

NUCLEOTIDE COMPOSITION AND APPLICATION IN FOOD THEREOF

The present application claims the priority of Chinese application No. 201510099850.6 with the title of “NUCLEOTIDE COMPOSITION AND APPLICATION IN FOOD THEREOF”
5 filed on March 6, 2015, of which the content is incorporated herein by reference in its entirety.

Technical field

Provided is a nucleotide composition as food additive. Provided is also a food comprising the nucleotide composition and use of the nucleotide composition for manufacture of a food.

Background

In the recent years, many experiments have been provided showing the important function of nucleotides in living body. For example, nucleotides may be used to enhance the functions of immune system and gastrointestinal tract of the body. Meanwhile, cowmilk-based milk substitute is an importance supplementary source of food for infant. Due to the low content of nucleotide and derivative thereof in cow milk, it is important to add foreign nucleotides into cowmilk-based milk substitute for growth and development (e.g. gastrointestinal tract development) and immune enhancement of the body, especially for that of the infant.

US 4,994,442 discloses addition of various nucleotides into baby formula food could enhance immune response of T cell. However, the nucleotide components (CMP, AMP, UMP, GMP and IMP) are each added into the formula food in an equal amount. EP 1549158 discloses a baby milk composition, comprising 3.2-15.4 mg/L of CMP, 1.8-11.0 mg/L of UMP, 1.8-8.0 mg/L of GMP, 0.1-2.2 mg/L of IMP and 2.5-13.2 mg/L of AMP. However, this composition is only useful for premature baby and none of possible effects was described.

On the basis of the prior art, to sufficiently exert the function of nucleotides on growth and development and optimize the effect of nucleotides as food additive, the present inventor has performed extensive experiments to adjust the components and proportions of nucleotides in nucleic acid composition so as to maximize the effect of the composition as nucleotides supplementary additive. Meanwhile, the present inventor surprisingly found that, as compared to the products of the prior art, the nucleotide composition according to the present invention has specific components and proportions and could provide better effects.

Summary

In the first aspect, provided is a nucleotide composition which is used as food additive.

In an embodiment, the nucleotide composition according to the invention substantially

consists of or consists of CMP, AMP, UMP, GMP and IMP, wherein on the weight basis, the proportions of the components are: CMP: 58~70%, AMP: 7.5~12.5%, UMP: 12~16.5%, GMP: 10~13% and IMP: 0~2.5%, provided that sum of the components is 100%.

- 5 In a preferable embodiment, the nucleotide composition according to the invention substantially consists of or consists of CMP, AMP, UMP, GMP and IMP, wherein on the weight basis, the proportions of the components are: CMP: 60~65%, AMP: 8~12%, UMP: 14~16%, GMP: 11~12% and IMP: 0~2%, provided that sum of the components is 100%.
- 10 In another embodiment, the nucleotide composition according to the invention substantially consists of or consists of CMP, AMP, UMP and GMP, wherein on the weight basis, the proportions of the components are: CMP: 58~72%, AMP: 6~14%, UMP: 10~18% and GMP: 8~14%, provided that sum of the components is 100%.
- 15 In a further embodiment, the nucleotide composition according to the invention substantially consists of or consists of CMP, AMP, UMP and GMP, wherein on the weight basis, the proportions of the components are: CMP: 60~70%, AMP: 8~12%, UMP: 12~16% and GMP: 10~12%, provided that sum of the components is 100%.
- 20 In a further embodiment, the nucleotide composition according to the invention substantially consists of or consists of CMP, AMP, UMP and GMP, wherein on the weight basis, the proportions of the components are: CMP: 60~65%, AMP: 10~12%, UMP: 14~16% and GMP: 11~12%, provided that sum of the components is 100%.
- 25 In a further embodiment, the nucleotide composition according to the invention substantially consists of or consists of CMP, AMP, UMP and GMP, wherein on the weight basis, the proportions of the components are: CMP: 65~70%, AMP: 8~10%, UMP: 12~14% and GMP: 10~11%, provided that sum of the components is 100%.
- 30 In a preferable embodiment, the nucleotide composition according to the invention substantially consists of or consists of CMP, AMP, UMP and GMP, wherein on the weight basis, the proportions of the components are: CMP: 60%, AMP: 12%, UMP: 16% and GMP: 12%.
- 35 In a preferable embodiment, the nucleotide composition according to the invention substantially consists of or consists of CMP, AMP, UMP and GMP, wherein on the weight basis, the proportions of the components are: CMP: 65%, AMP: 10%, UMP: 14% and GMP: 11%.

In a preferable embodiment, the nucleotide composition according to the invention substantially consists of or consists of CMP, AMP, UMP and GMP, wherein on the weight basis, the proportions of the components are: CMP: 70%, AMP: 8%, UMP: 12% and GMP: 10%.

In various embodiments of the first aspect, the food is preferably an infant food and more preferably the food is in the form of dairy product, for example the form of milk powder or liquid dairy product, such as the milk powder or liquid dairy product useful for infant.

In the second aspect, provided is a food comprising the nucleotide composition according to the invention. In a preferable embodiment, the food is an infant food. In a more preferable embodiment, the food is in the form of dairy product, for example the form of milk powder or liquid dairy product, such as the milk powder or liquid dairy product useful for infant.

In the third aspect, provided is a process for preparing a food, comprising adding the nucleotide composition according to the invention into the raw material of the food. In a preferable embodiment, the food is an infant food. In a more preferable embodiment, the food is in the form of dairy product, for example the form of milk powder or liquid dairy product, such as the milk powder or liquid dairy product useful for infant.

In the fourth aspect, provided is also use of the nucleotide composition according to the invention for the manufacture of food. In a preferable embodiment, the food is an infant food. In a more preferable embodiment, the food is in the form of dairy product, for example the form of milk powder or liquid dairy product, such as the milk powder or liquid dairy product useful for infant. In a preferable embodiment, the food is used to provide the effect of immunostimulation. In another preferable embodiment, the food is used to promote growth and development (such as, gastrointestinal tract development) and promote reparation of intestinal tract after damage (such as, promote reparation of cells after damage, particularly enterocytes). In a further preferable embodiment, the food is used to promote growth of intestinal beneficial microorganisms.

In the fifth aspect, provided is a method for enhancing immunity in a subject, promoting growth and development (such as, gastrointestinal tract development), promoting reparation of intestinal tract after damage (such as, promote reparation of cells, particularly enterocytes after damage, e.g. damage caused by oxidation) and/or promoting growth of intestinal beneficial microorganisms in a subject and/or any combination thereof, comprising administering the subject the nucleotide composition or food according to the invention. In a

preferable embodiment, the subject is human, preferably human infant.

Figures

Figure 1 shows the results of cell survival rate experiment.

5 Figure 2 shows the results of SOD activity experiment.

Figure 3 shows the results of LDH activity experiment.

Figure 4 shows the results of MDA content experiment.

Figure 5 shows the results of cell proliferative experiment.

Detailed description

Unless specifically defined otherwise, all the technical and scientific terms used herein have the same meanings as those commonly used for a person skilled in the relevant field. Unless specifically defined otherwise, the ratios, proportions (including percentages) used herein are calculated on weight basis.

15

Nucleotide

The term “nucleotide” used herein refers to the compound formed from purine or pyrimidine base, ribose or ribodesose and phosphate group. For example, according to the sugar group, nucleotides can be categorized as ribonucleotide and deoxyribonucleotide. For example, according to the base group, nucleotides can be categorized as adenine nucleotide, guanine nucleotide, cytosine nucleotide, uracil nucleotide, thymine nucleotide, hypoxanthine nucleotide and the like. When there is one phosphate group in nucleotide molecule, it is called monophosphate nucleotide (NMP). The phosphate group of 5'-nucleotide may be further phosphorylated to be diphosphate nucleotide (NDP) and triphosphate nucleotide (NTP).

25

The term “nucleotide” used herein also encompasses cytosine (C), uracil (U), adenine (A), guanine (G) and/or hypoxanthine (I) present in the nucleotide composition according to the invention in various forms, for example ribonucleoside, ribonucleotide, RNA phosphate and derivatives or precursors in any other forms, provided that they can be transferred or metabolized into their corresponding nucleotide forms *in vivo* or *in vitro*.

30

For example, in the field of food addition, the nucleotides used are mainly CMP (cytosine nucleotide), UMP (uracil nucleotide), AMP (adenine nucleotide), GMP (guanine nucleotide), IMP (hypoxanthine nucleotide) or the like. For example, the commercially available 5'-mixed nucleotides comprise 5'-adenosine monophosphate (AMP), 5'-cytidine monophosphate (CMP), 5'-guanosine monophosphate (GMP), 5'-uridine monophosphate (UMP) and 5'-inosine monophosphate (IMP). Generally, when they are used in combination, the 5'-mixed nucleotides may for example be present in two types: one type is that 5'- adenosine

35

monophosphate and 5'-cytidine monophosphate are in the forms of free acid while other three nucleotides are in the forms of sodium salt, i.e. two acid and three sodium type; the other is that all the nucleotides are in the form of sodium salt, i.e. five sodium type. Accordingly, the term "nucleotide" used herein encompasses its salt form, for example alkaline metal salts or alkaline earth metal salts, such as sodium salt, potassium salt, calcium salt or the like, e.g. monosodium salt, disodium salt or the like, such as CMPNa_2 , AMPNa_2 , UMPN_2 , GMPNa_2 , IMPNa_2 and CMPK_2 , AMPK_2 , UMPK_2 , GMPK , IMPK_2 or the like. It will be understood by a person skilled in the art, the components in the nucleotide composition according to the invention can be present each optionally and independently in the forms of various salts, comprising but not limited to the above-mentioned "two acid and three sodium type"; or the components and the salts thereof can be present in any combination, for example only GMP is in the form of salt or only CMP is in the form of salt. Optionally, all the components in the nucleotide composition according to the invention can be present in the form of salt, comprising but not limited to the above-mentioned "five sodium type".

Likewise, the term "nucleotide" used herein comprises the solvate thereof in various forms (such as hydrate). Accordingly, when the nucleotide composition according to the invention comprises other forms such as those listed above of the nucleotide components, the proportions of such forms in the composition shall be calculated according to their corresponding nucleotide molecules. For example, when CMP is present in the form of disodium salt (CMPNa_2), the weight ratio shall be calculated after being transferred into CMP.

Nucleotide composition

In an embodiment, the nucleotide composition according to the invention substantially consists of or consists of the following components: CMP, AMP, UMP, GMP and IMP. In another embodiment, the nucleotide composition according to the invention substantially consists of or consists of the following components: CMP, AMP, UMP and GMP.

In an embodiment, in the nucleotide composition according to the invention, CMP is present in an amount of 58~72% by weight, preferably 60~70% by weight, for example 60~65% by weight and 65~70% by weight. It will be understood that the ranges comprise all the point values therein, for example but not limited to 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71% or the like; as well as various subranges formed by such point values, for example but not limited to 60~63%, 60~68%, 63~70% or the like. In a specific embodiment, in the nucleotide composition according to the invention, CMP is present in an amount of 60%, 65% or 70% by weight.

In an embodiment, in the nucleotide composition according to the invention, AMP is present

in an amount of 6~14% by weight, preferably 8~12% by weight, for example 8~10% by weight and 10~12% by weight. It will be understood that the ranges comprise all the point values therein, for example but not limited to 7%, 8%, 9%, 10%, 11%, 12%, 13% or the like; as well as various subranges formed by such point values, for example but not limited to 8~9%, 8~11%, 9~12% or the like. In a specific embodiment, in the nucleotide composition according to the invention, AMP is present in an amount of 8%, 10% or 12% by weight.

In an embodiment, in the nucleotide composition according to the invention, UMP is present in an amount of 10~18% by weight, preferably 12~16% by weight, for example 12~14% by weight and 14~16% by weight. It will be understood that the ranges comprise all the point values therein, for example but not limited to 11%, 12%, 13%, 14%, 15%, 16%, 17% or the like; as well as various subranges formed by such point values, for example but not limited to 12~15%, 14~15% or the like. In a specific embodiment, in the nucleotide composition according to the invention, UMP is present in an amount of 12%, 14% or 16% by weight.

In an embodiment, in the nucleotide composition according to the invention, GMP is present in an amount of 8~14% by weight, preferably 10~12% by weight, for example 10~11% by weight and 11~12% by weight. It will be understood that the ranges comprise all the point values therein, for example but not limited to 9%, 10%, 11%, 12%, 13% or the like; as well as various subranges formed by such point values, for example but not limited to 8~11%, 10~14% or the like. In a specific embodiment, in the nucleotide composition according to the invention, GMP is present in an amount of 10%, 11% or 12% by weight.

In an embodiment, in the nucleotide composition according to the invention, IMP is present in an amount of 0~2.5% by weight, preferably 0~2% by weight, more preferably 0% (i.e. the nucleotide composition according to the invention does not comprise IMP). It will be understood that the ranges comprise all the point values therein, for example but not limited to 1%, 2% or the like as well as various subranges formed by such point values, for example but not limited to 0~1%, 1~2% or the like.

It will be understood by a person skilled in the art that, the ranges and specific values listed above for various components can be optionally combined and selected, provided that sum of the selected components is 100%. For example, in the nucleotide composition according to the invention, when the content of CMP is 60~70%, such as 60~65% or 65~70%, the content of AMP can be 8~12%, such as 8~10% or 10~12%. Likewise, when the contents of CMP and AMP are within any above ranges, the content of UMP can be 12~16%, such as 12~14% or 14~16%. Similarly, when the contents of CMP, AMP and UMP are within any above ranges, the content of GMP can be 10~12%, such as 10~11% or 11~12%. According to the same

principle, when the contents of CMP, AMP, UMP and GMP are within any above ranges, the content of IMP can be 0~2.5%, such as 0~2% or 0% (i.e. the nucleotide composition according to the invention does not comprise IMP). A person skilled in the art will understand that the above selections shall comply with the requirement that sum of the selected components is 100%.

The expression “substantially consist of” means in addition to the defined nucleotide components, if necessary, the nucleotide composition according to the invention can optionally comprise food or physiologically acceptable carrier, excipient or adjuvant, for example preservative, antioxidant, binder, thickener, diluent or the like and various impurities which possibly exist. The carrier, excipient or adjuvant and impurities are inert for the active ingredients in the composition (nucleotide components) and their presence or amount would not disturb or significantly disturb the functions of the active ingredients. Moreover, when calculating the proportions of the nucleotide components in the nucleotide composition according to the invention, the carrier, excipient or adjuvant and impurities which are optionally present will not be considered. In addition, a person skilled in the art will understand that the expression “substantially consist of ...” encompasses the meaning of “consist of ...”.

The term “optional” or “optionally” used herein refers a subject matter which may or may not exist. For example, “optionally comprising food or physiologically acceptable carrier, excipient or adjuvant” means the nucleotide composition according to the invention may or may not comprise such carrier, excipient or adjuvant.

Food

The term “food” used herein has the common meaning to a person skilled in the art and for example refers to edible or drinkable material for human, including the food which is processed, semi-processed, unprocessed or the like.

The term “infant food” used herein refers to the food, except breast milk, suitable for infant, which is added with various ingredients due to the requirement of infant’s growth and development, for example nucleotides, fatty acids, vitamins, hydrocarbons, vegetable oils, microelements or the like, such as the nucleotide composition according to the invention. Examples of the food are dairy product (e.g. milk powder and liquid dairy product), puree, rice flour or the like. According a specific embodiment, the infant food is in the form of dairy product, for example milk powder or liquid dairy product, such as infant milk powder or liquid dairy product added with the nucleotide composition according to the invention.

The term “infant” used herein generally refers to human subject of 0~3 years. Application of the nucleotide composition according to the invention, however, is not limited to this year range. If necessary, the nucleotide composition according to the invention or the food containing the same may be administered to older human subject, for example 4, 5, 6, 7, 8, 9, 10 or older.

In another aspect, provided is a process for preparing a food, comprising adding the nucleotide composition according to the invention into the food. In an embodiment, the food is an infant food. In a preferable embodiment, the food is in the form of dairy product, for example the forms of milk powder or liquid dairy product, such as the milk powder or liquid dairy product for infant.

Throughout the description, the terms “liquid dairy product” and “liquid milk” have the same meaning and are interchangeably used, referring to dairy product in liquid form and comprising various nutrient ingredient and energy useful for human, such as those listed below.

According to an embodiment, when preparing the milk powder according to the invention, the nucleotide composition according to the invention is used in an amount of 0.2~0.58 weight portion (based on 1000 weight portion of milk powder). For example, the milk powder, based on 1000 weight portion of the milk powder, comprise: skimmed milk powder 120~160 weight portion, lactose 240~280 weight portion, desalted whey powder 180~210 weight portion, whey protein powder (WPC34%) 90~120 weight portion, sunflower seed oil 155~180 weight portion, corn oil 35~55 weight portion, soybean oil 40~60 weight portion, nucleotide composition according to the invention 0.35~0.5 weight portion, soyabean lecithin 1~2.5 weight portion, bifidobacteria 0.1~0.15 weight portion, oligofructose powder 4~5 weight portion, galactooligosaccharide syrup 10~12 weight portion, nutriologically acceptable amount of vitamins and nutriologically acceptable amount of microelement.

In a specific embodiment, the main process for preparing the milk powder according to the invention mainly comprise: dosing, preheating, homogenizing, concentrating and sterilizing, spray-drying, dry-mixing and obtaining the final product, wherein the nucleotide composition according to the invention is added together with DHA, ARA and bifidobacteria into milk powder after spray-drying, which are then mixed.

According to another embodiment, the liquid dairy product according to the invention comprise the following ingredients (based on 100g liquid dairy product): protein beyond lactoferrin 1.75g~4.26g, fat 1.75g~4.97g, energy 250kJ~355kJ, vitamin A 42.5μg~

191.7μg RE, vitamin D 0.625μg~2.6625μg, vitamin E≥0.375mg α-TE, vitamin K1≥2.5μg, vitamin B1≥27.5μg, vitamin B2≥27.5μg, vitamin B6≥27.5μg, vitamin B12≥0.1μg, nicotinic acid (or nicotinamide)≥275μg, folic acid≥2.5μg, pantothenic acid≥175μg, vitamin C≥4.5mg, biotin≥1μg, sodium≤71mg, potassium 45mg ~ 244.95mg, copper 17.5μg ~ 124.25μg, magnesium ≥3.5mg, iron 0.625mg~1.775mg, zinc 0.25mg~1.065mg, calcium ≥42.5mg, phosphorus ≥20.75mg, iodine ≥3.5μg, chlorine ≤184.6mg, lactoferrin 5 ~ 13mg and hydrocarbon, wherein the amount of the nucleotides according to the invention is 2.64~7.66mg and the amount of hydrocarbon is such that the energy provided by hydrocarbon, protein and fat is 250kJ~355kJ.

10

The fat used is provided by one or more of anhydrous milk fat, soybean oil, corn oil, sunflower seed in any ratio and combination. In addition, to improve the properties of liquid milk system like stability, some food acceptable additive for example emulsion stabilizer can be added. Preferably, the food additive and the amount thereof herein comprise but not limited to one or more of carrageenan 0.005wt%~0.05wt%, glycerin monostearate 0.01wt%~1wt%, guar gum 0.01wt%~0.1wt%, locust bean gum 0.01wt%~0.1wt% or any combination thereof.

20

Finally, provided is use of the nucleotide composition according to the invention for the manufacture of food. In an embodiment, the food is an infant food. In a preferable embodiment, the food is in the form of dairy product, for example the form of milk powder or liquid dairy product, such as the milk powder or liquid dairy product useful for infant.

Immunostimulation

25

In a preferable embodiment, the nucleotide composition according to the invention and the food containing the same can provide the effect of immunostimulation after consumption. The effect of “immunostimulation” herein refers to the function of enhance the immunity in a subject, for example in the aspects of improving transformation function of lymphocytes, enhancing phagocytosis function of macrophagocytes, enhancing NK cells activity, improving immune response and antibody production or the like, such those provided in the Examples of the description. In a preferable embodiment, the subject is human and preferably human infant.

30

Promotion of growth and development and promotion of reparation of intestinal tract after damage

35

The nucleotide composition according to the invention and the food containing the same can promote growth and development (for example, promote development of gastrointestinal tract) and promote reparation of intestinal tract after damage in a subject after consumption. For

example, promotion of growth and development and mature of intestinal tract (e.g. small intestine), reparation of intestinal tract (e.g. small intestine) after damage, protection of intestinal tract (e.g. small intestine) cells from the attack of free radical, reduction of intestinal tract (e.g. small intestine) inflammation occurrence. The inventor, from the experiment *in vitro* found that the nucleotide composition according to the invention can promote growth of enterocyte, promote proliferation of enterocyte, and protect enterocyte and hepatocyte from damage. Such damage is for example oxidative damage, such as that cause by reactive oxygen species. In a preferable embodiment, the subject is human and preferably human infant.

10 Promotion of growth of intestinal beneficial microorganisms

The nucleotide composition according to the invention and the food containing the same can promote growth of intestinal beneficial microorganisms (e.g. bifidobacteria and lactobacillus) in a subject after consumption, for example in the aspects of being beneficial for growth of bifidobacteria, increase of contents of bifidobacteria and lactobacillus in intestinal tract and faeces (such bacterial flora can inhibit reproduction of acid-abominating pathogenic bacteria and E. coli.), reduction of proportion of intestinal tract harmful bacteria, thereby benefiting the health of the subject. The present inventor found that, addition of the composition into intestinal tract condition or a similar condition *in vitro*, by observing the multiplication amount of probiotics and lactobacillus in the environment, especially that of bifidobacteria, nucleotide composition according to the invention show significant growth promotion to intestinal beneficial microorganisms. In a preferable embodiment, the subject is human and preferably human infant.

Example

25 In the Examples, the test samples have the components and proportions as follows (on the weight basis):

Nucleotide composition according to the invention (also see claim 5):

Sample 1: CMP: 60%, AMP: 12%, UMP: 16% and GMP: 12%

Sample 2: CMP: 65%, AMP: 10%, UMP: 14% and GMP: 11%

30 Sample 3: CMP: 70%, AMP: 8%, UMP: 12% and GMP: 10%

Nucleotide composition of the prior art:

Comparative Sample 1: CMP: 33.7%, AMP: 20.3%, UMP: 23.1%, GMP: 7.6% and IMP: 15.3%

35 Comparative Sample 2: CMP: 60%, AMP: 14.5%, UMP: 18.2% and GMP: 7.3%

Comparative Sample 3: CMP: 42.7%, AMP: 13.4%, UMP: 24.2% and GMP: 19.7%

Example 1: immunostimulation effect (I)

The purpose of this example is to test the effect of immunostimulation of the nucleotide composition according to the invention and the principle and steps are based on the requirements and specification of “technical standards of examination and evaluation for healthcare food (2003)”, Second part “functional test method” Chapter I.

5

1. Materials and methods

1.1. Main reagents

Main reagents used in this example: RPMI-1640 medium (Gibco); fetal calf serum (Gibco); Concanavalin A (ConA) (Sigma); Lymphocyte Separation liquid, whole blood and tissue
10 diluent, cell wash solution (Tian Jin Hao Yang Biological Manufacture CO., LTD); 5% chicken red blood cell suspension (prepared by the lab); MTT kit, Hank's solution (Beyotime Institute of Biotechnology); Giemsa dye liquor (Zhuhai BASO Biotech CO., LTD); EDTA anticoagulant tube (BD); 96-well cell culture plate (Corning); Baicheng colostrum capsule (Shanghai Fuzheng Biotech CO., LTD); nucleotide samples (Nanjing Tongkaizhaoye Biotech
15 CO., LTD).

1.2. Main instruments

CO₂ incubator (Sanyo MCO-18AIC(UV)); clean bench (AIRTECH); inverted microscope (OLYMPUS); Hitachi horizontal centrifuge (himac-CT6EL); microplate reader (BIO-RAD
20 Model 680); OLYMPUS microscope (BX51); DNP thermostatic incubator (Shanghai Jinghong Lab instrument CO., LTD).

1.3. Animals

160 KM male mice of 5~6 weeks, provided by Shanghai SLAC Laboratory Animal Co., Ltd.
25

1.4. Animals grouping

The KM mice were adapted for 7 days and then randomly divided into 8 groups, 20 animals each group: blank control group, positive control group, high, medium and low dosage groups of Sample 1 and high, medium and low dosage groups of Comparative Sample 1. The animals
30 in each groups received gavage of sterile water dissolved with each sample (below) and the blank control group received gavage of sterile water at the same volume, one administration per day, for 30 days.

The high dosage group received a dose of 67.37 mg·kg⁻¹·d⁻¹, the medium dosage group
35 received a dose of 41.82 mg·kg⁻¹·d⁻¹, the low dosage group received a dose of 23.23 mg·kg⁻¹·d⁻¹ and the positive control group received a dose of 150.17 mg·kg⁻¹·d⁻¹.

The positive control group: colostrum capsule.

Test group: Sample 1 is nucleotide composition according the invention. Comparative Sample 1 is nucleotide composition which was prepared according to nucleotide components and proportions of a commercially available infant food added with nucleotides.

5

1.5. Assay

Lymphocyte transformation assay:

At 38th day, 10 mice were randomly taken from each group. Blood was taken by removal of eyeball with 2mL EDTA anticoagulant tube and lymphocytes were separated under
10 sterilization with Ficoll density gradient centrifugation. 1 mL anti-coagulate fresh blood was mixed uniformly with whole blood and tissue diluent at 1:1, which was carefully added to the liquid surface of lymphocyte separation liquid at the same volume. After centrifugation at 1500 rpm for 15 min with horizontal centrifuge, cyclic milk-white lymphocyte layer was collected, which was then washed twice with cell wash solution. RPMI-1640 full liquid
15 medium was used to prepare a concentration of 1×10^7 /mL, which was added into 96-well cell culture plate, 100 μ L each well and 6 wells per mice, wherein 3 wells were added with ConA (final concentration 5 μ g/mL) and other 3 wells without addition as control. After 68 h incubation in CO₂ incubator at 5%CO₂ and 37°C, MTT (5mg/mL) solution (10 μ L/well) was added, after a further incubation for 4 h, 100 μ L Formazan solution was added to each well,
20 followed by further incubation in CO₂ incubator at 37°C until full dissolution of Formazan. OD value at 570 nm was determined in microplate reader and Stimulation Index (SI) was calculated: SI = ConA stimulation tube OD average/control tube OD average.

Enterocoelia macrophage phagocytosis assay:

At 38th day, 10 mice were randomly taken from each group. Each mouse was injected 1 mL of
25 5% chicken red blood cell suspension at abdominal cavity. After 30 min, the animal was killed by cervical dislocation. To abdominal cavity was injected 1 mL normal saline and the abdomen was rubbed gently for 1 min. The skin of abdominal wall was cut with the open slot at muscular layer. A tubularis was used to suck 1 mL of peritoneal fluid from abdominal
30 cavity, which was placed dropwise on a clean slide. The slide was placed in an enamel box with wet gauze pad and then incubated in 37°C incubator for 30 min. The slide was taken out and washed to remove supernatant and cells which were not attached to the slide and dried in the air under room temperature. After fixation with 1:1 acetone/methanol solution, the slide was placed in Giemsa dye solution for 15~30 min and then washed and dried in the air.
35 Macrophages were counted under high magnification, and 100 cells were counted per slide to calculate phagocytosis percentage and phagocytosis index.

Phagocytosis percentage (%) = (number of macrophages taking chicken red blood cells /

number of counted macrophages) $\times 100$

Phagocytosis index = (total number of chicken red blood cells which have been taken / number of counted macrophages)

5

1.6. Statistical analysis

Data was shown as $\bar{x} \pm s$, variance analysis was performed with SPSS 13.0 statistical software, pairwise comparison was performed with SNK method, and test level is $\alpha=0.05$.

10 2. Results

2.1. Influence of samples on lymphocyte proliferation

Table 1: Influence of samples on mice lymphocyte proliferation ($\bar{x} \pm s$, n=10)

Group	Stimulation index (SI)
Blank control group	1.24 $\pm$ 0.19
Positive control group	2.13 $\pm$ 0.47*
Sample 1 high dosage group	2.09 $\pm$ 0.23*
Sample 1 medium dosage group	1.87 $\pm$ 0.26*
Sample 1 low dosage group	1.46 $\pm$ 0.34*
Comparative Sample 1 high dosage group	1.51 $\pm$ 0.18*
Comparative Sample 1 medium dosage group	1.30 $\pm$ 0.22
Comparative Sample 1 low dosage group	1.27 $\pm$ 0.15

Note: * significant as compared to blank control group (p<0.05)

15 According to table 1, SI values of high, medium and low dosage groups of Sample 1 were significantly higher than that of blank control group (p<0.05) and showed dose dependence. SI value of high dosage group of Comparative Sample 1 is significantly higher than that of blank control group (p<0.05), SI values of medium and low dosage groups of Comparative Sample 1 were not significantly distinguished from those of blank control group (p>0.05). SI
20 value of low dosage group of Sample 1 is significantly higher than that of medium dosage group of Comparative Sample 1 (p<0.05).

The results showed that the nucleotide composition according to the invention can significantly enhance mice lymphocyte proliferation and showed a better effect over
25 comparative sample.

2.2. Influence of samples on macrophages phagocytosis

Table 2: Influence of samples on mice macrophage phagocytosis ($\bar{x} \pm s$, n=10)

Grouping	Phagocytosis percentage (%)	Phagocytosis index
Blank control group	35.2±4.2	0.43±0.06
Positive control group	52.7±8.6*	0.62±0.10*
Sample 1 high dosage group	50.1±2.5*	0.59±0.01*
Sample 1 medium dosage group	48.7±3.2*	0.57±0.13*
Sample 1 low dosage group	46.7±3.1*	0.53±0.07*
Comparative Sample 1 high dosage group	49.8±2.0*	0.50±0.22*
Comparative Sample 1 medium dosage group	34.9±1.7	0.44±0.12
Comparative Sample 1 low dosage group	35.1±3.9	0.42±0.19

Note: * significant as compared to blank control group (p<0.05)

According to table 2, phagocytosis percentage and phagocytosis index of high, medium and low groups of Sample 1 are significantly higher than those of blank control group (p<0.05).

5 Phagocytosis percentage and phagocytosis index of high dosage group of Comparative Sample 1 are significantly higher than those of blank control group (p<0.05). Phagocytosis percentage and phagocytosis index of medium and low dosage groups of Comparative Sample 1 are not significantly distinguished from those of blank control group (p>0.05). Phagocytosis percentage and phagocytosis index of low dosage group of Sample 1 are significantly higher
10 than those of medium dosage group of Comparative Sample 1 (p<0.05).

The results showed that the nucleotide composition according to the invention can significantly enhance mice enterocoelia macrophage phagocytosis function and showed a better effect over comparative sample.

15

3. Conclusion

According to the experiment results, nucleotide composition according to the invention can significantly improve lymphocytes transformation function, enhance enterocoelia macrophage phagocytosis function, whereby showing the effect of enhance immunity function and such
20 immunoregulation effect is significantly better than comparative sample.

In another aspect, more potent immunoregulation effect means the same or similar effect can be achieved with less dosage, which shows a significant advantage with respect to cost in large scale industrial production.

25

On this basis, the above experiments are repeated, wherein Sample 1 were replaced with Sample 2 and Sample 3. As for Sample 2 and Sample 3, the results similar as Sample 1 were obtained.

Example 2: Immunostimulation effect (II)

The purpose of this example is to test the effect of immunostimulation of the nucleotide composition according to the invention and the principle and steps are based on the requirements and specification of “technical standards of examination and evaluation for healthcare food (2003)”, Second part “functional test method” Chapter I.

1. Materials and methods

1.1. Main instruments

high speed refrigerated centrifuge (SIGMA 3-30K), shaker (Vortex4 digital), pipettor (eppendorf, Germany), Whole blood cell analyzer (Urit, U-2900PLUS), CO₂ incubator (Thermo 311), microplate reader (Biotek H4), spectrophotometer (Shanghai JingKe 722s), clean bench (Shangyu Xingxing Instrument CO., LTD SW-CJ-2D), autoclave sterilizer (Sanyo Japan MLS-3781-PC 75L), cryogenic refrigerator (Hangzhou Aipu Instrument CO., LTD DW-40L058), inverted microscope (Nikon Japan Eclipse Ti-S), vernier caliper (Standard Gage, US), electronic scales (Sartorius BSA124S-CW), Toe Volume Meter (Jinan Yiyan Science Development CO., LTD YLS-7C), Hemolytic plaque automatic image analyzer (Beijing Antai Yongxin Medical Technology CO., LTD AT-Spot 5100), flow cytometry (BD, US FACSCalibur).

1.2 Main reagents

cyclophosphamide (Jiangsu Hengrui Medicine CO., LTD), Dipotassium ethylene diamine tetraacetate (Beijing Reagent factory), RpMI1640 liquid cell medium (Shanghai Yuanlong Biology Technology Company), bovine calf serum, 2-mercaptoethanol, penicillin, streptomycin, ConA solution (Beijing Mengyimei Biology Technology CO., LTD), sterile Hank's solution, BrdUp marker solution (Beijing Mengyimei Biology Technology CO., LTD), dinitrofluorobenzene (Beijing Qingshengda Chemical Engineering Technology CO., LTD), barium sulfide, sheep red blood cell (SRBC), guinea pig serum, Indian ink (Shanghai Yuanmu Biotechnology CO., LTD), YAC-1 cell (Shanghai Enzyme Research Biotechnology CO., LTD), Tris-HCL buffer (Beijing Mengyimei Biology Technology CO., LTD).

1.3 Animals

770 healthy male BALB/c mice of 3-4 years and weight of 11-13g were provided by Beijing Huafukang Bioscience CO., LTD and were accommodated in level 2 Animal house of Chinese Academy of Medical Science, Union Institute of Materia Medica: room temperature (25±2°C), relative humidity (55±2)%, 12h/12h illumination, free access to food and water, 4-5 animals per cage. The test was initiated after adaption for 3 days.

770 animals were fed in 5 big immune groups, 154 in each big group, which was randomly divided into 11 groups (high and low dosage for 5 nucleotide samples, 10 groups in total and one group as control group), 14 in each group. Immune group 1: delayed type hypersensitivity; Immune group 2: mice lymphocyte transformation assay, NK cell activity assay, ratio of the organs to body; Immune group 3: half value of hemolysis, antibody producing cell number; Immune group 4: carbon clearance test; Immune group 5: mice abdominal cavity macrophage taking fluorescent microsphere assay. Each sample was administered by gavage for 28 days, once per day, with the gavage volume as 0.2 mL/10g. Control group received distilled water by gavage. After administration, the mice were killed and determined for various immune indicators.

1.4 Test samples

Samples 1-3 were nucleotide composition according to the invention. Comparative Samples 2 and 3 were other two nucleotide compositions disclosed in the prior art. Each sample was set with low and high dosage groups as 120.7 mg/kg and 1207.0 mg/kg, respectively.

1.5 Test items and indicator

1.5.1 Organs/body weight ratio: The mice were weighed initially and at 28th day after administration as initial weight and final weight. The mice were killed by dislocation and spleen and thymus were taken and removed off fascia. The bloodiness on the surface was sucked by filter paper and the organs were weighed to calculate spleen/body weight ratio and thymus/body weight ratio.

1.5.2 Delayed type hypersensitivity(toe thickening, DTH): After successive administration for 28 days, each mouse was injected in abdominal cavity 2% packed-cell volume of SRBC (v/v, formulated in normal saline) SRBC 0.2 mL. Four days after sensitization, the thickness of left rear foot plantar was measured and average value was calculated according to 3 measurements at the same site. At the measuring site, 20 μ L of 20% SRBC was injected subcutaneously. 24 h after injection, the thickness of left rear foot plantar was measured and average value was calculated according to 3 measurements. The difference in thickness before and after the challenge (swelling degree of toe) was used to present DTH degree.

1.5.3 Mice lymphocytes transformation induced by ConA (MTT method): After successive administration for 28 days, the mice were killed. After sterilization in 75% alcohol in beaker, the spleen was taken under sterile condition and placed in a dish with four layers of 3 cm $\times$ 3 cm gauze (autoclaved), to which was added appropriate amount of Hank's solution. The spleen was packaged with gauze and ground with elbow forceps to prepare single cell suspension, which was washed twice with Hank's solution and centrifuged at 1000 rpm for 10

min. The cells were suspended in 2 mL full liquid medium and viable cells were counted to adjust the cell concentration to be $5 \times 10^6/\text{mL}$. The cell suspensions were added into 24 well culture plate in duplicated wells, 1 mL in each well. 75 μL of ConA solution (corresponding to 7.5 $\mu\text{g}/\text{mL}$) was added into one well and the other well was used as control. The cells were incubated at 5% CO_2 , 37°C for 72 h. 4 h prior to end of incubation, 0.7 mL supernatant was sucked gently from each well and then 0.7 mL of RPMI 1640 liquid culture medium free of bovine calf serum was added. At the same time, MTT (5 mg/mL) 50 $\mu\text{L}/\text{well}$ was added and incubation was continued for 4 h. After incubation, 1 mL acidic isopropanol was added to each well with beating for homogeneity such that purple crystal was dissolved completely. The system was split into 96 well culture plate, each well having 3 parallel wells. Optical density value at 570 nm was determined with enzyme linked immunosorbent detector. Proliferation capacity of lymphocyte was expressed as optical density value of well with ConA subtract that of the well without ConA.

1.5.4 Antibody producing cell assay: After successive administration for 28 days, sheep blood was washed three times with normal saline and each mouse was injected in abdominal cavity 2% packed-cell volume (v/v, formulated in normal saline) of SRBC 0.2 mL for immunization. Four days after immunization with SRBC, the mice were killed and spleen was taken to prepare a cell suspension of 5×10^6 cells/mL. Agarose was heated for dissolution and mixed with double amounts of Hank's solution, which was then split into tube, 0.5 mL in each tube. Into the tube was added 20% packed-cell volume (v/v, formulated in normal saline) of SRBC 50 μL , spleen cell suspension 200 μL . After quick mixing, the mixture was poured on 6 well plate coated with agarose thin layer. After solidification of the agar, the plate was placed in CO_2 incubator for 1h, then complement diluted with SA buffer (1:10) was added and incubation was performed for 2 h. Number of hemolytic plaque was counted.

1.5.5 Serum hemolysin half value of hemolysis (HC_{50}): After successive administration for 28 days, sheep blood was washed three times with normal saline and each mouse was injected in abdominal cavity 2% packed-cell volume (v/v, formulated in normal saline) of SRBC 0.2 mL for immunization. After four days, blood was taken by removal of eyeball into 1.5 mL centrifuge tube, which was placed at 4°C for 1 h to allow sufficient separation of serum. The tube was centrifuged at 2000rpm for 10 min and serum was collected. The serum was diluted 100 folds with SA buffer. The diluted serum was added into 96 well plate, 100 μL in each well, to which was successively added 10%(v/v) SRBC 50 μL , complement 100 μL diluted with SA buffer (1:8). The place was incubated in 37°C thermostatic water bath for 30min and then centrifuged at 1500 rpm for 10 min. 50 μL of supernatants from sample wells and blank control well were added into another 96 well plate, to which was added 150 μL of VanKampen-Zijlstra's reagent. At the same time, half hemolysis well was set, to which was

added 10%(v/v)SRBC 12.5 μ L and then VanKampen-Zijlstra's reagent to 200 μ L. The mixture was mixed homogenously with a shaker and allowed to stand for 10 min. Optical density values of each wells at 540 nm were determined with Automatic microplate reader.

- 5 The amount of hemolysin is shown as half value of hemolysis (HC_{50}) and calculated as:
Sample HC_{50} = (sample optical density value/ optical density value upon half hemolysis of SRBC) $\times$ dilution ratio

1.5.6 Mice abdominal cavity macrophage taking fluorescent microsphere assay: After
10 successive administration for 28 days, four days before end of gavage, each mice was injected 0.2 mL of 2% SRBC to activate macrophage. On the day of test, mice were killed with dislocation of the cervical spine, of which the abdominal cavity was injected Hank's solution added with bovine calf serum, 3mL/animal. The abdomen was kneaded softly for 20 times to wash macrophages from abdominal cavity sufficiently. The abdominal wall was cut with a
15 small open slot, and 2 mL of abdominal washing liquid was sucked and filtered into tube with 75 μ m filter. Macrophages number was adjusted to $4\sim6\times10^5$ /mL. A pipettor was used to transfer 1 mL abdominal washing liquid into 6 well culture well, to which was added preconditioned fluorescent microspheres (1×10^7 /plate). The plate was incubated in CO₂ incubator at 37°C in dark for 120 min. After incubation, the supernatant (containing cell
20 which were not adherent and excess fluorescent microspheres) was discarded. 1.0 mL of PBS buffer was used to wash gently for two times. After removal of supernatant, 0.3 mL PBS buffer at 4°C was added. The adherent cells were scraped with cell scraper and filtered with 75 μ m filter after gentle beating. The cells were ready for analysis.

- 25 The results were calculated as
phagocytosis percentage (%) = (number of macrophages taking fluorescent microspheres / counted number of macrophages) $\times 100$

phagocytosis index = (total number of fluorescent microsphere which have been taken /
30 counted number of macrophages)

1.5.7 NK cell activity assay (lactic dehydrogenase (LDH) method):

After successive administration for 28 days, 24 h before test, the target cells YAC-1 were subjected to passage culture. Before use, the cells were washed twice with Hank's solution
35 and adjusted to the concentration of 1×10^5 /mL(target cell) with RPMI 1640 full liquid medium containing 10% bovine calf serum. The mice were killed by cervical dislocation and spleen was taken under sterile condition to prepare spleen cell suspension, which was washed twice with Hank's solution, centrifuged at 1000 rpm for 10 min and resuspended with 2 mL of

RPMI 1640 full liquid medium containing 10% bovine calf serum. Trypan blue was used to perform viable cells dyeing counting (viable cells should be over 95%) and the cell concentration was adjusted to 1×10^7 /mL (effector cell) such that the ratio of effector cells to target cells was 100:1. 100 μ L of target cells and effector cells were respectively added into U shape 96 well culture plate. The target natural releasing well was added with target cells and liquid medium 100 μ L, respectively and the target maximum releasing well was added with target cells and 1% NP40 100 μ L, respectively. These wells were set with three parallel wells and incubated in 5% CO₂ incubator at 37°C for 4 h. 96 well culture plate was centrifuged at 1500 rpm for 5 min, 100 μ L of supernatant was sucked from each well and placed in flat 96 well culture plate, to which was added 100 μ L of LDH base solution. The reaction was performed for 3 min and to each well was added 30 μ L of 1 mol/L HCl solution to quench the reaction. OD value at 490 nm was determined with microplate reader and NK activity was calculated according to the following formula.

NK cell activity % = (reaction well OD – natural releasing well OD) / (maximum releasing well OD - natural releasing well OD) $\times$ 100%

1.5.8 Carbon clearance assay: The animals were successively administered for 28 days and weighed. Indian ink was injected through tail vein. 2, 10 min after ink injection, 20 μ L of blood was taken and added into 2 ml of 0.1% sodium carbonate solution and OD value at 600 nm was determined. The mice were killed and liver and spleen were taken, and the bloodiness on the surface was sucked by filter paper and the organs were weighed.

Phagocytosis index was used to present the mice carbon clearance capacity and calculated according to the following formula:

$$K = \frac{LgOD_1 - LgOD_2}{t_2 - t_1}$$

$$\text{phagocytosis index} = \frac{\text{bodyweight}}{\text{liverweight} + \text{spleenweight}} \times \sqrt[3]{K}$$

1.6 Criteria: As for the four major aspects of cellular immunity, humoral immunity, monocyte-macrophage function and NK cell activity, if any two aspects had positive results, the sample should be considered as having the function of enhance immunity. In cellular immunity function assay items, if two test results were both positive, or two dosage group of any one test had positive result, the cellular immunity function assay had positive result. In humoral immunity function assay items, if two test results were both positive, or two dosage group of any one test had positive result, the humoral immunity function assay had positive result. In monocyte-macrophage function assay items, if two test results were both positive, or two dosage group of any one test had positive result, the monocyte-macrophage function

assay had positive result. As for NK cell activity assay, if one or more dosage group had positive result, the NK cell activity assay had positive result.

2. Results:

- 5 After oral administration of test samples for 28 day, the immune function items measurements were as follows:

Body weight and organs ratios were not significantly distinguished from normal groups.

- 10 The positive and negative results were shown in Table 3.

Table 3

Grouping	cellular immunity		humoral immunity		Monocyte-macrophage function		NK cell activity
	Spleen lymphocyte transformation assay	Delayed type hypersensitivity	Antibody producing cells	Half value of hemolysis HC50	Carbon clearance assay	macrophage phagocytosis assay	
Sample 1 low dosage	positive	negative	positive	positive	positive	negative	positive
Sample 2 low dosage	negative	negative	negative	positive	positive	positive	positive
Sample 3 low dosage	negative	negative	negative	negative	negative	positive	negative
comparative Sample 2 low dosage	negative	negative	negative	negative	negative	negative	negative
comparative Sample 3 low dosage	negative	negative	negative	negative	negative	positive	positive
Sample 1 high dosage	positive	negative	positive	negative	positive	positive	positive
Sample 2 high dosage	negative	negative	negative	negative	positive	positive	positive
Sample 3 high dosage	negative	negative	negative	negative	positive	positive	positive
comparative Sample 2 high dosage	positive	negative	negative	negative	positive	negative	positive
comparative Sample 3 high dosage	positive	negative	negative	negative	positive	positive	positive

According to the results in Table 3, from the criteria for the four major aspects (i.e. the four major aspects in section 1.6), Samples 1, 2, 3 and Comparative Sample 3 were considered as positive and thus had the immunostimulation effect. On the contrary, Comparative Sample 2 was not considered as positive.

On this basis, as for Samples 1-3 and Comparative Sample 3, from point of the above dosage

groups (section 1.6), the nucleotide composition according to the invention had more immunostimulation positive indicators, showing more potent and more comprehensive immunostimulation effect. For example, 1) Sample 1 had the cellular immunity effect, while other samples did not; 2) Sample 1 had humoral immunity effect, while other samples did not; 3) Sample 1 and 2 were positive for carbon clearance assay, while other samples were not; 4) Samples 2, 3 and Comparative Sample 3 had macrophage phagocytosis function, while other samples did not; 5) Sample 1, 2, 3 and Comparative Sample 3 were positive for NK cell activity.

From the above results, the nucleotide composition according to the invention was effective in stimulation of immunity function and showed better effect over the compositions of the prior art. Likewise, as stated above in Example 1, more potent immunoregulation effect means the same or similar effect can be achieved with less dosage, which shows a significant advantage with respect to cost in large scale industrial production.

Example 3: Protection from damage and reparation of the nucleotide composition

The purpose of this example is to test the effect of the nucleotide composition according to the invention in providing protection from damage and reparation.

1. Materials and methods

1.1 Instruments and reagents

RPMI-1640 medium and DMEM high sugar medium were purchased from Gibco. 5'-AMP (A1752), 5'-CMP- Na_2 (C1006), 5'-GMP- Na_2 (G8377), 5'-UMP- Na_2 (U6375), Thiazolyl Blue (MTT), insulin were purchased from Sigma. Fetal calf serum was purchased from Sijiqin Hanzhou. The kits used in the example such as LDH, SOD, MDA kits were all purchased from Nanjing Jiancheng Bioengineering Institute. Trypsin, double-antibody, BCA protein assay kit were purchased from Beyotime. Other reagents such as dimethylsulfoxide (DMSO), hydrogen peroxide were domestic products with analytical purity and were purchased from Sinopharm Chemical Reagent CO., LTD, Shanghai. 25 cm² culture bottle, 96 well cell culture plate, 6 well cell culture plate, 60 mm cell culture dish, 50 mL centrifuge tube, 15 mL centrifuge tube were purchased from Corning, US. Disposable Syringe Filter was purchased from Millipore. The main instruments were Heal Force biosafety cabinet (Heal Force Bio-Meditech Holdings Limited, Hongkong, China), Countstar cell counter (Inno-Alliance Biotech, US), EVOS FL fluorescence microscope (Thermo Fisher), microplate reader (Finnpipette), 721 visible spectrophotometer (Shanghai INESA Analytical Instrument CO., LTD), thermostatic water bath (Shanghai Boxun), ultrasonic cracker (Ningbo Xinzhi Bioscience CO., LTD), inverted microscope (Nikon, Japan), refrigerated centrifuge (Thermo Fisher).

1.2 Materials

Rat small intestinal crypt epithelial cell (IEC-6 cell) was obtained from Chinese Academy of Medical Science, Cell Research Institute. Rat normal hepatocyte (BRL 3A Cell) was obtained from Chinese Academy of Sciences, Cell bank at Shanghai.

1.3 Test sample

Samples 1-3 were nucleotide composition according to the invention. Comparative Samples 2 and 3 were nucleotide compositions disclosed in the prior art (above). Each sample was set with three concentrations of low, medium and high (62.5, 250, 1000 $\mu\text{mol/L}$, respectively).

1.4 Model and indicators

Oxygen damage is one of the most common and typical damage to living body (e.g. intestinal tract damage). It has been known that many diseases like those of digestive system of human and animals are closely associated with radical and reactive oxygen species, for example, oxygen damage has been shown to be one of the causes of inflammatory bowel disease. The redox reaction in cell is kept balance under normal physiological status and oxygen radicals and antioxidant system in body are important for the balance. If oxygen radicals are produced greatly or the function of antioxidant system is reduced, damage will be caused to tissue cells. SOD (superoxide dismutase) is an important antioxidant, which can clear O_2^- produced by peroxide and protect cells from damage and thus is essential to oxidation/antioxidant balance in cell. As for the damage caused by oxide/peroxide, lactic dehydrogenase (LDH) is the indicator for cell membrane integrity or cell necrosis. In addition, oxide/peroxide in cells can attack polyunsaturated fatty acids in biofilms, which leads to lipid peroxidation and formation of lipid peroxide, for example malondialdehyde (MDA). Oxygen radicals not only cause cell damage by peroxidation of polyunsaturated fatty acids in biofilms, but lead to cell damage by decomposition product of lipid hydroperoxide, and thus the amount of MDA can generally reflect the degree of lipid peroxidation, thereby showing the degree of cell damage. Therefore, the concentrations, activities or changes thereof of the substances and cell survival rate can be used to evaluate the protective effects of the nucleotide composition according to the invention. See, "Study on Healing effect of *Rheum Tanguicum* Polysaccharides (RTP) on intestinal epithelial cell injury and its mechanism", Thesis of Ph. D. Liu Lin-na, Fourth Military Medical University, May 1, 2005; "Protection of Longyanshe Polysaccharide on CCl_4 – induced Damage of Primary Cultured Hepatocytes in Rats", Duan Xiaoqun et. al, China Pharmacy, 2006 vol 17, No 15, pp 1132-1143; "In vitro Protective Effects of Baoganning On Injury of Liver Cells Induced by Hydrogen Peroxide in Rats" Zhao Jinjun et. al, Journal of Guangzhou University of Traditional Chinese Medicine, 2002, vol 19, No 3, pp 211-213.

In addition, according to the inventor's experiments, H₂O₂ at 600 μmol/L will significantly lower survival rate of BRL 3A cells ($P<0.01$). Therefore, in oxidative damage test of BRL 3A, H₂O₂ at 600 μmol/L was used to induce cell oxidative damage. Similarly, H₂O₂ at 100 μmol/L was used to induce cell oxidative damage for IEC-6 cell.

According to the above information, in this example, the protective effect of the composition according to the invention on cell oxidative damage was investigated by treating cells with nucleotide composition and inducing cell oxidative damage via H₂O₂. For BRL 3A cell, cell survival rate and SOD activity were tested. For IEC-6 cell, LDH activity and MDA content were tested.

1.5 Cell culture and sample preparation

IEC-6 cell was cultured in 37°C, 5%CO₂ incubator with RPMI-1640 medium containing 5%FBS, 2 mg/L insulin and passage was performed every four days. BRL 3A was cultured in 37°C, 5%CO₂ incubator with DMEM high sugar medium containing 10%FBS and passage was performed every five days. The nucleotide composition was formulated as 50 mmol/L of stock solution with PBS, which was filtered for sterilization and stored at -20°C for use.

20 1.6 Procedures

As for cell survival rate, the cells at logarithmic phase were inoculated in 96 well plate at 5000 /well. After adherence, the cells were incubated with medium containing various concentrations of nucleotide compositions for 24 h (each concentration set with 6 repeats). In the test, there were normal control without H₂O₂ and positive control with H₂O₂ only. After 24 h, H₂O₂ was added to induce cell oxidative damage, respectively. After 2h, MTT method was used to determine cell survival rate. Cell survival rate = OD value of test group / OD value of normal control ×100%.

For other indicators, the cells at logarithmic phase were inoculated in 60 mm cell culture dish at 7×10⁵/dish. After adherence, the cells were incubated with medium containing various concentrations of nucleotide compositions for 24 h (each concentration set with 3 repeats). In the test, there were normal control without H₂O₂ and positive control with H₂O₂ only. After 24 h, H₂O₂ of a certain concentration was added to induce cell oxidative damage, respectively and culture was continued. After 2 h, liquid medium was sucked and centrifuged and the supernatant was taken for relevant analysis. The cells were digested with trypsin and washed with PBS once after centrifugation and then resuspended in 700 μl PBS, which were then cracked with supersonic and tested for all the indicators within 2 days.

1.7 Data analysis

All the data were shown as average $\pm$ standard deviation ($\bar{x} \pm s$) and statistics analysis was performed with SPSS17.0 software, inter-group difference comparisons utilized one-way analysis of variance. $P < 0.05$ means statistical significance and $P < 0.01$ means high statistical significance.

2. Results

2.1 Cell survival rate assay

Cell survival rate results of BRL 3A cell were shown in Figure 1. According to this Figure, H_2O_2 treatment significantly lowered the cell survival rate of BRL 3A ($P < 0.01$). Treating cells with nucleotide composition samples, cell survival rates were increased in various degrees, especially Samples 1 and 2. Statistical results showed, for all the concentration groups of Sample 1, low and medium concentration groups of Sample 2 and low concentration group of Sample 3, the cell survival rates were significantly higher than those of H_2O_2 control ($P < 0.01$ or $P < 0.05$). In addition, for all the concentration groups of Sample 1 and low concentration group of Sample 2, the cell survival rates were significantly higher than those of all the concentration groups of Comparative Samples 2 and 3 ($P < 0.01$ or $P < 0.05$). Accordingly, treatment of cells with the nucleotide composition according to the invention can effectively increase of survival rate the cell with oxidative damage, indicating protective effect against oxidative damage and further showing promotion reparation of cells after damage. Moreover, the protective effect is better than the nucleotide composition of the prior art.

2.2 SOD activity assay

SOD activity results of BRL 3A cell were shown in Figure 2. According to this Figure, H_2O_2 treatment significantly lowered the SOD activities in cells ($P < 0.01$). Treating cells with nucleotide composition samples, SOD activities in cells were increased in various degrees. For all the concentration groups of Sample 1 ($P < 0.05$), medium concentration group of Sample 2 ($P < 0.05$), medium and high concentration groups of Sample 3 ($P < 0.01$), the SOD activities in cells were significantly higher than those of H_2O_2 control. Moreover, for medium concentration group of Sample 3, the SOD activities in cells were significantly higher than those of medium and high concentration groups of Comparative Sample 2 ($P < 0.05$) and all concentration groups of Comparative Sample 3 ($P < 0.05$). As for high concentration group of Sample 3, SOD activities in cells were significantly higher than those of high concentration group of Comparative Sample 2 ($P < 0.05$) and low and high concentration groups of Comparative Sample 3 ($P < 0.01$). Accordingly, the nucleotide composition according to the invention can significantly increase concentration of antioxidative active substance (e.g. SOD) in cells with oxidative damage, indicating protective effect against oxidative damage.

Moreover, the protective effect is better than the nucleotide composition of the prior art.

2.3. LDH activity assay

LDH activity results of IEC-6 cell were shown in Figure 3. According to this Figure, H₂O₂ treatment significantly made LDH content in liquid medium supernatant higher than that of the normal control ($P < 0.01$). Treating cells with nucleotide composition, LDH contents in supernatant were lowered in various degrees. As for low concentration group of Sample 2 and medium concentration group of Sample 3, the LDH content decreased most significantly, showing no statistical difference as compared to normal control. Additionally, as compared to H₂O₂ control, in medium concentration group of Sample 1 ($P < 0.05$), low and high concentration groups of Sample 2 ($P < 0.01$ or $P < 0.05$), low and medium concentration groups of Sample 3 ($P < 0.01$), the LDH contents were lowered significantly. Moreover, as compared to high concentration of Comparative Sample 2, the LDH contents of low concentration group of Sample 2 ($P < 0.05$) and low and medium concentration group of Sample 3 ($P < 0.01$) were lowered significantly. Accordingly, the nucleotide composition according to the invention can effectively lower the concentration of the substance indicative of undesired indicator (e.g. LDH) in supernatant of cells with oxidative damage, indicating protective effect against oxidative damage. Moreover, the protective effect is better than the nucleotide composition of the prior art.

2.4. MDA concentration assay

MDA concentration results of IEC-6 cell were shown in Figure 4. According to this Figure, H₂O₂ treatment of cells significantly increased MDA contents in cells ($P < 0.01$). Treating cells with nucleotide composition samples, as for all concentration groups of Samples 1-3, MDA contents are decreased in some degree and were significantly lower than H₂O₂ control. On the contrary, medium and high concentration groups of Comparative Samples 2 and 3 were not significantly distinguished from H₂O₂ control. Accordingly, the nucleotide composition according to the invention can effectively lower the concentration of the substance indicative of undesired indicator (e.g. lipid peroxide MDA) in cells with oxidative damage, indicating protective effect against oxidative damage. Particularly, the nucleotide composition according to the invention can show significant and consistent protective effects under all the concentration ranges. On the contrary, the nucleotide composition in the prior art cannot show protective effect under relatively high concentrations (e.g. medium and high concentrations). This fact further shows advantage of the nucleotide composition according to the invention over the prior art.

3. Discussion

According to the above results, treatment with the nucleotide composition according to the

invention can increase survival rate of the cells with oxidative damage, increase activity/concentration of the factor with protective effect from oxidation (e.g. SOD) in cells, lower activity/concentration of the factor indicative of undesired effect (e.g. LDH and MDA). Such facts means the nucleotide composition according to the invention can provide protective effect against cell oxidative damage and the effect is better than the nucleotide composition in the prior art. Meanwhile, by increasing of the activity of the factor with protective effect and lower the content of undesired factor, the nucleotide composition can promote recovery of the balance condition of cell before damage, whereby promoting reparation after damage. Moreover, effects of the nucleotide composition according to the invention can not only be shown on enterocytes but also on other part of the digestive system (e.g. liver), thereby showing positive effect on the whole digestive system.

Example 4: Cell proliferative effect

The instruments and reagents used in this example refer to example 3, wherein IEC-6 cell was used to verify promotion of cell proliferation of the nucleotide composition according to the invention.

In this example, MTT method was used to detect the influence of nucleotide composition on IEC-6 cell. IEC-6 cells at logarithmic phase were digested with trypsin, which after centrifugation were prepared into cell suspension. Using cell counter, the cells were inoculated in 96 well plate at 5000/well, which was incubated overnight at 37°C, 5%CO₂ such that the cells were adherent. The liquid medium was exchanged the next day and each well was added with 200 µL of cell liquid medium containing various concentrations of nucleotide compositions (each sample was set with three concentrations of 62.5, 250, 1000 µmol/L). Each concentration of each sample was set with 6 repeats. Control group without addition of nucleotide composition and zero well without cells (only addition of normal medium) were also set. The liquid medium was exchanged every 24 h. At 24 h of nucleotide composition treatment, to each well of 96 well plate was added 10 µL of MTT with gentle shake and the plate was incubated in incubator for 4 h. The supernatant was discarded after 4 h and 150 µL/well of DMSO was added. The plate was shaken in dark. After dissolution of bluish violet formazan crystal, the plate was placed in microplate reader and OD value was determined at 490 nm wavelength. Zero was set with the blank group without cell and curve was drawn with OD value.

All the data were shown as average $\pm$ standard deviation ($\bar{x} \pm s$) and statistics analysis was performed with SPSS17.0 software, inter-group difference comparisons utilized one-way analysis of variance. $P < 0.05$ means statistical significance and $P < 0.01$ means high statistical significance.

The results were shown in Figure 5. According to this Figure, after addition of nucleotide sample, proliferation of IEC-6 cell was significantly changed as compared to control. As for high concentration group of Sample 1, all concentration groups of Sample 3, the cell proliferation speeds were significantly higher than those of normal control ($P<0.01$); the cell proliferation speed of medium concentration group of Sample 2 was faster than normal control ($P<0.05$). The cell proliferation speed of low concentration group of Comparative Sample 2 was significantly lower than those of high concentration group of Sample 1, low and medium concentration groups of Sample 2, low, medium and high concentration groups of Sample 3 ($P<0.01$). The cell proliferation speed of high concentration group of Sample 2 was significantly higher than that of low concentration group of Comparative Sample 2 ($P<0.05$). Accordingly, the nucleotide composition according to the invention can promote cell proliferation, especially promote intestinal tract cell proliferation and growth, thereby showing the effect of promotion of enterocytes growth and enhance enterocytes proliferation. The effect was better than the nucleotide composition in the prior art.

Example 5: Effect on intestinal tract beneficial bacterial flora

This example shows the nucleotide composition according to the invention has stimulation on intestinal tract beneficial bacterial flora. Each sample was set with 2 concentrations (low dosage: 1g/100ml; high dosage: 2g/100ml) and there was no nucleotide in control group.

1. Materials and methods

1.1. Main reagents

eosin methylene blue agar (EMB); Tryptose Sulfite Cycloserine Agar (TSC); Bifidobacterium Agar medium (BBL); sodium hydroxide; hydrochloric acid; Egg Yolk Emulsion 50%; D-cycloserine; nucleotide samples (Nanjing Tongkaizhaoye).

1.2. Main instruments

Automatic vertical steam sterilizer (LDZX-30FBS, Shanghai Shenan medical apparatus factory); CO₂ incubator (HH.CP-TW9, Shanghai Shenxian Thermostatic Equipment); electronic scales (XS1003S, Swiss); clean bench (VS-130L-U, Suzhou Antai Airtech CO., LTD.); Colony Counter (DIGITAL S 4905000, Selecta Spain); Sealed culture box (C-32, Mitsubishi Japan).

2. Procedures

2.1. Obtaining intestinal tract flora

In sterile bench, 0.5 g of faeces sample were taken from the anus of five SPF mice and placed in sterile tube, to which was added three sterile glass beads. The mixture was subjected to

vortex and then to gradient dilution to 10^{-2} and used as seed solution for further test.

2.2. Preparation of liquid medium

Beef extract peptone liquid medium was used as base medium, in which nucleotide samples at 1 g/100 ml, 2 g/100 ml were added and the seed solution prepared in section 2.1 was added at 1% (volume).

2.3. Bacterial flora counting

With specific selective medium (BBL agar), the Bifidobacteria in liquid medium was cultured under anaerobic condition for 48 h at $36\pm1^{\circ}\text{C}$ and then bacterial flora counting was performed and expressed as Lg CFU/ml.

2.4. Data statistic

Statistics analysis was performed with SPSS17.0 software and one-way analysis of variance was used to compare the data of various groups, wherein $\alpha=0.05$ was used as criterion for difference determination. The results were shown in Table 4.

Table 4

Grouping	Dosage	Bifidobacteria
Control group		7.00 ± 0.00
Sample 1	low dosage	7.15 ± 0.17
	high dosage	$8.30\pm0.35^*$
Sample 2	low dosage	$7.89\pm0.47^*$
	high dosage	$8.24\pm0.28^*$
Sample 3	low dosage	$7.63\pm0.17^*$
	high dosage	$7.80\pm0.23^*$

* Significant increase

Statistical results showed, taking Bifidobacteria as an example, after treatment with the nucleotide composition according to the invention, growth of Bifidobacteria was significantly increased as compared to control (Lg CFU), indicating that the nucleotide composition according to the invention can stimulate growth of intestinal tract beneficial bacterial flora.

Example 6: Infant milk powder preparation (1000 kg, dry addition)

Raw materials of the milk powder according to the invention were: skimmed milk powder 140 kg, lactose 260 kg, desalted whey powder 200 kg, whey protein powder (WPC34%) 100 kg, corn oil 52 kg, soybean oil 42 kg, sunflower seed oil 170 kg, oligofructose powder 4.8 kg, galactooligosaccharide syrup 12 kg, mixed nutrients 6.4 kg and soyabean lecithin 2 kg. After homogenous mixing, the above raw materials were subjected to pasteurization,

homogenization, evaporation and concentration and spray drying to form semiproduct as powder, to which was added 0.38 kg nucleotide composition according to the invention, 0.1 kg Bifidobacteria, 4.32 kg DHA, 6.0 kg ARA. After mixing with dry mixer, the uniformly mixed milk powder was packed with nitrogen to obtain the final product.

5

Example 7: Infant milk powder preparation (1000 kg, dry addition)

Skimmed milk powder 150 kg, lactose 250 kg, desalted whey powder 190 kg, whey protein powder (WPC34%) 110 kg, corn oil 44 kg, soybean oil 44 kg, sunflower seed oil 175 kg, oligofructose powder 4.5 kg, galactooligosaccharide syrup 11 kg, mixed nutrients 7 kg, 2 kg soyabean lecithin were mixed uniformly and then subjected to pasteurization, homogenization, evaporation and concentration and spray drying to form semiproduct as powder. Then 0.5 kg nucleotide composition according to the invention, 0.1 kg Bifidobacteria, 5.2 kg DHA and 6.7 kg ARA were added. After mixing with dry mixer, the uniformly mixed milk powder was packed with nitrogen to obtain the final product.

10
15

Example 8: Infant milk powder food preparation (1000 kg, wet addition)

Skimmed milk powder 150 kg, lactose 250 kg, desalted whey powder 190 kg, whey protein powder (WPC34%) 110 kg, corn oil 43 kg, soybean oil 44.7 kg, sunflower seed oil 178 kg, oligofructose powder 4.5 kg, galactooligosaccharide syrup 11 kg, mixed nutrients 7 kg, 0.4 kg nucleotide composition according to the invention and 2 kg soyabean lecithin were mixed uniformly and subjected to pasteurization, homogenization, evaporation and concentration and spray drying to form semiproduct as powder, to which were added 0.1 kg Bifidobacteria, 3.9 kg DHA and 5.4 kg ARA. After mixing with dry mixer, the uniformly mixed milk powder was packed with nitrogen to obtain the final product.

20
25

Example 9: Infant liquid milk preparation (based on 100g liquid milk)

The liquid milk according to the invention contained the follows nutritional ingredients:

Protein 2.2 g, fat 3.0g, lactose 9.0 g, vitamin A 80 µg RE, vitamin D 1.5 µg, vitamin E 1mg α-TE, vitamin K1 6 µg, vitamin B1 70 µg, vitamin B2 70 µg, vitamin B6 200 µg, vitamin B12 0.5 µg, nicotinic acid 350 µg, folic acid 3.5 µg, pantothenic acid 200 µg, vitamin C 7 mg, biotin 2 µg, sodium 50 mg, potassium 70 mg, copper 80 µg, magnesium 30 mg, iron 1 mg, zinc 0.7 mg, calcium 80 mg, phosphorus 45 mg, iodine 20 µg, chlorine 100 mg, lactoferrin 10 mg and 2.7 mg nucleotide composition according to the invention, wherein the energy is about 300kJ.

30
35

Fat is provided by anhydrous milk fat, soybean oil, corn oil, sunflower seed in any ratio and combination, wherein based on 100 g of total ingredients, 0.5 g of linoleic acid was included.

The preparing process was as follows (raw materials and process comply with relevant national standards):

1. Receiving, checking and optimizing the raw materials.

- 5 2. (1) Dissolving protein (including casein, whey protein, lactoferrin), B vitamins and vitamin C, and nucleotide composition according to the invention with warm water;
- (2) Dissolving hydrocarbons raw materials and food additives like carrageenan, glyceryl monostearate, guar gum and the like with warm water; dissolving minerals with warm water; dissolving other raw materials with fatty feed liquid at 40°C~50°C; and mixing
- 10 the above three dissolved feed liquids uniformly;
- (3) Subjecting the feed liquid obtained in step (1) to membrane filtration for sterilization;
- (4) Subjecting the feed liquid obtained in step (2) which has been mixed uniformly to UHT (ultra - high temperature instantaneous sterilization) for sterilization;
- (5) Mixing the feed liquids obtained in step (3) and step (4) under sterile condition and
- 15 metered to total amount of the ingredients with water;
- (6) Subjecting the above mixed feed liquids to sterile homogenization; and
- (7) Filling under sterile condition and packing.

Unless otherwise indicated, all numbers expressing quantities of ingredients, cell culture,

20 treatment conditions, and so forth used in the specification, including claims, are to be understood as being modified in all instances by the term “about”. Accordingly, unless otherwise indicated to the contrary, the numerical parameters are approximations and may vary depending upon the desired properties sought to be obtained by the present invention. Unless otherwise indicated, the term “at least” preceding a series of elements is to be

25 understood to refer to every element in the series. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the appended claims.

30 Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only and are not meant to be limiting in any way. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the appended claims.

35

Claims

[Claim 1]. Use of a nucleotide composition, for the manufacture of a medicament for promoting reparation of enterocytes after damage caused by oxidation, wherein the nucleotide composition consists of CMP, AMP, UMP and GMP, and the proportions of each of the nucleotides, on a weight basis relative to the total weight of the nucleotide composition, are CMP: 58-72%, AMP: 6-14%, UMP: 10-18% and GMP: 8-14%, provided that sum of the proportions is 100%.

[Claim 2]. The use according to claim 1, wherein the proportions of the components of the nucleotide composition are CMP: 60-70%, AMP: 8-12%, UMP: 12-16% and GMP: 10-12%.

[Claim 3]. The use according to claim 1, wherein the proportions of the components of the nucleotide composition are CMP: 60-65%, AMP: 10-12%, UMP: 14-16% and GMP: 11-12%.

[Claim 4]. The use according to claim 1, wherein the proportions of the components of the nucleotide composition are CMP: 65-70%, AMP: 8-10%, UMP: 12-14% and GMP: 10-11%.

[Claim 5]. The use according to claim 1, wherein the proportions of the components of the nucleotide composition are

CMP: 60%, AMP: 12%, UMP: 16% and GMP: 12%;

CMP: 65%, AMP: 10%, UMP: 14% and GMP: 11%; or

CMP: 70%, AMP: 8%, UMP: 12% and GMP: 10%.