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ORIGINAL ARTICLE

Microparticles coated with proteins in their natural state and *in vitro* gastrointestinal simulation

Micropartículas revestidas com proteínas em seu estado natural e simulação gastrointestinal in vitro

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Abstract

This study aimed to produce pectin and alginate microparticles by ionic gelation coated with different concentrations of bovine blood and egg white protein in their natural state. The coated microparticles were characterized, and their physical resistance and morphology were evaluated, as well as the released protein during *in vitro* gastrointestinal simulation. The highest protein adsorption (65.47%) was shown by pectin microparticles coated with bovine blood (10%), regardless of the protein type and concentration used. Likewise, higher amounts of adsorbed protein resulted as protein concentration increased, regardless of the type of microparticle. Nevertheless, the physical resistance of coated microparticles was affected more by the type of polysaccharide, being alginate microparticles more resistant. Adsorbed proteins on microparticles surface showed higher solubility values *in vitro* gastrointestinal simulation regardless of protein type. Bovine blood and egg white proteins in their natural state can be used as alternative coating materials for microparticles.

Keywords: Ionic gelation; Protein adsorption; Solubility; Bovine blood; Egg white; Microencapsulation.

Resumo

Nosso objetivo foi produzir micropartículas de alginato e pectina por gelificação iônica revestidas com diferentes concentrações de proteína de sangue bovino e clara de ovo em seu estado natural. As micropartículas revestidas foram caracterizadas e avaliadas quanto à sua resistência física, bem como em relação à proteína liberada durante

a simulação gastrointestinal *in vitro*. A maior adsorção de proteína (65,47%) foi demonstrada por micropartículas de pectina revestidas com sangue bovino (10%), independentemente da fonte da proteína e da concentração utilizada. Da mesma forma, maiores quantidades de proteína adsorvida resultaram no aumento da concentração da proteína, independentemente do tipo do polissacarídeo utilizado na micropartícula. No entanto, a resistência física das micropartículas revestidas foi mais afetada pelo tipo de polissacarídeo, sendo as micropartículas de alginato mais resistentes. As proteínas adsorvidas na superfície das micropartículas apresentaram maiores valores de solubilidade na simulação gastrointestinal, independentemente da fonte da proteína. As proteínas do sangue bovino e da clara de ovo em seu estado natural podem ser usadas como materiais de revestimento alternativos para micropartículas.

Palavras-chave: Gelificação iônica; Adsorção de proteínas; Solubilidade; Sangue bovino; Clara de ovo; Microencapsulação.

Highlights

- Proteins from bovine blood and egg white were used as a coating for microparticles
- Protein adsorption was higher for pectin microparticles
- Proteins from bovine blood and egg white were solubilized under intestinal conditions

1 Introduction

The microparticles can be defined as three-dimensional networks of polymer chains that can swell, absorb and retain water without dissolving in it (Silva Carvalho et al., 2019). One of the most used techniques for their production is associated with ionic gelation, which is a simple process that can be performed in mild conditions (without organic solvents or high temperatures) (Moura et al., 2018). This method enables the utilization of polysaccharides, such as alginate and pectin, which are biocompatible, biodegradable, accessible, and extracted from natural sources (Ali & Ahmed, 2018).

Nevertheless, this technique produces porous matrices with low physical resistance, thus limiting their application as a controlled release vehicle (Kurozawa & Hubinger, 2017). One of the proposed alternatives to approach this issue is the coating with opposite charge proteins through electrostatic interaction (Gbassi et al., 2011). Proteins carry a positive charge at pH values below their isoelectric point (De Kruif et al., 2004), allowing the interaction with negative charged carboxylic groups (COO-) available at the surface of microparticles (Ye, 2008). Accordingly, the resulting complexes reduce the porosity of the matrix improving its functionality and physical resistance (de Araújo Etchepare et al., 2020). Different proteins have been used to coat microparticles produced by ionic gelation which include whey proteins (Rezende et al., 2018), ovalbumin (Aguilar et al., 2015), Soybean Protein Concentrate (SPC) (Silverio et al., 2018), among others. According to these studies, the proteins used to coat microparticles underwent thermal processes that can decrease their functionality. Therefore, the utilization of proteins in their natural state (non-modified proteins by thermal or mechanical effects, recently obtained) is presented as an exploration alternative.

In Peru, the cattle slaughter produces large volumes of blood that are discarded directly to the sewage system becoming a polluting factor (Del Hoyo et al., 2008). Therefore, finding new applications that imply the industrial utilization of bovine blood is of great interest (Hsieh & Ofori, 2011). On the other hand, egg as a protein source has been studied by Aguilar et al. (2015), in the coating of microparticles with dehydrated egg white proteins, achieving up to 47.7% of adsorption and conferring protection to the encapsulated model oil. However, the use of egg white proteins in their natural state has not been reported yet.

Therefore, this study aimed to use Bovine Blood (BB) and Egg White (EW) (for comparison purposes) in their natural state to coat pectin (M_{PEC}) and alginate microparticles (M_{ALG}) produced by ionic gelation.

Moreover, this study also aimed to evaluate the physicochemical and morphological characteristics of coated microparticles, as well as their physical resistance and solubility of adsorbed proteins through *in vitro* gastrointestinal simulation.

2 Material and methods

2.1 Material

Pectin (PEC) of low methoxylation GENU® (CP Kelco, Brazil), alginate (ALG) (high molar mass, high content of guluronic acid, Manugel DMB, FCM Biopolymer, Brazil), and oil from *Plukenetia volubilis* L. (Arefon Laboratory, Tarapoto, Perú). BB (municipal slaughterhouse of Punchana, Perú) and EW from fresh eggs (Belen market, Perú). Pepsin from porcine gastric mucosa (activity > 250 units/mg solids), and pancreatin from porcine pancreas (activity 8/USP) from Sigma-Aldrich. Calcium chloride anhydrous, hydrochloric acid, chloroform, and sodium hydroxide from Merck.

2.2 Characterization of protein sources

BB and EW were characterized before the preparation of solutions regarding their moisture, ash, protein (Association of Official Analysis Chemists, 2012), and lipid contents (Bligh & Dyer, 1959).

2.3 Preparation of BB and EW solution

BB and EW were diluted with distilled water until reaching protein concentrations of 2, 4, 8, and 10% (w/w). These solutions were stirred at 27 °C for approximately three hours. Then, they were filtered, and the pH was adjusted to 4.0 using NaOH and HCl solutions. To avoid microorganism proliferation, after its collection BB was cooled in an ice bath between 2 and 5 °C with the addition of an EDTA solution (3 $g.L^{-1}$) to keep its characteristics until analysis.

2.4 Production of coated and uncoated microparticles

The microparticles production was performed by ionic gelation according to the methodology of Aguilar et al. (2015), with variations of the concentrations of polysaccharides and calcium chloride (pH 4.0) (Table 1). For the preparation of the emulsions, 100 mL of PEC and ALG solutions with oil of *P. volubilis* L. (model oil) were homogenized at 18000 rpm for 5 min using an Ultra Turrax homogenizer at 27 °C. Then M_{PEC} and M_{ALG} (40 g) were transferred to a 200 mL of protein solution of BB or EW in the different concentrations and kept under constant stirring for 30 min at 500 rpm. Then, they were washed with distilled water at pH 4.0 to remove the residues until the water appears transparent.

Table 1. Formulation of microparticles.

Microparticles	Polysaccharide concentration (%, w/w)	Oil (%, w/w)	Calcium chloride (%, w/w)
Pectin – M_{PEC}	2.50	1.75	2.50
Alginate – M_{ALG}	2.00	1.75	2.00

The microparticles were characterized regarding moisture, adsorbed protein, lipids, and Encapsulation Efficiency (EE) through the following Equation 1:

$$EE\ (\%) = \frac{Total\ oil\ in\ emulsion \frac{g}{total\ solids\ (g)}}{Initial\ oil \frac{g}{total\ solids\ (g)}} \times 100 \quad (1)$$

Likewise, the morphology and the mean size were also evaluated using an optical microscope (ZEISS – Primo Start, USA) coupled to a digital camera controlled by the Zen program 2.3 – Blue edition (Zen Imaging Software, Germany). Regarding mean size, images of 300 microparticles were used for each batch, which was measured and processed with the program Microcal Origin pro 9.1 (Microcal Software, Inc., MA, USA). The microstructure of microparticles was evaluated by scanning electron microscope (LEO 435 VP, Leo Electron Microscopy Ltd., Cambridge, England) with a voltage acceleration of 20 kV. Samples were coated with a gold layer of 30 nm.

2.5 *In vitro* gastrointestinal simulation

Uncoated and coated M_{PEC} and M_{ALG} with protein at 10%, which resulted in higher adsorption, were subjected to *in vitro* gastrointestinal simulation according to Mozzi et al. (2009). Optical microscopy was employed to capture the images at time zero (before simulation), after two hours (gastric simulation), and finally, after seven hours (intestinal simulation). For gastric simulation, 2 g of moist microparticles were added to a glass tube containing 20 mL of artificial gastric juice (AGJ) adjusted to pH 2.0. The glass tubes were incubated in a water bath with orbital shaking at 100 rpm, 37 °C for 2 h. The AGJ was obtained with 1.12 g/L KCl; 2 g/L NaCl; 0.11 $CaCl_2$; 0.4 g/L KH_2PO_4; 0.26 g/L of pepsin and HCl. For intestinal simulation, Artificial Intestinal Juice (AIJ) was prepared, for this purpose, the AGJ containing the microparticles was adjusted to pH 7 using a $NaHCO_3$ solution (20%, w/v) and pancreatin (1.95 g/L) was added. This system was incubated for an additional 5 h in a water bath at 37 °C with constant stirring. After 2 and 7 h of *in vitro* gastrointestinal simulation, 10 mL aliquots were separated and placed in an ice bath, where they remained immersed for 15 min to inactivate the enzymes. Thereafter, the aliquots were centrifuged for 20 min at 5000 rpm to evaluate the morphology and physical resistance of microparticles.

2.6 Protein released during gastrointestinal simulation

The protein released from microparticles coated with BB or EW, after 2 and 7 h of *in vitro* gastrointestinal simulation, was quantified. Likewise, a blank was prepared, containing only AGJ and pancreatin for nitrogen adjustment from the enzyme.

2.7 Statistical analysis

The significance of the differences between means was evaluated through an analysis of variance and a Tukey's test at a significance of 5% using the software SAS (Version 9.00, Cary, NC, USA). Since each type of microparticle was produced in three independent batches, the analysis was performed in triplicate.

3 Results and discussion

3.1 Characterization of protein sources

The sources showed a significant protein content which was higher for BB, and similar results were reported by Duarte et al. (1999) (Table 2).

Table 2. Characterization of protein sources.

Protein Sources	Centesimal composition (g· 100g^{-1})				
	Moisture	Proteins	Lipids	Carbohydrates (*)	Ashes
BB	80.69 ± 1.02	17.80 ± 0.44	0.38 ± 0.03	0.11 ± 0.01	1.02 ± 0.10
EW	87.63 ± 0.99	10.80 ± 0.77	0.18 ± 0.02	0.78 ± 0.11	0.61 ± 0.02

Mean values ± standard deviation (n=3). (*) By difference. BB = Bovine Blood; EW = Egg White.

3.2 Encapsulation efficiency (EE)

The lipid content for M_{ALG} and M_{PEC} was 41.17% and 42.85% (w/w total solids on a dry basis) with encapsulation efficiencies of 73.60% ± 3.62 and 88.27% ± 3.62, respectively. Previously, Benavides et al. (2016) obtained an EE of 85% when using ALG to encapsulate essential oil of thyme. Bušić et al. (2018) encapsulated phenolics extracted from dandelion in M_{ALG} and obtained from 82.44% to 84.38% of encapsulation. In another study, Menin et al. (2018), obtained up to 98% when encapsulating flax oil in M_{PEC}. These differences may be due to the core proportions used in relation to the polymers since 2.7 more oils were used. Likewise, the nature of the core may influence its behavior with the encapsulating agent regarding EE%. On the other hand, Silva et al. (2019) found 99% when encapsulating sacha inchi oil in ALG beads, this was due to the surfactant addition which led to the dimension reduction of the oil droplets size.

3.3 Adsorbed protein and mean size

The higher the concentration of protein in solution resulted in higher adsorption onto the surface of the microparticles (Figure 1A). Similar behaviors were reported by Schmidt et al. (2009) for different biomaterials. The amount of adsorbed protein onto M_{PEC} was significantly higher ($p < 0.05$) than those adsorbed onto M_{ALG}, regardless of the protein source and concentration used. The difference in the adsorbed protein values may be due to structural differences of the polysaccharides, where ALG has an ordered and stable structure (Yang et al., 2013) and PEC has a randomized structure of galacturonate residues sequences which are partially esterified, resulting in a disorganized structure (Guillotin, 2005). The ordered structure present in ALG results in a higher number of interactions between carboxylic groups and calcium ions during ionic gelation (Braccini & Pérez, 2001). Thus, when M_{ALG} are subjected to electrostatic interaction with proteins, there are not enough negative charges available, resulting in lower percentages of adsorbed protein. A different condition occurs with M_{PEC}, where the availability of carboxylic groups for interaction with BB and EW is higher. Tello et al. (2015), explained that this phenomenon is related to the electronegativity of M_{PEC} and M_{ALG} produced by ionic gelation which were – 3.8 and – 0.86 mV at pH 4.0 for, respectively. Therefore, higher quantities of adsorbed proteins at a constant pH 4.00 have a direct relationship with the electronegativity of M_{PEC} and M_{ALG}. Additionally, according to Haynes & Norde (1994) and Kim & Yoon (2002) other types of interactions can occur alongside electrostatic interactions, like hydrophobic interactions, hydrogen bonds, and Van der Waals forces.

In M_{PEC} more BB proteins (BBP) were adsorbed; however, in M_{ALG}, more EW proteins (EWP) were adsorbed (Figure 1A). Previously, Aguilar et al. (2015) reported a higher quantity of whey proteins adsorbed on M_{ALG} in comparison to EWP due to the high molar mass of ovalbumin (45 kDa) against β-lactoglobulin (18.5 kDa). The authors suggested that the higher the molar mass is, the lower the adsorbed quantity will be. This theory differs from the results found, since it was the BBP that were adsorbed in greater quantity even though its main protein, hemoglobin, has a molar mass of 64 kDa. Thus, based on our results, we suggested that protein adsorption was not only related to the protein molar mass but also the type of polysaccharide used as encapsulating agent. As previously mentioned, in M_{ALG} a few carboxylic groups are available on the surface after ionic gelation and, because of this, the contact surface with proteins would be lower. Therefore, higher adsorption of BBP onto M_{PEC} and EWP onto M_{ALG} occurred. Likewise, PEC would have a higher affinity for BBP, whereas ALG for EWP. Regardless of the quantities of adsorbed protein, it is possible to adsorb BBP and EWP on microparticles produced by ionic gelation.

At the same time, it was observed that protein adsorption modified the size of both microparticles with opposite behaviors, but without significant differences ($p > 0.05$) for the protein source and their concentration varying from 2 to 6% (Figure 1B). For M_{PEC}, the higher the protein adsorption is, the lower the size will be, in contrast for M_{ALG} in which the higher the protein adsorption is, the higher the size will be. This behavior would be related to the PEC and ALG chemical structure. For ALG, the ionic gelation is performed in three steps: mono-complexation; dimerization; and association of dimers. In contrast, ionic

gelation for PEC only involves mono-complexation and dimerization (Fang et al., 2007). Thus, the lateral association of dimers will make the M_{ALG} structure stronger and stable than M_{PEC} during ionic gelation. Becker et al. (2012) indicated that protein adsorption on charged microparticles and the constant release of counterions produce an osmotic pressure reduction and, consequently, the shrinkage of coated microparticles. Thus, the adsorbed protein exerted pressure on the fragile structure of M_{PEC} leading to water release and reducing their size.

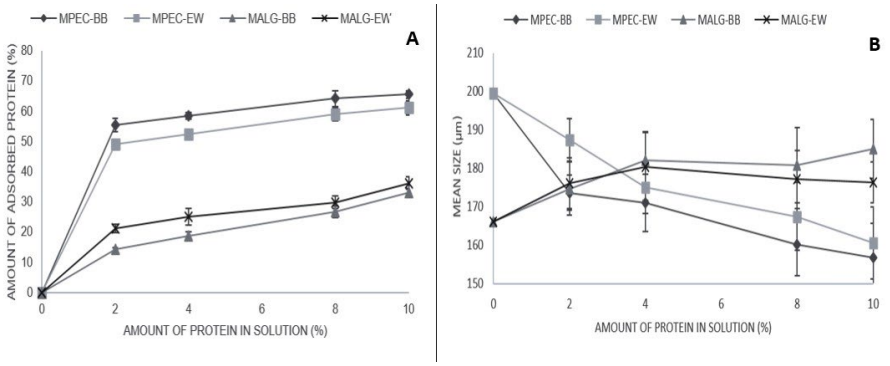

Figure 1. Amount of adsorbed proteins onto M_{PEC} and M_{ALG} coated with BB and EW (A). Mean size of coated microparticles (B).

3.4 Morphology and microstructure of coated microparticles

In Figure 2, images A and C represent moist coated M_{PEC}, whose oil droplets are distributed over the matrix, viewed by optical microscopy. Microparticles coated with BB showed a rough surface and with EW showed a smooth surface and spherical form. Likewise, in Figures 3 and 4, images A, D, and G belong to M_{PEC} and M_{ALG} with and without coating, using the highest protein concentration in solution (10%). It was observed that the protein coating did not affect the M_{ALG} shape. However, microparticles coated with BB showed a deformed surface in comparison to the smooth surface of microparticles with adsorbed proteins from the EW. In addition, it was observed that microparticles coated with BB acquired an intense brown color probably due to the blood oxidation process.

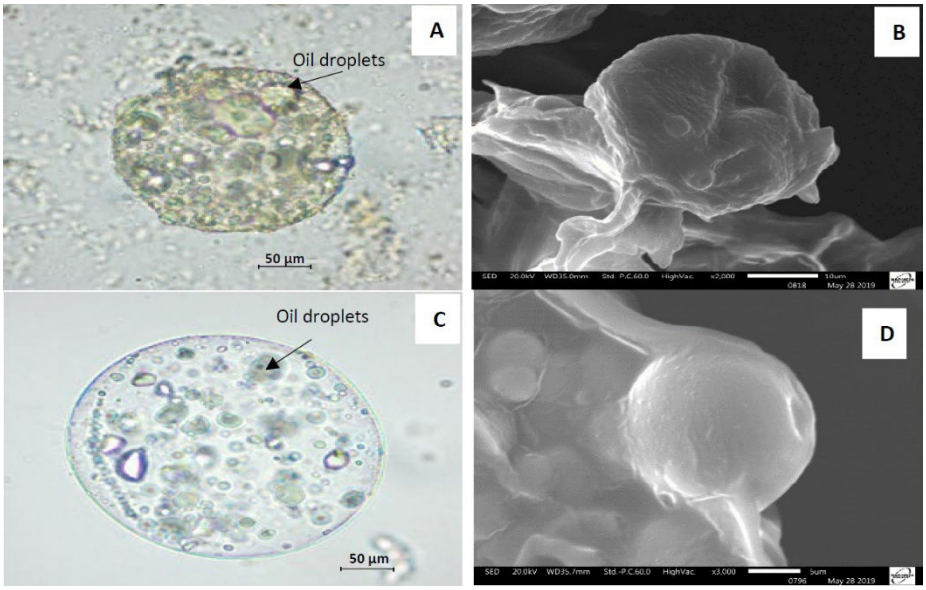

Figure 2. Optical microscopy (A and C) and scanning electron microscopy (B and D) of coated M_{PEC}. A and B, microparticles coated with BB. C and D, microparticles coated with EW. (A and B) OM bar = 50 µm, Objective = 40x; (C and D) SEM bar = 10 and 5 µm.

3.5 *In vitro* gastrointestinal simulation of coated microparticles

M_{PEC} without coating were affected by both gastric and intestinal conditions (Figures 3B and 3C) and were thoroughly destroyed in the second part of the process. However, M_{ALG} without coating showed greater resistance to the enzymatic action maintaining their spherical form (Figures 4B and 4C), although they swelled up approximately twice their initial size at intestinal conditions. The maintenance of the integrity of M_{ALG} structures may be related to the interaction between the carboxylic groups of ALG with calcium ions during the gelation process (Braccini & Pérez, 2001). These results are based on the principle that ALG can delay the enzymatic action in a range of digestive enzymes by limiting the enzyme availability to substrates (Brownlee et al., 2009). On the other hand, the disintegration of M_{PEC} in intestinal conditions may be explained by the presence of salts, which promote the electrostatic repulsion between carboxylic groups and ions (Lin et al., 2005).

The protein coating provided partial physical resistance to M_{PEC} subjected to gastric conditions (Figure 3: images B, E, and H), and the resistance was higher for M_{PEC} coated with BB. Nevertheless, in intestinal conditions. (Figure 3: images C, F, and I), the coating was not favorable, and disintegration thoroughly occurred. Similarly, Gebara et al. (2013) reported that whey protein coating did not protect the probiotic encapsulated in M_{PEC} when subjected to gastrointestinal simulation but did affect its release at these conditions. These findings would enable the use of pectin microparticles for the release of active compounds in the small intestine. On contrary, coated M_{ALG} (Figure 4: images B, E, and H) were not affected when subjected to gastric conditions. However, at intestinal conditions (Figure 4: images C, F, and I), their structure was weakened, and proteins were partially solubilized. Similar behavior was observed by Corstens et al. (2017) in M_{ALG} obtained by ionic gelation subjected to simulated gastrointestinal conditions. These findings showed that M_{ALG} had the potential to pass unbroken into the colon, where they would finally disintegrate by enzymatic activity from the microbiota, causing the total release of the encapsulated compound, either a type of drug or bioactive substances.

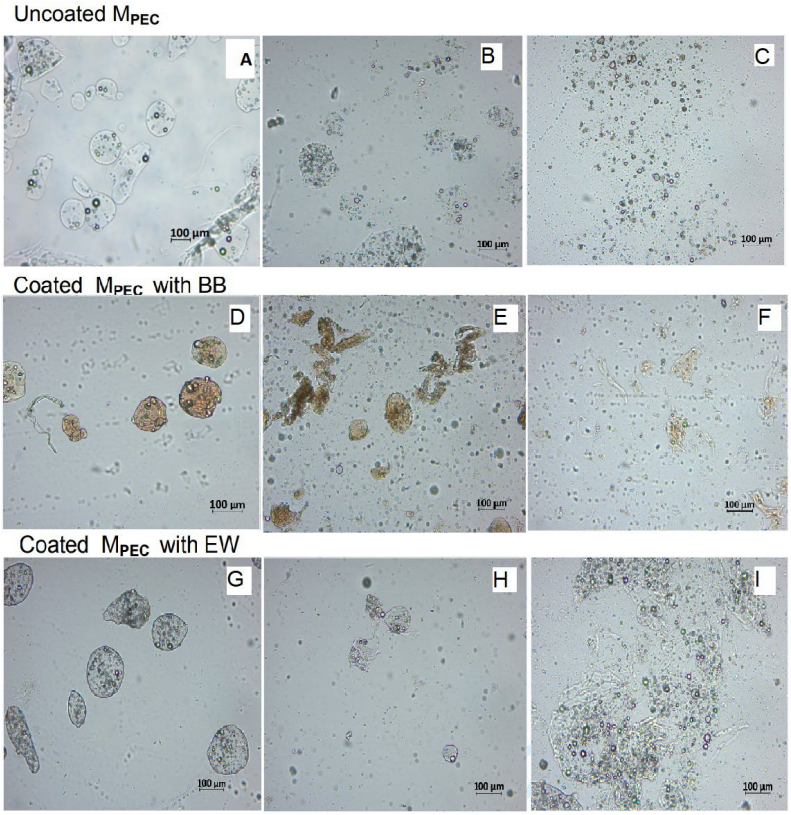

Figure 3. A, B, and C: Images obtained by optical microscopy (OM) of uncoated M_{PEC} subjected to gastrointestinal conditions. D, E, and F coated M_{PEC} with BB subjected to gastrointestinal conditions. G, H and I, coated M_{PEC} with EW subjected to gastrointestinal conditions. OM bar = 100 µm. Objective= 10x

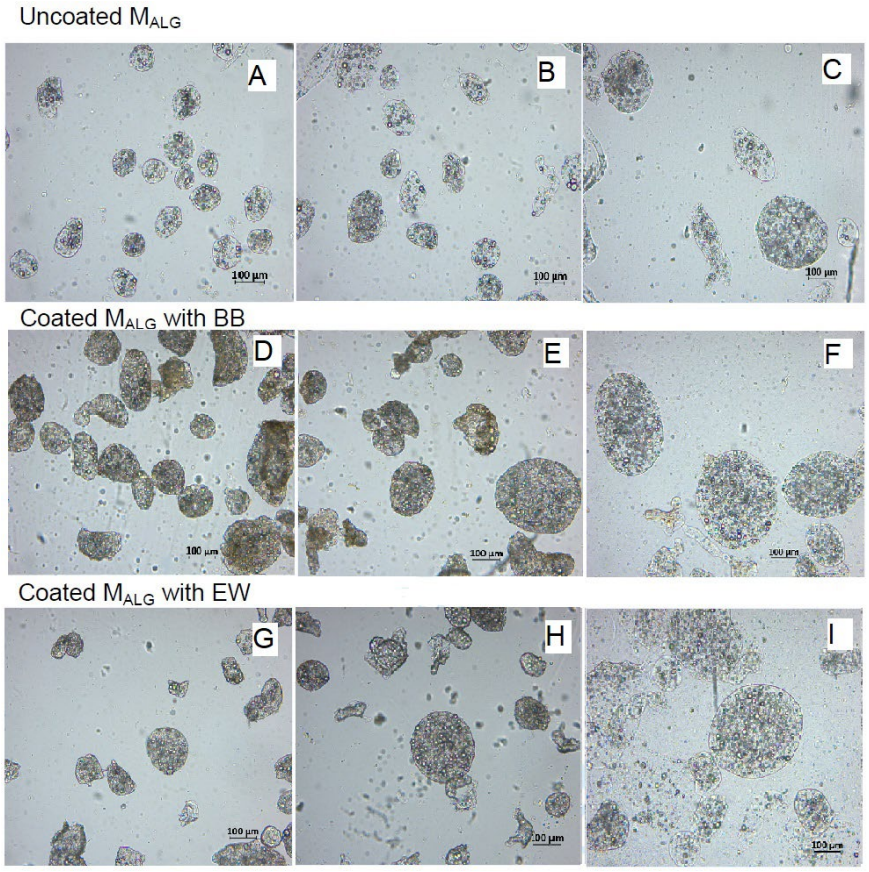

Figure 4. A, B, and C: Images obtained by OM of uncoated MALG subjected to gastrointestinal conditions. D, E, and F coated MALG with BB subjected to gastrointestinal conditions. G, H, and I, coated MALG with EW white subjected to gastrointestinal conditions. OM bar = 100 µm. Objective= 10x

3.6 Solubility of proteins adsorbed onto microparticles

Table 3 shows the solubility of proteins adsorbed on M_{PEC} and M_{ALG} after two and seven hours of exposure to *in vitro* gastrointestinal conditions. In gastric conditions, there were significant differences regarding the type of microparticle, seeing that M_{PEC} showed protein solubility higher than 30%. Thus, at the level of the small intestine, BBP and EWP were released from the surface of the microparticles, leading to the exposure of the porous structure of the microparticle, where the encapsulated compound would be released. This high solubility at the place of greatest nutrient absorption in the small intestine is desirable for the application of proteins in several food formulations (Etheridge et al., 1981).

Table 3. Solubility of proteins adsorbed onto M_{PEC} and M_{ALG} subjected to *in vitro* gastrointestinal conditions.

Microparticles	Protein sources	Solubility (%)	
		Gastric conditions (pH 2, 37 °C, 2h)	Intestinal conditions (pH 7, 37 °C, 5h)
M_{PEC}	BB	35.80 ± 1.34^{Ab}	95.16 ± 3.14^{Aa}
	EW	32.50 ± 2.14^{Ab}	94.45 ± 1.98^{Aa}
M_{ALG}	BB	15.71 ± 1.03^{Bb}	85.24 ± 2.60^{Ba}
	EW	10.02 ± 0.58^{Cb}	87.35 ± 0.95^{Ba}

Mean values ± standard deviation (n=3). BB = Bovine Blood; EW = Egg White. Means followed by different uppercase letters in the same column have significant differences between microparticle types in combination with the same protein source for each concentration ($p < 0.05$) by Tukey's Test. Means followed by different lowercase letters in the same row have significant differences between concentrations per protein source by microparticle types ($p < 0.05$) by Tukey's Test.

4 Conclusions

The ionic gelation produced high encapsulation efficiency using pectin and alginate with oil from *P. volubilis* L. High protein quantities from BB and EW were adsorbed onto the surface of the microparticles. The increase of protein in solution increased the adsorption, thus, we hypothesized that the proteins in their natural state possessed suitable characteristics for microparticles coating. Coated microparticles with BB and EW proteins at 10% were found to be highly soluble at the level of the small intestinal. Both microparticles had the potential to be used as delivery vehicles of active compounds, being M_{PEC} suitable to be released in the small intestine, and M_{ALG} in the large intestine and colon.

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