(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 October 2019 (17.10.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/199309 A1

(51) International Patent Classification:
A01K 67/033 (2006.01) *A23K 10/12* (2016.01)
A01K 67/04 (2006.01) *A23K 10/30* (2016.01)
A23K 50/90 (2016.01) *A23K 10/33* (2016.01)
A23K 10/00 (2016.01) *A23K 10/37* (2016.01)
A23K 10/10 (2016.01)

(21) International Application Number:
PCT/US2018/027252

(22) International Filing Date:
12 April 2018 (12.04.2018)

(25) Filing Language: English

(26) Publication Language: English

(71) Applicant: **THE TEXAS A&M UNIVERSITY SYSTEM** [US/US]; 3369 TAMU, College Station, TX 77843-3369 (US).

(72) Inventors: **TOMBERLIN, Jeffery, K.**; 3369 TAMU, College Station, TX 77843-3369 (US). **YANG, Fengchun**; 3369 TAMU, College Station, TX 77843-3369 (US). **CAMMACK, Jonathan, A.**; 3369 TAMU, College Station, TX 77843-3369 (US).

(74) Agent: **HANSON, Robert, E.**; Dentons US LLP, P.O Box 061080, Wacker Drive Station, Willis Tower, Chicago, IL 60606-1080 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*

(54) Title: BLACK SOLDIER FLY BULLET

(57) Abstract: The present disclosure provides compositions and methods for long-term storage and stable production of black soldier flies, capable of use in waste remediation or protein production. The invention overcomes drawbacks associated with current methods of using black soldier flies, significantly increasing the period during which black soldier fly neonates can be stored and the time in which larvae mature.

WO 2019/199309 A1

BLACK SOLDIER FLY BULLET

FIELD OF THE INVENTION

[0001] The present disclosure relates to the field of biotechnology. More specifically, the disclosure relates to compositions and methods for long-term storage and stable production of black soldier flies.

BACKGROUND

[0002] The production of black soldier flies as a protein source, or use of them to reduce waste is dependent on maintaining a robust population. Current methods rely on the inoculation of media or waste with black soldier fly eggs or neonates (recently hatched larvae). Development time from inoculation to harvest is approximately three weeks. The current system is susceptible to mass larval mortality, unpredictable larval size at harvest, and as a consequence, unpredictable larval harvest. Furthermore, current methods do not allow storage of eggs or larvae beyond 2 to 4 days, after which they die.

[0003] What is needed are new compositions and methods that allow for long-term storage and stable and reproducible production of black soldier flies.

SUMMARY OF THE INVENTION

[0004] The present disclosure provides a composition for storage of black soldier fly larvae, comprising a container, a layer of fermented nutrient source in the bottom of the container (the bottom layer), black soldier fly larvae on top of the layer of fermented nutrient source (the middle layer), a layer of dry nutrient source on top of the black soldier fly larvae (the top layer), and a cover on top of the container that allows air to pass into and out of the container.

[0005] In general, the container used in the composition can be of any desired size. In certain embodiments the container is about 300 ml, about 350 ml, about 400 ml, about 450 ml, about 500 ml, about 550 ml, about 600 ml, about 650 ml, about 700 ml, about 750 ml, about 800 ml, about 850 ml, about 900 ml, about 950 ml or about 100 ml in size. Although the size of the container can be based on volumetric values, as described above, in some embodiments the size can be based on other parameters, such as, but not limited to, weight. In various embodiments the container has tapered sides, with the taper increasing or decreasing from the bottom of the

container to the top of the container, straight sides, or irregular sides having a variety of angles and/or widths.

[0006] In some embodiments, once the layer of fermented nutrient source in the bottom of the container, the layer of black soldier fly larvae on top of the layer of fermented nutrient source, and the layer of dry nutrient source on top of the black soldier fly larvae has been added to the container, the container will also have a void volume between the top layer (the layer of dry nutrient source) and the top of the container after the cover or lid is placed on the container to close or seal the container. In general, the void volume should sufficient to maintain a relatively constant or relatively uniform moisture and temperature in the layer of fermented nutrient source (the bottom layer of the container). Since failure to maintain a relatively constant or relatively uniform moisture and temperature in the layer of fermented nutrient source can lead to spoilage of the fermented nutrient source, the void volume should be sufficient to avoid spoilage of the fermented nutrient source.

[0007] In particular embodiments the container used in the composition can have a solid bottom (such that the container will not leak or lose liquid through the bottom of the container) and an open or partially open top, for example a top that defines a single opening or a plurality of openings. In further embodiments the container is impermeable to gas, such as, but not limited to, air, or liquid, such as, but not limited to, water. In general the container can be made from, or comprise, a variety of different materials, or a combination of such materials. In various embodiments the container is made from, or comprised of, glass, plastic or metal, or any combination thereof. In other embodiments, the container is specifically designed to avoid the presence of microclimates in the container, or in the various layers within the container.

[0008] The same nutrient source can used in both the bottom layer (which is fermented) and the top layer (which is dry) of the container, although in some embodiments a first nutrient source is fermented and used in the bottom layer while a second nutrient source is used dry (without any added liquid) in the top layer of the container. Although any nutrient source that will support the long-term storage of black soldier fly larvae can be used in conjunction with the presently described compositions and methods, in certain embodiments the nutrient source is about 50% wheat bran, 30% alfalfa meal and 20% corn meal by volume.

[0009] In general, the fermented nutrient source is produced using a probiotic culture. In some embodiments the probiotic culture includes a single species of lactic acid bacteria, while in other embodiments the probiotic culture comprises a plurality of species of lactic acid bacteria. A large variety of lactic acid bacteria are well-known to those of skill in the art, and can be used in the presently disclosed probiotic culture. The probiotic culture can include, but is not limited to, *Bifidobacterium animalis*, *Bifidobacterium bifidum*, *Bifidobacteriumlongum, Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii* subsp *bulgaricus*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactococcus lactis* subsp *lactis* or *Streptococcus thermophilus*, or any combination thereof. In further embodiments, the probiotic culture comprises one or more species of lactic acid bacteria and a yeast, for example *Saccharomyces cerevisiae*, or a different bacteria, for example *Bacillus subtilis* or a member of the Bradyrhizobiaceae family of bacteria, or combinations thereof.

[0010] In certain embodiments the nutrient source is fermented for at least 24 hours, at least 48 hours, at least 72 hours, at least 96 hours, at least 5 days, at least 6 days, at least 1 week, at least 2 weeks, at least 3 weeks, at least 1 month, at least 2 months, at least 3 months, at least 4 months, at least 5 months, or at least 6 months or more. In some embodiments the nutrient source is fermented anaerobically. In other embodiments the nutrient source is fermented at about 65% moisture, at about 66% moisture, at about 67% moisture, at about 68% moisture, at about 69% moisture, at about 70% moisture, at about 71% moisture, at about 72% moisture, at about 73% moisture, at about 74% moisture, or at about 75% moisture. In additional embodiments the nutrient source is fermented at room temperature. As used herein throughout the present disclosure, the term "room temperature" means a temperature between about 22°C and about 29°C. Thus, in various embodiments "room temperature" means about 22°C, about 23°C, about 24°C, about 25°C, about 26°C, about 27°C, about 28°C, or about 29°C.

[0011] In general, the probiotic culture is activated (or expanded) prior to use in the fermentation of the nutrient source. In certain embodiments the probiotic culture is activated in a solution comprising a nutrient source or feedstock and water. In some embodiments the nutrient source or feedstock is molasses. Various types of molasses are known to those of skill in the art and can be used as the nutrient source or feedstock, including, but not limited to, light molasses, dark molasses, blackstrap molasses, sulphured molasses, or unsulphured molasses. Molasses is

generally made from sugarcane or sugar beets, and can vary by the amount of sugar, the method of extraction and the age of the plant. However, molasses made from other sources, including, but not limited to, sorghum, pomegranate, carob or dates, can be used in particular embodiments of the present disclosure.

[0012] The ratio of the probiotic culture to the nutrient source or feedstock can vary, but is generally about 1:1, but this ratio can vary by about 20% or so in certain embodiments. In addition, the ratio of the probiotic culture/nutrient source to water can also vary, but is generally about 1:20, although this ratio can also vary by about 20% or so in other embodiments. Thus, in particular embodiments the ratio of probiotic culture to molasses to water is about 1:1:20. In some embodiments the probiotic culture is activated or expanded for at least one week, while in various other embodiments the probiotic culture is activated or expanded for at least 2 weeks, at least 3 weeks, at least 1 month, at least 2 months, at least 3 months, at least 4 months, at least 5 months, or at least 6 months or more. In further embodiments the probiotic culture is activated or expanded anaerobically. In still further embodiments the probiotic culture is activated or expanded at room temperature.

[0013] In general neonate black soldier fly larvae are used in the presently disclosed compositions and methods. The term "neonate black soldier fly larvae," as used herein, means black soldier fly larvae from the time of eclosion (or hatching) until just before the black soldier fly larvae have molted. Once black soldier fly larvae molt, they are no longer considered to be "neonate black soldier fly larvae." Thus in some embodiments the black soldier fly larvae have not molted, and are used between eclosion and 36 hours of eclosion. In various other embodiments the black soldier fly larvae are used just after eclosion, 1 hour after eclosion, 2 hours after eclosion, 3 hours after eclosion, 4 hours after eclosion, 5 hours after eclosion, 6 hours after eclosion, 12 hours after eclosion, 18 hours after eclosion, 24 hours after eclosion, 30 hours after eclosion, 36 hours after eclosion, or just prior to the molting of the black soldier fly larvae.

[0014] The amount of black soldier fly larvae used as the middle layer in the container (on top of the layer of fermented nutrient source and under the layer of dry nutrient source) can vary, depending on the size and shape of the container. However, in general, the number of black soldier neonate larvae used as the middle layer of the composition is about 60, about 65, about 70, about 75, about 80, about 85 or about 90 or more neonate larvae per gram of fermented

nutrient source. Thus, in certain embodiments about 12,000, about 13,000, about 14,000, about 15,000, about 16,000, about 17,000 or about 18,000 black soldier fly larvae are used as the middle layer of the composition, on top of the bottom layer of fermented nutrient source.

[0015] The present disclosure also provides a method of storing, or long-term storage of, black soldier fly larvae, comprising placing black soldier fly larvae between a layer of fermented nutrient source and a layer of dry nutrient source in a container with a cover that allows air to pass into and out of the container. Since the art is devoid of any method of storing black soldier fly larvae, for any period of time, as used herein the term "long-term storage of black soldier fly larvae" means storage for any period of time between 1 week and 5 months or more. Thus, in various embodiments the black soldier fly larvae are stored for between about 2 weeks and about 5 months, between about 2 weeks and about 4 months, between about 2 weeks and about 3 months, between about 2 weeks and about 2 months, between about 2 weeks and about 1 month, between about 2 weeks and about 3 weeks, between about 3 weeks and about 5 months, between about 1 month and about 5 months, between about 2 months and about 5 months, between about 3 months and about 5 months, between about 4 months and about 5 months, between about 3 weeks and about 4 months, or between about 1 month and about 3 months. In still other embodiments the black soldier fly larvae are stored for about 1 week, about 2 weeks, about 3 weeks, about 1 months, about 2 months, about 3 months, about 4 months, or about 5 months or more. In certain embodiments the black soldier fly larvae are stored for between about 4 weeks and about 6 weeks. In some embodiments the black soldier fly larvae are stored at room temperature.

[0016] The present disclosure additionally provides a method of producing black soldier fly larvae, comprising inoculating organic material with a composition comprising a fermented nutrient source, black soldier fly larvae, and a dry nutrient source, incubating the inoculated organic material for about 6 days, about 7 days, about 8 days, about 9 days, or about 10 days or more, and collecting the black soldier fly larvae produced. In various embodiments the organic material can be organic waste to be digested (bioremediation) or an organic nutrient source or feedstock for use in protein production.

[0017] The present disclosure further provides a method of digesting organic waste, comprising inoculating organic waste with a composition comprising a fermented nutrient source, black

soldier fly larvae, and a dry nutrient source, and incubating the organic waste for about 6 days, about 7 days, about 8 days, about 9 days, or about 10 days or more.

[0018] The present disclosure also provides a media comprising a nutrient source and a probiotic culture comprising at least a first lactic acid bacteria. In certain embodiments the nutrient source comprises wheat bran, alfalfa meal and corn meal in a volume ratio of about 5:3:2. In some embodiments the nutrient source and the probiotic culture are allowed to ferment for at least 24 hours under anaerobic conditions prior to use.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] For a more complete understanding of the features and advantages of the present disclosure, reference is now made to the detailed description of the disclosure along with the accompanying figures. The drawings described herein are for illustration purposes only and are not intended to limit the scope of the present teachings in any way.

[0020] FIG. 1: Relationship between time from inoculation to harvest (days) and bullet age (days). The average bullet age of 14.31 days is represented by the dashed vertical line.

[0021] FIG. 2: Relationship between total pan output (g) and time from pan inoculation to harvest (d). The line represents a regression of the data.

[0022] FIG. 3: Relationship between total pan output (g) and age (d) of the Black Soldier Fly Bullet used to inoculate the pan. The line represents a regression of the data.

[0023] FIG. 4: Relationship between Black Soldier Fly Bullet age (d) and the amount of time from inoculation to harvest (d). The line represents a regression of the data.

[0024] FIG. 5: Larval development on swine manure when using the bullet system in comparison to a traditional experiment started with 4 d old larvae.

[0025] FIG. 6: Example of 500 ml container used for black soldier fly bullet preparation. The container shown has top diameter of about 11-12 cm, a bottom diameter of about 8 cm, and a height of about 8 cm.

DETAILED DESCRIPTION

[0026] The present disclosure describes compositions and methods for the long-term storage and stable production of black soldier flies. Mass production of the black soldier fly *Hermetia illucens* (Diptera: Stratiomyidae), relies on the production of eggs from the black soldier fly, hatching them, and using the larvae to recycle wastes or to produce protein and fat. Among the primary drawbacks with this system are: 1) unpredictable production of eggs; 2) unpredictable hatch time, necessitating use of larvae when produced from eggs; 3) unpredictable egg hatch rate; 4) unpredictable mortality rate of newly hatched larvae; and 5) unpredictable duration of larval development. These issues result in unpredictable mass production and inefficiencies. In addition, a major drawback of this system is the lack of the ability to store the larvae more than 2-4 days after hatching, after which they die.

[0027] The present disclosure overcomes these and other drawbacks associated with current methods of using black soldier flies. One aspect of the present disclosure is a container ("bullet") for storing black soldier fly neonates that can be prepared and stored at room temperature for up to 5 months or longer. Furthermore, when used to produce larvae, the larvae are mature in 5-10 days, as opposed to 20 days using current practices, with minimal mortality and predictable larval harvest. The present disclosure has the following advantages over the systems that are currently in use: 1) reduces concerns with adult fly maintenance, as the "bullets" can be stored long-term and are more durable for shipping; 2) accelerates conversion of food (media or waste) to larval protein/fat by 75%; and, 3) allows for predictable production of larval protein/fat.

[0028] In one embodiment, the bullet comprises a container, a layer of fermented nutrient source in the bottom of the container, black soldier fly larvae on top of the layer of fermented nutrient source, a layer of dry nutrient source on top of the black soldier fly larvae, and a cover on top of the container that allows air to pass into and out of the container. Certain aspects of the present disclosure are discussed in greater detail below.

[0029] I. Probiotic Culture and Activation

[0030] The present disclosure involves in part the use of a probiotic culture to ferment a nutrient source. In certain embodiments the probiotic culture comprises one or more lactic acid bacteria species. Such species can include, but are not limited to, *Bifidobacterium animalis*,

Bifidobacterium bifidum, *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii subsp bulgaricus*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactococcus lactis subsp lactis* or *Streptococcus thermophilus*. In some embodiments, the probiotic culture can include yeast, such as, but not limited to, *Saccharomyces cerevisiae*, or other bacteria, such as, but not limited to, *Bacillus subtilis*. In further embodiments, the probiotic culture can include a phototropic bacteria, including, but not limited to, members of the Bradyrhizobiaceae family of bacteria.

[0031] In addition, commercial sources of probiotic culture are available. Examples of commercially available probiotic cultures include, but are not limited to, EM-1®, which is available from TeraGanix, Inc. (Alto, TX), and ProBio Balance, which is available from SCD Probiotics (Kansas City, MO).

[0032] In general the probiotic culture is activated (or expanded) for at least one week, or up to 6 months or more, prior to use in fermentation of the nutrient source. In certain embodiments the probiotic culture is activated by incubating the probiotic culture with a nutrient source, commonly molasses (light, dark, blackstrap, sulphured, or unsulphured) with water at room temperature (about 24°C to 27°C) under anaerobic conditions. The ratio of probiotic culture to nutrient source to water can vary, but in various embodiments the ratio is about 1:1:10, 1:1:15, 1:1:20, 1:1:25 or 1:1:30. In certain embodiments each of the individual values of the above ratios can vary by about 20%.

[0033] II. Nutrient Source and Fermentation

[0034] The bullet composition disclosed herein generally comprises a base layer of a fermented nutrient source. The nutrient source is fermented using the expanded or activated probiotic culture for at least 24 hours, or up to 6 months or more, prior to use.

[0035] A variety of nutrient sources (feed) can be utilized in the present disclosure. In general, the nutrient source comprises wheat bran, alfalfa meal and corn meal. In certain embodiments the nutrient source comprises 50% wheat bran, 30% alfalfa meal and 20% corn meal by volume (volume ratio of about 5:3:2), or 28% wheat bran, 42% alfalfa meal and 30% corn meal by weight (weight ratio of about 2.8:4.2:3).

[0036] A number of different formulations of nutrient sources for black soldier flies are known to those of skill in the art, such as, but not limited to, Gainesville House Fly Diet (Hogsette, *J. Econ. Entomol.* 85:2291-2294, 1992). Some of these formulations are commercially available.

[0037] In general, some of the nutrient source is fermented by hydrating the nutrient source to about 70% moisture (± 2-3%) with water and the activated probiotic culture, and incubating at about room temperature (about 24°C to 27°C) under anaerobic conditions. However, certain aspects of the present disclosure also utilize "dry" nutrient source. As used herein the term "dry nutrient source" means nutrient source to which no additional water or liquid has been added after formulation of the nutrient source.

[0038] III. Production of Black Soldier Fly Larvae

[0039] Black soldier fly eggs are collected in strips of corrugated cardboard, essentially as previously described (Sheppard, *et al.*, *J. Med. Entomol.* 39:695-698, 2002, Tomberlin, *et al.*, *Ann. Entomol. Soc. Am.* 95:379-386, 2002). The eggs are held at about room temperature (about 24°C to 27°C) at between about 50% and about 70% relative humidity, for example at about 50%, about 55%, about 60%, about 65% or about 70% relative humidity, until eclosion. If the relative humidity is too low, the eggs will dry up and die. If the relative humidity is too high, the eggs will not hatch. The neonate larvae are collected at any time after eclosion up to 36 hours after eclosion for addition to the bullet. Larvae are designated as neonate larvae if they have not yet molted.

[0040] IV. Bullet Specifications

[0041] The black soldier fly bullet is prepared in a container that is impermeable to gas and liquids, but with a breathable top that seals the container and allows air to pass into and out of the sealed container. In general the container will comprise a void volume near the top of the container, sufficient to maintain a uniform or constant moisture and temperature throughout the fermented media in the bottom of the container. Failure to maintain a uniform or constant moisture and temperature throughout the fermented media in the bottom of the container can lead to spoilage of the fermented media. Therefore the void volume is sufficient to avoid the presence of microclimates in the container, and in the fermented media.

[0042] A base layer of the probiotic fermented nutrient source is placed in the bottom of the container. Then neonate larvae are placed on top of the fermented nutrient source, and dry (unfermented) nutrient source is placed on top of the larvae. In general, the ratio of fermented nutrient source to dry nutrient source is about 5:1, about 6:1 or about 7:1, depending on the container used to create the bullet. The amount of black soldier neonate larvae added to the bullet can vary somewhat, but in general is about 70, about 75, or about 80 or more neonate larvae per gram of fermented nutrient source.

[0043] The container can generally be of any configuration, for example tapered from narrower at the bottom to wider at the top, tapered from wider at the bottom to narrower at the top, or with straight sides, of any shape, for example generally circular, generally square, or generally oval, and made from any material, such as, but not limited to, plastic, glass or metal, or combinations thereof, so long as the container is impermeable to gas and liquid.

[0044] The bullet with the fermented nutrient source, neonate black soldier fly larvae and dry nutrient source are then stored uncovered for about 2-4 days, for example about 3 days at about room temperature (about 24°C to 27°C) at a relative humidity of about between about 50% and about 70%, for example about 50%, about 55%, about 60%, about 65% or about 70% relative humidity. Then the bullet is covered (sealed) with the breathable lid or top, and stored for up to 6 months or more at about room temperature (about 24°C to 27°C). The present disclosure thus provides for the first time stable young (immature) larvae that can be stored for up to 6 months or more.

[0045] The bullet can be used to inoculate the desired organic material (organic waste for bioremediation, or other organic material for protein production) any time after 5 days of storage. In general, for bioremediation (or bioconversion) of organic waste, a high bullet to feed stock ratio is preferable, while a low bullet to feed stock ration is preferable when the black soldier fly larvae are to be used as a protein source.

EXAMPLES

[0046] The following examples are included to demonstrate preferred embodiments of the present disclosure. It should be appreciated by those of skill in the art that the techniques disclosed in the examples, which follow represent techniques discovered by the inventors to

function well in the practice of the present disclosure, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments, which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the present disclosure.

Example 1: Preparation of Probiotic Culture

[0047] The probiotic culture is prepared by mixing concentrated EM-1® Microbial Inoculant (TeraGanix, Inc., Alto, TX, USA), Golden Barrel Unsulfured Blackstrap Molasses (Zook Molasses Company, Honey Brook, PA, USA), and warm water at a ratio of 1:1:20. Ingredients are combined and kept at room temperature (~27°C), under anaerobic conditions (using an air lock), for at least one week prior to use.

Example 2: Preparation and Fermentation of Feed

[0048] The feed is composed of the Gainesville House fly Diet (Hogsette, *J. Econ. Entomol.* 85:2291-2294, 1992), which is 50% wheat bran, 30% alfalfa meal, and 20% corn meal, by volume, hydrated to approximately 70% moisture. Approximately 22.7 kg of Gainesville diet is mixed with 56 L of water and 150 ml of expanded probiotic culture, and allowed to ferment anaerobically at room temperature (~27°C) for at least 24 hours prior to use.

Example 3: Collection and Incubation of Black Soldier Fly Eggs and Neonates

[0049] Eggs are collected in strips of corrugated cardboard using essentially the method as previously described (Sheppard, *et al.*, *J. Med. Entomol.* 39:695-698, 2002, Tomberlin, *et al.*, *Ann. Entomol. Soc. Am.* 95:379-386, 2002), and held at about 27°C and 55% relative humidity until eclosion. Within 24 hours of eclosion, neonate larvae are harvested and aliquoted for inoculation into the "bullets."

Example 4: Black Soldier Fly Bullet Preparation

[0050] A Black Soldier Fly bullet is prepared by placing 200 g of probiotic fermented Gainesville Diet (at 70% moisture) into a 500 ml container (example shown in FIG. 6). In the container shown in FIG. 6, this is about 4-5 cm of fermented feed at the bottom of the container. Approximately 15,000 neonate larvae less than 24 hours old are placed on top of the diet, using a level, 0.61 ml scoop (1/8 tsp, ~0.2 g). An additional 35 g of dry Gainesville Diet is then placed

on top of the wet diet/larvae (in the container shown in FIG. 6, this is about 1 cm of dry feed). The additional dry feed on top of the larvae serves two purposes: 1) to absorb any excess moisture that accumulates around the edges of the wet diet and prevent neonate drowning; and 2) to provide a dry barrier that prevents neonate larvae from escaping from the container. The bullet is then stored uncovered for 3 days at about 27°C and 55% relative humidity. It is then covered with a breathable lid that allows air to transfer into and out of the container, and can be used for colony production or waste management at a minimum age of 5 to 6 days.

Example 5: Results Using a Black Fly Soldier Bullet

[0051] When Black Soldier Fly Bullets were used to inoculate pans of organic material, the average time from inoculation to harvest was 6.51 days, for data collected from 460 pans. These 460 pans resulted in the digestion of approximately 3.2 metric tons of organic material and the production of approximately 405 kg of live larvae. For 318 of these pans, data was collected on both time to harvest and bullet age, and the mean age of a bullet used for these pans was 14.31 days (FIG. 1).

[0052] Although the average time to harvest was approximately 6.5 days, there was a significantly positive ($p<0.0001$) relationship between time to harvest and pan output (FIG. 2), with pans producing approximately 150 g more larvae when reared for an additional three days. FIG. 3 shows the relationship between bullet age and pan output; there was no significant relationship ($p=0.15$) between these two variables. There was also no significant relationship ($p=0.73$) between bullet age (days) and time from inoculation to harvest (days) (FIG. 4).

[0053] When reared on swine manure, larvae from the presently disclosed bullets develop faster and grow larger when compared to experiments started with 4 day old larvae (FIG. 5). Larval weight peaks on day 6 when using the presently disclosed bullet system, indicating this is the best point at which to harvest the larvae for maximum biomass production. This corresponds well with an embodiment of harvesting larvae at approximately 6.5 days after inoculation into the food resource.

[0054] Although FIG. 2 indicates that allowing the larvae to feed longer increases the total output per pan, the benefit of a 17% increase in production does not outweigh the cost of feeding the larvae for 50% more time, especially from an industrial standpoint where time and space

would be at a premium. During that same amount of time, new pans of larvae could be digesting waste, and would already be in a phase of growing exponentially, as indicated from days 1 to 5 in FIG. 5.

[0055] The lack of a significant relationship between bullet age and output supports the ability of this method to allow for long term storage of larvae that can then be used for organic waste digestion, protein production, or black soldier fly colony maintenance. FIG. 1, FIG. 3 and FIG. 4 also provide support for the ability to use this method to buffer against inconsistent egg production or waste intake for up to 40-60 days or even longer, when the bullets are stored at approximately 22°C.

[0056] When compared to what is currently known about black soldier fly biology and production, the ability to achieve larval harvest in 6.51 days when using the presently disclosed system is a significant improvement over current methods used in both research and industry. For example, in experiments on Gainesville Diet started with 8-day-old larvae, larvae required an average of 13 days of feeding to mature, which is double that achieved when using the presently disclosed Black Soldier Fly Bullet System. A previous study conducted on dairy manure, starting with 4 day old larvae, also recorded long development times, with larvae requiring 29.3-31.5 days to complete development (Myers, *et al.*, *Environ. Entomol.* 37:11-15, 2008). In another study where black soldier fly larvae were reared on mixtures of food processing by-products, larvae required 21-37 days to mature (Oonincx, *et al.*, *PLoS One* 10:20, 2015).

[0057] The term "about" is used herein to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value. The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and to "and/or." When not used in conjunction closed wording in the claims or specifically noted otherwise, the words "a" and "an" denote "one or more."

[0058] The terms "comprise," "have," and "include" are open-ended linking verbs. Any forms or tenses of one or more of these verbs, such as "comprises," "comprising," "has," "having," "includes," and "including," are also open-ended. For example, any method that "comprises," "has" or "includes" one or more steps is not limited to possessing only those one or more steps and also covers other unlisted steps. Similarly, any cell that "comprises," "has" or "includes" one

or more traits is not limited to possessing only those one or more traits and covers other unlisted traits.

[0059] While the disclosure has been described in connection with specific embodiments thereof, it will be understood that the present disclosure is capable of further modifications by one of skill in the art. It is to be understood that, unless otherwise indicated, the present disclosure is not limited to particular materials, reagents, reaction materials, manufacturing processes, or the like, as such can vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting. It is also possible in the present disclosure that steps can be executed in different sequence where this is logically possible. The present disclosure is therefore intended to encompass any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features herein before set forth.

[0060] All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this disclosure have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

[0061] All publications, patents, and patent publications cited are incorporated by reference herein in their entirety for all purposes.

What Is Claimed Is:

Claim 1. A composition for storage of black soldier fly larvae, comprising:

a) a container;

b) a layer of fermented nutrient source in the bottom of the container;

c) black soldier fly larvae on top of the layer of fermented nutrient source;

d) a layer of dry nutrient source on top of the black soldier fly larvae; and

e) a cover on top of the container that allows air to pass into and out of the container.

Claim 2. The composition of claim 1, wherein the container is about 500 ml in size.

Claim 3. The composition of claim 1, wherein the container has tapered sides.

Claim 4. The composition of claim 1, wherein the container has straight sides.

Claim 5. The composition of claim 1, wherein the container has a void volume above the layer of dry nutrient source sufficient to maintain a constant moisture and temperature in the layer of fermented nutrient source.

Claim 6. The composition of claim 1, wherein the container is impermeable to gas or liquid.

Claim 7. The composition of claim 1, wherein the container is comprised of glass, plastic or metal.

Claim 8. The composition of claim 1, wherein the nutrient source is about 50% wheat bran, 30% alfalfa meal and 20% corn meal by volume.

Claim 9. The composition of claim 1, wherein the fermented nutrient source is produced using a lactic acid bacteria to ferment the nutrient source.

Claim 10. The composition of claim 9, wherein the fermented nutrient source is produced using a plurality of species of lactic acid bacteria to ferment the nutrient source.

Claim 11. The composition of claim 9, wherein the lactic acid bacteria is selected from the group consisting of *Bifidobacterium animalis*, *Bifidobacterium bifidum*, *Bifidobacteriumlongum, Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii* subsp *bulgaricus*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactococcus lactis* subsp *lactis* and *Streptococcus thermophilus*.

Claim 12. The composition of claim 9, wherein the nutrient source is fermented for at least 24 hours.

Claim 13. The composition of claim 9, wherein the nutrient source is fermented anaerobically.

Claim 14. The composition of claim 9, wherein the nutrient source is fermented at about 70% moisture.

Claim 15. The composition of claim 9, wherein the nutrient source is fermented at about 27°C.

Claim 16. The composition of claim 1, wherein the lactic acid bacteria is activated using molasses and water.

Claim 17. The composition of claim 16, wherein the ratio of lactic acid bacteria to molasses to water is about 1:1:20.

Claim 18. The composition of claim 16, wherein the lactic acid bacteria is activated for at least one week.

Claim 19. The composition of claim 16, wherein the lactic acid bacteria is activated anaerobically.

Claim 20. The composition of claim 1, wherein about 15,000 black soldier fly larvae are used on top of the layer of fermented nutrient source.

Claim 21. The composition of claim 1, wherein the black soldier fly larvae are between eclosion and 36 hours of eclosion.

Claim 22. The composition of claim 1, wherein the black soldier fly neonate larvae have not molted.

Claim 23. A method of storing black soldier fly larvae, comprising placing black soldier fly larvae between a layer of fermented nutrient source and a layer of dry nutrient source in a container with a cover that allows air to pass into and out of the container.

Claim 24. The method of claim 23, wherein the black soldier fly larvae are stored for between about 2 weeks and about 5 months.

Claim 25. The method of claim 24, wherein the black soldier fly larvae are stored for between about 4 weeks and about 6 weeks.

Claim 26. The method of claim 23, wherein the black soldier fly larvae are stored at about 27°C.

Claim 27. A method of producing black soldier fly larvae, comprising:

a) inoculating organic material with a composition comprising:

i) a fermented nutrient source;

ii) black soldier fly larvae; and

iii) a dry nutrient source;

b) incubating the organic material for about 6 days; and

c) collecting the black soldier fly larvae produced.

Claim 28. A method of digesting organic waste, comprising:

a) inoculating organic waste with a composition comprising:

i) a fermented nutrient source;

ii) black soldier fly larvae; and

iii) a dry nutrient source; and

b) incubating the organic waste for about 6 days.

Claim 29. A media comprising a nutrient source and a probiotic culture comprising at least a first lactobacillus.

Claim 30. The media of claim 29, wherein the nutrient source comprises wheat bran, alfalfa meal and corn meal in a volume ratio of about 5:3:2.

Claim 31. The media of claim 29, wherein the nutrient source and the probiotic culture are allowed to ferment for at least 24 hours under anaerobic conditions prior to use.

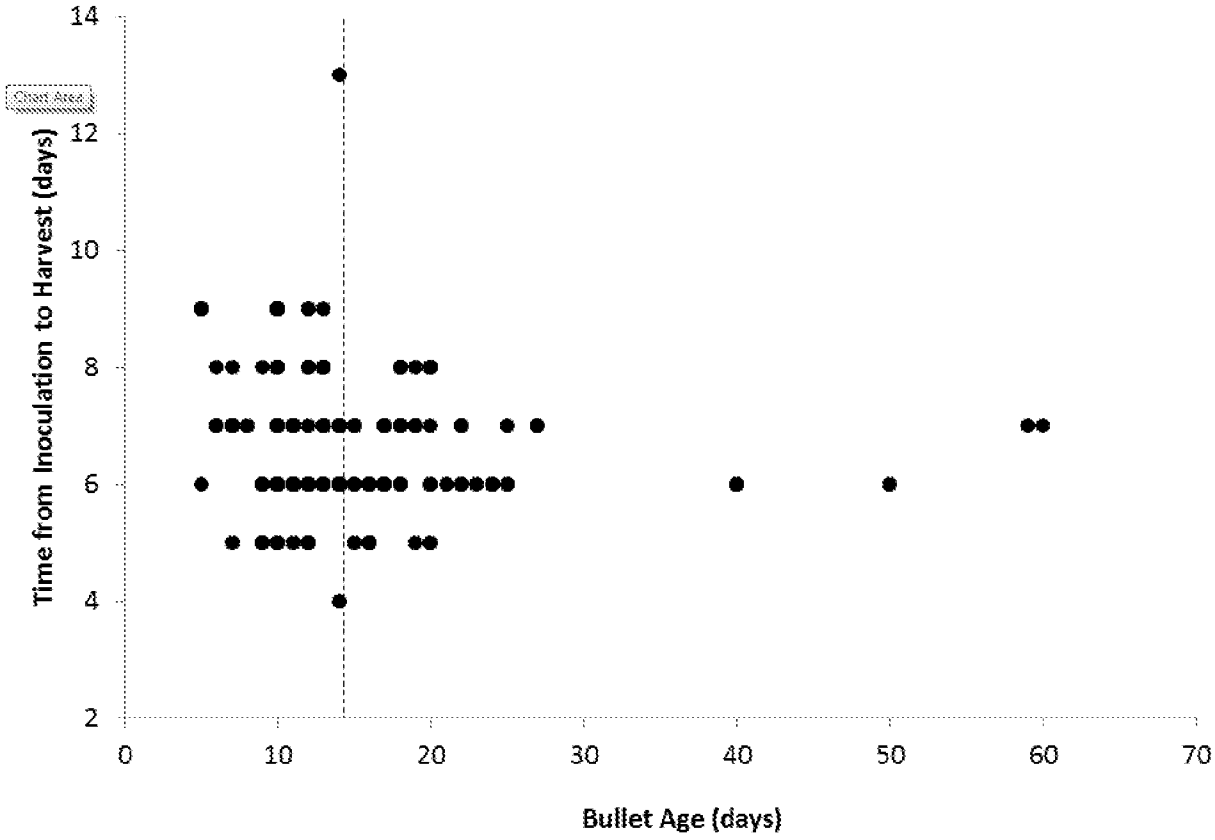

Time from Inoculation to Harvest (days)
14
12
10
8
6
4
2
0
10
20
30
40
50
60
70
Bullet Age (days)

FIG. 1

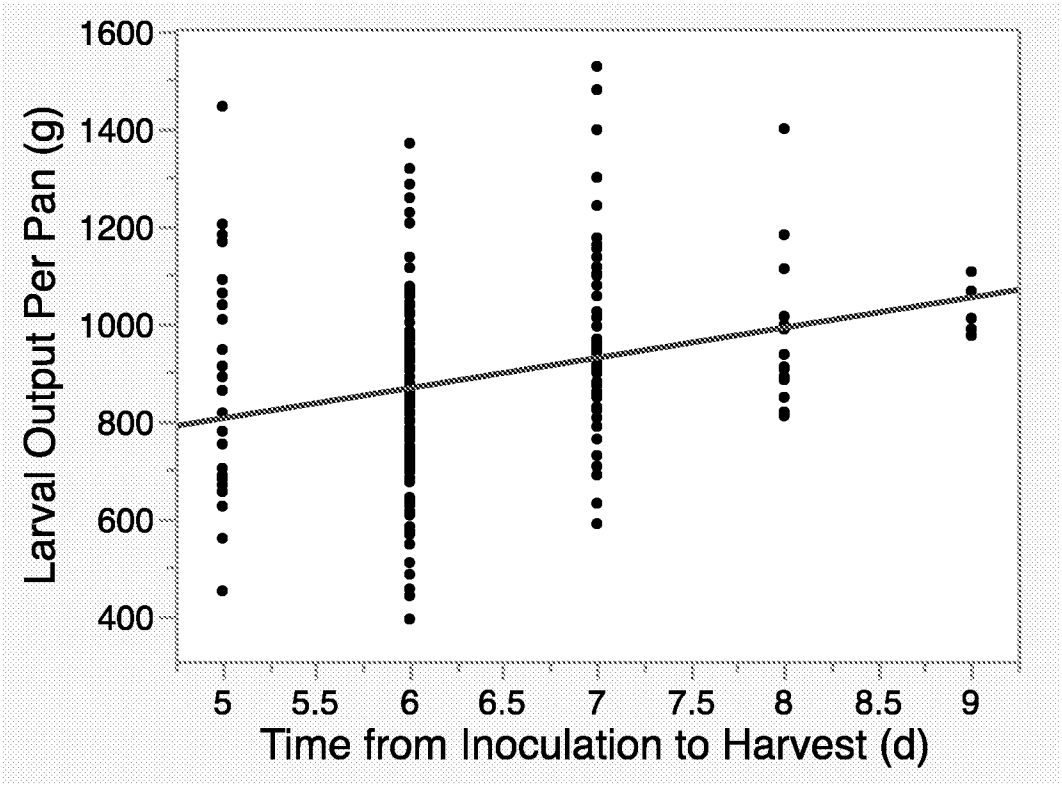

Larval Output Per Pan (g)
1600
1400
1200
1000
800
600
400
5
5.5
6
6.5
7
7.5
8
8.5
9
Time from Inoculation to Harvest (d)

FIG. 2

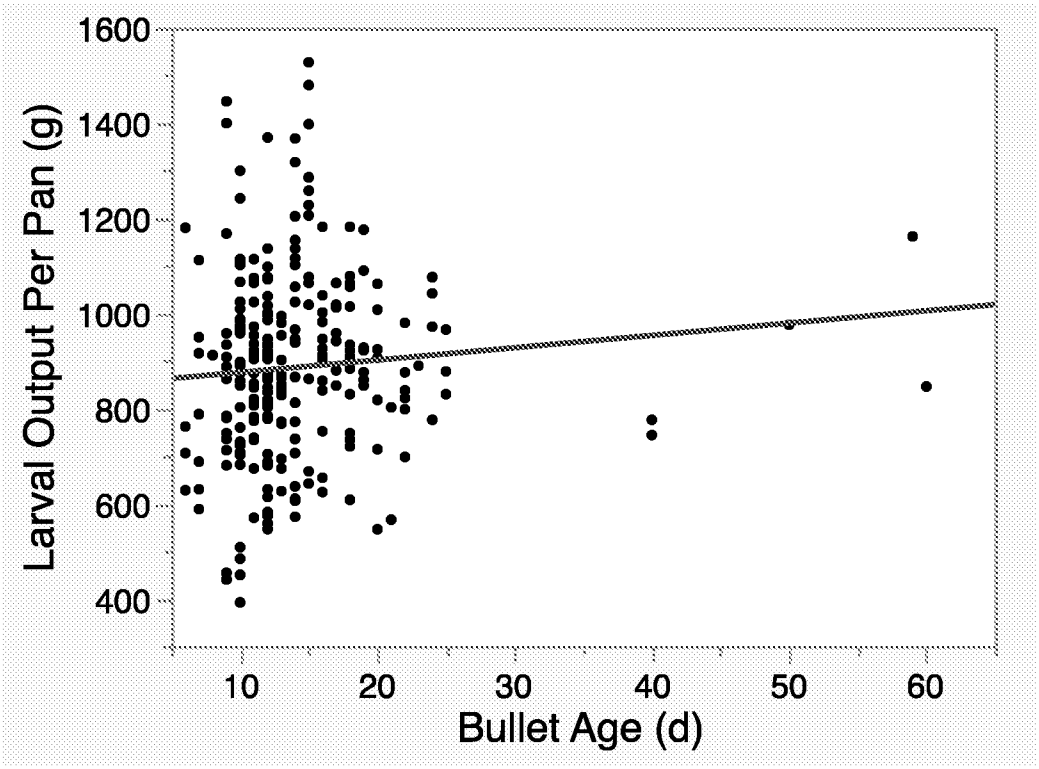

1600
1400
1200
1000
800
600
400
Larval Output Per Pan (g)
10
20
30
40
50
60
Bullet Age (d)

FIG. 3

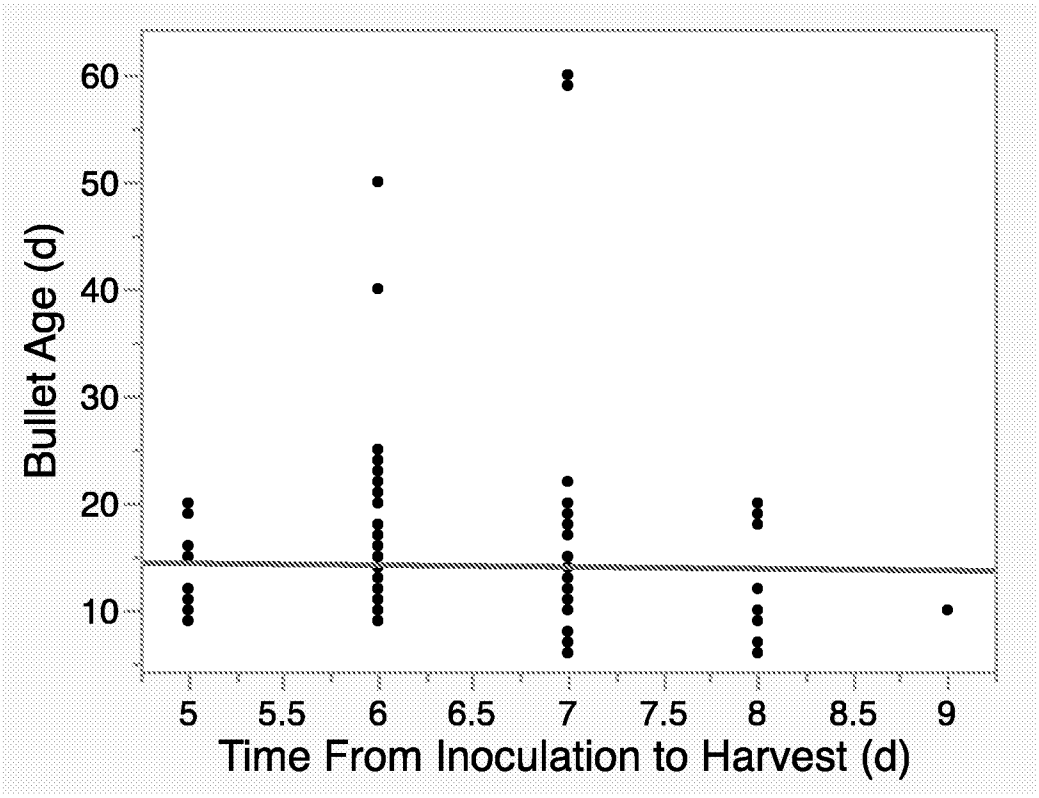

Bullet Age (d)
60
50
40
30
20
10
5
5.5
6
6.5
7
7.5
8
8.5
9
Time From Inoculation to Harvest (d)

FIG. 4

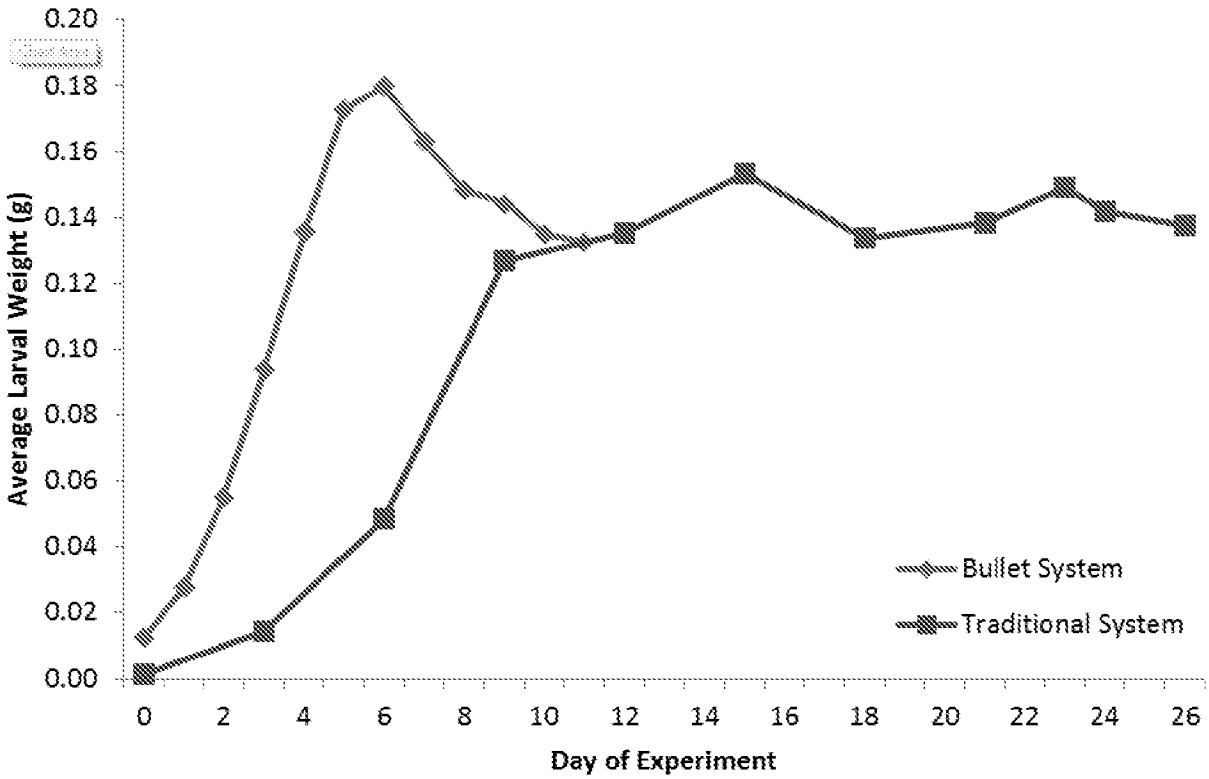

0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Average Larval Weight (g)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Day of Experiment
Bullet System
Traditional System

FIG. 5

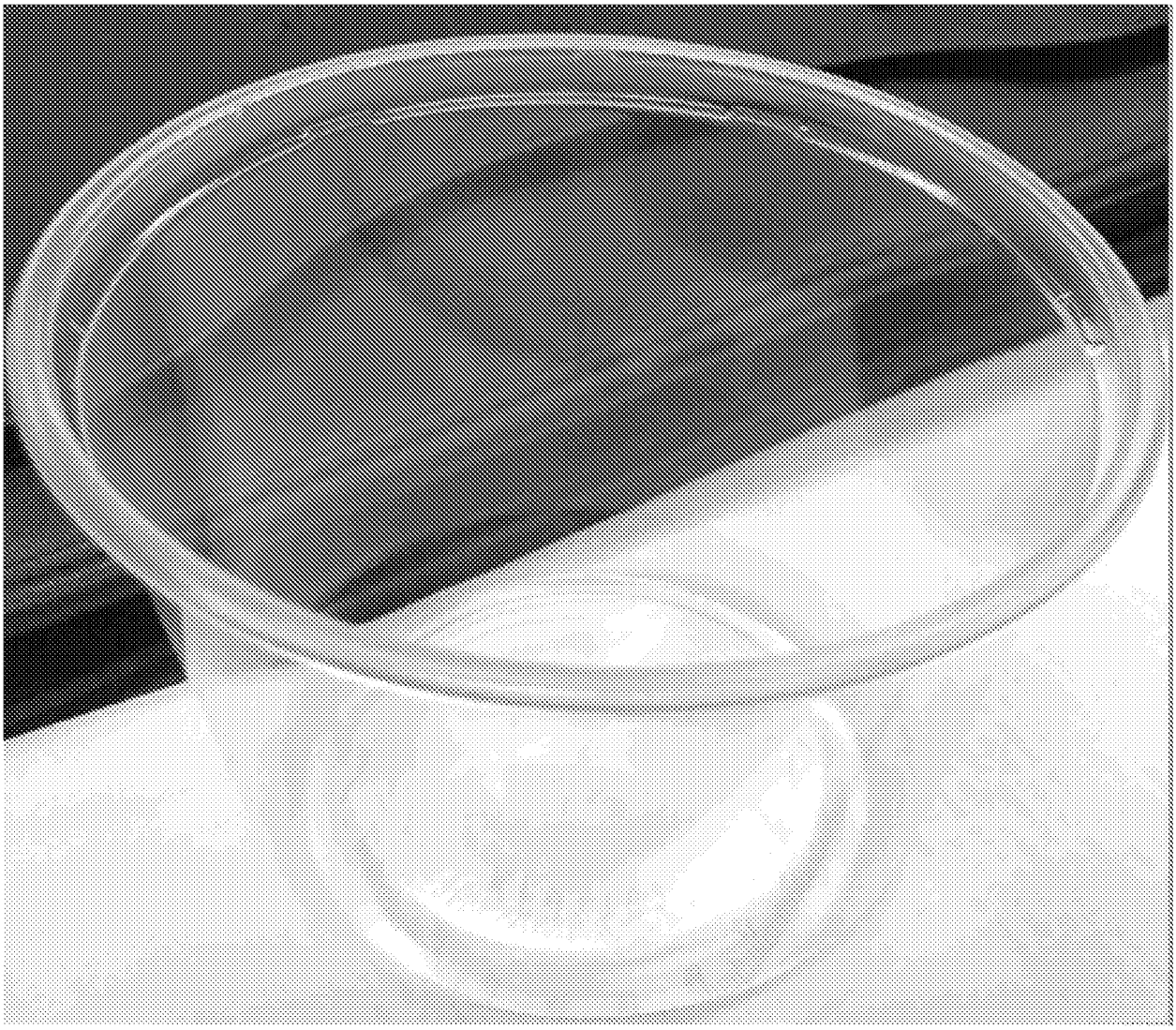


FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/27252

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A01K 67/033, 67/04; A23K 50/90, 10/00, 10/10, 10/12, 10/30, 10/33, 10/37 (2018.01)

CPC - A01K 67/033, 67/04; A23K 50/90, 10/00, 10/10, 10/12, 10/30, 10/33, 10/37

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)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	(BRITS, D) Improving feeding efficiencies of black soldier fly larvae, Hermetia illucens (L., 1758) (Diptera: Stratiomyidae: Hermetiinae) through manipulation of feeding conditions for industrial mass rearing. March 2017. [Retrieved from the internet on 21 June 2018]. <URL: http://ir.nrf.ac.za/bitstream/handle/10907/1248/brits_improving_2017.pdf?sequence=1&isAllowed=y>; pages 49-50, 88, 112-113, 119	1, 6-7, 20-24, 26 --- 2-5, 8-19, 25
Y	(SHEPPARD, DC et al.) Rearing Methods for the Black Soldier Fly (Diptera: Stratiomyidae). Journal of Medical Entomology, Vol,. 29, No. 4, pp. 695-698, 10 July 2002; page 696	2
Y	US 2016/0219887 A1 (ENTERRA FEED CORPORATION) 04 August 2016; figure 1; paragraphs [0056], [0062], [0064]	3, 25
Y	(HOGSETTE, JA) New Diets for Production of House Flies and Stable Flies (Diptera: Muscidae) in the Laboratory. Journal of economic entomology, Vol. 85, No. 6, pp. 2291-2294, 01 December 1992; pages 2292-2294	4, 8
Y	CN 103,548,780 B (CHONGQING ACADEMY CHINESE MED) 13 January 2016; see machine translation; abstract; claim 4	5
Y	CN 106,359,850 A (GUO SHUYANG) 01 February 2017; see machine translation; abstract; claim 1; paragraphs [0004]-[0005]	9, 11-15
Y	WO 2016/024910 A1 (CONCELLAE AB) 18 February 2016; abstract; page 5, lines 20-22; page 8, lines 15-23; page 14, lines 18-20; page 15, lines 6-1	9-10

☒ 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 but 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	Date of mailing of the international search report
21 June 2018 (21.06.2018)	13 AUG 2018
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/27252

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	(ZIMMERMAN, I et al.) Overview of variety of trials on agricultural applications of effective microorganisms = em. AGRICOLA, pp. 17-26, 2008; pages 17-18	16-19
Y	CN 105,851,719 A (YINGSHANG COUNTY NONGBO AGRICULTURE DEV CO LTD) 17 August 2016; see machine translation; abstract; paragraphs [0002], [0038], [0041]	13-15
Y	(KOK, FS) Effects of pH and temperature on the growth and β-glucosidase activity of Lactobacillus rhamnosus NRRL 442 in anaerobic fermentation. International Review of Chemical Engineering, Vol. 4, No. 3, pp. 293-299, May 2012; page 293	19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/27252

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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

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

2. ☐ Claims 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. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-26 are directed toward a composition and a method for storing black soldier fly larvae.
Group II: Claims 27-28 are directed towards a method of producing black larvae.
Group III: Claims 29-31 is directed towards a media having a first lactobacillus.

-***-Continued Within the Next Supplemental Box-***-

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 additional fees, this Authority did not invite payment of additional fees.

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.:
1-26

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/27252

-***-Continued from Box III: Observations where unity of invention is lacking-***-

The special technical features of Group I include placing black soldier fly larvae between a layer of fermented nutrient source and a layer of dry nutrient source in a container with a cover that allows air to pass into and out of the container, which are not present in Groups II-III; the special technical features of Group II include p a) inoculating organic material with a composition; and b) incubating the organic waste for about 6 days, which are not present in Groups I and III; and the special technical features of Group III include a media comprising a probiotic culture comprising at least a first lactobacillus, which are not present in Groups I-II.

The common technical feature between Groups I-III is a nutrient source.

The common technical feature is disclosed by US 8,815,539 B1 to River Road Research, Inc. (hereinafter "Popa).
Popa discloses a nutrient source (a source of nutrients; abstract).

Since the common technical feature is previously disclosed by Popa, these common features are not special and so Groups I-III lack unity.