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Identity and Safety of a Novel *Aurantiochytrium sp.* for Terrestrial Heterotrophic Docosahexaenoic Acid Production

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Abstract

The objective of the studies in this paper was to expand on the published toxicological assessment of *Aurantiochytrium limacinum* (AURA) with further strain characterization and to investigate the potential for the biomass or extracted oil to have antimicrobial properties or undesirable substances. AURA is being investigated as a novel source of the omega-3 long-chain polyunsaturated fatty acid docosahexaenoic acid (DHA) for enriching foods of animal origin by means of feed supplementation. In the first studies, we provided the 18S rRNA identification of the novel marine isolated thraustochytrid, established the nutritional composition of AURA biomass for application as a food or feed ingredient including proximate analysis and fatty acid profiling, and confirmed the DHA production potential of the strain. We determined through minimum inhibitory concentration (MIC) analysis that the unextracted AURA biomass was safe, showing no antimicrobial influence and no evidence of any deleterious effects of this product or its extracts at concentrations up to 1% w/w on the reference human intestinal bacteria tested. This would indicate that AURA should not stimulate selective pressure on the commensal microbiota and is therefore unlikely to aid development of antimicrobial resistance and the concomitant harm to humans and animals. Further analysis revealed that the AURA biomass produced through industrial heterotrophic fermentation was free from undesirables; toxic marine microalgal metabolites, heavy metals, pesticides, microbial contaminants, and mycotoxins. Including heterotrophically-grown AURA in food or feed, up to 1% w/w, is a safe and environmentally beneficial strategy for DHA supplementation.

Keywords

Thraustochytrid, Docosahexaenoic Acid, Fatty Acids, *Aurantiochytrium*

limacinum, Minimum Inhibitory Concentration, Antimicrobial Resistance

1. Introduction

The challenges of feeding a growing global population continue to be met by the food industry worldwide. However, it is true that changing environmental considerations must influence the origin of our food sources. Wild-caught seafood is the primary source of dietary long chain polyunsaturated fatty acids in the human diet through the direct consumption of seafood, nutritional supplements or through inclusion of fish products into the diets of other aquatic or terrestrial animals [1]. Supplementation of ruminant diets with fish oil has been shown to promote the prevalence of unsaturated fatty acids in meat and dairy products [2] [3], a fact that may have implications for human health where a reduction in saturated fat consumption and an accompanying increase in unsaturated fats can reduce the incidence of heart disease and other associated conditions [4] [5]. Harvesting from the marine environment to feed aquaculture and terrestrial agriculture is unlikely to be sustainable in the long term, and appropriate alternatives must be sought [6] [7]. When considering the source of unsaturated fatty acids, there are a range of safety factors to consider. Bioaccumulation of heavy metals, persistent organic contaminants and toxins derived from marine microalgae can result in high levels of contaminants in fish oils owing to the low metabolic capacity of fish [8] [9]. Reduction or removal of these contaminants is vital to prevent introduction into the food chain as their toxicity can offset the benefits of inclusion in food and feed to humans and animals alike [10].

Oils derived from terrestrial heterotrophic fermentation using marine sourced protists have a number of proven advantages over fish oil; terrestrial production is scalable, it is more sustainable owing to growth on simple carbon sources, there is no direct impact on the marine environment, and it can produce a more consistent and cost-effective product [11] [12]. Frequently referred to as an algae or marine microalgae, *Aurantiochytrium limacinum* (AURA) is actually a member of the Stramenopile group, recently described as athraustochytrid or protist, exhibiting non-photosynthetic fungal physiological traits [13]. This oleaginous unicellular organism has proven safe through recent toxicological and genotoxicity testing [14] and useful for the production of docosahexaenoic acid (DHA) rich biomass for dietary application in food-producing animals. Such dietary supplementation has demonstrated efficacy in elevating levels of DHA and EPA (eicosapentaenoic acid) as well as other omega-3 fatty acids in milk [15], meat [16] [17] [18] and eggs [19] [20], a valuable means of supplementing the human diet.

From a safety perspective it is notable that primary desirable components of fish/algal oil supplements (DHA for example) have shown some antimicrobial activity [4]. There is often an overlap between compounds regarded as toxins and those seen as antimicrobials. Secondary metabolites (SM) derived from cel-

lular processes can give an organism an advantage against its competitors, penicillin is an example of such a compound [21]. Marine microalgae are known producers of bioactive SMs such as Saxitoxin, Domoic acid and a variety of polyketides [9] [22]. Polyketides are SMs associated with fatty acid synthesis and are often of clinical importance with roles in immunosuppression, cancer treatment and as antimicrobial agents [23] [24] [25]. In the context of mitigating risks associated with the introduction of a novel feed material, it is prudent to determine the overall safety of the material and assess the potential for antimicrobial activity, or for antimicrobial residues to be suspended within the material.

The goal with this set of studies was to provide complementary information to our recent safety assessment of AURA [14]; including more detailed information on the identity and product composition; safety information in relation to contaminants and undesirables: mycotoxins, heavy metals and pesticides; a hazard characterization confirming that unextracted *Aurantiochytrium*, its biomass and metabolites, are safe with respect to antimicrobial resistance to a reasonable certainty of no harm to humans; and finally test whether residues or metabolites from the unextracted *Aurantiochytrium* biomass, or extracted oil, could result in putative residues in edible tissues that may affect human intestinal flora. Our studies followed the United States Food and Drug Administration Center for Veterinary Medicine (CVM) Guidance for Industry (GFI) #3 "*General Principles for Evaluating the Safety of Compounds Used in Food-Producing Animals*" [26] for the residue chemistry portion; GFI #152, "*Evaluating the Safety of Antimicrobial New Animal Drugs With Regard to Their Microbiological Effects on Bacteria of Human Health Concern*" [27] for antimicrobial resistance testing, and GFI #159 "*Studies to Evaluate the Safety of Residues of Veterinary Drugs in Human Food: General Approach to Establish a Microbiological ADI*" [28] for guidance on test procedures to interrogate potential impacts of AURA on the human intestinal microflora.

2. Materials and Methods

2.1. Biochemical Profiling

A heterotrophically grown, unextracted AURA biomass powder (CCAP 4087/2) was provided to Minnesota Valley Testing Laboratory (MVTL, New Ulm, MN, USA) by Alltech Inc. (Nicholasville, KY, USA). The analytical composition of the biomass product was determined in compliance with current Good Laboratory Practices (GLP) guidelines (21 CFR part 58): crude protein (AOAC 990.03 [29]), crude fat (AOAC 954.02 [30]), Fatty acid composition (AOAC 996.06 [31]), moisture (AOAC 930.15 [32]), and ash (AOAC 942.05 [33]).

2.2. 18S rRNA Culture Identification

A purified monoculture of metabolically active culture was supplied to the Culture Collection of Algae at the University of Texas (Austin, Texas, USA) by All-

tech Inc. (Nicholasville, KY, USA) for identification and characterisation within the College of Natural Sciences (University of Texas at Austin, College of Natural Sciences, Austin, TX, USA). Colonies were isolated and multiplied in Medium-H for the following analyses: 18S rRNA identification, Minimum inhibitory concentration and polyketide analysis (Sections 2.3, 2.4 and 2.5). DNA was extracted from the micro-organism using a modification of the JGI protocol for CTAB mediated bacterial genomic DNA isolation (outlined in [34]) and PCR amplification was performed with universal 18S primer sets. Reading produced a partial 18S sequence which was compared to the NCBI BLAST database to determine sequence similarity.

2.3. Minimum Inhibitory Concentration (Disc Diffusion)

Compounds, extraction and bacterial strains

Whole cells were selected during mid-exponential phase and concentrated through centrifugation. The growth-medium supernatant was separated and stored for later solid-phase extraction while the remaining cells were prepared for solvent extraction. Solvent extraction involved repeated suspension in cold acetone followed by centrifugation and decantation to remove pigmentation. Remaining cellular mass was then extracted with four methanol washes before being homogenized in 80% 1-propanol/ water solution. Homogenate underwent centrifugation to separate cellular material and the subsequent supernatant joined the methanol extracts. This homogenization process was repeated a second time with the 80% 1-propanol/water solution. The extract mixture was evaporated to remove solvent and resuspended in 2 ml of water. 2 ml of ethyl acetate was used to de-fat this mixture; the aqueous phase was de-fatted a total of four times and again dried. In order to elute the desired analytes, cellular extract was suspended in 10 ml of 25% 1-propanol/water and passed through a chromatography cartridge, washed with 45% 1-propanol/water solution, eluted in 80% 1-propanol/water solution and dried. 30 ml of growth-medium supernatant was mixed with 10 ml of 25% 1-propanol/water solution and the same solid-phase extraction process was followed. The resulting dried cellular extract and supernatants were suspended in 5% methanol/water solution for use in MIC analysis [35]. DH5a *E. coli* culture was used as the reference culture for the MIC analyses.

Minimum inhibitory concentration determination

The experiment was carried out in accordance with CLSI M02-A12 guidelines using the Disc diffusion method [36]. Whole cells or extracts were tested as follows.

Nine discs were placed onto sterile LB agar plates to accommodate the cell extracts. Eight discs were placed around the plate and one disc was applied to the center of the plate. The central disc received a dose of 5% Methanol solution as a negative control. Four discs received concentrations of the test solutions (**Table 1**) while the remaining four discs had positive controls applied. For analysis with

Table 1. Disc diffusion test and positive control concentrations.

Test/positive control solution	Conc. Applied (Replicate 1)	Conc. Applied (Replicate 2)
Cellular extract	10 µg and 20 µg	40 µg and 80 µg
Supernatant extract	10 µg and 20 µg	40 µg and 80 µg
Ampicillin	100 µg and 200 µg	1 mg and 2 mg
Streptomycin	100 ng and 200 ng	400 ng and 800 ng

whole cells, the four test discs were replaced by spotting four 10 µl drops of dense AURA culture directly onto the agar). After test and control solutions had been applied and the plates allowed to dry, *E. coli* (DH5a) culture was inoculated onto the agar by means of a misting nebulizer. Duplicate plates were incubated at 37˚C for 12 - 18 hours. Observation of plates established if zones of clearance were present or absent.

2.4. Minimum Inhibitory Concentration (Broth Microdilution)

The following bacterial stains were selected as per VICH GL36 (R) guidelines [28] and procured from the American Type Culture Collection (Manassas, VA, USA) (**Table 2**).

Minimum inhibitory concentration determination

The experiments were carried out in accordance with CLSI M07-A9 guidelines using the Broth macro-dilution method at ABC Laboratories (Gainesville, FL, USA) [37]. Each product was tested separately with each microorganism under aerobic, anaerobic or microaerobic growth conditions as appropriate. Inocula were initially prepared on solid media, Trypticase Soy Agar (TSA) under aerobic conditions for *E. coli* and *E. faecalis*, TSA with 5% Sheep blood (SBA) under anaerobic organisms for *B. fragilis*, *B. longum*, C. *aerofaciens*, *F. canifelinum*, *P. anaerobius* and *C. difficile* while *L. brevis* was prepared on de Mann, Rogosa Sharpe (MRS) agar under microaerobic conditions. Colonies were selected and suspended in sterile 0.85% saline in order to obtain optical densities of 2.0 McFarland prior to the experiment.

Aerobic analysis

Serial dilutions (1:1) (n = 13) of algal powder and algal oil suspensions were separately prepared in Mueller Hinton Broth (MHB) to obtain 1% down to 0.0002441406% test concentrations. Positive and negative controls were similarly prepared in MHB. 10 µl volumes were inoculated into each broth suspension to form an initial concentration of ca. 1×10^5 CFU/ml. Dilution tubes were incubated aerobically at 35˚C ± 2˚C for 18 - 20 hours.

Anaerobic analysis

Serial dilutions (1:1) (n = 13) of algal powder and algal oil suspensions were separately prepared in Brucella Broth with Hemin and Vitamin K^1 to obtain 1% down to 0.0002441406% test concentrations. Positive and negative controls were similarly prepared in supplemented Brucella Broth. 10 µl volumes were inoculated

Table 2. Strains selected for broth macro dilution MIC analysis.

Organism	Source of Isolation
Bacteroides fragilis ATCC® 25285™	Appendix abscess
Bifidobacterium longum ATCC® 15707™	Intestine of Adult
Clostridium difficile ATCC® 700057™	Not specified; application: susceptibility testing
Enterococcus faecalis ATCC® 51299™	Peritoneal fluid
Escherichia coli ATCC® 25922™	Clinical isolate
Collinsella aerofaciens ATCC® 29738™	Human faeces
Fusobacterium canifelinum ATCC® BAA-689™	Clinical specimen, human, dog-bite wound
Lactobacillus brevis ATCC® 14869™	Human faeces
Peptostreptococcus anaerobius ATCC® 27337™	Not specified; application: QC testing

into each broth suspension to form an initial concentration of ca. 1×10^5 CFU/ml. Dilution tubes were incubated anaerobically at 36˚C ± 1˚C for 46 - 48 hours (*B. fragilis* and *B. longum*) or at 36˚C ± 1˚C for 70 - 72 hours (*C. difficile*, *C. aerofaciens*, *F. canifelinum* and *P. anaerobius*).

Microaerobic analysis

Serial dilutions (1:1) (n = 13) of algal powder and algal oil suspensions were separately prepared in MRS Broth to obtain 1% down to 0.0002441406% test concentrations. Positive and negative controls were similarly prepared in MRS Broth. 10 µl volumes were inoculated into each broth suspension to form an initial concentration of ca. 3×10^5 CFU/ml. Dilution tubes were incubated microaerobically at 30˚C ± 2˚C for 46 - 48 hours.

Following incubation, tubes containing MHB, supplemented Brucella Broth and MRS Broth were examined and scored positive or negative for turbidity. The MIC was determined from the lowest concentration of test material that acted to completely inhibit bacterial growth.

2.5. Algal Toxin Analysis

Polyketide analysis

The presence of "Golden algae" polyketides was carried out by LC/MS metabolic fingerprinting. Whole cells were selected during mid-exponential phase and concentrated through centrifugation. Cellular extracts and supernatant extracts were prepared as per [35]. Extract fractions were suspended in 5% methanol solution before being diluted to 1 µg/µl for LC/MS analysis. Spectra were collated and peaks in the 900 - 1200 m/z regions were compared to peaks for known polyketides.

Domoic acid and epi-domoic acid analysis

Unextracted *Aurantiochytrium limacinum* (CCAP 4087/2) biomass (produced by Alltech Inc. KY, USA) was supplied to the Marine Institute (Galway, Ireland). Samples were prepared in 50:50 methanol /water solution and extracted through ultrasonication for 15 minutes. HPLC-DAD and UHPLC-DAD analysis for the

presence of domoic acid toxins was carried out according to in-house protocols.

2.6. Contaminants, Residues and Undesirables

These analyses were carried out by Minnesota Valley Testing Laboratories Inc (Minnesota, USA) in compliance with current Good Laboratory Practices (GLP) guidelines (21 CFR part 58) according to the methods indicated in Table 3.

3. Results

3.1. Biochemical Profiling

Proximate analysis confirmed the oleaginous nature of the unextracted *Aurantiochytrium* biomass with an average 72.85% crude fat in the sample (Table 4). Percentages of crude protein, moisture and ash are all low (11.44%, 3.10% and 3.39% respectively), indicating significant conversion of substrate to desirable fatty acids. Table 5 reveals the fatty acid profile and the lipid composition. The major components are palmitic acid (average 36.35% w/w) and docosahexaenoic acid (average 18.17% w/w). The total polyunsaturated fat content in the AURA biomass is 18.83% w/w of which DHA constitutes the greatest proportion.

3.2. 18S rRNA Identification

The partial 18S sequence assembled from the genetic identification can be seen in Figure 1. When compared to the NCBI BLAST database, the test strain was determined to be most comparable to the following six protists; *Aurantiochytrium* Sp. ST-2012 clone BJ61 (Genbank: JQ982491.1), *Aurantiochytrium* Sp. TF81 (Genbank: KM023695.1), *Aurantiochytrium* Sp. TF23 (Genbank: KM023689.1), *Aurantiochytrium* Sp. LY-2012 isolate PKU#Sed1 (Genbank: JX847370.1), *Aurantiochytrium* Sp. LY-2012 isolate PKU#MN7 (Genbank: JX847363.1), *Aurantiochytrium* Sp.

TGTGTNNGTATAAGCGATTGTACTGTGAGACTGCGAACGGCTCATTATATCAGTAATAATTTCTTCGGTAGTT
TCTTTTATATGGATACCTGCAGTAATTCTGGAAATAATACATGCTGTAAGAGCCCTGTATGGGGCTGCACTTAT
TAGATTGAAGCCGATTTTATTGGTGAATCATGATAATTGAGCAGATTGACTTTTT *
TCGATGAATCGTTTGAGTTTCTGCCCCATCAGTTGTCGACGGTAGTGTATTGGACTACGGTGACTATAACGGG
TGACGGAGAGTTAGGGCTCGACTCCGGAGAGGGAGCCTGAGAGACGGCTACCATATCCAAGGATAGCNNNN
GGCGCGTA *
ATCTTTCACTGTAATCAAAGCAGAGTGTTCCAAGCAGGTCGTATGACCGGTATGTTTATTATGGGATGATAAG
ATAGGACTTGGGTGCTATTTTGTTGGTTTGCACGCCTGAGTAATGGTTAATAGGAACAGTTGGGGGTATTCGT
ATTTAGGAGCTAGAGGTGAAATTCTNGGATTTCCGAAAGACGAACNAGAGCGAAGGC *
GGAATTGAGTGCTTGGTCGGAAGGCCTGGCTAATCCTTGGAACGCTCATCGTGCTGGGGCTAGATTTTTGCAA
TTATTAATCTCCAACGAGGAATTCCTAGTAAACGCAAGTCATCAGCTTGCATTGAATACGTCCCTGCCCTTTGT
ACACACCGCCCGTCGCACCTACCGATTGAACGGTCCGATGAAACCATGGGANNTNNNTGTTTGGATNNATTT
TNGGACAGAGGCAGAACNNNGGGNNANTCTTA

***Indicates gapped region**

Figure 1. Analysis of the lipid composition and fatty acid profile of unextracted *Aurantiochytrium limacinum* biomass (AURA) in accordance with GLP principles.

Table 3. Analytes and analytical methods for evaluation of contaminants, residues and other undesirables in unextracted AURA biomass following heterotrophic fermentation.

Analyte	Method
Minerals	
Copper (mg/kg)	AOAC 985.01 [38]
Calcium (mg/kg)	AOAC 985.01 [38]
Iron (mg/kg)	AOAC 985.01 [38]
Magnesium (mg/kg)	AOAC 985.01 [38]
Manganese (mg/kg)	AOAC 985.01 [38]
Potassium (mg/kg)	AOAC 985.01 [38]
Sodium (mg/kg)	AOAC 985.01 [38]
Sulfur (mg/kg)	AOAC 985.01 [38]
Zinc (mg/kg)	AOAC 985.01 [38]
Selenium (mg/kg)	9.2669 ISU
Fluoride (mg/kg)	AOAC 975.08 [39]
Heavy Metals	
Antimony (mg/kg)	SW-846 6020 [40]
Arsenic (mg/kg)	SW-846 6020 [40]
Cadmium (mg/kg)	SW-846 6020 [40]
Lead (mg/kg)	SW-846 6020 [40]
Mercury (mg/kg)	ASTM D6722 [41]
Microbial	
Aerobic Plate count (cfu/g)	FDA/BAM Chapter 3 [42]
Coliform Count (cfu/g)	FDA/BAM Chapter 4 [43]
Escherichia coli (cfu/g)	FDA/BAM Chapter 4 [43]
Staphylococci Confirm (cfu/g)	FDA/BAM Chapter 12 [44]
Mould Count (cfu/g)	FDA/BAM Chapter 18 [45]
Yeast Count (cfu/g)	FDA/BAM Chapter 18 [45]
Pseudomonas (cfu/g)	Chromagar/Difco BBL
Clostridium (cfu/g)	CMMEF chapter 34 [46]
Salmonella(/25g)	FDA/BAM Chapter 5 [47]
Listeria (cfu/g)	FDA/BAM Chapter 10 [48]
Spoilage	
Peroxide Value (meq/kg fat)	AOAC 965.33 [49]
Mycotoxins	
Aflatoxin B1 (ppb)	"Vicam Manual Mod" (modification of the BAM (Chapter 18) method [45]).
Aflatoxin G1 (ppb)	
Aflatoxin B2 (ppb)	
Aflatoxin G2 (ppb)	
Deoxynivalenol (Vomitoxin) (ppm)	
Ochratoxin A (ppb)	
Zearalenone (ppm)	
Pesticides	
Residues as indicated in Table 11.	Modification of EPA 8081 method [50]

9.2669 ISU—internal method code for Iowa State University; Chromagar/Difco BBL—proprietary enumeration method on chromogenic agar.

Table 4. Proximate analysis by weight of unextracted *Aurantiochytrium limacinum* biomass (AURA) in accordance with GLP principles (n = 5).

Analyte	Batch Analysis Results (N = 5)	
	Range (%)	Average (%)
Crude Protein	8.32 - 15.8	11.44
Crude Fat	70.21 - 77.25	72.85
Moisture	2.27 - 5.57	3.10
Ash	2.41 - 3.92	3.39

Table 5. Analysis of the lipid composition and fatty acid profile of unextracted *Aurantiochytrium limacinum* biomass (AURA) in accordance with GLP principles.

Fatty acid	Batch Analysis Results (N = 5)	
	Range (%)	Average (%)
C10:0 (Capric acid)	0.010 - 0.017	0.013
C12:0 (Lauric acid)	0.112 - 0.156	0.128
C13:0 (Tridecanoic acid)	0.016 - 0.021	0.019
C14:0 (Myristic acid)	3.020 - 3.702	3.475
C15:0 (Pentadecanoic acid)	1.050 - 1.283	1.135
C16:0 (Palmitic acid)	34.512 - 38.228	36.351
C16:1 (Palmitoleic acid)	0.062 - 0.146	0.101
C17:0 (Magaric acid)	0.308 - 0.440	0.363
C18:0 (Stearic acid)	0.982 - 1.197	1.069
C18:1 (Oleic acid)	0.099 - 0.274	0.174
C18:2 (t-Octadecadienoic acid)	0.026 - 0.062	0.043
C18:2 (Linoleic acid)	0.004 - 0.466	0.098
C20:0 (Arachidic acid)	0.103 - 0.186	0.145
C18:3 (g-Linolenic acid)	0.020 - 0.035	0.025
C18:3 (Linolenic acid)	0.014 - 0.129	0.045
C22:0 (Behenic acid)	0.058 - 0.095	0.077
C20:3 (g-Eicosatrienoic acid)	0.061 - 0.101	0.075
C20:3 (Eicosatrienoic acid)	0.353 - 0.723	0.554
C20:4 (Arachidonic acid)	0.050 - 0.067	0.054
C22:2 (Docosadienoic acid)	0.005 - 0.296	0.162
C24:0 (Lignoceric acid)	0.057 - 0.317	0.161
C20:5 (Eicosapentaenoic acid)	0.212 - 0.338	0.271
C22:3 (Docosatrienoic acid)	0.006 - 0.053	0.029
C22:5 (Docosapentaenoic acid)	0.030 - 0.070	0.046
C22:6 (Docosahexaenoic acid)	16.920 - 19.837	18.174
Total saturated fatty acids	39.380 - 42.330	40.916
Total monounsaturated fatty acids	0.170 - 0.370	0.280
Total polyunsaturated fatty acids	17.320 - 20.520	18.834
Total trans fatty acids	0.040 - 0.090	0.066
Total fatty acids	58.15 - 61.40	60.096
Total fat as triglycerides	60.83 - 63.61	62.874

Results are reported in terms of weight of ingredient.

isolate SL1101 (Genbank: JN986842.1). Furthermore, taking into account the fatty acid profile, the protist reveals itself to be similar to *Aurantiochytrium* sp. as determined by [51].

3.3. Minimum Inhibitory Concentration (Disc Diffusion)

As revealed by the results in Table 6, uninhibited growth of *E. coli* (DH5a) was observed at all test application points. Concurrently, inhibition was observed in the presence of the positive control organisms while no inhibition was observed with the negative controls. These results indicate that at low concentrations, *Aurantiochytrium* cells and cellular extracts thereof pose no observable antimicrobial threat to the reference organism through DHA production or the presence of secondary metabolites.

3.4. Minimum Inhibitory Concentration (Broth Microdilution)

The experimental results manifested in Table 7 and Table 8 display the turbidity scores for each microorganism against the respective concentration of test material. Turbidity was evident in test suspensions between 1% and 0.0625% concentrations (owing to the presence of the test materials) prior to incubation and was noted to have increased following incubation. All 13 dilution tubes for each microorganism and test material combination were positively scored following incubation, indicating that none of the nine intestinal microorganisms were inhibited by DHA-rich algae powder nor the extracted oil at concentrations up to at least 0.03125% (owing to the initial turbidity at concentrations above 0.03125%).

3.5. Algal Toxin Analysis

LC/MS spectra for both the cell fraction extract and the supernatant fraction extracts (Figure 2 and Figure 3) were compared to the Golden Algae spectra in

Table 6. Minimum inhibitory concentration (MIC) of *Aurantiochytrium* extracts or whole cell culture in the presence of *E. coli* (DH5a).

Analyte	Test concentration			
Test materials				
Whole cell culture	Drop 1 (+)	Drop 2 (+)	Drop 3 (+)	Drop 4 (+)
Cellular extracts	10 µg (+)	20 µg (+)	40 µg (+)	80 µg (+)
Supernatant extract	10 µg (+)	20 µg (+)	40 µg (+)	80 µg (+)
Positive controls				
Ampicillin	100 µg (−)	200 µg (−)	1 mg (−)	2 mg (−)
Streptomycin	100 µg (−)	200 µg (−)	400 µg (−)	800 µg (−)
Negative control	5% Methanol (+)		5% Methanol (+)	

(+) indicates growth around the treatment point; (−) indicates inhibition of growth around the treatment point.

Table 7. Minimum Inhibitory Concentration (MIC) of unextracted *Aurantiochytrium limacinum* powder in the presence of selected microorganisms.

Test Tube	AURA powder concentration (%)	*E. coli*	*E. faecalis*	*B. fragilis*	*B. longum*	*C. difficile*	*F. canifelinum*	*C. aerofaciens*	*P. anaerobius*	*L. brevis*
1	1	+	+	+	+	+	+	+	+	+
2	0.5	+	+	+	+	+	+	+	+	+
3	0.25	+	+	+	+	+	+	+	+	+
4	0.125	+	+	+	+	+	+	+	+	+
5	0.0625	+	+	+	+	+	+	+	+	+
6	0.03125	+	+	+	+	+	+	+	+	+
7	0.015625	+	+	+	+	+	+	+	+	+
8	0.0078125	+	+	+	+	+	+	+	+	+
9	0.00390625	+	+	+	+	+	+	+	+	+
10	0.001953125	+	+	+	+	+	+	+	+	+
11	0.000976563	+	+	+	+	+	+	+	+	+
12	0.000488281	+	+	+	+	+	+	+	+	+
13	0.000244141	+	+	+	+	+	+	+	+	+
Positive control		+	+	+	+	+	+	+	+	+
Negative control		–	–	–	–	–	–	–	–	–

+ indicates turbidity (growth) in the culture medium; – indicates an absence of turbidity (no growth) in the culture medium.

Table 8. Minimum Inhibitory Concentration (MIC) of extracted *Aurantiochytrium limacinum* powder in the presence of selected microorganisms.

Test Tube	AURA powder concentration (%)	*E. coli*	*E. faecalis*	*B. fragilis*	*B. longum*	*C. difficile*	*F. canifelinum*	*C. aerofaciens*	*P. anaerobius*	*L. brevis*
1	1	+	+	+	+	+	+	+	+	+
2	0.5	+	+	+	+	+	+	+	+	+
3	0.25	+	+	+	+	+	+	+	+	+
4	0.125	+	+	+	+	+	+	+	+	+
5	0.0625	+	+	+	+	+	+	+	+	+
6	0.03125	+	+	+	+	+	+	+	+	+
7	0.015625	+	+	+	+	+	+	+	+	+
8	0.0078125	+	+	+	+	+	+	+	+	+
9	0.00390625	+	+	+	+	+	+	+	+	+
10	0.001953125	+	+	+	+	+	+	+	+	+
11	0.000976563	+	+	+	+	+	+	+	+	+
12	0.000488281	+	+	+	+	+	+	+	+	+
13	0.000244141	+	+	+	+	+	+	+	+	+
Positive control		+	+	+	+	+	+	+	+	+
Negative control		–	–	–	–	–	–	–	–	–

+ indicates turbidity (growth) in the culture medium; – indicates an absence of turbidity (no growth) in the culture medium.

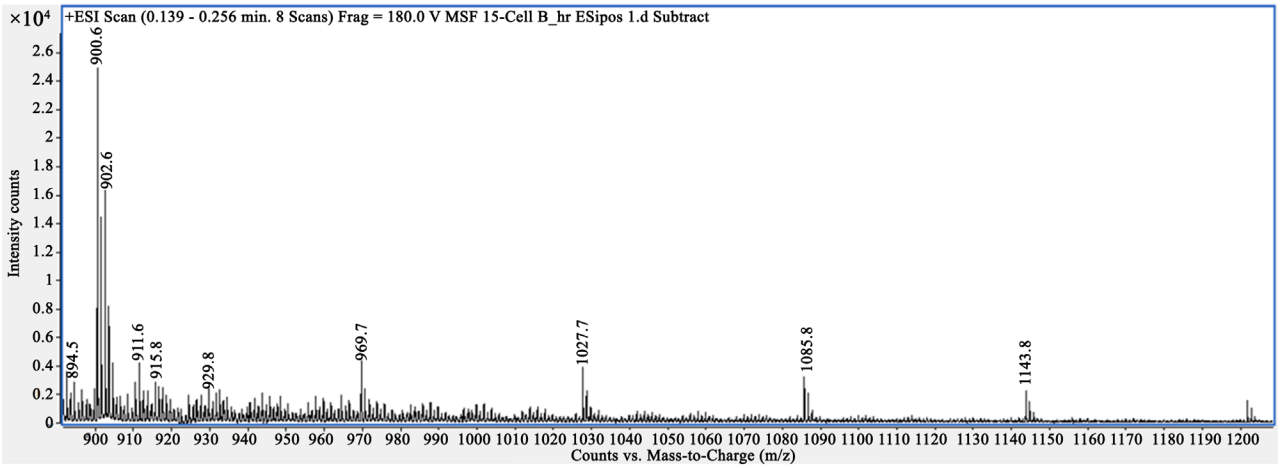

Figure 2. Spectra form LC/MS analysis of *Aurantiochytrium limacinum* cell fraction extract.

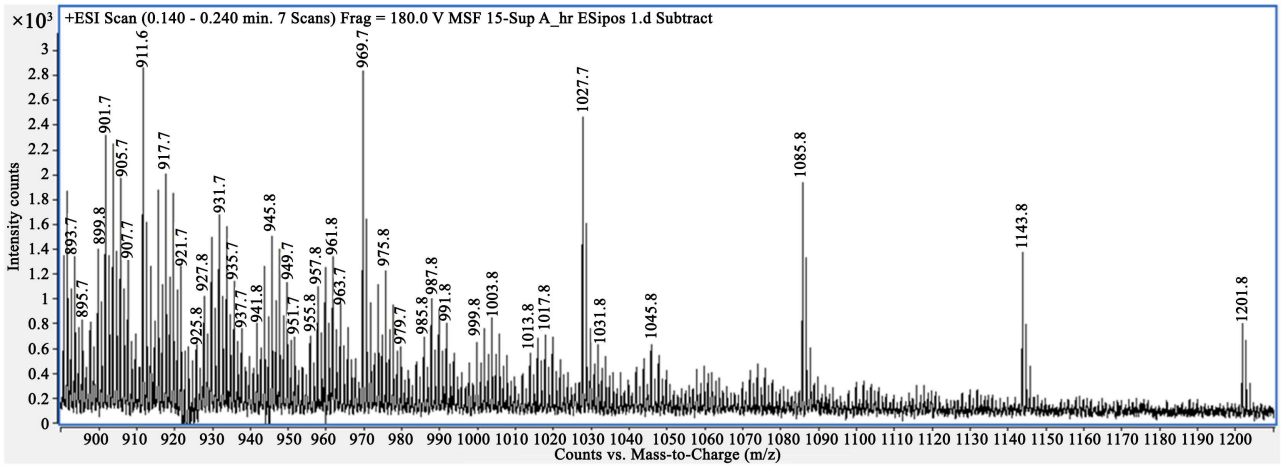

Figure 3. Spectra form LC/MS analysis of *Aurantiochytrium limacinum* supernatant fraction extract.

[35]. Polyketide prymnesinspectral peaks, based on spiked controls, would have been evident at 985.89^{2+} m/z and 1141.44^{2+} m/z for Prym1 or 919.88^{2+} m/z and 1141.44^{2+} m/z for Prym2. While phenotypically similar, *Aurantiochytrium limacinum* is phylogenetically distant from golden algae and proved to have a dissimilar metabolic fingerprint when compared to the spectra of polyketide producing *Prymnesium parvum.* None of the spectral peaks aligned with known polyketides, indicating that this organism is unlikely to pose a threat in terms of production of such algal toxins. Moreover, as evidenced by the additional analyses for the presence of Domoic and epi-domoic acids in unextracted AURA-biomass samples (**Table 9**); *Aurantiochytrium limacinum* has not been found to produce any of the frequently observed marine algal toxins.

3.6. Contaminants, Residues and Undesirables

The results of the further assessment for heavy metals, pesticides and other undesirables from industrial fermentation can be seen in **Table 10** and **Table 11**. These analyses are significant in that they demonstrate that the *Aurantiochytrium*

Table 9. Domoic and epi-domoic acid analysis of unextracted *Aurantiochytrium limacinum biomass* (*AURA*).

Batch#	Source of Isolation
1	<0.25 μg/g
2	<0.25 μg/g
3	<0.25 μg/g
4	<0.25 μg/g
5	<0.25 μg/g

Table 10. Mineral, heavy metal, microbial pathogen, spoilage and toxin analysis of unextracted *Aurantiochytrium limacinum* biomass (AURA) in accordance with GLP principles.

Analyte	Batch Analysis Results (N = 5)	
	Range	Average
Minerals		
Copper (mg/kg)	-	<1.24
Calcium (mg/kg)	2736 - 3810	3353.2
Iron (mg/kg)	-	<12.5
Magnesium (mg/kg)	2482 - 3312	2833.6
Manganese (mg/kg)	-	<1.25
Potassium (mg/kg)	1633 - 3208	2228.4
Sodium (mg/kg)	1104 - 1539	1277.8
Sulfur (mg/kg)	5248 - 7047	6013.2
Zinc (mg/kg)	3.74 - 36.81	20.722
Selenium (mg/kg)	<0.02	<0.02
Fluoride (mg/kg)	<5 - 8.49	<5.794
Heavy Metals		
Antimony (mg/kg)	<0.025	<0.025
Arsenic (mg/kg)	0.192	0.259
Cadmium (mg/kg)	<0.005	<0.005
Lead (mg/kg)	<0.025	<0.025
Mercury (mg/kg)	<0.02	<0.02
Microbial		
Aerobic Plate count (cfu/g)	40 - 450	354
Coliform Count (cfu/g)	<10	<10
Escherichia coli (cfu/g)	<10	<10
Staphylococci Confirm (cfu/g)	<10	<10
Mould Count (cfu/g)	<10 - 20	14*
Yeast Count (cfu/g)	<10	<10

Continued

Pseudomonas (cfu/g)	<1	<1
Clostridium (cfu/g)	<100	<100
Salmonella (/25g)	Negative	Negative
Listeria (cfu/g)	Negative	Negative
Spoilage		
Peroxide Value (meq/kg fat)	<0.2 - 18.5	3.86
Mycotoxins		
Aflatoxin B1 (ppb)	<3	<3
Aflatoxin G1 (ppb)	<15	<15
Aflatoxin B2 (ppb)	<1	<1
Aflatoxin G2 (ppb)	<5	<5
Deoxynivalenol (Vomitoxin) (ppm)	<0.2	<0.2
Ochratoxin A (ppb)	<12.5	<12.5
Zearalenone (ppm)	<0.1	<0.1

cfu = colony forming units; ND = not detected; meq = milliequivalent; ppb = parts *per* billion; ppm = parts *per* million; *Assuming ND = 0.

Table 11. Pesticides in unextracted *Aurantiochytrium limacinum* biomass (AURA) in accordance with GLP principles.

Pesticide	Result (ppm)
Aldrin	<0.01
alpha-benzene hexachloride	<0.01
beta-benzene hexachloride	<0.01
delta-benzene hexachloride	<0.01
Carbophenothion (Trithion)	<0.15
alpha-Chlordane	<0.01
gamma-Chlordane	<0.01
Diazinon	<0.14
Dieldrin	<0.02
Disulfoton	<0.15
Endosulfan (Thiodan)	<0.02
Endrin	<0.03
Ethion	<0.14
Heptachlor	<0.01
Heptachlor epoxide	<0.01
Hexachlorobenzene	<0.01
Lindane	<0.01
Malathion	<0.01
Methoxychlor	<0.05

Continued

Methyl parathion	<0.14
Mirex	<0.01
Parathion	<0.12
Phorate (Thimet)	<0.15
Ronnel	<0.13
Toxaphene	<0.05
2,4'-DDD	<0.03
4,4'-DDD	<0.02
2,4'-DDE	<0.11
4,4'-DDE	<0.02
2,4'-DDT	<0.03
4,4'-DDT	<0.03

DDD = Dichlorodiphenyldichloroethane; DDE = Dichlorodiphenyldichloroethylene; DDT = dichlorodiphenyltrichloroethane.

production process itself is safe and fit for purpose. Industrial scale heterotrophic fermentation did not show any indications of elevated heavy metal accumulation, hygiene indicator organisms and microbial pathogens were all absent and mycotoxin contaminant levels were below the limits of detection. Similarly, pesticide residue levels were below the limits of detection for these analytical methods. Copper and selenium levels were shown to be low, which is pertinent as AURA is intended to be used as a DHA rich feed material for food producing animals, many of which can be sensitive to the presence of high concentrations of these minerals.

4. Discussion and Conclusions

Originally isolated from the marine environment off the Floridian coast, the oleaginous protist studied here showed early promise as a source of polyunsaturated fatty acids. Biochemical profiling revealed not only that the organism produced in excess of 70% crude fat, but that 18% of the fatty acid content was docosahexaenoic acid (DHA). This compared favorably with alternative terrestrial sources [52] and placed the protist as a viable source of DHA, and as an alternative to fish-oil derived DHA for dietary inclusion in aquaculture and animal nutrition. Microscopic and biochemical analyses had identified the organism as a member of the Thraustochytriaceae family. As phenotypic classification could not resolve the species identity, this was confirmed as *Aurantiochytrium limacinum* through 18S rRNA profiling.

Having established the potential of AURA as a commercial DHA source, attention was focused on the safety of the organism. Every novel isolate has the potential to be a producer of bioactive secondary metabolites and other organisms from the marine environment are known to produce toxic metabolites.

"Golden algae" are a group of algal protists that are morphologically similar to *Aurantiochytrium* and are producers of toxic polyketides such as prymnesin-1 (prym1) and prymnesin-2 (prym2) [35]. LC/MS spectra from *Aurantiochytrium* extracts revealed no correlation with spectral signatures of known polyketides. Similarly, the analysis for domoic and epi-domoic acid production revealed AURA not to be a producer of these neurotoxic compounds. Having established biochemically that known toxins were absent, analysis was carried out to ascertain whether cellular mass or extracts thereof would have inhibitory effects on an indicator organism. Despite dosing with up to 80 μg of AURA extracts, no inhibition was observed in the *E. coli* (DH5a) population. Cumulatively therefore, there is no evidence that this strain of *Aurantiochytrium limacinum* poses any particular threat to an animal or to the microflora therewith.

Ascertaining that there was no production of known microalgal toxins nor gross antimicrobial effects, attention was turned to the primary desirable component (DHA) and the effect it may have on the microbiota of food-producing animals. Details are limited on the degree to which DHA supplementation into the diet can influence commensal bio-flora populations, however some inference can be made from the effect DHA can have on microorganisms from other environments. MICs for an extracted DHA exist for a range of foodborne pathogens; *Bacillus subtilis* (350 μg/ml), *Listeria monocytogenes* (350 μg/ml), *Staphylococcus aureus* (500 μg/ml), *Pseudmonas aeruginosa* (250 μg/ml), *E. coli* O157:H7 (1650 μg/ml), *Enterobacter aerogenes* (4800 μg/ml), *Salmonella enteritidis* (1650 μg/ml) and *Salmonella typhimurium* (1650 μg/ml) [53]. Owing to the potential clinical application in the treatment of oral health conditions, MICs have been determined for a range of oral pathogens; *Streptococcus mutans* (625 μg/ml), *Candida albicans* (1250 μg/ml), *Porphyromonas gingivalis* (9.76 μg/ml), *Aggregatibacter actinomycetemcimitans* (625 μg/ml), *Aggregatibacter segnis* (19.53 μg/ml), *Fusobacterium nucleatum subsp. vinventi* (39.06 μg/ml), *fusobacterium nucleatum subsp. polymorphum* (39.06 μg/ml), *Prevotella intermedia* (78.12 μg/ml) [54]. It is noteworthy that different strains can exhibit different sensitivities towards DHA; Sun *et al.* determined MICs for oral pathogens; *P. gingivalis* (4.11 μg/ml), *F. nucleatum* (>32.85 μg/ml) [55] and *S. mutans* (32.85 μg/ml) [56]. DHA has shown a tendency to change population dynamics within the rumen through growth inhibition of sensitive organisms. For example, growth of *Butyrvibrio fibrisolvens* has shown growth inhibition at low concentrations (50 μg/ml) [4].

There is evidence of some antimicrobial or bacteriostatic effects induced by polyunsaturated fatty acids on specific microorganisms [4] [53] [54] [55] [56], and by extension, there is the possibility that DHA derived from supplementation of animal feeds with unextracted *Aurantiochytrium limacinum* powder or extracted oil, may influence microbial populations in the gut. However, the available experimental data regarding susceptibility of a range of microorganisms to antimicrobial effects of DHA, indicate that the majority of these organisms have an MIC ≥ 312.5 μg/ml. There is no body of evidence that suggests

DHA can promote development of antimicrobial resistance within the food animals or in the realm of public health, in fact [54] states that "to date, no bacterial resistance to free fatty acids has been encountered and no resistance phenotype has emerged". As the US Food and Drug Administration established that the Acceptable daily intake (ADI) for DHA in humans is 3000 mg per day (the European Food Safety Authority set the ADI at 5000 mg per day), it is unlikely that DHA obtained from consumption of food products derived from animals whose diet has been supplemented with AURA, will pose a threat to humans either directly or through promotion of antimicrobial resistance.

The final screening for the presence of microbial pathogens, pesticides and heavy metals showed through their absence that the production process was safe, fit for purpose, and free from manufacturing contaminants. DHA yielded by extracted heterotrophically-grown *Aurantiochytrium limacinum* (CCAP 4087/2) shows promise to be a safe and environmentally acceptable alternative to traditional marine-harvested fish oils.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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