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Schizochytrium limacinum SR-21 as a source of docosahexaenoic acid: optimal growth and use as a dietary supplement for laying hens

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Abstract. Culture conditions for the marine fungus *Schizochytrium limacinum* SR-21 (SR-21) to produce microbial docosahexaenoic acid (DHA) were evaluated, and the practicality of using this fungus product as a dietary supplement for laying Leghorn hens was investigated. The data showed that the cultured fungus produced high biomass and DHA. It generated 584 mg DHA/L of culture at the end of a 6-day culture. The fungus grew better at 25°C than at 20°C or 30°C. With an increase in glucose concentration from 1% to 5% in the culture medium, biomass and DHA production were enhanced. A 6% glucose treatment reduced the biomass production compared with 5% glucose. A bioreactor was used to mass-produce SR-21. The biomass was increased from 1.12 g/L at Day 0 to 12 g/L at Day 4. We established optimal culture conditions of 5% glucose, 2% sea salt, and 1% yeast extract for SR-21. Three concentrations of dried fungal meal (0, 1, or 3% in the diet) were fed to birds over a 3-week period. There were no negative effects of 1 and 3% dietary SR-21 on egg production, egg weight, and egg yolk weight. The DHA content of yolk was increased by the dietary supplementation with the fungal meal both in the 1 and 3% treatments. Dietary fungal meal treatments increased the DHA concentrations of liver and plasma. However, dietary DHA enrichment had no effect on the expression of hepatic lipogenic genes in laying hens.

Additional keywords: laying Leghorn hens, lipid metabolism.

Introduction

Dietary *n*-3 polyunsaturated fatty acids (PUFA) such as linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) may help prevent coronary artery diseases, hypertension, diabetes, and inflammatory disorders (Innis 1992; Lewin *et al*. 2000; Simopoulos 2000). These beneficial effects of dietary *n*-3 PUFA on human health have stimulated interest in supplementation of animal feeds with dietary fish oil, fish meal, marine algae, linseed meal, or flaxseed meal to increase levels of *n*-3 PUFA (Navarro *et al*. 1972; Hargis *et al*. 1991; López-Ferrer *et al*. 2001).

The fatty acid (FA) profile of the egg yolk can be affected by the FA profile of the hen diets, particularly PUFA (Cruickshank 1934; Hargis *et al*. 1991; Cheng *et al*. 2004). Therefore, many researchers have tried to increase the levels of *n*-3 PUFA in egg yolk by dietary supplementation. By supplementing laying hen diets with 3–7% fish oil, it was possible to increase *n*-3 PUFA/egg to more than 200 mg (Hargis *et al*. 1991). Eggs from hens fed 50 g fish oil/kg, contained significantly more DHA (5.27% of total fatty acids) than ordinary eggs (0.44% of total fatty acids; Farrell 1998).

Although fish oil or fishmeal can be used to enrich the DHA content of egg yolk, fish odour is a concern with increasing amounts of dietary fish oil (Nash *et al*. 1995; de Swaaf *et al*. 1999). An inclusion of 6% fish oil in the diet has been reported to create a fishy flavour in *n*-3 PUFA-enriched eggs (Adams *et al*. 1989). Similar flavour in hard-cooked eggs from hens fed flaxseed meal was also reported (Scheideler and Froning 1996). Therefore, alternative *n*-3 PUFA sources are of interest, especially from marine algae and fungi that contain considerable amounts of EPA and DHA (Bajpai *et al*. 1992; Cohen 1994; Li and Ward 1994; Singh and Ward 1996; Chen and Vazhappilly 1998).

Schizochytrium limacinum SR-21 (SR-21) is a marine fungus isolated from the coral reef area of the Yap islands in the Federated States of Micronesia by Nakahara *et al*. (1996). The strain accumulates a high content of DHA and is a potential candidate for use as a DHA-rich ingredient in poultry diets (Nakahara *et al*. 1996; Yokochi *et al*. 1998). Recently, DHA-rich oil has been produced commercially from a similar microalga, *Schizochytrium* sp., and another strain, *Crypthecodinium cohnii*, by Martek Biosciences Co.

In the present study, culture conditions of SR-21 for producing maximal microbial DHA were evaluated. We also evaluated the use of the fungus as an alternative source of DHA in the diets for laying Leghorn hens to study the effect of dietary DHA enrichment on DHA accumulation in the egg.

10.1071/AR05099 0004-9409/06/010013

Materials and methods

Microorganism and cell culture

The SR-21 was obtained from the Institute for Fermentation, Osaka (IFO), Japan, strain number IFO 32693. The organism was plate-cultured with the GPY medium recommended by IFO, containing 20 g glucose, 10 g peptone, 5 g yeast extract, and 15 g agar in 1 L of 2% salinity artificial sea water (added sea salt 20 g/L, Sigma, St Louis, MO) at 25°C. After 5 days of culture, an agar block (1 × 1 cm) containing growing cells was cut from the plate and put into a 150-mL Erlenmeyer flask containing 50 mL of a basal medium consisting of 2.0% glucose, 1.0% yeast extract, and 2.0% sea salt. The flask was incubated at 25°C on an orbital shaker set at 150 rpm for 5 days, to produce an inoculum for further studies.

Optimisation of biomass and DHA yield

The effects of different culture conditions on cell biomass, lipid, and DHA yield for SR-21 were examined using batch cultures in 250-mL Erlenmeyer flasks using orbital shaking as described above. Each flask contained 150 mL medium and all cultures were inoculated with 5% (v/v) (7.5 mL) inoculum of cells from a culture grown in basal medium in shaken flasks for 5 days. Every experiment was repeated 3 times in the present study, unless indicated otherwise. Experimental conditions are described in detail in the relevant tables and figures.

Determination of cell biomass

Cells from each 250-mL flask were harvested by centrifugation (4000*G*, 5 min) with 2 washes with distilled water. The washed cell pellet was lyophilised until constant weight was achieved. The dry cell weight was the cell biomass yield for each sample.

Lipid extraction and fatty acid analysis

Lipids were extracted from 0.5 g lyophilised cells by using the method of Bligh and Dyer (1959) with 30 min sonication of the cells in 2 : 1 (v/v) chloroform : methanol mixture. The extracted lipids were weighed and then converted to fatty acid methyl esters (FAMEs) to determine the fatty acid profile of the sample following the procedure described by Hsu *et al*. (2004). The FAMEs were extracted with n-hexane and then the fatty acid profile was determined by gas chromatography (model 263–30, Dani Educational, Italy) equipped with a 60 m*0.53 mm ID, 1.00 µm film thickness, Supelcowax-10 capillary column (Supelco Inc., USA) and a flame-ionisation detector. Nitrogen was used as the carrier gas. Initial column temperature was set at 120°C, which was subsequently raised to 250°C at 5°C/min. The injector was kept at 250°C with an injection volume of 1 µL under splitless mode. The detector temperature was set at 270°C. Individual FA was identified by chromatographic comparison with authentic standards (Sigma, St Louis, MO) and the FA contents were quantified by comparing their peak areas with that of the internal standard (heptadecaenoic acid, C17:0).

Animals and diets

Fifteen 80-week-old white laying Leghorn hens (Hyline) of equal egg production were housed in individual cages with a light regimen of 16 h light : 8 h darkness. They were randomly divided into 3 groups. Diets were based on corn–soybean meal (ME, 2751 kcal/kg; CP, 15.5%; Ca, 3.4%; Table 1) supplemented with 0, 1.0, or 3.0% of SR-21 fungal meal. Vitamin E (37.5 IU/kg of diet) in the premix served as the antioxidant, and the diets were made up for the 3-week experimental period and stored in a 4°C refrigerator to prevent lipid oxidation. The major fatty acids of the fungal meal were palmitic acid (55.9%) and DHA (32.7%) and the fatty acid profile is listed in Table 2. The dietary FA composition is listed in Table 3. Feed and water were provided *ad libitum*. After feeding the hens with a control diet (containing 1.8% tallow) for 1 week to adapt to the experimental diet, the birds were fed with the experimental diets for 3 weeks. During the feeding period, eggs were collected every day and stored at −20°C for further analysis. Egg production rate, egg weight, yolk weight, egg mass, and DHA concentration were measured on the 0, 7th, 14th, and 21th day of feeding. At the end of the 3-week feeding period, the feed intake and feed conversion ratio (g feed/g egg) were calculated. The blood samples were obtained from the brachial vein (*V. Ulnaris*) using EDTA as anticoagulant. After the hens were sacrificed by cervical dislocation, 3 g of liver tissue were obtained for determination of DHA concentration and the remaining liver sample was used for RNA extraction. Liver samples were removed, wrapped in foil, frozen in liquid nitrogen, and stored at −70°C. Egg yolk was freeze-dried for further analysis. Plasma, dry egg yolk, and diet samples were frozen at −70°C until analysis of DHA concentration.

Table 1. Composition of experimental diets

Ingredients	Control	1% Fungal meal (%)	3% Fungal meal
Yellow corn	62.47	62.47	62.47
Soybean meal	23.16	23.16	23.16
Tallow	1.8	1.5	0.9
Fungal meal	–	1.0	3.0
Cellulose	2.1	1.4	–
Dicalcium phosphate	1.5	1.5	1.5
Limestone, pulverised	8.0	8.0	8.0
Iodised salt	0.3	0.3	0.3
Vitamin premix[A]	0.3	0.3	0.3
Mineral premix[B]	0.2	0.2	0.2
DL-Methionine	0.17	0.17	0.17
Total	100.00	100.00	100.00
Analysed lipid content (%)	10.5	10.6	10.6
Crude protein (%)	15.50	15.50	15.50
ME (kcal/kg)	2751	2751	2751
Calcium (%)	3.4	3.4	3.4

[A] Supplied per kg of diet: Vit. A, 11250 IU; Vit. D_3, 1200 IU; Vit. E, 37.5 IU; Vit. K, 2 mg; Vit. B_1, 2.6 mg; Vit. B_2, 8 mg; Vit. B_6, 3 mg; pantothenic acid, 15 mg; niacin, 60 mg; biotin, 0.2 mg; folic acid, 0.65 mg; Vit. B_{12}, 0.013 mg.
[B] Supplied per kg of diet: Cu, 10 mg; Fe, 100 mg; Mn, 60 mg; Zn, 65 mg; Se, 0.15 mg.

RNA analysis

Following the procedure described by Hsu and Ding (2003), total RNA was extracted from the powdered, frozen livers, separated by denatured electrophoresis, blotted to nylon membranes, and hybridised with radiolabelled cDNA probes in Ultrahyb solution (Ambion Inc., Austin, TX, USA). The duck genes of fatty acid synthase (FAS)

Table 2. Fatty acid composition (% of total fatty acids) of *Schizochytrium limacinum* SR-21 fungal meal

Fatty acid composition							
C16:0	C16:1	C18:0	C18:1	C18:2 (*n*-6)	C20:5 (*n*-3)	C22:5 (*n*-6)	C22:6 (*n*-3)
55.9	0.1	1.5	0.1	0.1	0.6	4.9	32.7

Table 3. Fatty acid compositions of experimental diets expressed as percentage of total identified fatty acids

Fatty acid profile	Control	1% Fungal meal	3% Fungal meal
C16:0	17.75	18.62	21.64
C16:1	5.87	5.54	4.83
C18:0	8.95	8.65	8.03
C18:1	36.66	36.02	31.40
C18:2 *n*-6	24.99	23.91	23.83
C18:3 *n*-3	4.43	3.77	3.59
C20:4 *n*-6	1.37	1.39	1.42
C20:5 *n*-3	0	0.02	0.08
C22:6 *n*-3	0	2.13	5.28

and lipogenesis-related transcriptional factors, sterol response element binding protein 1 (SREBP1) and SREBP2 (Yen *et al*. 2005), were used to generate probes for northern analysis of these genes. The FAS cDNA fragment is 490 bp with 91% homology with the chicken FAS (GenBank, J04485). The SREBP1 cDNA fragment is 283 bp with 93% homology with the chicken SREBP1 (GenBank, AJ310768). The SREBP2 cDNA fragment is 386 bp with 83% homology with the chicken SREBP2 (GenBank, AJ310769). The primer sequences to generate the gene fragments are listed in Table 4. The blots after hybridisation were quantified by phosphor-image analysis. The densitometric value for an individual transcript in a sample lane was normalised to the densitometric value for the 18S rRNA in the same lane.

Statistical analysis

Statistical analyses of experimental results were made using analysis of variance. Duncan's multiple range test was applied to detect differences among means using SAS statistical software (SAS Institute 2001). A probability level of 0.05 was used to show statistical significance.

Results

The cell growth (Table 5) increased as the concentration of glucose in the medium rose from 1% (biomass of 4.58 g/L) to 4.0% (biomass of 7.27 g/L), but the cell mass decreased at 5.0% and 6.0% glucose (biomass of 7.10 and 5.87 g/L). The lipid content of the biomass ranged from 28.79 to 45.84% in different treatment groups. The lipid yield varied from 1.32 to 3.26 g/L for different treatments. Culture at 5.0% glucose resulted in the highest lipid yield. The DHA concentration of total fatty acids ranged from 27.42 to 28.38% for different treatments and there were no significant differences among treatments ($P > 0.05$). The DHA productivity increased as the glucose concentration in the medium rose from 1.0% (373 mg/L) to 5.0% (874 mg/L), and decreased with 6.0% glucose.

Because the SR-21 is a marine fungus, we investigated the effect of different concentrations of sea salt (0–3.0%) on cell growth and DHA productivity. As shown in Table 6, 0% sea salt treatment generated the lowest biomass yield (5.18 g/L), whereas the 1–3% treatments sustained much better cell growth that was not related to concentration. The lipid content in biomass among treatments ranged from 21.75 to 34.66%. The 1 and 2% sea salt treatments resulted in a greater total lipid yield (3.33 and 3.10 g/L, respectively) than 0% sea salt. The DHA concentration of total fatty acids was not affected by sea salt concentration. However, numerically the maximal DHA productivity was obtained at 2.0% sea salt concentration.

The effects of initial culture pH on cell growth and DHA productivity were tested with the pH ranging from 5.0 to 8.0 (Fig. 1). Biomass yield and DHA productivity were not affected by initial pH ($P > 0.05$). The biomass yield increased from 4.94 g/L at 2 days to reach a plateau of 9.66 g/L at 8 days, and then declined after 10 days of incubation (8.75 g/L, Fig. 2). The DHA productivity increased from 2 to 6 days, and then decreased from 6 to 10 days.

Table 4. Primers for FAS, transcription factors, SREBP1, and SREBP2

Cloned gene	Sequence of primer	Size (bp)	Origin
FAS	Forward: 5′-ATTGACACAGCCTGCTCCTC-3′ Reverse: 5′-ACGGCTCTCTCTCACATTGG-3′	490	Goose
SREBP1	Forward: 5′-GCGCTACCGCTATCCATCA-3′ Reverse: 5′-GGTCGGCATCTCCATCACCT-3′	283	Chicken
SREBP2	Forward: 5′-TAATACGACTCACTATAGCG-3′ Reverse: 5′-TCAAGTCCTTCAGCCTCAAG-3′	386	Human

Table 5. Effect of glucose concentration on biomass yield (g/L), lipid content (%, d.w.), lipid yield (g/L), DHA content of total fatty acids (%) and DHA productivity (mg/L) of *S. limacinum* SR-21

Culture condition: 25°C, 150 rpm, 5 days. Within columns, means followed by the same letter are not significantly different at $P = 0.05$

Glucose conc.	Biomass yield	Lipid content	Lipid yield	DHA content	DHA productivity
1.0%	4.58 ± 0.45b	28.79 ± 1.26c	1.32 ± 0.06d	28.27 ± 1.27a	373 ± 17c
2.0%	6.19 ± 0.19a	39.91 ± 2.90ab	2.47 ± 0.18bc	27.89 ± 2.49a	688 ± 61b
3.0%	6.50 ± 0.75a	40.12 ± 5.49ab	2.61 ± 0.36b	27.44 ± 3.39a	716 ± 89b
4.0%	7.27 ± 0.26a	41.28 ± 1.44ab	3.00 ± 0.10a	28.38 ± 3.67a	852 ± 110a
5.0%	7.10 ± 0.10a	45.84 ± 0.64a	3.26 ± 0.05a	28.32 ± 3.89a	874 ± 43a
6.0%	5.87 ± 0.12a	37.05 ± 5.44b	2.18 ± 0.32c	27.42 ± 1.48a	602 ± 29b

Table 6. Effect of sea salt concentration on biomass yield (g/L), lipid content (%, d.w.), lipid yield (g/L), DHA concentration of total fatty acids (%), and DHA productivity (mg/L) of *S. limacinum* SR-21
Culture condition: 5% glucose, 1% yeast extract, 150 rpm, 5 days at 25°C. Within columns, means followed by the same letter are not significantly different at $P = 0.05$

Sea salt conc.	Biomass yield	Lipid content	Lipid yield	DHA conc.	DHA productivity
0%	5.18 ± 0.91b	31.59 ± 2.69ab	1.47 ± 0.12b	27.53 ± 7.76a	300 ± 42b
1.0%	9.90 ± 1.12a	34.66 ± 2.16a	3.33 ± 0.63a	27.69 ± 6.51a	413 ± 18ab
2.0%	10.20 ± 1.79a	27.05 ± 0.23bc	3.10 ± 0.01a	35.18 ± 0.46a	447 ± 20a
3.0%	10.89 ± 0.78a	21.75 ± 0.07c	2.48 ± 0.05ab	35.26 ± 6.14a	382 ± 44ab

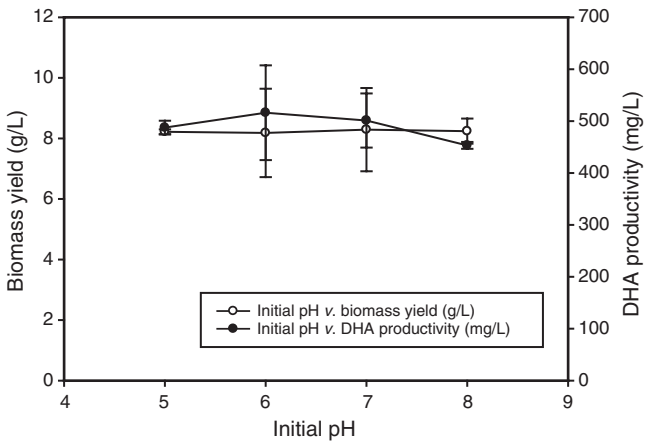

Fig. 1. Effects of the initial pH of the growth medium on biomass yield and docosahexaenoic acid (DHA) productivity of *S. limacinum* SR-21. The SR-21 was cultured in medium containing 5% glucose, 2% sea salt, and 1% yeast extract, for 5 days at 25°C. The initial pH ranged from 5 to 8.

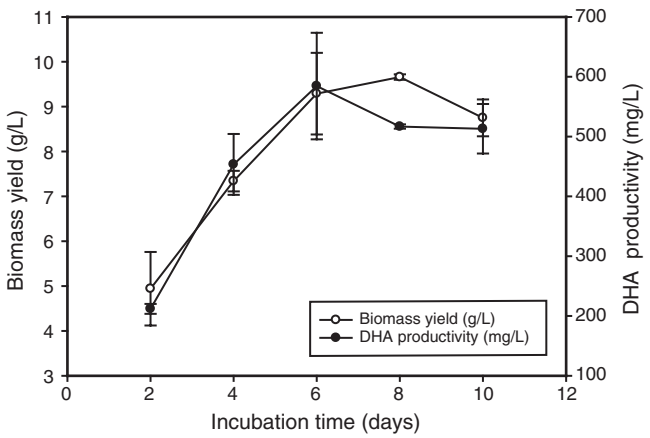

Fig. 2. Effects of the incubation time on biomass yield and docosahexaenoic acid (DHA) productivity of *S. limacinum* SR-21. The SR-21 was cultured in medium containing 5% glucose, 2% sea salt, 1% yeast extract, and with an initial pH of 6 and temperature at 25°C. The cells were harvested every 2 days up to 10 days.

The maximal DHA productivity (584 mg/L) was obtained from the 6-day incubation.

Although the SR-21 when cultured at 20°C generated greater DHA concentration than cultured at other temperatures (25°C or 30°C), the total biomass yield, lipid content in biomass, and overall DHA productivity were not significantly affected by temperature ($P > 0.05$, Table 7). After a 5-day incubation, the DHA productivity at 25°C was 601 mg/L, whereas it was 552 and 468 mg/L for treatments of 20 and 30°C, respectively.

The egg production, egg weight, yolk weight, egg mass, feed intake, and feed conversion ratio of the laying hens were not significantly affected by the fungal meal (containing 27% lipid and 33% of the total FA as DHA) supplementation in the diet ($P > 0.05$). During the experimental period, the egg production rate was 68–86%, egg weights were 58.13–66.83 g, and yolk weights were 15.86–18.02 g for different dietary treatments. The feed intake was 98–105 (g/day), and the feed conversion ratio (g/g) was 2.19–2.35 (feed/egg) for different dietary treatments. The egg mass was 46.8–53.4 g/hen.day for different dietary treatments.

The DHA concentrations of yolk, liver, and plasma were significantly increased by the dietary fungal meal supplementation ($P < 0.05$, Tables 8, 9). The DHA concentrations in yolks were 0.36%, 1.17%, and 1.81% of total fatty acids for 0%, 1%, and 3% fungal meal treatments for 3 weeks, respectively (Table 8). The liver DHA concentrations of laying hens were 1.76%, 4.82%, and 7.11% of total fatty acids for 0%, 1%, and 3% fungal meal treatments ($P < 0.05$; Table 9).

The hepatic FAS, SREBP1, and SREBP2 mRNA concentrations were not affected by the 3 dietary treatments ($P > 0.05$; Fig. 3). The hepatic lipogenesis-related genes of the laying hens were not affected by the dietary fungal meal supplementation.

Discussion

Several species that belong to the genus close to *Thraustochytrium* (Labyrinturomycota) have been shown to contain large quantities of DHA in their cytoplasm, and have been used as alternative sources for commercial DHA

Table 7. Effect of culture temperature (°C) on biomass yield (g/L), lipid content (%, d.w.), lipid yield (g/L), DHA concentration of total fatty acids (%) and DHA productivity (mg/L) of *S. limacinum* SR-21
Culture condition: 5% glucose, 1% yeast extract, and 150 rpm for 5 days. Within columns, means followed by the same letter are not significantly different at $P = 0.05$

Temp.	Biomass yield	Lipid content	Lipid yield	DHA conc.	DHA productivity
20	10.99 ± 1.69a	21.39 ± 1.38a	2.30 ± 0.57a	40.39 ± 2.25a	552 ± 58a
25	12.23 ± 0.80a	25.84 ± 0.64a	3.13 ± 0.17a	24.28 ± 1.83b	601 ± 35a
30	8.73 ± 1.71a	19.84 ± 1.09a	1.67 ± 0.48a	23.22 ± 2.37b	468 ± 95a

Table 8. Effect of dietary *S. limacinum* SR-21 fungal meal on DHA levels of laying Leghorn hen egg yolk
Values are means ± s.d. of 5 eggs. Within columns, means followed by the same letter are not significantly different at $P = 0.05$

Time	% of total fatty acid			
	0 Week	1 Week	2 Week	3 Week
Control	0.42 ± 0.12a	0.37 ± 0.10b	0.41 ± 0.08c	0.36 ± 0.10c
1.0% Fungal meal	0.35 ± 0.07a	0.50 ± 0.12b	1.03 ± 0.24b	1.17 ± 0.22b
3.0% Fungal meal	0.40 ± 0.12a	1.08 ± 0.18a	1.74 ± 0.12a	1.81 ± 0.26a

Table 9. Effect of dietary *S. limacinum* SR-21 fungal meal on DHA levels in the plasma and liver of laying Leghorn hens
Values are means ± s.d. of 5 hens. Within columns, means followed by the same letter are not significantly different at $P = 0.05$

	% of total fatty acids	
	Plasma	Liver
Control	1.02 ± 0.09c	1.76 ± 0.17c
1.0% Fungal meal	4.41 ± 0.93b	4.82 ± 0.60b
3.0% Fungal meal	7.26 ± 1.24a	7.11 ± 1.19a

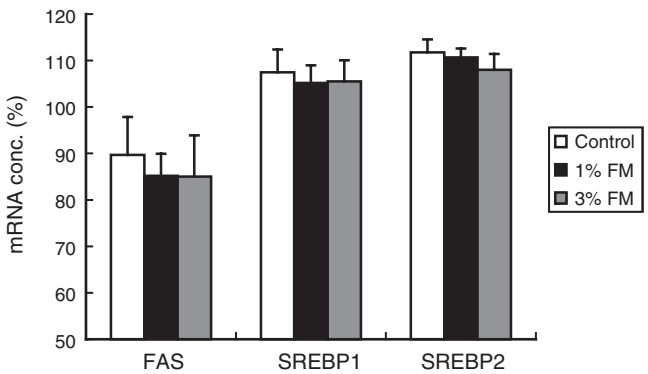

Fig. 3. The effect of dietary SR-21 fungal meal (FM) supplementation on the abundance of FAS, SREBP1, and SREBP2 mRNA in the livers of laying Leghorn hens. Leghorn hens were fed diets containing either 0%, 1%, or 3% *S. limacinum* SR-21 fungal meal for 21 days (6 hens per group). On the day of sampling, hens were killed 2 h after feeding. The mRNA concentrations of FAS, SREBP1, and SREBP2, and concentration of 18S rRNA were determined by Northern analysis. The mRNA values were normalised to 18S rRNA content with $n = 6$ replicates/treatment. There was no treatment effect detected ($P > 0.05$).

production (Yongmanitchai and Ward 1992). In the present study, SR-21, a member of the *Thraustochytrium* family, also contained a high DHA concentration of about 30% of total fatty acids. Similar results were observed by Yokochi *et al*. (1998) who established standard culture conditions for *S. limacinum* SR-21. They found that about 30% of total FA was DHA when cultured with glucose, fructose, and glycerol. When cultured with these monosaccharides and glycerol as carbon sources, there was a greater dry cell weight than with di- or polysaccharides such as maltose and starch. Generally, excess energy sources in fungi culture will be metabolised to lipids (Evans and Ratledge 1984). Therefore, increasing the concentration of the carbon source will increase the lipid content in the culture medium. In the present study, the optimal glucose concentration for DHA production was 5.0%. With 5.0% glucose, the DHA productivity reached a plateau at 874 mg/L. Above 5.0% glucose, there was a reduction in culture biomass production, resulting in an overall decline in DHA productivity (Table 4). The high glucose concentration may create an inadequate osmotic pressure that represses cell growth. These results are not in agreement with the observations of Yokochi *et al*. (1998) who found that the maximal cell growth and DHA yield were obtained at 9% glucose. The discrepancy may due to different nitrogen sources and concentrations used in the 2 different experiments. In the present study, the nitrogen source was 1.0% yeast extract, whereas in Yokochi *et al*. (1998), it was 2.0% corn steep liquor.

Observations from Yokochi *et al*. (1998) showed that the SR-21 can tolerate a wide range of salinity. Although the fungus can grow at zero salinity, addition of 2% sea salt resulted in greater biomass and increased DHA productivity (Yokochi *et al*. 1998; Table 5).

A range of initial pH from 5 to 8 in the culture medium did not have significant influence on the biomass yield or DHA productivity. The strain can grow well at a pH range of 5–8. Numerically, an initial pH of 6 was optimal for DHA production. An adequate initial pH in media for fungi or algae to produce EPA or DHA ranged from 6.0 to 7.6 (Bajpai *et al*. 1992; Yongmanitchai and Ward 1992). Li and Ward (1994) examined the DHA production of another marine fungus, *Thraustochytrium roseum* ATCC 28210, and found that an initial pH of 6.0 was optimal for maximum biomass and DHA production.

Moreton (1987) indicated that the major lipid accumulation in the cytoplasm of the cells was in the late-logarithmic growth phase and stationary phase, but not in the early logarithmic growth phase. In the current study, the logarithmic growth of strain SR-21 was observed within the first 6 days of incubation. Biomass yield in the medium reached the plateau after 8 days of cultivation and then decreased. The production of DHA increased in parallel with the cell growth and reached a plateau on the 6th day, and then decreased gradually.

The optimal temperature for growing SR-21 was found to be 25°C (Yokochi *et al*. 1998). We also found that this fungus grew well when cultured at 25°C, and the DHA content of total FA was decreased when the culture temperature was increased. Other species, *T. aureum* and *T. roseum*, have the same optimal culture temperature of 25°C (Bajpai *et al*. 1991; Li and Ward 1994). Bowles *et al*. (1999) showed that Thraustochytrid species isolated from a subtropical mangrove produced higher levels of biomass, but the DHA fraction of the fatty acids was relatively lower than from SR-21 (23.79%). Lower temperatures have been found to result in an increase in lipid unsaturation (Kates and Hagen 1964; Wassef 1977). Similar to what was found in the current study, the lower temperature resulted in lower biomass, but produced higher levels of DHA in the total fatty acids (45.73%). Taken together, we suggest that the optimal culture conditions for SR21 are 5% glucose, 1% yeast extract, and 2% sea salt with a pH of 6.0 and incubation for 6 days at 25°C.

Fish oil has been used in the diet of laying hens to enrich the EPA or DHA content of eggs. We used the DHA-enriched fungus, SR-21, as a replacement for fish oil. This fungal meal enriched DHA in the egg yolk and also increased the DHA concentrations in livers and plasma of the laying hens. Several groups have indicated that egg production, egg weight, or yolk size was decreased by dietary PUFA (Whitehead *et al*. 1993; van-Elswyk *et al*. 1994; Scheideler *et al*. 1994), whereas other reports indicated no effect (Hargis *et al*. 1991; Ferrier *et al*. 1995; Meluzzi *et al*. 2000; Chen and Hsu 2003). We showed that egg production, egg weight, yolk size, and egg mass of the laying Leghorn hens were not affected by supplementation with 1 or 3% fungal meal in the diet for 3 weeks.

Nash *et al*. (1995) found that feeding laying hens diets containing 12% herring meal increased plasma DHA from 1.27% to 2.73% of total FA, or to 2.69% of total FA in another experiment. We observed (Table 9) a much greater increase (about 4- to 7-fold) of plasma DHA with dietary supplementations of 1 or 3% SR-21 fungal meal for laying hens. Addition of 1% dietary menhaden oil for 3 weeks increased the yolk DHA content to 2.98% compared with 0.53% in the control group (Huang *et al*. 1990). Chen and Hsu (2003) found that incorporation of dietary DHA into egg yolk lipids reached a plateau after 2 weeks of feeding with fish-oil-containing diets. Therefore, an enrichment of dietary DHA will increase yolk DHA concentration. We also found that after 3 weeks of feeding with SR-21 fungal meal, the yolk DHA concentration was increased 5-fold compared with the control group. Perhaps a greater yolk DHA concentration could be achieved if we fed the hens with more than 3% fungal meal.

In the current study, the DHA concentration in the liver was increased after 3 weeks of feeding with fungal meal, confirming data in the literature (Huang *et al*. 1990; Leskanich and Noble 1997; López-Ferrer *et al.* 2001).

In poultry, liver is the major site for FA and cholesterol synthesis (Leveille *et al*. 1968, 1975). Two key enzymes involved in these processes are FAS and 3-hydroxyl-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), which are regulated by 2 transcription factors, SREBP1 and SREBP2, respectively (Back *et al*. 1986; Semenkovich 1997; Horton *et al*. 1998; Gondret *et al*. 2001). Recent researchers have shown that PUFA reduce the SREBP1 expression in rodent and pig livers (Xu *et al*. 1999; Yahagi *et al*. 1999; Hsu *et al*. 2004). Unlike the mammalian model, we did not find any effect of dietary DHA on the expression of the FAS gene or of these lipogenic transcription factors in the liver of the laying hens (Fig. 3). These findings are similar to what was reported by Cheng *et al*. (2004). Physiologically, the *de novo* lipogenesis in the liver of laying hens is high in order to generate yolk fat. Such cellular function may be related to a high oestrogen concentration, a factor that may induce fatty liver in aged laying hens (Harms *et al*. 1972; Polin and Wolford 1977; Dashti *et al*. 1983). Perhaps in the laying hens, the hormonal regulation is actively increasing lipogenesis and the hen is relatively unresponsive to dietary DHA content. Alternatively, the supplement levels of the fungal meal may not be high enough to affect the expression of these genes.

In conclusion, the present study demonstrates that dietary DHA-enriched fungal meal obtained from *S. limacinum* SR-21, can be digested and incorporated into eggs, plasma, and liver in the laying hens. Even though the expression of the FAS gene and the 2 transcription factors (SREBP1 and SREBP2) in the livers of laying hens was not affected by dietary DHA enrichments, the enrichment of yolk DHA may improve the value of the eggs for human consumption.

Acknowledgments

This work was funded by the Council of Agriculture in Taiwan [Grant number 93AS-3.1.4-U1 (7)]. We thank Y. T. Kao for her technical assistance and Harry Mersmann for his input in enhancing this publication.

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Manuscript received 19 March 2005, accepted 7 September 2005