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(71) Applicant and
(72) Inventor: HAREL, Moti [IL/US]; 2012 Masters Drive, Baltimore, MD 21209 (US).

(74) Agent: GARRETT, Arthur, S.; Finnegan, Henderson, Farabow, Garrett & Dunner, L.L.P., 1300 I Street, NW, Washington, DC 20005-3315 (US).

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(54) Title: ZOOPLANKTON ENRICHMENT WITH PROBIONTS AND PREBIONTS AND USES THEREOF

(57) Abstract: Probiont- and prebiont-loaded zooplankton, such as Brachionus plicatilis and Artemia nauplii, are obtained by culturing zooplankton in the presence of the probionts or prebionts. Probionts or prebionts are loaded into zooplankton in such a manner as to maintain their viability and/or bioactivity. Zooplankton are cultured with free or microencapsulated probionts and/or prebionts, and used as a vector for probiont and/or prebiont delivery to larval and juvenile fish, and crustaceans. The survival rate in breeding larvae and juveniles can be remarkably improved by feeding the probiont- and/or prebiont-containing zooplankton to the larvae or juveniles.

ZOOPLANKTON ENRICHMENT WITH PROBIONTS AND PREBIONTS AND USES THEREOF

[001] This application is related to provisional application 60/387,421, filed in the United States Patent and Trademark Office on June 11, 2002, the disclosure of which is herein incorporated by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

[002] The present invention relates to probiont- and prebiont-loaded zooplankton for improving health, growth, and survival rates of larval fish, fry fish, and crustaceans, by feeding them probiont- or prebiont-loaded zooplankton.

Probionts and Prebionts in the Gastrointestinal Tract of Aquatic Animals

[003] It is well known that inside the gastrointestinal tract of each aquatic animal resides a microbial flora, which is a rich ecosystem of enormous complexity containing trillions of bacteria divided into more than 400 species, in addition to yeasts, and other organisms. In addition to the normal microbial flora (resident microflora) established in the gastrointestinal tract, bacteria and yeast are routinely introduced into culture medium as a normal part of food consumption (transient microflora) or as contaminants (accidental microflora).

[004] The bacteria in the aquatic animal gastrointestinal tract can be divided into several functional groups; important among these are probionts, commensals and pathogens. The probionts are beneficial, since they participate in a wide variety of positive and health promoting activities in fish physiology (Gildberg et al., 1997; Hansen, 2000; Irianto et al., 2000). Commensals establish a community within the gut and provide continuous interaction with the intestinal contents and the animal. The pathogens are harmful, as they disrupt the normal gut flora, or invade the animal

to cause disease. Normally the probionts and commensals predominate within the gut, providing normal function. The balance between beneficial and harmful microorganisms is essential for a properly functioning digestive tract, which is crucial to the optimal health of an aquatic animal. The number and efficacy of the proper probionts and commensals to maintain proper function declines as fish or crustacean health fails.

[005] The major benefits probiotics render to fish are as follows:

- The presence and adherence of probiotics, especially many lactic acid producing bacteria, to the mucous membrane of the intestine from the ileo-caecal region downward, constitute a formidable natural biologic barrier for many pathogenic bacteria.
- The probiotics compete with disease-causing bacteria for villi attachment sites and nutrients.
- Many kinds of acids are produced by probionts, such as lactic acid, acetic acid, and benzoic acid, to create an acidic environment unfriendly to harmful bacteria, viruses and yeasts.
- The probionts produce many bioactive compounds, such as bacteriocins, hydrogen peroxide, lactocidin, acidophilin, and bulgarican, which have antibiotic properties inhibiting or modifying the growth of a wide spectrum of undesirable organisms, pathogens and potential pathogens.
- The metabolic products of probionts provide many useful enzymes and volatile or non-volatile fatty acids to help digestion and absorption of certain minerals, such as phosphorus, calcium, and iron.
- Probiotics contribute to a longer intestinal transit time as well, which improves nutrient assimilation.

- Many probionts also produce B complex vitamins, such as niacin (B3), pyridoxine (B6), folic acid, and biotin, for the needs of the host fish.
- The lactic acid-producing bacteria confer significant protection for the fish through various immune mechanisms, which include interactions between the microbes and the host, as well as interactions among microbes.

[005] Prebiotics do not colonize the gut of the target animal yet can deliver many of the same benefits derived from probionts. Prebionts deliver pre-made enzymes, acids, polysaccharides, secondary metabolites, and other metabolic products that influence the composition of the intestine, as well as the overall health of the target organism.

[006] In the past two decades, although much progress has been made in the field of probiotic research, problems exist. First of all, although some benefits of probiotics are well documented, not all of the varied hypotheses have been substantiated. The problems are, in part, related to the fact that most health effects of probiotics are species, or even strain, specific. Therefore, varying results may occur when using different strains of the same species or even different preparations of the same strain. There is also large variability in the physiology of individual fish, and large populations must be studied to provide valid results. In addition, many bacterial species, such as *Lactobacillus*, are not viable for a great length of storage time. Also, a majority of the probiotics becomes rapidly inactivated in the fish upper gastrointestinal tract, where the pH is in the range of about 2.0 to 2.9, before they reach the lower intestines where colonization occurs. Therefore, oral application of probionts and prebionts poses problems related to stability, viability, and storage.

[007] Cultured zooplankton, such as *Brachionus plicatilis* and *Artemia nauplii*, have been fed as live feeds for breeding fry, larval, and juvenile fish, and crustaceans. In natural surroundings, hatched larval and juvenile fish feed on a wide variety of microbe-containing crustacean plankton. Artificially cultured zooplankter, such as *Brachionus plicatilis,* differ greatly from naturally occurring crustacean plankton in their microbial content. Therefore, cultured fish larvae may not be able to establish gastrointestinal flora suitable for optimal growth.

Related Art

[008] Crustaceans, rotifers, nematodes, and caldocerans have been used extensively as live feeds for aquaculture, mostly for larval culture of crustaceans and finfish (Abu-Rezq et al., 2002; Chu and Ozkizilcik, 1995; Harel et al., 1999; Shields et al., 1999). While many groups are searching for alternatives to live feeds, commercial aquaculture still relies heavily on live feeds for larviculture.

[009] *Artemia nauplii* and rotifers have been used for enrichment in essential nutrients for larval culture in fish and crustaceans (Curé et al., 1996). In this use, essential nutrients such as carotenoids and long-chain polyunsaturated acids (*e.g.*, docosahexaenoic and arachidonic acids) have been delivered successfully to fish and crustacean larvae to improve survival rates (Harel et al., 1999). Methods for delivery of specific carotenoids have also been developed for aquacultural applications (Abe et al., 1998).

[010] Bioencapsulation of drugs using *Artemia* has also been accomplished for application to larval culture (Verpraet et al., 1992). In this format, drugs are delivered to the live food and accumulated in the *Artemia,* then the *Artemia* are

consumed by the fish or crustacean larvae as a drug delivery vector (Dixon et al., 1995).

[011] Algae have often been explored for their ability to make compounds that might be useful as drugs (Faulkner, 2002; Jones, 1988; Kumvan et al., 2001; Metting and Pyne, 1986; Neves et al., 2001; Prati et al., 2002). Very few examples can be found of whole cells being used as feeds because their bioactivity is vulnerable to the acidic pH of the upper gastrointestinal tract.

SUMMARY OF THE INVENTION

[012] In recent years, the importance of commercial aquaculture has increased, and the survival rate for the breeding of larval and juvenile fish has been an important economic factor. The survival rate of larval and juvenile fish bred by the traditional existing methods is sometimes considerably lowered due to disease and improper nutrition. In these circumstances, a method for the stable breeding of larval and juvenile fish at a high yield must be developed. The present invention provides an additional tool to solve this problem.

[013] The present invention relates to both a composition of zooplankton loaded with probiotic or prebiotic organisms, and a method for using these specifically loaded zooplankton for aquaculture, including larviculture, in the production of crustaceans (*e.g.*, shrimp, lobsters, red claw, and crab), mollusks (oysters and abalone), and fish (*e.g.*, striped bass, sea bass, sea bream, carp, tilapia, catfish, trout, salmon, halibut, and yellow flounder). Probiotic organisms, such as *Bacillus subtilis* or *Saccharomyces cerevisiae*) are fed to zooplankton live feeds, such as *Artemia franciscana* or *Brachionus plicatilis,* either as single cells or as

microencapsulated cells or cell aggregates. The feeding method depends on the maintenance of viability needs for specific probionts in the gut of the zooplankton being fed. Types of microencapsulation could be a coating, such as, but not limited to, lipid, chitosan, alginate, or starch. Prebiotic organisms are envisioned in this invention, and can be delivered in a manner analogous to that described for probionts, with the limitation on encapsulation being the maintenance of bioactivity provided by the specific prebiont.

[014] Specifically, the invention provides for zooplankton comprising at least one microencapsulated, viable, bioactive prebiotic or probiotic.

[015] The zooplankton can comprise crustaceans, including those of the *Artemia* species, e.g., *Artemia salina.*

[016] The zooplankton can also comprise brine shrimp, fairy shrimp, *Daphina* sp., *Daphina magna, Streptocephalus* sp., *Streptocephalus dichotomus*, *Branchinella* sp., *Artemia salina*, *Artemia* sp., *Artemia parthenogenetica, Artemia franciscana,* or *Triops granaries.*

[017] The invention also provides that the zooplankton can comprise rotifers, including those of the *Brachionus* species, e.g., *Brachionus plicatilis, Brachionus rotundiformis*, and *Brachionus calyciflorus.*

[018] The invention further provides that the zooplankton can comprise Copepods, including *Eurytemora velox.*

[019] The invention yet further provides that the zooplankton can comprise nematodes.

[020] The invention provides that the zooplankton can comprise *Panagellus redivivus.*

[021] The invention also provides that the zooplankton can comprise caldocerans.

[022] The invention further provides that the zooplankton can comprise a *Moina* species.

[023] The invention yet further provides that the probiotic and or prebiotic can be selected from fish intestinal aerobic or anaerobic microbial flora, and crustacean intestinal aerobic or anaerobic microbial flora.

[024] The invention provides that the probiotic and or prebiotic can comprise a *Clostridium* species.

[025] The invention also provides that the probiotic and or prebiotic can be selected from *Aeromonas* sp., *Aeromonas media*, *Bacteriodies* sp., *Carnobacterium* sp., *Carnobacterium divergens*, *Carnobacterium* sp. strain K1, *Clostridium* sp., *Clostridium difficile*, *Fusobacterium* sp., *Lactobacillus* sp., *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Listonella* sp., *Listonella anguillarum*, *Bacillus* sp., *Bacillus cereus*, *Bacillus cereus var. toyoi*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus polymxa*, *Bacillus subtilis*, *Streptococcus* sp., *Streptococcus lactis*, *Streptococcus thermophilus*, *Bifidobacterium* sp., *Bifidobacterium breve*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Vibrio* sp., *Vibrio alginolyticus*, *Vibrio anguillarum*, *Vibrio carchariae*, *Vibrio salmonicida-like*, *Peptostreptococcus* sp., *Photobacterium* sp., *Pseudomonas* sp., *Pseudomonas corrugata*, and *Pseudomonas fluorescens*.

[026] The invention also provides that the probiotic and or prebiotic can be selected from *Saccharomyces* sp., *Saccharomyces cerevisiae*, *Pfaffia* sp., *Pfaffia rhodomonas*, *Debaryomyces* sp., and *Debaryomyces hansenii* HF1.

[027] The invention further provides that the probiotic and or prebiotic can be selected from *Aspergillus* sp., *Aspergillus oryzae*, *Morteriella* sp., and *Morteriella alpina*.

[028] The invention further provides that the probiotic and or prebiotic can be selected from *Tetraselmis* sp., *Tetraselmis suecica*, *Myrmecia* sp., *Myrmecia bissecta*, *Lyngbya* sp., *Lyngbya majuscula*, *Cytospora* sp., *Scenedesmus* sp., *Scenedesmus obliquus*, *Scytonema* sp., *Scytonema hofmanni*, *Nostoc* sp., *Nostoc weissfloggia*, *Chaetoceros* sp., *Chaetoceros lauderi*, *Ecklonia* sp., *Ecklonia maxima*, *Duneliella* sp., *Duneliella salina*, *Duneliella tertiolecta*, *Duneliella bardiwal*, *Pseudoanabaena* sp., *Anabaena* sp., *Prorocentrum* sp., *Prorocentrum minimum*, *Polysiphonia* sp., *Polysiphonia denudata*, *Spirulina* sp., *Arthrospira* sp., *Spirulina platensis*, *Aphanotheae* sp., *Aphanotheae nidulans*, *Hydrodictyon* sp., *Hydrodictyon reticulatum*, *Navicula* sp., *Navicula delongei*, *Phaeodactylum* sp., *Phaeodactylum tricornutum*, *Pseudonitzschia* sp., *Nitzschia* sp., *Nitzschia navis-varingica*, *Chlorella* sp., *Chlorella pyrenoidosa*, *Chlorella vulgaris*, *Chlamydomonas* sp., *Chlamydomonas reinhardii*, *Prennioporia* sp., *Prennioporia medullaeparis*, *Pandorina* sp., and *Pandorina morum*.

[029] The invention provides a composition comprising the zooplankton of the invention, further comprising a component selected from alginate, chitosan, starch, cornstarch, and potato starch.

[030] In another aspect, the invention provides a method for delivery of live, bioactive probionts or prebionts to larval fish, juvenile fish, and crustaceans, by providing the probionts or prebionts; and feeding them to said larval fish, juvenile fish, and crustaceans a feed or feed supplement comprising zooplankton further comprising the probionts or prebionts.

[031] The invention also provides a method wherein the zooplankton are selected from crustaceans, rotifers, caldocerans, and nematodes.

[032] The invention further provides a method wherein the crustaceans are selected from *Artemia* sp., *Daphina* sp., *Daphina magna*, *Streptocephalus* sp., *Streptocephalus dichotomus*, *Branchinella* sp., *Artemia salina*, *Artemia* sp., *Artemia parthenogenetica, Artemia franciscana*, and *Triops granaries*.

[033] The invention yet further provides a method wherein the zooplankton are rotifers.

[034] The invention provides a method wherein the rotifers are selected from *Brachionus* sp., *Brachionus plicatilis, Brachionus rotundiformis*, and *Brachionus calyciflorus*.

[035] The invention provides a method wherein the probionts or prebionts are processed in a manner that maintains their viability.

[036] The invention also provides a method wherein the probionts or prebionts are microencapsulated.

[037] The invention further provides a method wherein the probionts or prebionts are mixed with alginate, chitosan, starch, cornstarch, or potato starch.

[038] The invention yet further provides a method wherein the probionts or prebionts are cross-linked for stability.

[039] The invention provides a method wherein the probionts or prebionts are in an encysted or resting stage.

[040] The invention provides a method wherein the microcapsules with the probionts or prebionts are sized to a diameter of from about 2 to about 20 microns.

[041] The invention also provides a composition wherein the zooplankton are produced by culturing on nutrient culture media comprising probiont- or prebiont-containing microcapsules.

[042] The invention also provides a method of culturing the zooplankton on nutrient culture media comprising probiont- or prebiont-containing microcapsules.

[043] The invention is not limited to the particular described embodiments. The terminology used herein is for the purpose of describing particular embodiments, and is not limiting. The scope of the invention is limited only by the claims.

DETAILED DESCRIPTION OF THE EMBODIMENTS

[044] As contemplated herein, a "probiont" is an organism that grows stably or transiently in the intestine of the target animal. A "probiotic" is a general term applied to the use of probionts to deliver benefit to the target animal.

[045] "Prebiotic" as contemplated herein, refers to an organism grown in such a method to contain one or several compounds that provide a specific health or nutritional benefit to the target animal.

[046] A "target animal" as contemplated herein refers to a cultured animal to which a probiotic and/or prebiotic is developed and which provides some health or nutritional benefit.

[047] A “zooplankton” (plural) or “zooplankter” (singular) as contemplated herein, is an organism or organisms belonging to the animal kingdom, found in the water, including either a fresh water or seawater column,. Examples of zooplankton include, but are not limited to, crustaceans, *Artemia*, rotifers, caldocerans, nematodes, copepods, and ciliates. Zooplankton can adapt to pelagic (open water), littoral (vegetated), and benthic (bottom) environments, and are widespread in both freshwater and marine environments.

[048] A “crustacean” is any organism of the class Crustacea.

[049] A "rotifer" is any of a class of minute, usually microscopic, but many-celled aquatic invertebrate animals having the anterior end modified into a retractile disk bearing circles of strong cilia that often give the appearance of rapidly revolving wheels. Rotifers are used as a source of live feed for aquatic animals during their early larval stage.

[050] *"Artemia"* include any strain belonging to the phylum Arthropoda in the class Crustacea. They are also referred to as “brine shrimp.” These tiny crustaceans can be found in salt lakes and brine ponds throughout the world, and are used as a source of live feed for aquatic animals.

[051] A “copepod” is any organism of the subclass Copepoda.

[052] A "nematode" is any organism of the phylum Nematoda.

[053] "Aerobic" means living or occurring only in the presence of oxygen.

[054] "Anerobic" means living or occurring in the absence of oxygen.

[055] "Viability" is the ability to live, develop, or germinate.

[056] "Bioactivity" is the ability to affect a living organism.

[057] An "encysted" stage is a period of time in the course of a process in which an organism is enclosed in a small capsule or sac that encloses it in its dormant or larval stage. A "resting stage" is not equivalent to the encysted stage since capsule formation is not included in the cells preparation for lower metabolism. In the resting stage the cell has lowered metabolism compared to actively growing organisms.

[058] A "caldoceran" is an organism formerly grouped in the branchiopod crustacean group. The caldocerans are in the class Branchiopoda, subclass diplostraca, order caldocera and family Daphniidae mainidae. Examples of caldocerans are *Daphnina magana, D. galeata, D. pulex* and *Bosmina logirostins*.

[059] "Microencapsulation" as contemplated herein, refers to coating, containing, or incorporating in a matrix an organism or organisms in another compound or mixture of compounds such that a cell or aggregate of cells (homogeneous or a mixture) is sealed within a matrix (liquid, solid or gel) that may be used to conveniently deliver the cell or cell aggregate as described in this invention. Any process that binds up the materials to be provided to the zooplankter for eventual consumption by the target organism is contemplated herein.

[060] The term "load" is used synomously with the term "enrich" for the purposes of this invention. Here zooplankter are fed materials that are taken orally but are not meant for metabolism by the zooplankter, rather to be maintained intact in the gastrointestinal tract and consumed by the target species as a mode of delivery of the material provided to the zooplankter.

Example 1. *Clostridium difficile* spores fed directly to *Artemia* nauplii.

[061] *Clostridium difficile* is cultured using standard anaerobic techniques and allowed to form spores (Holdeman and Moore, 1975). Anaerobic medium for growth of *Clostridium* is made using the following recipe: 1.75 g K_2HPO_4, 0.01 g $CaCl_2 \cdot 2H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 2.0 g L-arginine HCl, 2.0 g L-lysine HCl, 5.0 g yeast extract, 2.0 g sodium formate, 2.0 g NH_4Cl, 0.002 g methylene blue, and 1 L tap water. Medium is mixed and sterilized, and then 0.03% $Na_2S \cdot 9H_2O$ is added. The culture is allowed to grow anaerobically with agitation (either by shaking or by bubbling with O_2 free CO_2 gas) until late stationary phase wherein spore formation is maximal. Cells are then harvested by centrifugation under an inert gas atmosphere (Helium or Nitrogen, all O_2-free). Spores can then be directly fed to *Artemia* as a wet suspension or partially dried under vacuum (using a VIRTIS laboratory freeze drier or a spray drier) to affect more uniform distribution. The spores are mixed with an enrichment supplement for *Artemia* (such as AquaGrow Enhance®) as a dried mix or provided as an oil emulsion (mixed with water/oil/Tween 80 and spores in a Beadbeater® or sonicator) and fed to *Artemia* nauplii (Curé et al., 1996).

Example 2. *Clostridium difficile* spores delivered to rotifers in bioencapsulated format.

[062] *Clostridium difficile* spores are produced as in Example 1. In a reduced medium, alginate is added at from 1 to 8% and dissolved. Calcium chloride at 2-20% is added to induce cross-linking of the gel and the microbeads are spray dried through a fine nozzle to form microencapsulated beads containing spores. Once cured, these can be stored under nitrogen for prolonged viability of the spores. Microencapsulated spores can be added to zooplankton cultures as a top dressing to

existing feeds or mixed directly into the culture medium for *Artemia* consumption. Once the medium has been cleared, typically in 4-16 hours, preloaded *Artemia* can be used to introduce viable *C. difficile* spores into larval fish and crustaceans by direct feeding using the *Artemia* of this invention as live feed.

Example 3. Prebiont delivery to rotifers.

[063] *Clostridium difficile* spores are produced as in Example 1. Rotifers are cultured in seawater or artificially made seawater at a salinity of 15-40 psu and temperature between 20-30°C. Rotifers are fed a mixture of bakers yeast and concentrated microalgae (*Isochrysis* sp., *Chlorella* sp., *Nanochloropsis* sp., *Tetraselmis* sp.). Up to 30% of the rotifers are harvested daily from the culture tank, rinsed with fresh water and transferred to enrichment tanks at concentration of 500,000 rotifers per liter. The enrichment tanks are then inoculated with *Clostridium difficile* spores every 4 hours. Enrichment of essential nutrients with 0.05 g per liter of AquaGrow DHA, ARA, and Enhance are also carried out at 4-hour intervals. Rotifers are harvested after 8-16 hours, rinsed with seawater, and delivered to aquatic larvae.

Example 4. Microencapsulation of *Lactobacillus fermentum* in alginate.

[064] Lactobacillus *fermentum* spores are mixed with 1-3% of an emulsifying agent such as phosphatidyl choline, Tween 80, or Santone, and vortexed vigorously to produce a fine emulsion. The emulsion is blended at 10-50% into sodium alginate (1-3%) and cornstarch (1-5%) in deionized water. The resultant probiont in gel blend is then atomized through a spray nozzle assembly pressurized with nitrogen gas, into a bath of chilled solution containing 2-20% calcium chloride

and 1-5% sodium chloride. The resultant microcapsules are sieved through 20μm mesh, and microcapsules <20 μm are collected to ensure optimal size for consumption by *Artemia* and rotifers. These <20 μm microcapsules are freeze-dried and packed under vacuum for longer periods of preservation.

Example 5. Microencapsulation of *Vibrio alginolyticus* in alginate plus additional components.

[065] *Vibrio alginolyticus* are produced as in Example 4, microencapsulated and dried into a fine powder. Four homogeneous mixtures containing AquaGrow DHA or AquaGrow Advantage or AquaGrow Enhanced or AquaGrow ARA and 2-20% of fine powder of *Vibrio alginolyticus* are formulated and packed under nitrogen for later use as zooplankton enrichment feed.

Example 6. Feeding probiont-loaded rotifers to shrimp larvae.

[066] Newly hatched Pacific white shrimp (*Litopenaeus vannamei*) are stocked at 100 larvae per liter of seawater. Larvae are fed four times daily with rotifers (at concentration of 5 rotifers per ml) loaded with probionts as described in examples 1-3. Larvae are also given a daily mixture of the live algae *Tetraselmis* sp. and *Chaetoceros* sp., at concentrations of 10,000 and 5,000 cells per ml, respectively. Zoea-1 larvae are introduced with *Artemia* nauplii loaded with probionts as described in examples 1-3. Four rations of *Artemia* at a concentration of 10 nauplii per ml are given daily until larvae reach post larvae stage (PL1). Post larvae shrimps are then counted in each tank for survivorship and sampled for average weight.

Example 7. Feeding prebiont-loaded *Artemia* to sea bream larvae.

[067] *Artemia* are loaded with probionts as described in Examples 2 and 5. Sea bream eggs are stocked in a larvae rearing system at a concentration of 100 eggs per liter and allowed to hatch in full seawater (32-40 ppt) at a temperature of about 17-19°C. Larvae are commenced feeding 3 days post hatching with rotifers enriched with AquaGrow DHA and an algal supplement as described in Koven et al. (Koven et al., 2001). Fourteen days post hatch, larvae are offered *Artemia* nauplii enriched with AquaGrow DHA and loaded with probionts as outlined in Example 5. Larvae are fed 3 times daily at concentration of 10 nauplii per ml until day 36 post hatch. Tanks are then harvested and counted individually for survivorship and sampled for overage weight.

Example 8. Feeding probiont-loaded *Artemia* to sea bream larvae.

[068] *Artemia* are loaded with probionts as described in Examples 2, 4 and 5. Sea bream eggs are stocked in larvae-rearing system at a concentration of 100 eggs per liter, and allowed to hatch in full seawater (32-40 ppt) at a temperature of about 17-19°C. Larvae are commenced feeding 3 days post hatching with rotifers enriched with AquaGrow DHA and algal supplement as described in Koven et al. (Koven et al., 2001). Fourteen days post hatch larvae are offered *Artemia* nauplii enriched with AquaGrow DHA and loaded with probionts as outlined in Example 5. Larvae are fed 3 times daily at concentration of 10 nauplii per ml until day 36 post hatch. Tanks are then harvested and counted individually for survivorship and sampled for overage weight.

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We claim:

1. Zooplankton comprising at least one microencapsulated, viable, bioactive prebiotic or probiotic.

2 Zooplankton as in Claim 1, wherein the zooplankton further comprises crustaceans.

3. Zooplankton as in Claim 2, wherein the crustaceans further comprise *Artemia*.

4. Zooplankton as in Claim 3, wherein the *Artemia* further comprise *Artemia salina*.

5. Zooplankton as in Claim 2, wherein the zooplankton is selected from brine shrimp, fairy shrimp, *Daphina* sp., *Daphina magna, Streptocephalus sp., Streptocephalus dichotomus*, *Branchinella* sp., *Artemia salina*, *Artemia* sp., *Artemia parthenogenetica, Artemia franciscana,* and *Triops granaries*.

6. Zooplankton as in Claim 1, wherein the zooplankton further comprises rotifers.

7. Zooplankton as in Claim 6, wherein the rotifers further comprises *Brachionus*.

8. Zooplankton as in Claim 7, wherein the *Brachionus* further comprises *Brachionus plicatilis*.

9. Zooplankton as in Claim 7, wherein the *Brachionus* further comprises *Brachionus rotundiformis*.

10. Zooplankton as in Claim 7, wherein the *Brachionus* further comprises *Brachionus calyciflorus*.

11. Zooplankton as in Claim 1, wherein the zooplankton further comprises Copepods.

12. Zooplankton as in Claim 11, wherein the copepods further comprise *Eurytemora velox.*

13. Zooplankton as in Claim 1, wherein the zooplankton further comprises nematodes.

14. Zooplankton as in Claim 1, wherein the nematodes further comprise *Panagellus redivivus.*

15. Zooplankton as in Claim 1, wherein the zooplankton further comprises caldocerans.

16. Zooplankton as in Claim 1, wherein the caldocerans further comprise *Moina.*

17. Zooplankton as in claims 1-16, wherein the prebiotic or probiotic is selected from fish intestinal aerobic or anaerobic microbial flora, and crustacean intestinal aerobic or anaerobic microbial flora.

18. Zooplankton as in claims 1-16, wherein the probiotic and or prebiotic further comprises *Clostridium.*

19. Zooplankton as in claims 1-16, wherein the probiotic and or prebiotic are selected from *Aeromonas* sp., *Aeromonas media, Bacteriodies* sp., *Carnobacterium* sp., *Carnobacterium divergens*, *Carnobacterium* sp. strain K1, *Clostridium* sp., *Clostridium difficile, Fusobacterium* sp., *Lactobacillus* sp., *Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus plantarum, Listonella* sp., *Listonella anguillarum,* , *Bacillus* sp., *Bacillus cereus, Bacillus cereus* var. *toyoi*, *Bacillus licheniformis,*

Bacillus megaterium, Bacillus polymxa, Bacillus subtilis, Streptococcus sp., *Streptococcus lactis, Streptococcus thermophilus, Bifidobacterium* sp., *Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium longum, Vibrio* sp., *Vibrio alginolyticus, Vibrio anguillarum, Vibrio carchariae, Vibrio salmonicida*-like, *Peptostreptococcus* sp., *Photobacterium* sp., *Pseudomonas* sp., *Pseudomonas corrugata,* and *Pseudomonas fluorescens.*

20. Zooplankton as in Claims 1-16, wherein the probiotic and or prebiotic are selected from *Saccharomyces* sp., *Saccharomyces cerevisiae, Pfaffia* sp., *Pfaffia rhodomonas, Debaryomyces* sp., and *Debaryomyces hansenii* HF1.

21. Zooplankton as in Claims 1-16, wherein the probiotic and or prebiotic are selected from *Aspergillus* sp., *Aspergillus oryzae, Morteriella* sp., and *Morteriella alpina.*

22. Zooplankton as in Claims 1-16, wherein the probiotic and or prebiotic are selected from *Tetraselmis* sp., *Tetraselmis suecica, Myrmecia* sp., *Myrmecia bissecta, Lyngbya* sp., *Lyngbya majuscula, Cytospora* sp., *Scenedesmus* sp., *Scenedesmus obliquus, Scytonema* sp., *Scytonema hofmanni, Nostoc* sp., *Nostoc weissfloggia, Chaetoceros* sp., *Chaetoceros lauderi, Ecklonia* sp., *Ecklonia maxima, Duneliella* sp., *Duneliella salina, Duneliella tertiolecta, Duneliella bardiwal, Pseudoanabaena* sp., *Anabaena* sp., *Prorocentrum* sp., *Prorocentrum minimum, Polysiphonia* sp., *Polysiphonia denudata, Spirulina* sp., *Arthrospira* sp., *Spirulina platensis, Aphanotheae* sp., *Aphanotheae nidulans, Hydrodictyon* sp., *Hydrodictyon reticulatum, Navicula* sp., *Navicula delongei, Phaeodactylum* sp., *Phaeodactylum tricornutum, Pseudonitzschia* sp., *Nitzschia* sp., *Nitzschia navis-varingica, Chlorella* sp., *Chlorella pyrenoidosa, Chlorella vulgaris, Chlamydomonas* sp.,

Chlamydomonas reinhardii, Prennioporia sp., *Prennioporia medullaeparis, Pandorina* sp., and *Pandorina morum.*

23. A composition comprising the zooplankton of Claims 1-22, and further comprising a component selected from alginate, chitosan, starch, cornstarch, and potato starch.

24. A method for delivery of live, bioactive probionts or prebionts to larval fish, juvenile fish, and crustaceans, comprising

(a) providing the probionts or prebionts; and

(b) feeding the probionts or prebionts to said larval fish, juvenile fish, and crustaceans as a feed or feed supplement comprising zooplankton further comprising the probionts or prebionts.

25. A method as in Claim 24, wherein the zooplankton are selected from crustaceans, rotifers, caldocerans, and nematodes.

26. A method as in Claim 25, wherein the crustaceans are selected from *Artemia* sp., *Daphina* sp., *Daphina magna, Streptocephalus sp., Streptocephalus dichotomus, Branchinella* sp., *Artemia salina, Artemia* sp., *Artemia parthenogenetica, Artemia franciscana,* and *Triops granaries.*

27. A method as in Claim 24, wherein the zooplankton are rotifers.

28. A method as in Claim 28, wherein the rotifers are selected from *Brachionus* sp., *Brachionus plicatilis, Brachionus rotundiformis*, and *Brachionus calyciflorus.*

29. A method as in Claims 24-28, further comprising processing the probionts or prebionts in a manner that maintains their viability.

30. A method as in Claim 29, wherein the probionts or prebionts are microencapsulated.

31. A method as in Claim 30, further comprising mixing the probionts or prebionts with a component chosen from alginate, chitosan, starch, cornstarch, and potato starch.

32. A method as in Claim 31, wherein the components are cross-linked for stability.

33. A method as in Claim 29, wherein the probionts or prebionts are in an encysted or resting stage.

34. A method as in claims 24-33, further comprising sizing the microcapsules to a diameter of from about 2 to about 20 microns.

35. A composition as in claims 1-23 wherein said zooplankton are produced by culturing on nutrient culture media comprising probiont- or prebiont-containing microcapsules.

36. A method as in claims 24-34, further comprising culturing the zooplankton on nutrient culture media comprising probiont- or prebiont-containing microcapsules.

AMENDED CLAIMS

**[Received by the International Bureau on 17 November 2003 (17.11.03):
original claims 2-3, 6-24, 29-30, and 33-36 replaced by amended claims 2-3, 6-24, 29-30, and 33-36
original claims 1, 4-5, 25-28, and 31-32 are unchanged (5 pages)]**

I claim:

1. Zooplankton comprising at least one microencapsulated, viable, bioactive prebiotic or probiotic.

2 Zooplankton as in Claim 1, wherein the zooplankton further comprise one or more crustaceans.

3. Zooplankton as in Claim 2, wherein the crustaceans further comprise one or more *Artemia*.

4. Zooplankton as in Claim 3, wherein the *Artemia* further comprise *Artemia salina*.

5. Zooplankton as in Claim 2, wherein the zooplankton is selected from brine shrimp, fairy shrimp, *Daphina* sp., *Daphina magna, Streptocephalus sp., Streptocephalus dichotomus*, *Branchinella* sp., *Artemia salina*, *Artemia* sp., *Artemia parthenogenetica, Artemia franciscana,* and *Triops granaries*.

6. Zooplankton as in Claim 1, wherein the zooplankton further comprise one or more rotifers.

7. Zooplankton as in Claim 6, wherein the rotifers further comprise one or more *Brachionus*.

8. Zooplankton as in Claim 7, wherein the *Brachionus* is *Brachionus plicatilis*.

9. Zooplankton as in Claim 7, wherein the *Brachionus* is *Brachionus rotundiformis*.

10. Zooplankton as in Claim 7, wherein the *Brachionus* is *Brachionus calyciflorus*.

11. Zooplankton as in Claim 1, wherein the zooplankton further comprise one or more Copepods.

12. Zooplankton as in Claim 11, wherein the copepods further comprise one or more *Eurytemora velox.*

13. Zooplankton as in Claim 1, wherein the zooplankton further comprise one or more nematodes.

14. Zooplankton as in Claim 1, wherein the nematodes further comprise one or more *Panagellus redivivus.*

15. Zooplankton as in Claim 1, wherein the zooplankton further one or more caldocerans.

16. Zooplankton as in Claim 1, wherein the caldocerans further comprise one or more *Moina.*

17. Zooplankton as in any of Claims 1-16, wherein the prebiotic or probiotic is selected from fish intestinal aerobic or anaerobic microbial flora, and crustacean intestinal aerobic or anaerobic microbial flora.

18. Zooplankton as in any of Claims 1-16, wherein the probiotic and/or prebiotic further comprises *Clostridium.*

19. Zooplankton as in any of Claims 1-16, wherein the probiotic and/or prebiotic are selected from *Aeromonas* sp.*, Aeromonas media, Bacteriodies* sp., *Carnobacterium* sp., *Carnobacterium divergens, Carnobacterium* sp. strain K1, *Clostridium* sp., *Clostridium difficile, Fusobacterium* sp., *Lactobacillus* sp., *Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus plantarum, Listonella* sp., *Listonella anguillarum, Bacillus* sp., *Bacillus cereus, Bacillus cereus* var. *toyoi, Bacillus licheniformis, Bacillus megaterium, Bacillus*

AMENDED SHEET (ARTICLE 19)

polymxa, *Bacillus subtilis*, *Streptococcus* sp., *Streptococcus lactis*, *Streptococcus thermophilus*, *Bifidobacterium* sp., *Bifidobacterium breve*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Vibrio* sp., *Vibrio alginolyticus*, *Vibrio anguillarum*, *Vibrio carchariae*, *Vibrio salmonicida*-like, *Peptostreptococcus* sp., *Photobacterium* sp., *Pseudomonas* sp., *Pseudomonas corrugata*, and *Pseudomonas fluorescens*.

20. Zooplankton as in any of Claims 1-16, wherein the probiotic and/or prebiotic are selected from *Saccharomyces* sp., *Saccharomyces cerevisiae*, *Pfaffia* sp., *Pfaffia rhodomonas*, *Debaryomyces* sp., and *Debaryomyces hansenii* HF1.

21. Zooplankton as in any of Claims 1-16, wherein the probiotic and/or prebiotic are selected from *Aspergillus* sp., *Aspergillus oryzae*, *Morteriella* sp., and *Morteriella alpina*.

22. Zooplankton as in any of Claims 1-16, wherein the probiotic and/or prebiotic are selected from *Tetraselmis* sp., *Tetraselmis suecica*, *Myrmecia* sp., *Myrmecia bissecta*, *Lyngbya* sp., *Lyngbya majuscula*, *Cytospora* sp., *Scenedesmus* sp., *Scenedesmus obliquus*, *Scytonema* sp., *Scytonema hofmanni*, *Nostoc* sp., *Nostoc weissfloggia*, *Chaetoceros* sp., *Chaetoceros lauderi*, *Ecklonia* sp., *Ecklonia maxima*, *Duneliella* sp., *Duneliella salina*, *Duneliella tertiolecta*, *Duneliella bardiwal*, *Pseudoanabaena* sp., *Anabaena* sp., *Prorocentrum* sp., *Prorocentrum minimum*, *Polysiphonia* sp., *Polysiphonia denudata*, *Spirulina* sp., *Arthrospira* sp., *Spirulina platensis*, *Aphanotheae* sp., *Aphanotheae nidulans*, *Hydrodictyon* sp., *Hydrodictyon reticulatum*, *Navicula* sp., *Navicula delongei*, *Phaeodactylum* sp., *Phaeodactylum tricornutum*, *Pseudonitzschia* sp., *Nitzschia* sp., *Nitzschia*

navis-varingica, Chlorella sp., *Chlorella pyrenoidosa, Chlorella vulgaris, Chlamydomonas* sp., *Chlamydomonas reinhardii, Prennioporia* sp., *Prennioporia medullaeparis, Pandorina* sp., and *Pandorina morum.*

23. A composition comprising the zooplankton of any of Claims 1-22, and further comprising a component selected from alginate, chitosan, starch, cornstarch, and potato starch.

24. A method for delivery of live, bioactive probionts or prebionts to larval fish, juvenile fish, and crustaceans, comprising

(a) providing the probionts or prebionts; and

(b) feeding the probionts or prebionts to said larval fish, juvenile fish, and/or crustaceans as a feed or feed supplement comprising zooplankton further comprising the probionts or prebionts.

25. A method as in Claim 24, wherein the zooplankton are selected from crustaceans, rotifers, caldocerans, and nematodes.

26. A method as in Claim 25, wherein the crustaceans are selected from *Artemia* sp., *Daphina* sp., *Daphina magna, Streptocephalus sp., Streptocephalus dichotomus, Branchinella* sp., *Artemia salina, Artemia* sp., *Artemia parthenogenetica, Artemia franciscana,* and *Triops granaries.*

27. A method as in Claim 24, wherein the zooplankton are rotifers.

28. A method as in Claim 28, wherein the rotifers are selected from *Brachionus* sp., *Brachionus plicatilis, Brachionus rotundiformis*, and *Brachionus calyciflorus.*

29. A method as in any of Claims 24-28, further comprising processing the probionts or prebionts in a manner that maintains their viability.

30. A method as in Claim 29, wherein the probionts or prebionts comprise microcapsules.

31. A method as in Claim 30, further comprising mixing the probionts or prebionts with a component chosen from alginate, chitosan, starch, cornstarch, and potato starch.

32. A method as in Claim 31, wherein the components are cross-linked for stability.

33. A method as in Claim 29, wherein the probionts or prebionts are in an encysted and/or resting stage.

34. A method as in any of Claims 29-33, further comprising sizing the microcapsules to a diameter of from about 2 to about 20 microns.

35. A composition as in any of Claims 1-22, wherein said zooplankton are produced by culturing on nutrient culture media comprising probiont- or prebiont-containing microcapsules.

36. A method as in any of Claims 24-34, further comprising culturing the zooplankton on nutrient culture media comprising probiont- or prebiont-containing microcapsules.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/15408

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 35/66, 35/68, 35/70, 35/72, 35/74, 35/76, 47/00; A01N 63/00, 63/02; C12N 1/00, 1/12, 1/20
US CL : 424/ 93.4, 439, 780; 435/170, 252.1, 822

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/ 93.4, 439, 780; 435/170, 252.1, 822

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,698,246 A (VILLAMAR) 16 December 1997 (16.12.1997), see abstract and column 3, lines 45-50.	1-22, 24-33 ----------- 1-22, 24-33
X --- Y	US 6,399,118 B1 (ZEMACH et al) 04 June 2002 (04.06.2002), see abstract and column 3, all lines, column 6, all lines.	1-22, 24-33 ----------- 1-22, 24-33

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
22 August 2003 (22.08.2003)

Date of mailing of the international search report
15 SEP 2003

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
Facsimile No. (703)305-3230

Authorized officer
Felicia D. Roberts for
Deborah K. Ware
Telephone No. 703-308-0196

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claim Nos.: 23 and 34-36
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.