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High protein microparticles produced by ionic gelation containing *Lactobacillus acidophilus* for feeding pacu larvae

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ABSTRACT

Microparticles with high protein content can be used as diets to mimic the proximate composition of *Artemia* nauplii. After production, the particles were characterized with respect to their proximate composition, mean size, morphology, and rehydration behavior after drying. The protein content, lipid content and the particle moisture were similar to *Artemia* nauplii, with mean values of 50, 23, and 85%, respectively. Additionally, the particles were used in a pacu (*Piaractus mesopotamicus*) larval growth experiment. Also, the probiotic *Lactobacillus acidophilus* was added to one of the diets, and the effects of the diets were evaluated on larvae growth and stress resistance. Larvae fed the experimental diets had lower growth than larvae fed with *Artemia* nauplii or a commercial diet. All of the evaluated diets, including the experimental ones, showed high ingestion rates (>90%). In the stress test by air exposure, larvae fed with the microparticle without probiotic exhibited a significantly higher mortality than those fed the commercial diet or those fed with *Artemia* nauplii. The low growth rates may have been due to a potential nutritional inadequacy with respect to the low mineral/vitamin content of the experimental diets.

1. Introduction

Microencapsulation aims to protect different materials from adverse conditions of the environment, improve undesirable sensory aspects that a substance may present and also control the release of the active core materials (Anal & Stevens, 2005; Chanasattru, Jones, Decker, & McClements, 2009). The composition of the capsules varies according to the practical application and different wall materials can be used to produce the microparticle (Arshady, 1993).

The ionic gelation technique is simple and does not involve the use of organic solvents or extreme pH and temperature, which reduces its cost compared to other techniques, such as liposomes (Langdon, 2003). The adsorption of polyions and proteins on gelled particles through electrostatic interactions has been studied in detail for physiological applications (de Vos, Faas, Strand, & Calafiore, 2006; Souza et al., 2012) and for creating a protective layer to increase the viability of encapsulated probiotics (Annan, Borza, & Hansen, 2008; Gbassi, Vandamme, Ennahar, & Marchioni, 2009). Furthermore, this technique provides good protein retention and a high moisture content that is similar to that of *Artemia* nauplii (Mukai-Correa, Prata, Alvim, & Grosso, 2004).

Microalgae, rotifers, and especially *Artemia* nauplii are commonly used to feed fish larvae, although their costs are high. Formulated diets produced by different methods have been studied with the aim of replacing live foods (Holt, Webb, & Rust, 2011). The microencapsulation technique addresses some of the difficulties associated with microparticle diets, such as acceptability, size, and buoyancy, and greatly improves the leaching of nutrients and controlled release of the encapsulated material (Cahu & Zambonino Infante, 2001; Langdon, 2003). Several factors have been discussed, including the difficulty of digestion, the types of ingredients used, the nutritional composition, and the attractiveness of the diets (Langdon, 2003).

Formulated diets produced by microencapsulation using carrageenan or alginate that was obtained by ionic gelation have been evaluated in fish. These diets have shown high ingestion rates, water stability, and with a retention of 90–98% of the crystalline amino acids (López-

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Alvarado, Langdon, Teshima, & Kanazawa, 1994), in addition to enabling growth and development of marine fish larvae (Yúfera, Fernández-Díaz, & Pascual, 2005).

The success of fish larvae feeding depends on the nutritional composition of the diet and its attractiveness. The diets must have an attractive color, shape, size, decanting speed, and release properties that favor capture and ingestion by larvae. Additionally, the larvae must be capable of digesting and assimilating the diet nutrients for satisfactory growth and survival (Kvåle et al., 2006).

Modern aquaculture is increasingly based on the use of microbial organisms that promote the health or welfare of the fish or aquatic environment. The Food and Agriculture Organization/World Health Organization (FAO/WHO, 2001) defines probiotics as microbial cell preparations that have beneficial effects on the health and welfare of the host when administered in adequate amounts. The incorporation of probiotics in particles produced by ionic gelation, associated with electrostatic interactions, such as coating, was evaluated in in vitro gastrointestinal simulation tests. These tests showed a protective effect of probiotic microorganisms because the probiotics remained viable (Annan et al., 2008; Gbassi et al., 2009; Krasaekoopt, Bhandari, & Deeth, 2006), and therefore, the use of encapsulated probiotics can be a promising alternative in aquaculture, particularly in the larval stage.

The development of a microparticle containing high quantity of protein for feeding fish larvae has been studied but without success (Rodrigues, 2012). Because of this, the aim of this study was to produce gel microparticles coated with whey proteins containing the probiotic microorganism *Lactobacillus acidophilus* and to evaluate their use as food in pacu (*Piaractus mesopotamicus*) larviculture.

2. Material and methods

2.1. Materials

The materials used to produce the particles were low methoxyl amidated pectin — LMAP (CPKelco, mixture of various lots, Limeira, São Paulo, Brazil), deodorized salmon oil, containing Vit. E as antioxidant, food grade, provided by DDK Import and Export Trades Ltd., mineral and vitamin mixture (Fri-Ribe, Pitangueiras, S.P., Brazil) (composition provided by the supplier: 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, and biotin 0.10 mg), whey protein concentrate — WPC (Lacprodan, lote Lac804U17601, 76, Portenã, Cordoba Province, Argentina), fish hydrolyzed CPSP 90 (Anorel, Le Portel, France). The commercial diet used was Hatchfry Encapsulon III (Argent Laboratories, Redmond, WA, USA) (composition provided by the supplier: 50% of protein, 12% of fat, 3% of fiber, 12% of ash, 6% of moisture and size 250–450 μm). The culture used was probiotic microorganism *L. acidophilus* (La-05, Christian Hansen, Valinhos, S.P., Brazil), mucin (M1778), pepsin (P7012) and pancreatin (P1625) from Sigma-Aldrich Co. (St. Louis, MI, USA). Anhydrous calcium chloride (lot 36308, Dinamica, Diadema, S.P., Brazil), MRS agar, MRS broth and GasPak® anaerobic system (Becton, Dickinson and Company, Franklin Lakes, USA) were used for the microbiological analyses. Distilled and deionized water and other reagents of analytical grade were used.

2.2. Zeta potential

The zeta potential was evaluated in the solutions (0.5% w/w) – WPC, LMAP – by using the Zetasizer model Nano-Z (Malvern Instruments, Malvern, Worcestershire, WR, UK). The analyses were carried out in triplicate and the zeta potential was measured at pH intervals from 3.0 to 7.0.

2.3. Microparticles

2.3.1. Production of microparticles

The microparticles were prepared with a solution of 2% pectin (LMAP) (w/w of solution) emulsified with salmon oil (25%, w/w of total solids) and a mineral and vitamin mixture (0.5%, w/w of solution) using an Ultra-Turrax homogenizer (14,000 rpm/3 min, IKA, R.J., Brazil). For diets containing probiotic microorganisms, the probiotics (prepared according to Section 2.3.2) were added to the emulsion to obtain a concentration of approximately 10^5 CFU g^{-1} of freeze-dried particles. The emulsion was pulverized by a double fluid atomizer (Φ 1.5 mm) with compressed air flow (0.125 kgf cm^{-2}) positioned 12 cm from the calcium chloride solution (2% w/w, pH 4.0 — the pH was adjusted with a solution of 0.1 M of hydrochloric acid) under constant magnetic stirring. The microcapsules were kept in the calcium chloride solution for 30 min (hardening time) to ensure complete gelation. After the hardening time, the microcapsules were washed with water at pH 4.0 on sieves (Φ 125 μm) and dispersed in a 12% solution of whey protein concentrate (WPC) (pH 4.0) and 0.1% (w/v) CPSP 90 commercial hydrolyzed fish under constant magnetic stirring for 30 min. The particles were again transferred to sieves (Φ 125 μm) and washed with water at pH 4.0.

2.3.2. Maintenance and preparation of probiotic microorganisms

The commercial probiotic culture of *L. acidophilus* that was used in the production of microcapsules was rehydrated in 11% (w/w) reconstituted sterilized skimmed milk with 10% glycerol, aliquot into Eppendorf microtubes, and stored frozen (−18 °C) until use. To obtain a cell suspension of *L. acidophilus* La-5, a frozen sample was thawed, and the culture was activated in MRS broth (Oxoid) and later incubated for 15 h at 37 °C. The culture was centrifuged at 7974 *g* for 10 min at 4 °C (RC5C centrifuge, Sorvall Instruments, Du Pont, Wilmington, USA). The cell concentrate was resuspended in 0.1% peptonized water to wash the cells, followed by another centrifugation step under the previous conditions. After the cells were washed, they were resuspended in 0.1% peptonized water. The cell count was determined after the use of successive dilutions in 0.1% peptonized water and plating on Petri dishes with MRS agar. The plates were incubated at 37 °C for 72 h under anaerobic conditions using the anaerobic atmosphere generation system Anaerogen (Oxoid, Cambridge, UK). The cell suspension was subsequently used to produce microcapsules (2% v/v). To count the encapsulated microorganisms, the microcapsules were rehydrated in peptonized water (pH 4.0) for 15 min, disrupted by exposure to sodium citrate solution (2% w/v) under vigorous stirring for 5 min at pH 7.0, and counted as described above.

2.3.3. Characterization of diets and materials

The LMAP was characterized according to FAO (2009), containing 81.3 ± 1.2% galacturonic acid, 30.4 ± 1.6% degree of esterification and 10.4 ± 1.0% degree of amidation. The total content of protein, moisture, ash (AOAC, 2006), and lipids (Bligh & Dyer, 1959) was determined for the material used (LMAP, WPC, CPSP 90) and for the moist diets. Also, the protein content was determined for the rehydrated particles. All measurements were performed in triplicate. A portion of the moist particles was frozen and freeze dried (Mod. 501, Edwards Pirani, Crawley, West Sussex, UK) at an initial temperature of −40 °C, a pressure of 0.1 mm Hg, a final temperature of 25 °C/2 h, a total cycle of 48 h and then, was kept under refrigeration.

For protein quantification, the Kjeldahl methodology was applied, with correction factors of 6.38 and 6.25 for nitrogen to protein conversion in the WPC and the other protein material, respectively. In all analyses, a particle without protein coating was used as a blank to correct for the non-protein nitrogen content present in LMAP.

For lipid content extraction from the diets, sodium citrate was added (2% w/w) to 5 g of moist capsules for complete dissolution. The pH of the solution was adjusted to 3, and 1.5 mg of pepsin was added. The