

MIMICRY OF FISH LARVAE LIVE FOOD WITH PARTICLES OBTAINED BY IONIC GELATION AND COATED WITH PROTEINS THROUGH ELECTROSTATIC INTERACTION

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In an attempt to mimic the proximate composition found in *Artemia's* nauplii, a type of food commonly used in intensive rearing of fish larvae, inert diets were produced by ionic gelation using either low methoxyl amidated pectin or sodium alginate and subsequently coated with whey proteins by electrostatic interaction. The particles were morphologically characterized by optical microscopy and scanning electron microscopy, showing spherical shape when moist or rehydrated. The resulting particles were characterized according to their proximate composition, average size, and rehydration behavior after freeze drying. The coated pectin and alginate particles, had sizes averaging $256.7 \mu\text{m} \pm 17.1$ and $241.9 \mu\text{m} \pm 7.5$, respectively, with no statistical difference. In relation to the contents of lipid, protein, dry-matter, and ash, the resulting particles were similar to the live food. However, an *in vivo* evaluation with fish larvae becomes necessary to confirm their suitability and functionality.

KEY-WORDS: IONIC GELATION; ELECTROSTATIC INTERACTION; PECTIN; ALGINATE; WHEY PROTEIN; MICROPARTICLES.

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1 INTRODUCTION

The encapsulation technology is a delivery method that allows the controlled release of ingredients under specific conditions, such as heating, hydration, diffusion, mechanical pressure and biodegradation (PEPPAS & BRANNON-PEPPAS, 1996; ANAL & STEVENS, 2005; KAILASAPATHY & MASONDOLE, 2005).

Currently, the encapsulation is used in different areas including pharmaceutical and food industries. In the food industry, it can be used as a protection against environmental adversity conditions (temperature, pH, light, moisture), to improve stability of flavors, enzymes, microorganisms, vitamins, fatty acids, minerals and peptides, and as well as to mask undesirable taste (DZIEZAK, 1988; LISERRE, RÉ & FRANCO, 2007). Furthermore, microencapsulation have being used for incorporating bioactive compounds in fortified foods (AUGUSTIN *et al.*, 2011).

Microencapsulation has also being investigated for the substitution or reduction of live food, such as rotifers, and *Artemia*, commonly used in larviculture (LANGDON, 2003). The inert micro diets have the ability to provide a full diet to the larva, preventing nutrient loss and deterioration of water tanks, among others. However, despite these positive characteristics, microencapsulated diets developed for feeding fish larvae, have yielded low growth and low survival rates (HAMLIN & KLING, 2001). Many authors related this fact to the low acceptance, assimilation and digestion of these diets, both of which are directly related to the intrinsic nature of the particle, such as texture, taste, color, design, size, density and stability in water (CAHU & ZAMBONINO INFANTE, 2001; KOLKOVSKI, 2001). The *Artemia* nauplii is generally composed of 9.2 % dry matter and 55-60 % of protein in dry basis (BASKERVILLE & KLING, 2000). However, most micro diet developed as live food replacement with adequate levels of protein also contain high amounts of dry matter (> 90 %) (BASKERVILLE & KLING, 2000; GUTHRIE *et al.*, 2000).

Alginate and pectin are examples of natural polymers, non-toxic, biodegradable and biocompatible (CHAN, LEE & HENG, 2002). They are used in microencapsulation processes for biomedical applications, and also for the production of diets for feeding fish larvae (GUTHRIE *et al.*, 2000). Ionic gelation is used in particles production; it happens under mild conditions, it is low cost, simple, and fast. It involves an aqueous polymer solution, which when in contact with low molecular mass ions, interacts forming a complex. Alginate and low methoxyl pectin are widely used as wall materials, and the calcium ion is the most used reticulation agent. The electrostatic characteristics, and the gels formation make these polysaccharides good wall source material for the particles formation (MESTDAGH & AXELOS, 1998).

Despite these positive characteristics, the ionic gelled particles are generally very porous, and possess low holding capacity when compared to other techniques for encapsulating water soluble compounds of low molecular mass (MUKAI-CORREA *et al.*, 2004). To work around this problem it is possible to associate the ionic gelation, and the complex coacervation techniques, which besides improving the porosity and release of encapsulated hydrophilic material, allows greater protein retention. By adding a polycation (proteins or chitosan) to the particle obtained by ionic gelation, it is possible to induce the formation of polyanionic-polycationic complex stabilizing the hydrogel network; thus reducing the permeability of the alginate or pectin particles, and improving the chemical and mechanical stability of the same particles (HUGUET & DELLACHERIE, 1996; ALLAN-WOJTAS *et al.*, 1999; CHANDRAMOULI *et al.*, 2004; DAI, WANG & ZHAO, 2005). Gbassi *et al.* (2011) combined the ionic gelation with alginate, and covered the particles with whey protein by complex coacervation, to produce gastric protection to the encapsulated microorganisms.

In this study, the objective was to produce diets that mimic live food (*Artemia franciscana*) commonly used in intensive larviculture, through ionic gelation using low methoxyl amidated pectin or alginate with high guluronic acid content, and subsequently coated with whey protein through electrostatic interaction. The particles were characterized for the proximate composition, morphology, swelling and the average size. Additionally, the polysaccharides and protein solutions

were evaluated with respect to the zeta potential at different pH values.

2 MATERIAL AND METHODS

2.1 MATERIAL

The following materials were used: sodium alginate (AG) DMB (high molecular mass, high content of guluronic groups (lot G3512301 MANUGEL, FMC Biopolymer, Brazil), citrus pectin, low methoxyl amidated pectin - LMAP (CPKelco, mixture of different lots, Limeira, São Paulo, Brazil), galacturonic acid (GA) $85.9\% \pm 1.9$, degree of esterification (DE) $34.1\% \pm 1.3$ and degree of amidation (DA) $5.5\% \pm 0.4$ as determined (FAO, 2009), whey protein concentrate – WPC (Lacprodan, lote Lac804U17601, 76, Porteña, Provincia de Córdoba, Argentina; moisture content $6.9 \pm 0.1\%$; protein content 81.0 ± 1.0 ; ash content 2.77 ± 0.06 and lipids content 16.2 ± 0.5 determined according to AOAC, 2006, anhydrous calcium chloride P.A. (Dinâmica, lot 36308, P.M. 110.99), cold pressed sunflower oil (Viapaxbio, Joinville, Santa Catarina, Brazil), vitamin and mineral mixture (Fri-Ribe, Pitangueiras, Brazil), according to the manufacturer, containing the following amounts per 100 g of the mixture: folic acid 1 mg, pantothenic acid 20 mg, BHT antioxidant 125 mg, choline 150 mg, copper 10 mg, iron 100 mg, iodine 5 mg, manganese 70 mg, selenium 0.15 mg, vitamin A 3000 UI.kg⁻¹, vitamin B1 6 mg, vitamin B12 20 mg, vitamin B2 8 mg, vitamin B6 3 mg, vitamin C 350 mg, vitamin D3 3000 UI.kg⁻¹, vitamin E 200 mg, vitamin K 6 mg, zinc 150 mg, niacin 100 mg, biotin 0.10 mg), and other analytical grade reagents.

2.2 ZETA POTENTIAL OF THE MATERIALS

The determination of the zeta potential of the WPC, LMAP and GA solutions (0.1 % w/w), was carried out using a Zetasizer (Malvern, Worcestershire, UK) in the 3-7 pH ranges. Measurements were independently prepared for two samples. Duplicate readings were performed for each sample, and the results expressed as means \pm standard deviation (SOUZA *et al.* 2012).

2.3 PRODUCTION OF PARTICLES

The particles were prepared with a solution of LMAP or alginate (2 % w/w of the total emulsion) emulsified with sunflower oil (25 % w/w total solids) and vitamin and mineral complex (4.5 % w/w total solids) using an Ultra Turrax homogenizer (IKA, Brazil) at 14.000 rpm/3 min. The emulsion was sprayed with a double fluid atomizer (diameters 1.0 and 1.5 mm for pectin and alginate, respectively) with compressed air flow (0.125 and 0.375 kgf/cm² of pectin and alginate, respectively), positioned at 12 cm from the calcium chloride solution level (2 % w/w, pH 4.0) under constant magnetic stirring. For the full gelation, the particles were held for 30 min (curing time) in a calcium chloride solution. After the curing time, the particles were washed in sieves with water adjusted to pH 4.0 ($\phi = 125 \mu\text{m}$), and dispersed in solutions of non-denatured WPC (12 % w/w, pH 4.0) under constant magnetic stirring for 30 min. The particles were again transferred to sieves ($\phi = 125 \mu\text{m}$) and washed with water adjusted to pH 4.0 (SOUZA *et al.*, 2012). The moisture content was determined for both, the PEM particles (particles produced with pectin) and the ALM particles (particles produced with alginate) and covered with whey proteins. The adsorbed protein levels were also determined, after the deduction of the LMAP nitrogen content. Then, the particles were dried in a freeze dryer (Edwards Pirani 501 West Sussex, UK). The temperature was reduced to $-40\text{ }^{\circ}\text{C}$, under pressure of 0.1 mmHg. The final drying was performed at $25\text{ }^{\circ}\text{C}$ temperature. The dried material was packed in lidded jars, and stored at $-18\text{ }^{\circ}\text{C}$. The particles were produce in nine independent batches. After drying out, the batches were mixed and characterized.

2.4 CHARACTERIZATION OF THE PARTICLES

2.4.1 Proximate composition

Moist particles, were characterized in regard to the protein, moisture and ash contents, according to AOAC (2006); and lipids according Bligh & Dyer (1959). A preliminary digestion of the protein adsorbed on the particle was needed for the determination of lipid content, allowing for a complete lipid extraction, because of its retention in the protein fraction. Sodium citrate solution (2 % w/w) was added to 5 g of moist particles, to allow the dissolution of the particles. Then 0.3 mg of pepsin/g was added, the pH corrected to 3.0, stirred for 1 min, and maintained in water bath (37 °C) with agitation for 4 h. The pH was corrected to 7.0, and 0.3 mg/g of pancreatin was added, stirred for 1 min, and maintained in bath (37 °C) for 12 h. Following these steps, the extraction followed the Bligh & Dyer methodology (1959).

2.4.2 Average size and size distribution

The average size (d 0.5) of moist and rehydrated particles at pH 7.0, were determined using a Mastersizer 2000 (Malvern, Worcestershire, UK). With fast stirring, the dry particles were rehydrated with water (pH 7.0), and immediately followed by the size determination (SOUZA *et al.*, 2012).

2.4.3 Morphology

The morphology of both, moist and rehydrated particles (pH 7.0) was observed by optical microscopy (Jenaval, Carl Zeiss, Germany). The images were captured with digital camera, fitted with 12.5 objectives, 1.25-x optvar and the EDN-2 program - Image Processing Microscopy System (SOUZA *et al.*, 2012). The freeze dried particles microstructure was observed in a scanning electron microscope (SEM) Jeol T300 (Tokyo, Japan) using 15 kV. The samples were previously fixed on stubs with double-sided metallic tape and coated with a thin gold layer (40 mA/150 s) employing a Baltzer SCD50 evaporator (Baltec, Liechtensten).

3 RESULTS AND DISCUSSION

3.1 ZETA POTENTIAL OF THE BIOPOLYMERS

The zeta potential determines the amount of free charge in polysaccharides, and whey protein solutions; thus affording the assessment not only of a possible electrostatic interaction between the biopolymers but also of the pH range liable to contain the highest interaction. As expected, the zeta potential of the pectin and the alginate solutions (Figure 1) had a negative charge in the studied pH range; probably, because the low methoxyl amidated pectin presents a pKa of 2.9, as reported in the literature (RALET *et al.*, 2001) and above this pH, the polysaccharide solution will be negatively charged. Conversely, the alginate contains a pKa from 3.2 to 3.6; below these values, there is suppression of the carboxylic dissociation (SIMSEK-EGE, BOND & STRINGER, 2003). Furthermore, the alginate presented higher levels of negative charge in relation to the pectin.

The pH chosen for the particles' production was adjusted to 4.0, as described previously in the literature, when beet pectin and β -lactoglobulin were used for nanoparticles production by electrostatic interaction (SANTIPANICHWONG *et al.*, 2008). The choice of pH is justified because the isoelectric point (pI) found to whey proteins was of approximately 4.5 (Figure 1); thus, indicating that at pH levels below 4.5 and above 2.9 and 3.2 it is possible to achieve electrostatic interaction between the protein and polysaccharides. In connection with the whey protein, the isoelectric point (pI) reported before in the literature for the β -lactoglobulin, the major protein in whey, varies from 4.5 to 5.2 (WALKENSTRÖM & HERMANSSON, 1997; LY *et al.*, 2008) and at pH lower than the pI, the protein solution will be positively charged as shown in Figure 1.

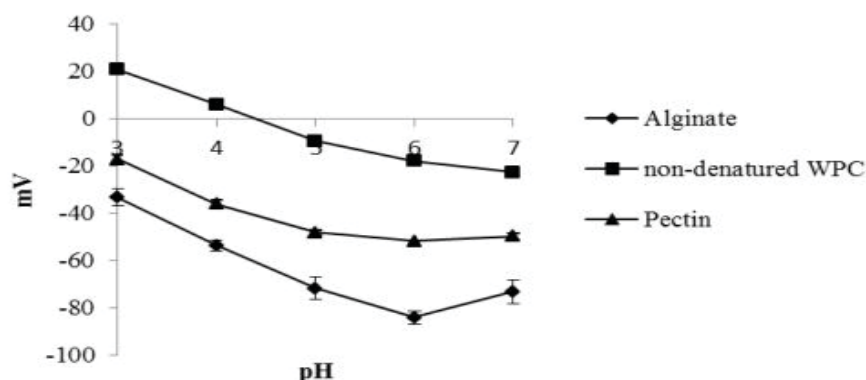


FIGURE 1 - THE pH EFFECT ON THE ELECTRIC CHARGE (Z POTENTIAL, mV) OF THE SOLUTIONS (0.1 % w/w) OF PECTIN, ALGinate, AND NON-DENATURED WPC

3.2 PARTICLE SIZES

Table 1 shows the mean diameters (d 0.5) of moist and rehydrated particles determined by light scattering using a Mastersizer meter.

TABLE 1 - MEAN DIAMETER (μm) AND STANDARD DEVIATION OF PARTICLES, OBTAINED BY IONIC GELATION CONTAINING PROTEIN ADSORBED BY ELECTROSTATIC INTERACTION, MOIST AND REHYDRATED (pH 7.0)

Treatments	Moist size	Rehydrated size
PEM	256.7 \pm 17.1 ^{aA*}	227.3 \pm 16.2 ^{aB}
ALM	241.9 \pm 7.5 ^{aA}	221.9 \pm 16.4 ^{aB}

PEM = particles produced from pectin and covered with WPC; ALM = particles produced from alginate and covered with WPC*. Means followed by same letters (uppercase in the column and lowercase on the line) did not differ according to the Tukey test ($p > 0.05$).

The particle sizes obtained by ionic gelation using polymers, i.e., pectin or alginate, are affected by various parameters such as the diameter of the atomizer's needle, solution concentration, distance between the needle and the cationic solution (GOMBOTZ & WEE, 1998; SILVA *et al.*, 2006).

The particle average sizes obtained when pectin was used were: 243.3 $\mu\text{m} \pm 9.3$ for the uncoated moist particle and 256.7 $\mu\text{m} \pm 17.1$ for the coated particles. When using alginate, the values were 230.7 $\mu\text{m} \pm 5.0$ for the uncoated moist particles and 241.9 $\pm 7.5 \mu\text{m}$ for the coated particles. These results show that the coating did not affect the particle size, although, there is a tendency to size increase after coating. Although, there are differences between the sizes of the particles freshly rehydrated and the moist ones (Table 1), there is a high rehydration rate meaning that placing the dried particle in aqueous systems will result in quick rehydration. This rapid rate of rehydration was previously observed in dry particles containing alginate or alginate-chitosan (POLK *et al.*, 1994).

The sizes obtained are compatible with living foods commonly used to feed larvae ranging from 125 to 300 μm in size, for rotifers such as the *Brachionus plicatilis* and from 350 to 517 μm

for the *Artemia* spp. (EMMERSON, 1984; YÚFERA, RODRIGUEZ & LUBIÁN, 1984; GENODEPA, ZENG & SOUTHGATE, 2004). Cahu & Zambonino Infante (2001) used a 50-125 µm micro diet during the first larval stage; from day 14 to day 25, the diets were increased to 125-200 µm, and 200-400 µm thereafter until the 40th day. Tang, Chen & Wu (2010), used 150-250 µm micro diet from 1-9 days after the Cobia larvae eclosion (*Rachycentron canadum*). Particularly small diets (< 50 µm) can not be easily detected by the larvae, whereas the ingestion of the large ones is difficult and can lead to blockage of the digestive system (WALFORD, LIM & LAM, 1991).

3.3 MORPHOLOGY

The morphology of the particles when observed by light microscopy shows the formation of multi nucleated matrixes, with a defined and continuous wall around the microparticle (Figure 2). The lipid content was uniformly distributed in the pectin or alginate matrixes, showing the distribution of lipid droplets of different sizes along the length of the microparticle. The particles were, mostly spherical or approximately spherical. The light microscopy images indicated that both the moist (Figure 2A to 2E) and the rehydrated particles presented similar formation after rehydration (Figure 2D and 2H). This effect was previously observed in dried particles produced by ionic gelation, which showed a considerable rehydration after contact with water (MUKAI-CORREA *et al.*, 2005).

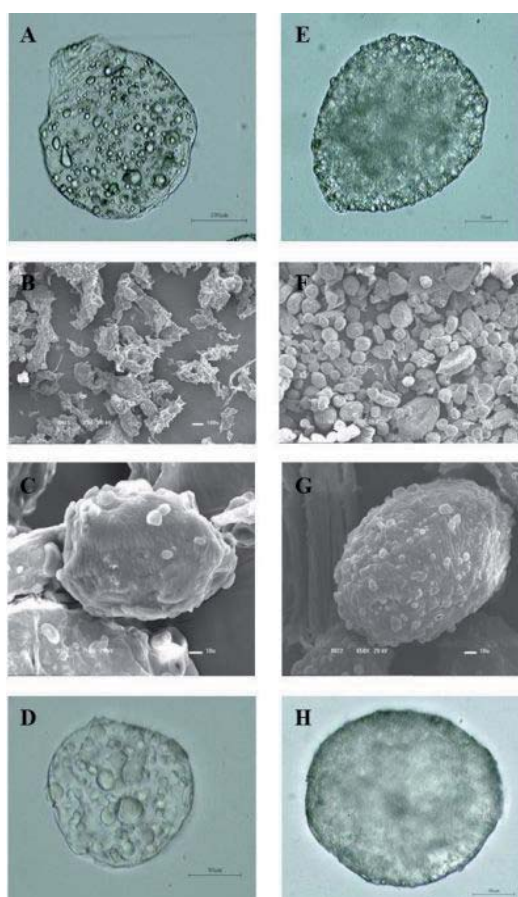


FIGURE 2 – MICROGRAPHS OF PEM PARTICLES (PARTICLES PRODUCED WITH PECTIN AND COATED WITH WPC) LOCATED IN THE LEFT COLUMN AND ALM PARTICLES (PARTICLES PRODUCED WITH ALGinate AND COATED WPC) LOCATED IN THE RIGHT COLUMN. A AND E - MOIST PARTICLES, B, F, C AND G – FREEZE DRIED PARTICLES OBSERVED BY SCANNING ELECTRON MICROSCOPY, D AND H - REHYDRATED PARTICLES

The microscopy of the dried particles using SEM (Figure 2C and Figure 2G) showed that the particles had similar form and remained partly intact after the drying process, having a spherical contour, with lipid droplets in the wall material. Agglomeration was observed between pectin particles (Figure 2B) after the freeze drying. This behavior is different from the alginate particles, which after freeze drying retained the spherical shape (Figure 2F) with whole and non-agglomerated particles. Despite the clustering observed, the pectin particles after rehydration regain a nearly spherical form, and with continuous walls without any cracking (Figure 2D and Figure 2H).

3.4 PROXIMATE COMPOSITION

Table 2 shows the protein, lipid, ash and moisture contents of the particles produced by ionic gelation and after electrostatic interaction with the whey proteins. The averages of the protein, lipid, moisture and ash contents were not significantly different ($p>0.05$) between treatments.

Rodrigues (2012) evaluated WPC coated particles with concentrations ranging from 4 to 12 %, observing no significant differences in the protein adsorption with WPC concentrations of 6, 8 and 12 %, obtaining particles protein levels above 50 %. Furthermore, the average size of the particles increased with the rising protein concentration. In this study, the choice of the whey protein concentration used for coating the particles, was based on the possibility to obtain particles with high content of adsorbed protein associated with an appropriate size. The WPC 12 % concentrations was chosen for the particles production, also considering that the *Artemia* nauplii average size are between 350-530 μm (EMMERSON, 1984; YÚFERA, RODRIGUEZ & LUBIÁN, 1984).

TABLE 2 - PROXIMATE COMPOSITION OF THE PARTICLES PRODUCED BY IONIC GELATION, FOLLOWED BY ELECTROSTATIC INTERACTION WITH WHEY PROTEINS

Diets	Proximate Composition % (dry base)			
	Moisture	Protein	Lipid	Ashes
PEM	85.49 \pm 0.59 ^{a*}	49.08 \pm 0.47 ^a	21.22 \pm 0.65 ^a	4.14 \pm 0.28 ^a
ALM	85.39 \pm 0.53 ^a	49.01 \pm 0.60 ^a	20.98 \pm 0.35 ^a	4.26 \pm 0.14 ^a

PEM = particles produced from pectin cover with WPC; ALM = particles produced from alginate covered with WPC*. Means followed by same letters did not differ according to the Tukey test ($p>0.05$).

Regardless the type of polysaccharide used, the particles showed high levels of protein adsorbed (~50 %). In comparison with the *Artemia* nauplii proximate composition found in the literature (BASKERVILLE & KLING, 2000) the two particles presented a similar protein composition. In previous studies seeking to mimic the *Artemia* nauplii, the attempt to incorporate the protein inside a microparticle produced using ionic gelation, was not met accordingly with respect to the microencapsulation efficiency (MUKAI-CORREA, 2008), reaching the maximum of 35 % protein incorporation (dry basis).

The lipid contents in the PEM and the ALM particles (21 %) are similar to those found in the *Artemia* nauplii (BASKERVILLE & KLING, 2000; RODRIGUES, 2012). Lipids are known for playing an important role in the feeding, providing the energy which supports the structural integrity of the biologic membranes and act as precursors of important steroids (CORRAZE, 2001). Diets formulated

for sea bream and sole larvae contained from 18 and 25 % lipids respectively, similar values to those usually found in live foods (SALHI *et al.*, 1999; FURUITA, TAKEUCHI & UEMATSU, 1998). The particles ash contents (~4 %) were similar to that found in the *Artemia* nauplii and later found in the diet produced for *pacu* larvae feeding (RODRIGUES, 2012). The protein and lipid contents were similar to the microencapsulated diet developed by Chu & Ozkizilcik (1999), which contained 53 % of protein and 21 % of lipids, respectively.

The diets moisture contents were comparable to the moisture contents observed for the *Artemia*, unlike the commercial and experimental diets, whose moisture content is quite low, averaging 10 % (YÚFERA; PASCUAL & FERNÁNDEZ-DIAZ, 1999; BASKERVILLE & KLING, 2000; KVÅLE *et al.*, 2006). This high moisture content can contribute to the better digestion of the diet, since the large amount of dry matter is one of the factors contributing for difficulty in the digestion of formulated diets (KOLKOVSKI, 2001).

All particles (Table 2) showed high levels of moisture (> 85.0 %), similar to the particles obtained by Mukai-Correa *et al.* (2005), which have shown moisture levels superior to 90 %. Such high values are characteristic of particles produced with gel forming polysaccharides, due to its high water retention capabilities. Moreover, the disadvantage of a high moisture content is a rapid deterioration of the microparticle; however all the particles remained intact after the freeze drying process that would minimize the moist particles short shelf life issue, besides the excellent rehydration capacity.

4 CONCLUSION

The results showed that the particles produced using ionic gelation followed by the electrostatic interaction with whey proteins, allowed for the production of particles with protein (~50 %), lipid (~20 %) and moisture (~85 %) compositions, similar as found in the *Artemia*, using either low methoxyl amidated pectin or alginate with high contents of guluronic acid and whey proteins. The particles were partly spherical with homogeneous lipids distribution, average size ranging from $256.7 \mu\text{m} \pm 17.1$ and $241.9 \mu\text{m} \pm 7.5$ for pectin and alginate, respectively with no statistical difference, similar to the mean size of rotifers and close to the mean size of *Artemia*, both used for intensive rearing of fish larvae.

The incorporation of a high content of minerals and vitamins (~4 %) produces a nutritionally balanced microparticle; therefore suitable as a partial replacement for the live food. Although, the composition is very similar to the live food, it becomes necessary to perform *in vivo* tests with fish larvae to evaluate the use of particles as artificial diet and, if necessary, their suitability.

RESUMO

MIMETIZAÇÃO DE ALIMENTO VIVO DE LARVAS DE PEIXES COM PARTÍCULAS OBTIDAS POR GELIFICAÇÃO IÔNICA E RECOBERTAS COM PROTEÍNA POR INTERAÇÃO ELETROSTÁTICA

Na tentativa de mimetizar a composição centesimal encontrada em náuplios de *Artemia*, alimentação comumente utilizada na criação intensiva de larvas de peixes, foram produzidas dietas inertes por gelificação iônica utilizando pectina de baixo teor de esterificação amidada ou alginato de sódio, posteriormente recobertas com proteínas de soro de leite por interação eletrostática. As partículas foram caracterizadas morfolologicamente por microscopia ótica e microscopia eletrônica de varredura e apresentaram forma esférica quando úmidas ou reidratadas. As partículas após produção foram caracterizadas com relação a sua composição centesimal, tamanho médio e comportamento de reidratação após secagem por liofilização. Partículas de pectina e alginato recobertas apresentaram tamanhos médios de $256,7 \mu\text{m} \pm 17,1$ e $241,9 \mu\text{m} \pm 7,5$, respectivamente, sem diferença estatística. Em relação ao conteúdo lipídico, proteico, de matéria-seca e cinzas as partículas produzidas eram semelhantes ao alimento vivo. No entanto, avaliação *in vivo* com larvas de peixes torna-se necessária para confirmar a sua adequação e funcionalidade.

PALAVRAS-CHAVE: GELIFICAÇÃO IÔNICA; INTERAÇÃO ELETROSTÁTICA; PECTINA; ALGINATO; CONCENTRADO PROTEICO DE SORO DE LEITE; MICROPARTÍCULAS.

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