ORIGINAL SUBMISSION

#513

Algaeon, Inc.
8440 West 82nd St.
Indianapolis, IN 46278
Phone: (317) 225-4180
www.Algaeon-inc.com

April 9, 2014

GRN 000513

RECEIVED
APR 17 2014
OFFICE OF FOOD ADDITIVE SAFETY

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Pkwy.
College Park, MD 20740-3835

RE: GRAS NOTICE FOR *Euglena gracilis* CONTAINING β-1,3-GLUCAN

Greetings,

Please find included herein a Form FDA-3667 and two documents:

1. GRAS NOTICE FOR *Euglena gracilis* CONTAINING β-1,3-GLUCAN FOR USE AS A HUMAN FOOD SUPPLEMENT

and

2. GRAS PANEL REVIEW: *Euglena gracilis* CONTAINING β-1,3-GLUCAN

Also included is a CD containing the PDF/electronic files for the FDA-3667, these two documents, and a collection of reference materials cited in these documents. This package is intended to provide you with the information used by our independent panel to ascertain that *Euglena gracilis* biomass containing paramylon (beta-1,3,glucan) is generally recognized as safe (GRAS) for consumption as a food supplement or food additive by human beings on the basis of available literature.

Please do let me know if you have questions, feedback, or comments about our findings or have any information that would bear on the accuracy of our findings. Our mission is (as is yours) to ensure that our product is safe for human consumption, and we welcome any feedback you might have. Thank you for your efforts in this regard.

Sincerely yours

(b) (6)

(b) (6)

John A. Kassebaum
Chief Technical Officer - Algaeon, Inc.

Form Approved: OMB No. 0910-0342; Expiration Date: 02/29/2016
(See last page for OMB Statement)

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration, 5100 Paint Branch Pkwy., College Park, MD 20740-3835.

PART I INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

☒ New ☐ Amendment to GRN No._____ ☐ Supplement to GRN No. _____

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3a. For New Submissions Only: Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd)*: _____

3b. For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd)*: _____

PART II INFORMATION ABOUT THE NOTIFIER

1a. Notifier			
Name of Contact Person John A. Kassebaum		Position Chief Technical Officer	
Company *(if applicable)* Algaeon, Inc.			
Mailing Address *(number and street)* 8440 West 82nd St.			
City Indianapolis	State or Province Indiana	Zip Code/Postal Code 46278	Country United States of America
Telephone Number (317) 225-4180	Fax Number	E-Mail Address John.Kassebaum@Algaeon-inc.com	

1b. Agent or Attorney *(if applicable)*			
Name of Contact Person		Position	
Company *(if applicable)*			
Mailing Address *(number and street)*			
City	State or Province	Zip Code/Postal Code	Country
Telephone Number	Fax Number	E-Mail Address	

000003

PART III GENERAL ADMINISTRATIVE INFORMATION

1. Name of Substance

Euglena gracilis biomass containing paramylon (β-1,3-glucan)

2. Submission Format: *(Check appropriate box(es))*

☐ Electronic Submission Gateway

☒ Paper

☒ Electronic files on physical media with paper signature page

If applicable give number and type of physical media

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in FDA's files by reference? *(Check one)*

☒ Yes *(Proceed to Item 5)* ☐ No *(Proceed to Item 6)*

5. The submission incorporates by reference information from a previous submission to FDA as indicated below *(Check all that apply)*

☒ a) GRAS Notice No. GRN 413

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☒ e) Other or Additional *(describe or enter information as above)* 21CFR172.809, GRN 309, GRN 239, GRN 412

6. Statutory basis for determination of GRAS status *(Check one)*

☒ Scientific Procedures *(21 CFR 170.30(b))* ☐ Experience based on common use in food *(21 CFR 170.30(c))*

7. Does the submission (including information that you are incorporating by reference) contain information that you view as trade secret or as confidential commercial or financial information?

☐ Yes *(Proceed to Item 8)*

☒ No *(Proceed to Part IV)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, see attached Designation of Confidential Information

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

PART IV INTENDED USE

1. Describe the intended use of the notified substance including the foods in which the substance will be used, the levels of use in such foods, the purpose for which the substance will be used, and any special population that will consume the substance *(e.g., when a substance would be an ingredient in infant formula, identify infants as a special population).*

Euglena gracilis biomass containing paramylon (β-1,3-glucan) is intended to be used as a human food supplement. The paramylon (β-1,3-glucan) is intended as a source of fermentable β-1,3-glucan fiber in food. Based on an expected average intake of about 1277 grams of solid food per day by adult humans, supplementation is expected to be 125mg to 650mg of paramylon (β–1,3–glucan) per day (not to exceed 2.5g per day) or 250mg to 1300mg of *Euglena gracilis* biomass per day (not to exceed 5g per day).

2. Does the intended use of the notified substance include any use in meat, meat food product, poultry product, or egg product? *(Check one)*

☐ Yes ☒ No

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PART V IDENTITY

1. Information about the Identity of the Substance

	Name of Substance[1]	Registry Used *(CAS, EC)*	Registry No.[2]	Biological Source *(if applicable)*	Substance Category *(FOR FDA USE ONLY)*
1	Paramylon (β-1,3-glucan)	CAS	9051-97-2	Euglena gracilis	
2					
3					

[1] Include chemical name or common name. Put synonyms *(whether chemical name, other scientific name, or common name)* for each respective item (1 - 3) in Item 3 of Part V *(synonyms)*

[2] Registry used e.g., CAS *(Chemical Abstracts Service)* and EC *(Refers to Enzyme Commission of the International Union of Biochemistry (IUB), now carried out by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB))*

2. Description

Provide additional information to identify the notified substance*(s)*, which may include chemical formula*(s)*, empirical formula*(s)*, structural formula*(s)*, quantitative composition, characteristic properties *(such as molecular weight(s)),* and general composition of the substance. For substances from biological sources, you should include scientific information sufficient to identify the source *(e.g., genus, species, variety, strain, part of a plant source (such as roots or leaves), and organ or tissue of an animal source)*, and include any known toxicants that could be in the source.

The basic ingredient is *Euglena gracilis* biomass containing paramylon (a β-1,3-glucan). Paramylon is a linear (unbranched) β–1,3–glucan polysaccharide polymer with high molecular weight. Curdlan, which is essentially identical to paramylon, is a permitted addition to food for human consumption under the *U.S. FDA Code of Federal Regulations Title 21*, section 172.809. The only difference between curdlan and paramylon is the length of the polymer chain (molecular weight) of the polysaccharide polymer (100 kDa for curdlan, 500 kDa for paramylon). Curdlan is identified with the CAS Registry Number 54724-00-4.

3. Synonyms

Provide as available or relevant:

1	
2	
3	

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PART VI OTHER ELEMENTS IN YOUR GRAS NOTICE

(check list to help ensure your submission is complete – check all that apply)

- ☒ Any additional information about identity not covered in Part V of this form
- ☒ Method of Manufacture
- ☒ Specifications for food-grade material
- ☒ Information about dietary exposure
- ☒ Information about any self-limiting levels of use *(which may include a statement that the intended use of the notified substance is not-self-limiting)*
- ☒ Use in food before 1958 *(which may include a statement that there is no information about use of the notified substance in food prior to 1958)*
- ☒ Comprehensive discussion of the basis for the determination of GRAS status
- ☒ Bibliography

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☒ Yes ☐ No

Did you include this other information in the list of attachments?

☒ Yes ☐ No

PART VII SIGNATURE

1. The undersigned is informing FDA that Algaeon, Inc.
(name of notifier)

has concluded that the intended use*(s)* of Euglena gracilis biomass containing paramylon (β-1,3-glucan)
(name of notified substance)

described on this form, as discussed in the attached notice, is (are) exempt from the premarket approval requirements of section 409 of the Federal Food, Drug, and Cosmetic Act because the intended use(s) is (are) generally recognized as safe.

2. ☒ Algaeon, Inc. *(name of notifier)* agrees to make the data and information that are the basis for the determination of GRAS status available to FDA if FDA asks to see them.

Algaeon, Inc. *(name of notifier)* agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so.

8440 West 82nd St., Indianapolis, IN 46278
(address of notifier or other location)

Algaeon, Inc. *(name of notifier)* agrees to send these data and information to FDA if FDA asks to do so.

OR

☐ The complete record that supports the determination of GRAS status is available to FDA in the submitted notice and in GRP No. ----------
(GRAS Affirmation Petition No.)

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
com.apple.idms.appleid.prd.2f366246716a554d4a5778664239776767627 86d7a773d3d Digitally signed by com.apple.idms.appleid.prd.2f366246716a554d4a5778664239776767627 86d7a773d3d DN: cn=com.apple.idms.appleid.prd.2f366246716a554d4a5778664239776767627 86d7a773d3d Date: 2014.04.07 10:51:06 -05'00'	John A. Kassebaum, Chief Technical Officer	04/07/2014

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PART VI – OTHER ELEMENTS IN YOUR GRAS NOTICE

(check list to help ensure your submission is complete – check all that apply)

- ☒ Any additional information about identity not covered in Part V of this form
- ☒ Method of Manufacture
- ☒ Specifications for food-grade material
- ☒ Information about dietary exposure
- ☒ Information about any self-limiting levels of use *(which may include a statement that the intended use of the notified substance is not-self-limiting)*
- ☒ Use in food before 1958 *(which may include a statement that there is no information about use of the notified substance in food prior to 1958)*
- ☒ Comprehensive discussion of the basis for the determination of GRAS status
- ☒ Bibliography

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☒ Yes ☐ No

Did you include this other information in the list of attachments?

☒ Yes ☐ No

PART VII – SIGNATURE

1. The undersigned is informing FDA that Algaeon, Inc. *(name of notifier)*

has concluded that the intended use(s) of Euglena gracilis biomass containing paramylon (β-1,3-glucan) *(name of notified substance)*

described on this form, as discussed in the attached notice, is (are) exempt from the premarket approval requirements of section 409 of the Federal Food, Drug, and Cosmetic Act because the intended use(s) is (are) generally recognized as safe.

2. ☒ Algaeon, Inc. *(name of notifier)* agrees to make the data and information that are the basis for the determination of GRAS status available to FDA if FDA asks to see them.

Algaeon, Inc. *(name of notifier)* agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so.

8440 West 82nd St., Indianapolis, IN 46278 *(address of notifier or other location)*

Algaeon, Inc. *(name of notifier)* agrees to send these data and information to FDA if FDA asks to do so.

OR

☐ The complete record that supports the determination of GRAS status is available to FDA in the submitted notice and in GRP No. ---------- *(GRAS Affirmation Petition No.)*

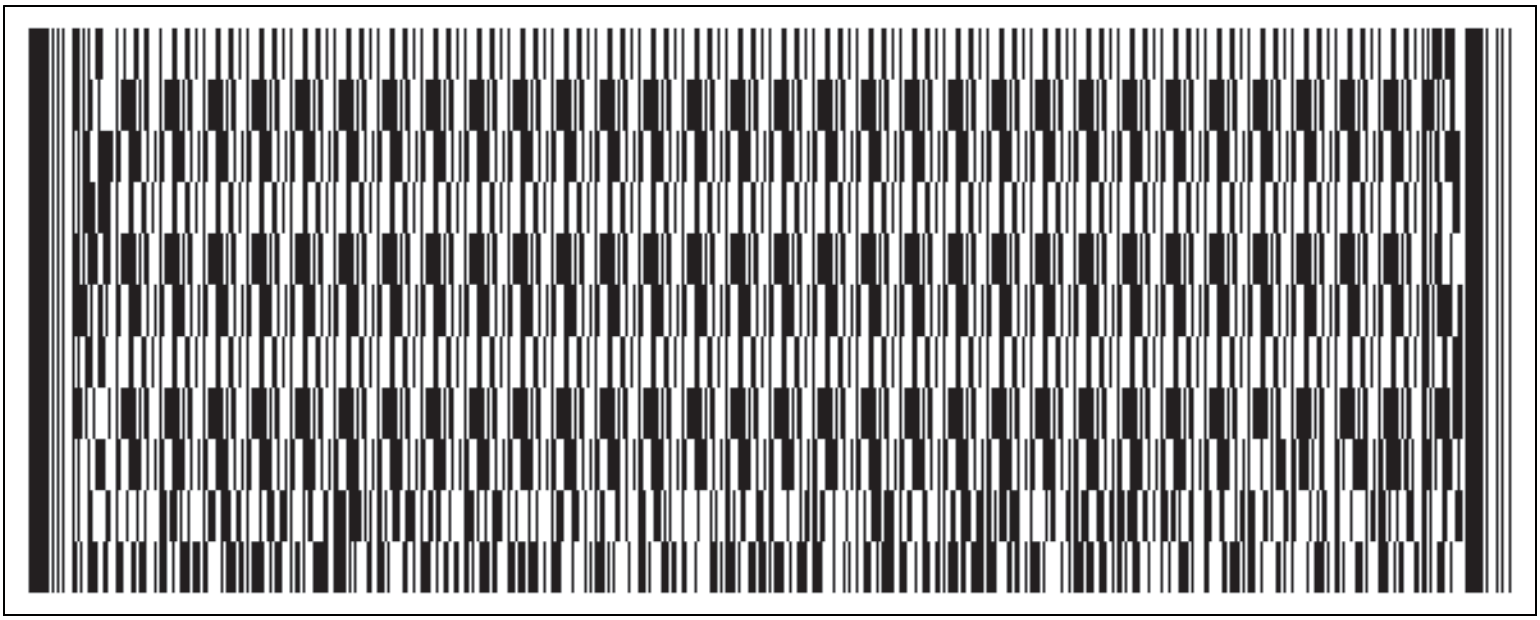
3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
(b) (6) (b) (6)	John A. Kassebaum, Chief Technical Officer	04/07/2014

PART VIII LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Euglena-GRAS-Notice.pdf	Submission
	Euglena-GRAS-Panel.pdf	Submission

OMB Statement: Public reporting burden for this collection of information is estimated to average 150 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services,Food and Drug Administration, Office of Chief Information Officer, 1350 Piccard Drive, Room 400, Rockville, MD 20850. (Please do NOT return the form to this address.). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.



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Algaeon

RECEIVED
APR 17 2014
OFFICE OF
FOOD ADDITIVE SAFETY
GRN 000513

GRAS NOTICE FOR *Euglena gracilis* CONTAINING β-1,3-GLUCAN FOR USE AS A HUMAN FOOD SUPPLEMENT

Dr. John A. Kassebaum, CTO
John.Kassebaum@Algaeon-Inc.Com

1+ (317) 225-4180 (Main)

Algaeon, Inc.
8440 W 82nd St.
Indianapolis, IN 46278

GRAS NOTICE FOR *Euglena gracilis* CONTAINING β-1,3-GLUCAN FOR USE AS A HUMAN FOOD SUPPLEMENT

Dr. John A. Kassebaum, CTO
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1+ (317) 225-4180 (Main)

Algaeon, Inc.
8440 W 82nd St.
Indianapolis, IN 46278

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1. About This Submission

The information presented in this section is a reiteration of the information presented on the attached Form FDA-3667_V13(2.13).

This is a new submission. No previous meetings with FDA have been held.

2. The Notifier

The information presented in this section is a reiteration of the information presented on the attached Form FDA-3667_V13(2.13).

The notifier is on behalf of Algaeon Inc. (the Company) is John A. Kassebaum, the Chief Technical Officer of Algaeon. John can be reached at the Company's headquarters - currently 8440 West 82nd St., Indianapolis, IN 46278 in the United States of America. The Company's main telephone number is (317) 225-4180. John may also be reached by email to 'John.Kassebaum@Algaeon-inc.com'.

3. Administrative Information

The information presented in this section is a reiteration of the information presented on the attached Form FDA-3667_V13(2.13).

The substance that is the subject of this GRAS notice submission is "*Euglena gracilis* biomass containing paramylon (β–1,3–glucan)."

This submission is in paper form, and consists of Form FDA-3667_V13(2.13), this GRAS notice document, and a GRAS Panel Review Report.

There are citations in this document which refer to information from FDA's files - specifically from: (a) *U.S. FDA Code of Federal Regulations Title 21*, section 172.809 [1]; (b) GRN 413 [2]; (c) GRN 309 [3]; (d) GRN 239 [4]; and (e) GRN 412 [5].

The determination of GRAS status was made primarily via scientific procedures by an independent panel of experts and relies on existing literature. Regardless, experience does exist based on the the presence of substantially similar substances (β–1,3–glucans) in food and feed prior to 1958 [2–9, 9, 10, 10, 11, 11, 11–13].

The current submission does not include any information that the Company views as a trade secret or as confidential commercial or financial information. There are two reports mentioned in the notice and accompanying panel report regarding Company sponsored feeding trials that were made available to the GRAS Panel, but which are *not* included in this submission. Those reports *will* be provided to the FDA upon request if the FDA should wish to examine them, but since each of those trials were conducted under non-disclosure agreements with customers of the Company, the Company must consider them confidential commercial information. No redacted copy of the current submission is currently necessary and none have been included.

4. Intended Use

Euglena gracilis biomass containing paramylon (β–1,3–glucan) to be used as a human food supplement. The paramylon (β–1,3–glucan) is intended as a source of fermentable β–1,3–glucan fiber. Supplementation is expected to be 125mg to 650 mg of paramylon (β–1,3–glucan) per day (not to exceed 2.5g per day) or 250 to 1300 mg of Euglena gracilis biomass per day (not to exceed 5g per day). This is based on an expected average intake of about 1277 grams of solid food per day by adult humans [14].

This notice is *not* intended to establish any medical functionality as an intended use of paramylon (β–1,3–glucan). However, the preponderance of literature regarding β–1,3–glucans (including paramylon) is focused on observations of β–1,3–glucan functionality as a supporter of immune function or its beneficial effects upon various conditions or injuries in humans and/or animals. Because the available literature is mainly focussed on functionality, studies of functionality will be cited - but our intent is only to establish safety for consumption.

5. Identity

The basic ingredient is *Euglena gracilis* biomass containing paramylon (a β-1,3-glucan). Paramylon is delivered either in lysed cells, or as an extracted product with greater purity.

5.1 Paramylon - the β–1,3–glucan from *Euglena gracilis*

Paramylon is a linear (unbranched) β–1,3–glucan polysaccharide polymer with high molecular weight (Figure 6.1). There are other linear (unbranched) β–1,3–glucans that are well known: curdlan, pachyman, conidiobolus and entomophthora β-1,3-glucan, and callose [7]. Curdlan, which is essentially identical to paramylon, is a permitted addition to food for human consumption under the *U.S. FDA Code of Federal Regulations Title 21*, section 172.809 [1]. Curdlan is produced by pure culture fermentation from the non-pathogenic and nontoxicogenic bacterium Alcaligenes faecalis var. myxogenes [1, 7]. Curdlan has been broadly investigated, and is universally considered to be safe for consumption [15, 16]. The only difference between curdlan and paramylon is the length of the polymer chain (molecular weight) of the polysaccharide polymer [7]. Curdlan has an average molecular weight of 100 kDa (a polymer length $n \approx 554$), while paramylon has an average molecular weight of 500 kDa (a polymer length $n \approx 2776$) [17]. Paramylon has been identified with the CAS Registry Number 9051-97-2 (which has sometimes been applied to some yeast-derived β–glucans as well), while curdlan is identified with the CAS Registry Number 54724-00-4.

6. Other Elements

6.1 Additional Identity Information

The basic ingredient is paramylon (a β-1,3-glucan), derived from *Euglena gracilis* biomass. Paramylon is delivered either in lysed cells, or as an extracted product with greater purity.

6.1.1 Paramylon - the β–1,3–glucan from *Euglena gracilis*

Paramylon is a linear (unbranched) β–1,3–glucan polysaccharide polymer with high molecular weight (Figure 6.1). There are other linear (unbranched) β–1,3–glucans that are well known: curdlan, pachyman, conidiobolus and entomophthora β-1,3-glucan, and callose [7]. Curdlan, which is essentially identical to paramylon, is a permitted addition to food for human consumption under the *U.S. FDA Code of Federal Regulations Title 21*, section 172.809 [1]. Curdlan is produced by pure culture fermentation from the non-pathogenic and nontoxicogenic bacterium Alcaligenes faecalis var. myxogenes [1, 7]. Curdlan has been broadly investigated, and is universally considered to be safe for consumption [15, 16]. The only difference between curdlan and paramylon is the length of the polymer chain (molecular weight) of the polysaccharide polymer [7]. Curdlan has an average molecular weight of 100 kDa (a polymer length $n \approx 554$), while paramylon has an average molecular weight of 500 kDa (a polymer length $n \approx 2776$) [17]. Paramylon has been identified with the CAS Registry Number 9051-97-2 (which has sometimes been applied to some yeast-derived β–glucans as well), while curdlan is identified with the CAS Registry Number 54724-00-4.

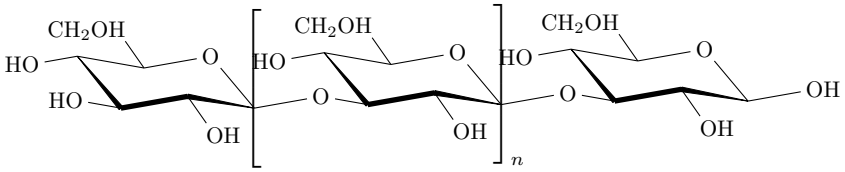
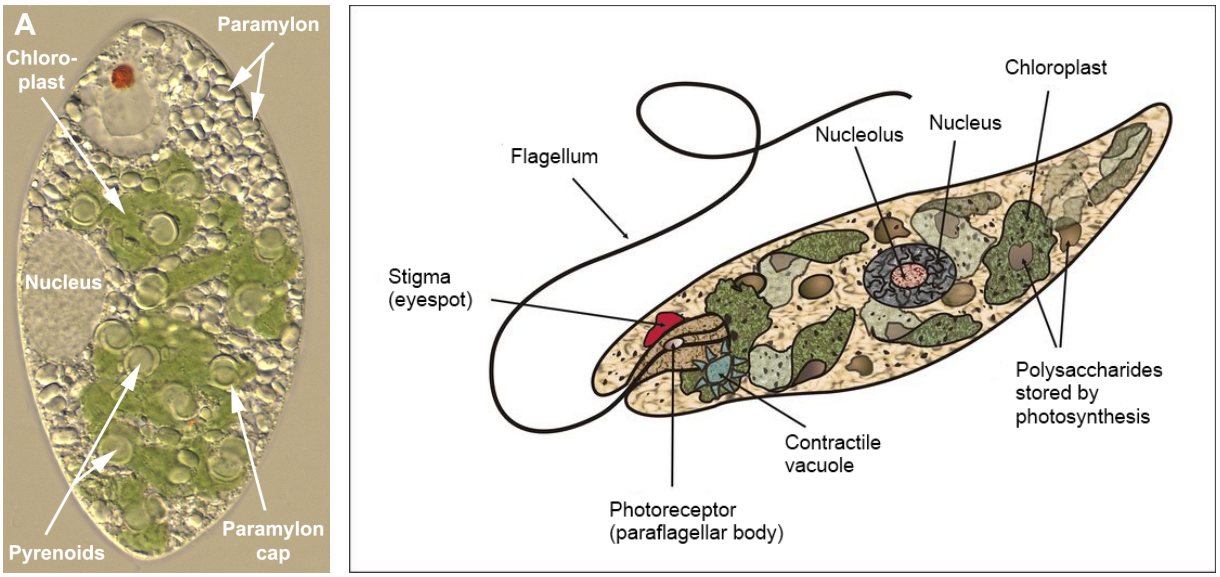


Figure 6.1: Molecular structure of the linear (unbranched) β–1,3–glucans paramylon and curdlan [7]

Other common sources of dietary β–glucans include: (a) branch-on-branch β–(1,3;1,6)–glucans from the cell walls of yeast [7]; (b) linear β–(1,3;1,6)–glucans found in grasses and cereals (such as oats and barley) [7]; and (c) linear β–1,3–glucans with β–(1,6)–glucosyl or oligoglucosyl side chains (lentinan) found in mushrooms such as *Lentinus edodes* (Shiitake) [7]. In all of these cases, paramylon has less variety since it consists wholly of β–1,3–glucan type bonds. Paramylon lacks β–(1,6), β–(1,4), and β–(1,2) bonds as well as side branching structures of any kind. The result is a molecule which is less soluble and more homogeneous. As a result, the long safety record of yeasts and grains in both feed and human food apply directly to paramylon.



(a) Pyrenoids and paramylon (b) Euglena Diagram

Figure 6.2: Cellular Structures of Euglena
(a) ©Angelika Preisfeld and David Patterson (b) [18]

6.1.2 *Euglena gracilis* Ecology

Euglena gracilis (see Figure 6.2 on this page) is a member of the micro-algae division *Euglenophyceae*, within the *Euglenales* family [18]. The division *Euglenophyceae* includes about 75 species of autotrophic and heterotrophic species. Most are found in enriched freshwaters, particularly shallow water rivers, lakes, and ponds. At least two species can live in saline waters. Blooms of these algae occur during late summer-fall in temperate systems. The strain used by the Company was obtained from the Culture Collection of Algae and Protozoa (CCAP) in Glasgow, Scotland - *Euglena gracilis* (CCAP 1224/5Z).

Paramylon is the principal energy-storage compound for Euglenoids, comparable to starch or oils/fats in other algal divisions. Paramylon is produced in the pyrenoids and stored as granules in the cytoplasm. The paramylon granules in *Euglena gracilis* are about 2 μm in diameter [19]. Midwest Laboratories Inc analyzed cultured *Euglena gracilis* and reported chemical composition (Table 6.1 on the next page). This same lot was also independently quantitated at 58.1% paramylon by Dr. Kyle Newman of Venture Laboratories in Lexington Kentucky (Report No. 13599 - Lot No. EG107.0008). Composition detail is also available in the literature [20, 21].

6.2 Method of Manufacture

Euglena gracilis and its paramylon is manufactured according to current Good Manufacturing Practices (cGMP) - see Figure 6.3). Cultures will be grown in closed bioreactors that are cleaned and sanitized to kill micro-organisms prior to inoculation and growout. *Euglena gracilis* stock cultures that are maintained in controlled laboratory conditions will be used as inoculum. The *Euglena gracilis* inoculum is aseptically transferred to the culture vessels (bioreactors) where it is grown in large quantities. Algal cultures will be grown in liquid cultures using agriculture grade fertilizers. At harvest, the finished culture is then dewatered to separate solids (*Euglena* biomass) from culture media. The resulting paste is then pasteurized to kill the *Euglena gracilis* and any biological contaminants that may be present. Thereafter, the biomass is dried to NMT 10% moisture and lightly ground to form a powder. This is the first form of the product - a cracked (lysed) *Euglena gracilis* biomass as a free-flowing powder.

Table 6.1: Component Analysis of Euglena performed by Midwest Laboratories Inc. (Report #13-157-9434).

Component	*Dry Weight*	*Meas. Limit*	*Method*
Protein (crude)	11.0%	0.20%	AOAC 990.03
Fat (crude)	11.3%	0.10%	AOAC 2003.05
Fiber (acid detergent)	0.5%	0.5%	ANKOM Tech. Method
Fiber (neutral detergent)	n.d.	1.0%	ANKOM Tech. Method
Ash	0.74%	0.10%	AOAC 942.05
Starch (total)	n.d.	0.10%	AACC 76-11 (mod)
Nitrogen Free Extract (NFE)	76.46%	-	-
— Paramylon (β–1,3–glucan)	58.1%	-	-
Sulfur (total)	0.11%	0.01%	AOAC 985.01 (mod)
Phosphorus (total)	0.65%	0.01%	AOAC 985.01 (mod)
Potassium (total)	0.05%	0.01%	AOAC 985.01 (mod)
Magnesium (total)	0.04%	0.01%	AOAC 985.01 (mod)
Calcium (total)	0.01%	0.01%	AOAC 985.01 (mod)
Sodium (total)	n.d.	0.01%	AOAC 985.01 (mod)
Iron (total)	341 ppm	5.0 ppm	AOAC 985.01 (mod)
Manganese (total)	5.2 ppm	1.0 ppm	AOAC 985.01 (mod)
Copper (total)	18.9 ppm	1.0 ppm	AOAC 985.01 (mod)
Zinc (total)	34.4 ppm	1.0 ppm	AOAC 985.01 (mod)

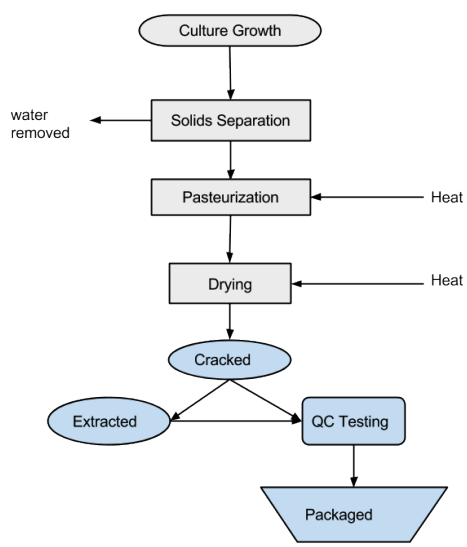


Figure 6.3: Manufacturing Process Diagram

A second product requires chemical extraction of the cracked *Euglena gracilis* biomass. Several chemical extraction methods are available [22–28] including sodium dodecyl sulfate (SDS) in water for extraction of lipids and pigments followed by washing with either water or ethanol. The material can then be further isolated either by the application of a sodium hydroxide solution to dissolve the paramylon, or an acid solution to remove materials other than paramylon. In either case, the further isolated mass of paramylon is neutralized, washed, dried, and milled to produce a free-flowing powder. The extraction process may modify the paramylon granules in the biomass by increasing their exposed surface area. The extracted paramylon may be more bioavailable than the paramylon in either uncracked or cracked *Euglena gracilis*.

The final paramylon product(s) are then packaged to reduce exposure to moisture and oxygen. The bags

are stored frozen in sealed containers prior to shipment.

6.3 Specifications for Food-grade Material

Two products are envisioned by this definition: (a) cracked *Euglena gracilis* biomass (lysed cells); and (b) extracted/purified paramylon. Product specifications are presented in Table 6.2 on this page.

Table 6.2: Paramylon Product Specifications

1. Quality Specifications
   (a) Cracked *Euglena gracilis* biomass – NLT 50% paramylon by dry weight.
   (b) Extracted/Purified Paramylon – NLT 80% paramylon by dry weight.
2. Physical Properties
   (a) Appearance: beige to light grey/brown powder
   (b) Moisture content: NMT 10%
3. Inorganic Contaminants
   (a) Inorganic Arsenic: NMT 3 ppm
   (b) Total Arsenic: NMT 10 ppm
   (c) Cadmium: NMT 1 ppm
   (d) Lead: NMT 3 ppm
   (e) Mercury: NMT 0.25 ppm
4. Biological Contaminants
   (a) Total Aerobic Count: NMT 10^5 per gram
   (b) Total Yeast and Mold: NMT 10^2 per gram
   (c) E Coli/Coliforms: NMT 10^1 per gram (but no detectable fecal coliforms)
5. Pesticides and Plasticizer Contaminants
   (a) Free of measurable dioxins
   (b) Free of measurable melamine
6. Handling Recommendations
   (a) Material is somewhat hygroscopic - store in sealed container at NMT 70% relative humidity.
   (b) Material susceptible to mold - it is acceptable to refrigerate or freeze product for longer shelf life.

Paramylon becomes hygroscopic at relative humidities greater than 70% and will increase in weight by up to 10% upon hydration, and then is difficult to re-dry [22, 29, 30]. As a result, paramylon (in purified form) should be kept in dry conditions (NMT 70% relative humidity) to avoid formation of a hydrate. Undissolved paramylon, in lysed cell form, would require heating to $140°C$ in water to become hydrated [22, 29, 30]. Nonetheless, the *Euglena* biomass will hydrate as well, so it should also be kept in dry conditions.

6.4 Dietary Exposure

Intended and expected dietary exposure to paramylon (β-1,3-glucan) is in the range of 0.01% to 0.05% (100 to 500 mg of paramylon per kilogram of food) but is not expected to exceed to 0.2% (2000 mg of paramylon per kilogram of food). In the case of lysed *Euglena gracilis* cells, with a minimum paramylon content of 50%, the intended and expected dietary exposure to *Euglena gracilis* biomass containing paramylon (β-1,3-glucan) is in the range of 0.02% to 0.1% (200 to 1000 mg of *Euglena gracilis* biomass per kilogram of food) but is not expected to exceed 0.4% (4000 mg of *Euglena gracilis* biomass per kilogram of food). Based on an expected average intake of about 1277 grams of solid food per day by adult humans [14], supplementation is expected to be 125mg to 650 mg of paramylon (β–1,3–glucan) per day (not to exceed 2.5g per day) or 250 to 1300 mg of Euglena gracilis biomass per day (not to exceed 5g per day).

6.5 Self-limiting Levels of Use

A toxicological assessment of curdlan, which has identical molecular structure to paramylon [7], showed that even at 15% of the diet there was no evidence of adverse effects on the nutritional status of the animals [16]. Likewise, the U.S. FDA and the U.N World Health Organization have recognized curdlan as safe at 30% of the diet of mice, rats, and dogs [1, 15]. Acute toxicity levels (LD_{50}) for oral administration were shown to be greater than 10,000 milli-grams per kilogram of body weight for both mice and rats [15, 16]. Curdlan was *not* found to have genotoxicity [15]. Studies in humans at dosages up to 50 grams of curdlan per day showed no evidence of toxicity [15].

If we add the above assessment to the other known sources of β–1,3–glucans present in oats, barley, mushrooms, and other long-known food and feed sources [2–9, 9, 10, 10, 11, 11, 11–13], it is clear that any conceivable use of paramylon (up to 15% of an human or animal diet) is unlikely to be able to achieve toxicity. The Company, therefore, is confident that paramylon from *Euglena gracilis* is safe for consumption up to a paramylon level of 15% of a human diet.

6.6 Common use in food before 1958 (if applicable)

Although this notice is based upon scientific study, it is a fact that β–1,3–glucans are present in oats, barley, mushrooms, and other long-known food and feed sources in use before 1958 [2–9, 9, 10, 10, 11, 11, 11–13].

6.7 Basis for GRAS status

In order to determine whether Euglena gracilis could be considered GRAS by use of scientific procedures under 21 CFR 170.30(b), the Company convened an independent panel of experts into a GRAS review panel. The review panel found that *paramylon*, as a β-1,3-glucan polysaccharide polymer, is safe for the proposed uses based on: (a) the long history of safe use of β-1,3-glucans contained in many common food & feed sources such as oats and grains; (b) existing GRAS filings and FDA regulations in regard to β-1,3-glucans; (c) the large number of peer reviewed studies which have demonstrated no untoward effects from *paramylon* and β-1,3-glucans while purporting improved functionality in immune function; and (d) the broadly understood safety of curdlan [1, 15, 16]. The review panel believes that *Euglena gracilis* is safe because: (a) it contains nutrients such as proteins, lipids, and carbohydrates; (b) it is nontoxogenic and nonpathogenic based on long use in industry as a laboratory microbe; (c) the Company's own analysis (using an independent lab) found no traces of biotoxins; (d) a Company sponsored fish trial that showed no significant differences in mortality between control and treated fish over a 12 week period; (e) a Company sponsored poultry trial that showed no significant differences in mortality between control and treated poultry over a 2 week period; and (f) there is a company in the international market selling *Euglena gracilis* for human

consumption, and another company in the US. market selling a *Euglena gracilis* derived product for animal feed.

There are companies other than Algaeon already selling *Euglena gracilis* containing *paramylon* for both human and animal consumption with no untoward effects. The Euglena Company has been working since 2005 to make Euglena a human food source and dietary supplement in Japan. They are focussed on *Euglena gracilis* itself (rather than on *paramylon*) and offer a number of foods and supplements derived from *Euglena gracilis*. There is also an approved human use β-1,3-glucan [1] and several β–(1,3;1,6)–glucans that have GRAS notices filed with the FDA [2–4]. Many brands of human food supplements containing β–(1,3;1,6)–glucans are readily available to the public at pharmacies, groceries, and health food stores.

The preponderance of literature regarding β–1,3–glucans is focused on observations of β–1,3–glucan as a supporter of immune function or its beneficial effects upon various conditions or injuries in humans or animals. This notice is *not* intended to establish any human medical claims with the use of paramylon. However, since much of the available literature is focused on functionality, these studies will be cited in support of safe use.

The paramylon derived from *Euglena gracilis* is similar to β–1,3–glucans from other sources such as yeast, bacteria, mushrooms and grains [6, 17, 31–34]. The differences and advantages in the β-1,3-glucans from *Euglena gracilis* come from: (a) the purity of the paramylon β–1,3–glucan bond structure (with no branching or 1-2, 1-4 or 1-6 bond types) [23, 24, 27, 35, 36]; (b) the size and shape of the β-1,3-glucan crystals produced by Euglena [19, 37]; and (c) the molecular weight of the β-1,3-glucan chains [17, 33]. Paramylon, the β-1,3-glucan from *Euglena gracilis*, has been shown to be safe in numerous trials [6, 17, 31–34]. A quantifiably superior effect of paramylon from *Euglena gracilis* over the β–1,3–glucans from other sources has also been observed [17]. Substantially pure paramylon from *Euglena gracilis* is also usable as an adjuvant in vaccines [27, 38–42]. All of this strongly supports a broad safety profile for paramylon.

6.7.1 Safety Reports

The safety of β–1,3–glucans in food is best viewed in the context of their long safe use as a constituent of oat and barley grains [6–12]. The same polysaccharide polymers are present in other food-acceptable sources, including mushrooms [2, 9–11, 13], yeasts [3, 4, 11], and even molds [5]. Newer sources of dietary β–1,3–glucans include *Euglena gracilis* micro-algae (an AAFCO listed metal-polysaccharide - a zinc β–1,3–glucan compound [35]) and bacteria (permitted human food additive - Curdlan [1, 15, 16]). As curdlan is chemically identical to paramylon, safety data for curdlan are directly referable. A subchronic toxicity study of mushroom β–(1,3;1,6)–glucan in rats found no adverse effect levels with a no observed-adverse-effect level (NOAEL) for β–(1,3;1,6)–glucans fed at 2,000 mg per kg of body weight per day [13]. β–1,3–glucans caused no adverse effects in nonhuman primates and appeared to have positive effects on the overall health of the test animals [39] characterized by: (a) supporting immunity to a diverse group of microbial and tumor antigens; and (b) nonspecifically enhanced host resistance to neoplastic, bacterial, and fungal diseases. An assessment of in vitro safety and tolerability in human cells at different concentrations demonstrated no adverse events [43]. β–1,3–glucans were also shown to be hepatoprotective in rats given carbon tetrachloride [32]. Further, inhalation of β–1,3–glucan by healthy humans caused markedly less inflammatory response than bacterial endotoxin [44]. β–1,3–glucans also appear to have radio-protective properties (reducing some deleterious effects of ionizing radiation) in animals and people [45–48]. An extensive review of over 1000 papers revealed no adverse effects in humans or animals from β–1,3–glucan ingestion or administration.

6.7.2 Impurities and Contaminants

Euglenophycin is not produced by *Euglena gracilis*

Euglena species are generally not producers of toxins (nontoxogenic), but there is a member of the division - *Euglena sanguinea* - that produces a known ichthyotoxin, euglenophycin [49, 50]. *Euglena* species, and

specifically the species *Euglena gracilis* utilized by the Company, are widely considered to be nonpathogenic and nontoxogenic. To further test and confirm this, the Company has engaged in two in-vivo tests: 1) a feeding trial in yellow perch using dried *Euglena gracilis* biomass at varying dosages vs. a control; and 2) an assay of the Company's *Euglena gracilis* culture and dried product (conducted by Dr. Paul Zimba, author of [49, 50]) for the presence of euglenophycin. The fish trial found no significant differences in mortality between control and treated fish over a 12 week feeding period. The assays of the culture and the dried *Euglena gracilis* biomass product showed no measurable content of euglenophycin in either. A brief report on the fish trial and the euglenophycin assay results are available from the Company upon request.

6.7.3 Safe for Human Consumption and Handling

Paramylon is chemically identical to curdlan that has a long history of safe use [1, 15, 16]. β–1,3–glucans have been shown to support human immune function (for instance, against viral infections such as the common cold) [9, 10, 51–53] and oral β–1,3–glucans have demonstrated some benefit to cardiovascular health [54] without any serious averse events. Oral β–1,3–glucans have also shown anti-tumor effects and may contribute to patient survival [6, 43]. β–1,3–glucans have demonstrated apparent radio-protective properties (reducing some deleterious effects of ionizing radiation) in animals and people [45–48]. In contrast to known inflammatory agents, such as bacterial endotoxin, inhalation of β–1,3–glucans do not cause an acute inflammatory response [44]. An assessment of human safety and tolerability of soluble β–1,3–glucans fed at 100mg/day, 200mg/day or 400mg/day for 4 consecutive days demonstrated no adverse events [43]. An extensive investigation of the literature revealed no adverse effects in humans or animals from β–1,3–glucan ingestion, administration, or inhalation.

6.7.4 Paramylon is Safe for Animal Consumption

Multiple studies affirm safety in many animal species which can be used to support safety as a constituent of human food.

Swine

β–1,3–glucans have been shown to enhance innate antiviral immunity (via T-cell function) in weanling pigs [17, 55–58]. Effects of β–1,3–glucans on immune function did not contribute to a systemic inflammatory response, but instead appeared to prime immune defenses by altering subsequent responses to infection [9, 10, 58–61]. Oral β–1,3–glucans also appeared to support cardiovascular health of swine [62]. Furher, oral β–1,3–glucans improved the composition of the intestinal microbiome [61, 63]. This may play a role in β–1,3–glucans improving weight gain performance in weanling pigs [58]. Based on this evidence, β–1,3–glucans (such as Paramylon from *Euglena gracilis*) appear to be safe as a food additive for swine and piglets.

Poultry

Dietary benefits of β-1,3-glucan have been demonstrated in poultry health [64–68]. One study notes that dietary β-1,3-glucan is effective in promoting growth in broiler chickens [65, 66]. Many of the studies show significant growth-promoting and immune-enhancing effects in poultry in response to dietary β-1,3-glucan [64–66, 69–75]. Under a Salmonella challenge, oral β-1,3-glucans also improved poultry gut morphology (increased villus height and villus height to crypt depth ratio) and inhibited cecal *Salmonella* colonization levels [75]. The Company, in colloboration with one of its potential customers, had a trial conducted with varying treatment levels of paramlyon for poultry. The test showed no significant differences in mortality between control and treated chicks over the trial period of two (2) weeks. Based on all this evidence, β–1,3–glucans (such as paramylon from *Euglena gracilis*) appear to be safe as a food additive for poultry.

Cattle

β-1,3-glucans have also been shown to support immune function and improve responses to stressful events, such as transport, while enhancing feed consumption in cattle [12, 34, 64, 76, 77]. These benefits seem to be further enhanced by dietary antioxidants such as vitamin-C (ascorbic acid) [77]. Apparent digestibility of β–1,3–glucans in steers was 98.6% [12]. Thus, β–1,3–glucans (such as paramylon from *Euglena gracilis*) appear to be safe as a food additive for cattle.

Fish

β-1,3-glucans have been shown to support immune function in fish, shellfish and live-feeds (such as artemia and rotifers) [23, 31, 34, 78–84]. Paramylon also appears to support immune function of rainbow trout fry when dissolved or suspended in the water [78]. Immersion of fish in β-1,3-glucans provides some limited protection, but dietary intake of β–1,3–glucan was much better at increasing resistance of fish to bacterial pathogens [85]. The Company, in collaboration with one of its potential customers, had a trial conducted with varying treatment levels of paramlyon in fish (yellow perch). The test showed no significant differences in mortality between control and treated fish over the trial period of twelve (12) weeks. Thus, β–1,3–glucans (such as paramylon from *Euglena gracilis*) appear to be safe as a food additive for fish and in live-feeds.

Companion Animals

β–1,3–glucans were able to support immune function in dogs in one study [86] with apparent positive effects on inflammatory bowel disease and osteoarthritis [87, 88]. β–1,3–glucan administration also demonstrated similar benefit as levamisole in mares and foals by support of the foal immune system via colostrum trasnfer [89]. β–1,3–glucans have demonstrated no harm in companion animals and appear to decrease the incidence of certain disease states [90].

6.7.5 Environmental Safety

Paramylon from *Euglena gracilis* is nontoxic. Both *Euglena gracilis* and parmylon are natural materials that are completely biodegradable and pose negligible environmental hazard. Further, they do not pose a biohazard risk for shipping or handling.

Bibligraphy

[1] "Curdlan," *U.S. FDA Code of Federal Regulations Title 21*, vol. 3, no. 172.809, Apr. 2013. [Online]. Available: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=172.809

[2] E. A. Steele, "Notification of GRAS Determination for β-glucans derived from mushroom ," *U.S. Food and Drug Administration, GRAS Notice*, no. 413, pp. 1–33, Jan. 2012. [Online]. Available: http://www.accessdata.fda.gov/scripts/fcn/gras_notices/GRN000413.pdf

[3] Glucan Corporation Ltd., "GRAS Notice for Black Yeast Beta-Glucan (Polycan®)," *U.S. Food and Drug Administration, GRAS Notice*, no. 309, pp. 1–125, Jun. 2010. [Online]. Available: http://www.accessdata.fda.gov/scripts/fcn/gras_notices/grn_309.pdf

[4] D. J. Cox, "GRAS Notice for Yeast Beta-Glucan," *U.S. Food and Drug Administration, GRAS Notice*, no. 239, pp. 1–91, Jul. 2008. [Online]. Available: http://www.accessdata.fda.gov/scripts/fcn/gras_notices/grn000239.PDF

[5] V. Maquet, "GRAS Notice for Chitin-Glucan," *U.S. Food and Drug Administration, GRAS Notice*, no. 412, pp. 1–122, Dec. 2011. [Online]. Available: http://www.accessdata.fda.gov/scripts/fcn/gras_notices/GRN000412.pdf

[6] R. Wismar, S. Brix, H. Frøkiaer, and H. N. Laerke, "Dietary fibers as immunoregulatory compounds in health and disease," *Annals of the New York Academy of Sciences*, vol. 1190, no. 1, pp. 70–85, Mar. 2010.

[7] B. A. Stone, "Chemistry of β-Glucans," in *Chemistry, Biochemistry and Biology of (1→3)-β-glucans and Related Polysaccharides*, A. Bacic, G. B. Fincher, and B. A. Stone, Eds. Academic Press, Jan. 2009, pp. 5–46.

[8] P. J. Harris and G. B. Fincher, "Distribution, Fine Structure and Function of (1,3;1,4)-β-Glucans in the Grasses and Other Taxa," in *Chemistry, Biochemistry and Biology of (1→3)-β-glucans and Related Polysaccharides*, A. Bacic, G. B. Fincher, and B. A. Stone, Eds. Academic Press, Jan. 2009, pp. 621–654.

[9] J. Volman, *Immune modulation by dietary glucans from oat and mushrooms: results from* in vitro*, animal and human studies*, ser. PhD Thesis. Universiteit Maastricht, 2010.

[10] J. J. Volman, J. D. Ramakers, and J. Plat, "Dietary modulation of immune function by β-glucans," *Physiology & Behavior: Traces in Metabolism and Nutrition*, vol. 94, no. 2, pp. 276–284, May 2008. [Online]. Available: http://www.sciencedirect.com/science/article/pii/S0031938407004702

[11] D. Akramiene, A. Kondrotas, J. Didziapetriene, and E. Kevelaitis, "Effects of β-glucans on the immune system." *Medicina (Kaunas, Lithuania)*, vol. 43, no. 8, pp. 597–606, 2007. [Online]. Available: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=17895634&retmode=ref&cmd=prlinks

[12] D. F. Engstrom, G. W. Mathison, and L. A. Goonewardene, "Effect of β-glucan, starch, and fibre content and steam vs. dry rolling of barley grain on its degradability and utilisation by steers," *Animal Feed Science and Technology*, vol. 37, no. 1, pp. 33–46, 1992. [Online]. Available: http://www.sciencedirect.com/science/article/pii/037784019290118P

[13] S. N. Chen, F. H. Nan, S. Chen, J. F. Wu, C. L. Lu, and M. G. Soni, "Safety assessment of mushroom β-glucan: subchronic toxicity in rodents and mutagenicity studies." *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, vol. 49, no. 11, pp. 2890–2898, Nov. 2011. [Online]. Available: http://linkinghub.elsevier.com/retrieve/pii/S027869151100411X

[14] S. Bingham, N. I. McNeil, and J. H. Cummings, "The diet of individuals: a study of a randomly-chosen cross section of British adults in a Cambridgeshire village." *British Journal of Nutrition*, vol. 45, no. 1, pp. 23–35, Jan. 1981. [Online]. Available: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=6258625&retmode=ref&cmd=prlinks

[15] M. E. von Apeldoorn and G. J. A. Speijers, "968. Thickener: Curdlan (WHO Food Additives Series 44)," *inchem.org*, 2000. [Online]. Available: http://www.inchem.org/documents/jecfa/jecmono/v44jec04.htm

[16] E. J. Spicer, E. I. Goldenthal, and T. Ikeda, "A toxicological assessment of curdlan." *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, vol. 37, no. 4, pp. 455–479, Apr. 1999. [Online]. Available: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=10418959&retmode=ref&cmd=prlinks

[17] E. Sonck, E. Stuyven, B. Goddeeris, and E. Cox, "The effect of β-glucans on porcine leukocytes," *Veterinary Immunology and Immunopathology*, vol. 135, no. 3-4, pp. 199–207, Jun. 2010. [Online]. Available: http://dx.doi.org/10.1016/j.vetimm.2009.11.014

[18] B. S. Leander, "Euglenida. euglenids or euglenoids. Version 10 November 2012." in *The Tree of Life Web Project. http://tolweb.org/*, 2012. [Online]. Available: http://tolweb.org/Euglenida/97461/2012.11.10

[19] A. K. Monfils, R. E. Triemer, and E. F. Bellairs, "Characterization of paramylon morphological diversity in photosynthetic euglenoids (Euglenales, Euglenophyta)," *Phycologia*, vol. 50, no. 2, pp. 156–169, Mar. 2011. [Online]. Available: http://www.phycologia.org/doi/pdf/10.2216/09-112.1

[20] E. Kempner and J. Miller, "The Molecular Biology of *Euglena gracilis*: 1. Growth Conditions and Cellular Composition," *Biochimica Et Biophysica Acta*, vol. 104, pp. 11–17, 1965. [Online]. Available: http://www.sciencedirect.com/science/article/pii/030441656590214X

[21] A. Tolivia, V. Conforti, O. Córdoba, and L. Flores, "Chemical constituents and biological activity of Euglenagracilis extracts," *JOPR: Journal of Pharmacy Research*, vol. 7, no. 3, pp. 209–214, Apr. 2013. [Online]. Available: http://dx.doi.org/10.1016/j.jopr.2013.03.019

[22] J. Z. Kiss, E. M. Roberts, R. M. Brown, and R. E. Triemer, "X-ray and Dissolution Studies of Paramylon Storage Granules from *Euglena*," *Protoplasma*, vol. 146, pp. 150–156, Nov. 1988. [Online]. Available: http://link.springer.com/article/10.1007/BF01405924#page-1

[23] L. Barsanti, R. Vismara, V. Passarelli, and P. Gualtieri, "Paramylon (β-1, 3-glucan) content in wild type and WZSL mutant of *Euglena gracilis*. Effects of growth conditions," *Journal of Applied Phycology*, vol. 13, no. 1, pp. 59–65, 2001.

[24] B. Šantek, M. Felski, K. Friehs, M. Lotz, and E. Flaschel, "Production of paramylon, a β-1,3-glucan, by heterotrophic cultivation of *Euglena gracilis* on a synthetic medium," *Engineering in Life Sciences*, vol. 9, no. 1, pp. 23–28, Feb. 2009.

[25] B. Šantek, K. Friehs, M. Lotz, and E. Flaschel, "Production of paramylon, a β-1,3-glucan, by heterotrophic growth of *Euglena gracilis* on potato liquor in fed-batch and repeated-batch mode of cultivation," *Engineering in Life Sciences*, vol. 12, no. 1, pp. 89–94, 2011. [Online]. Available: http://onlinelibrary.wiley.com/doi/10.1002/elsc.201100025/pdf

[26] A. Sugiyama, S. Hata, K. Suzuki, E. Yoshida, R. Nakano, S. Mitra, R. Arashida, Y. Asayama, Y. Yabuta, and T. Takeuchi, "Oral Administration of Paramylon, a β-1,3-D-glucan Isolated from *Euglena gracilis Z* Inhibits Development of Atopic Dermatitis-Like Skin Lesions in NC/Nga Mice." *The Journal of veterinary medical science the Japanese Society of Veterinary Science*, vol. 72, no. 6, pp. 755–763, Feb. 2010. [Online]. Available: https://www.jstage.jst.go.jp/article/jvms/72/6/72_09-0526/_pdf

[27] D. Tuse, L. Marquez, and L. A. Hokama, "Production of Beta-1,3-Glucan in Euglena," *U.S. Patent*, pp. 1–9, 1992. [Online]. Available: http://www.google.co.il/patents/US5084386?printsec=abstract#v=onepage&q&f=false

[28] J. Rodríguez-Zavala, M. Ortiz-Cruz, G. Mendoza-Hernández, and R. Moreno-Sánchez, "Increased synthesis of α-tocopherol, paramylon and tyrosine by *Euglena gracilis* under conditions of high biomass production," *Journal of Applied Microbiology*, vol. 109, no. 6, pp. 2160–2172, Sep. 2010.

[29] R. H. Marchessault and Y. Deslandes, "Fine structure of (1$\rightarrow$3)-β-D-glucans: curdlan and paramylon," *Carbohydrate Research*, vol. 75, pp. 231–242, 1979. [Online]. Available: http://www.sciencedirect.com/science/article/pii/S000862150084642X

[30] K. Kobayashi, S. Kimura, E. Togawa, M. Wada, and S. Kuga, "Crystal transition of paramylon with dehydration and hydration," *Carbohydrate Polymers*, vol. 80, no. 2, pp. 491–497, Apr. 2010. [Online]. Available: http://dx.doi.org/10.1016/j.carbpol.2009.12.009

[31] R. Vismara, S. Vestri, A. Frassanito, L. Barsanti, and P. Gualtieri, "Stress resistance induced by paramylon treatment in Artemia sp." *Journal of Applied Phycology*, vol. 16, no. 1, pp. 61–67, 2004.

[32] A. Sugiyama, K. Suzuki, S. Mitra, R. Arashida, E. Yoshida, R. Nakano, Y. Yabuta, and T. Takeuchi, "Hepatoprotective effects of paramylon, a beta-1, 3-D-glucan isolated from Euglena gracilis Z, on acute liver injury induced by carbon tetrachloride in rats." *The Journal of veterinary medical science the Japanese Society of Veterinary Science*, vol. 71, no. 7, pp. 885–890, 2009.

[33] E. Sonck, B. Devriendt, B. Goddeeris, and E. Cox, "Varying Effects of Different β-Glucans on the Maturation of Porcine Monocyte-Derived Dendritic Cells," *Clinical and Vaccine Immunology*, vol. 18, no. 9, pp. 1441–1446, Sep. 2011.

[34] R. Jaafar, J. Skov, P. Kania, and K. Buchmann, "Dose Dependent Effects of Dietary Immunostimulants on Rainbow Trout Immune Parameters and Susceptibility to the Parasite Ichthyophthirius Multifiliis," *J Aquac Res Development S*, vol. 3, p. 2, Nov. 2011. [Online]. Available: http://www.omicsonline.org/2155-9546/2155-9546-S3-001.pdf

[35] G. Horst, "Use of β-(1,3)-D-Glucan in the Feed Industry," *Algal Scientific Corporation Technical Report*, pp. 1–5, Feb. 2013. [Online]. Available: http://www.algalscientific.com/algal-scientific-beta-glucan-white-paper.pdf

[36] J. Cook, "Quantitative measurement of paramylum in *Euglena gracilis*," *Journal of Eukaryotic Microbiology*, vol. 14, no. 4, pp. 634–636, 1967. [Online]. Available: http://onlinelibrary.wiley.com/doi/10.1111/j.1550-7408.1967.tb02053.x/abstract

[37] R. Howard, S. Wright, and B. Grant, "Structure and Some Properties of Soluble 1, 3-β-Glucan Isolated from the Green Alga *Caulerpa simpliciuscula*," *Plant Physiology*, vol. 58, no. 4, p. 459, 1976. [Online]. Available: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC543239/pdf/plntphys00165-0016.pdf

[38] W. Suwelack, P. Tewes-Schwarzer, D. Baumer, and U. Kahmann, "Freeze-Dried Agent Containing Paramylon, Its Production and Use," *US Patent #2003/0203016 A1*, pp. 1–15, Oct. 2012.

[39] J. A. Reynolds, M. D. Kastello, D. G. Harrington, C. L. Crabbs, C. J. Peters, J. V. Jemski, G. H. Scott, and N. R. DiLuzzio, "Glucan-Induced Enhancement of Host Resistance to Selected Infectious Diseases," *Infection and Immunity*, vol. 30, no. 1, pp. 51–57, Oct. 1980. [Online]. Available: http://iai.asm.org/content/30/1/51.full.pdf

[40] J. A. Cook, T. W. Holbrook, and W. J. Dougherty, "Protective Effect of Glucan Against Visceral Leishmaniasis in Hamsters," *Infection and Immunity*, vol. 37, no. 3, pp. 1261–1269, Aug. 1982. [Online]. Available: http://iai.asm.org/content/37/3/1261.full.pdf

[41] J. B. Jorgensen, H. Lunde, and B. Robertsen, "Peritoneal and head kidney cell response to intraperitoneally injected yeast glucan in Atlantic salmon, Salmo salar L." *Journal of Fish Diseases*, vol. 16, no. 4, pp. 313–325, 1993. [Online]. Available: http://dx.doi.org/10.1111/j.1365-2761.1993.tb00865.x

[42] D. Chen and A. J. Ainsworth, "Glucan administration potentiates immune defence mechanisms of channel catfish, *Ictalurus punctatus* Rafinesque," *Journal of Fish Diseases*, vol. 15, no. 4, pp. 295–304, 1992. [Online]. Available: http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2761.1992.tb00667.x/full

[43] G. Chan, W. Chan, and D. Sze, "The effects of β-glucan on human immune and cancer cells," *Journal of Hematology & Oncology*, vol. 2, no. 25, 2009. [Online]. Available: http://www.jhoonline.org/content/2/1/25

[44] J. Thornca, L. Beijer, and R. Rylander, "Effects after inhalation of (1→3)-β-D-glucan in healthy humans," *Mediators of Inflammation*, vol. 10, no. 4, pp. 173–178, Aug. 2001. [Online]. Available: ttp://downloads.hindawi.com/journals/mi/2001/573670.pdf

[45] M. Patchen, M. D'Alesandro, M. Chirigos, and J. Weiss, "Radioprotection by biological response modifiers alone and in combination with WR-2721," *Armed Forces Radiobiology Research Institute*, 1989. [Online]. Available: http://www.dtic.mil/dtic/tr/fulltext/u2/a207914.pdf

[46] P. J. Rice, E. L. Adams, T. Ozment-Skelton, A. J. Gonzalez, M. P. Goldman, B. E. Lockhart, L. A. Barker, K. F. Breuel, W. K. DePonti, J. H. Kalbfleisch, H. E. Ensley, G. D. Brown, S. Gordon, and D. L. Williams, "Oral Delivery and Gastrointestinal Absorption of Soluble Glucans Stimulate Increased Resistance to Infectious Challenge," *Journal of Pharmacology and Experimental Therapeutics*, vol. 314, no. 3, pp. 1079–1086, Sep. 2005. [Online]. Available: http://jpet.aspetjournals.org/content/314/3/1079.full.pdf+html

[47] S. Salama, "β-Glucan Ameliorates Gamma–Rays Induced Oxidative Injury in Male Swiss Albino Rats," *Pakistan J. Zool*, vol. 43, no. 5, pp. 933–939, 2011. [Online]. Available: http://zsp.com.pk/pdf43/933-939%20(16)%20PJZ-601-11%20page%20prof.pdf

[48] D. K. Meena, P. Das, M. S. Akhtar, S. M, and S. Kumar, "Beta-glucans In Aquaculture," *Ascot International*, pp. 1–9, May 2012. [Online]. Available: http://aquafind.com/articles/Beta-glucans-in-Aquaculture.php

[49] P. V. Zimba, P. D. Moeller, K. Beauchesne, H. E. Lane, and R. E. Triemer, "Identification of euglenophycin - A toxin found in certain euglenoids," *Toxicon*, vol. 55, no. 1, pp. 100–104, Jul. 2009. [Online]. Available: http://dx.doi.org/10.1016/j.toxicon.2009.07.004

[50] P. V. Zimba, M. Rowan, and R. Triemer, "Identification of euglenoid algae that produce ichthyotoxin(s)," *Journal of Fish Diseases*, vol. 27, pp. 115–117, Feb. 2004. [Online]. Available: http://naldc.nal.usda.gov/download/42131/PDF

[51] H.-J. Graubaum, "A Double-Blind, Randomized, Placebo-Controlled Nutritional Study Using an Insoluble Yeast Beta-Glucan to Improve the Immune Defense System," *Food and Nutrition Sciences*, vol. 03, no. 06, pp. 738–746, Jun. 2012. [Online]. Available: http://www.scirp.org/journal/PaperDownload.aspx?DOI=10.4236/fns.2012.36100

[52] B. M. Ley, *Discover the Beta Glucan Secret: For Immune Enhancement, Cancer Prevention & Treatment, Cholesterol Reduction, Glucose Regulation, and Much More!*, 2nd ed. Detroit Lakes, MN: BL Publications, Feb. 2001.

[53] A. B. Onderdonk, R. L. Cisneros, P. Hinkson, and G. Ostroff, "Anti-infective effect of poly-beta 1-6-glucotriosyl-beta 1-3-glucopyranose glucan in vivo." *Infection and Immunity*, vol. 60, no. 4, pp. 1642–1647, Apr. 1992. [Online]. Available: http://iai.asm.org/content/60/4/1642.full.pdf

[54] E. Aarsæther, M. Rydningen, R. Einar Engstad, and R. Busund, "Cardioprotective effect of pretreatment with β-glucan in coronary artery bypass grafting," *Scandinavian Cardiovascular Journal*, vol. 40, no. 5, pp. 298–304, Jan. 2006. [Online]. Available: http://informahealthcare.com/doi/abs/10.1080/14017430600868567

[55] Z. Xiao, C. Trincado, and M. Murtaugh, "β-Glucan enhancement of T cell IFNγ response in swine," *Veterinary Immunology and Immunopathology*, vol. 102, no. 3, pp. 315–320, 2004.

[56] E. Stuyven, E. Cox, S. Vancaeneghem, S. Arnouts, P. Deprez, and B. Goddeeris, "Effect of β-glucans on an ETEC infection in piglets," *Veterinary Immunology and Immunopathology*, vol. 128, no. 1-3, pp. 60–66, 2009.

[57] Z. Wang, Y. Guo, J. Yuan, and B. Zhang, "Effect of Dietary β-1,3/1,6-glucan Supplementation on Growth Performance, Immune Response and Plasma Prostaglandin, E_2, Growth Hormone and Ghrelin in Weanling Piglets," *Asian Australasian Journal Of Animal Sciences*, vol. 21, no. 5, p. 707, 2008. [Online]. Available: http://www.ajas.info/Editor/manuscript/upload/21-99.pdf

[58] T.-W. T. Hahn, J. D. J. Lohakare, S. L. S. Lee, W. K. W. Moon, and B. J. B. Chae, "Effects of supplementation of β-glucans on growth performance, nutrient digestibility, and immunity in weanling pigs." *Journal of Animal Science*, vol. 84, no. 6, pp. 1422–1428, Jun. 2006. [Online]. Available: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=16699099&retmode=ref&cmd=prlinks

[59] M. T. Ryan, C. B. Collins, J. V. O'Doherty, and T. Sweeney, "Effects of dietary β-glucans supplementation on cytokine expression in porcine liver." *Journal of Animal Science*, vol. 90 Suppl 4, pp. 40–42, Dec. 2012. [Online]. Available: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=23365278&retmode=ref&cmd=prlinks

[60] J. Li, D. F. Li, J. J. Xing, Z. B. Cheng, and C. H. Lai, "Effects of β-glucan extracted from *Saccharomyces cerevisiae* on growth performance, and immunological and somatotropic responses of pigs challenged with *Escherichia coli* lipopolysaccharide." *Journal of Animal Science*, vol. 84, no. 9, pp. 2374–2381, Sep. 2006. [Online]. Available: http://www.journalofanimalscience.org/cgi/doi/10.2527/jas.2004-541

[61] T. X. Zhou, J. H. Jung, Z. F. Zhang, and I. H. Kim, "Effect of dietary β-glucan on growth performance, fecal microbial shedding and immunological responses after lipopolysaccharide challenge in weaned pigs," *Animal Feed Science and Technology*, vol. 179, no. 1-4, pp. 85–92, Jan. 2013. [Online]. Available: http://linkinghub.elsevier.com/retrieve/pii/S0377840112003495

[62] E. E. Aarsæther, E. E. Straumbotn, A. A. Rösner, and R. R. Busund, "Oral β-glucan reduces infarction size and improves regional contractile function in a porcine ischaemia/reperfusion model." *European Journal of Cardio-thoracic Surgery*, vol. 41, no. 4, pp. 919–925, Apr. 2012. [Online]. Available: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=22228849&retmode=ref&cmd=prlinks

[63] J. Shimizu, N. Tsuchihashi, K. Kudoh, M. Wada, T. Takita, and S. Innami, "Dietary curdlan increases proliferation of bifidobacteria in the cecum of rats," *Bioscience, Biotechnology, and Biochemistry*, vol. 65, no. 2, pp. 466–469, 2001. [Online]. Available: https://www.jstage.jst.go.jp/article/bbb/65/2/65_2_466/_pdf

[64] S. D. Eicher, "Immunological Measures of Animal Well-Being," in *The Science and Ethics Behind Animal Well-Being Assessment: Future Trends in Animal Agriculture*, R. Reynnells, Ed. United States Department of Agriculture, May 2003, pp. 13–15. [Online]. Available: http://digitalcorpora.org/corp/nps/files/govdocs1/355/355121.pdf#page=15

[65] B. Rathgeber, K. Budgell, J. MacIsaac, and J. Mirza, "Yeast Beta Glucans and Broiler Chicken Growth," *Atlantic Poultry Research Institute*, no. 30, pp. 1–2, Aug. 2007. [Online]. Available: http://nsac.ca/apri/apri%20folder/APRI%20Fact%20Sheets/2007/30%20FACTSHEET%20Yeast%20Beta%20Glucans%20and%20Broiler%20Chicken%20Growth.pdf

[66] B. Rathgeber, K. Budgell, J. MacIsaac, M. Mirza, and K. Doncaster, "Growth performance and spleen and bursa weight of broilers fed yeast beta-glucan," *Canadian Journal of Animal Science*, vol. 88, no. 3, pp. 469–473, 2008. [Online]. Available: http://pubs.aic.ca/doi/pdf/10.4141/CJAS07101

[67] C. M. Cox and R. A. Dalloul, "Beta-glucans as immunomodulators in poultry: use and potential applications," *Avian Biology Research*, vol. 3, no. 4, pp. 171–178, Dec. 2010. [Online]. Available: http://www.ingentaconnect.com/content/stl/abr/2010/00000003/00000004/art00003

[68] B. Chae, J. Lohakare, W. Moon, S. Lee, Y. Park, and T. Hahn, "Effects of supplementation of β-glucan on the growth performance and immunity in broilers," *Research in veterinary science*, vol. 80, no. 3, pp. 291–298, 2006. [Online]. Available: http://www.sciencedirect.com/science/article/pii/S0034528805001566

[69] Y. Guo, R. Ali, and M. Qureshi, "The Influence of β-Glucan on Immune Responses in Broiler Chicks," *Immunopharmacology and immunotoxicology*, vol. 25, no. 3, pp. 461–472, 2003. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/19180808

[70] B. An, B. Cho, S. You, H. Paik, H. Chang, S. Kim, C. Yun, and C. Kang, "Growth performance and antibody response of broiler chicks fed yeast derived β-glucan and single-strain probiotics," *Asian-australas. J. Anim. Sci*, vol. 21, pp. 1027–1032, 2008. [Online]. Available: http://www.ajas.info/Editor/manuscript/upload/21-143.pdf

[71] V. Lowry, M. Farnell, P. Ferro, C. Swaggerty, A. Bahl, and M. Kogut, "Purified β-glucan as an abiotic feed additive up-regulates the innate immune response in immature chickens against *Salmonella enterica* serovar *Enteritidis*," *International journal of food microbiology*, vol. 98, no. 3, pp. 309–318, 2005. [Online]. Available: http://naldc.nal.usda.gov/download/39276/PDF

[72] B. Zhang, Y. Guo, and Z. Wang, "The modulating effect of β-1, 3/1, 6-glucan supplementation in the diet on performance and immunological responses of broiler chickens," *Asian Australasian Journal Of Animal Sciences*, vol. 21, no. 2, p. 237, 2008. [Online]. Available: http://www.ajas.info/Editor/manuscript/upload/21-35.pdf

[73] Y. Cheng, D. Lee, C. Wen, and C. Weng, "Effects of beta-glucan supplementation on lymphocyte proliferation, macrophage chemotaxis and specific immune responses in broilers," *Asian Australasian Journal Of Animal Sciences*, vol. 17, no. 8, pp. 1145–1149, 2004. [Online]. Available: http://140.127.198.19:8080/ir/bitstream/310360000Q/12723/2/Beta-glucan%20broiler%20AJAS.pdf

[74] G. Huff, W. Huff, N. Rath, and G. Tellez, "Limited Treatment with β-1,3/1,6-Glucan Improves Production Values of Broiler Chickens Challenged with *Escherichia coli*," *Poultry Science*, vol. 85, no. 4, pp. 613–618, 2006. [Online]. Available: http://naldc.nal.usda.gov/download/3799/PDF

[75] Y. Shao, Y. Guo, and Z. Wang, "β-1,3/1,6-Glucan alleviated intestinal mucosal barrier impairment of broiler chickens challenged with *Salmonella enterica* serovar Typhimurium," *Poultry Science*, vol. 92, no. 7, pp. 1764–1773, Jun. 2013. [Online]. Available: http://ps.fass.org/cgi/doi/10.3382/ps.2013-03029

[76] S. Eicher, "Nutritional Intervention During Transportation of Heifers," *Tri-State Dairy Nutrition Conference*, pp. 133–142, 2001. [Online]. Available: http://vaca.agro.uncor.edu/~pleche/material/Material%20II/A%20archivos%20internet/Alimentacion/3estadosconferencias/2001%2520Proceedings.pdf#page=140

[77] S. D. Eicher, I. V. Wesley, V. K. Sharma, and T. R. Johnson, "Yeast cell-wall products containing β-glucan plus ascorbic acid affect neonatal *Bos taurus* calf leukocytes and growth after a transport stressor," *Journal of Animal Science*, vol. 88, no. 3, pp. 1195–1203, 2010. [Online]. Available: http://www.journalofanimalscience.org/content/88/3/1195

[78] J. K. Chettri, P. W. Kania, and K. Buchmann, "Immunomodulation of rainbow trout (*Oncorhynchus mykiss*) fry by bath exposure to a β-glucan from *Euglena gracilis*," *Aquaculture Research*, pp. 1–9, Apr. 2012.

[79] M. T. Cook, P. J. Hayball, W. Hutchinson, B. F. Nowak, and J. D. Hayball, "Administration of a commercial immunostimulant preparation, EcoActiva as a feed supplement enhances macrophage respiratory burst and the growth rate of snapper (*Pagrus auratus*, Sparidae (Bloch and Schneider)) in winter," *Fish & Shellfish Immunology*, vol. 14, no. 4, pp. 333–345, Apr. 2003. [Online]. Available: http://scsagr.com/upimg/2008530143254.pdf

[80] A. Siwicki, D. Anderson, and G. Rumsey, "Dietary intake of immunostimulants by rainbow trout affects non-specific immunity and protection against furunculosis," *Veterinary Immunology and Immunopathology*, vol. 41, no. 1-2, pp. 125–139, 1994.

[81] J. Burgents, K. Burnett, and L. Burnett, "Disease resistance of Pacific white shrimp, Litopenaeus vannamei, following the dietary administration of a yeast culture food supplement," *Aquaculture*, vol. 231, no. 1, pp. 1–8, 2004. [Online]. Available: http://burnettl.people.cofc.edu/research/pubs/Burgents,%20Burnett%20%26%20Burnett,%20Aquacult%202003.pdf

[82] J. Rodríguez and G. Le Moulac, "State of the art of immunological tools and health control of penaeid shrimp," *Aquaculture*, vol. 191, pp. 109–119, 2000. [Online]. Available: http://www.vliz.be/imisdocs/publications/33355.pdf

[83] F. Vargas-Albores and G. Yepiz-Plascencia, "Beta glucan binding protein and its role in shrimp immune response," *Aquaculture*, vol. 191, no. 1-3, pp. 13–21, 2000. [Online]. Available: http://www.vliz.be/imisdocs/publications/33357.pdf

[84] S. Soltanian, "Protection of gnotobiotic Artemia against Vibrio campbellii using baker's yeast strains and extracts." *PhD Thesis*, pp. 1–235, Nov. 2007. [Online]. Available: http://lib.ugent.be/fulltxt/RUG01/001/238/083/RUG01-001238083_2010_0001_AC.pdf#page=153

[85] N. J. N. Guselle, R. J. F. R. Markham, and D. J. D. Speare, "Intraperitoneal administration of β-1,3/1,6-glucan to rainbow trout, *Oncorhynchus mykiss* (Walbaum), protects against *Loma salmonae*." *Journal of Fish Diseases*, vol. 29, no. 6, pp. 375–381, Jun. 2006. [Online]. Available: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=16768719&retmode=ref&cmd=prlinks

[86] E. Stuyven, F. Verdonck, I. Van Hoek, S. Daminet, L. Duchateau, J. P. Remon, B. M. Goddeeris, and E. Cox, "Oral administration of β-1,3/1,6-glucan to dogs temporally changes total and antigen-specific IgA and IgM." *Clinical and Vaccine Immunology*, vol. 17, no. 2, pp. 281–285, Feb. 2010. [Online]. Available: http://cdli.asm.org/cgi/doi/10.1128/CVI.00344-09

[87] A. C. Beynen and E. Legerstee, "Influence of dietary beta-1, 3/1, 6-glucans on clinical signs of canine osteoarthritis in a double-blind, placebo-controlled trial," *American Journal of Animal and Veterinary Sciences*, vol. 5, no. 2, pp. 97–101, 2010. [Online]. Available: http://thescipub.com/abstract/10.3844/ajavsp.2010.97.101

[88] A. Rychlik, R. Nieradka, M. Kander, M. Nowicki, M. Wdowiak, and A. Kołodziejska-Sawerska, "The effectiveness of natural and synthetic immunomodulators in the treatment of inflammatory bowel disease in dogs," *Acta Veterinaria Hungarica*, vol. 61, no. 3, pp. 297–308, Sep. 2013. [Online]. Available: http://www.google.com/search?client=safari&rls=10_7_4&q=The+effectiveness+of+natural+and+synthetic+immunomodulators+in+the+treatment+of+inflammatory+bowel+disease+in+dogs&ie=UTF-8&oe=UTF-8

[89] L. L. Krakowski, J. J. Krzyzanowski, Z. Z. Wrona, and A. K. A. Siwicki, "The effect of nonspecific immunostimulation of pregnant mares with 1,3/1,6 glucan and levamisole on the immunoglobulins levels in colostrum, selected indices of nonspecific cellular and humoral immunity in foals in neonatal and postnatal period." *Veterinary Immunology and Immunopathology*, vol. 68, no. 1, pp. 1–11, Mar. 1999.

[Online]. Available: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=10231947&retmode=ref&cmd=prlinks

[90] K. S. Swanson and G. C. Fahey, Jr, "The role of yeasts in companion animal nutrition," in *Re-imagining the feed industry: Nutritional Biotechnology in the Feed and Food Industries*, T. P. Lyons and K. A. Jacques, Eds., Department of Animal Sciences, University of Illinois, Urbana, Illinois, USA. Proceedings of Alltech's Twentieth Annual Symposium, Dec. 2004, pp. 475–484. [Online]. Available: http://www.anslab.iastate.edu/Class/AnS536w/08%20Gastrointestinal%20Physiology/heinrichs%20and%20Kehoe%202004%20pp%20194-203.pdf#page=449

7. Signature

The information presented in this section is a reiteration of the information presented on the attached Form FDA-3667_V13(2.13).

I, the undersigned, is informing FDA that **Algaeon, Inc.** has concluded that the intended use of ***Euglena gracilis* biomass containing paramylon (β–1,3–glucan)** described in this document is exempt from the premarket approval requirements of section 409 of the Federal Food, Dug, and Cosmetic Act because the intended use is generally recognized as safe.

The complete record that supports the determination of GRAS status is available to FDA in this submitted notice.

(b) (6)

February 13, 2014

John A. Kassebaum, Chief Technical Officer date

8. List of Attachments

The following documents are a part of this GRAS submission:

- Form FDA-3667_V13(2.13)
- GRAS NOTICE FOR *Euglena gracilis* CONTAINING β–1,3–GLUCAN FOR USE AS A HUMAN FOOD SUPPLEMENT
- GRAS PANEL REVIEW: *Euglena gracilis* CONTAINING β–1,3–GLUCAN

GRAS Panel Review: *Euglena gracilis* containing β-1,3-glucan

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GRAS PANEL REVIEW: *Euglena gracilis* CONTAINING β-1,3-GLUCAN

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1. Submission Summary

Paramylon is a high molecular weight polysaccharide polymer of glucose (β-1,3-D-glucan; CAS No. 9051-97-2) produced by members of the micro-algae division *Euglenophyceae* [1, 2]. This report specifically addresses production using a pure culture of *Euglena gracilis*, a nonpathogenic and nontoxogenic member of the family *Euglenales* in the micro-algae division *Euglenophyceae*. *Euglena gracilis* biomass is primarily intended as a dietary source of β-1,3-glucans (specifically paramylon). Paramylon may be extracted from *Euglena* biomass or may be delivered in *Euglena* biomass. On a dry weight basis, paramylon concentration in biomass exceeds 50%, while extracted paramylon concentration exceeds 80%. Paramylon is a minimally soluble linear (unbranched) β-1,3-glucan high molecular weight polysaccharide polymer that is intended as a source of fermentable fiber (fermented by bifidobacteria in the gut [3, 4]). The non-paramylon remainder of *Euglena gracilis* biomass provides other commonly useful nutrients such as proteins, vitamins, and minerals.

This submission is intended to assert that *Euglena gracilis* biomass containing paramylon is safe for use as an additive to food and feed (for both animal and human consumption).

The review panel believes that paramylon, as a β-1,3-glucan polysaccharide polymer, is safe for addition to food and feed based on: (a) the long history of safe use of β-1,3-glucans contained in many common food & feed sources such as oats and grains; (b) existing GRAS filings and FDA regulations in regard to β-1,3-glucans; and (c) the large number of peer reviewed studies which show no harm from paramylon and β-1,3-glucans while purporting functionality in supporting immune function. The review panel believes that *Euglena gracilis* biomass is safe for addition to food and feed because: (a) it contains nutrients such as proteins, lipids, and carbohydrates; (b) it is nontoxogenic and nonpathogenic based on long use as a laboratory microbe; (c) it is devoid of euglenophycin based on analysis in an independent lab; (d) a Company sponsored fish trial that showed no significant differences in mortality between control and treated fish over a 12 week period; (e) a Company sponsored poultry trial that showed no significant differences in mortality between control and treated poultry over a 2 week period; and (f) existing use of *Euglena gracilis* for human consumption by one international company, and existing use of *Euglena gracilis* for animal feed by another company in the US market.

2. Identity

The basic ingredient is paramylon (a β-1,3-glucan), derived from *Euglena gracilis* biomass. Paramylon is delivered either in lysed cells, or as an extracted product with greater purity.

2.1 Paramylon - the β–1,3–glucan from *Euglena gracilis*

Paramylon is a linear (unbranched) β–1,3–glucan polysaccharide polymer with high molecular weight (Figure 2.1). There are other linear (unbranched) β–1,3–glucans that are well known: curdlan, pachyman, conidiobolus and entomophthora β-1,3-glucan, and callose [5]. Curdlan, which is essentially identical to paramylon, is a permitted addition to food for human consumption under the *U.S. FDA Code of Federal Regulations Title 21*, section 172.809 [6]. Curdlan is produced by pure culture fermentation from the nonpathogenic and nontoxicogenic bacterium Alcaligenes faecalis var. myxogenes [5, 6]. Curdlan has been broadly investigated, and is universally considered to be safe for consumption [7, 8]. The only difference between curdlan and paramylon is the length of the polymer chain (molecular weight) of the polysaccharide polymer [5]. Curdlan has an average molecular weight of 100 kDa (a polymer length $n \approx 554$), while paramylon has an average molecular weight of 500 kDa (a polymer length $n \approx 2776$) [9]. Paramylon has been identified with the CAS Registry Number 9051-97-2 (which has sometimes been applied to some yeast-derived β–glucans as well), while curdlan is identified with the CAS Registry Number 54724-00-4.

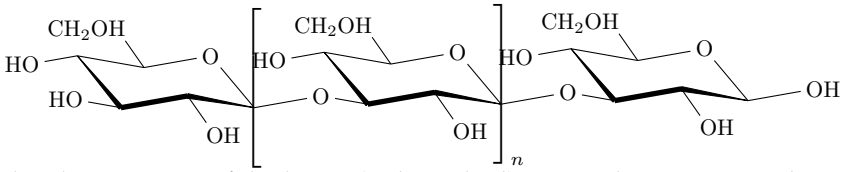
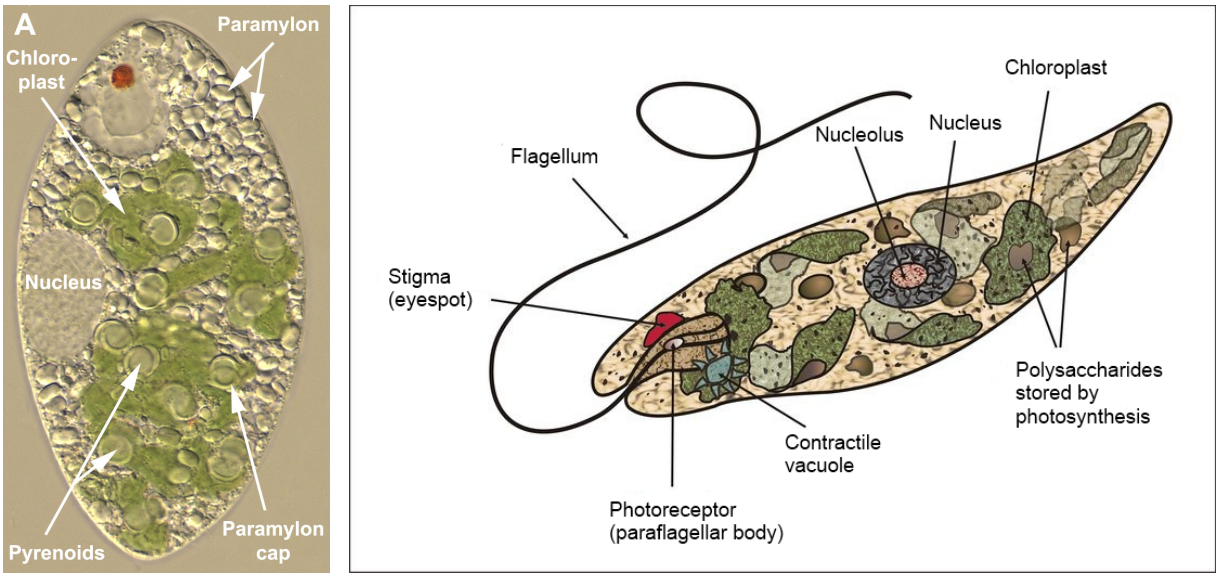


Figure 2.1: Molecular structure of the linear (unbranched) β–1,3–glucans paramylon and curdlan [5]

Other common sources of dietary β–glucans include: (a) branch-on-branch β–(1,3;1,6)–glucans from the cell walls of yeast [5]; (b) linear β–(1,3;1,6)–glucans found in grasses and cereals (such as oats and barley) [5]; and (c) linear β–1,3–glucans with β–(1,6)–glucosyl or oligoglucosyl side chains (lentinan) found in mushrooms such as *Lentinus edodes* (Shiitake) [5]. In all of these cases, paramylon has less variety since it consists wholly of β–1,3–glucan type bonds. Paramylon lacks β–(1,6), β–(1,4), and β–(1,2) bonds as well as side branching structures of any kind. The result is a molecule which is less soluble and more homogeneous. As a result, the long safety record of yeasts and grains in both feed and human food apply directly to paramylon.

2.2 *Euglena gracilis* Ecology

Euglena gracilis (see Figure 2.2 on the following page) is a member of the micro-algae division *Euglenophyceae*, within the *Euglenales* family [2]. The division *Euglenophyceae* includes about 75 species of autotrophic and heterotrophic species. Most are found in enriched freshwaters, particularly shallow water



(a) Pyrenoids and paramylon (b) Euglena Diagram

Figure 2.2: Cellular Structures of Euglena
(a) ©Angelika Preisfeld and David Patterson (b) [2]

rivers, lakes, and ponds. At least two species can live in saline waters. Blooms of these algae occur during late summer-fall in temperate systems. The strain used by the Company was obtained from the Culture Collection of Algae and Protozoa (CCAP) in Glasgow, Scotland - *Euglena gracilis* (CCAP 1224/5Z).

Paramylon is the principal energy-storage compound for Euglenoids, comparable to starch or oils/fats in other algal divisions. Paramylon is produced in the pyrenoids and stored as granules in the cytoplasm. The paramylon granules in *Euglena gracilis* are about 2 μm in diameter [1]. Midwest Laboratories Inc analyzed

Table 2.1: Component Analysis of Euglena performed by Midwest Laboratories Inc. (Report #13-157-9434).

Component	*Dry Weight*	*Meas. Limit*	*Method*
Protein (crude)	11.0%	0.20%	AOAC 990.03
Fat (crude)	11.3%	0.10%	AOAC 2003.05
Fiber (acid detergent)	0.5%	0.5%	ANKOM Tech. Method
Fiber (neutral detergent)	n.d.	1.0%	ANKOM Tech. Method
Ash	0.74%	0.10%	AOAC 942.05
Starch (total)	n.d.	0.10%	AACC 76-11 (mod)
Nitrogen Free Extract (NFE)	76.46%	-	-
— Paramylon (β–1,3–glucan)	58.1%	-	-
Sulfur (total)	0.11%	0.01%	AOAC 985.01 (mod)
Phosphorus (total)	0.65%	0.01%	AOAC 985.01 (mod)
Potassium (total)	0.05%	0.01%	AOAC 985.01 (mod)
Magnesium (total)	0.04%	0.01%	AOAC 985.01 (mod)
Calcium (total)	0.01%	0.01%	AOAC 985.01 (mod)
Sodium (total)	n.d.	0.01%	AOAC 985.01 (mod)
Iron (total)	341 ppm	5.0 ppm	AOAC 985.01 (mod)
Manganese (total)	5.2 ppm	1.0 ppm	AOAC 985.01 (mod)
Copper (total)	18.9 ppm	1.0 ppm	AOAC 985.01 (mod)
Zinc (total)	34.4 ppm	1.0 ppm	AOAC 985.01 (mod)

cultured *Euglena gracilis* and reported chemical composition (Table 2.1 on the previous page). This same lot was also independently quantitated at 58.1% paramylon by Dr. Kyle Newman of Venture Laboratories in Lexington Kentucky (Report No. 13599 - Lot No. EG107.0008). Composition detail is also available in the literature [10, 11].

2.3 Product/Material Specification

Two products are envisioned by this definition: (a) cracked *Euglena gracilis* biomass (lysed cells); and (b) extracted/purified paramylon. Product specifications are presented in Table 2.2 on this page.

Table 2.2: Paramylon Product Specifications

1. Quality Specifications
   (a) Cracked *Euglena gracilis* biomass – NLT 50% paramylon by dry weight.
   (b) Extracted/Purified Paramylon – NLT 80% paramylon by dry weight.
2. Physical Properties
   (a) Appearance: beige to light grey/brown powder
   (b) Moisture content: NMT 10%
3. Inorganic Contaminants
   (a) Inorganic Arsenic: NMT 3 ppm
   (b) Total Arsenic: NMT 10 ppm
   (c) Cadmium: NMT 1 ppm
   (d) Lead: NMT 3 ppm
   (e) Mercury: NMT 0.25 ppm
4. Biological Contaminants
   (a) Total Aerobic Count: NMT 10^5 per gram
   (b) Total Yeast and Mold: NMT 10^2 per gram
   (c) E Coli/Coliforms: NMT 10^1 per gram (but no detectable fecal coliforms)
5. Pesticides and Plasticizer Contaminants
   (a) Free of measurable dioxins
   (b) Free of measurable melamine
6. Handling Recommendations
   (a) Material is somewhat hygroscopic - store in sealed container at NMT 70% relative humidity.
   (b) Material susceptible to mold - it is acceptable to refrigerate or freeze product for longer shelf life.

Paramylon becomes hygroscopic at relative humidities greater than 70% and will increase in weight by up to 10% upon hydration, and then is difficult to re-dry [12–14]. As a result, paramylon (in purified form) should be kept in dry conditions (NMT 70% relative humidity) to avoid formation of a hydrate. Undissolved paramylon, in lysed cell form, would require heating to $140°C$ in water to become hydrated [12–14]. Nonetheless, the *Euglena* biomass will hydrate as well, so it should also be kept in dry conditions.

3. Manufacturing Procesess

Euglena gracilis and its paramylon is manufactured according to current Good Manufacturing Practices (cGMP) - see Figure 3.1). Cultures will be grown in closed bioreactors that are cleaned and sanitized to kill micro-organisms prior to inoculation and growout. *Euglena gracilis* stock cultures that are maintained in controlled laboratory conditions will be used as inoculum. The *Euglena gracilis* inoculum is aseptically transferred to the culture vessels (bioreactors) where it is grown in large quantities. Algal cultures will be grown in liquid cultures using agriculture grade fertilizers. At harvest, the finished culture is then de-watered to separate solids (*Euglena* biomass) from culture media. The resulting paste is then pasteurized to kill the *Euglena gracilis* and any biological contaminants that may be present. Thereafter, the biomass is dried to NMT 10% moisture and lightly ground to form a powder. This is the first form of the product - a cracked (lysed) *Euglena gracilis* biomass as a free-flowing powder.

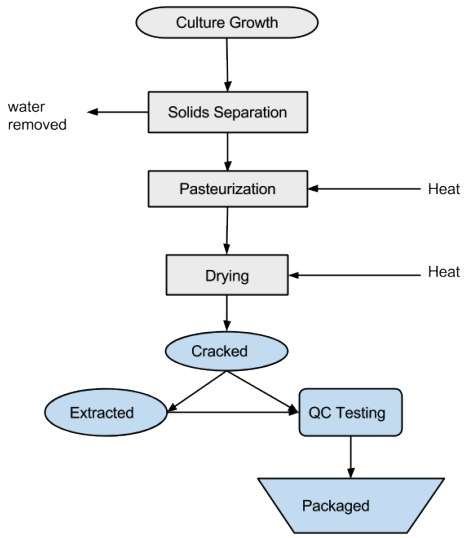


Figure 3.1: Manufacturing Process Diagram

A second product requires chemical extraction of the cracked *Euglena gracilis* biomass. Several chemical extraction methods are available [13, 15–20] including sodium dodecyl sulfate (SDS) in water for extraction of lipids and pigments followed by washing with either water or ethanol. The material can then be further isolated either by the application of a sodium hydroxide solution to dissolve the paramylon, or an acid solution to remove materials other than paramylon. In either case, the further isolated mass of paramylon is neutralized, washed, dried, and milled to produce a free-flowing powder. The extraction process may modify the paramylon granules in the biomass by increasing their exposed surface area. The extracted paramylon may be more bioavailable than the paramylon in either uncracked or cracked *Euglena gracilis*.

The final paramylon product(s) are then packaged to reduce exposure to moisture and oxygen. The bags are stored frozen in sealed containers prior to shipment.

4. Purpose of the Ingredient

Paramylon is a minimally soluble linear (unbranched) β-1,3-glucan high molecular weight polysaccharide polymer (β-1,3-D-glucan; CAS No. 9051-97-2) that is intended as a source of fermentable fiber and as a thickener and/or stabilizer in feed. Paramylon is fermented by bifidobacteria in the gut of most animals [3, 4]. Paramylon, although not as good a thickener as curdlan due to its higher crystallinity, may also be useful as a thickener and/or stabilizer to soften pelletized foods making them more palatable [7, 8, 12, 21].

The other components of *Euglena gracilis* biomass (see Table 2.1 on page 7 and reference [10]) are ordinary macro nutrients (fats and proteins), minerals, and vitamins. *Euglena gracilis* biomass as cracked (lysed) cells, or as extracted/purified paramylon is primarily intended as a dietary source of β-1,3-glucans (specifically paramylon).

This report is *not* intended to establish any medical functionality as an intended use of paramylon. However, the preponderance of literature regarding β–1,3–glucans (including paramylon) is focused on observations of β–1,3–glucan functionality as a supporter of immune function or its beneficial effects upon various conditions or injuries in humans and/or animals. Because the available literature is mainly focussed on functionality, studies of functionality will be cited - but our intent is only to establish safety for consumption.

5. Use limitations

A toxicological assessment of curdlan, which has identical molecular structure to paramylon [5], showed that even at 15% of the diet there was no evidence of adverse effects on the nutritional status of the animals [8]. Likewise, the U.S. FDA and the U.N World Health Organization have recognized curdlan as safe at 30% of the diet of mice, rats, and dogs [6, 7]. Acute toxicity levels (LD_{50}) for oral administration were shown to be greater than 10,000 milli-grams per kilogram of body weight for both mice and rats [7, 8]. Curdlan was *not* found to have genotoxicity [7]. Studies in humans at dosages up to 50 grams of curdlan per day showed no evidence of toxicity [7].

If we add the above assessment to the other known sources of β–1,3–glucans present in oats, barley, mushrooms, and other long-known food and feed sources [5, 22–24, 24, 25, 25, 26, 26, 26–32], it is clear that any conceivable use of paramylon (up to 15% of an animal diet) is unlikely to be able to achieve toxicity. The Company, therefore, is confident that paramylon from *Euglena gracilis* is safe for consumption up to a paramylon level of 15% of an animal diet.

6. Intended Use Data

Euglena gracilis biomass containing paramylon is intended to be used as an additive to animal feed and as a supplement to human food. *Euglena gracilis* biomass can be lysed in the stomach of most animals by the action of pepsin on *Euglena*'s proteinaceous pellicle [33]. It is further lysed in the gut of most creatures by the action of trypsin on the pellicle [33].

Once the cellular components are released, the paramylon (β-1,3-glucan) is catabolized along multiple paths: (a) paramylon is taken up at the Peyer's patches [22, 34, 35] and possibly mucosa-draining lymph nodes [36]; (b) paramylon is fermented by bifidobacteria in the gut of most animals [3, 4]; and (c) any unabsorbed and non-fermented paramylon, as an insoluble fiber, is excreted along with all other undigested fibers.

Paramylon, although not as good a thickener as curdlan due to its higher crystallinity, may also be usable as a thickener and/or stabilizer to soften pelletized foods to make them more palatable [7, 8, 12, 21]. Curdlan, which is the same molecule as paramylon [5], is known to be safe [6–8].

7. Safety Assessment

The review panel believes that *paramylon*, as a β-1,3-glucan polysaccharide polymer, is safe for the proposed uses based on: (a) the long history of safe use of β-1,3-glucans contained in many common food & feed sources such as oats and grains; (b) existing GRAS filings and FDA regulations in regard to β-1,3-glucans; (c) the large number of peer reviewed studies which have demonstrated no untoward effects from *paramylon* and β-1,3-glucans while purporting improved functionality in immune function; and (d) the broadly understood safety of curdlan [6–8]. The review panel believes that *Euglena gracilis* is safe because: (a) it contains nutrients such as proteins, lipids, and carbohydrates; (b) it is nontoxogenic and nonpathogenic based on long use in industry as a laboratory microbe; (c) the Company's own analysis (using an independent lab) found no traces of biotoxins; (d) a Company sponsored fish trial that showed no significant differences in mortality between control and treated fish over a 12 week period; (e) a Company sponsored poultry trial that showed no significant differences in mortality between control and treated poultry over a 2 week period; and (f) there is a company in the international market selling *Euglena gracilis* for human consumption, and another company in the US. market selling a *Euglena gracilis* derived product for animal feed.

There are companies other than Algaeon already selling *Euglena gracilis* containing *paramylon* for both human and animal consumption with no untoward effects. The Euglena Company has been working since 2005 to make Euglena a human food source and dietary supplement in Japan. They are focussed on *Euglena gracilis* itself (rather than on *paramylon*) and offer a number of foods and supplements derived from *Euglena gracilis*. The Algal Scientific Corporation (ASC) is selling *Euglena gracilis* enriched to have a high fraction of *paramylon*. They also have produced a white-paper describing their offerings and its benefits [37]. One of their products is an AAFCO listed metal-polysaccharide (a zinc β–1,3–glucan compound) [37].

The preponderance of literature regarding β–1,3–glucans is focused on observations of β–1,3–glucan as a supporter of immune function or its beneficial effects upon various conditions or injuries in humans or animals. The current report is *not* intended to establish any veterinary or human medical claims with the use of paramylon. However, since much of the available literature is focused on functionality, these studies will be cited in support of safe use.

The paramylon derived from *Euglena gracilis* is similar to β–1,3–glucans from other sources such as yeast, bacteria, mushrooms and grains [9, 22, 38–41]. The differences and advantages in the β-1,3-glucans from *Euglena gracilis* come from: (a) the purity of the paramylon β–1,3–glucan bond structure (with no branching or 1-2, 1-4 or 1-6 bond types) [15, 16, 19, 37, 42]; (b) the size and shape of the β-1,3-glucan crystals produced by Euglena [1, 43]; and (c) the molecular weight of the β-1,3-glucan chains [9, 40]. Paramylon, the β-1,3-glucan from *Euglena gracilis*, has been shown to be safe in numerous trials [9, 22, 38–41]. A quantifiably superior effect of paramylon from *Euglena gracilis* over the β–1,3–glucans from other sources has also been observed [9]. Substantially pure paramylon from *Euglena gracilis* is also usable as an adjuvant in vaccines [19, 44–48]. All of this strongly supports a broad safety profile for paramylon.

7.1 Safety Reports

The safety of β–1,3–glucans in food and feed is best viewed in the context of their long safe use as a constituent of oat and barley grains [5, 22–27]. The same polysaccharide polymers are present in other food-acceptable sources, including mushrooms [24–26, 28, 29], yeasts [26, 30, 31], and even molds [32]. Newer

sources of dietary β–1,3–glucans include *Euglena gracilis* micro-algae (an AAFCO listed metal-polysaccharide - a zinc β–1,3–glucan compound [37]) and bacteria (permitted human food additive - Curdlan [6–8]). As curdlan is chemically identical to paramylon, safety data for curdlan are directly referable. A subchronic toxicity study of muchroom β–(1,3;1,6)–glucan in rats found no adverse effect levels with a no observed-adverse-effect level (NOAEL) for β–(1,3;1,6)–glucans fed at 2,000 mg per kg of body weight per day [29]. β–1,3–glucans caused no adverse effects in nonhuman primates and appeared to have positive effects on the overall health of the test animals [45] characterized by: (a) supporting immunity to a diverse group of microbial and tumor antigens; and (b) nonspecifically enhanced host resistance to neoplastic, bacterial, and fungal diseases. An assessment of in vitro safety and tolerability in human cells at different concentrations demonstrated no adverse events [49]. β–1,3–glucans were also shown to be hepatoprotective in rats given carbon tetrachloride [39]. Further, inhalation of β–1,3–glucan by healthy humans caused markedly less inflammatory response than bacterial endotoxin [50]. β–1,3–glucans also appear to have radio-protective properties (reducing some deleterious effects of ionizing radiation) in animals and people [35, 51–53]. An extensive review of over 1000 papers revealed no adverse effects in humans or animals from β–1,3–glucan ingestion or administration.

7.2 Impurities and Contaminants

7.2.1 Euglenophycin is not produced by *Euglena gracilis*

Euglena species are generally not producers of toxins (nontoxogenic), but there is a member of the division - *Euglena sanguinea* - that produces a known ichthyotoxin, euglenophycin [54, 55]. *Euglena* species, and specifically the species *Euglena gracilis* utilized by the Company, are widely considered to be nonpathogenic and nontoxogenic. To further test and confirm this, the Company has engaged in two in-vivo tests: 1) a feeding trial in yellow perch using dried *Euglena gracilis* biomass at varying dosages vs. a control; and 2) an assay of the Company's *Euglena gracilis* culture and dried product (conducted by Dr. Paul Zimba, author of [54, 55]) for the presence of euglenophycin. The fish trial found no significant differences in mortality between control and treated fish over a 12 week feeding period. The assays of the culture and the dried *Euglena gracilis* biomass product showed no measurable content of euglenophycin in either. A brief report on the fish trial and the euglenophycin assay results are available from the Company upon request.

7.3 Paramylon is Safe for Animals Consumption

7.3.1 Swine

β–1,3–glucans have been shown to enhance innate antiviral immunity (via T-cell function) in weanling pigs [9, 56–59]. Effects of β–1,3–glucans on immune function did not contribute to a systemic inflammatory response, but instead appeared to prime immune defenses by altering subsequent responses to infection [4, 24, 25, 59–61]. Oral β–1,3–glucans also appeared to support cardiovascular health of swine [62]. Furher, oral β–1,3–glucans improved the composition of the intestinal microbiome [3, 4]. This may play a role in β–1,3–glucans improving weight gain performance in weanling pigs [59]. Based on this evidence, β–1,3–glucans (such as Paramylon from *Euglena gracilis*) appear to be safe as a food additive for swine and piglets.

7.3.2 Poultry

Dietary benefits of β-1,3-glucan have been demonstrated in poultry health [63–67]. One study notes that dietary β-1,3-glucan is effective in promoting growth in broiler chickens [64, 65]. Many of the studies show significant growth-promoting and immune-enhancing effects in poultry in response to dietary β-1,3-glucan [63–65, 68–74]. Under a Salmonella challenge, oral β-1,3-glucans also improved poultry gut morphology

(increased villus height and villus height to crypt depth ratio) and inhibited cecal *Salmonella* colonization levels [74]. The Company, in colloboration with one of its potential customers, had a trial conducted with varying treatment levels of paramlyon for poultry. The test showed no significant differences in mortality between control and treated chicks over the trial period of two (2) weeks. Based on all this evidence, β–1,3–glucans (such as paramylon from *Euglena gracilis*) appear to be safe as a food additive for poultry.

7.3.3 Cattle

β-1,3-glucans have also been shown to support immune function and improve responses to stressful events, such as transport, while enhancing feed consumption in cattle [27, 41, 63, 75, 76]. These benefits seem to be further enhanced by dietary antioxidants such as vitamin-C (ascorbic acid) [76]. Apparent digestibility of β–1,3–glucans in steers was 98.6% [27]. Thus, β–1,3–glucans (such as paramylon from *Euglena gracilis*) appear to be safe as a food additive for cattle.

7.3.4 Fish

β-1,3-glucans have been shown to support immune function in fish, shellfish and live-feeds (such as artemia and rotifers) [15, 38, 41, 77–83]. Paramylon also appears to support immune function of rainbow trout fry when dissolved or suspended in the water [77]. Immersion of fish in β-1,3-glucans provides some limited protection, but dietary intake of β–1,3–glucan was much better at increasing resistance of fish to bacterial pathogens [84]. The Company, in collaboration with one of its potential customers, had a trial conducted with varying treatment levels of paramlyon in fish (yellow perch). The test showed no significant differences in mortality between control and treated fish over the trial period of twelve (12) weeks. Thus, β–1,3–glucans (such as paramylon from *Euglena gracilis*) appear to be safe as a food additive for fish and in live-feeds.

7.3.5 Companion Animals

β–1,3–glucans were able to support immune function in dogs in one study [36] with apparent positive effects on inflammatory bowel disease and osteoarthritis [85, 86]. β–1,3–glucan administration also demonstrated similar benefit as levamisole in mares and foals by support of the foal immune system via colostrum trasnfer [87]. β–1,3–glucans have demonstrated no harm in companion animals and appear to decrease the incidence of certain disease states [88].

7.4 Safe for Human Consumption and Handling

Paramylon is chemically identical to curdlan that has a long history of safe use [6–8]. β–1,3–glucans have been shown to support human immune function (for instance, against viral infections such as the common cold) [24, 25, 89–91] and oral β–1,3–glucans have demonstrated some benefit to cardiovascular health [92] without any serious averse events. Oral β–1,3–glucans have also shown anti-tumor effects and may contribute to patient survival [22, 49]. β–1,3–glucans have demonstrated apparent radio-protective properties (reducing some deleterious effects of ionizing radiation) in animals and people [35, 51–53]. In contrast to known inflammatory agents, such as bacterial endotoxin, inhalation of β–1,3–glucans do not cause an acute inflammatory response [50]. An assessment of human safety and tolerability of soluble β–1,3–glucans fed at 100mg/day, 200mg/day or 400mg/day for 4 consecutive days demonstrated no adverse events [49]. An extensive investigation of the literature revealed no adverse effects in humans or animals from β–1,3–glucan ingestion, administration, or inhalation.

7.5 Environmental Safety

Paramylon from *Euglena gracilis* is nontoxic. Both *Euglena gracilis* and parmylon are natural materials that are completely biodegradable and pose negligible environmental hazard. Further, they do not pose a biohazard risk for shipping or handling.

REFERENCES

References

[1] A. K. Monfils, R. E. Triemer, and E. F. Bellairs, "Characterization of paramylon morphological diversity in photosynthetic euglenoids (Euglenales, Euglenophyta)," *Phycologia*, vol. 50, no. 2, pp. 156–169, Mar. 2011. [Online]. Available: http://www.phycologia.org/doi/pdf/10.2216/09-112.1

[2] B. S. Leander, "Euglenida. euglenids or euglenoids. Version 10 November 2012." in *The Tree of Life Web Project. http://tolweb.org/*, 2012. [Online]. Available: http://tolweb.org/Euglenida/97461/2012.11.10

[3] J. Shimizu, N. Tsuchihashi, K. Kudoh, M. Wada, T. Takita, and S. Innami, "Dietary curdlan increases proliferation of bifidobacteria in the cecum of rats," *Bioscience, Biotechnology, and Biochemistry*, vol. 65, no. 2, pp. 466–469, 2001. [Online]. Available: https://www.jstage.jst.go.jp/article/bbb/65/2/65_2_466/_pdf

[4] T. X. Zhou, J. H. Jung, Z. F. Zhang, and I. H. Kim, "Effect of dietary β-glucan on growth performance, fecal microbial shedding and immunological responses after lipopolysaccharide challenge in weaned pigs," *Animal Feed Science and Technology*, vol. 179, no. 1-4, pp. 85–92, Jan. 2013. [Online]. Available: http://linkinghub.elsevier.com/retrieve/pii/S0377840112003495

[5] B. A. Stone, "Chemistry of β-Glucans," in *Chemistry, Biochemistry and Biology of (1→3)-β-glucans and Related Polysaccharides*, A. Bacic, G. B. Fincher, and B. A. Stone, Eds. Academic Press, Jan. 2009, pp. 5–46.

[6] "Curdlan," *U.S. FDA Code of Federal Regulations Title 21*, vol. 3, no. 172.809, Apr. 2013. [Online]. Available: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=172.809

[7] M. E. von Apeldoorn and G. J. A. Speijers, "968. Thickener: Curdlan (WHO Food Additives Series 44)," *inchem.org*, 2000. [Online]. Available: http://www.inchem.org/documents/jecfa/jecmono/v44jec04.htm

[8] E. J. Spicer, E. I. Goldenthal, and T. Ikeda, "A toxicological assessment of curdlan." *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, vol. 37, no. 4, pp. 455–479, Apr. 1999. [Online]. Available: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=10418959&retmode=ref&cmd=prlinks

[9] E. Sonck, E. Stuyven, B. Goddeeris, and E. Cox, "The effect of β-glucans on porcine leukocytes," *Veterinary Immunology and Immunopathology*, vol. 135, no. 3-4, pp. 199–207, Jun. 2010. [Online]. Available: http://dx.doi.org/10.1016/j.vetimm.2009.11.014

[10] E. Kempner and J. Miller, "The Molecular Biology of *Euglena gracilis*: 1. Growth Conditions and Cellular Composition," *Biochimica Et Biophysica Acta*, vol. 104, pp. 11–17, 1965. [Online]. Available: http://www.sciencedirect.com/science/article/pii/030441656590214X

[11] A. Tolivia, V. Conforti, O. Córdoba, and L. Flores, "Chemical constituents and biological activity of Euglenagracilis extracts," *JOPR: Journal of Pharmacy Research*, vol. 7, no. 3, pp. 209–214, Apr. 2013. [Online]. Available: http://dx.doi.org/10.1016/j.jopr.2013.03.019

[12] R. H. Marchessault and Y. Deslandes, "Fine structure of (1→3)-β-D-glucans: curdlan and paramylon," *Carbohydrate Research*, vol. 75, pp. 231–242, 1979. [Online]. Available: http://www.sciencedirect.com/science/article/pii/S000862150084642X

[13] J. Z. Kiss, E. M. Roberts, R. M. Brown, and R. E. Triemer, "X-ray and Dissolution Studies of Paramylon Storage Granules from *Euglena*," *Protoplasma*, vol. 146, pp. 150–156, Nov. 1988. [Online]. Available: http://link.springer.com/article/10.1007/BF01405924#page-1

[14] K. Kobayashi, S. Kimura, E. Togawa, M. Wada, and S. Kuga, "Crystal transition of paramylon with dehydration and hydration," *Carbohydrate Polymers*, vol. 80, no. 2, pp. 491–497, Apr. 2010. [Online]. Available: http://dx.doi.org/10.1016/j.carbpol.2009.12.009

[15] L. Barsanti, R. Vismara, V. Passarelli, and P. Gualtieri, "Paramylon (β-1, 3-glucan) content in wild type and WZSL mutant of *Euglena gracilis*. Effects of growth conditions," *Journal of Applied Phycology*, vol. 13, no. 1, pp. 59–65, 2001.

[16] B. Šantek, M. Felski, K. Friehs, M. Lotz, and E. Flaschel, "Production of paramylon, a β-1,3-glucan, by heterotrophic cultivation of *Euglena gracilis* on a synthetic medium," *Engineering in Life Sciences*, vol. 9, no. 1, pp. 23–28, Feb. 2009.

[17] B. Šantek, K. Friehs, M. Lotz, and E. Flaschel, "Production of paramylon, a β-1,3-glucan, by heterotrophic growth of *Euglena gracilis* on potato liquor in fed-batch and repeated-batch mode of cultivation," *Engineering in Life Sciences*, vol. 12, no. 1, pp. 89–94, 2011. [Online]. Available: http://onlinelibrary.wiley.com/doi/10.1002/elsc.201100025/pdf

[18] A. Sugiyama, S. Hata, K. Suzuki, E. Yoshida, R. Nakano, S. Mitra, R. Arashida, Y. Asayama, Y. Yabuta, and T. Takeuchi, "Oral Administration of Paramylon, a β-1,3-D-glucan Isolated from *Euglena gracilis Z* Inhibits Development of Atopic Dermatitis-Like Skin Lesions in NC/Nga Mice." *The Journal of veterinary medical science the Japanese Society of Veterinary Science*, vol. 72, no. 6, pp. 755–763, Feb. 2010. [Online]. Available: https://www.jstage.jst.go.jp/article/jvms/72/6/72_09-0526/_pdf

[19] D. Tuse, L. Marquez, and L. A. Hokama, "Production of Beta-1,3-Glucan in Euglena," *U.S. Patent*, pp. 1–9, 1992. [Online]. Available: http://www.google.co.il/patents/US5084386?printsec=abstract#v=onepage&q&f=false

[20] J. Rodríguez-Zavala, M. Ortiz-Cruz, G. Mendoza-Hernández, and R. Moreno-Sánchez, "Increased synthesis of α-tocopherol, paramylon and tyrosine by *Euglena gracilis* under conditions of high biomass production," *Journal of Applied Microbiology*, vol. 109, no. 6, pp. 2160–2172, Sep. 2010.

[21] L. N. Sadar, *Rheological and textural characteristics of copolymerized hydrocolloidal solutions containing curdlan gum*. Master's Thesis, University of Maryland, 2004. [Online]. Available: http://drum.lib.umd.edu/handle/1903/1850

[22] R. Wismar, S. Brix, H. Frøkiaer, and H. N. Laerke, "Dietary fibers as immunoregulatory compounds in health and disease," *Annals of the New York Academy of Sciences*, vol. 1190, no. 1, pp. 70–85, Mar. 2010.

[23] P. J. Harris and G. B. Fincher, "Distribution, Fine Structure and Function of (1,3;1,4)-β-Glucans in the Grasses and Other Taxa," in *Chemistry, Biochemistry and Biology of (1→3)-β-glucans and Related Polysaccharides*, A. Bacic, G. B. Fincher, and B. A. Stone, Eds. Academic Press, Jan. 2009, pp. 621–654.

[24] J. Volman, *Immune modulation by dietary glucans from oat and mushrooms: results from* in vitro*, animal and human studies*, ser. PhD Thesis. Universiteit Maastricht, 2010.

[25] J. J. Volman, J. D. Ramakers, and J. Plat, "Dietary modulation of immune function by β-glucans," *Physiology & Behavior: Traces in Metabolism and Nutrition*, vol. 94, no. 2, pp. 276–284, May 2008. [Online]. Available: http://www.sciencedirect.com/science/article/pii/S0031938407004702

[26] D. Akramiene, A. Kondrotas, J. Didziapetriene, and E. Kevelaitis, "Effects of β-glucans on the immune system." *Medicina (Kaunas, Lithuania)*, vol. 43, no. 8, pp. 597–606, 2007. [Online]. Available: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=17895634&retmode=ref&cmd=prlinks

[27] D. F. Engstrom, G. W. Mathison, and L. A. Goonewardene, "Effect of β-glucan, starch, and fibre content and steam vs. dry rolling of barley grain on its degradability and utilisation by steers," *Animal Feed Science and Technology*, vol. 37, no. 1, pp. 33–46, 1992. [Online]. Available: http://www.sciencedirect.com/science/article/pii/037784019290118P

[28] E. A. Steele, "Notification of GRAS Determination for β-glucans derived from mushroom ," *U.S. Food and Drug Administration, GRAS Notice*, no. 413, pp. 1–33, Jan. 2012. [Online]. Available: http://www.accessdata.fda.gov/scripts/fcn/gras_notices/GRN000413.pdf

[29] S. N. Chen, F. H. Nan, S. Chen, J. F. Wu, C. L. Lu, and M. G. Soni, "Safety assessment of mushroom β-glucan: subchronic toxicity in rodents and mutagenicity studies." *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, vol. 49, no. 11, pp. 2890–2898, Nov. 2011. [Online]. Available: http://linkinghub.elsevier.com/retrieve/pii/S027869151100411X

[30] Glucan Corporation Ltd., "GRAS Notice for Black Yeast Beta-Glucan (Polycan®)," *U.S. Food and Drug Administration, GRAS Notice*, no. 309, pp. 1–125, Jun. 2010. [Online]. Available: http://www.accessdata.fda.gov/scripts/fcn/gras_notices/grn_309.pdf

[31] D. J. Cox, "GRAS Notice for Yeast Beta-Glucan," *U.S. Food and Drug Administration, GRAS Notice*, no. 239, pp. 1–91, Jul. 2008. [Online]. Available: http://www.accessdata.fda.gov/scripts/fcn/gras_notices/grn000239.PDF

[32] V. Maquet, "GRAS Notice for Chitin-Glucan," *U.S. Food and Drug Administration, GRAS Notice*, no. 412, pp. 1–122, Dec. 2011. [Online]. Available: http://www.accessdata.fda.gov/scripts/fcn/gras_notices/GRN000412.pdf

[33] J. Kirk, "The effect of trypsin on the pellicle of Euglena gracilis," *Journal of Eukaryotic Microbiology*, vol. 11, no. 3, pp. 435–437, 1964. [Online]. Available: http://onlinelibrary.wiley.com/doi/10.1111/j.1550-7408.1964.tb01776.x/abstract

[34] M. Ye, J. Bak, C. An, H. Jin, J. Kim, H. Kweon, D. Choi, P. Park, Y. Kim, and B. Lim, "Dietary β-Glucan Regulates the Levels of Inflammatory Factors, Inflammatory Cytokines, and Immunoglobulins in Interleukin-10 Knockout Mice," *Journal of Medicinal Food*, vol. 14, no. 5, pp. 468–474, 2011. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/21434779

[35] P. J. Rice, E. L. Adams, T. Ozment-Skelton, A. J. Gonzalez, M. P. Goldman, B. E. Lockhart, L. A. Barker, K. F. Breuel, W. K. DePonti, J. H. Kalbfleisch, H. E. Ensley, G. D. Brown, S. Gordon, and D. L. Williams, "Oral Delivery and Gastrointestinal Absorption of Soluble Glucans Stimulate Increased Resistance to Infectious Challenge," *Journal of Pharmacology and Experimental Therapeutics*, vol. 314, no. 3, pp. 1079–1086, Sep. 2005. [Online]. Available: http://jpet.aspetjournals.org/content/314/3/1079.full.pdf+html

[36] E. Stuyven, F. Verdonck, I. Van Hoek, S. Daminet, L. Duchateau, J. P. Remon, B. M. Goddeeris, and E. Cox, "Oral administration of β-1,3/1,6-glucan to dogs temporally changes total and antigen-specific IgA and IgM." *Clinical and Vaccine Immunology*, vol. 17, no. 2, pp. 281–285, Feb. 2010. [Online]. Available: http://cdli.asm.org/cgi/doi/10.1128/CVI.00344-09

[37] G. Horst, "Use of β-(1,3)-D-Glucan in the Feed Industry," *Algal Scientific Corporation Technical Report*, pp. 1–5, Feb. 2013. [Online]. Available: http://www.algalscientific.com/algal-scientific-beta-glucan-white-paper.pdf

[38] R. Vismara, S. Vestri, A. Frassanito, L. Barsanti, and P. Gualtieri, "Stress resistance induced by paramylon treatment in Artemia sp." *Journal of Applied Phycology*, vol. 16, no. 1, pp. 61–67, 2004.

[39] A. Sugiyama, K. Suzuki, S. Mitra, R. Arashida, E. Yoshida, R. Nakano, Y. Yabuta, and T. Takeuchi, "Hepatoprotective effects of paramylon, a beta-1, 3-D-glucan isolated from Euglena gracilis Z, on acute liver injury induced by carbon tetrachloride in rats." *The Journal of veterinary medical science the Japanese Society of Veterinary Science*, vol. 71, no. 7, pp. 885–890, 2009.

[40] E. Sonck, B. Devriendt, B. Goddeeris, and E. Cox, "Varying Effects of Different β-Glucans on the Maturation of Porcine Monocyte-Derived Dendritic Cells," *Clinical and Vaccine Immunology*, vol. 18, no. 9, pp. 1441–1446, Sep. 2011.

[41] R. Jaafar, J. Skov, P. Kania, and K. Buchmann, "Dose Dependent Effects of Dietary Immunostimulants on Rainbow Trout Immune Parameters and Susceptibility to the Parasite Ichthyophthirius Multifiliis," *J Aquac Res Development S*, vol. 3, p. 2, Nov. 2011. [Online]. Available: http://www.omicsonline.org/2155-9546/2155-9546-S3-001.pdf

[42] J. Cook, "Quantitative measurement of paramylum in *Euglena gracilis*," *Journal of Eukaryotic Microbiology*, vol. 14, no. 4, pp. 634–636, 1967. [Online]. Available: http://onlinelibrary.wiley.com/doi/10.1111/j.1550-7408.1967.tb02053.x/abstract

[43] R. Howard, S. Wright, and B. Grant, "Structure and Some Properties of Soluble 1, 3-β-Glucan Isolated from the Green Alga *Caulerpa simpliciuscula*," *Plant Physiology*, vol. 58, no. 4, p. 459, 1976. [Online]. Available: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC543239/pdf/plntphys00165-0016.pdf

[44] W. Suwelack, P. Tewes-Schwarzer, D. Baumer, and U. Kahmann, "Freeze-Dried Agent Containing Paramylon, Its Production and Use," *US Patent #2003/0203016 A1*, pp. 1–15, Oct. 2012.

[45] J. A. Reynolds, M. D. Kastello, D. G. Harrington, C. L. Crabbs, C. J. Peters, J. V. Jemski, G. H. Scott, and N. R. DiLuzzio, "Glucan-Induced Enhancement of Host Resistance to Selected Infectious Diseases," *Infection and Immunity*, vol. 30, no. 1, pp. 51–57, Oct. 1980. [Online]. Available: http://iai.asm.org/content/30/1/51.full.pdf

[46] J. A. Cook, T. W. Holbrook, and W. J. Dougherty, "Protective Effect of Glucan Against Visceral Leishmaniasis in Hamsters," *Infection and Immunity*, vol. 37, no. 3, pp. 1261–1269, Aug. 1982. [Online]. Available: http://iai.asm.org/content/37/3/1261.full.pdf

[47] J. B. Jorgensen, H. Lunde, and B. Robertsen, "Peritoneal and head kidney cell response to intraperitoneally injected yeast glucan in Atlantic salmon, Salmo salar L." *Journal of Fish Diseases*, vol. 16, no. 4, pp. 313–325, 1993. [Online]. Available: http://dx.doi.org/10.1111/j.1365-2761.1993.tb00865.x

[48] D. Chen and A. J. Ainsworth, "Glucan administration potentiates immune defence mechanisms of channel catfish, *Ictalurus punctatus* Rafinesque," *Journal of Fish Diseases*, vol. 15, no. 4, pp. 295–304, 1992. [Online]. Available: http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2761.1992.tb00667.x/full

[49] G. Chan, W. Chan, and D. Sze, "The effects of β-glucan on human immune and cancer cells," *Journal of Hematology & Oncology*, vol. 2, no. 25, 2009. [Online]. Available: http://www.jhoonline.org/content/2/1/25

[50] J. Thornca, L. Beijer, and R. Rylander, "Effects after inhalation of (1$\rightarrow$3)-β-D-glucan in healthy humans," *Mediators of Inflammation*, vol. 10, no. 4, pp. 173–178, Aug. 2001. [Online]. Available: ttp://downloads.hindawi.com/journals/mi/2001/573670.pdf

[51] M. Patchen, M. D'Alesandro, M. Chirigos, and J. Weiss, "Radioprotection by biological response modifiers alone and in combination with WR-2721," *Armed Forces Radiobiology Research Institute*, 1989. [Online]. Available: http://www.dtic.mil/dtic/tr/fulltext/u2/a207914.pdf

[52] S. Salama, "β-Glucan Ameliorates Gamma–Rays Induced Oxidative Injury in Male Swiss Albino Rats," *Pakistan J. Zool*, vol. 43, no. 5, pp. 933–939, 2011. [Online]. Available: http://zsp.com.pk/pdf43/933-939%20(16)%20PJZ-601-11%20page%20prof.pdf

[53] D. K. Meena, P. Das, M. S. Akhtar, S. M, and S. Kumar, "Beta-glucans In Aquaculture," *Ascot International*, pp. 1–9, May 2012. [Online]. Available: http://aquafind.com/articles/Beta-glucans-in-Aquaculture.php

[54] P. V. Zimba, P. D. Moeller, K. Beauchesne, H. E. Lane, and R. E. Triemer, "Identification of euglenophycin - A toxin found in certain euglenoids," *Toxicon*, vol. 55, no. 1, pp. 100–104, Jul. 2009. [Online]. Available: http://dx.doi.org/10.1016/j.toxicon.2009.07.004

[55] P. V. Zimba, M. Rowan, and R. Triemer, "Identification of euglenoid algae that produce ichthyotoxin(s)," *Journal of Fish Diseases*, vol. 27, pp. 115–117, Feb. 2004. [Online]. Available: http://naldc.nal.usda.gov/download/42131/PDF

[56] Z. Xiao, C. Trincado, and M. Murtaugh, "β-Glucan enhancement of T cell IFNγ response in swine," *Veterinary Immunology and Immunopathology*, vol. 102, no. 3, pp. 315–320, 2004.

[57] E. Stuyven, E. Cox, S. Vancaeneghem, S. Arnouts, P. Deprez, and B. Goddeeris, "Effect of β-glucans on an ETEC infection in piglets," *Veterinary Immunology and Immunopathology*, vol. 128, no. 1-3, pp. 60–66, 2009.

[58] Z. Wang, Y. Guo, J. Yuan, and B. Zhang, "Effect of Dietary β-1,3/1,6-glucan Supplementation on Growth Performance, Immune Response and Plasma Prostaglandin, E_2, Growth Hormone and Ghrelin in Weanling Piglets," *Asian Australasian Journal Of Animal Sciences*, vol. 21, no. 5, p. 707, 2008. [Online]. Available: http://www.ajas.info/Editor/manuscript/upload/21-99.pdf

[59] T.-W. T. Hahn, J. D. J. Lohakare, S. L. S. Lee, W. K. W. Moon, and B. J. B. Chae, "Effects of supplementation of β-glucans on growth performance, nutrient digestibility, and immunity in weanling pigs." *Journal of Animal Science*, vol. 84, no. 6, pp. 1422–1428, Jun. 2006. [Online]. Available: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=16699099&retmode=ref&cmd=prlinks

[60] M. T. Ryan, C. B. Collins, J. V. O'Doherty, and T. Sweeney, "Effects of dietary β-glucans supplementation on cytokine expression in porcine liver." *Journal of Animal Science*, vol. 90 Suppl 4, pp. 40–42, Dec. 2012. [Online]. Available: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=23365278&retmode=ref&cmd=prlinks

[61] J. Li, D. F. Li, J. J. Xing, Z. B. Cheng, and C. H. Lai, "Effects of β-glucan extracted from *Saccharomyces cerevisiae* on growth performance, and immunological and somatotropic responses of pigs challenged with *Escherichia coli* lipopolysaccharide." *Journal of Animal Science*, vol. 84, no. 9, pp. 2374–2381, Sep. 2006. [Online]. Available: http://www.journalofanimalscience.org/cgi/doi/10.2527/jas.2004-541

[62] E. E. Aarsæther, E. E. Straumbotn, A. A. Rösner, and R. R. Busund, "Oral β-glucan reduces infarction size and improves regional contractile function in a porcine ischaemia/reperfusion model." *European Journal of Cardio-thoracic Surgery*, vol. 41, no. 4, pp. 919–925, Apr. 2012. [Online]. Available: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=22228849&retmode=ref&cmd=prlinks

[63] S. D. Eicher, "Immunological Measures of Animal Well-Being," in *The Science and Ethics Behind Animal Well-Being Assessment: Future Trends in Animal Agriculture*, R. Reynnells, Ed. United States Department of Agriculture, May 2003, pp. 13–15. [Online]. Available: http://digitalcorpora.org/corp/nps/files/govdocs1/355/355121.pdf#page=15

[64] B. Rathgeber, K. Budgell, J. MacIsaac, and J. Mirza, "Yeast Beta Glucans and Broiler Chicken Growth," *Atlantic Poultry Research Institute*, no. 30, pp. 1–2, Aug. 2007. [Online]. Available: http://nsac.ca/apri/apri%20folder/APRI%20Fact%20Sheets/2007/30%20FACTSHEET%20Yeast%20Beta%20Glucans%20and%20Broiler%20Chicken%20Growth.pdf

[65] B. Rathgeber, K. Budgell, J. MacIsaac, M. Mirza, and K. Doncaster, "Growth performance and spleen and bursa weight of broilers fed yeast beta-glucan," *Canadian Journal of Animal Science*, vol. 88, no. 3, pp. 469–473, 2008. [Online]. Available: http://pubs.aic.ca/doi/pdf/10.4141/CJAS07101

[66] C. M. Cox and R. A. Dalloul, "Beta-glucans as immunomodulators in poultry: use and potential applications," *Avian Biology Research*, vol. 3, no. 4, pp. 171–178, Dec. 2010. [Online]. Available: http://www.ingentaconnect.com/content/stl/abr/2010/00000003/00000004/art00003

[67] B. Chae, J. Lohakare, W. Moon, S. Lee, Y. Park, and T. Hahn, "Effects of supplementation of β-glucan on the growth performance and immunity in broilers," *Research in veterinary science*, vol. 80, no. 3, pp. 291–298, 2006. [Online]. Available: http://www.sciencedirect.com/science/article/pii/S0034528805001566

[68] Y. Guo, R. Ali, and M. Qureshi, "The Influence of β-Glucan on Immune Responses in Broiler Chicks," *Immunopharmacology and immunotoxicology*, vol. 25, no. 3, pp. 461–472, 2003. [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/19180808

[69] B. An, B. Cho, S. You, H. Paik, H. Chang, S. Kim, C. Yun, and C. Kang, "Growth performance and antibody response of broiler chicks fed yeast derived β-glucan and single-strain probiotics," *Asian-australas. J. Anim. Sci*, vol. 21, pp. 1027–1032, 2008. [Online]. Available: http://www.ajas.info/Editor/manuscript/upload/21-143.pdf

[70] V. Lowry, M. Farnell, P. Ferro, C. Swaggerty, A. Bahl, and M. Kogut, "Purified β-glucan as an abiotic feed additive up-regulates the innate immune response in immature chickens against *Salmonella enterica* serovar *Enteritidis*," *International journal of food microbiology*, vol. 98, no. 3, pp. 309–318, 2005. [Online]. Available: http://naldc.nal.usda.gov/download/39276/PDF

[71] B. Zhang, Y. Guo, and Z. Wang, "The modulating effect of β-1, 3/1, 6-glucan supplementation in the diet on performance and immunological responses of broiler chickens," *Asian Australasian Journal Of Animal Sciences*, vol. 21, no. 2, p. 237, 2008. [Online]. Available: http://www.ajas.info/Editor/manuscript/upload/21-35.pdf

[72] Y. Cheng, D. Lee, C. Wen, and C. Weng, "Effects of beta-glucan supplementation on lymphocyte proliferation, macrophage chemotaxis and specific immune responses in broilers," *Asian Australasian Journal Of Animal Sciences*, vol. 17, no. 8, pp. 1145–1149, 2004. [Online]. Available: http://140.127.198.19:8080/ir/bitstream/310360000Q/12723/2/Beta-glucan%20broiler%20AJAS.pdf

[73] G. Huff, W. Huff, N. Rath, and G. Tellez, "Limited Treatment with β-1,3/1,6-Glucan Improves Production Values of Broiler Chickens Challenged with *Escherichia coli*," *Poultry Science*, vol. 85, no. 4, pp. 613–618, 2006. [Online]. Available: http://naldc.nal.usda.gov/download/3799/PDF

[74] Y. Shao, Y. Guo, and Z. Wang, "β-1,3/1,6-Glucan alleviated intestinal mucosal barrier impairment of broiler chickens challenged with *Salmonella enterica* serovar Typhimurium," *Poultry Science*, vol. 92, no. 7, pp. 1764–1773, Jun. 2013. [Online]. Available: http://ps.fass.org/cgi/doi/10.3382/ps.2013-03029

[75] S. Eicher, "Nutritional Intervention During Transportation of Heifers," *Tri-State Dairy Nutrition Conference*, pp. 133–142, 2001. [Online]. Available: http://vaca.agro.uncor.edu/~pleche/material/Material%20II/A%20archivos%20internet/Alimentacion/3estadosconferencias/2001%2520Proceedings.pdf#page=140

[76] S. D. Eicher, I. V. Wesley, V. K. Sharma, and T. R. Johnson, "Yeast cell-wall products containing β-glucan plus ascorbic acid affect neonatal *Bos taurus* calf leukocytes and growth after a transport stressor," *Journal of Animal Science*, vol. 88, no. 3, pp. 1195–1203, 2010. [Online]. Available: http://www.journalofanimalscience.org/content/88/3/1195

[77] J. K. Chettri, P. W. Kania, and K. Buchmann, "Immunomodulation of rainbow trout (*Oncorhynchus mykiss*) fry by bath exposure to a β-glucan from *Euglena gracilis*," *Aquaculture Research*, pp. 1–9, Apr. 2012.

[78] M. T. Cook, P. J. Hayball, W. Hutchinson, B. F. Nowak, and J. D. Hayball, "Administration of a commercial immunostimulant preparation, EcoActiva as a feed supplement enhances macrophage respiratory burst and the growth rate of snapper (*Pagrus auratus*, Sparidae (Bloch and Schneider)) in winter," *Fish & Shellfish Immunology*, vol. 14, no. 4, pp. 333–345, Apr. 2003. [Online]. Available: http://scsagr.com/upimg/2008530143254.pdf

[79] A. Siwicki, D. Anderson, and G. Rumsey, "Dietary intake of immunostimulants by rainbow trout affects non-specific immunity and protection against furunculosis," *Veterinary Immunology and Immunopathology*, vol. 41, no. 1-2, pp. 125–139, 1994.

[80] J. Burgents, K. Burnett, and L. Burnett, "Disease resistance of Pacific white shrimp, Litopenaeus vannamei, following the dietary administration of a yeast culture food supplement," *Aquaculture*, vol. 231, no. 1, pp. 1–8, 2004. [Online]. Available: http://burnettl.people.cofc.edu/research/pubs/Burgents,%20Burnett%20%26%20Burnett,%20Aquacult%202003.pdf

[81] J. Rodríguez and G. Le Moulac, "State of the art of immunological tools and health control of penaeid shrimp," *Aquaculture*, vol. 191, pp. 109–119, 2000. [Online]. Available: http://www.vliz.be/imisdocs/publications/33355.pdf

[82] F. Vargas-Albores and G. Yepiz-Plascencia, "Beta glucan binding protein and its role in shrimp immune response," *Aquaculture*, vol. 191, no. 1-3, pp. 13–21, 2000. [Online]. Available: http://www.vliz.be/imisdocs/publications/33357.pdf

[83] S. Soltanian, "Protection of gnotobiotic Artemia against Vibrio campbellii using baker's yeast strains and extracts." *PhD Thesis*, pp. 1–235, Nov. 2007. [Online]. Available: http://lib.ugent.be/fulltxt/RUG01/001/238/083/RUG01-001238083_2010_0001_AC.pdf#page=153

[84] N. J. N. Guselle, R. J. F. R. Markham, and D. J. D. Speare, "Intraperitoneal administration of β-1,3/1,6-glucan to rainbow trout, *Oncorhynchus mykiss* (Walbaum), protects against *Loma salmonae*." *Journal of Fish Diseases*, vol. 29, no. 6, pp. 375–381, Jun. 2006. [Online]. Available: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=16768719&retmode=ref&cmd=prlinks

[85] A. C. Beynen and E. Legerstee, "Influence of dietary beta-1, 3/1, 6-glucans on clinical signs of canine osteoarthritis in a double-blind, placebo-controlled trial," *American Journal of Animal and Veterinary Sciences*, vol. 5, no. 2, pp. 97–101, 2010. [Online]. Available: http://thescipub.com/abstract/10.3844/ajavsp.2010.97.101

[86] A. Rychlik, R. Nieradka, M. Kander, M. Nowicki, M. Wdowiak, and A. Kołodziejska-Sawerska, "The effectiveness of natural and synthetic immunomodulators in the treatment of inflammatory bowel disease in dogs," *Acta Veterinaria Hungarica*, vol. 61, no. 3, pp. 297–308, Sep. 2013. [Online]. Available: http://www.google.com/search?client=safari&rls=10_7_4&q=The+effectiveness+of+natural+and+synthetic+immunomodulators+in+the+treatment+of+inflammatory+bowel+disease+in+dogs&ie=UTF-8&oe=UTF-8

[87] L. L. Krakowski, J. J. Krzyzanowski, Z. Z. Wrona, and A. K. A. Siwicki, "The effect of nonspecific immunostimulation of pregnant mares with 1,3/1,6 glucan and levamisole on the immunoglobulins levels in colostrum, selected indices of nonspecific cellular and humoral immunity in foals in neonatal and postnatal period." *Veterinary Immunology and Immunopathology*, vol. 68, no. 1, pp. 1–11, Mar. 1999. [Online]. Available: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=10231947&retmode=ref&cmd=prlinks

[88] K. S. Swanson and G. C. Fahey, Jr, "The role of yeasts in companion animal nutrition," in *Re-imagining the feed industry: Nutritional Biotechnology in the Feed and Food Industries*, T. P. Lyons

and K. A. Jacques, Eds., Department of Animal Sciences, University of Illinois, Urbana, Illinois, USA. Proceedings of Alltech's Twentieth Annual Symposium, Dec. 2004, pp. 475–484. [Online]. Available: http://www.anslab.iastate.edu/Class/AnS536w/08%20Gastrointestinal%20Physiology/heinrichs%20and%20Kehoe%202004%20pp%20194-203.pdf#page=449

[89] H.-J. Graubaum, "A Double-Blind, Randomized, Placebo-Controlled Nutritional Study Using an Insoluble Yeast Beta-Glucan to Improve the Immune Defense System," *Food and Nutrition Sciences*, vol. 03, no. 06, pp. 738–746, Jun. 2012. [Online]. Available: http://www.scirp.org/journal/PaperDownload.aspx?DOI=10.4236/fns.2012.36100

[90] B. M. Ley, *Discover the Beta Glucan Secret: For Immune Enhancement, Cancer Prevention & Treatment, Cholesterol Reduction, Glucose Regulation, and Much More!*, 2nd ed. Detroit Lakes, MN: BL Publications, Feb. 2001.

[91] A. B. Onderdonk, R. L. Cisneros, P. Hinkson, and G. Ostroff, "Anti-infective effect of poly-beta 1-6-glucotriosyl-beta 1-3-glucopyranose glucan in vivo." *Infection and Immunity*, vol. 60, no. 4, pp. 1642–1647, Apr. 1992. [Online]. Available: http://iai.asm.org/content/60/4/1642.full.pdf

[92] E. Aarsæther, M. Rydningen, R. Einar Engstad, and R. Busund, "Cardioprotective effect of pretreatment with β-glucan in coronary artery bypass grafting," *Scandinavian Cardiovascular Journal*, vol. 40, no. 5, pp. 298–304, Jan. 2006. [Online]. Available: http://informahealthcare.com/doi/abs/10.1080/14017430600868567

SIGNATURES

Signatures

Signatures - page 1 of 4

We the undersigned members of the panel approve this report and attest to its accuracy and completeness with respect to the safety of *Euglena gracilis* biomass and it's β–1,3–glucan, paramylon, for use in food and feed as described herein. We have independently and collectively evaluated the attached referenced information and offer this GRAS declaration, with reasonable certainty of no harm under the intended conditions of use. It is our position that human and animal exposure to *Euglena gracilis* and it's β–1,3–glucan, paramylon, is generally recognized as safe (GRAS).

(b) (6)

Paul V. Zimba, PhD

Signatures - page 2 of 4

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(b) (6)

1/21/14

Paul B. Brown, PhD

Signatures - page 3 of 4

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(b) (6)

[redacted], 1-17-2014

Eric van Heugten, PhD

Signatures - page 4 of 4

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(b) (6)

[redacted]

21 Jan 2014

Jeffery A. Engelhardt, DVM, PhD

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