *in vitro* fermentation showed significant differences ($P \leq 0.05$) depending on the type of capsule, where the M-capsules (36.33% ± 3.6%) were less digested than the D-capsules (46.33 ± 3.6%).

In case of *in situ* digestibility test, the AGM (56.2 ± 3.2%) increases its degradation and presents significant differences against AAM (64.3 ± 3.2%), this was due to factors such as peristaltic movements and non-cultivable microorganisms *in vitro* medium among others (Bokkhim *et al*., 2016; Campos-Montiel *et al*., 2008; Carlson *et al*., 2016). These results suggest that the formulation with guar gum is resistant to ruminal digestibility, due to a better interaction between the polysaccharide chains and their low branching reducing the enzyme-binding sites when there are no bioactive agents in the polymer matrix (Carlson *et al*., 2016). In the case of ACD and ACM, it is possible that polymeric digestibility during the *in situ* tests was increased (Table 4) by the action of other ruminal microorganisms such as fungi and protozoa (Campos-Montiel *et al*., 2008; Newbold *et al*., 2015).

The formulations that include gums behave similarly in both types of capsules and fermentations (*in situ* and *in vitro*) of these the AXD formulation stands out (43.8 ± 3%), and presents significant differences with respect to the rest of the capsules with the exception of AGD, and the same way happens in the M-capsules; of these, the AAM formulation (64.3 ± 3%) is the one with the highest digestibility, these results suggest that the gum Arabic has the least effective cross-linking with alginate (da S. Gulão *et al*., 2014; Sanchez-Herrera *et al*., 2018).

The M-capsules did not show significant differences ($p \leq 0.05$) between *in vitro* fermentation formulations, while in the ALM *in situ* digestibility (45.7 ± 3%) presented the highest resistance to

digestibility (Table 4). This also suggests that chlorophyll interferes to a lesser extent in the arrangement of alginate chains due to the absence of other materials during gelation (Jones *et al*., 2012). Thus, significant differences are also observed (P≤0.05), between particles with the same wall material in the fermentation without substrate oats hay, these results suggest that the ruminal microorganisms can better degrade polymer formulations with interfering substances in their matrix such as chlorophyll. In the case of interaction between digestibility and morphometric parameter, all capsules showed a statistically significant correlation (p <0.05) between morphometric parameters respect to the percentage of digestibility.

3.4 Chlorophyll release in the medium during fermentation in vitro with oat hay

Data of chlorophyll contained in fed capsules and chlorophyll released during the fermentation *in vitro* with oat hay are exhibited in Table 5. In the *in vitro* fermentation, the results showed significant differences (p <0.05) depending on the capsule type and the biopolymeric formulation. The ACD, AAD, AAM and AXM exhibited chlorophyll release higher 90%. The D-capsules presented the lowest release and of these, the AGD (0.01%) stands out. When M-capsules exceed 60% digestibility, they completely release their content, while in the D-capsules the release-digestibility relationship between formulations, derived from the thickness of the capsule wall which is directly related to the dispersion viscosity (Jones *et al*., 2012). The observed increase in the amount of chlorophyll in the medium may be due to the forage degraded by the growth of the bacterial population (Myer *et al*., 2015). The AGD exhibited the greatest resistance to chlorophyll release (Table 5), are also the capsules with a residual tail generated during its formation and preserved during its gelation in calcium chloride.

Table 5. Percentage of chlorophyll release in fermentation *in vitro* conditions with Oak hay.

Capsule	Chlorophyll contained in capsules (µg/g)	Concentration of chlorophyll fed* (µg/mL)	Chlorophyll released in the ruminal liquid (µg/mL)**	Chlorophyll release (%)
ALD	13.23	0.218±0.01^{a}	0.103±0.03^{a}	47.2^{A}
ACD	13.34	0.219±0.01^{a}	0.214±0.03^{b}	98^{A}
AAD	13.75	0.229±0.01^{a}	0.567±0.03^{b}	100^{A}
AXD	14.62	0.234±0.01^{a}	0.161±0.03^{c}	68^{A}
AGD	11.11	0.186±0.01^{b}	0.11E-6±0.03^{d}	0^{A}
ALM	6.48	0.318±0.01^{a}	0.191±0.03^{a}	60^{B}
ACM	7.97	0.392±0.01^{a}	0.215±0.03^{a}	54.8^{B}
AAM	6.02	0.292±0.01^{a}	2.431±0.03^{b}	100^{A}
AXM	5.92	0.299±0.01^{a}	3.074±0.03^{c}	100^{B}
AGM	6.10	0.299±0.01^{a}	0.175±0.03^{a}	58.5^{B}

* 0.016g of chlorophyll in D-capsules, 0.05g of chlorophyll in M-capsules. (a) Show differences between formulations and oat hay. Significant difference between capsules and wall materials (p ≤ 0.05). Lower letters show differences between formulations per group of particles, capital letters show the contrast between particles with the same formulation.

It could be providing a vast area for bacterial adhesion, also explaining the increase in percentage digestibility during *in vitro* fermentation without affecting the release.

Formulations with higher viscosity (Table 1) produce thicker-looking capsular walls and low deformation during rehydration unlike the rest of the capsules, this may be the cause of its lower release and high resistance which would explain the significant differences ($P < 0.05$) between formulations. This resistance could be due to better gelation, which points to a better interaction between guar and xanthan gums with alginate compared to the other materials, additional the bacterial groups that can better digest gum Arabic than the forage, as well as a non-homogeneous digestibility of the wall in these capsules causing their rupture and release, but not the digestibility of the material (da S. Gulão *et al*., 2014). However, more tests are necessary, since the *in situ* fermentation test shows a greater digestibility of the capsules which could improve their release.

Conclusions

The main resistance to digestion is derived from the type of particle more than the materials used in all formulation, being the D-capsules the most resistant to digestibility and the release of the bioactive, in contrast, M-capsules are highly susceptible to digestibility and release (more than 50% in the ruminal fluid). The maximum resistance to ruminal fermentation was achieved using the alginate, and alginate-guar gum formulation. This means the more resistant a material is in fermentation *in situ* or *in vitro*, the less digestible it will be, being a good option to be used in post-ruminal stage, however, it is necessary to perform other tests to confirm the release or overprotection of this material in the animal intestine. If the capsules are expected to remain less than 24 hours exposed to the ruminal microenvironment, a proposal is to take in count the size particle (1-2 mm), materials (alginate, alginate-guar gum) and type of capsule (deposit type) in order to reduce both the percentage of digestibility and the bioactive release.

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Abbreviations

a_w	Water activity
AA	sodium alginate and Arabic gum
AAD	sodium alginate -Arabic gum capsules in deposit-type
AAM	sodium alginate -Arabic gum capsules in deposit-type
AC	sodium alginate and collagen
ACD	sodium alginate -collagen in deposit-type
ACM	sodium alginate -collagen in matrix-type
AG	sodium alginate and guar gum
AGD	sodium alginate -guar gum in deposit-type
AGM	sodium alginate -guar gum in matrix-type
AGV	accumulated gas volume
AL	sodium alginate
ALD	sodium alginate capsules in deposit-type
ALM	sodium alginate capsules in matrix-type
AX	sodium alginate and xanthan gum
AXD	sodium alginate and xanthan gum in deposit-type
AXM	sodium alginate and xanthan gum in matrix-type
D-capsules	deposit-type capsules
FD	Feret diameter, mm
L	delay time or Lag phase, h
M-capsules	matrix-type capsules
S	gas production rate, h-1
SF	Sphericity factor, mm
V	maximum volume of gas produced, mL/g dry matter

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