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Nutritional Enhancement of n-3 and n-6 Fatty Acids in Rotifers and *Artemia* Nauplii by Feeding spray-dried *Schizochytrium* sp.,

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Abstract

A Docosahexaenoic acid (DHA), 22:6(n-3), rich strain of *Schizochytrium* sp. Was used in a spray-dried form to evaluate the enhancement of highly unsaturated fatty acids (HUFAs) in *Artemia franciscana* nauplii (Utah biotype) and the rotifer *Brachionus plicatilis*. This heterotrophic microalga was selected because of its high concentration of the longest chain HUFA's in the n-3 and n-6 series, DHA and docosapentaenoic acid (DPA), 22:5(n-6), respectively. When 24-h-old *Artemia* nauplii were fed 400 mg/L of the algae for 24 h, the DHA content of the nauplii went from undetectable levels to 0.8% of dry weight and the omega-3 HUFA eicosapentaenoic acid (EPA), 20:5n-3, content went from 0.1% to 0.5% of dry weight in the nauplii. Similarly, 22:5(n-6) increased in the nauplii from undetectable levels to 0.4% of dry weight, with a concomitant increase in arachidonic acid, (20:4n-6) from trace to 0.3% of dry weight even though there was no arachidonic acid in the algal biomass. Similar enrichment patterns were observed in rotifers. The results suggest that spray-dried cells of *Schizochytrium* sp. are effective in enriching *Artemia* nauplii and rotifers in both n-3 and n-6 HUFAs. The results also suggest that *Artemia* nauplii and rotifers are capable of readily retroconverting 22:6(n-3) to 20:5(n-3) and 22:5(n-6) to 20:4(n-6) through the process of B-oxidation, a well-known process in mammals.

Enrichment of rotifers and *Artemia* nauplii with n-3 HUFAs prior to feeding the nauplii to larval fish and shrimp is a common procedure in the aquaculture industry. These fatty acids are essential for the normal development of larval fish and shrimp, but most of the commonly available strains of *Artemia* and rotifers used as food for these larvae have only a very small amount of these HUFAs (Watanabe et al. 1978). Enrichment techniques currently in use include: 1) microencapsulated oils containing high concentrations of n-3 HUFAs (Sakamoto et al. 1982; Ozkizilcik and Chu 1994); 2) emulsified marine oils rich in omega-3 HUFAs (Watanabe et al. 1980; Leger et al. 1987; Kissil and Coven 1990; Sorgeloos and Leger 1992; Ozkizilcik and Chu 1994); and 3) live microalgae (Watanabe et al. 1980, 1982; Millamena et al. 1988; Whyte and Nagata 1990; Ozkizilcik and Chu 1994).

The importance of Docosahexaenoic acid (DHA), 22:6(n-3), facilitating the normal development of larval fish and oyster spat have been noted by many investigators including Langdon and Waldock (1981), Ostrowski and Divakaran (1990), Watanabe (1993), and Ozkizilcik and Chu (1994). However, few of the existing enrichment methods produce significant increases in the DHA content of *Artemia* nauplii or rotifers. Menhaden oil, the fish oil most commonly used in microencapsulated or emulsified oils, generally has a DHA content of less than 12% total fatty

acids. *Artemia* nauplii enriched with these products generally have undetectable levels of DHA. (Ozkizilcik and Chu 1994). Furthermore, data from previous studies suggests that *Artemia* cannot effectively elongate eicosapentaenoic acid (EPA), 20:5(n-3) to DHA (Watanabe et al. 1978), and rotifers appear to have only a limited capacity for this elongation (Whyte and Nagata 1990).

Two of the best microalgae for aquaculture feeds are *Isochrysis galbana* and *Chaetoceros gracilis*. Their effectiveness is due in part to their small size and n-3 HUFA content. However, an overlooked and unique attribute of these species is that they contain a significant portion of their n-3 HUFAs as DHA (10% in C. gracilis and 95% in I. Galbana) while additionally containing significant quantities of long chain omega-6 fatty acids, arachidonic acid, 20:4(n-6), in C. gracilis and docospentaenoic acid (DPA), 22:5n-6, in I. Galbana (Webb and Chu 1982; Napolitano et al. 1988; Mourente et al. 1990). Much focus has been placed on the essential role of long-chain n-3 fatty acids especially in early nervous system development of fish and shrimp, but the n-6 HUFA, arachidonic acid is also important as the precursor of some prosta-glandins and other biologically active compounds which regulate growth and reproductive functions (Stanley-Samuelson 1987; De Petrocellis and Di Marzo 1994). Napolitano et al. (1988) suggested that the n-6 fatty acid content of the algae used as feed in the culture of marine bivalves may be critical for normal bivalve development and reproduction. They noted that studies have demonstrated the biosynthesis and activity of eicosanoids derived from both the n-3 and n-6 HUFA series in bivalve molluscs (Christ and Van Dorp 1972; Nomura and Ogata 1976).

A drawback of utilizing microalgae in aquaculture feeds is the microalgae are very expensive to produce in the moderate scale production facilities in most aquaculture nurseries (Barclay et al. 1987). Species of heterotrophic microalgae that could be grown in conventional fermentation systems might be produced at a much lower cost than microalgae produced in outdoor ponds. Furthermore, the production controls inherent in fermentation systems have the potential to facilitate production of heterotrophic algae with an improved and more consistent biochemical quality (Jones et al. 1993).

Previous attempts have been made to untilize spray-dried heterotrophic microalgae as aquaculture feeds. The strains employed, however, were selected primarily for their heterotrophic mass production attributes, with only a secondary concern for their nutritional profile, especially in terms of their n-3 and n-6 HUFAs. As a result, they generally performed poorly as feed for larval marine organisms (Laing and Verdugo 1991).

The purpose of this study was to determine the effectiveness of enriching rotifers and *Artemia* nauplii with long chain fatty acids by using a spray-dried heterotrophic strain of microalgae, *Schizochytrium* sp., rich in both n-3 and n-6 long chain fatty acids. A secondary focus of the study determined if enrichment of rotifers and *Artemia* nauplii with the longest chain fatty acids in the n-3 and n-6 series would lead to a significant increase in shorter chain bioactive fatty acids in the n-3 and n-6 series through the process of retroconversion.

Materials and Methods

Schizochytrium sp. (American Type Culture Collection 20888) biomass was produced in a 400-L fermenter following the general procedures in Barclay (1994) and Barclay et al. (1994). At the end of the fermentation, the cells were concentrated by centrifugation, spray-dried, and vacuum sealed in foil packets. The spray-dried product is also available commercially as AlgaMac-2000 (Aquafauna Bio-Marine, Hawthorne, California, USA).

For proximate analysis of the spray-dried microalgae, total protein was determined using the Kjeldahl method (Strickland and Parsons 1968). Total carbohydrate was determined by the phenol/sulfuric acid method (Strickland and Parsons 1968). Total fat was reported as total fatty acid methyl esters (FAME) as determined by gas-liquid chromatography. Fatty acids in whole cells were methylated by a one-step process employing 4% sulfuric acid in methanol (100 C for 1

h). The fatty acid methyl esters were then separated and quantified on a Hewlett Packard 5890 Series II Plus gas-liquid chromatograph equipped with a Hewlett-Packard 3365 Series II ChemStation, a flame ionization detector, and a 30 m x 0.25 mm (I.D.) DB-225 fused silica capillary column (J & W Scientific, Folsom, California, USA). Nu-Chek-Prep (Elysian, Minnesota, USA) fatty acid standards were employed as external standards both to identify and quantify the fatty acid peaks observed. The sterol content of the biomass was measured by gas liquid chromatography on a Hewlett -Packard Model 5830A gas-liquid chromatograph with a model 18850A integrator, a flame ionization detector and 3% OV-17 on a 80/100 Supelcoport packed column (Supelco Inc., Bellefonte, Pennsylvania, USA).

The particle size of the spray-dried microalgae was measured using an Olympus CH phase contrast microscope with calibrated micrometer eyepiece. In order to quantify settling characteristics of the algae, spray-dried samples were pre-hydrated in deionized water, synthetic tap water, or 20 parts per thousand (ppt) seawater. The pre-hydration procedure involved mixing the sample at a concentration of 1 mg/mL in a blender for 1 min. The pre-hydrated whole-cell suspension was then diluted in the appropriate water type to achieve a concentration of 0.1 mg/L. The solution was thoroughly mixed and an aliquot was transferred to a 1-cm cuvette and the absorbance monitored over time at 660 nm.

Brine shrimp nauplii (*Artemia franciscana*, Utah biotype) were produced by hatching brine shrimp cysts (premium grade, Sanders Brine Shrimp Co., Ogden, Utah, USA) in 20 ppt artificial seawater (Reef Crystals, Aquarium Systems, Mentor, Ohio, USA) for 24 h at 30 C. The 24-h old nauplii were separated from the cyst shells and diluted to a density of 100 nauplii/mL. All enrichment trials were conducted at 30 C in 500-mL polycarbonate bottles provided with vigorous aeration. A 250-mL aliquot of the nauplii suspension was placed in each bottle (with duplicate bottles for each treatment) and the test enrichment food was then added at the desired density. In the first experiment *Schizochytrium* sp. cells and a microencapsulated fish oil product (FRIPPAK Booster, INVE, Belgium) were evaluated at 100mg/L. After 24-h enrichment, the nauplii were harvested by pouring them through a 100-um mesh nylon screen followed by rinsing with 20 ppt artificial seawater and finally deionized water. They were then dried at 100 C for 24 h and analyzed for their fatty acid content. Fatty acids in the dry nauplii were methylated in 4% sulfuric acid in methanol (100 C for 1 h). Fatty acids were quantified as outlined previously and calculated as % totally fatty acids and as % dry weight of the nauplii.

A second enrichment trial was conducted to examine the effect of different densities of *Schizochytrium* sp. cells on the resulting enrichment of n-3 fatty acids in the *Artemia* nauplii. This enrichment trial (100 nauplii/mL) was conducted as previously described except that pre-hydrated *Schizochytrium* sp. cells were added at 0, 50, 100, 200, and 400 mg/L. Each treatment was conducted in duplicate.

A culture of the rotifer *Brachionus plicatilis* was obtained from Aquaculture Supply (Dade City, Florida, USA) and maintained in the experiments at 26 C in 20 ppt artificial seawater. To evaluate the enrichment of rotifers, 600-mL portions of rotifers at a density of 400/mL were placed in 1-L glass beakers and provided with mild aeration. Rotifer cultures undergoing enrichment were fed 70 mg of spray-dried *Schizochytrium* sp. at the beginning of the enrichment period and again after 4 h. Similarly, control rotifer cultures were fed 70 mg of dry brewers yeast at the beginning of the enrichment period and again after 4 h. After 8 h, the rotifers were collected by pouring the cultures through a 53-micron mesh screen, followed by brisk rinsing, first with 20 ppt artificial seawater and then de-ionized water to remove food and salts. The rotifers were then lyophilized and their fatty acid content determined by the method outlined previously. An additional experiment with rotifers was also conducted as outlined above except that the rotifers were fed *Schizochytrium* sp. for 24 h to observe the effects of longer enrichment periods on their long chain HUFA profile.

Results

The proximate composition, and fatty acid and sterol profile of the spray-dried microalga *Schizochytrium* sp. used in the study is presented in Table 1. The fat content (as % total fatty acids) of the algal cells was 32% of dry weight. The HUFA content of the fatty acids was 37.5% of total fatty acids consisting of 24.0% DHA(n-3), 0.6% EPA(n-3) and 12.9% DPA(n-6) (all as % total fatty acids).

The results of the particle size analysis indicated that the size of the spray-dried material ranged from 3-18 um with an average particle size of 7.5 um, the approximate size of an individual *Schizochytrium* sp. cell. The settling characteristics of the spray-dried material is illustrated in Fig. 1. The spray-dried cells readily remained in suspension in all three water types. Approximately 50% of the cells remained suspended after 6 h of static conditions (no mixing or aeration) in all three treatments. There were no significant differences in the settling rates of spray-dried *Schizochytrium* sp. cells suspended in seawater, tap water, or deionized water.

The results of the enrichment trial comparing the n-3 fatty acid enrichment potential of *Schizochytrium* sp. and microencapsulated fish oil are presented in Table 2. Control nauplii contained 0.1% of dry weight as EPA. No DHA was detected in the control nauplii. *Artemia* nauplii enriched with encapsulated fish oil for 24 h contained 0.3% of dry weight as EPA and no DHA was detected . Nauplii enriched with *Schizochytrium* sp. for 24 h had an EPA content of 0.4% of dry weight and a DHA content of 0.1%. The fat content of the *Schizochytrium* sp. enriched nauplii was also higher than the encapsulated fish oil or control nauplii. The *Schizochytrium* sp. enriched nauplii exhibited a fat content (estimated as fatty acid methyl ester % of dry weight) of 7.9% of dry weight, while the encapsulated fish oil and control nauplii fat contents were 6.8 and 5.5% of dry weight respectively.

The results of the trial examining the n-3 fatty acid enrichment of *Artemia* nauplii in different densities of *Schizochytrium* are shown in Table 3. The highest enrichment level of long chain n-3 fatty acids occurred at 400 mg/mL of *Schizochytrium*. The EPA content in the nauplii in this treatment was 0.5% of dry weight and the DHA content was 0.8%. The EPA and DHA content of the starved controls was 0.1% and 0.0% of dry weight, respectively. In addition to long chain n-3 fatty acid enrichment, the nauplii in this treatment were also enriched with the long chain n-6 fatty acid, DPA. As a result, the nauplii in this treatment were enriched with a total 1.3% of dry weight as n-3 HUFAs and 0.7% of dry weight as n-6 HUFAs. The total fat of the nauplii also increased with an increasing content of *Schizochytrium* employed in the enrichment process. The highest fat content achieved in the *Artemia* nauplii was 10.2% of dry weight. This occurred in the treatment fed 400 mg/L of *Schizochytrium*. Control nauplii exhibited a fat content of only 6.1% of dry weight.

The results of the rotifer enrichment trial are presented in Table 4. The only long chain HUFA in the control rotifers was 20:4(n-6) at a concentration of 0.6 mg/g dry weight. On the other hand, the enriched rotifers contained all three HUFAs in the n-3 series, 20:5(n-3) (0.9 mg/g dry weight), 22:5(n-3) (0.9 mg/g of dry weight) and 22:6(n-3) (13.7 mg/g dry weight). Additionally, the enriched rotifers contained a higher amount of 20:4(n-6) (1.0 mg/g dry weight) than the control rotifers plus an additional 5.8 mg/g dry weight of 22:5(n-6). The fat content of the enriched rotifers (7.5 mg/g dry weight) was also three times that of the control rotifers (2.5 mg/g dry weight).

Rotifers fed *Schizochytrium* sp. for 24 h (16 h longer than the industry standard enrichment period of 8 h) do not exhibit further increased concentrations of DHA in their fatty acids but do exhibit increased EPA, and arachidonic acid concentrations. Rotifers fed *Schizochytrium* sp. for 24 h have DHA, EPA, and arachidonic acid contents 17.7%, 5.7% and 6.5% of total fatty acids respectively. As illustrated in Table 4, rotifers fed *Schizochytrium* sp. for only 8 h had DHA, EPA, and arachidonic acid contents of 18.3%, 0.3% and 1.4% of total fatty acids.

Discussion

Microorganisms in the genus *Schizochytrium* were originally classified as fungi in part due to their heterotrophic nature. Their taxonomic history and present placement with the golden algae have been summarized in Barclay et al. (1994) and supported by more recent data based on analysis of rRNA molecular weights and 5S rRNA sequences (Izzo et al. 1994).

The results of this study indicate that spray-dried cells of the heterotrophic alga *Schizochytrium* sp. can be utilized to effectively enrich *Artemia* nauplii and rotifers with long chain HUFAs. The nutritional requirements of marine finfish for the essential n-3 HUFAs are estimated to range from 0.5 - 2.0% of dry weight in their feed, with many species requiring about 1.0% of dry weight in their feed as n-3 HUFA (Watanabe 1993). In this study, *Artemia* nauplii enriched by feeding with *Schizochytrium* cells (400 mg/L; 100 nauplii/mL; 24 h) exhibited a n-3 HUFA content of 1.3% of dry weight. Enriched rotifers achieved a n-3 HUFA content of 1.6% of dry weight. The effectiveness of enrichment achieved with this strain of microalgae is likely due to several factors: 1) the high content of n-3 HUFA in the spray-dried cells; 2) the small size of the cells which readily facilitated ingestion by *Artemia* nauplii and rotifers; and 3) the excellent suspension characteristics exhibited by the spray-dried cells in seawater which kept them available for ingestion.

Of primary importance with regards to rotifer and *Artemia* enrichment is that the results of this study indicate that *Schizochytrium* can be used as a feed to increase both the DHA and EPA content of *Artemia* nauplii and *Brachionus* prior to feeding them to larval fish and shrimp. In contrast, fish oil-enriched nauplii generally only have enhanced contents of EPA with a trace amount of DHA.

Enrichment of both EPA and arachidonic acid in *Artemia* nauplii and rotifers fed Schizochytrium for 24 h may be the result of the retroconversion of these fatty acids from their longer chain forms. Retroconversion of 22:6(n-3) to 20:5(n-3) and 22:5(n-6) to 20:4(n-6) through the process of B-oxidation has long been known to occur in mammals (Stoffel et al. 1970; Kunau and Bartnik 1974; Kunau and Couzens 1971; Hagve and Christopherson 1986). The process, which occurs in peroxisomes of mitochondria, involves two reactions: 1) the docosapolyenoic acid (e.g. 22:6(n-3) or 22:5(n-6)) loses its double bond in position 4, a reaction involving the enzyme 4-enol-CoA reductase, while the carbon chain length remains unchanged; and 2) chain shortening then occurs (Kunau and Bartnik 1974). Thus 22:6(n-3) is first converted to 22:5(n-3) and then converted to 20:5(n-3). Similarly, 22:5(n-6) is converted to 22:4(n-6) and the to 20:4(n-6).

The importance of retroconversion of HUFAs in aquaculture nutrition has often been overlooked. Early work on the need to enrich *Artemia* with long chain n-3 fatty acids focused on EPA in part because the menhaden oil used in the enrichment studies was rich in EPA. In more recent studies, enrichment of *Artemia* nauplii with DHA-rich oil has proven more effective than the use of EPA-rich fish oil. For example, Watanabe (1993) reported that enrichment of *Artemia* nauplii with 99% pure EPA (as ethyl ester) resulted in an increase of EPA in the nauplii but with no DHA detected. However, his data indicated that nauplii enriched with 99% pure DHA (as ethyl ester) resulted in increases in both EPA and DHA in a 1:4 ratio respectively. When evaluated as food for a variety of larval fish, the fish fed the DHA ethyl ester-enriched nauplii generally exhibited better growth, survival and vitality. Watanabe (1993) suggested that the data indicated that feeding fish larvae enriched only in EPA may result in lower viability fish larvae because of a resulting imbalance in EPA to DHA in the fish's biomembranes causing changes in membrane fluidity or phospholipid function. However, Watanabe's data and the data developed in the present study suggest an alternative explanation. DHA is an important component of developing nervous systems in both invertebrates and vertebrates, and as such is an essential fatty acid for normal development (Castell et al. 1994). Enrichment of *Artemia* nauplii with DHA prior to feeding them to larval fish helps to provide this essential fatty acid for the fish larvae during a critical phase in their development. Enrichment of *Artemia* nauplii only with EPA does not provide the larvae with an essential DHA, and many types of marine fish are apparently incapable of

elongating the EPA to DHA (Ostrowski and Divakaran 1990); Watanabe 1993). Additionally, feeding rotifers and *Artemia* nauplii with DHA results in EPA enhancement providing eicosanoids which positively enhance immunocompetence in fish larvae (Bell et al. 1994).

The results of this study, in conjunction with those of Watanabe (1993), suggest that brief feeding of rotifers and *Artemia* nauplii with DHA-rich microalgae such as *Schizochytrium* sp. may provide the best strategy for n-3 HUFA enrichment of live food organisms used in aquaculture. This is because it facilitates enrichment of both DHA and EPA in the brine shrimp nauplii and rotifers. The data also suggest that a similar process may occur with enrichment of n-6 HUFA. Therefore, spray-dried *Schizochytrium* is effective as a feed for enriching rotifers and *Artemia* nauplii with all of the bioactive HUFAs in the n-3 and n-6 series.

Higher levels of n-3 HUFA enrichment have been reported in rotifers and brine shrimp nauplii fed emulsified oil products (Sorgeloos and Leger 1992), but the quantity of n-3 HUFA enrichment may not be as important as achieving an appropriate ratio of n-3/n-6 HUFAs. Furthermore, spray-dried microalgae in their naturally encapsulated form provide a dry source of HUFAs which may be easier to use than emulsified oil products and minimizes the contamination of enrichment media with bacteria that often occurs with emulsified oils (Ozkizilcik and Chu 1994). Whole-cell microalgae also provides a broader profile of other natural nutrients (protein, sterols, vitamins, trace elements) than is available in manufactured oil-based products.

Spray-dried *Schizochytrium* with its unique n-3 and n-6 HUFA profile may also be a candidate for replacing much of the live algae used in the culture of panaeid shrimp larvae. However, it will first be necessary to demonstrate that panaeid larvae are capable of directly retroconverting 22:6(n-3) to 20:5(n-3) and 22:5(n-6) to 20:4(n-6) as suggested by the brine shrimp nauplii and rotifers in this study.

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