

DESCRIPTION**NUTRITIONAL COMPOSITIONS CONTAINING A PEPTIDE COMPONENT AND USES THEREOF****TECHNICAL FIELD**

[0001] The present disclosure relates to nutritional compositions that include a peptide component that may reduce the incidence of autoimmune disease. More specifically, the nutritional composition may reduce the incidence of diabetes mellitus, including type 1 diabetes mellitus ("T1D") and type 2 diabetes mellitus ("T2D"). The nutritional compositions described herein are suitable for administration to adult and pediatric subjects. The peptide component of the nutritional composition includes selected peptides disclosed herein.

[0002] Additionally, the disclosure relates to methods of reducing the incidence of autoimmune disease by providing a nutritional composition comprising the peptide component described herein.

BACKGROUND ART

[0003] Autoimmune disease arises from an inappropriate immune response of the body against certain tissues present in the body. This autoimmune response may be restricted to certain organs, for example the pancreas as in diabetes mellitus, or may affect only certain membranes of certain tissues and organs. One of the functions of the immune system is to protect the body by responding to invading microorganism, such as viruses or bacteria, by producing antibodies or sensitized lymphocytes. Under normal conditions, an immune response cannot be triggered against the cells of one's own body. However, autoimmune diseases are characterized in that the body's immune system may make a mistake and attack the cells they are meant to protect. Therefore, autoimmune disease encompasses a broad category of related diseases in which the body's immune system attacks its own tissue.

[0004] An autoimmune disease is any disease caused by immune cells that become misdirected at healthy cells and/or tissues of the body. Currently, autoimmune disease affects 3% of the U.S. Population and likely a similar percentage of the industrialized world population. Autoimmune diseases are characterized by T and B lymphocytes that aberrantly target self-proteins, -polypeptides, -peptides, and/or other self-molecules causing injury and or malfunction of an organ, tissue or cell type within the body. Thus, autoimmune disease occur when there is some interruption of the normal control process, which allows lymphocytes to avoid suppression, or when there is an alteration in some body tissue so that it is no longer recognized as "self" and thus is attacked. The exact mechanism causing autoimmune disease may differ but may be triggered by many factors including: bacteria, viruses, toxins, drugs, and genetic predisposition.

[0005] Autoimmune diseases include diseases that affect specific tissues as well as diseases that can affect multiple tissues. The characteristic feature of tissue-specific autoimmunity is the selective targeting of a single tissue or individual cell type. However, certain autoimmune diseases that target ubiquitous self-proteins can also affect specific tissues.

[0006] Particular autoimmune diseases are often classified into organ-specific disorders and non-organ-specific types. For example, Hashimoto's disease affects the thyroid gland, Addison's disease affects the adrenal glands, and Type 1 diabetes mellitus affects the pancreas. Examples of non-organ-specific autoimmune diseases include rheumatoid arthritis, systemic lupus erythematosus and dermatomyositis. More than 30 autoimmune diseases are presently known, including rheumatoid arthritis, insulin-dependent diabetes mellitus, multiple sclerosis, myasthenia gravis, systemic lupus erythematosus, and scleroderma. Additional nonlimiting examples of autoimmune disease include: acute disseminated encephalomyelitis, Addison's disease, amyotrophic lateral sclerosis, autoimmune hepatitis, autoimmune lymphoproliferative syndrome, Berger's disease, Blau syndrome, certain types of cancer, celiac disease, Chagas disease, chronic recurrent multifocal osteomyelitis, Churg-Strauss syndrome, Cogan syndrome, cold agglutinin disease, Crohn's disease, Cushing's syndrome, Diabetes Mellitus type 1, Evan's syndrome, Grave's disease, Hashimoto's encephalopathy, Kawasaki's disease, Lou Gehrig's disease, Meniere's disease, multiple sclerosis, neuromyotonia, ocular cicatricial pemphigoid, psoriasis, psoriatic arthritis, Reynaud phenomenon, Reiter's syndrome, restless leg syndrome, rheumatoid arthritis, Sjogren's syndrome, temporal arteritis, transverse myelitis, vaculitis, and Wegener's granulomatosis.

[0007] Diabetes mellitus is a generic term for metabolic disorders characterized by persistence of a hyperglycemic state due to the deficiency of insulin action. Generally, diabetes mellitus is classified roughly into insulin-dependent diabetes mellitus ("IDDM") and non-insulin-dependent diabetes mellitus ("NIDDM"). One form of IDDM includes type 1 diabetes mellitus ("T1D"), which is a form of diabetes mellitus that results from the autoimmune destruction of insulin-producing beta cells of the pancreas.

[0008] Insulin is a biological material that suppresses elevated blood glucose levels. Insulin is a hormone secreted by the pancreas that can promote carbohydrate metabolism in the liver and enhance the uptake of glucose into muscle cells and fat cells in order to lower an elevated blood glucose level. A lack of insulin, which leads to an increase in blood and urine glucose, is observed both while the beta cells of the pancreas are being destroyed and when all the beta cells have been destroyed. As such, diabetes mellitus is characterized by recurrent or persistent hyperglycemia.

[0009] T1D may be induced by many different factors including genetic factors, environmental factors, dietary factors, an individual's overall susceptibility, diabetogenic

triggers and/or exposure to a driving antigen. T1D is currently understood to be a polygenic disease, meaning that different genes can contribute to the onset of T1D.

[0010] Typical symptoms of T1D include polyuria (frequent urination), polydipsia (increased thirst), xerostomia (dry mouth), polyphagia (increased hunger), fatigue and weight loss. Often times, untreated T1D can lead to diabetic ketoacidosis. Diabetic ketoacidosis is a potentially life-threatening complication that occurs when the body experiences a shortage of insulin and thus begins breaking down fatty acids for energy. The breakdown of the fatty acids produces acidic ketone bodies that cause many of the symptoms described herein.

[0011] Another form of diabetes mellitus includes T2D, which is a type of diabetes mellitus in which hyperglycemia is manifested due to insufficient insulin secretion and insulin resistance caused by uncertain and diverse factors such as aging, stress, and diet. T2D is characterized as NIDDM. Typically, about 90% of all individuals with diabetes mellitus fall under NIDDM.

[0012] Rapid increases in blood glucose levels after meals and its continuance for many years is known to exacerbate diabetes mellitus. Exacerbation of diabetes mellitus is often accompanied by promotion of angiopathy, which can lead to development of neurosis, nephropathy and retinopathy and to further complications of myocardial infarction and apoplexy.

[0013] For individuals prone to developing or suffering from diabetes mellitus often, certain types of diet and exercise therapies are adopted. Adopting certain exercise and diet patterns can help stabilize blood glucose levels and may improve overall carbohydrate metabolism.

[0014] Evidence has shown that tight glycemic control is a major factor in the prevention of complications associated with diabetes mellitus. However, currently available agents generally fail to maintain adequate glycemic control in the long term due to progressive deterioration of hyperglycemia, resulting from progressive loss of pancreatic cell function. Therefore, optimal glycemic control by drugs, therapeutic regimens, nutritional supplements and/or nutritional compositions is an important approach for the treatment of diabetes mellitus. While diabetes mellitus may be treated by insulin or the administration of oral hypoglycemic drugs, there is a need for a safe and effective nutritional supplement or nutritional composition for reducing the incidence of diabetes mellitus.

[0015] Autoimmune diseases may be treated with immunosuppressive agents, hormone replacement therapy or blood transfusion, in the case of autoimmune blood disorders. These treatments include several unwanted side effects and can be costly for the target subject. Secondly, when administering immunosuppressive agents, there is a delicate balance between diminishing the activity of the immune system while allowing the immune

system's ability to fight disease in general. Further, immunosuppressive agents may cause unwanted side effects such as bone loss and/or bone and tissue deterioration.

[0016] Accordingly, the present disclosure provides a nutritional composition comprising a peptide component that may reduce the incidence of autoimmune disease. Further, the present disclosure provides a nutritional composition comprising a peptide component that may reduce the incidence of T1D and T2D by providing a nutritional composition including the peptide component disclosed herein to target subject.

DISCLOSURE OF THE INVENTION

[0017] Briefly, the present disclosure is directed, in an embodiment, to a nutritional composition comprising a protein equivalent source including a peptide component comprising SEQ ID NO 4, SEQ ID NO 13, SEQ ID NO 17, SEQ ID NO 21, SEQ ID NO 24, SEQ ID NO 30, SEQ ID NO 31, SEQ ID NO 32, SEQ ID NO 51, SEQ ID NO 57, SEQ ID NO 60, and SEQ ID NO 63. In some embodiments, the peptide component comprises at least 10 additional peptides selected from Table 1.

[0018] In some embodiments the peptide component may comprise at least 5 peptides selected from Table 1 and at least additional 3 peptides selected from Table 2. In still other embodiment, the peptide component may comprise at least 10 additional peptides selected from Table 1.

[0019] In some embodiments 20% to 80% of the protein equivalent source comprises the protein component described herein and 20% to 80% of the protein equivalent source comprises intact protein, hydrolyzed protein, including partially hydrolyzed protein, and combinations thereof.

[0020] The nutritional composition(s) of the present disclosure may further comprise an infant formula. In some embodiments, the nutritional composition(s) of the present disclosure may comprise a pediatric nutritional composition, nutritional supplement or adult nutritional composition.

[0021] In some embodiments, the disclosure is directed to a method for reducing the incidence of autoimmune disease, the method includes providing a nutritional composition comprising a protein equivalent source including the peptide component disclosed herein. In still other embodiments, the disclosure provides a method for reducing the incidence of diabetes mellitus by providing a nutritional composition including the peptide component disclosed herein.

[0022] It is to be understood that both the foregoing general description and the following detailed description present embodiments of the disclosure and are intended to provide an overview or framework for understanding the nature and character of the disclosure as it is claimed. The description serves to explain the principles and operations of the claimed

subject matter. Other and further features and advantages of the present disclosure will be readily apparent to those skilled in the art upon a reading of the following disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023] Fig. 1 illustrates the pro-inflammatory cytokine secretion of IL12p70 by activated primary human dendritic cells exposed to extensively hydrolyzed casein.

[0024] Fig. 2 illustrates the pro-inflammatory cytokine secretion of IL12p70 by activated primary human dendritic cells exposed to a greater than 500 Da fraction of extensively hydrolyzed casein.

[0025] Fig. 3 illustrates the pro-inflammatory cytokine secretion of Interferon-gamma by activated human dendritic cells exposed to extensively hydrolyzed casein.

[0026] Fig. 4 illustrates the pro-inflammatory cytokine secretion of Interferon-gamma by activated primary human dendritic cells exposed to a greater than 500 Da fraction of extensively hydrolyzed casein.

[0027] Fig. 5 illustrates the pro-inflammatory cytokine secretion of Interleukin-8 by activated human dendritic cells exposed to extensively hydrolyzed casein.

[0028] Fig. 6 illustrates the pro-inflammatory cytokine secretion of Interleukin-8 by activated primary human dendritic cells exposed to a greater than 500 Da fraction of extensively hydrolyzed casein.

[0029] Fig. 7 illustrates the pro-inflammatory cytokine secretion of Tumor Necrosis Factor-alpha by activated human dendritic cells exposed to extensively hydrolyzed casein.

[0030] Fig. 8 illustrates the pro-inflammatory cytokine secretion of Tumor Necrosis Factor-alpha by activated primary human dendritic cells exposed to a greater than 500 Da fraction of extensively hydrolyzed casein.

[0031] Fig. 9 illustrates the pro-inflammatory cytokine secretion of Interleukin-6 by activated human dendritic cells exposed to extensively hydrolyzed casein.

[0032] Fig. 10 illustrates the pro-inflammatory cytokine secretion of Interleukin-6 by activated primary human dendritic cells exposed to a greater than 500 Da fraction of extensively hydrolyzed casein.

[0033] Fig. 11 illustrates the pro-inflammatory cytokine secretion of Interleukin-1 β by activated human dendritic cells exposed to extensively hydrolyzed casein.

[0034] Fig. 12 illustrates the pro-inflammatory cytokine secretion of Interleukin-1 β by activated human dendritic cells exposed to a greater than 500 Da fraction of extensively hydrolyzed casein.

[0035] Fig. 13 shows the secretion of Tumor Necrosis Factor-alpha of activated primary human macrophages when exposed to extensively hydrolyzed casein.

[0036] Fig. 14 illustrates the secretion of Tumor Necrosis Factor-alpha of activated primary human macrophages when exposed to a greater than 500 Da fraction of extensively hydrolyzed casein.

[0037] Fig. 15 illustrates the secretion of Interleukin-1 (IL1) in the ileum of NOD mice, BALB/C mice and NOD mice fed an extensively hydrolyzed casein diet.

[0038] Fig. 16 illustrates the secretion of Interleukin-18 (IL18) in the ileum of NOD mice, BALB/C mice and NOD mice fed an extensively hydrolyzed casein diet.

[0039] Fig. 17 illustrates the secretion of Interleukin-6 (IL6) in the ileum of NOD mice, BALB/C mice and NOD mice fed an extensively hydrolyzed casein diet.

[0040] Fig. 18 illustrates the secretion of Interleukin-17 (IL17) in the ileum of NOD mice, BALB/C mice and NOD mice fed an extensively hydrolyzed casein diet.

[0041] Fig. 19 illustrates the secretion of IFN-gamma of gut-draining lymph node T cells of NOD mice, BALB/C mice and NOD mice fed an extensively hydrolyzed casein diet.

[0042] Fig. 20 illustrates the secretion of Interleukin-4 of gut-draining lymph node T cells of NOD mice, BALB/C mice and NOD mice fed an extensively hydrolyzed casein diet.

[0043] Fig. 21 illustrates the secretion of Interleukin-17 of gut-draining lymph node T cells of NOD mice, BALB/C mice and NOD mice fed an extensively hydrolyzed casein diet.

[0044] Fig. 22 illustrates the pro-inflammatory cytokine secretion of Interleukin-12p40 from mouse macrophages exposed to extensively hydrolyzed casein, non-sterile filtered extensively hydrolyzed casein, and a greater than 500 Da fraction of extensively hydrolyzed casein.

[0045] Fig. 23 illustrates the pro-inflammatory cytokine secretion of Interleukin-1 β from mouse macrophages exposed to extensively hydrolyzed casein, non-sterile filtered extensively hydrolyzed casein, and a greater than 500 Da fraction of extensively hydrolyzed casein.

[0046] Fig. 24 illustrates the pro-inflammatory cytokine secretion of Interleukin-6 from mouse macrophages exposed to extensively hydrolyzed casein, non-sterile filtered extensively hydrolyzed casein, and a greater than 500 Da fraction of extensively hydrolyzed casein.

[0047] Fig. 25 illustrates the pro-inflammatory cytokine secretion of Tumor necrosis factor-alpha from mouse macrophages exposed to extensively hydrolyzed casein, non-sterile filtered extensively hydrolyzed casein, and a greater than 500 Da fraction of extensively hydrolyzed casein.

[0048] Fig. 26 illustrates the pro-inflammatory cytokine secretion of Interleukin-12p40, Interleukin-1 β , Interleukin-6, and Tumor Necrosis Factor-alpha from mouse macrophages

exposed to extensively hydrolyzed casein, non-sterile filtered extensively hydrolyzed casein, and a greater than 500 Da fraction of extensively hydrolyzed casein.

[0049] Fig. 27 illustrates the effect of extensively hydrolysed casein on insulin secretion (n=4) upon exposure to IL-1 β .

[0050] Fig. 28 illustrates the corresponding effects on insulin secretion (n=4) upon exposure to IL-1 β for a greater than 500 Da fraction of the extensively hydrolyzed casein.

[0051] Fig. 29 illustrates the effect of hydrolysate samples on insulin secretion (n=4) upon exposure to IFN- γ .

[0052] Fig. 30 illustrates the effect of extensively hydrolyzed casein on insulin secretion (n=4) upon exposure to IL-23.

[0053] Fig. 31 illustrates the corresponding effects with respect to IL-17 for a >500Da fraction of the extensively hydrolysed casein.

BEST MODE FOR CARRYING OUT THE INVENTION

[0054] Reference now will be made in detail to the embodiments of the present disclosure, one or more examples of which are set forth hereinbelow. Each example is provided by way of explanation of the nutritional composition of the present disclosure and is not a limitation. In fact, it will be apparent to those skilled in the art that various modifications and variations can be made to the teachings of the present disclosure without departing from the scope of the disclosure. For instance, features illustrated or described as part of one embodiment, can be used with another embodiment to yield a still further embodiment.

[0055] Thus, it is intended that the present disclosure covers such modifications and variations as come within the scope of the appended claims and their equivalents. Other objects, features and aspects of the present disclosure are disclosed in or are apparent from the following detailed description. It is to be understood by one of ordinary skill in the art that the present discussion is a description of exemplary embodiments only and is not intended as limiting the broader aspects of the present disclosure.

[0056] The present disclosure relates generally to nutritional compositions comprising a peptide component. Additionally, the disclosure relates to methods of reducing the incidence of autoimmune disease and/or diabetes mellitus by providing a target subject a nutritional composition containing the peptide component described herein.

[0057] "Nutritional composition" means a substance or formulation that satisfies at least a portion of a subject's nutrient requirements. The terms "nutritional(s)", "nutritional formula(s)", "enteral nutritional(s)", and "nutritional supplement(s)" are used as non-limiting examples of nutritional composition(s) throughout the present disclosure. Moreover, "nutritional composition(s)" may refer to liquids, powders, gels, pastes, solids, concentrates, suspensions, or ready-to-use forms of enteral formulas, oral formulas, formulas for infants,

formulas for pediatric subjects, formulas for children, growing-up milks and/or formulas for adults.

[0058] The term "enteral" means deliverable through or within the gastrointestinal, or digestive, tract. "Enteral administration" includes oral feeding, intragastric feeding, transpyloric administration, or any other administration into the digestive tract.

"Administration" is broader than "enteral administration" and includes parenteral administration or any other route of administration by which a substance is taken into a subject's body.

[0059] The term "peptide" as used herein describes linear molecular chains of amino acids, including single chain molecules or their fragments. The peptides described herein include no more than 50 amino acids total. Peptides may further form oligomers or multimers consisting of at least two identical or different molecules. The corresponding higher order structures of such multimers are, correspondingly, termed homo- or heterodimers, homo- or heterotrimers etc. Furthermore, peptidomimetics of such peptides where amino acid(s) and/or peptide bond(s) have been replaced by functional analogs are also encompassed by the term "peptide". Such functional analogues may include, but are not limited to, all known amino acids other than the 20 gene-encoded amino acids such as selenocysteine.

[0060] The term "peptide" may also refer to naturally modified peptides where the modification is effected, for example, by glycosylation, acetylation, phosphorylation and similar modification which are well known in the art. Further, peptides may, for example, be produced recombinantly, semi-synthetically, synthetically, or obtained from natural sources such as after hydrolysis of proteins, all according to methods known in the art.

[0061] The term "degree of hydrolysis" refers to the extent to which peptide bonds are broken by a hydrolysis method. The peptides present after hydrolysis may be hydrolyzed to various degrees. For example, the protein equivalent source of the present disclosure may, in some embodiments comprise a protein having a degree of hydrolysis of no greater than 40%. This means that no greater than 40% of the peptide bonds of the protein have been broken by a hydrolysis method.

[0062] The term "partially hydrolyzed" means having a degree of hydrolysis which is greater than 0% but less than 50%.

[0063] The term "extensively hydrolyzed" means having a degree of hydrolysis which is greater than or equal to 50%.

[0064] The term "molar mass distribution" when used in reference to a hydrolyzed protein or protein hydrolysate pertains to the molar mass of each peptide present in the protein hydrolysate. For example, a protein hydrolysate having a molar mass distribution of greater

than 500 Daltons means that each peptide included in the protein hydrolysate has a molar mass of at least 500 Daltons. Accordingly, in some embodiments, the peptides disclosed in Table 1 and Table 2 are derived from a protein hydrolysate having a molar mass distribution of greater than 500 Daltons. To produce a protein hydrolysate having a molar mass distribution of greater than 500 Daltons, a protein hydrolysate may be subjected to certain filtering procedures or any other procedure known in the art for removing peptides, amino acids, and/or other proteinaceous material having a molar mass of less than 500 Daltons. For the purposes of this disclosure, any method known in the art may be used to produce the protein hydrolysate having a molar mass distribution of greater than 500 Dalton.

[0065] The term “protein equivalent” or “protein equivalent source” includes any protein source, such as soy, egg, whey, or casein, as well as non-protein sources, such as peptides or amino acids. The protein equivalent source can be any used in the art, e.g., nonfat milk, whey protein, casein, soy protein, hydrolyzed protein, amino acids, and the like. Bovine milk protein sources useful in practicing the present disclosure include, but are not limited to, milk protein powders, milk protein concentrates, milk protein isolates, nonfat milk solids, nonfat milk, nonfat dry milk, whey protein, whey protein isolates, whey protein concentrates, sweet whey, acid whey, casein, acid casein, caseinate (*e.g.* sodium caseinate, sodium calcium caseinate, calcium caseinate), soy bean proteins, and any combinations thereof. The protein equivalent source can, in some embodiments comprise hydrolyzed protein, including partially hydrolyzed protein and extensively hydrolyzed protein. The protein equivalent source may, in some embodiments, include intact protein.

[0066] The term “protein equivalent source” also encompasses free amino acids. In some embodiments, the amino acids may comprise, but are not limited to, histidine, isoleucine, leucine, lysine, methionine, cysteine, phenylalanine, tyrosine, threonine, tryptophan, valine, alanine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, proline, serine, carnitine, taurine and mixtures thereof. In some embodiments, the amino acids may be branched chain amino acids. In certain other embodiments, small amino acid peptides may be included as the protein component of the nutritional composition. Such small amino acid peptides may be naturally occurring or synthesized.

[0067] “Pediatric subject” means a human less than 13 years of age. In some embodiments, a pediatric subject refers to a human subject that is between birth and 8 years old. In other embodiments, a pediatric subject refers to a human subject between 1 and 6 years of age. In still further embodiments, a pediatric subject refers to a human subject between 6 and 12 years of age. The term “pediatric subject” may refer to infants (preterm or fullterm) and/or children, as described below.

[0068] "Infant" means a human subject ranging in age from birth to not more than one year and includes infants from 0 to 12 months corrected age. The phrase "corrected age" means an infant's chronological age minus the amount of time that the infant was born premature. Therefore, the corrected age is the age of the infant if it had been carried to full term. The term infant includes low birth weight infants, very low birth weight infants, and preterm infants. "Preterm" means an infant born before the end of the 37th week of gestation. "Full term" means an infant born after the end of the 37th week of gestation.

[0069] "Child" means a subject ranging in age from 12 months to about 13 years. In some embodiments, a child is a subject between the ages of 1 and 12 years old. In other embodiments, the terms "children" or "child" refer to subjects that are between one and about six years old, or between about seven and about 12 years old. In other embodiments, the terms "children" or "child" refer to any range of ages between 12 months and about 13 years.

[0070] "Children's nutritional product" refers to a composition that satisfies at least a portion of the nutrient requirements of a child. A growing-up milk is an example of a children's nutritional product.

[0071] "Infant formula" means a composition that satisfies at least a portion of the nutrient requirements of an infant. In the United States, the content of an infant formula is dictated by the federal regulations set forth at 21 C.F.R. Sections 100, 106, and 107. These regulations define macronutrient, vitamin, mineral, and other ingredient levels in an effort to simulate the nutritional and other properties of human breast milk.

[0072] The term "growing-up milk" refers to a broad category of nutritional compositions intended to be used as a part of a diverse diet in order to support the normal growth and development of a child between the ages of about 1 and about 6 years of age.

[0073] "Nutritionally complete" means a composition that may be used as the sole source of nutrition, which would supply essentially all of the required daily amounts of vitamins, minerals, and/or trace elements in combination with proteins, carbohydrates, and lipids. Indeed, "nutritionally complete" describes a nutritional composition that provides adequate amounts of carbohydrates, lipids, essential fatty acids, proteins, essential amino acids, conditionally essential amino acids, vitamins, minerals and energy required to support normal growth and development of a subject.

[0074] Therefore, a nutritional composition that is "nutritionally complete" for a preterm infant will, by definition, provide qualitatively and quantitatively adequate amounts of carbohydrates, lipids, essential fatty acids, proteins, essential amino acids, conditionally essential amino acids, vitamins, minerals, and energy required for growth of the preterm infant.

[0075] A nutritional composition that is “nutritionally complete” for a full term infant will, by definition, provide qualitatively and quantitatively adequate amounts of all carbohydrates, lipids, essential fatty acids, proteins, essential amino acids, conditionally essential amino acids, vitamins, minerals, and energy required for growth of the full term infant.

[0076] A nutritional composition that is “nutritionally complete” for a child will, by definition, provide qualitatively and quantitatively adequate amounts of all carbohydrates, lipids, essential fatty acids, proteins, essential amino acids, conditionally essential amino acids, vitamins, minerals, and energy required for growth of a child.

[0077] As applied to nutrients, the term “essential” refers to any nutrient that cannot be synthesized by the body in amounts sufficient for normal growth and to maintain health and that, therefore, must be supplied by the diet. The term “conditionally essential” as applied to nutrients means that the nutrient must be supplied by the diet under conditions when adequate amounts of the precursor compound is unavailable to the body for endogenous synthesis to occur.

[0078] “Prebiotic” means a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the digestive tract that can improve the health of the host.

[0079] “Probiotic” means a microorganism with low or no pathogenicity that exerts at least one beneficial effect on the health of the host.

[0080] The term “inactivated probiotic” means a probiotic wherein the metabolic activity or reproductive ability of the referenced probiotic organism has been reduced or destroyed. The “inactivated probiotic” does, however, still retain, at the cellular level, at least a portion its biological glycol-protein and DNA/RNA structure. As used herein, the term “inactivated” is synonymous with “non-viable”. More specifically, a non-limiting example of an inactivated probiotic is inactivated *Lactobacillus rhamnosus* GG (“LGG”) or “inactivated LGG”.

[0081] All percentages, parts and ratios as used herein are by weight of the total formulation, unless otherwise specified.

[0082] The nutritional composition of the present disclosure may be substantially free of any optional or selected ingredients described herein, provided that the remaining nutritional composition still contains all of the required ingredients or features described herein. In this context, and unless otherwise specified, the term “substantially free” means that the selected composition may contain less than a functional amount of the optional ingredient, typically less than 0.1% by weight, and also, including zero percent by weight of such optional or selected ingredient.

[0083] All references to singular characteristics or limitations of the present disclosure shall include the corresponding plural characteristic or limitation, and vice versa, unless otherwise specified or clearly implied to the contrary by the context in which the reference is made.

[0084] All combinations of method or process steps as used herein can be performed in any order, unless otherwise specified or clearly implied to the contrary by the context in which the referenced combination is made.

[0085] The methods and compositions of the present disclosure, including components thereof, can comprise, consist of, or consist essentially of the essential elements and limitations of the embodiments described herein, as well as any additional or optional ingredients, components or limitations described herein or otherwise useful in nutritional compositions.

[0086] As used herein, the term "about" should be construed to refer to both of the numbers specified as the endpoint(s) of any range. Any reference to a range should be considered as providing support for any subset within that range.

[0087] Autoimmune diseases, including diabetes mellitus, are widespread, chronic diseases that have no cure. The incidence and prevalence of autoimmune diseases is increasing exponentially. For example, diabetes mellitus is among the most common metabolic disorders in developed and developing countries. Further, immunosuppressive agents used to treat autoimmune diseases have unwanted side effects and may diminish the immune system's ability to fight off infection.

[0088] Diabetes mellitus is associated with hyperglycemia, hypercholesterolemia and hyperlipidemia. Uncontrolled hyperglycemia is associated with increase and premature mortality due to an increased risk for microvascular and macrovascular diseases, including nephropathy, neuropathy, retinopathy, hypertension, stroke, and heart disease.

[0089] Accordingly, the present disclosure relates generally to nutritional compositions comprising a protein equivalent source, wherein the protein equivalent source includes a peptide component comprising SEQ ID NO 4, SEQ ID NO 13, SEQ ID NO 17, SEQ ID NO 21, SEQ ID NO 24, SEQ ID NO 30, SEQ ID NO 31, SEQ ID NO 32, SEQ ID NO 51, SEQ ID NO 57, SEQ ID NO 60, and SEQ ID NO 63. In some embodiments, the peptide component may comprise additional peptides disclosed in Table 1. For example, the composition may include at least 10 additional peptides disclosed in Table 1. In some embodiments, 20% to 80% of the protein equivalent source comprises the peptide component, and 20% to 80% of the protein equivalent source comprises an intact protein, a partially hydrolyzed protein, and combinations thereof. In some embodiments, the term "additional" means selecting different peptides than those enumerated.

[0090] In another embodiment 20% to 80% of the protein equivalent source includes a peptide component comprising at least 5 peptides selected from Table 1 and at least 3 additional peptides selected from Table 2; and 20% to 80% of the protein equivalent source comprises an intact protein, a partially hydrolyzed protein, or combinations thereof.

[0091] Without being bound by any particular theory the peptide component comprising peptides identified in Table 1 and/or Table 2 may prevent the elevation of blood glucose levels, promote glycogen storage, enhance physical strength and/or promote glycogen storage. Additionally, providing a nutritional composition including certain peptides from Table 1 and/or Table 2 may prevent or reduce the incidence of autoimmune disease and/or diabetes mellitus.

[0092] Table 1 below identifies the specific amino acid sequences of the peptides that may be included in the peptide component disclosed herein.

TABLE 1

SEQ ID	Amino Acid Sequence									(aa)
1	Ala	Ile	Asn	Pro	Ser	Lys	Glu	Asn		8
2	Ala	Pro	Phe	Pro	Glu					5
3	Asp	Ile	Gly	Ser	Glu	Ser				6
4	Asp	Lys	Thr	Glu	Ile	Pro	Thr			7
5	Asp	Met	Glu	Ser	Thr					5
6	Asp	Met	Pro	Ile						4
7	Asp	Val	Pro	Ser						4
n/a	Glu	Asp	Ile							3
n/a	Glu	Leu	Phe							3
n/a	Glu	Met	Pro							3
8	Glu	Thr	Ala	Pro	Val	Pro	Leu			7
9	Phe	Pro	Gly	Pro	Ile	Pro				6
10	Phe	Pro	Gly	Pro	Ile	Pro	Asn			7
11	Gly	Pro	Phe	Pro						4
12	Gly	Pro	Ile	Val						4
13	Ile	Gly	Ser	Glu	Ser	Thr	Glu	Asp	Gln	9
14	Ile	Gly	Ser	Ser	Ser	Glu	Glu	Ser		8
15	Ile	Gly	Ser	Ser	Ser	Glu	Glu	Ser	Ala	9
16	Ile	Asn	Pro	Ser	Lys	Glu				6
17	Ile	Pro	Asn	Pro	Ile					5

18	Ile	Pro	Asn	Pro	Ile	Gly						6
19	Ile	Pro	Pro	Leu	Thr	Gln	Thr	Pro	Val			9
20	Ile	Thr	Ala	Pro								4
21	Ile	Val	Pro	Asn								4
22	Lys	His	Gln	Gly	Leu	Pro	Gln					7
23	Leu	Asp	Val	Thr	Pro							5
24	Leu	Glu	Asp	Ser	Pro	Glu						6
25	Leu	Pro	Leu	Pro	Leu							5
26	Met	Glu	Ser	Thr	Glu	Val						6
27	Met	His	Gln	Pro	His	Gln	Pro	Leu	Pro	Pro	Thr	11
28	Asn	Ala	Val	Pro	Ile							5
29	Asn	Glu	Val	Glu	Ala							5
n/a	Asn	Leu	Leu									3
30	Asn	Gln	Glu	Gln	Pro	Ile						6
31	Asn	Val	Pro	Gly	Glu							5
32	Pro	Phe	Pro	Gly	Pro	Ile						6
33	Pro	Gly	Pro	Ile	Pro	Asn						6
34	Pro	His	Gln	Pro	Leu	Pro	Pro	Thr				8
35	Pro	Ile	Thr	Pro	Thr							5
36	Pro	Asn	Pro	Ile								4
37	Pro	Asn	Ser	Leu	Pro	Gln						6
38	Pro	Gln	Leu	Glu	Ile	Val	Pro	Asn				8
39	Pro	Gln	Asn	Ile	Pro	Pro	Leu					7
40	Pro	Val	Leu	Gly	Pro	Val						6
41	Pro	Val	Pro	Gln								4
42	Pro	Val	Val	Val	Pro							5
43	Pro	Val	Val	Val	Pro	Pro						6
44	Ser	Ile	Gly	Ser	Ser	Ser	Glu	Glu	Ser	Ala	Glu	11
45	Ser	Ile	Ser	Ser	Ser	Glu	Glu					7
46	Ser	Ile	Ser	Ser	Ser	Glu	Glu	Ile	Val	Pro	Asn	11
47	Ser	Lys	Asp	Ile	Gly	Ser	Glu					7
48	Ser	Pro	Pro	Glu	Ile	Asn						6
49	Ser	Pro	Pro	Glu	Ile	Asn	Thr					7
50	Thr	Asp	Ala	Pro	Ser	Phe	Ser					7
51	Thr	Glu	Asp	Glu	Leu							5

52	Val	Ala	Thr	Glu	Glu	Val					6
53	Val	Leu	Pro	Val	Pro						5
54	Val	Pro	Gly	Glu							4
55	Val	Pro	Gly	Glu	Ile	Val					6
56	Val	Pro	Ile	Thr	Pro	Thr					6
57	Val	Pro	Ser	Glu							4
58	Val	Val	Pro	Pro	Phe	Leu	Gln	Pro	Glu		9
59	Val	Val	Val	Pro	Pro						5
60	Tyr	Pro	Phe	Pro	Gly	Pro					6
61	Tyr	Pro	Phe	Pro	Gly	Pro	Ile	Pro			8
62	Tyr	Pro	Phe	Pro	Gly	Pro	Ile	Pro	Asn		9
63	Tyr	Pro	Ser	Gly	Ala						5
64	Tyr	Pro	Val	Glu	Pro						5

[0093] Table 2 below further identifies a subset of specific amino acid sequences of Table 1 that may be included and/or comprise the peptide component disclosed herein.

TABLE 2

SEQ ID	Amino Acid Sequence										(aa)
4	Asp	Lys	Thr	Glu	Ile	Pro	Thr				7
13	Ile	Gly	Ser	Glu	Ser	Thr	Glu	Asp	Gln		9
17	Ile	Pro	Asn	Pro	Ile	Gly					6
21	Ile	Val	Pro	Asn							4
24	Leu	Glu	Asp	Ser	Pro	Glu					6
30	Asn	Gln	Glu	Gln	Pro	Ile					6
31	Asn	Val	Pro	Gly	Glu						5
32	Pro	Phe	Pro	Gly	Pro	Ile					6
51	Thr	Glu	Asp	Glu	Leu						5
57	Val	Pro	Ser	Glu							4
60	Tyr	Pro	Phe	Pro	Gly	Pro					6
63	Tyr	Pro	Ser	Gly	Ala						5

[0094] In some embodiments, the peptide component may be present in the nutritional composition in an amount from about 0.2 g/100 kcal to about 5.6 g/100 kcal. In other embodiments the peptide component may be present in the nutritional composition in an amount from about 1 g/100 kcal to about 4 g/100 kcal. In still other embodiments, the

peptide component may be present in the nutritional composition in an amount from about 2 g/100 kcal to about 3 g/100 kcal.

[0095] The peptide component may be formulated with other ingredients in the nutritional composition to provide appropriate nutrient levels for the target subject. In some embodiments, the peptide component is included in a nutritionally complete formula that is suitable to support normal growth.

[0096] In some embodiments, the peptide component may comprise a nutritional supplement that can be added to other nutritional formulations, foodstuffs or beverages. For the purposes of this disclosure, "nutritional supplement" includes a concentrated source of nutrient, for example the peptides identified herein, or alternatively other substances with a nutritional or physiological effective whose purpose is to supplement the normal diet.

[0097] The peptide component may be provided as an element of a protein equivalent source. In some embodiments, the peptides identified in Tables 1 and 2, may be provided by a protein equivalent source obtained from cow's milk proteins, including but not limited to bovine casein and bovine whey. In some embodiments, the protein equivalent source comprises hydrolyzed bovine casein or hydrolyzed bovine whey. Accordingly, in some embodiments, the peptides identified in Table 1 and Table 2 may be provided by a casein hydrolysate. Such peptides may be obtained by hydrolysis or may be synthesized in vitro by methods known to the skilled person.

[0098] A nonlimiting example of a method of hydrolysis is disclosed herein. In some embodiments, this method may be used to obtain the protein hydrolysate and peptides of the present disclosure. The proteins are hydrolyzed using a proteolytic enzyme, Protease N. Protease N "Amano" is commercially available from Amano Enzyme U.S.A. Co., Ltd., Elgin, Ill. Protease N is a proteolytic enzyme preparation that is derived from the bacterial species *Bacillus subtilis*. The protease powder is specified as "not less than 150,000 units/g", meaning that one unit of Protease N is the amount of enzyme which produces an amino acid equivalent to 100 micrograms of tyrosine for 60 minutes at a pH of 7.0. To produce the infant formula of the present invention, Protease N can be used at levels of about 0.5% to about 1.0% by weight of the total protein being hydrolyzed.

[0099] The protein hydrolysis by Protease N is typically conducted at a temperature of about 50° C. to about 60° C. The hydrolysis occurs for a period of time so as to obtain a degree of hydrolysis between about 4% and 10%. In a particular embodiment, hydrolysis occurs for a period of time so as to obtain a degree of hydrolysis between about 6% and 9%. In another embodiment, hydrolysis occurs for a period of time so as to obtain a degree of hydrolysis of about 7.5%. This level of hydrolysis may take between about one half hour to about 3 hours.

[0100] A constant pH should be maintained during hydrolysis. In the method of the present invention, the pH is adjusted to and maintained between about 6.5 and 8. In a particular embodiment, the pH is maintained at about 7.0.

[0101] In order to maintain the optimal pH of the solution of whey protein, casein, water and Protease N, a caustic solution of sodium hydroxide and/or potassium hydroxide can be used to adjust the pH during hydrolysis. If sodium hydroxide is used to adjust the pH, the amount of sodium hydroxide added to the solution should be controlled to the level that it comprises less than about 0.3% of the total solid in the finished protein hydrolysate. A 10% potassium hydroxide solution can also be used to adjust the pH of the solution to the desired value, either before the enzyme is added or during the hydrolysis process in order to maintain the optimal pH.

[0102] The amount of caustic solution added to the solution during the protein hydrolysis can be controlled by a pH-stat or by adding the caustic solution continuously and proportionally. The hydrolysate can be manufactured by standard batch processes or by continuous processes.

[0103] To better ensure the consistent quality of the protein partial hydrolysate, the hydrolysate is subjected to enzyme deactivation to end the hydrolysis process. The enzyme deactivation step may consist include at heat treatment at a temperature of about 82° C. for about 10 minutes. Alternatively, the enzyme can be deactivated by heating the solution to a temperature of about 92° C. for about 5 seconds. After enzyme deactivation is complete, the hydrolysate can be stored in a liquid state at a temperature lower than 10° C.

EXAMPLE 1

[0104] Example 1 further illustrates a method for producing a protein partial hydrolysate. Initially, 60.3 kg non-milk solids (milk powder) and 37.4 kg whey protein concentrate (60%) were intermixed in a tank containing water at 54° C. The slurry had a total solids content of between 20% and 23%. The pH of the slurry was then measured. Sodium and potassium hydroxide were added to the slurry to adjust the pH of the slurry to 7.0. After adjusting the pH, 0.5 kg of Amano N enzyme was added to the slurry. Following the addition of Amano N to the slurry, the pH was continuously adjusted to a pH of 7.0 using sodium hydroxide and potassium hydroxide. The total amount of sodium hydroxide added to the slurry was 0.3 kg. The total amount of potassium hydroxide added to the slurry was 1.5 kg.

[0105] The hydrolysis was permitted to occur for 90 minutes, the time starting with the addition of Amano N enzyme to the slurry. At the end of 90 minutes, the slurry was heat treated to inactivate the enzyme. The heat treatment consisted of raising the temperature of the slurry to 82° C. for 10 minutes. The degree of hydrolysis obtained in this example was

between 6% and 9%. The slurry was then cooled and spray dried to obtain a powdered hydrolysate.

EXAMPLE 2

[0106] Example 2 provides a non-limiting method of determining the molecular weight distribution of the hydrolysate peptides.

[0107] Size exclusion chromatography (SEC) was used to determine the molecular weight distribution of the hydrolysate peptides created by the presently-described hydrolysis process. Specifically, a sufficient amount of the powdered infant formula was weighed out to provide 0.5 grams of protein into a 50 ml conical centrifuge tube. Water was added to bring the tube to a volume of 45 ml. The mixture was placed in a Sarstedt D-5223 Mixer and mixed for one hour. After mixing, a 1% protein solution was created by adding another 5 ml of water to the tube. A stock standard was prepared and mixed for one hour as well.

[0108] Separately, 14.91 grams potassium chloride (KCl) was added to a 1000 ml beaker. The KCl was dissolved by adding 700 ml of water to the beaker. 250 ml acetonitrile and 1.0 ml trifluoroacetic acid were then added to the KCl solution (eluent). The pH was adjusted to 3.0 using a 0.2M K₂HPO₄ solution.

[0109] An HPCL reagent bottle was filled and the bottle was washed with eluent, reserving about 50 ml for dilution of samples and standards. The Hitachi L-6200 A Intelligent Pump lines were purged with eluent and the columns were equilibrated with eluent for one hour.

[0110] After the samples were mixed for one hour, 5.0 ml of each sample was pipetted into glass screw-cap tubes. 5.0 ml Dichloromethane was also pipetted into each tube. The tubes were capped and mixed by inversion four times. The samples were then centrifuged for five minutes at 200×g.

[0111] While the samples were in the centrifuge, the stock standards 1-5 were diluted with eluent (800 ul+3200 ul). Approximately 1 ml of each standard was pipetted into each of two autosampler vials and capped.

[0112] The upper (aqueous) layer of the centrifuged samples 1-10 were diluted with eluent (100 ul+900 ul). The vials were loaded into the autosampler tray as follows: blank, standard, samples and second standard. The tray was placed in the Hitachi autosampler. The total number of vials to be run were entered into the autosampler program using the keys on the front of the autosampler and the samples were run. The results indicated the molecular weight profile of the protein.

[0113] In some embodiments, the protein equivalent source comprises a partially hydrolyzed protein, such as casein. In some embodiments the protein equivalent source comprises a hydrolyzed protein including peptides having a molar mass distribution of greater than 500 Daltons. In some embodiments, the hydrolyzed protein comprises peptides having a molar

mass distribution in the range of from about 500 Daltons to about 1,500 Daltons. Still, in some embodiments the hydrolyzed protein may comprise peptides having a molar mass distribution range of from about 500 Daltons to about 2,000 Daltons.

[0114] In some embodiments, the protein equivalent source may comprise the peptide component, intact protein, hydrolyzed protein, including partially hydrolyzed protein, and combinations thereof. In some embodiments, 20% to 80% of the protein equivalent source comprises the peptide component disclosed herein. In some embodiments, 40% to 70% of the protein equivalent source comprises the peptide component disclosed herein. In still other embodiments, 50% to 60% of the protein equivalent source comprises the peptide component.

[0115] In some embodiments, 20% to 80% of the protein equivalent source comprises intact protein, partially hydrolyzed protein, or combinations thereof. In some embodiments, 40% to 70% of the protein equivalent source comprises intact proteins, partially hydrolyzed proteins, or a combination thereof. In still further embodiments, 50% to 60% of the protein equivalent source may comprise intact proteins, partially hydrolyzed protein, or a combination thereof.

[0116] In some embodiments the protein equivalent source comprises partially hydrolyzed protein having a degree of hydrolysis of less than 40%. In still other embodiments, the protein equivalent source may comprise partially hydrolyzed protein having a degree of hydrolysis of less than 25%, or less than 15%.

[0117] In some embodiments, the nutritional composition comprises between about 1 g and about 7 g of a protein equivalent source per 100 kcal. In other embodiments, the nutritional composition comprises between about 3.5 g and about 4.5 g of protein equivalent source per 100 kcal.

[0118] The protein equivalent source including the peptide component may be added or incorporated into the nutritional composition by any method well known in the art. In some embodiments, the peptide component may be added to a nutritional composition to supplement the nutritional composition. For example, in one embodiment, peptide component may be added to a commercially available infant formula. For example, Enfalac, Enfamil®, Enfamil® Premature Formula, Enfamil® with Iron, Enfamil® LIPIL®, Lactofree®, Nutramigen®, Pregestimil®, and ProSobee® (available from Mead Johnson & Company, Evansville, IN, U.S.A.) may be supplemented with suitable levels of the peptide component, and used in practice of the present disclosure.

[0119] The nutritional composition(s) of the present disclosure may be administered in one or more doses daily. Any orally acceptable dosage form is contemplated by the present disclosure. Examples of such dosage forms include, but are not limited to pills, tablets,

capsules, soft-gels, liquids, liquid concentrates, powders, elixirs, solutions, suspensions, emulsions, lozenges, beads, cachets, and combinations thereof.

[0120] In some embodiments, the protein equivalent source including the peptide component described herein may be added to a more complete nutritional product. In this embodiment, the nutritional composition may contain protein, fat, and carbohydrate components and may be used to supplement the diet or may be used as the sole source of nutrition.

[0121] In some embodiments, the nutritional composition comprises at least one carbohydrate source. The carbohydrate source can be any used in the art, e.g., lactose, glucose, fructose, corn syrup solids, maltodextrins, sucrose, starch, rice syrup solids, and the like. The amount of the carbohydrate component in the nutritional composition typically can vary from between about 5 g/100 kcal and about 25 g/100 kcal. In some embodiments, the amount of carbohydrate is between about 6 g/100 kcal and about 22 g/100 kcal. In other embodiments, the amount of carbohydrate is between about 12 g/100 kcal and about 14 g/100 kcal. In some embodiments, corn syrup solids are preferred. Moreover, hydrolyzed, partially hydrolyzed, and/or extensively hydrolyzed carbohydrates may be desirable for inclusion in the nutritional composition due to their easy digestibility. Specifically, hydrolyzed carbohydrates are less likely to contain allergenic epitopes.

[0122] Non-limiting examples of carbohydrate materials suitable for use herein include hydrolyzed or intact, naturally or chemically modified, starches sourced from corn, tapioca, rice or potato, in waxy or non-waxy forms. Non-limiting examples of suitable carbohydrates include various hydrolyzed starches characterized as hydrolyzed cornstarch, maltodextrin, maltose, corn syrup, dextrose, corn syrup solids, glucose, and various other glucose polymers and combinations thereof. Non-limiting examples of other suitable carbohydrates include those often referred to as sucrose, lactose, fructose, high fructose corn syrup, indigestible oligosaccharides such as fructooligosaccharides and combinations thereof.

[0123] The nutritional composition may be protein-free in some embodiments and comprise free amino acids as an element of the protein equivalent source. In some embodiments, the amino acids may be branched chain amino acids. In certain other embodiments, small amino acid peptides may be included as the protein component of the nutritional composition. Such small amino acid peptides may be naturally occurring or synthesized. The amount of free amino acids in the nutritional composition may vary from about 1 g/100 kcal to about 5 g/100 kcal.

[0124] The nutritional composition may also comprise a fat source. Suitable fat or lipid sources for the nutritional composition of the present disclosure may be any known or used in the art, including but not limited to, animal sources, e.g., milk fat, butter, butter fat, egg

yolk lipid; marine sources, such as fish oils, marine oils, single cell oils; vegetable and plant oils, such as corn oil, canola oil, sunflower oil, soybean oil, palm olein oil, coconut oil, high oleic sunflower oil, evening primrose oil, rapeseed oil, olive oil, flaxseed (linseed) oil, cottonseed oil, high oleic safflower oil, palm stearin, palm kernel oil, wheat germ oil; medium chain triglyceride oils and emulsions and esters of fatty acids; and any combinations thereof.

[0125] In some embodiment the nutritional composition comprises between about 1.3 g/100 kcal to about 7.2 g/100 kcal of a fat source. In other embodiments the fat source may be present in an amount from about 2.5 g/100 kcal to about 6.0 g/100 kcal. In still other embodiments, the fat source may be present in the nutritional composition in an amount from about 3.0 g/100 kcal to about 4.0 g/100 kcal.

[0126] The nutritional composition of the present disclosure may also contain a source of long chain polyunsaturated fatty acids ("LCPUFAs"). Suitable LCPUFAs include, but are not limited to DHA, eicosapentaenoic acid ("EPA"), ARA, linoleic (18:2 n-6), γ -linolenic (18:3 n-6), dihomo- γ -linolenic (20:3 n-6) acids in the n-6 pathway, α -linolenic (18:3 n-3), stearidonic (18:4 n-3), eicosatetraenoic (20:4 n-3), eicosapentaenoic (20:5 n-3), and docosapentaenoic (22:6 n-3).

[0127] The amount of LCPUFA in the nutritional composition is advantageously at least about 5 mg/100 kcal, and may vary from about 5 mg/100 kcal to about 100 mg/100 kcal, more preferably from about 10 mg/100 kcal to about 50 mg/100 kcal.

[0128] Sources of LCPUFAs include dairy products like eggs and butterfat; marine oils, such as cod, menhaden, sardine, tuna and many other fish; certain animal fats, lard, tallow and microbial oils such as fungal and algal oils, or from any other resource fortified or not, from which LCPUFAs could be obtained and used in a nutritional composition. The LCPUFA could be part of a complex mixture obtained by separation technology known in the art aimed at enrichment of LCPUFAs and the derivatives or precursors of LCPUFAs in such mixtures.

[0129] The LCPUFAs may be provided in the nutritional composition in the form of esters of free fatty acids; mono-, di- and tri-glycerides; phosphoglycerides, including lecithins; and/or mixtures thereof. Additionally, LCPUFA may be provided in the nutritional composition in the form of phospholipids, especially phosphatidylcholine.

[0130] In an embodiment, especially if the nutritional composition is an infant formula, the nutritional composition is supplemented with both DHA and ARA. In this embodiment, the weight ratio of ARA:DHA may be between about 1:3 and about 9:1. In a particular embodiment, the weight ratio of ARA:DHA is from about 1:2 to about 4:1.

[0131] DHA is advantageously present in the nutritional composition, in some embodiments, from at least about 17 mg/100 kcal, and may vary from about 5 mg/100 kcal to about 75

mg/100 kcal. In some embodiments, DHA is present from about 10 mg/100 kcal to about 50 mg/100 kcal.

[0132] The nutritional composition may be supplemented with oils containing DHA and/or ARA using standard techniques known in the art. For example, DHA and ARA may be added to the composition by replacing an equivalent amount of an oil, such as high oleic sunflower oil, normally present in the composition. As another example, the oils containing DHA and ARA may be added to the composition by replacing an equivalent amount of the rest of the overall fat blend normally present in the composition without DHA and ARA.

[0133] If utilized, the source of DHA and/or ARA may be any source known in the art such as marine oil, fish oil, single cell oil, egg yolk lipid, and brain lipid. In some embodiments, the DHA and ARA are sourced from single cell Martek oils, DHASCO® and ARASCO®, or variations thereof. The DHA and ARA can be in natural form, provided that the remainder of the LCPUFA source does not result in any substantial deleterious effect on the infant. Alternatively, the DHA and ARA can be used in refined form.

[0134] In an embodiment, sources of DHA and ARA are single cell oils as taught in U.S. Pat. Nos. 5,374,567; 5,550,156; and 5,397,591, the disclosures of which are incorporated herein in their entirety by reference. However, the present disclosure is not limited to only such oils.

[0135] Furthermore, some embodiments of the nutritional composition may mimic certain characteristics of human breast milk. However, to fulfill the specific nutrient requirements of some subjects, the nutritional composition may comprise a higher amount of some nutritional components than does human milk. For example, the nutritional composition may comprise a greater amount of DHA than does human breast milk. The enhanced level of DHA of the nutritional composition may compensate for an existing nutritional DHA deficit.

[0136] The nutritional composition may also contain one or more prebiotics (also referred to as a prebiotic source) in certain embodiments. Prebiotics can stimulate the growth and/or activity of ingested probiotic microorganisms, selectively reduce pathogens found in the gut, and favorably influence the short chain fatty acid profile of the gut. Such prebiotics may be naturally-occurring, synthetic, or developed through the genetic manipulation of organisms and/or plants, whether such new source is now known or developed later. Prebiotics useful in the present disclosure may include oligosaccharides, polysaccharides, and other prebiotics that contain fructose, xylose, soya, galactose, glucose and mannose.

[0137] More specifically, prebiotics useful in the present disclosure may include polydextrose, polydextrose powder, lactulose, lactosucrose, raffinose, gluco-oligosaccharide, inulin, fructo-oligosaccharide, isomalto-oligosaccharide, soybean oligosaccharides, lactosucrose, xylo-oligosaccharide, chito-oligosaccharide, manno-oligosaccharide, aribino-oligosaccharide, siallyl-oligosaccharide, fuco-oligosaccharide, galacto-oligosaccharide, and

gentio-oligosaccharides. In some embodiments, the total amount of prebiotics present in the nutritional composition may be from about 0.1 g/100 kcal to about 1 g/100 kcal. In certain embodiments, the total amount of prebiotics present in the nutritional composition may be from about 0.3 g/100 kcal to about 0.7 g/100 kcal. Moreover, the nutritional composition may comprise a prebiotic component comprising polydextrose ("PDX") and/or galacto-oligosaccharide ("GOS"). In some embodiments, the prebiotic component comprises at least 20% GOS, PDX or a mixture thereof.

[0138] If PDX is used in the prebiotic composition, the amount of PDX in the nutritional composition may, in an embodiment, be within the range of from about 0.1 g/100 kcal to about 1 g/100 kcal. In another embodiment, the amount of polydextrose is within the range of from about 0.2 g/100 kcal to about 0.6 g/100 kcal. And in still other embodiments, the amount of PDX in the nutritional composition may be from about 0.1 mg/100 kcal to about 0.5 mg/100 kcal.

[0139] If GOS is used in the prebiotic composition, the amount of GOS in the nutritional composition may, in an embodiment, be from about 0.1 g/100 kcal to about 1 g/100 kcal. In another embodiment, the amount of GOS in the nutritional composition may be from about 0.2 g/100 kcal to about 0.5 g/100 kcal. In other embodiments, the amount of GOS in the nutritional composition may be from about 0.1 mg/100 kcal to about 1.0 mg/100 kcal or from about 0.1 mg/100 kcal to about 0.5 mg/100 kcal.

[0140] In a particular embodiment of the nutritional composition, PDX is administered in combination with GOS. In this embodiment, PDX and GOS can be administered in a ratio of PDX:GOS of between about 9:1 and 1:9. In another embodiment, the ratio of PDX:GOS can be between about 5:1 and 1:5. In yet another embodiment, the ratio of PDX:GOS can be between about 1:3 and 3:1. In a particular embodiment, the ratio of PDX to GOS can be about 5:5. In another particular embodiment, the ratio of PDX to GOS can be about 8:2.

[0141] In a particular embodiment, GOS and PDX are supplemented into the nutritional composition in a total amount of at least about 0.2 mg/100 kcal or about 0.2 mg/100 kcal to about 1.5 mg/100 kcal. In some embodiments, the nutritional composition may comprise GOS and PDX in a total amount of from about 0.6 to about 0.8 mg/100 kcal.

[0142] In one embodiment, the nutritional composition may contain one or more probiotics. Any probiotic known in the art may be acceptable in this embodiment. In a particular embodiment, the probiotic may be selected from any *Lactobacillus* species, *Lactobacillus rhamnosus* GG (ATCC number 53103), *Bifidobacterium* species, *Bifidobacterium longum* BB536 (BL999, ATCC: BAA-999), *Bifidobacterium longum* AH1206 (NCIMB: 41382), *Bifidobacterium breve* AH1205 (NCIMB: 41387), *Bifidobacterium infantis* 35624 (NCIMB:

41003), and *Bifidobacterium animalis subsp. lactis* BB-12 (DSM No. 10140) or any combination thereof.

[0143] If included in the composition, the amount of the probiotic may vary from about 1×10^4 to about 1.5×10^{10} cfu of probiotics per 100 kcal, more preferably from about 1×10^6 to about 1×10^9 cfu of probiotics per 100 kcal. In certain other embodiments the amount of probiotic may vary from about 1×10^7 cfu/100 kcal to about 1×10^8 cfu/100 kcal.

[0144] In an embodiment, the probiotic(s) may be viable or non-viable. As used herein, the term "viable", refers to live microorganisms. The term "non-viable" or "non-viable probiotic" means non-living probiotic microorganisms, their cellular components and/or metabolites thereof. Such non-viable probiotics may have been heat-killed or otherwise inactivated, but they retain the ability to favorably influence the health of the host. The probiotics useful in the present disclosure may be naturally-occurring, synthetic or developed through the genetic manipulation of organisms, whether such source is now known or later developed.

[0145] In some embodiments, rather than a probiotic itself, the nutritional composition(s) of the present disclosure may comprise a culture supernatant from a late-exponential growth phase of a probiotic batch-cultivation process (hereinafter referred to as the "culture supernatant"); in specific embodiments, the probiotic is LGG. Batch cultivation culture supernatant (which can also be referred to as "spent medium") may possess protection against pathogen infection, including infection by *C. sakazakii*. Specifically the harvested culture supernatant may prevent the invasion of *C. sakazakii* to organs such as the brain and reduce mortality associated with *C. sakazakii*.

[0146] In some embodiments, the nutritional composition comprises a culture supernatant from a late-exponential growth phase of a probiotic batch-cultivation process, for use in the treatment or prevention of pathogen infection. In certain embodiments, the probiotic is LGG, and the pathogen is *C. sakazakii*.

[0147] Without wishing to be bound by theory, it is believed that the activity of the culture supernatant can be attributed to the mixture of components (including proteinaceous materials, and possibly including (exo)polysaccharide materials) as found released into the culture medium at a late stage of the exponential (or "log") phase of batch cultivation of LGG. The chemical composition of the culture supernatant is believed to be a mixture of a plurality of amino acids, oligo- and polypeptides, and proteins, of various molecular weights. The culture supernatant may further comprise polysaccharide structures and/or nucleotides. In some embodiments the culture supernatant pertains to the entire, i.e. unfractionated culture supernatant. Further, in some embodiments the culture supernatant pertains to the entire, i.e. unfractionated culture supernatant.

[0148] The stages recognized in batch cultivation of bacteria are known to the skilled person. These are the "lag," the "log" ("logarithmic" or "exponential"), the "stationary" and the "death" (or "logarithmic decline") phases. In all phases during which live bacteria are present, the bacteria metabolize nutrients from the media, and secrete (exert, release) materials into the culture medium. The composition of the secreted material at a given point in time of the growth stages is not generally predictable.

[0149] In some embodiments, a composition according to the disclosure and/or embodiments thereof is obtainable by a process comprising the steps of (a) subjecting a probiotic such as LGG to cultivation in a suitable culture medium using a batch process; (b) harvesting the culture supernatant at a late exponential growth phase of the cultivation step, which phase is defined with reference to the second half of the time between the lag phase and the stationary phase of the batch-cultivation process; (c) optionally removing low molecular weight constituents from the supernatant so as to retain molecular weight constituents above 5 kiloDaltons (kDa) or even above 6kDa; (d) removing liquid contents from the culture supernatant so as to obtain the composition.

[0150] In the present disclosure and embodiments thereof, secreted materials are harvested from a late exponential phase. The late exponential phase occurs in time after the mid exponential phase (which is halftime of the duration of the exponential phase, hence the reference to the late exponential phase as being the second half of the time between the lag phase and the stationary phase). In particular, the term "late exponential phase" is used herein with reference to the latter quarter portion of the time between the lag phase and the stationary phase of the batch-cultivation process. In some embodiments of the present disclosure, harvesting of the culture supernatant is at a point in time of 75% to 85% of the duration of the exponential phase, and most preferably is at about $\frac{5}{6}$ of the time elapsed in the exponential phase.

[0151] The term "cultivation" or "culturing" refers to the propagation of micro-organisms, in this case LGG, on or in a suitable medium. Such a culture medium can be of a variety of kinds, and is particularly a liquid broth, as customary in the art. A preferred broth, e.g., is MRS broth as generally used for the cultivation of lactobacilli. MRS broth generally comprises polysorbate, acetate, magnesium and manganese, which are known to act as special growth factors for lactobacilli, as well as a rich nutrient base. A typical composition comprises (amounts in g/liter): peptone from casein 10.0; meat extract 8.0; yeast extract 4.0; D(+)-glucose 20.0; dipotassium hydrogen phosphate 2.0; Tween® 80 1.0; triammonium citrate 2.0; sodium acetate 5.0; magnesium sulphate 0.2; manganese sulphate 0.04.

[0152] In some embodiments, the culture supernatant of the present disclosure may be included in a nutritional composition that is an infant formula. The harvesting of secreted

bacterial products brings about a problem that the culture media cannot easily be deprived of undesired components. This specifically relates to nutritional products for relatively vulnerable subjects, such as infant formula or clinical nutrition. This problem is not incurred if specific components from a culture supernatant are first isolated, purified, and then applied in a nutritional product. However, it is desired to make use of a more complete cultural supernatant. This would serve to provide a composition better reflecting the natural action of the probiotic (i.e. LGG). One cannot, however, just use the culture supernatant itself as a basis for non-viable probiotic materials to be specifically used in infant formula and the like.

[0153] In some embodiments, the culture supernatant harvested from LGG cultivation does not contain components (as may be present in the culture medium) that are not desired, or generally accepted, in nutritional compositions, such as an infant formula. With reference to polysorbate regularly present in MRS broth, media for the culturing of bacteria may include an emulsifying non-ionic surfactant, e.g. on the basis of polyethoxylated sorbitan and oleic acid (typically available as Tween® polysorbates, such as Tween® 80). While these surfactants are frequently found in food products, e.g. ice cream, and are generally recognized as safe, they are not in all jurisdictions considered desirable, or even acceptable for use in nutritional products for relatively vulnerable subjects, such as infant formula or clinical nutrition.

[0154] The present disclosure thus, in some embodiments utilizes a culture media in which the aforementioned polysorbates can be avoided. To this end, a culture medium of the disclosure is devoid of polysorbates such as Tween 80. In a preferred embodiment of the disclosure and/or embodiments thereof the culture medium may comprise an oily ingredient selected from the group consisting of oleic acid, linseed oil, olive oil, rapeseed oil, sunflower oil and mixtures thereof. It will be understood that the full benefit of the oily ingredient is attained if the presence of a polysorbate surfactant is essentially or entirely avoided.

[0155] The culture supernatant, in some embodiments, may have a neutral pH, such as a pH of between pH 5 and pH 7, preferably pH 6.

[0156] In addition to the foregoing, it should be noted that the batch cultivation of lactobacilli, including LGG, is common general knowledge available to the person skilled in the art. These methods thus do not require further elucidation here. The culture supernatant of the present disclosure can be harvested by any known technique for the separation of culture supernatant from a bacterial culture. Such techniques are well-known in the art and include, e.g., centrifugation, filtration, sedimentation, and the like.

[0157] The disclosed nutritional composition(s) may be provided in any form known in the art, such as a powder, a gel, a suspension, a paste, a solid, a liquid, a liquid concentrate, a reconstitutable powdered milk substitute or a ready-to-use product. The nutritional

composition may, in certain embodiments, comprise a nutritional supplement, children's nutritional product, infant formula, human milk fortifier, growing-up milk or any other nutritional composition designed for an infant or a pediatric subject. Nutritional compositions of the present disclosure include, for example, orally-ingestible, health-promoting substances including, for example, foods, beverages, tablets, capsules and powders. Moreover, the nutritional composition of the present disclosure may be standardized to a specific caloric content, it may be provided as a ready-to-use product, or it may be provided in a concentrated form. In some embodiments, the nutritional composition is in powder form with a particle size in the range of 5 μm to 1500 μm , more preferably in the range of 10 μm to 300 μm .

[0158] If the nutritional composition is in the form of a ready-to-use product, the osmolality of the nutritional composition may be between about 100 and about 1100 mOsm/kg water, more typically about 200 to about 700 mOsm/kg water.

[0159] In certain embodiments, the nutritional composition is hypoallergenic. In other embodiments, the nutritional composition is kosher and/or halal. In still further embodiments, the nutritional composition contains non-genetically modified ingredients. In an embodiment, the nutritional formulation is sucrose-free. The nutritional composition may also be lactose-free. In other embodiments, the nutritional composition does not contain any medium-chain triglyceride oil. In some embodiments, no carrageenan is present in the composition. In other embodiments, the nutritional composition is free of all gums.

[0160] The nutritional composition of the present disclosure is not limited to compositions comprising nutrients specifically listed herein. Any nutrients may be delivered as part of the composition for the purpose of meeting nutritional needs and/or in order to optimize the nutritional status in a subject.

[0161] Moreover, in some embodiments, the nutritional composition is nutritionally complete, containing suitable types and amounts of lipids, carbohydrates, proteins, vitamins and minerals to be a subject's sole source of nutrition. Indeed, the nutritional composition may optionally include any number of proteins, peptides, amino acids, fatty acids, probiotics and/or their metabolic by-products, prebiotics, carbohydrates and any other nutrient or other compound that may provide many nutritional and physiological benefits to a subject. Further, the nutritional composition of the present disclosure may comprise flavors, flavor enhancers, sweeteners, pigments, vitamins, minerals, therapeutic ingredients, functional food ingredients, food ingredients, processing ingredients or combinations thereof.

[0162] The nutritional composition of the present disclosure may be standardized to a specific caloric content, it may be provided as a ready-to-use product, or it may be provided in a concentrated form.

[0163] In some embodiments, the nutritional composition of the present disclosure is a growing-up milk. Growing-up milks are fortified milk-based beverages intended for children over 1 year of age (typically from 1-3 years of age, from 4-6 years of age or from 1-6 years of age). They are not medical foods and are not intended as a meal replacement or a supplement to address a particular nutritional deficiency. Instead, growing-up milks are designed with the intent to serve as a complement to a diverse diet to provide additional insurance that a child achieves continual, daily intake of all essential vitamins and minerals, macronutrients plus additional functional dietary components, such as non-essential nutrients that have purported health-promoting properties.

[0164] The exact composition of a nutritional composition according to the present disclosure can vary from market-to-market, depending on local regulations and dietary intake information of the population of interest. In some embodiments, nutritional compositions according to the disclosure consist of a milk protein source, such as whole or skim milk, plus added sugar and sweeteners to achieve desired sensory properties, and added vitamins and minerals. The fat composition is typically derived from the milk raw materials. Total protein can be targeted to match that of human milk, cow milk or a lower value. Total carbohydrate is usually targeted to provide as little added sugar, such as sucrose or fructose, as possible to achieve an acceptable taste. Typically, Vitamin A, calcium and Vitamin D are added at levels to match the nutrient contribution of regional cow milk. Otherwise, in some embodiments, vitamins and minerals can be added at levels that provide approximately 20% of the dietary reference intake (DRI) or 20% of the Daily Value (DV) per serving. Moreover, nutrient values can vary between markets depending on the identified nutritional needs of the intended population, raw material contributions and regional regulations.

[0165] One or more vitamins and/or minerals may also be added in to the nutritional composition in amounts sufficient to supply the daily nutritional requirements of a subject. It is to be understood by one of ordinary skill in the art that vitamin and mineral requirements will vary, for example, based on the age of the child. For instance, an infant may have different vitamin and mineral requirements than a child between the ages of one and thirteen years. Thus, the embodiments are not intended to limit the nutritional composition to a particular age group but, rather, to provide a range of acceptable vitamin and mineral components.

[0166] In embodiments providing a nutritional composition for a child, the composition may optionally include, but is not limited to, one or more of the following vitamins or derivations thereof: vitamin B₁ (thiamin, thiamin pyrophosphate, TPP, thiamin triphosphate, TTP, thiamin hydrochloride, thiamin mononitrate), vitamin B₂ (riboflavin, flavin mononucleotide, FMN, flavin adenine dinucleotide, FAD, lactoflavin, ovoflavin), vitamin B₃ (niacin, nicotinic acid,

nicotinamide, niacinamide, nicotinamide adenine dinucleotide, NAD, nicotinic acid mononucleotide, NicMN, pyridine-3-carboxylic acid), vitamin B₃-precursor tryptophan, vitamin B₆ (pyridoxine, pyridoxal, pyridoxamine, pyridoxine hydrochloride), pantothenic acid (pantothenate, panthenol), folate (folic acid, folacin, pteroylglutamic acid), vitamin B₁₂ (cobalamin, methylcobalamin, deoxyadenosylcobalamin, cyanocobalamin, hydroxycobalamin, adenosylcobalamin), biotin, vitamin C (ascorbic acid), vitamin A (retinol, retinyl acetate, retinyl palmitate, retinyl esters with other long-chain fatty acids, retinal, retinoic acid, retinol esters), vitamin D (calciferol, cholecalciferol, vitamin D₃, 1,25,-dihydroxyvitamin D), vitamin E (α-tocopherol, α-tocopherol acetate, α-tocopherol succinate, α-tocopherol nicotinate, α-tocopherol), vitamin K (vitamin K₁, phyloquinone, naphthoquinone, vitamin K₂, menaquinone-7, vitamin K₃, menaquinone-4, menadione, menaquinone-8, menaquinone-8H, menaquinone-9, menaquinone-9H, menaquinone-10, menaquinone-11, menaquinone-12, menaquinone-13), choline, inositol, β-carotene and any combinations thereof.

[0167] In embodiments providing a children's nutritional product, such as a growing-up milk, the composition may optionally include, but is not limited to, one or more of the following minerals or derivations thereof: boron, calcium, calcium acetate, calcium gluconate, calcium chloride, calcium lactate, calcium phosphate, calcium sulfate, chloride, chromium, chromium chloride, chromium picolinate, copper, copper sulfate, copper gluconate, cupric sulfate, fluoride, iron, carbonyl iron, ferric iron, ferrous fumarate, ferric orthophosphate, iron trituration, polysaccharide iron, iodide, iodine, magnesium, magnesium carbonate, magnesium hydroxide, magnesium oxide, magnesium stearate, magnesium sulfate, manganese, molybdenum, phosphorus, potassium, potassium phosphate, potassium iodide, potassium chloride, potassium acetate, selenium, sulfur, sodium, docusate sodium, sodium chloride, sodium selenate, sodium molybdate, zinc, zinc oxide, zinc sulfate and mixtures thereof. Non-limiting exemplary derivatives of mineral compounds include salts, alkaline salts, esters and chelates of any mineral compound.

[0168] The minerals can be added to growing-up milks or to other children's nutritional compositions in the form of salts such as calcium phosphate, calcium glycerol phosphate, sodium citrate, potassium chloride, potassium phosphate, magnesium phosphate, ferrous sulfate, zinc sulfate, cupric sulfate, manganese sulfate, and sodium selenite. Additional vitamins and minerals can be added as known within the art.

[0169] In an embodiment, the children's nutritional composition may contain between about 10 and about 50% of the maximum dietary recommendation for any given country, or between about 10 and about 50% of the average dietary recommendation for a group of countries, per serving, of vitamins A, C, and E, zinc, iron, iodine, selenium, and choline. In another embodiment, the children's nutritional composition may supply about 10 – 30% of

the maximum dietary recommendation for any given country, or about 10 – 30% of the average dietary recommendation for a group of countries, per serving of B-vitamins. In yet another embodiment, the levels of vitamin D, calcium, magnesium, phosphorus, and potassium in the children's nutritional product may correspond with the average levels found in milk. In other embodiments, other nutrients in the children's nutritional composition may be present at about 20% of the maximum dietary recommendation for any given country, or about 20% of the average dietary recommendation for a group of countries, per serving.

[0170] The nutritional composition(s) of the present disclosure may optionally include one or more of the following flavoring agents, including, but not limited to, flavored extracts, volatile oils, cocoa or chocolate flavorings, peanut butter flavoring, cookie crumbs, vanilla or any commercially available flavoring. Examples of useful flavorings include, but are not limited to, pure anise extract, imitation banana extract, imitation cherry extract, chocolate extract, pure lemon extract, pure orange extract, pure peppermint extract, honey, imitation pineapple extract, imitation rum extract, imitation strawberry extract, grape and or grape seed extracts, apple extract, bilberry extract or vanilla extract; or volatile oils, such as balm oil, bay oil, bergamot oil, cedarwood oil, cherry oil, cinnamon oil, clove oil, or peppermint oil; peanut butter, chocolate flavoring, vanilla cookie crumb, butterscotch, toffee, and mixtures thereof. The amounts of flavoring agent can vary greatly depending upon the flavoring agent used. The type and amount of flavoring agent can be selected as is known in the art.

[0171] The nutritional compositions of the present disclosure may optionally include one or more emulsifiers that may be added for stability of the final product. Examples of suitable emulsifiers include, but are not limited to, lecithin (*e.g.*, from egg or soy or any other plant and animal sources), alpha lactalbumin and/or mono- and di-glycerides, and mixtures thereof. Other emulsifiers are readily apparent to the skilled artisan and selection of suitable emulsifier(s) will depend, in part, upon the formulation and final product.

[0172] The nutritional compositions of the present disclosure may optionally include one or more preservatives that may also be added to extend product shelf life. Suitable preservatives include, but are not limited to, potassium sorbate, sodium sorbate, potassium benzoate, sodium benzoate, calcium disodium EDTA, and mixtures thereof.

[0173] The nutritional compositions of the present disclosure may optionally include one or more stabilizers. Suitable stabilizers for use in practicing the nutritional composition of the present disclosure include, but are not limited to, gum arabic, gum ghatti, gum karaya, gum tragacanth, agar, furcellaran, guar gum, gellan gum, locust bean gum, pectin, low methoxyl pectin, gelatin, microcrystalline cellulose, CMC (sodium carboxymethylcellulose), methylcellulose hydroxypropyl methyl cellulose, hydroxypropyl cellulose, DATEM (diacetyl

tartaric acid esters of mono- and diglycerides), dextran, carrageenans, CITREM, and mixtures thereof.

[0174] The present disclosure further provides a method for reducing the incidence of autoimmune disease, including diabetes mellitus, by providing a nutritional composition comprising the peptide component described herein to a target subject. The method includes providing a nutritional composition comprising a carbohydrate source, a protein equivalent source, and a fat source, wherein the protein equivalent source includes a peptide component comprising SEQ ID NO 4, SEQ ID NO 13, SEQ ID NO 17, SEQ ID NO 21, SEQ ID NO 24, SEQ ID NO 30, SEQ ID NO 31, SEQ ID NO 32, SEQ ID NO 51, SEQ ID NO 57, SEQ ID NO 60, and SEQ ID NO 63, to a target subject.

[0175] In some embodiments, the nutritional composition administered to a target subject comprises a protein equivalent source wherein 20% to 80% of the protein equivalent source comprises the peptide component described herein and 20% to 80% of the protein equivalent source comprises intact protein, partially hydrolyzed protein or combinations thereof.

[0176] In some embodiments the target subject may be a pediatric subject. Further, in one embodiment, the nutritional composition provided to the pediatric subject may be an infant formula. The peptide component described herein and added to the infant formula may be selected from a specific source and concentrations thereof may be adjusted to maximize health benefits. In another embodiment of this method, the nutritional composition comprising the peptide component described that is provided to a pediatric subject is a growing up milk.

[0177] Examples 3-7 illustrate the reduction in proinflammatory cytokines in certain in vitro and in vivo models.

EXAMPLE 3

[0178] Example 3 is directed to the inhibition of pro-inflammatory cytokines in primary human dendritic cell assays by extensively hydrolyzed casein and a greater than 500 Da fraction thereof. Specifically, the pro-inflammatory cytokines inhibited include, Interleukin-12p70 ("IL12p70"), Interferon-gamma (IFN γ), Interleukin-8 ("IL8"), Tumor Necrosis Factor-alpha ("TNF α "), Interleukin-6 ("IL6"), and Interleukin-1beta ("IL1 β ").

[0179] Isolated CD14⁺ monocytes were suspended in RPMI medium and supplemented with 10% heat-inactivated fetal bovine serum ("FBS"), penicillin/streptomycin, 500 IU/ml recombinant Interleukin-4 ("rhIL-4"), and 800 IU/ml rhGM-CSF and cultured in tissue culture flasks at 37 °C and 5% CO₂ for 5 days, to allow differentiation into dendritic cells.

[0180] On day 5, the cells were seeded in the same medium at 20,000 cells/well in 96-well U bottom plates, followed by addition of the hydrolysate samples including extensively

hydrolyzed casein and a greater than 500 Da fraction thereof at a final concentration of 0.007, 0.02, 0.08 and 0.2 % m/v. Dexamethasone ("Dex") was used at a final concentration of 1 µg/ml as a positive control for inhibition of cytokine production. All samples were tested in biological duplicate.

[0181] On day 6, a refreshment of the cells was performed with hydrolysate samples in medium (RPMI + 10% FBS + pen/strep) without GM-CSF and IL-4. One hour after hydrolysate addition, CD40L + enhancer (Alexis) was added to a final concentration of 0.5 µg/ml.

[0182] The cells were then incubated at 37 °C and 5% CO₂ for 24 hours and subsequently supernatants were collected and stored at -20°C until quantification of cytokines using the Meso Scale Discovery pro-inflammatory cytokine multiplex assay according to the manufactures instructions.

[0183] As shown in Figs. 1-2, pro-inflammatory cytokine secretion of Interleukin-12p70 (IL12p70) by activated primary human dendritic cells is inhibited by extensively hydrolyzed casein and a greater than 500Da fraction of the extensive casein hydrolysate.

[0184] As shown in Figs. 3-4, pro-inflammatory cytokine secretion of Interferon-gamma (IFNγ) by activated primary human dendritic cells is inhibited by extensively hydrolyzed casein and a greater than 500 Da fraction of the extensive casein hydrolysate.

[0185] As shown in Figs. 5-6, pro-inflammatory cytokine secretion of Interleukin-8 (IL8) by activated primary human dendritic cells is inhibited by extensively hydrolyzed casein and a greater than 500 Da fraction of the extensive casein hydrolysate.

[0186] As shown in Figs. 7-8, pro-inflammatory cytokine secretion of Tumor Necrosis Factor-alpha (TNFα) by activated primary human dendritic cells is inhibited by extensively hydrolyzed casein and a greater than 500 Da fraction of the extensive casein hydrolysate.

[0187] As shown in Figs. 9-10, pro-inflammatory cytokine secretion of Interleukin-6 (IL6) by activated primary human dendritic cells is inhibited by extensively hydrolyzed casein and a greater than 500 Da fraction of the extensive casein hydrolysate.

[0188] As shown in Figs. 11-12, pro-inflammatory cytokine secretion of Interleukin-1beta (IL1β) by activated primary human dendritic cells is inhibited by extensively hydrolyzed casein and a greater than 500 Da fraction of the extensive casein hydrolysate.

EXAMPLE 4

[0189] Example 4 is directed to the inhibition of pro-inflammatory cytokine Tumor Necrosis Factor-alpha ("TNFα") in a primary human macrophage assay by extensively hydrolyzed casein hydrolysate and a greater than 500 Da fraction thereof.

[0190] Isolated monocytes were suspended in RPMI medium supplemented with 10 % heat-inactivated fetal bovine serum (FBS) and penicillin/streptavidin. Recombinant human M-CSF

was added to a final concentration of 100 ng/ml and cells were incubated at 37 °C and 5% CO₂ for 5 days.

[0191] On day 5, the hydrolysate samples including extensively hydrolyzed casein and a greater than 500 Da fraction thereof at a final concentration of 0.007, 0.02, 0.08 and 0.2 % m/v were added in RPMI medium with M-CSF.

[0192] On day 6, the medium including the hydrolysate samples was removed and cells were refreshed with hydrolysate samples in medium without M-CSF. One hour after hydrolysate addition, LPS (*E. Coli*(0111:84) was added to a final concentration of 10 ng/ml. The cells were then incubated at 37 °C and 5% CO₂ for 24 hours and subsequently supernatants were collected and stored at -20 °C until quantification of TNF α using the Meso Scale Discovery TNF α assay according to the manufactures instructions

[0193] As shown in Figs. 13-14, pro-inflammatory cytokine secretion of Tumor Necrosis Factor-alpha (TNF α) of activated primary human macrophages is inhibited by extensively hydrolyzed casein and a greater than 500 Da fraction of the extensive casein hydrolysate.

EXAMPLE 5

[0194] Example 5 is directed to the evaluation of pro-inflammatory cytokine messenger RNA expression in the ileum of Non-Obese Diabetic (NOD) mice.

[0195] Briefly, age-matched female BALB/c mice were used as the healthy control mice. BALB/c mice were kept on regular chow if not otherwise stated, whereas NOD mice were kept either on regular chow or extensively hydrolyzed casein (CH)-containing dietary formulation starting at the time of weaning.

[0196] RNA isolation from ileum samples was performed by an RNA isolation kit. cDNA was synthesized and cytokine expression was assessed by quantitative real-time PCR. Absolute copy numbers of RNA (molecules/ μ g) were calculated by running standards and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as loading control.

[0197] As can be seen in Figs. 15-18, pro-inflammatory cytokine secretion of IL1, IL6, IL18 and IL17 in the ileum of NOD mice is elevated as compared to age-matched healthy (BALB/C) control mice. These elevated levels are decreased in NOD mice fed an extensively hydrolyzed casein diet.

EXAMPLE 6

[0198] Example 6 is directed to the evaluation of pro-inflammatory cytokine profiles from T cell isolated from gut-draining lymph nodes of Non-Obese Diabetic (NOD) mice.

[0199] Briefly, age-matched female BALB/c mice were used as the healthy control mice. BALB/c mice were kept on regular chow if not otherwise stated, whereas NOD mice were kept either on regular chow or an extensively hydrolyzed casein (CH)-containing dietary formulation starting at the time of weaning.

[0200] Isolated mesenteric lymph node cells from each group of mice, were stimulated with platebound anti-CD3 in complete medium (DMEM with 10% (vol./vol.) FBS, L-glutamine 2 mmol/l, 100 U/ml penicillin plus streptomycin). Supernatant fractions were collected 48 h later for cytokine analysis with multiplex cytokine bead arrays according to the manufactures instructions.

[0201] As shown in Figs. 19-21, secretion of pro-inflammatory cytokines IFN γ , IL4, and IL17 of gut-draining lymph node T cells from NOD mice is elevated as compared to age-matched healthy (BALB/C) control mice. These elevated levels were decreased in NOD mice by an extensively hydrolyzed casein diet.

EXAMPLE 7

[0202] Example 7 is directed to cytokine secretion analysis in a murine macrophage cell line.

[0203] The J774A.1 murine macrophage cell line was maintained in RPMI-1640 supplemented with 10% (v/v) fetal calf serum, 50 IU/ml penicillin and 0.5mg/ml streptomycin. Cells were incubated at 37°C with 5% CO₂.

[0204] Casein hydrolysate samples including extensively hydrolysed casein and a greater than 500Da fraction thereof were added to sterile water at a concentration of 100mg/ml and filter sterilised through 0.2 μ m nylon filters. As an additional control also non-filtered sterilized extensively hydrolysed casein was tested. Cells were plated at 1x10⁶ cells/ml and incubated in triplicate with a final concentration of 1 mg/ml hydrolysate for 1 hour. Cells were then either treated with 100ng/ml LPS or left untreated for 24 hours before collecting supernatants by centrifugation and storing at -20°C.

[0205] Supernatants were assessed for each cytokine concentration using individual ELISA assays for IL-1 β , IL-6, IL12p40, and TNF- α according to the manufactures instructions. Samples were tested in triplicate and significant differences were calculated using the ordinary one-way ANOVA analysis on graphpad prism 6 software. p<0.05 was considered significant.

[0206] As can be seen from Figs. 22-25 pro-inflammatory cytokine secretion IL-12p40 and IL-1 β from mouse macrophages stimulated with LPS was decreased by extensively hydrolyzed casein, both with or without sterile filtration prior to incubation of the cells (* p<0.05). Secretion of the pro-inflammatory cytokine IL6 was significantly decreased by extensively hydrolyzed casein (* p<0.05). Secretion of the pro-inflammatory cytokine TNF α was significantly decreased by the greater than 500 Da fraction of the extensive casein hydrolysate (* p<0.05). The results are also shown in Table format in Fig. 26.

EXAMPLE 8

[0207] Example 8 illustrates the effect of insulin secretion of extensively hydrolyzed casein and a greater than 500 Da fraction of extensively hydrolyzed casein.

[0208] Materials and Methods: Insulin secreting (BRIN-BD11) cells were maintained in Gibco® RPMI-1640 medium containing 11.1 mM glucose, supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 50 IU/mL penicillin, 0.05 mg/mL streptomycin and incubated at 37 °C in a humidified atmosphere containing 5% CO₂ and 95% air.

For insulin secretion experiments, BRIN BD 11 cells were incubated with 1.1 mmol/L glucose Krebs for 40 minutes. All wells were then treated for 20 minutes with (1) 16.7 mmol/L glucose Krebs and 10 mmol/L Alanine or (2) 16.7 mM glucose and 1 mg/mL of extensively hydrolyzed casein. Following this treatment the supernatant was removed and frozen until insulin was analyzed. Insulin secreted was measured using the Mercodia Ultrasensitive Rat Insulin ELISA kit (Mercodia AB, Uppsala, Sweden). The protein content was measured using a bicinchoninic acid (BCA) protein assay and the concentration expressed as ng insulin/mg protein as previously described (Wallace et al 2012). Cellular viability was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay.

[0209] Selected cytokines interleukin 1 β (IL-1 β), interleukin-23 (IL-23), interferon- γ (IFN- γ) with known detrimental effects on beta-cell functionality and viability were added at a final concentration of 10ng/mL. Concentrations were chosen so as to not induce cytotoxic effects but to cause mild effects in order to allow investigation of potential rescue effects of the extensively hydrolysed casein.

[0210] For the insulin secretion experiments, 10mM alanine and 16.7 mM glucose served as a positive control. A sample inducing insulin secretion above this positive control is considered a potent secretagogue.

[0211] The effect of extensively hydrolysed casein on insulin secretion (n=4) upon exposure to IL-1 β are depicted in Fig. 27. Values are averages $\pm$ SEM. Glu represents 16.7 mM glucose, and Glu/Ala represents 16.7 mM glucose and 10 mM alanine (positive control). Anova analysis was performed with an overall significance of $P < 0.001$. Posthoc analysis revealed significant differences from the positive control (* $P < 0.05$). Extensively hydrolyzed casein demonstrated the ability to significantly promote insulin secretion to a higher extent than the positive control.

[0212] Fig. 28 indicates corresponding effects for a >500Da fraction of the extensively hydrolyzed casein. Glu -IL-1Beta represents glucose control without exposure to IL-1 β , G/A -IL-1Beta represents glucose/alanine control without exposure to IL-1 β , Glu: glucose represents control with exposure to IL-1 β , and G/A: glucose/alanine represents control with exposure to IL-1 β .

[0213] Fig. 29 depicts the effect of hydrolysate samples on insulin secretion (n=4) upon exposure to IFN- γ . Values are averages $\pm$ SEM. Glu represents 16.7 mM glucose, and Glu/Ala represents 16.7 mM glucose and 10 mM alanine (positive control). Anova analysis

was performed with an overall significance of $P < 0.001$. Posthoc analysis revealed significant differences from the positive control ($** P < 0.01$). The extensively hydrolyzed casein demonstrated the ability to significantly promote insulin secretion to a higher extent than the positive control.

[0214] Fig. 30 depicts the effect of extensively hydrolyzed casein on insulin secretion ($n=4$) upon exposure to IL-23. Values are averages $\pm$ SEM. Glu represents 16.7 mM glucose, and Glu/Ala represents 16.7 mM glucose and 10 mM alanine (positive control). Anova analysis was performed with an overall significance of $P < 0.001$. Posthoc analysis revealed significant differences from the positive control ($** P < 0.01$). Extensively hydrolyzed casein demonstrated the ability to significantly promote insulin secretion to a higher extent than the positive control.

[0215] Fig. 31 right indicates corresponding effects with respect to IL-17 for a >500 Da fraction of the extensively hydrolysed casein. G/A –IL-17: glucose/alanine represents control without exposure to IL-17, Glu: glucose represents control with exposure to IL-17, and G/A: glucose/alanine represents control with exposure to IL-17).

FORMULATION EXAMPLES

[0216] Table 3 provides an example embodiment of a peptide component including 5 peptides selected from Table 1 and 3 peptides selected from Table 2 that may comprise the peptide component described herein that can be incorporated or added to the nutritional compositions described herein.

Table 3. Nutrition profile of an example peptide component

Example of Selected Peptides for Peptide Component
SEQ ID NO 5
SEQ ID NO 24
SEQ ID NO 33
SEQ ID NO 56
SEQ ID NO 64
SEQ ID NO 13
SEQ ID NO 24
SEQ ID NO 60

[0217] Table 4 provides an example embodiment of a peptide component including 5 peptides selected from Table 1, 3 peptides selected from Table 2, and at least 10 additional

peptides from Table 1 that may comprise the peptide component described herein that can be incorporated or added to the nutritional compositions.

Table 4. Nutrition profile of an example peptide component

Example of Selected Peptides for Peptide Component
SEQ ID NO 13
SEQ ID NO 24
SEQ ID NO 60
SEQ ID NO 5
SEQ ID NO 11
SEQ ID NO 22
SEQ ID NO 25
SEQ ID NO 33
SEQ ID NO 45
SEQ ID NO 46
SEQ ID NO 47
SEQ ID NO 48
SEQ ID NO 52
SEQ ID NO 34
SEQ ID NO 36
SEQ ID NO 61
SEQ ID NO 62
SEQ ID NO 64

[0218] Table 5 provides an example embodiment of a nutritional composition according to the present disclosure and describes the amount of each ingredient to be included per 100 kcal serving.

Table 5. Nutrition profile of an example nutritional composition

	per 100 kcal	
Nutrient		
	Minimum	Maximum
Protein equivalent source (g)	1.8	6.8
Fat (g)	1.3	7.2
Carbohydrates (g)	6	22
Prebiotic (g)	0.3	1.2

DHA (g)	4	22
Beta glucan (mg)	2.9	17
Probiotics (cfu)	9.60×10^5	3.80×10^8
Vitamin A (IU)	134	921
Vitamin D (IU)	22	126
Vitamin E (IU)	0.8	5.4
Vitamin K (mcg)	2.9	18
Thiamin (mcg)	63	328
Riboflavin (mcg)	68	420
Vitamin B6 (mcg)	52	397
Vitamin B12 (mcg)	0.2	0.9
Niacin (mcg)	690	5881
Folic acid (mcg)	8	66
Panthothenic acid (mcg)	232	1211
Biotin (mcg)	1.4	5.5
Vitamin C (mg)	4.9	24
Choline (mg)	4.9	43
Calcium (mg)	68	297
Phosphorus (mg)	54	210
Magnesium (mg)	4.9	34
Sodium (mg)	24	88
Potassium (mg)	82	346
Chloride (mg)	53	237
Iodine (mcg)	8.9	79
Iron (mg)	0.7	2.8
Zinc (mg)	0.7	2.4
Manganese (mcg)	7.2	41
Copper (mcg)	16	331

[0219] All references cited in this specification, including without limitation, all papers, publications, patents, patent applications, presentations, texts, reports, manuscripts, brochures, books, internet postings, journal articles, periodicals, and the like, are hereby incorporated by reference into this specification in their entireties. The discussion of the references herein is intended merely to summarize the assertions made by their authors and

no admission is made that any reference constitutes prior art. Applicants reserve the right to challenge the accuracy and pertinence of the cited references.

[0220] Although embodiments of the disclosure have been described using specific terms, devices, and methods, such description is for illustrative purposes only. The words used are words of description rather than of limitation. It is to be understood that changes and variations may be made by those of ordinary skill in the art without departing from the spirit or the scope of the present disclosure, which is set forth in the following claims. In addition, it should be understood that aspects of the various embodiments may be interchanged in whole or in part. For example, while methods for the production of a commercially sterile liquid nutritional supplement made according to those methods have been exemplified, other uses are contemplated. Therefore, the spirit and scope of the appended claims should not be limited to the description of the versions contained therein.

CLAIMS

What is claimed is:

1. A nutritional composition comprising:
 - (i) a carbohydrate source;
 - (ii) a fat source; and
 - (iii) a protein equivalent source,wherein
 - a) 20% to 80% of the protein equivalent source includes a peptide component comprising SEQ ID NO 4, SEQ ID NO 13, SEQ ID NO 17, SEQ ID NO 21, SEQ ID NO 24, SEQ ID NO 30, SEQ ID NO 31, SEQ ID NO 32, SEQ ID NO 51, SEQ ID NO 57, SEQ ID NO 60, and SEQ ID NO 63; and
 - b) 20% to 80% of the protein equivalent source comprises an intact protein, a partially hydrolyzed protein, or combinations thereof.
2. The nutritional composition of claim 1, wherein the peptide component is present in an amount from about 0.2 g/100 kcals to about 5.6 g/100 kcals.
3. The nutritional composition of claim 1, wherein the peptide component further comprises at least 10 additional peptides selected from Table 1.
4. The nutritional composition of claim 1, wherein the protein equivalent source comprises partially hydrolyzed protein having a degree of hydrolysis of less than 40%.
5. The nutritional composition of claim 1, further comprising at least one long-chain polyunsaturated fatty acid.
6. The nutritional composition of claim 5, wherein the at least one long-chain polyunsaturated fatty acid is selected from the group consisting of docosahexaenoic acid and arachidonic acid.
7. The nutritional composition of claim 1, further comprising a culture supernatant from a late-exponential growth phase of a probiotic batch-cultivation process, for use in the treatment or prevention of infection by a pathogen.
8. The nutritional composition of claim 1, further comprising a probiotic.
9. The nutritional composition of claim 1, further comprising a prebiotic.
10. The nutritional composition of claim 1, wherein when administered to a subject, the nutritional composition reduces the incidence of autoimmune disease.
11. A nutritional composition, comprising:
 - (i) a carbohydrate source;
 - (ii) a fat source; and
 - (iii) a protein equivalent source;wherein

- a) 20% to 80% of the protein equivalent source includes a peptide component comprising at least 5 peptides selected from Table 1 and at least 3 additional peptides selected from Table 2; and
 - b) 20% to 80% of the protein equivalent source comprises an intact protein, a partially hydrolyzed protein, or combinations thereof.
- 12. The nutritional composition of claim 11, comprising at least 10 additional peptides selected from Table 1.
 - 13. The nutritional composition of claim 11, wherein the protein equivalent source comprises casein hydrolysate having a molar mass distribution of greater than 500 Daltons.
 - 14. The nutritional composition of claim 11, wherein the protein equivalent source comprises partially hydrolyzed protein having a degree of hydrolysis of less than 40%.
 - 15. The nutritional composition of claim 11, further comprising a prebiotic.
 - 16. The nutritional composition of claim 11, further comprising a culture supernatant from a late-exponential growth phase of a probiotic batch-cultivation process, for use in the treatment or prevention of infection by a pathogen.
 - 17. A method for reducing the incidence of autoimmune disease, comprising providing to the subject, a nutritional composition comprising:
 - a carbohydrate source,
 - a fat source, and
 - a protein equivalent source,wherein 20% to 80% of the protein equivalent source includes a peptide component comprising SEQ ID NO 4, SEQ ID NO 13, SEQ ID NO 17, SEQ ID NO 21, SEQ ID NO 24, SEQ ID NO 30, SEQ ID NO 31, SEQ ID NO 32, SEQ ID NO 51, SEQ ID NO 57, SEQ ID NO 60, and SEQ ID NO 63; and wherein 20% to 80% of the protein equivalent source comprises intact protein, partially hydrolyzed protein or combinations thereof.
 - 18. The method of claim 17, wherein the peptide component further comprises at least 10 additional peptides selected from Table 1.
 - 19. The method of claim 17, wherein the protein equivalent source comprises a partially hydrolyzed protein having a degree of hydrolysis of less than 40%.
 - 20. The method of claim 17, wherein the nutritional composition reduces the incidence of autoimmune disease.