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Utility of fatty acid profile and in vitro immune cell activation for chemical and biological standardization of Arthrospira/ Limnospira

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Utility of fatty acid profile and *in vitro* immune cell activation for chemical and biological standardization of *Arthrospira*/*Limnospira*

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Abstract

Commercially cultivated *Limnospira* (species formerly classified to genus *Arthrospira*) is a popular food/supplement consumed by millions of people worldwide for health benefits. The objective of the current research was to advance the standardization technology for *Limnospira*. Quantitative methods were established to detect fatty acids as potential chemical markers and immune-enhancing activity. Analysis of 20 different batches of biomass obtained from one commercial grower demonstrated that there was a statistically significant relationship between the sum of two fatty acids (linoleic and γ-linolenic) and Toll-like receptor (TLR)2/TLR1-dependent activation (R^2=0.48, p=0.0007). Investigation of 12 biomass samples sourced from growers in 10 different countries demonstrated that fatty acid content was again significantly correlated with biological activity (R^2=0.72, p=0.0005) and the content of fatty acids varied by 2-fold and activity by 12.5-fold. This large variation between different samples confirms the need to use the present standardization methods to ensure consistent and properly characterized biomass for consumers and for future scientific research.

Materials and Methods

Biomass Material. The biomass that was selected and taxonomically identified for reference material was obtained from Dongtai Cibainian Biological Engineering Company, LTD (established in 1994 and one of the largest producers of *Limnospira* in China). *Limnospira* dried powder (supplier lot # 20190501 and 20190820B) was used for sequence analysis. Live biomass was collected at the cultivation facility (located in Dongtai City, Jiangsu Province), and preserved in 2.5% formaldehyde for morphological analysis. The light microscopy analysis was performed on an Olympus BX53 microscope using bright field and differential interference contrast.

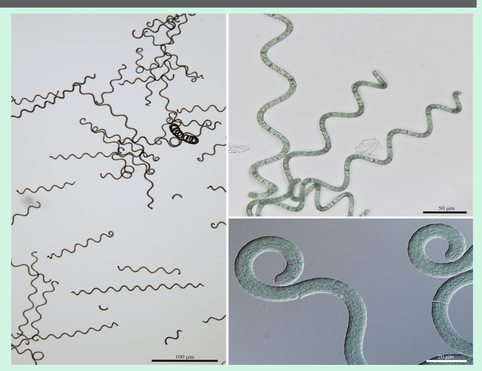

Figure 1. Morphology of material obtained from Dongtai Cibainian Biological Engineering Co. used as reference in this study.

Twenty different batches of *Limnospira* dried samples were obtained from Dongtai Cibainian Biological Engineering over 14 years (2007 – 2021). Dried biomass samples were stored in the dark at room temperature in sealed containers at the National Center for Natural Products Research (NCNPR) and all samples were analyzed together in June and September, 2021 for fatty acid content and activation of the TLR2/TLR1 pathway , respectively.

From November 2020 through January 2021, twelve *Limnospira* dried samples were purchased from commercial growers worldwide (Table 1) that sell this biomass for food or use as a botanical dietary supplement. Biomass samples were stored in the dark at room temperature in sealed containers at the NCNPR, and all samples were analyzed together in March and May, 2021 for activation of the TLR2/TLR1 pathway and fatty acid content, respectively. Immulina extract was provided by Scandinavian Clinical Nutrition (Lot 2290006) and ChromaDex (Lots 2290020 and 2290021).

The samples of powdered biomass listed in Table 1 were resuspended in distilled water and the reference sample were examined by a light microscopy for their morphological features, which were compared with known taxa listed in. Subsequently their DNA was extracted using the Ultra Clean Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). Plasmid twelves were purified and commercially sequenced in both directions with the use of primers T7 (5′-TAA TAC GAC TCA CTA TAG GG-3′) and SP6 (5′-TAT TTA GGT GAC ACT ATA G-3′). Sequences were then submitted to the NCBI GenBank database with the accession numbers listed in Table 1. The percent identities of the 16S rRNA gene sequences were calculated in Geneious Prime 2020.2.4 (http://www.geneious.com).

Sample Preparation for Evaluation of TLR2/TLR1-Dependent Activity

Crude extracts were prepared from the *Limnospira* dried biomass samples to enrich the levels of Braun-type lipoproteins. Raw material (1g) was extracted with 7mL of 50% ethanol at 80 ° C for 45 minutes. Following centrifugation, the supernatant was collected, ethanol concentration adjusted to 72.5% by adding 1 volume of cold 95%, and the sample incubated at -20 ° C overnight. Precipitable material was collected by centrifugation and subsequently washed with cold 95% ethanol and the final extract dried. For TLR2/TLR1-dependent activity analysis, extracts were dissolved in Milli-Q water containing 2% sodium dodecyl sulfate at 25 mg/mL.

Receptor Cell Lines

Selective detection of pathogen recognition receptors (PRR) activation was accomplished using HEK-Blue hTLR2, HEK-Blue hTLR4, HEK-Blue hTLR2-TLR1, and HEK-Blue hTLR2 KO-TLR1/6 cells (InvivoGen, San Diego CA USA). These cell lines are engineered to stably co-express a PRR gene and an NF-kappa B inducible secreted embryonic alkaline phosphatase (SEAP) gene. Activation is detected by evaluating SEAP levels in the culture medium using the QUANTI-Blue reagent with optical density measurement at 635nm. Experiments were performed according to manufacturer instructions. Positive controls were purchased from InvivoGen and included Pam_3CSK_4 (TLR2/TLR1 agonist) and Pam_2CSK_4 (TLR2/TLR6 agonist).

Derivatization of *Limnospira* Biomass and Immulina

To characterize the overall lipid composition, dried biomass of *Limnospira* or its patented extract, Immulina, was subjected to saponification followed by esterification to convert free fatty acids into their corresponding methyl esters, hereafter referred to as fatty acid methyl esters (FAMES). All derivatization experiments (method A: **Saponification and Esterification** or method B: **Transesterification**, Table 3) were repeated at least twice, and each sample was injected twice into the GC-MS, resulting in at least quadruplicate results for every sample.

GC-MS Analysis and Quantitation of FAMES

The samples prepared from derivatization experiments (Method A or B) were vortexed for 30 seconds to ensure sample homogeneity. Next, 480 µL of the sample was removed and transferred to a labeled GC vial. To the vial, 20 µL of the previously prepared internal standard (IS, methyl myristate) solution was added to the sample vial for a final concentration of 80 µg/mL. The vial was vortexed for 30 seconds and then analyzed by GC-MS.

Selective Ion Monitoring and Confirmation

The 5977A mass spectrometer was operated with an electron energy of 70 eV, and the MS was operated in both scan and selective ion monitoring (SIM) modes. All scan mass spectra data were recorded at a rate of 5 Hz from m/z 40 to 400 after a 5-minute solvent delay. The selective ions, m/z 69 for methyl oleate; m/z 74 for palmitate, palmitoleate, and stearate methyl esters; m/z 79 for a-linolenate and g-linolenate methyl esters; and m/z 81 for linoleate methyl ester were identified as suitable ions in SIM mode with enhanced detection sensitivity and quantification purposes. Data were acquired utilizing Agilent MassHunter software (version B7.06.274), while quantification was achieved with Agilent MassHunter Quantitative Analysis (version 10.0.707.0). The NIST database (version 2.3) was utilized for tentative compound identification. The 6 quantifiable fatty acids were later confirmed with analytical reference standards.

Quantitative Analyses

Methyl myristate in heptane was utilized as an IS, and the 36 reference standards were obtained either from Sigma-Aldrich (St. Louis, MO USA), Thermo Fisher Scientific (Waltham, MA USA), or Cayman Chemicals (Ann Arbor, MI USA). The six quantifiable fatty acid methyl esters (FAMES) in *Limnospira* biomass, methyl palmitate, methyl palmitoleate, methyl stearate, methyl oleate, methyl linoleate, and methyl g-linolenate were quantified using the GC-MS method. Methyl a-linolenate was also evaluated since this fatty acid was included in the USP method and it also served as a marker for detection of chlorella contamination. A series of standard solutions were prepared for each FAME analyte to establish the calibration curves using 0.1, 0.45, 0.75, 1, 4.5, 10, 25, 45, 75, 100, 200, 300, and 450 µg/mL solutions from a corresponding 5000 µL/mL solution in heptane and spiked with methyl myristate as IS with a final concentration of 80 µg/mL. Next, duplicate injections of each calibration curve solution were analyzed by GC-MS.

Results

Table 1. Variation in total fatty acid content and immune-enhancing activity (TLR2/TLR1 activation) in biomass samples obtained from various commercial growers throughout the world. Biomass identification is based on the company literature, 16S rRNA and 16S-23S ITS region sequences were obtained in this study. EC_{50} values represent the concentration (µg/mL) of biomass material required to induce activation for the TLR2/TLR1 signaling pathway to levels 50% of those achieved by Pam_3CSK_4 (100ng/mL).

	Company	Country	Lot Number	Biomass	Total Fatty Acids (mg/g)	TLR2/TLR1 (EC_{50} value)	16S rRNA, 16S-23S ITS
1	Cyanotech Corporation	USA	1400036543	Arthrospira platensis	44.91	5.99	OM419146–8
2	Earthrise Nutritionals	USA	41739	Arthrospira platensis	59.47	3.62	OM419149
3	Solarium Biotechnology S.A.	Chile	not available	Arthrospira maxima	46.15	7.01	OM419150–2
4	TAAU Australia Pty Ltd	Australia	not available	Arthrospira maxima	35.83	45.29	OM419153–5
5	FEBICO	Taiwan	20200309001 02	Spirulina platensis	51.30	5.43	OM419156–8
6A	EID - Parry	India	SPEPF2001	Arthrospira platensis	50.77	6.65	OM419159
6B	EID - Parry (sold by Triquetra Health)	India	1952	Arthrospira platensis	48.15	18.72	OM419160
7	Dongtai Cibainian Biological Engineering	China	20190501	Arthrospira platensis	51.34	5.23	OM419161–3
8	Spirulina Nigrita	Greece	148	Arthrospira platensis	31.35	39.16	OM419164–6
9	Flora	Mongolia	180968	Arthrospira platensis	54.10	6.90	OM419167–9
10	Akal Food	France	2048/SOL	Arthrospira platensis	43.74	9.03	OM419170
11	Akal Food	Burkina Faso	2029	Arthrospira platensis	49.88	4.03	OM419171–3

Table 2. Linear regressions between TLR2/TLR1 extract activity and content of individual fatty acids in biomass obtained from Dongtai Cibainian Biological Engineering (20 samples) and commercial growers in 10 different countries (12 samples, details summarized in Table 1). Highlighting indicates regressions that are statistically significant.

	20 samples (single source)		12 samples (10 countries)	
Fatty Acid	R-Squared	*p* value	R-Squared	*p* value
Methyl Palmitate	0.160	0.080	0.388	0.030
Methyl Palmitoleate	0.075	0.243	0.355	0.041
Methyl Stearate	0.167	0.073	0.004	0.847
Methyl Oleate	0.003	0.817	0.014	0.717
Methyl Linoleate	0.689	<0.0001	0.660	0.001
Methyl γ Linolenate	0.359	0.005	0.506	0.009

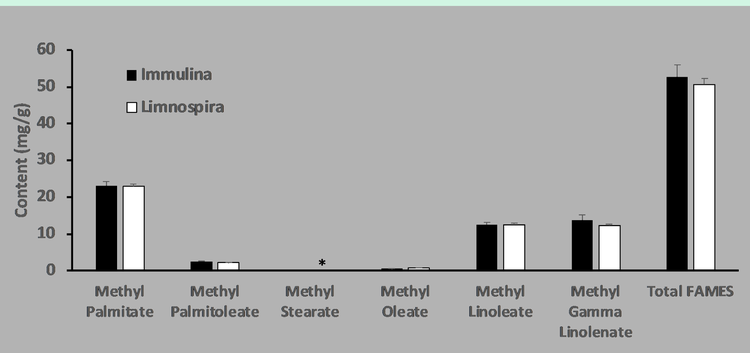

Figure 2. Comparative content of each FAME in dried *Limnospira* and commercial extract, Immulina, extracted from the same batch of dried biomass. Bars represent the average ± s.d. of three samples from the same batch of material. Each sample was analyzed in duplicate in the GC-MS. No statistically significant differences were observed between sample means, except for methyl stearate (compared by paired t-tests). *p = 0.04.

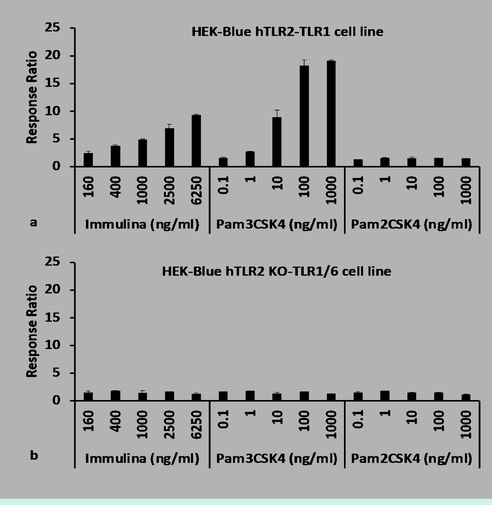

Figure 3. Immulina extract activates the TLR2/TLR1 signaling pathway. Activity evaluated in HEK-Blue hTLR2-TLR1 cells (a) and control HEK-Blue hTLR2 KO-TLR1/6 cells (b). Response ratio ± s.d. is defined as OD of sample / OD of untreated cells. Synthetic triacylated lipopeptide Pam_3CSK_4 was used as a positive control, and the synthetic diacylated lipopeptide Pam_2CSK_4 served as the negative control.

Table 3. Comparison of the USP method (monograph # 2181) for *Limnospira* biomass with the newly developed method. Chemical marker values for the new method represent average (range) values from 32 samples.

	USP (Method A)	Newly Developed (Method B)	
Analytical System	GC	GC-MS with SIM	
• Derivatization method (esterification)	Two-step	Single-step	
• Column	0.25mm × 30m fused silica capillary; 0.25 µm film of phase G16 coating	0.25mm × 60m fused silica capillary; 0.2 µm film of HP-88 ((88% cyanopropyl)aryl-polysiloxane) coating	
• Coefficients of variation (%)	Inter-assay: 16.70 Intra-assay: 8.32	Inter-assay: 3.40 Intra-assay: 2.62	
Chemical Markers	% of total (range)	% of total average ± s.d. (range)	Content (mg/g biomass) average ± s.d. (range)
• Palmitic acid	35-60	47.7±5.0 (38-57)	21.6±2.0 (15-27)
• Palmitoleic acid	2-8	4.4±1.5 (2.0-9.0)	2.0±0.7 (0.5-3.5)
• Stearic acid	1-5	0.2±0.1 (0.0-1.0)	0.1±0.0 (0.0-0.5)
• Oleic acid	1-7	1.5±1.0 (0.0-4.0)	0.7±0.5 (0.0-1.7)
• Linoleic acid	13-25	20.7±1.8 (14.5-24.0)	9.5±1.8 (4.5-13.5)
• Gamma linoleic acid	13-27	25.6±5.4 (16-38)	11.9±3.7 (5.5-21.0)
• Alpha linolenic acid	<0.5	Undetected	Undetected
• Total fatty acids			45.7±6.5 (31.0-60.0)
Utility	Identification	Identification and Standardization	

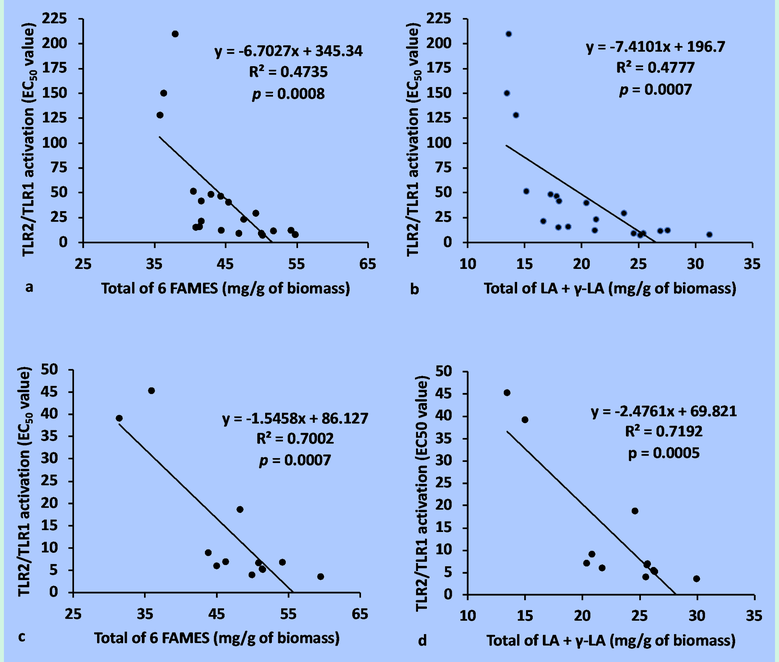

Figure 4. Correlations between TLR2/TLR1 extract activity and content of fatty acids in biomass material obtained from Dongtai Cibainian Biological Engineering (20 batches, a and b) and commercial growers in 10 different countries (12 batches, c and d). The total content of 6 fatty acids represents the combined total of palmitate, palmitoleate, stearate, oleate, linoleate, and γ linolenate in each sample (a, c). "LA" and "g-LA" represent linoleate and gamma linolenate, respectively. EC_{50} values represent the concentration (µg/mL) of biomass material required to induce activation for the TLR2/TLR1 signaling pathway to levels 50% of those achieved by Pam_3CSK_4 (100ng/mL).

Conclusion

- **We have identified an *in vitro* bioassay (detection of TLR2/TLR1 activity) and an analytical method (detection of FAME content) that can serve as a relevant approach for standardizing the immune-enhancing activity of *Limnospira* botanical dietary supplements and food products.**
- **Measurement of TLR2/TLR1-dependent activity detects Braun-type lipoprotein activity, the predominant compounds responsible for the *in vitro* macrophage activation potential of *Limnospira*.**
- **FAME content is correlated with activity and therefore expands the utility of this chemical marker to standardization efforts in addition to its original use in the USP method as a component of product identification.**
- **Utilizing the combination of an analytical method and bioassay provides an orthogonal approach to botanical standardization. The value of this combination approach has been highlighted for other botanicals such as hops (*Humulus lupulus*) and red clover (*Trifolium pratense*).**
- **Widespread adoption of chemicobiological standardization techniques to the botanical field would be expected to greatly enhance product quality and well-defined material for clinical studies on the efficacy of these products.**

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