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Oil Bodies as Novel Astaxanthin Carrier and Potential Aquafeed Ingredient for Aquaculture

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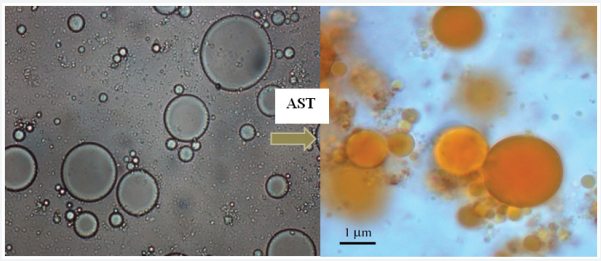
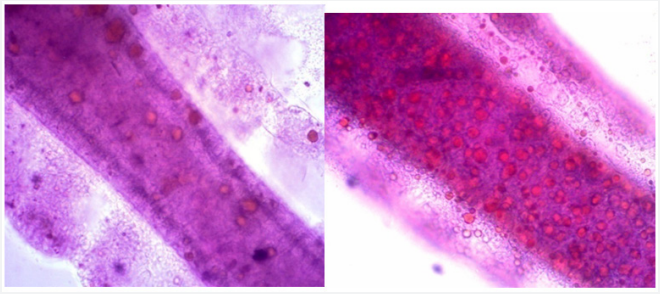
Abstract

This study presents preliminary evaluations for the potential use of Oil Bodies (OBs), spherical intracellular organelles that store lipids in the rapeseed *Brassica napus*, as a dietary supplement in aquaculture feeds. Objectives are straightforward: to evaluate OBs capacity to microencapsulate the highly valued but liable lipid-soluble pigment astaxanthin, used as feed supplement for appropriate coloration and to promote the health of aquatic animals. Likewise, to test the ability of *Artemia*, the biological model for micronutrient encapsulation, to safely uptake OBs and maintain astaxanthin stability over time in different *Artemia* bioencapsulation trials. Finally, to evaluate OBs qualitative lipid contribution. Oil bodies were extracted from rapeseeds with over 99% astaxanthin encapsulation efficiency. Astaxanthin was already visible one hourafter being encapsulated by *Artemia*, peaked at 24 hours, and remained non-metabolized into canthaxanthin for 48 hours. Significant differences were observed between the control group encapsulating astaxanthin-free OBs, and experimental groups, i.e., OBs enriched with four astaxanthin concentrations. Basal lipid content of *Artemia* increased qualitatively over time. It is concluded that OBs offer promise as a renewable and cost-effective agricultural carrier of astaxanthin and as alipid sourceto be potentially transferred to fish larvae via the live diet *Artemia*.

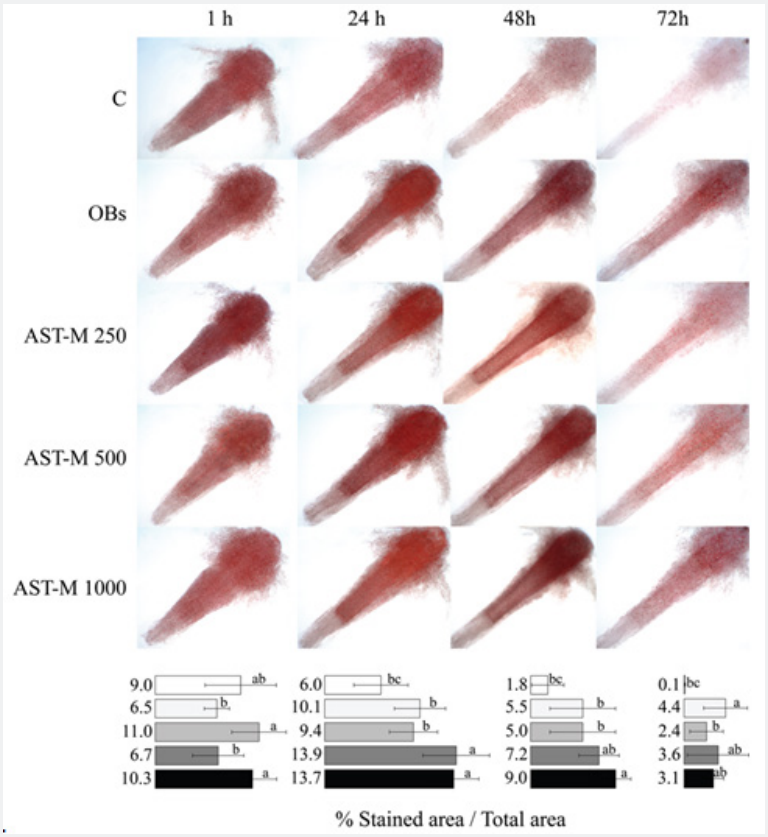
Graphical Abstract



A. Oil bodies (OBs) extracted from rapeseeds B. Astaxanthin microencapsulated in OBs (AST-M)



B. Qualitative assessment of lipid content in the gut of *Artemia nauplii* (Oil Red O stain), the biological model of encapsulation and live diet to transfer nutraceutics to fish larvae. Left, control. Right Oil Bodies ingested.



C. Bioencapsulation trials to track OBs ingestion over time, lipid contribution in *Artemia nauplii* and astaxanthin stability (not shown).

Keywords: Oil bodies; Rapeseed; Astaxanthin; Microencapsulation; *Artemia*

Abbreviations: OBs: Oil Bodies; AST: Astaxanthin; AST-M: Astaxanthin Microencapsulated within Oil Bodies

Introduction

Feedstuffs are finite and expensive ingredients which limits aquaculture diversification and expansion worldwide [1]. Fish oil represents a highly demanded but limited source of essential fatty acids used for dietary supplements in larval rearing (larviculture) of aquaculture species in captivity. Likewise, astaxanthin (AST) and other similar carotenoid pigments are valued but scarce feed supplements which are required for appropriate coloration and as a potent antioxidant to improve health status, since aquatic species exhibit poor ability to synthesize astaxanthin *de novo*. In the marine food chain, astaxanthin is primarily biosynthesized in microalgae and transferred to zooplankton and fish grazing on microalgae [2]. During marine plankton larvi culture, the natural diet of marine fish larvae is conveniently replaced by the microcrustacean *Artemia* whose *nauplii* (free swimming stages) are the live diet of choice during the critical period of metamorphosis, when marine fish larvae need to overcome radical molecular, physiological and morphological changes [3,4]. As a non-natural diet, *Artemia* often does not meet the nutritional requirements of fish larvae, and so a variety of boosters, or *Artemia* enrichment products are traded in the aquaculture market [5].

These products are bio-encapsulated in *Artemia nauplii* thanks to their ability to filter particles of less than 30µm. Lipid emulsions often produce such particle sizes when mixed with water, and these oil droplets are rich inessential fatty acids (EFA) such as eicosapentaenoic acid (EPA, 20:5 n-3), docosa hexaenoic acid (DHA, 22:6 n-3) and arachidonic acid (ARA, 20:4n-6), or a combination of these [3,6-8], depending on the booster used and bioencapsulation conditions [9]. Alternative micro diets like liposomes containing pigments, vitamins and free amino acids are also traded in the aquaculture market [10]. However, they are costly and short-lived as lipids oxidize easily. Additionally, the use of these boosters is unsustainable because they depend on fish oil which is increasingly scarce. Therefore, alternative plant oil sources have gained interest as they are globally available, inexpensive and can at least partially satisfy requirements offish larva [11,12].

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Plant oils are generally rich in n-6 and n-9 types, mainly linoleic acid (18:2n-6) and oleic acid (18:1n-9), with moderate to low levels of n-3 such as α-linolenic acid (18:3n-3) (except linseed oil) [13]. With some limitations, rapeseed oils potentially emerge as suitable microdiets, or as potentially safe carriers of liable dietary supplements [14]. In oleaginous plants like rape seed (*Brassica napus*), oils are generally stored in spherical intracellular organelles referred to as oil bodies (OBs). They consist of an oil core with a matrix of triacylglycerol (TAG) bound by a phospholipid monolayer embedded with proteins known as oleosins [15]. Moreover, tocopherol and polyphenols are reported to be intrinsically bound to OB structures in *Brassica napus* seeds [16]. Therefore, OBs may be considered a natural protection system against fatty acid oxidation because they can safely store lipids in seeds, in the form of TAGs, for long periods. Due to their spherical shape and small size (0.5 to 2.0μm in diameter), OBs are considered the natural equivalent of liposomes [17], and may be useful for the development of safe and efficient carriers to deliver bioactive molecules for food and/or pharmaceutical purposes.

The aims of this study were to evaluate the ability of OBs to microencapsulate and safely carry the highly valued but liable lipid-soluble pigment astaxanthin, used as feed supplement for appropriate coloration and to promote health inaquatic animals. Likewise, to test the ability of *Artemia*, the biological model for micronutrient encapsulation, to safely uptake OBs and maintain astaxanthin stability over time in different bioencapsulation trials. Finally, to evaluate OBs qualitative lipid contribution.

Materials and Methods

Preparation of astaxanthin microencapsulated within oil bodies

Isolation of OBs from *Brassica napus* seeds: Mature *Brassica napus* seeds obtained from local producers (Gorbea, La Araucanía Region, Chile) were dried at 30 °C for 24h. Isolation was done following methods of Chen et al. [18] with slight modifications. Briefly, 50g of seeds were ground in a blender (Moulinex Super 96 Junior "S"); the sample was then mixed with 250mL of buffer solution (3mM concentration of $MgCl_2$ and 100mM Tris-HCl, pH 8.5) and homogenized (200rpm, 3h, 4 °C, orbital shaker). The homogenate was filtered through three layers of Miracloth and the remaining filter cake was pressed. The collected slurry was then centrifuged (10,000×g, 30min, 4 °C) twice. The upper layer ("cream layer") was isolated using a chilled metal spatula. The resulting slurry was filtered and centrifuged with what was collected after the first extraction step at 10,000×g for 30min at 4 °C. The cream layer containing crude oil bodies was collected and re-suspended (10% w/w in buffer solution 10mM Tris-HCl pH 8.5 containing sodium azide 0.02M) and stored at 4 °C until further use. Sodium azide was added to prevent microbial spoilage of the OB suspension.

Astaxanthin microencapsulation within oil bodies

Different astaxanthin solutions dissolved in dimethylsulfoxide were prepared (250, 500 and 1000ppm) according to Acevedo et al. [19]. An aliquot (10mL) of each solution was mixed with 10mL of OB suspension, and stirred at 200rpm for 5.3h. The orange cream layer containing AST microencapsulated or loaded within OBs (AST-M) was collected and washed twice with buffer solution (10mMTris-HCl pH 8.6). After microencapsulation, samples were centrifuged (6,793×g, 15min) for phase separation as described by Acevedo et al. [19]; the orange cream layer (AST-M) was recovered using a chilled metal spatulaand stored at 4 °C until further use [20-23].

OBs bioencapsulation by Artemia nauplii

Artemia cysts from Great Salt Lake (GSL, USA) were decapsulated and hatched according to the standard protocol by Sorgeloos et al. [24]. *Nauplii* were collected and distributed (~180,000 *nauplii*) in five 250mL conical tanks with 250mL of artificial seawater (35ppt) at 25 °C. Each tank was illuminated and subject to high aeration in order to homogenize *nauplii* distribution in the water column. Experimental groups (three tanks) consisted of *Artemia nauplii* fed OBs loaded with different astaxanthin concentrations (AST-M: 250, 500 or 1000ppm). For this, aliquots (1.8mL) from a 10% w/w OBs suspension were added in a single dose at the start of the bioencapsulation experiment. Control groups (2 tanks) corresponded to starved *nauplii* and a group fed free AST-M. Independent samples (1.0mL each) were taken at 1, 6, 12, 24, 48 and 72 hours for analysis: two for fluorescence microscopic analysis (fixed in 25% v/v acetic acid), and two for qualitative lipid staining (fixed in 4% v/v formalin) by means of the Oil Red Olipid staining technique. Fixed samples were stored at 4 °C for further analysis.

Quantification of astaxanthin concentration in Artemia nauplii by HPLC

Five groups of *Artemia nauplii* with three replicates each were independently compared: i) control (starved *nauplii*); ii) *nauplii* fed OB suspension (10% w/w); iii) *nauplii* enriched with AST-M: 250; 500 and 1000ppm. A single OB dose (1.5mL in 250mL artificial seawater) was provided for enrichment and temporal monitoring was done in two groups, one tested at 1, 6 and 12h, and the other at 24 and 48h. *Nauplii* mortality was visually checked to avoid manipulating the enrichment process, and to keep a reasonable number of *nauplii* for analysis. Mortality in the experimental groups was insignificant compared to the starved control, in which dead *nauplii* sank to the bottom of the tank. *Nauplii* samples (20mg wet weight) from each group were taken for HPLC analysis at 0,1,6,12,24 and 48 hours. Time zero corresponded to *nauplii* sampled at the beginning of the enrichment experiments (24h post-hatch). The collected *nauplii* were thoroughly washed with distilled water in a mesh net to

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remove salt and extra-corporal OBs, then weighed and stored at -20 °C until HPLC analysis. *Nauplii* biomass was completely homogenized in 350µL of acetone using a glass homogenizer. Samples were centrifuged for 1min at 13,000rpm, and 20µL of each organic extract was immediately analyzed by HPLC-DAD. The RP-column was from Kromasil (C-8, 50 x 4.6mM, 2.5µm particle size). The high-performance liquid chromatograph used was from Jasco (Japan), with a quaternary pump (PU-2089 Plus model) and a diode array detector (MD-2015 Plus model). Chrompass software (Jasco) was used for the chromatogram analysis. Solvents used to develop the gradient were (A) Milli-Q water and (B) methanol. Solvent gradient in volumetric ratios for solvents A and B was as follows: 0-5.6min, 12A/ 88B; 5.6-7.6min, 0A/100B; 7.6-7.8min, 0A/100B; 7.8-9.5min, 12A/88B. Astaxanthin and canthaxanthin carotenoids were detected at 478nm absorption, and quantified by comparing the peak areas with the respective calibration curves (external standards, both from Sigma Chem. Co.). No internal standard was used because the extraction procedure was quantitative, reproducible, and required only one extraction step. Retention times for AST and canthaxanthin were 3.13min and 5.65min, respectively. Flow rate was set to 0.7mL/min.

Qualitative lipid assessment

Triglyceride-specific Oil Red O histological staining [20] was used to visualize lipid presence in the gut of *Artemia nauplii*. Formalin-fixed samples were rinsed with 60% v/v isopropanol and centrifuged at 12,000 rpm for 2min. Samples were stained with freshly prepared Oil Red O (0.3% v/v in isopropanol) working solution for 20min, and then rinsed with 60% v/v isopropanol and distilled water. After centrifugation at 12,000rpm for 2min, samples were stained with violet crystal (contrast), diluted twice and rinsed with 60% v/v isopropanol and distilled water. Finally, samples were suspended with 100µL distilled water. Image J 1.48v software was used to quantify the stained area of *Artemia nauplii* images (http://imagej.nih.gov/ij/docs/examples/stained-sections/index.html). Average (±SD; 10 individual *nauplii*) ratio of the stained area *vs.* total area was obtained for each group. The median image was taken from each group (Image/Stacks/Images to stack) using "Z Project".

Statistical significance of AST incorporated in *Artemia nauplii* was analyzed by a multivariate ANOVA using the "R" software, with Tukey's post-test. Four diets (AST free-OBs and OBs enriched with different astaxanthin concentration, AST-M diets) and five enrichment times (1, 6, 12, 24 and 48h) were compared. Lipid accumulation-related differences were analyzed using one-way ANOVA. Differences between means were compared using Duncan's test. A significance level of 95% (p<0.05) was used throughout.

Result and Discussion

Oil bodies were extracted from *Brassica napus* seeds with a yield of about 30% (50gr of rapeseed initially grinded). Figure 1 shows selected OBs loaded with astaxanthin (AST-M). The encapsulation efficiency of AST with OBs was over 99% as determined by the methodology of Acevedo et al. [19]. In a scenario of scarce basic aquafeed ingredients, the aquaculture industry has switched to plants as more sustainable alternative sources [1]. This study showed some practical advantages of OBs such as easy and cheap extraction, as well as being a renewable and available agricultural product [19]. Additionally, OBs were easily enriched with different astaxanthin concentrations. As hydrophobic molecule, astaxanthin exhibits high affinity for the triacylglycerol core of OBs which facilitates its microencapsulation by passive diffusion in a simple, fast (less than 6 hours) and efficient process, as previously shown (over 99% encapsulation efficiency) [19].

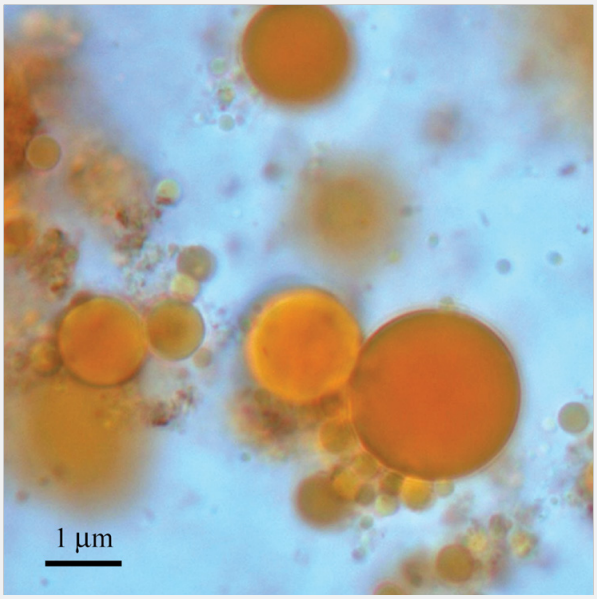

Figure 1: Astaxanthin microencapsulated within oil bodies (AST-M) isolated from *Brassica napus* seeds.

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Figure 2A illustrates the results of *Artemia* bioencapsulation experiments in which OBs loaded with different astaxanthin concentrations (AST-M) were monitored over time in *Artemianauplii*. Astaxanthin was visible in all groups except control (starved *nauplii*), one hour after encapsulation experiments began, and peaked at 24hours in all experimental groups. Astaxanthin concentration was higher in *Artemia nauplii* taking up OBs with higher astaxanthin concentration. Statistically significant ($p<0.05$) differences were observed between control, bioencapsulating AST-free OBs, and the AST-M experimental groups (with 250, 500, 1000ppm) (Figure 2A). No statistically significant ($p>0.05$) differences were observed in the *Artemia*-endogenous content of canthaxanthin (Figure 2B) according to the diet provided, implying that astaxanthin was not metabolized into canthaxanthin under these study conditions. Optimal AST microencapsulation time was 24hours.

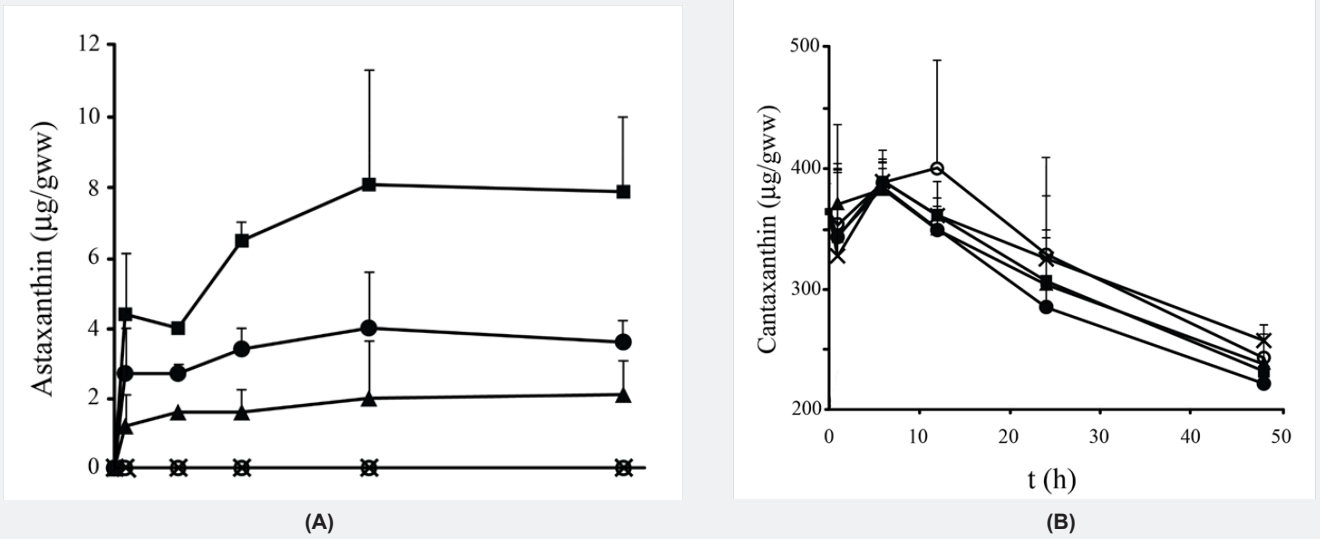

Figure 2: A) Astaxanthin concentration (µg/g wet weight) in nauplii fed different quality microdiets: (○) starved nauplii (control); (×) OB-enriched nauplii, (▲) AST-M 250ppm, (●) AST-M 500ppm and (■) AST-M 1000ppm-enriched nauplii. Means of detected AST concentrations±SD (n=3) are shown. Concentration "zero" of astaxanthin corresponds to "not detected" by HPLC analyses. Content of astaxanthin in *Artemia* is expressed as µg/g wet weight (at 0, 1, 6, 12, 24 and 48h).
B) Endogenous canthaxanthin content in *Artemia* nauplii fed different quality microdiets: (○) Control (starved nauplii); (•) nauplii fed OBs, (■) enrichment with AST-M 250ppm, (□) 500ppm and (×) 1000ppm. Means of detected canthaxanthin±SD (n=3) are shown. Canthaxanthin content in nauplii is expressed as µg/g wet weight of *Artemia* (at time 0, 1, 6, 12, 24 and 48h).

An initial concern was OB size (average diameter 0.5 to 2.0µm), which is below the range of 7-28µm filtered by *Artemia nauplii* [21], with an optimum around 16µm. This study made it clear that *Artemia nauplii* can ingest particles below the pre-established size limit, though bioencapsulation efficiency depends on several experimental conditions [9]. Astaxanthin stability was tested for at least 48hours in *Artemia* which confirmed the hypothesisthat OBs are safe astaxanthin carriers [19]. This is not normally the case with emulsions used to boost *Artemia*, which tend to be quickly digested, and explains why the bio encapsulation process is often relatively fast [22]. Thus, an extra benefit of bio encapsulating AST-M is that, unlike unprotected supplements, enriched *nauplii* can be kept stable for relatively longer periods without affecting biological properties of the ingested substance. By extending the pre-established window time for efficient *Artemia* bio encapsulation (around 24 hours), OBs provide a chance for *nauplii* to grow beyond the size normally reached at 24hours, and hence be available for larger fish larvae (greater mouth opening). Protection provided by OBs would explain why astaxanthin was not metabolized into can thaxanthin, the most abundant carotenoid in *Artemia*. Isomeric canthaxanthin forms (cis and trans) vary during the *Artemia* life cycle, depending on the availability of its metabolic precursors: echinenone and beta-carotene [23].

Figure 3 shows Oil Red O staining for *Artemia nauplii* individuals after 1 to 72 hours post encapsulation for the following groups: 1) control group (starved *nauplii*) in which stain intensity decayed over time; 2) *nauplii* bioencapsulated with AST-free OBs, which exhibited higher intensity and 3) *nauplii* that bio encapsulated OBs loaded with different astaxanthin concentrations. As expected, there are no clear visual differences between groups 2 and 3 since the Oil Red O stain does not show differences in AST concentration. Image quantification shows the ratio of stained area *vs* total area (in percent) increased as bioencapsulation proceeded, from 6.0% at 24 hours in the control, 10.1% in the group fed OBs and 13.9 and 13.7% in those *nauplii* fed OBs with increasing astaxanthin concentration (500 and 1000ppm). This means that *nauplii* reached the maximum stain intensity at 24hours, which is a proxy of the basal lipid quantity and/or OBs concentration in the gut of *Artemia nauplii*.

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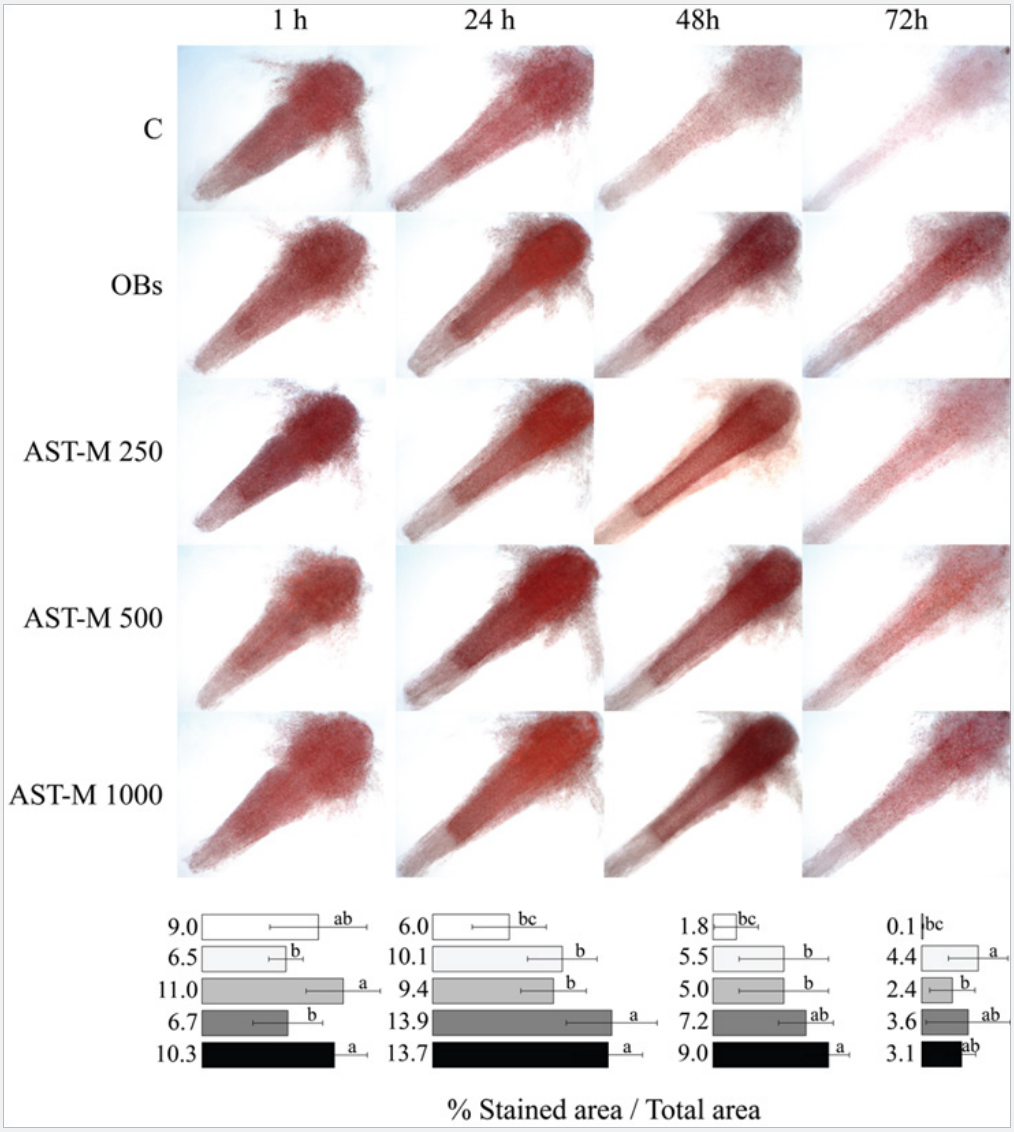

Figure 3: Oil Red O staining images of *Artemia* nauplii guts in different bioencapsulation trials (1, 24, 48 72 hours). C (control): starved nauplii; OBs: *Artemia* nauplii enriched with pure Oil Bodies; AST-M: *Artemia* nauplii enriched with OBs with different astaxanthin concentrations (200, 500, 1000ppm).The average value (n=10 nauplii) for the ratio percentage of stained area: total area is provided in the lower part of the Figure. Different letters represent significant differences.

Testing the safe OBs uptake by *Artemia nauplii* is a pre-condition to transfer AST to marine fish larvae, owing to the role this microcrustacean plays in the food chain of animals reared in captivity. However, Oil Bodies enriched with astaxanthin could be directly provided to fish larvae, or even to shellfish larvae that are also boosted with dietary supplements [24]. The advantage of OBs as a lipid source would be that they are already naturally emulsified after the extraction, with remarkable physical and chemical stability and potentially high nutritional value, depending on the species targeted [25].

Conclusion

A. OBs are cheap and renewable agriculture products that can potentially be used as microdiets to safely carry lipid-soluble pigments like AST, which is highly valued for aquatic species due to its nutritive and biological properties. In this study OBs were efficiently microencapsulated with different astaxanthin concentrations [26].

B. OBs were encapsulated by *Artemia nauplii*, the biological model for encapsulation that is additionally used as live diet in the food chain of marine fish reared in captivity. However, OBs could also be delivered directly to fish or shellfish, in the latter case as a complementary or partial replacement for lipid emulsions.

C. Artemia nauplii ingested and stored OBs loaded with AST for over 24 hours, the operational time defined for optimal *Artemia* enrichment, thus extending the enrichment

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time window to allow larger fish larvae to feed on *Artemia*. Astaxanthin remained non-metabolized in the gut of *Artemia nauplii* for longer times, likely protected inside OBs lipid core [27].

D. OBs supplemented the basal lipid composition of *Artemia nauplii*, and since they are rich in a variety of lipids, they appear to be a naturally good provider of such an essential diet ingredient.

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