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(54) Title: BROTH COMPOSITIONS AND THEIR USE AS PREBIOTICS

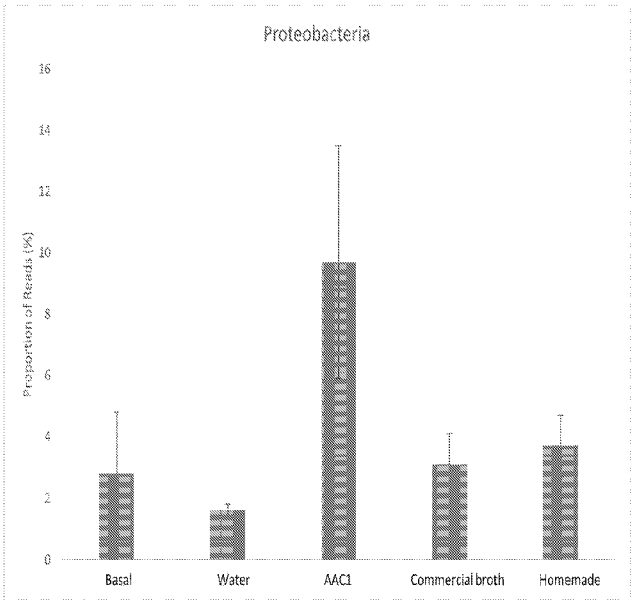

FIG. 5

(57) Abstract: Broth compositions prepared from poultry are shown to act as prebiotics by enhancing the probiotic microbiota of an individual. Methods of preparing these broth compositions from selected poultry raw materials and methods of using the broth compositions are disclosed.

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BROTH COMPOSITIONS AND THEIR USE AS PREBIOTICS

RELATED APPLICATION

[0001] This application claims priority to U.S. Patent application 62/108,008, filed January 26, 2015, the entire content of which is hereby incorporated by reference into this application.

BACKGROUND

1. Field of the Invention

[0002] This disclosure relates to the field of nutritional and dietary supplement as well as the general field of microbiology.

2. Description of Related Art

[0003] Microorganisms are known to colonize the gastrointestinal tract of mammals. Some microorganisms have certain benefits for the host. These beneficial microorganisms are also known as "probiotic microorganisms" or "probiotics." Beneficial bacteria (or "good" bacteria) are also referred to as "probiotic bacteria." Some probiotic microorganisms may exist naturally in the gut of the host. Others may be introduced into the host by dietary supplement.

[0004] The term "prebiotic" generally refers to substances that induce the growth (and hence the live count or CFU) or activity of probiotic microorganisms (*e.g.*, bacteria or fungi). However, most, if not all prebiotics are carbohydrate based and may contain gluten from various plant sources.

SUMMARY

[0010] The present disclosure advances the art by providing a composition prepared from poultry that may be used as a prebiotic for enhancing the microbiome of an individual. More particularly, in one embodiment, the disclosed prebiotic composition is gluten-free.

[0011] In one embodiment, the composition may be prepared from poultry, such as chicken, turkey, or other birds. In another embodiment, the disclosed composition may be prepared using parts from other animal sources. In another embodiment, the disclosed composition may be in the form of broth, stock, extract, or powder.

[0012] In another embodiment, methods are disclosed for enhancing one or more probiotic bacteria in an individual by administering to the individual an effective amount of a composition prepared from poultry parts. In one aspect, the effective amount is an amount effective in enhancing at least one probiotic bacterium of the individual. In another aspect, enhancing a probiotic bacterium means improving the well-being or increasing the live count (i.e., colony-forming unit or CFU) of the probiotic bacterium in the gastrointestinal tract (or gut) of the individual. The CFU of a bacterium in the individual may be measured directly or indirectly. Indirect measurements may include but are not limited to measuring the CFU of the bacterium in fecal samples from the individual. In one aspect, the method of metagenomic sequence counts may be used to measure the CFU of a bacterium in a sample.

[0013] In another embodiment, the effective amount to be administered may be an amount of the broth composition that increases the live count of at least one probiotic bacterium in the gut of the individual by 2-200 folds, by 5-200 folds, by 10-200 folds, by 20-200 folds, or by about 200-folds, after the composition is administered to the individual. In another aspect, the effective amount may be an amount of the broth composition that increases the proportion (or percentage) of at least one probiotic bacterium (i.e., selectively increases the count of at least one beneficial probiotic bacterium) in the gut microbiome of the individual. In another aspect, the effective amount may be an amount of the broth composition that increases the diversity of the gut microbiome in the individual.

[0014] In one embodiment, the disclosed broth composition may be administered to the individual consecutively at the effective amount every day for 1 day, 2, 3, 4, 5, 6, 7 days, or 14 or more days. In another embodiment, the composition may be administered to the individual non-consecutively, for example, every other day over a period of 4, 6, 8, 10, 12, 14 or more days.

[0015] In one embodiment, the compositions prepared according to the disclosed methods may have higher concentration of certain beneficial components. In one aspect, the compositions may contain soluble collagen fibers. For purpose of this disclosure, the term "soluble collagen fiber" is used to refer to fragments of collagen protein that are soluble in a broth after being subject to the broth-making process. In one aspect, the fragments may represent full-length or partial-length of the collagen protein. In another aspect, the fragments may contain small peptides or single amino acids derived

from the collagen. In another aspect, at least 20%, 30%, 40%, or at least 50% of the soluble collagen fibers contains fragments or peptides of 20 or more amino acids long. In another aspect, the soluble collagen fibers may act as prebiotics in enhancing or promoting the probiotic bacteria in the individual. In another aspect, the soluble collagen fibers are primarily derived from the cartilage of poultry. In another aspect, the soluble collagen fibers constitute at least 20% (w/w on solid basis) of the final broth composition. In another aspect, the soluble collagen fibers may constitute at least 30%, 40%, 50%, 60%, 70%, 80%, or at least 90% (w/w on solid basis), or as high as 100% of the final broth composition. In another aspect, the soluble collagen fibers may be derived from Type I, II or III collagen.

[0016] In another embodiment, certain beneficial compounds may be enriched in the disclosed compositions to a greater extent as compared to other broth products currently available on the market.

[0017] In another embodiment, one or more ingredients may be supplemented in the broth to achieve certain benefits that are not generally associated with chicken broth. Examples of such supplements may include but are not limited to ginger, coffee extract, ginseng, green tea, other botanicals such as willow bark or boswellia, curcumin/turmeric, omega-3 fatty acids, fish oil, krill oil, algal oil, Pycnogenol French maritime pine bark extract, grape seed extract, flax seed extract, ribonuclease, angiogenin, lactoferrin, ribonuclease-enriched lactoferrin, S-adenosyl methionine, collagen, collagen proteins (e.g., Type I, II or III collagens) or collagen peptides, gelatin, avocado/soybean unsaponifiables (ASU), extract of hops cones, egg shell membrane, polypeptides derived from milk, such as casein or whey, MSM (Methylsulfonylmethane), Yucca, Devils claw, Bromelain, glutamic acid, cocoa, stinging nettle, Vitamin E, Vitamin D3, walnut extract, etc. In one aspect, the disclosed broth may contain significant amount of collagen, collagen proteins and/or collagen peptides. In another aspect, significant amount of the collagen, collagen proteins and/or collagen peptides may be present naturally in the disclosed broth. In another aspect, significant amount of the collagen, collagen proteins and/or collagen peptides may be added into the disclosed broth as a supplement.

[0018] In another embodiment, according to the disclosed methods, an individual who is in need of increased count of a probiotic bacterium is first identified before the broth composition is administered to the individual. Such individuals who are in need of the disclosed broth composition may include but are not limited to those who

have unbalanced microbiome in their GI system, and those who need higher number (CFU) of certain desirable probiotic bacteria, such as lactic acid producing bacteria. Examples of such desirable bacteria may include but are not limited to *Lactobacillus, Parasutterella*, *Morganella*, *Oscillospira*, *Coprococcus*, *Oscillibacter*, *Odoribacter*, *Gastranaerophilales*, *Allabaculum*, or *Faecalibacterium*. In another embodiment, one example of desirable probiotic bacteria may be *Lactobacillus rhamnosus*. In another embodiment, the broth compositions provide not only proteins (and amino acids) as nutrients, but also proteins (and amino acids) in the form of collagen fibers which help increase the number and percentage of probiotic bacteria in the gut microbiome of the individual. Thus, in one aspect of the present disclosure, administration of the disclosed broth compositions may eliminate the need for continuous supplementation of probiotics to an individual who would otherwise have to take probiotic supplement regularly. The term "regularly" means at least once a month, once a week or at least once a day.

[0019] For purpose of this disclosure, the term "enhance" means increase the growth (and hence the live count or CFU) or activity of a microorganism. In one embodiment, the disclosed composition may enhance one or more probiotic bacteria that are native in an individual. The term "native" means the microorganism naturally exist in the gut of the individual. In another embodiment, the disclosed composition may enhance one or more probiotic bacteria that are not native in an individual. Both native or non-native probiotic bacteria may be supplemented to an individual.

[0020] In one aspect, the disclosed composition may be administered to the individual by oral feeding. In another aspect, the disclosed composition may be administered in conjunction with one or more probiotic bacteria. In another aspect, the disclosed composition may be mixed with one or more probiotic bacteria and administered to an individual as a mixture.

[0021] In another aspect, the prebiotic composition may be prepared according to the process disclosed herein and then be prepared into a liquid or solid (e.g., powder) form to be taken as a dietary supplement.

[0022] In one embodiment, the one or more probiotic bacteria that are enhanced by the disclosed composition may include at least a lactic acid producing bacterium. In another embodiment, the lactic acid producing bacteria may include, by way of example, a *Lactobacillus* bacterium. In another embodiment, the lactic acid producing bacteria may include *Lactobacillus rhamnosus*.

[0023] In home-style cooking, chicken or turkey broths may be made by cooking for extended period of time in an open vessel or under high temperature in a pressurized vessel. By contrast, this disclosure provides broth products having unique composition and methods of preparing the same. Also disclosed here are methods of selecting and preparing raw materials, cooking raw materials, and separating and purifying the resultant broths.

[0024] In one embodiment, raw materials from poultry or other animal sources may be comminuted to a great extent to maximize extraction of various beneficial compounds. In one aspect, the raw materials may be reduced to a size of less than about 2cm, 1 cm, 5mm, 4mm, 3mm, 2mm, or less than 2mm. Mechanical processing of the poultry parts to small sized particles prior to cooking may help maximizing extraction of certain compounds. In another embodiment, because the poultry parts have been mechanically processed to very small-sized particles prior to cooking, more gentle cooking condition (e.g., at a temperature of 60-100°C) and shorter cooking time (from about 8 minutes to 300 minutes) may be used to obtain the broth from the processed poultry parts. Such gentler and shorter cooking may help prevent inactivation of certain compounds that would be otherwise inactivated if conventional processing and cooking methods are used. Preservation of such compounds may explain the superior benefits observed when the disclosed broths are tested against other broth products that are not prepared according to the instantly disclosed methods.

[0025] In another embodiment, poultry parts with bones and cartilage may be mechanically separated and comminuted to fine pieces of less than 5mm (millimeter), 4mm, 3mm, 2mm, or 1 mm in size. In one aspect, no steps are taken to remove the residual meat from the bones. These small pieces may be cooked at about 70°C, 100°C, 110°C, 120°C, or as high as 150°C, for at least 8 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, or 6 hours or longer to maximize the extraction of certain broth fraction and/or compounds.

[0026] In another embodiment, the broth prepared according to the disclosed methods show different protein profiles from those obtained from other commercial broth products when analyzed by SDS-PAGE. In one aspect, at least 10%, 20%, 30%, 40%, 50%, 70%, or 90% of the total proteins in the disclosed compositions have a molecular weight of between 10 kD (kilo-Dalton) and 70 kD. In another aspect, at least 95% of the proteins in the disclosed compositions have a molecular weight of less than 100 kD. In

another aspect, the methods may be used to further reduce molecular weight of the proteins in the broth to less than 10 kD, 5 kD, or even lower than 3 KD to enhance assimilation by a subject. By way of example, one or more enzymes that digest proteins may be used to reduce the molecular weight of the proteins in the broth.

[0027] In another embodiment, the disclosed composition may contain at least 40% protein by weight of total solids. By way of example, the disclosed composition may contain 50%, 60%, 70%, or 80% or more protein by weight of total solids in the composition. In another embodiment, the disclosed composition may contain less than 10% carbohydrate by weight of total solids in the composition. By way of example, the disclosed composition may contain 8%, 5%, 2%, or 1% or less carbohydrate by weight of total solids in the composition. In another embodiment, the disclosed composition is prepared from an animal source (e.g., poultry) and does not contain any proteins from a plant source. In another embodiment, the disclosed prebiotic composition does not contain detectable amount of gluten. In another embodiment, the disclosed prebiotic composition does not contain significant amount of gluten that would cause adverse reaction in an individual sensitive or allergic to gluten.

[0028] In another embodiment, the disclosed composition may contain the amino acids proline and histidine, wherein the ratio between proline and histidine is at least 4:1 by weight. In one aspect, the proline is present in the composition by at least 8% (w/w), 10% or greater on solid basis. In another aspect, the ratio between the proline and the histidine is at least 6:1 by weight.

[0029] In another embodiment, the disclosed composition may contain the amino acids glycine and histidine, wherein the ratio between the glycine and histidine is at least 6:1 by weight. In one aspect, the glycine is present in the total amino acids of the disclosed compositions by at least 12%, 14% or greater (w/w) on solid basis. In another aspect, the ratio between the glycine and the histidine is at least 10:1 by weight.

[0030] In another embodiment, the disclosed composition may contain the amino acids hydroxyproline and histidine, wherein the ratio between the hydroxyproline and histidine is at least 4:1 by weight. In one aspect, the hydroxyproline is present in the total amino acids of the compositions by at least 7%, 8% or greater (w/w) on solid basis. In another aspect, the ratio between the hydroxyproline and the histidine is at least 6:1 by weight.

[0031] In another embodiment, the disclosed composition may contain proline, glycine, hydroxyproline and histidine. In one aspect, the ratio between the glycine and the histidine is at least 6:1 by weight. In another aspect, the glycine is present in the composition by at least 12% (w/w) on solid basis. In another aspect, the ratio between the proline and the histidine is at least 4:1 by weight. In another aspect, the ratio between the hydroxyproline and the histidine is at least 6:1 by weight.

[0032] In another embodiment, the disclosed composition may contain proline and other amino acids, and the amount of the proline is at least 8%, 10% or greater by weight of the total amino acids in the composition. In another embodiment, the disclosed composition may contain glycine and other amino acids, and the amount of the glycine is at least 20% by weight of the total amino acids in the composition. In another embodiment, the disclosed composition may contain hydroxyproline and other amino acids, and the amount of the hydroxyproline is at least 8% by weight of the total amino acids in the composition.

[0033] In another embodiment, the disclosed composition may contain one or more branched chain amino acids (BCAA) (e.g., valine, leucine and isoleucine), and the BCAAs in the composition are present at a higher level than the level of BCAAs in other broth products. In one aspect, the amount of total BCAAs, including valine, leucine and isoleucine, is at least 5%, 6%, or 7% by weight of total amino acids in the composition.

[0034] In another embodiment, the broth products prepared according to this disclosure may have a moisture protein ratio (MPR) of between 999:1 and 1:999, or between 200:1 and 10:1. By way of example, a MPR of 200:1 means the product contains 200 parts water and 1 part meat (protein). In another embodiment, the broth products prepared according to this disclosure may have a moisture protein ratio (MPR) of between 150:1 and 40:1, or between 135:1 and 67:1.

[0035] Chondroitin sulfate (CS) is rich in cartilage and has been reported to be beneficial for joint health. The selection of raw materials and the process by which the broth is prepared may contribute to the relatively higher levels of chondroitin sulfate in the disclosed composition. In one embodiment of the present disclosure, the broth composition may contain at least 6%, 7%, 8%, or 10% of chondroitin sulfate by weight of total dry solids in the composition, as measured by using enzymatic digestion and LC-UV detection assay. *See e.g.*, Ji et al., Journal of AOAC International, Vol. 90, No. 3, 659-69 (2007), which is hereby incorporated by reference into this disclosure.

[0036] In one aspect, the composition may be in a liquid form ready to be consumed or it may be in a concentrated liquid form such as a stock. In another aspect, the composition may be in a solid form, such as a powder or a paste.

[0037] In one embodiment, the composition may contain one or more polyphenols, wherein the concentration of the polyphenols is at least 4,000 µg/ml GAE (Gallic Acid Equivalent) based on Folin-Ciocalteu assay.

[0038] In another embodiment, the composition may comprise amino acids glycine, proline and hydroxyproline, and wherein glycine constitutes at least 12% (w/w), proline constitutes at least 8% (w/w), and hydroxyproline constitutes at least 7% (w/w) of total amino acids of the composition.

[0039] Probiotic bacteria are typically grown on culture media before they are harvested and used as dietary supplement. In one embodiment, the disclosed prebiotic composition may be used for culturing a probiotic bacterium. Probiotic bacteria may be grown on a medium containing, among others, the disclosed prebiotic composition prepared from poultry. In one aspect, the disclosed prebiotic composition contains soluble collagen fiber prepared from poultry.

[0040] The broth compositions disclosed herein may be characterized by the unique composition as described herein. The broth compositions may also be characterized by the methods by which they are prepared. Moreover, the disclosed compositions are also unique in the benefits they may provide to a subject. Subjects may be divided into two groups, one group ingests (or consumes) an effective amount of the disclosed broth product each day, while the other group ingests water, or other broth products. The various indicators may then be measured and compared between the two groups.

BRIEF DESCRIPTION OF THE DRAWINGS

[0041] Figure 1 shows the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram from fecal samples.

[0042] Figure 2 shows the UPGMA dendrogram from caecal samples.

[0043] Figure 3 shows DGGE (Denaturing Gradient Gel Electrophoresis) profile generated for the selection of DNA bands representative of species influenced by the addition of AAC1 into the diet. Numbered bands indicate potential regions of interest. Lanes are as follows: (1) Caecal – Water; (2) Caecal – AAC1; (3) Caecal –

Commercial product; (4) Caecal – Homemade; (5) Fecal – Water; (6) Fecal – AAC1; (7) Fecal – Commercial product.

[0044] Figure 4 shows that the different chicken broth products do not significantly alter the Bacteroidetes : Firmicutes ratio.

[0045] Figure 5 shows that AAC1 selects for *Proteobacteria* in the lower GI tract.

[0046] Figure 6 shows that AAC1 selects for or maintains *Lactobacillus* in the lower GI tract.

[0047] Figure 7 shows that AAC1 selects for or maintains *Lactobacillus* in the cecum.

[0048] Figure 8 shows that AAC1 increases the diversity of microbiota in the gut.

DETAILED DESCRIPTION

[0049] Chicken soup and chicken broth have been available for human consumption for centuries. Studies have shown various benefits of chicken soup or broth. According to conventional home-style method, chicken soup or broth is prepared by boiling whole chicken or large chicken parts in a pot of water for an extended period of time. The soup or broth prepared according to this home-style method may not have maximized the health promoting effects of the soup or broth because some of the compounds may be lost during cooking or during processing, while other compounds may not have been extracted from the chicken parts.

[0050] In one embodiment, this disclosure provides methods of using chicken broth as a prebiotic. Prebiotics differ from probiotics (i.e., so called "good bacteria") in that prebiotics are not live organisms but are ingredients that can support or enhance probiotics. Prebiotics typically bypass the upper digestive system and reach the intestine intact after ingestion. It is also possible that prebiotics may influence bacteria in the upper GI section such that these bacteria in the upper GI section begin to ferment nutrients, which, in turn, provide metabolites that selectively support the "good bacteria" in the lower section of the gut. Once they reach the intestine, prebiotics may play an important role in fermentation by the normal gut microbiota. The fermentation process may stimulate and promote the proliferation of the “good bacteria,” such as *Lactobacillus*.

[0051] As used herein, the term "probiotic" may refer to a number of microorganisms which can be taken by an individual as a supplement to benefit the individual. The term "probiotic" may also refer to a number of different microorganisms that naturally live in the body of a host and provide benefit to the host. Examples of such desirable bacteria may include but are not limited to *Lactobacillus, Parasutterella*, *Morganella*, *Oscillospira*, *Coprococcus*, *Oscillibacter*, *Odoribacter*, *Gastranaerophilales*, *Allabaculum*, or *Faecalibacterium*. For instance, many *Lactobacillus* bacteria have been shown to be beneficial to human. *Faecalibacterium* bacteria have also been shown to be anti-inflammatory. See "Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients." Proceedings of the National Academy of Sciences of the United States of America. 105(43):16731-6 (2008).

[0052] The disclosed methods may help preserve certain components by processing chicken parts prior to cooking and by controlling the processing temperature and cooking time to maximize the extraction of beneficial compounds while, concomitantly, minimizing loss of activity due to harsh processing conditions.

[0053] Specially selected raw materials from poultry may be processed according to the disclosed methods to obtain a broth having high protein content. Certain amino acids are present in relatively higher concentration as compared to a home-made broth or other commercial products. In one aspect, as shown in various animal studies described herein, the disclosed broth compositions may prove effective in enhancing the well-being of probiotics of the host. In another aspect, the disclosed broth compositions may increase the live count of probiotics in the host. In another aspect, the disclosed broth compositions may selectively enhance certain microorganisms, but not others. In another aspect, the disclosed broth compositions may increase the diversity of microorganisms in the gut of the host.

[0054] The terms "broth" and "soup" refer to a liquid composition containing at least one solute and may also be used to refer to a ready to serve form, a concentrate, a stock in either liquid or solid form.

[0055] The poultry broth of the disclosure may contain a significant amount of different amino acids. Such amino acids may be present in the form of a protein or as free amino acids in the broth. Total amino acids include both amino acids present in the form of proteins and those present as free amino acids. For purpose of this disclosure, the

ratio of different amino acids in a broth composition refers to the ratio between total amino acids.

[0056] The term "dry solid" or "solid" as used herein refers to the components of a liquid composition that remain after all free liquid is removed from the liquid composition. In the case of an aqueous broth, the free liquid is water.

[0057] The term "administer" means delivering of a material to an individual, such as by oral ingestion.

[0058] The term "not detectable" means a chemical or ingredient that may be present in such a small amount that it cannot be measured by currently available analytic instruments.

[0059] The term "subject" or "individual" is used to refer to a mammal, including a human being.

[0060] The term "substantially" means by at least 10-20%.

Examples

[0061] The following examples are provided for purposes of illustration of the embodiments only and are not intended to be limiting. The raw materials, reagents, chemicals, and other materials are presented as exemplary components or reagents, and various modifications may be made in view of the foregoing discussion within the scope of this disclosure. Unless otherwise specified in this disclosure, components, reagents, protocol, and other methods used in the system and the assays, as described in the Examples, are for the purpose of illustration only.

Example 1 Preparation of broth from turkey parts

[0062] In this example, turkey parts were used to prepare a broth and the overall quality and potential benefits of the broth were determined. Briefly, raw turkey was mechanically separated and the parts were finely comminuted to less than 2mm in size to maximize extraction. The small-sized parts from the turkey were gently cooked to 195°F in steam for about 15 minutes or less. The broth was then separated from the insoluble fraction by decanting. Freed fat in the broth was also removed from the broth by a centrifugal separator. The broth was concentrated in a commercial evaporator, chilled, and packaged for sale.

Example 2 Preparation of broth from chicken parts

[0063] In this study, chopped chicken parts containing chicken bones and cartilage were cooked in water at a temperature greater than 250°F for more than 6 hours to maximize the extraction of certain broth fraction and/or compounds. The broth obtained from this process was designated "AAC1" for internal reference. More particularly, USDA inspected chopped raw chicken bones remaining after major muscles were removed were cooked in water in a large commercial stainless steel cooking tank. After cooking, the liquid broth portion was separated from the chicken solids by decanting. The broth was concentrated in a commercial evaporator then spray dried and packed in labeled containers.

Example 3 Comparing the protein and amino acid profiles of different broths

[0064] Proteins are large molecules composed of one or more chains of amino acids that perform a wide array of functions in biological systems including, for example, functioning as enzymes, facilitating cell communication, and providing structural support to cells. Humans, as well as other animals, obtain essential amino acids from protein consumed as part of their diet since they lack enzymes needed to synthesize them. The objective of this study was to determine the concentration and size range of proteins in chicken broth AAC1 as compared to a home-made product.

[0065] One sample prepared according to this disclosure (AAC1) and another sample prepared according to home-style cooking methods (Homemade) were compared. The percent solid (w/v) for AAC1 was 8% solid, while the percentage (w/v) for Homemade was 1.7% solid.

[0066] Prior to determining the amount of protein by the Bradford method, each sample was diluted in distilled water to a final 1% w/v solution. A standard curve was prepared using bovine serum albumin (0-3.5µg/µL). All samples were analyzed in triplicate. The amount of total protein was determined using a plate reader at a wavelength of 595 nm. Results are shown in Table 1.

Table 1 Amount of total protein in broth samples

Sample	Values	Results	Mean Results	Concentration	SD	CV
AAC1	0.446	1.558	1.691	1.691	0.132	7.8
	0.461	1.693				
	0.474	1.821				
Homemade	0.544	2.496	2.52	2.52	0.034	1.3
	0.549	2.544				

[0067] To correct for differences in the percent solids in AAC1 (8%) and Homemade (1.7%) samples, the protein values based on a 1% solution for AAC1 and Homemade were multiplied by their respective starting % solids. The final adjusted amount of total protein for AAC1 and Homemade are shown in Table 2.

Table 2 Adjusted total protein concentration in AAC1 and homemade broth

Sample	Adjusted Final Concentration	% of AAC1
AAC1	13.6 µg/ul	-
Homemade	4.3 µg/ul	31.6%

[0068] To determine the protein profile of each sample, equal volumes of the AAC1 and Homemade samples (15.6µl) were each mixed with Laemmli's sample buffer and a reducing agent, heated at 95°C for 5 minutes, and separated on a 4-12% Bis Tris gel. The relative size range of the proteins was determined by comparison to a commercially available protein standard (ranging from 4.5 kDa to 300 kDa). A constant voltage of 150V was applied to the gel for 30 minutes, allowing for separation of proteins in each sample. The proteins were visualized in the gel using SimplyBlue™ SafeStain.

[0069] The results are shown in Fig. 1. Lane 1 shows the protein profile for homemade broth, lane 2 for the AAC1 broth, and lane 3 is molecular weight standard. Based on the protein profiles, AAC1 is composed of approximately 3 times more protein than a homemade product. Proteins in the homemade product displayed a wider range of molecular weight distribution, containing both large (up to about 300-500 kD) and small proteins (about 5-15kD). By contrast, the majority (i.e., greater than 50%) of proteins in AAC1 has molecular weight of between 70 kDa and 15 kDa.

[0070] Individual amino acid content was also analyzed. Table 3 below shows the individual amino acid content of the broth compositions prepared according to the disclosed methods as compared to those prepared using home-style methods as well as other commercial products.

Table 3 Amino acid composition of different broths

Table Two: Content values above calculated to 100% solids basis:	3823	Commercial product	Home-made-1	Home-made-2	AAC1-1	AAC1-2	AAC1-3
-	W/W%	%					
TAURINE	2.50						
ASPARTIC ACID	3.63	2.44	3.63	3.47	7.04	5.34	5.38
THREONINE	1.44	0.76	1.52	1.42	1.75	2.13	1.79
SERINE	1.56	1.34	1.96	1.89	2.08	1.88	2.40
GLUTAMIC ACID	10.25	6.98	9.44	9.26	10.52	9.50	10.14
PROLINE	3.53	3.05	5.93	5.57	9.59	10.66	11.07
HYDROXYPROLINE	2.19	2.99	5.31	4.92	na	7.00	9.39
GLYCINE	5.97	7.38	8.79	9.47	18.02	15.41	17.68
Lanthionine	0.06	0.00	0.00	0.00	0.00	0.00	0.00
ALANINE	3.91	3.37	4.60	4.58	8.06	7.53	7.53
CYSTINE	0.44	0.29	0.34	0.40	0.00	0.25	0.17
VALINE	**1.44**	**0.73**	**1.43**	**1.42**	**2.44**	**2.19**	**2.21**
ISOLEUCINE	**1.13**	**0.47**	**1.18**	**1.02**	**1.54**	**1.59**	**1.59**
LEUCINE	**2.72**	**1.51**	**2.48**	**2.32**	**3.38**	**3.47**	**3.43**
METHIONINE	0.81	0.52	0.93	0.77	1.01	1.06	1.10
TYROSINE	0.88	0.44	0.81	0.65	1.09	1.00	0.90
PHENYLALANINE	1.00	0.73	9.53	7.03	2.30	2.16	1.98
HYDROXYLYSINE	0.16	0.00	0.00	0.00	0.00	0.00	0.00
HISTIDINE	2.03	2.56	2.70	1.73	1.32	1.16	1.00
ORNITHINE	2.25	0.00	0.00	0.00	0.00	0.00	0.00
LYSINE	3.75	2.67	3.07	3.00	3.96	3.66	3.05
ARGININE	3.06	2.79	4.01	3.75	8.24	6.69	6.61
TRYTOPHAN	0.16	0.06	0.12	0.09		0.22	0.15
Total	54.84	41.08	67.80	62.79	82.35	82.88	87.55

Example 4 Comparing levels of chondroitin sulfate (CS) in different broths

[0071] Chondroitin sulfate (CS) is an important structural compound found in cartilage and is implicated in joint health. CS has been shown to reduce the levels of many inflammatory mediators such as iNOS, PGE2, COX-2 (Gwendolyn, Spine 2011), and NFkB (Vallières, Osteoarthritis Cartilage, 2010). The goal of this study was to determine the amount of CS in various chicken broth samples. Chicken broths were analyzed for total CS using enzymatic digestion and LC-UV detection (Ji, Journal of AOAC International, 2007). Results were calculated as area on the curve compared to standard samples and the limit of quantification as 8 mg chondroitin sulfate/g dry material

(Table 4). Among all chicken broths tested, AAC1 had the greatest amount of CS (8.797% w/w).

Table 4 Amounts of chondroitin sulfate (CS)

Samples	Average (mg)	Dry mg/g	Dry % w/w
Powdered Chicken Broth AAC1	17.480	87.971	8.797
Powdered Chicken Broth H814	15.443	67.553	6.755
Frozen Concentrate TSN	10.886	52.059	5.206
Frozen Concentrate IDF Chicken 823	8.627	42.104	4.210
Home Style Broth from Back Bones	5.866	28.258	2.826
Frozen Concentrate Turkey 824	5.243	26.227	2.623
Powdered Chicken Broth HML 34511	4.979	24.335	2.434
Powdered Chicken Broth A1004	3.817	18.305	1.830
Powdered Chicken Broth P1301	3.075	15.054	1.505
Home Style Broth from Chicken Parts	2.185	10.99	1.09
Home Style Broth from Chicken Necks	1.937	9.361	0.936

Example 5 Comparison of concentration of polyphenols in different chicken broths

[0072] The concentration of polyphenols in different chicken broths was determined using a modified Folin-Ciocalteau method (Slinkard, American Journal of Enology and Viticulture, 1977). Polyphenols are a class of chemical compounds known to have anti-oxidant and anti-inflammatory properties. While both AAC1 and home-style broths contained polyphenols, AAC1 contained 4828.8 μg/mL GAE of polyphenols, which is approximately 7 times more GAE of polyphenols than a homemade broth (687.7 μg/mL GAE) made from the same kind of chicken parts.

Example 6 Effects of broth feeding on microbiome in the lower GI

[0073] Various broth compositions were fed to rodents and fecal samples were collected at Day 0 (basal) and Day 14. Denaturing gradient gel electrophoresis (DGGE)

was used to analyze the changes in the microbial profiles. Broth compositions tested include: the disclosed broth AAC1, water (as negative control), homemade-styled broth and a commercial broth product. The DGGE profiles of the microbiome at Day 0 and Day 14 after the animals were fed different broth compositions were compared.

[0074] All of the basal samples showed a relatively high degree of similarity. At most, there was a 9% difference between the basal samples of the AAC1 and water groups. The mean difference between all basal samples was 6.1%. The introduction of the various chicken broths, as well as the two-week time period, resulted in additional variability between the samples. At most, AAC1 and the commercial broth product differed by 15%. In contrast, Homemade broth differed from water and the commercial broth by 9%. The mean difference between all samples was 12%.

[0075] All of the samples changed in composition between the basal Day 0 and Day 14 time points. For instance, the difference between the water control on Day 0 (basal) and Day 14 was 7%. This 7% change demonstrated a relatively stable gut microbiome over a period of two weeks and serves as an excellent control. The average variance among all samples from the basal to day 14 time point was 13.5%.

[0076] The different profiles were grouped and classified based on banding similarities (i.e., presence/absence of DNA bands) in DGGE. The animals receiving AAC1 chicken broth experienced a distinct shift in their gut microbiomes when compared to animals receiving water or the other two broths. This result is most clearly demonstrated in Figure 1, as hierarchical clustering reveals that the dissimilarities observed in the AAC1 fed animals are unique to this administration. The animals fed AAC1 at day 14 do not cluster with any of the basal or other day 14 groups. This indicated that the AAC1 broth causes a unique shift in the rodent gut microbiome. The scale at the bottom of Figure 1 indicates percent similarity between clusters.

Example 7 Effects of broth feeding on microbiome in the cecum

[0077] Caecal samples for each feeding group were also collected at Day 14 and were analyzed according to the same protocols described above. Although many microorganisms that are found in the feces are also found in the cecum, the contents in the cecum offer an additional look into the gut microbiome and any associated changes that occur within the animal due to the different function/conditions of the organ. The downside to caecal analysis is that the animal must be sacrificed for collection, and therefore no basal samples were taken. DGGE analysis from caecal materials reveals a

high degree of similarity between the water, homemade and commercial broth fed animals (average 4.6% difference among all three samples). Interestingly, the AAC1 group was over 14% dissimilar from the water, homemade and the commercial broth group.

[0078] The different profiles were grouped and classified based on banding similarities (i.e., presence/absence of bands) in DGGE. The scale at the bottom of Figure 2 indicates percent similarity between clusters.

[0079] Results from analysis of the caecal materials mirror those of the fecal samples. While no baseline information as to the composition of the caecal microbiota is available, 14 days of ingesting the AAC1 broth produced a unique caecal composition (Figure 2).

[0080] In an effort to compare shifts between fecal and caecal materials, the gel of Figure 3 was created. This gel allowed for the direct comparison across all caecal and select fecal samples. From this result, eleven regions of interest were selected, each corresponding to a unique bacterial species. Direct sequencing of the DNA from the bands allowed for the identification of several species that are altered as a result of AAC1 ingestion (Table 5). Due to the tremendous diversity of the gut microbiota, several of the species could not be identified, as they have never been previously characterized.

Table 5 Results from DNA sequencing of bands altered by AAC1 feeding

Band	Identity	Relationship to AAC1 Administration	Summary of Species	Phylum
1	*Acholeplasma sp.*	Found in all fecal and caecal samples, but appears to be decreased in AAC1 feces.	Saprotrophic or pathogenic. Little known about the role in the gut	Firmicutes
2	Uncultured Lachnospiraceae	Unique to AAC1 fecal and caecal samples. Not found in any other treatment groups or controls.	Closely related to *Clostridium.*	Firmicutes
3	*Lactobacillus rhamnosus*	Unique to AAC1 cecum. Not found in any other samples, fecal or caecal.	Popular probiotic. Certain strains have been shown to produce a number of beneficial effects.	Firmicutes
4	Uncultured Lachnospiraceae	Unique to AAC1 fecal and caecal samples. Not found in any other treatment groups or controls.	Closely related to *Clostridium.*	Firmicutes
5	Mixed Sample/No known sequence	N/D	N/A	N/A

6	Uncultured *Clostridium sp.*	Unique to AAC1 cecum. Not found in any other samples, fecal or caecal.	Common residents of the gut microbiota. Metabolically diverse.	Firmicutes
7	Mixed Sample/No known sequence	N/D	N/A	N/A
8	Uncultured Clostridiales	Found in all fecal and caecal samples, but is apparently increased in AAC1 feces.	Common residents of the gut microbiota. Metabolically diverse.	Firmicutes
9	Mixed Sample/No known sequence	Unique to AAC1 fecal and caecal samples. Not found in any other treatment groups or controls.	N/A	N/A
10	Mixed Sample/No known sequence	Unique to AAC1 cecum. Not found in any other samples, fecal or caecal.	N/A	N/A
11	*Clostridium polysaccharolyticum*	Present in all fecal samples, but also appears in AAC1-treated cecum. Does not appear in any other caecal samples.	Able to degrade a wide variety of complex polysaccharides, including cellulose and xylan.	Firmicutes

[0081] A sequence that is of special interest is band three, which was identified as *Lactobacillus rhamnosus*. This species was only found in the caecal contents of the animals receiving AAC1 broth. *L. rhamnosus* is a popular, commercially-available probiotic, but is also a native resident of the gut microbiota. Several studies have indicated that numerous benefits are associated with the ingestion of certain strains of *L. rhamnosus*. For example, research has shown that ingestion of *L. rhamnosus* by mice can lead to lower levels of anxiety and stress hormones.

Example 8 Use of next generation sequencing to identify probiotic bacteria in fecal samples

[0082] In a healthy human adult, 80% of the identified fecal microbiota may be classified into three dominant phyla: Bacteroidetes, Firmicutes and Actinobacteria. The Firmicutes to Bacteroidetes ratio has been regarded as a good indicator of gut microbiota composition and balance. See Ley et al. (2006). The Firmicutes to Bacteroidetes ratio was measured for the groups of rodents fed with different broth products. As shown in Figure 4, the Firmicutes to Bacteroidetes ratio did not change significantly among the different groups.

[0083] The profiles of fecal microbiota serve as good indicators of microbiota in the lower GI tract. Figure 5 showed the relative amount of Proteobacteria in the lower

GI tract of animals fed with different broth compositions. AAC1 selected for Proteobacteria in the lower GI tract of the animals. The relative amounts of *Lactobacillus* in the lower GI tract of the animals fed with different broth compositions were also measured. As shown in Figure 6, AAC1 selected for (or specifically maintained) *Lactobacillus* in the lower GI tract of the animals. In addition, one genus, *Faecalibacterium*, was only found in the group fed with AAC1 for 14 days, but not in other groups or in the basal measurement. Three genera, *Parasutterella*, *Morganella* and *Oscillospira,* were present at significantly higher levels after 14 days on AAC1 as compared to the basal level.

Example 9 Use of next generation sequencing to identify probiotic bacteria in caecal samples

[0084] The profiles of caecal microbiota in the groups of rodents fed with different broth products were measured. As shown in Figure 7, AAC1 selected for (or specifically maintained) *Lactobacillus* in the cecum of the animals. In addition, four genera, *Odoribacter*, *Gastranaerophilales*, *Faecalibacterium* and *Allabaculum*, were only found in the group fed with AAC1 for 14 days, but not in other groups or in the basal measurement. Three genera, *Lactobacillus*, *Coprococcus* and *Oscillibacter,* were present at significantly higher levels after 14 days on AAC1 as compared to the basal level. One genus, *Intestimonas,* was present at significantly lower levels after 14 days on AAC1 as compared to the basal level.

Example 10 The disclosed composition enhance biodiversity in the gut

[0085] Another important consideration in NGS data analysis is biodiversity. It is generally agreed upon in the literature that a more diverse gut microbiota is beneficial. Two of the most commonly used measurements of biodiversity in NGS analysis are the Chao and Shannon-Weiner indices. Each index produces a value that is representative of how diverse a microbiota is. Figure 8 demonstrates that the degree of diversity after 14 days was much higher in animals fed AAC1 as compared to the diversity in groups fed Homemade, another commercial product and water control groups.

[0086] All animal studies described herein were performed using approved protocols in compliance with government rules and regulations. Changes may be made in the above methods and systems without departing from the scope hereof. It should thus be noted that the matter contained in the above description or shown in the accompanying

drawings should be interpreted as illustrative and not in a limiting sense. The following claims are intended to cover generic and specific features described herein, as well as statements of the scope of the present method and system, which, as a matter of language, might be said to fall there between.

[0087] Although each of the embodiments described above has been illustrated with various components having particular respective orientations, it should be understood that the system and methods as described in the present disclosure may take on a variety of specific configurations with the various components being located in a variety of positions and mutual orientations and still remain within the spirit and scope of the present disclosure. Furthermore, suitable equivalents may be used in place of or in addition to the various components, the function and use of such substitute or additional components being held to be familiar to those skilled in the art and are therefore regarded as falling within the scope of the present disclosure. Therefore, the present examples are to be considered as illustrative and not restrictive, and the present disclosure is not to be limited to the details given herein but may be modified within the scope of the appended claims.

[0088] All references cited in this disclosure, including patents, patent applications, scientific papers and other publications, are hereby incorporated by reference into this application.

References

All references cited in the specification or listed below are hereby incorporated into this disclosure as if fully reproduced herein.

Arumugam, M., et al. "Enterotypes of the human gut microbiota." *Nature* (2011).

Bravo, J. A., et al. "Ingstion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve." *Proceedings of the National Academy of Science* (2011): doi: 10.1073/pnas.1102999108.

Larsen, N., et al. "Gut Microbiota in Human Adults with Type 2 Diabetes Differs from Non-Diabetic Adults." *PLOS One* (2010).

Ley, R. E., et al. "Obesity alters gut microbial ecology." *Proceedings of the National Academy of Science* (2005): 11070-11075.

Mariat, D., et al. "The Firmicutes/Bacteroides ratio of the human microbiota changes with age." *BMC Microbiology* (2009).

Ley RE, Turnbaugh P, Klein S, Gordon JI: Microbial ecology: human gut microbes associated with obesity. Nature 2006, 444:1022-1023.

Claims

We claim

1. A method for enhancing one or more probiotic bacteria in an individual, comprising administering to said individual a composition in an amount effective in enhancing said one or more probiotic bacteria, wherein said composition is prepared from poultry.

2. The method of claim 1, wherein said composition comprises soluble collagen fiber from poultry.

3. The method of any one of the preceding claims, wherein said composition comprises at least 40% protein by weight of total solids.

4. The method of any one of the preceding claims, wherein said composition comprises less than 10% carbohydrate by weight of total solids.

5. The method of any one of the preceding claims, wherein said composition is administered to said individual at said effective amount each day for at least 14 days.

6. The method of any one of the preceding claims, wherein said probiotic-enhancing effective amount is the amount of said composition that increases the live count of said one or more probiotic bacteria in the gastrointestinal system of said individual when administered to said individual.

7. The method of any one of the preceding claims, wherein said composition increases microbial diversity in the gastrointestinal system of said individual.

8. The method of any one of the preceding claims, further comprising identifying an individual in need of increased count of said one or more probiotic bacteria.

9. The method of any one of the preceding claims, further comprising measuring the live count of said one or more probiotic bacteria in the gastrointestinal system of said individual after administering the composition to the individual at said effective amount each day for at least 14 days.

10. The method of any one of the preceding claims, wherein said one or more probiotic bacteria comprise a lactic acid producing bacterium.

11. The method of any one of the preceding claims, wherein said composition increases the live count of said one or more probiotic bacteria in the gastrointestinal system of said individual as compared to a comparable individual not receiving said composition, said one or more probiotic bacteria comprising at least one member selected

from the group consisting of *Parasutterella*, *Morganella*, *Oscillospira*, *Faecalibacterium* and combination thereof.

12. The method of any one of the preceding claims, wherein said individual takes a probiotic supplement regularly before the administration of said composition, wherein said administration eliminates the need for said individual to take probiotic supplement regularly.

13. The method of any one of the preceding claims, wherein said composition increases the live count of one or more probiotic bacteria in the upper and/or middle sections of the intestine of said individual, said at least one probiotic bacterium comprising at least one member selected from the group consisting of *Lactobacillus*, *Coprococcus*, *Oscillibacter*, *Odoribacter*, *Gastranaerophilales*, *Faecalibacterium*, *Allabaculum*, and combination thereof.

14. The method of any one of the preceding claims, wherein said one or more probiotic bacteria comprise a *Lactobacillus* bacterium.

15. The method of any one of the preceding claims, wherein said one or more probiotic bacteria comprises *Lactobacillus rhamnosus*.

16. The method of any one of the preceding claims, wherein said composition is administered to the individual as a mixture with one or more probiotic bacteria.

17. The method of any one of the preceding claims, wherein said composition comprises amino acids glycine, proline and hydroxyproline, wherein glycine constitutes at least 12% (w/w), proline constitutes at least 8% (w/w), and hydroxyproline constitutes at least 7% (w/w) of total amino acids of said composition.

18. The method of any one of the preceding claims, wherein said composition does not contain detectable amount of gluten.

19. The method of any one of the preceding claims, wherein said composition is prepared from poultry parts by a process comprising the steps of:

(a) cooking chicken pieces in water at a temperature of between 70 ºC and 150 ºC for about 8 minutes to about 24 hours, said chicken pieces being prepared from whole chicken or chicken parts, and

(b) removing insoluble from step (a) to obtain said composition in a liquid form.

20. A method of culturing a probiotic bacterium, comprising growing the probiotic bacterium on a medium, said medium comprising a prebiotic composition prepared from

poultry, said prebiotic composition comprising soluble collagen fiber prepared from poultry.

21. The method of claim 20, wherein said prebiotic composition is prepared from poultry parts by a process comprising the steps of:

(a) cooking chicken pieces in water at a temperature of between 70 °C and 150 °C for about 8 minutes to about 24 hours, said chicken pieces being prepared from whole chicken or chicken parts, and

(b) removing insoluble from step (a) to obtain said composition in a liquid form.

22. A composition to be administered to an individual in need of probiotic bacteria, said composition comprising soluble collagen fiber and a probiotic bacterium.

23. The composition of claim 22, wherein said composition is prepared from poultry parts by a process comprising the steps of:

(a) cooking chicken pieces in water at a temperature of between 70 °C and 150 °C for about 8 minutes to about 24 hours, said chicken pieces being prepared from whole chicken or chicken parts, and

(b) removing insoluble from step (a) to obtain said composition in a liquid form.

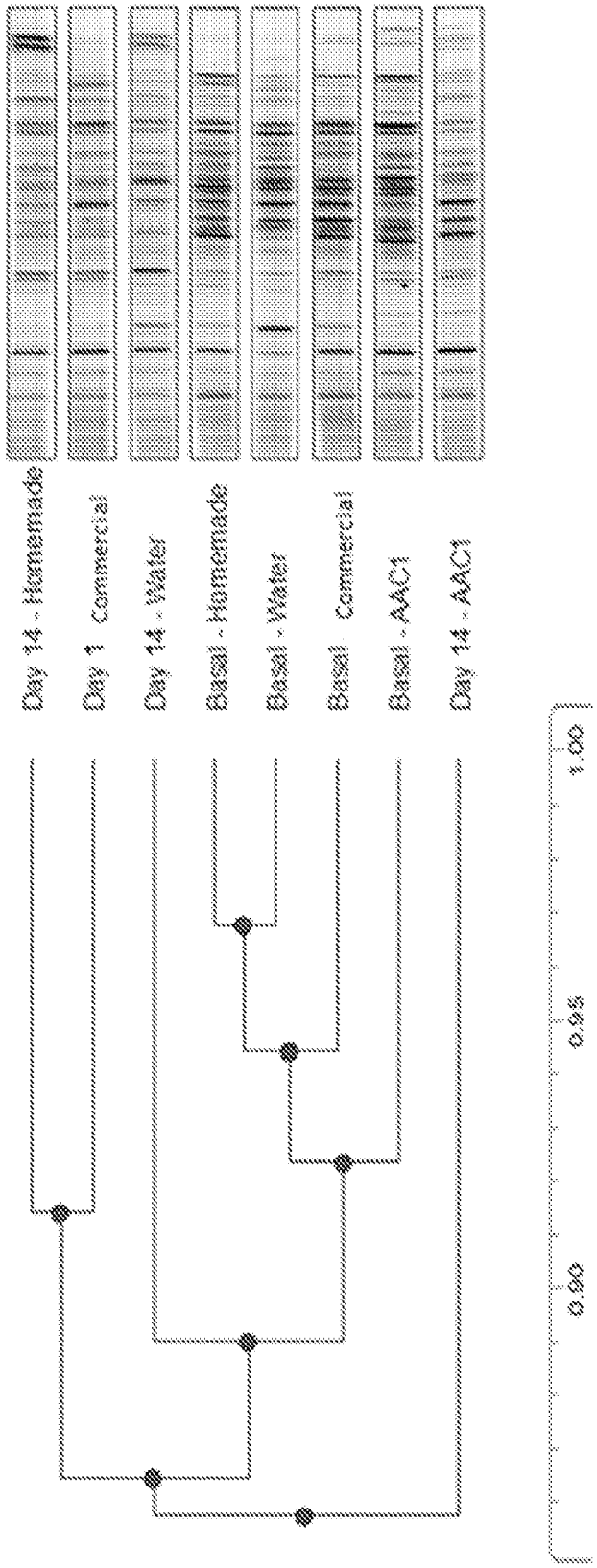
Day 14 - Homemade
Day 1 Commercial
Day 14 - Water
Basal - Homemade
Basal - Water
Basal Commercial
Basal - AAC1
Day 14 - AAC1
1.00
0.95
0.90

FIG. 1

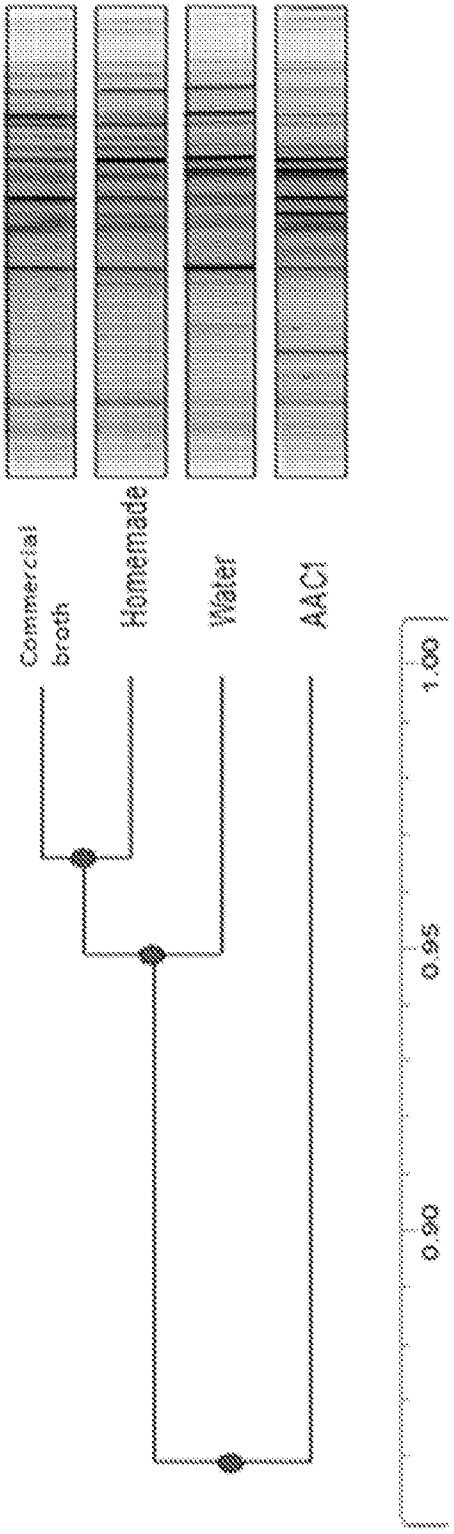

FIG. 2

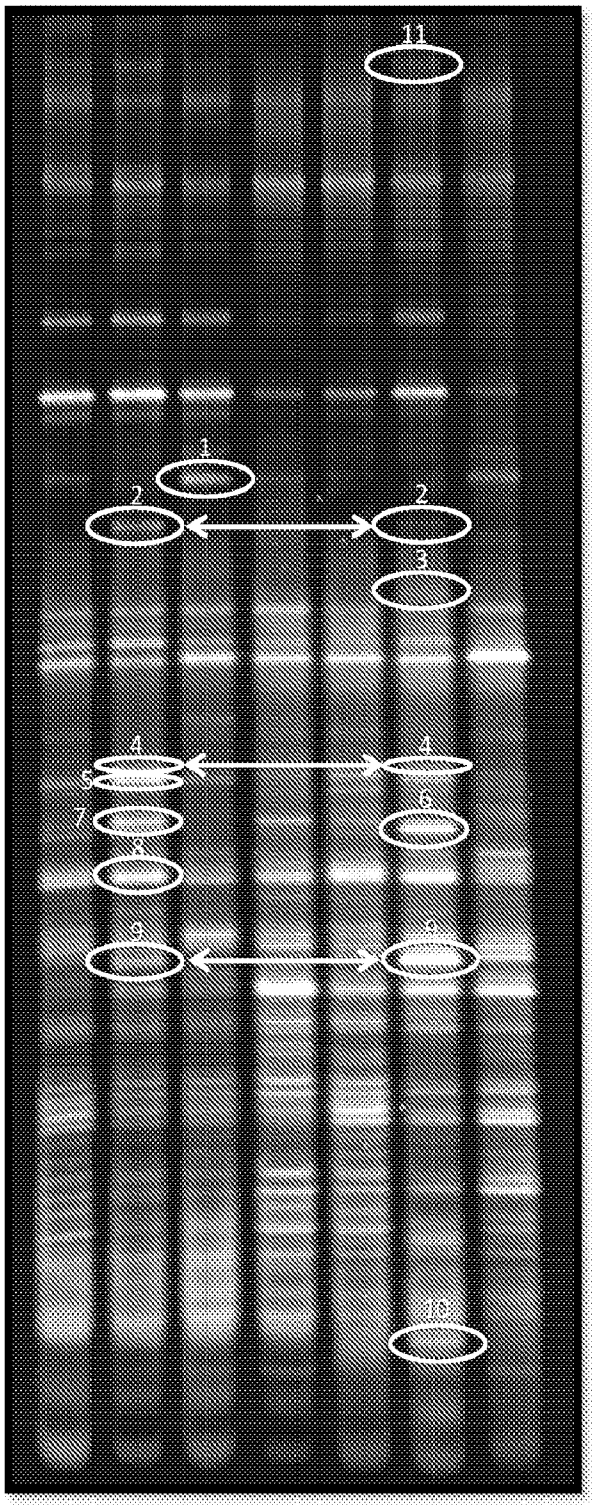

FIG. 3

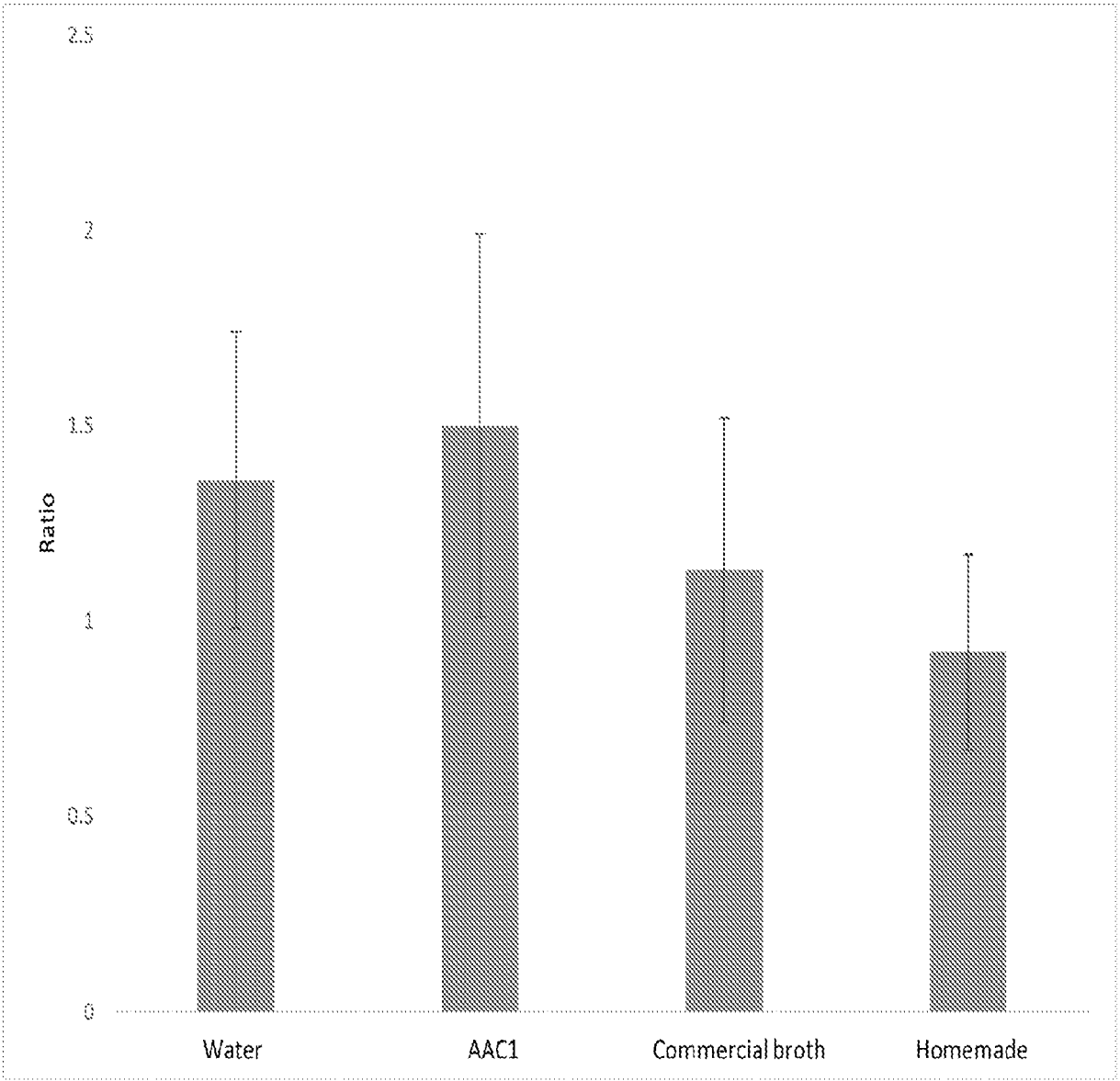

FIG. 4

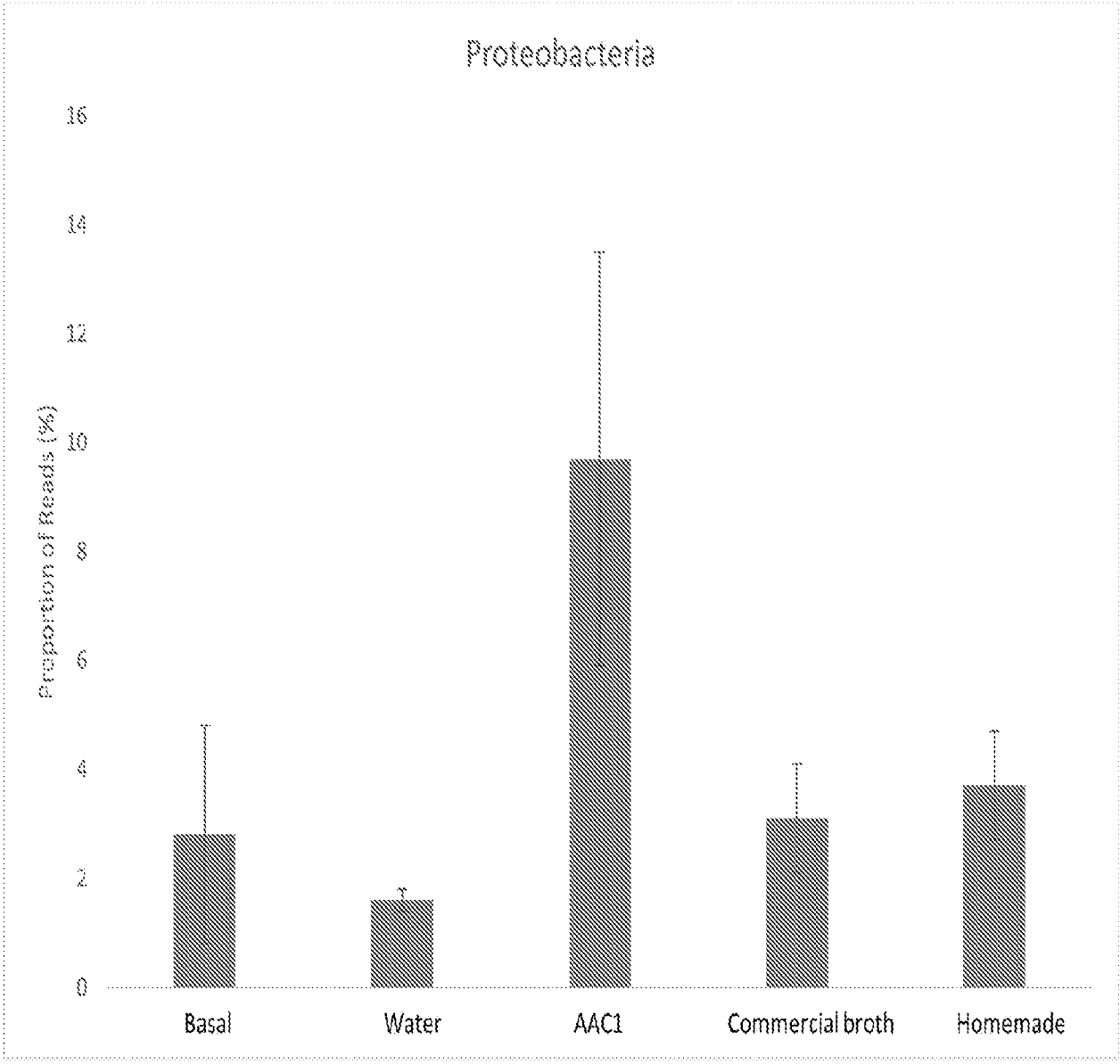
Proteobacteria
Proportion of Reads (%)
16
14
12
10
8
6
4
2
0
Basal
Water
AAC1
Commercial broth
Homemade

FIG. 5

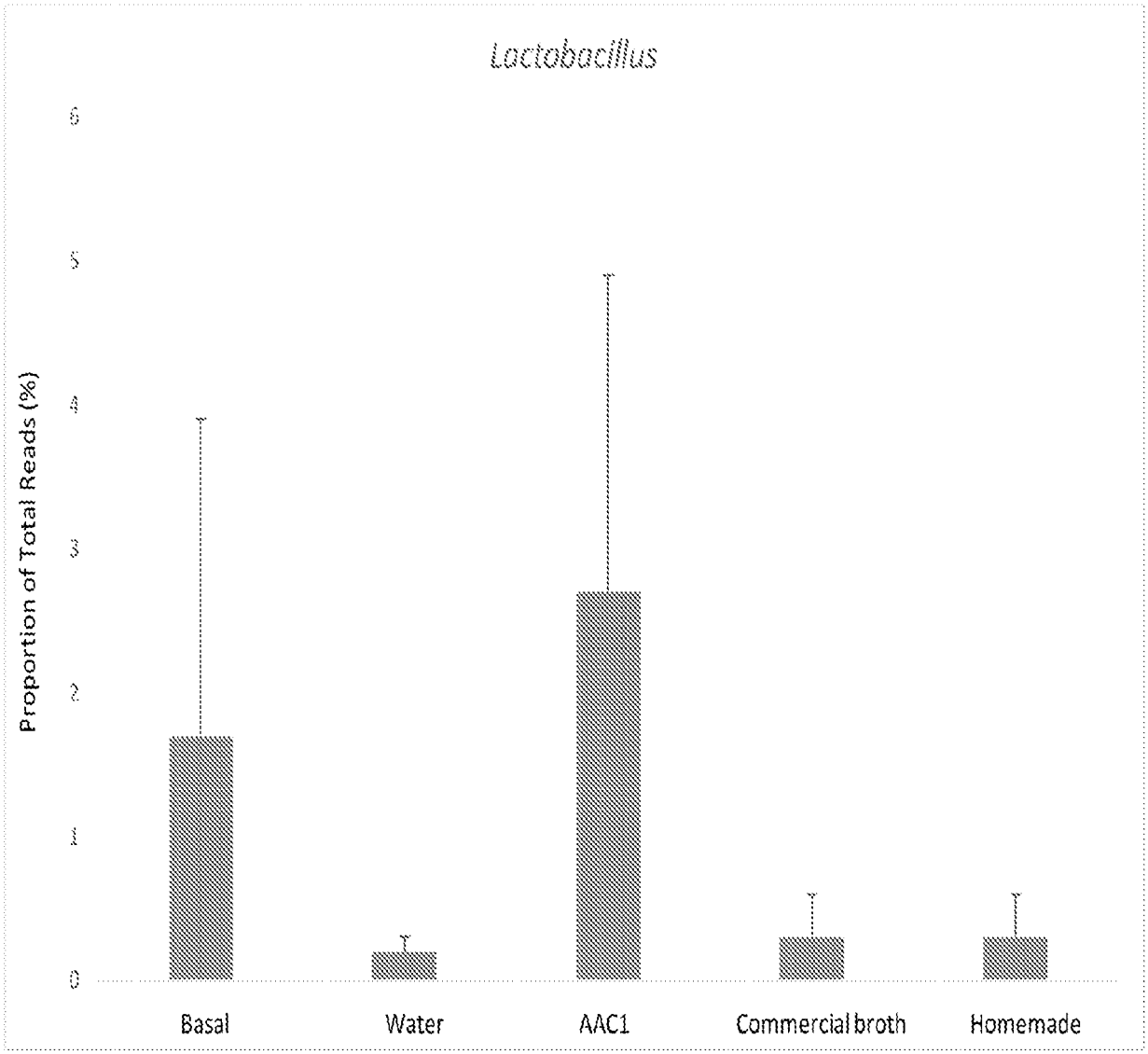
Lactobacillus
Proportion of Total Reads (%)
6
5
4
3
2
1
0
Basal
Water
AAC1
Commercial broth
Homemade

FIG. 6

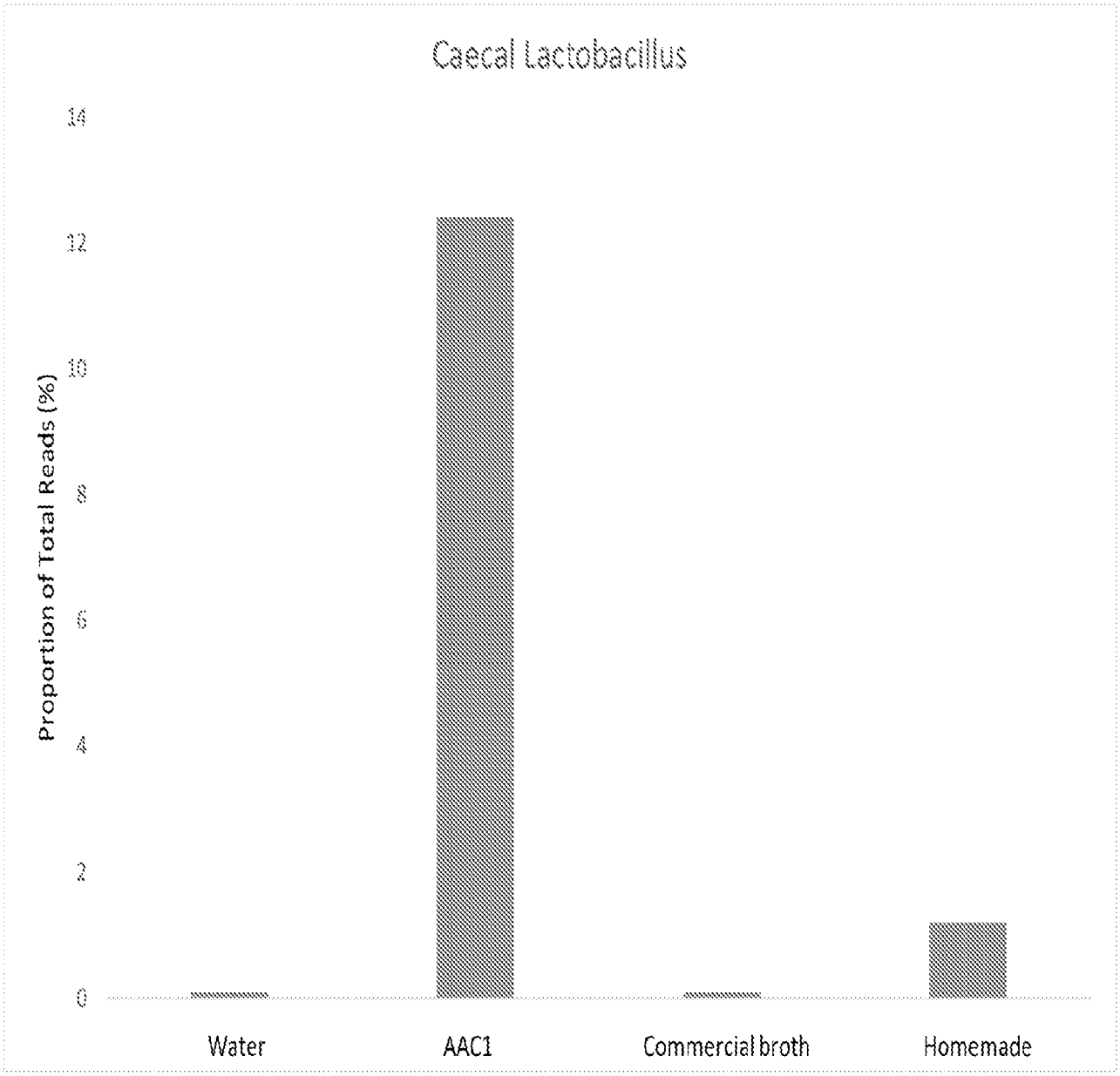
Caecal Lactobacillus
Proportion of Total Reads (%)
14
12
10
8
6
4
2
0
Water
AAC1
Commercial broth
Homemade

FIG. 7

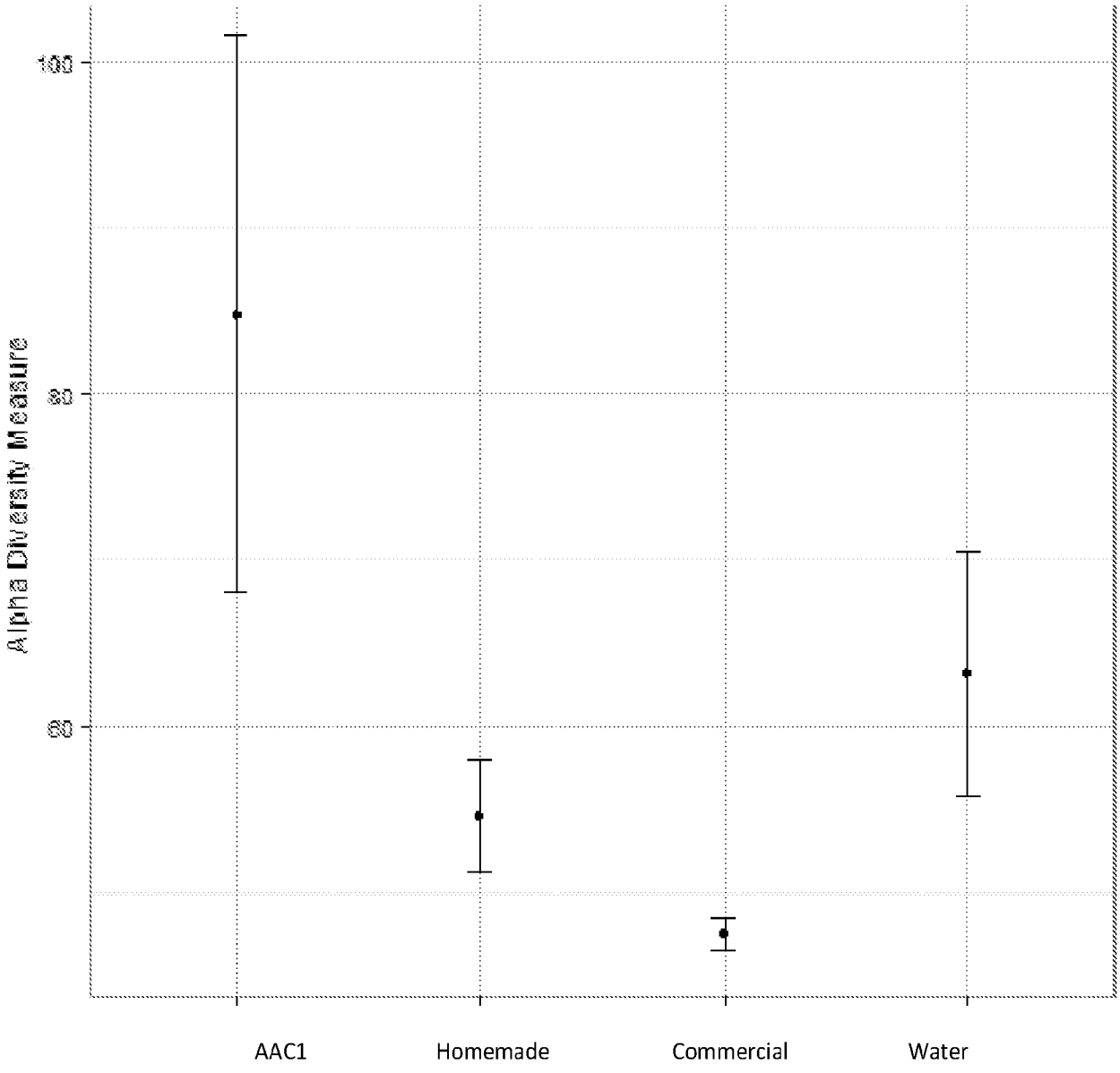

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US16/14825

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 1/20; A61K 35/66 (2016.01)
CPC - A61K 45/06, 35/744
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)
IPC(8): C12N 1/20; A61K 35/66 (2016.01)
CPC: A61K 45/06, 35/744

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); ProQuest; Scifinder; Google/Google Scholar; KEYWORDS: probiotic, prebiotic, broth, chicken, poultry, increase, enhance, collagen, fiber

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2012/0263758 A1 (CHINACHOTI, P et al.) 18 October 2012; paragraphs [0005], [0012]-[0015], [0020], [0133], [0148]	1-2, 3/1-2, 20-23
Y	CN 102948621 A1 (UNIVERSITY NORTHEAST AGRICULTURAL) 06 March 2013; English translation; paragraphs [0077]-[0090]; claim 1	1-2, 3/1-2, 20-21
Y	US 2015/0011500 A1 (INTERNATIONAL DEHYDRATED FOODS, INC.) 08 January 2015; paragraph [0056]; claims 1, 15-16, 20	2, 3/2, 20-23
A	US 2009/0263542 A1 (LIN, CH et al.) 22 October 2009; entire document	1-2, 3/1-2, 20-23
A	WO 2014/049023 A1 (AQUILON CYL SANIDAD ANIMAL) 03 April 2014; abstract; page 2, lines 21-24; page 7, lines 28-33	1-2, 3/1-2, 20-23

☐ 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
08 March 2016 (08.03.2016)	08 APR 2016
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/US16/14825

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.: 4-19
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:

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.:

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.