

COMPOSITION FOR ENHANCING UROLITHIN PRODUCTION IN A HUMAN SUBJECT

TECHNICAL FIELD

The present invention relates to a composition comprising 1) a preparation of *Punica granatum* L. and/or
5 another source of ellagitannins and/or ellagic acid and 2) one or more human milk oligosaccharides (HMOs).
Also disclosed herein is a method of improving the production of urolithin A and/or other urolithins in the
gut by administering said composition to a human subject.

BACKGROUND OF THE INVENTION

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Urolithins are gut microbiota-derived metabolites resulting from the catabolization of ellagitannins and/or
ellagic acid by the gut microbiota. Rich sources of ellagitannins are pomegranate and certain fruits and nuts,
such as raspberry, strawberry, blackberry and walnut.

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Ellagitannins are hydrolyzed in the gut to release ellagic acid, which is further processed by the gut microbiota
into urolithins through the loss of one of its two lactones and by successive removal of hydroxyl groups.

In humans, urolithin A, urolithin B and isourolithin A are the measurable final metabolites of the catabolic
conversion of ellagitannins and/or ellagic acid (see Figure 1). Urolithin A is the main metabolite observed in
human plasma and urine, where it mainly occurs as conjugate forms with glucuronic acid and/or sulfate.

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Urolithins A, B, C and D have all shown the ability to extend the lifespan of the nematode *Chaenorhabditis*
elegans (between 19.0% and 45.4%). Urolithin A activated mitophagy throughout the duration of the
treatment. Mitophagy is the process by which damaged or superfluous mitochondria are removed by
autophagy. The results further suggested that urolithin A treatment activated mitochondrial biogenesis in
older worms. Pharyngeal pumping rate and mobility was improved by urolithin A treatment during aging.
Similar effects were observed with urolithin B, whereas urolithin C and urolithin D showed mild to no benefit
25 (Ryu et al. 2016). Treatment with urolithin A has shown improved endurance capacity (42% greater running
endurance) and increased muscle function (57% greater level of spontaneous exercise) in old mice. Lean
muscle mass did not increase, suggesting that urolithin A improves muscle cell quality rather than quantity.
At the molecular level, treatment with urolithin A led to a general tendency for a higher expression of
autophagy and mitophagy transcripts (Ryu et al. 2016).

Furthermore, urolithin A has shown promising effects on mitochondrial health in a clinical trial. Urolithin A stimulated mitochondrial biogenesis in the skeletal muscle of humans following 28 days of oral administration at doses of 500 mg/d and 1000 mg/d (Andreux et al. 2019).

Mitochondria are the central energy producers of the cell and play a vital role in a wide range of cellular processes, including regulation of cell homeostasis. An intricate regulatory network, balancing the generation of new mitochondria (mitochondrial biogenesis) and removal of damaged mitochondria (mitophagy), forms the basis of healthy aging and longevity.

Mitophagy efficiency decline with age, leading to progressive accumulation of damaged and/or superfluous mitochondria, deterioration of cellular function, and ultimately cell death. Most aging-related diseases, particularly neurodegenerative diseases, have a mitochondrial involvement. Mitochondrial dysfunction has been associated with such diverse diseases as diabetes, cancer, cardiovascular disease, Alzheimer's Disease, Parkinson's Disease, multiple sclerosis, amyotrophic lateral sclerosis, age-related macular degeneration, chronic inflammation and autoimmune disorders (Haas 2019). The significance of impaired or defective mitophagy is well-known for the development of Alzheimer's Disease (Fang et al. 2019) and Parkinson's Disease (Srivastava 2017).

Poor mitochondrial function in the skeletal muscle has been closely linked to slow walking speed and poor muscle strength in elderly individuals. Decline in muscle function in the elderly is often part of a larger syndrome, termed frailty, which is an important risk factor for disability, hospitalization and mortality. A decrease in mitophagy-related gene expression was reported to correlate with slower walking speed in frail elderly (Drummond et al. 2014).

Emerging evidence suggests that enhanced mitophagy promotes healthy aging as well as delays the onset and progression of various age-related pathologies. Thus, mitophagy modulation may serve as a potential therapeutic approach to alleviate age-associated weaknesses.

There is thus substantial evidence suggesting that urolithins have beneficial effects in relation to delaying certain aging processes linked to mitochondrial function, and therefore urolithins might be a valuable food supplement for the aging population. However, isolated urolithins may in general not be administrated directly to humans due to pending regulatory approval procedures. Urolithin A has been GRAS approved in the US, but not authorized as novel food in EU yet (Djedjibegovic et al. 2020).

An alternative to the consumption of isolated urolithins would be to use natural sources of ellagitannins such as pomegranate and certain fruits and nuts, such as raspberry, strawberry, blackberry and walnut, and rely on the *in vivo* catabolization of ellagitannins and/or ellagic acid by the gut microbiota to urolithins.

However, ingesting natural sources of ellagitannins such as pomegranate is not a generally applicable alternative, as not all individuals have the appropriate gut microbiota to produce the desired urolithin metabolites. As reported in a number of clinical trials, 5-30% of a given population are not able to convert ellagitannins or ellagic acid to urolithins, or only produce traces hereof (Tomas-Barberan et al. 2014; Li et al. 2015). Among urolithin producers, two main metabotypes have been distinguished: individuals who produce only urolithin A and individuals who produce isourolithin A and/or urolithin B in addition to urolithin A (Tomas-Barberan et al. 2014). Thus, three different urolithin metabotypes are generally referred to in the scientific literature: exclusive urolithin A-producers (UM-A), mixed urolithin producers (UM-B) and non-producers (UM-0).

It remains yet to be established which gut microorganisms are responsible for the complete catabolic conversion of ellagitannins to urolithins. A number of clinical trials and *in vitro* studies have contributed to shedding light on this question, so that at least some bacteria groups, genera and species have been recognized as playing a role in the transformation. The genus *Gordonibacter*, belonging to the family Eggerthellaceae and the phylum Actinobacteria, has been linked to urolithin production in several clinical studies conducted in Spain (González-Sarriás et al. 2017; Cortés-Martín et al. 2019; Romo-Vaquero et al. 2015).

The bacteria species *Akkermansia muciniphila* (Akkermaniaceae, Verrucomicrobia) is capable of producing ellagic acid from pomegranate ellagitannins *in vitro*. This species was found at 33-fold and 47-fold higher concentrations in stool samples of urolithin A-producers compared to non-producers at baseline and after 4 weeks of pomegranate extract administration, respectively (Li et al. 2015).

Lactic acid bacteria and *Bifidobacteria* have been reported to decrease in UM-B individuals (but not UM-A individuals) upon intake of pomegranate extract (González-Sarriás et al. 2017). On the other hand, another clinical study reported that *Bifidobacterium* increased exclusively in UM-B subjects upon walnut intake (García-Mantrana et al. 2019). Further, a higher abundance of *Bifidobacterium* (Bifidobacteriaceae, Actinobacteria) was found in UM-A postpartum women than in UM-B individuals (Cortés-Martín et al. 2019).

Similar cases of conflicting reports on gut microbiota composition under certain conditions and in certain metabotypes seem to be more the rule than the exception. In short, the breakdown of ellagitannins to urolithins has only been partly elucidated and it is likely that more routes exist, when it comes to the involved microorganisms.

At present, there is an unmet need for a means of activating urolithin production among the group of non-producers, since these individuals do not benefit from the health improving effect of urolithins.

SUMMARY OF THE INVENTION

It is an objective of the present invention to enable urolithin non-producers, particularly men and women above 50 years of age, to benefit from the physiological advantages of urolithin in terms of healthy aging due to improved mitochondrial function. Health benefits of improved mitochondrial function include retention of muscle strength, preservation of a healthy cardiac and immune system and avoidance of neurodegenerative disorders.

It has now been found by the inventors that administering an oral composition comprising a preparation of the fruit of *Punica granatum* in combination with an effective dose of one or more human milk oligosaccharides (HMOs), leads to an activation of urolithin production in individuals who are considered “non-producers”. As defined herein, non-producers do not produce any of the so-called final urolithin metabolites, *i.e.* urolithin A, urolithin B and isourolithin A, or only minimal amounts thereof, upon ingesting food sources containing ellagitannins and/or ellagic acid. A true non-producer cannot unequivocally be determined due to the inherent limit of detection (LoD) of analytical equipment used for the measurement of urolithins (or any other compound) in body fluids or tissue. Herein we define non-producers as individuals with a maximum amount of final urolithins in the urine of $<5 \mu\text{g}/\text{mg}$ creatinine. This definition is in accordance with the findings by Li et al. (2015), *supra*, since urolithin A non-producers are technically identical with final urolithin non-producers. Li et al. (2015) reported that the group of individuals with “no baseline UA production” comprises individuals with a baseline urolithin A production up to $2.9 \mu\text{g}/\text{mg}$ creatinine in urine (Li et al., 2015). Thus, a maximum limit of $5 \mu\text{g}$ final urolithins/mg creatinine in urine seems to be a reasonable dividing line between non-producers and producers of final urolithins.

The increase in the production of urolithins following administration of an oral composition comprising a preparation of the fruit of *Punica granatum* in combination with an effective dose of one or more human milk oligosaccharides (HMOs) referred to above may not be achieved by a corresponding amount of pomegranate preparation alone or another source of ellagitannins or ellagic acid.

In a first aspect, the present invention thus provides a composition comprising a preparation of the fruit of *Punica granatum* together with an effective dose of one or more human milk oligosaccharides (HMOs). The combined effect of these substances is an increased production of final urolithins by the gut microbiota.

The invention also relates to a method for the identification and treatment of non-producers in order to make these individuals capable of benefitting from the positive health effects of improved mitochondrial

function, including increased mitophagy. This is especially true for middle-aged and elderly individuals (+50). The identification is based on analyzing a urine sample obtained from an individual after said individual has ingested a standardized dose of ellagitannins for three consecutive days.

In a second aspect, the present invention thus provides a composition according to the first aspect for use in the treatment of a human subject identified as being a non-producer, said identification comprising the following steps:

- a) Orally administering to the human subject a standardized dose of ellagitannins for three consecutive days and,
- b) Obtaining a urine sample from the human subject 72-88 hours after the human subject has ingested the first standardized dose of ellagitannins,
- c) Analyzing the urine sample for the presence and levels of urolithin A, urolithin B and isourolithin A,
- d) Comparing the level of urolithin A, urolithin B and isourolithin A with metabotype reference levels for UA-producers and mixed producers, respectively;
- e) Determining whether the subject is a UA-producer, mixed producer or non-producer, based on the comparison of the subject levels with the reference levels,

wherein the presence of urolithin A, urolithin B and isourolithin A in a total amount less than 5µg/mg creatinine in urine classifies the subject as a non-producer.

In a third aspect, the invention also relates to a method of treatment of a human subject identified as being a non-producer, said method comprising

- a) Orally administering to the human subject a standardized dose of ellagitannins for three consecutive days,
- b) Obtaining a urine sample from the human subject 72-88 hours after the human subject has ingested the first standardized dose of ellagitannins,
- c) Analyzing the urine sample for the presence and levels of urolithin A, urolithin B and isourolithin A,
- d) Comparing the level of urolithin A, urolithin B and isourolithin A with metabotype reference levels for UA-producers and mixed producers, respectively;
- e) Determining whether the subject is a UA-producer, mixed producer or non-producer, based on the comparison of the subject levels with the reference levels,
- f) administering a therapeutically effective dose of a composition according to the first aspect comprising a preparation of the fruit of *Punica granatum* together with one or more human

milk oligosaccharides (HMOs), to any human subject identified as belonging to non-producers;
and

- g) optionally monitoring said identified subject for an increase in the level of final urolithins over a period of at least 30 days, such as 30 days, 60 days or preferably 90 days.

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In a fourth aspect, the present invention also relates to a method for increasing the production of urolithins, in particular final urolithins, in the human gut, said method comprising administering a composition according to the first aspect as described above to a human subject, in particular a middle-aged or elderly (50+) human subject. The composition may be administered as an ingestible medicament, as a medical device, a dietary supplement, a food additive or a medical food or a food for special medical purpose. Preferably, the one or more HMOs in the composition is at a dosage of between 500 mg and 10000 mg HMOs/day, such as at least 500 mg HMOs/day, such as at least 750 mg HMOs/day, such as at least 1000 mg HMOs/day, such as at least 2000 mg HMOs/day, such as at least 5000 mg HMOs/day.

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15 BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Gut microbiota catabolism of pomegranate ellagitannins (punicalagin) to urolithins, and differences between UA-producers (Metabotype A) and mixed producers (Metabotype B). The transient intermediate metabolite luteic acid has not yet been detected. Final urolithins are urolithin A, urolithin B and isourolithin A. All other urolithins are defined as intermediary urolithins (García-Villalba et al. 2017).

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Figure 2: Production of urolithin A during two timeframes of the incubation (0-24h and 0-48h) upon fermentation of three different substrates, i.e. 3 g/L HMO, 1 g/L pomegranate extract (PG) and 3g/L HMO + 1g/L PG. A negative control (blank) was also included.

Figure 3: Production of isourolithin A during two timeframes of the incubation (0-24h and 0-48h) upon fermentation of three different substrates, i.e. 3 g/L HMO, 1 g/L pomegranate extract (PG) and 3g/L HMO + 1g/L PG. A negative control (blank) was also included.

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Figure 4: Production of urolithin C during two timeframes of the incubation (0-24h and 0-48h) upon fermentation of three different substrates, i.e. 3 g/L HMO, 1 g/L pomegranate extract (PG) and 3g/L HMO + 1g/L PG. A negative control (blank) was also included.

Figure 5: Average production of urolithin A across 9 donors during two timeframes of the incubation (0-24h and 0-48h) upon fermentation of three different substrates, i.e. 3 g/L HMO, 1 g/L pomegranate extract (PG) and 3g/L HMO + 1g/L PG. A negative control (blank) was also included.

DEFINITIONS

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Autophagy: Autophagy is the regulated digestion within a cell of cytoplasmic elements which it no longer requires.

Bifidogenic: A substance or compound that promotes the growth of bifidobacteria in the intestinal tract is said to be bifidogenic.

10 **Biogenesis:** The production of new living organisms or organelles.

Catabolization: The process in which molecules are broken down into smaller units through a set of metabolic pathways.

Conjugate: A compound formed by the joining of two or more chemical compounds.

15 **Decoy receptor:** A receptor that is able to recognize and bind specific growth factors or cytokines efficiently but is not structurally able to signal or activate the intended receptor complex.

Ellagitannin: The ellagitannins are a diverse class of hydrolysable tannins, a type of polyphenol formed primarily from the oxidative linkage of galloyl groups in 1,2,3,4,6-pentagalloyl glucose. Ellagitannins yield ellagic acid on hydrolysis. Examples of ellagitannins found in pomegranate fruit are: punicalagins A and B, punicalins, punicalin isomers.

20 **Final urolithins:** Final urolithins comprise urolithin A, urolithin B and isourolithin A. These urolithins are the measurable final metabolites of the catabolic conversion of ellagitannins and/or ellagic acid in the human gut.

Fucosylated molecule: A molecule to which one or more fucose sugar units have been added.

Gut microbiota: The gut microbiota is the microbial communities inhabiting the gastrointestinal tract.

25 **Homeostasis:** The state of steady internal, physical and chemical conditions maintained by living systems.

Human milk oligosaccharide (HMO): Human milk oligosaccharide, abbreviated as HMO, are complex carbohydrates found in human breast milk. The HMOs have a core structure comprising a lactose unit at the reducing end that can be elongated by one or more β -N-acetyl-lactosaminyl and/or one or more β -lacto-N-

biosyl units, and which core structure can be substituted by an α -L-fucopyranosyl and/or an α -N-acetyl-neuraminyl (sialyl) moiety. In this regard, the non-acidic (or neutral) HMOs are devoid of a sialyl residue, and the acidic HMOs have at least one sialyl residue in their structure. The non-acidic (or neutral) HMOs can be fucosylated or nonfucosylated.

- 5 **Husk:** The husk is a usually dry or membranous outer covering of various seeds and fruits. The pomegranate husk is comprised of two parts: the pericarp, which provides an outer cuticle layer and fibrous mat, and the mesocarp, which is the spongy tissue and inner fruit wall where the fleshy arils are attached.

Hydrolysis: The chemical process in which a molecule is cleaved into two parts by the addition of a molecule of water.

- 10 **Intermediary urolithins:** Intermediary urolithins comprise urolithin M-5, urolithin M-6, urolithin M-7, urolithin C, urolithin D and urolithin E. These urolithins are the measurable intermediary metabolites of the catabolic conversion of ellagitannins and/or ellagic acid to the final metabolites urolithin A, urolithin B and isourolithin A in the human gut.

- 15 **Metabolite:** A metabolite is any substance produced during metabolism, i.e. digestion or other physiological chemical processes.

Metabotype: A metabolic phenotype, i.e. the kind of metabolism characterizing an individual.

Microbiome: The microbiome comprises all of the genetic material within a microbiota, which is the entire collection of microorganisms in a specific niche, such as the human gut.

- 20 **Mitochondria:** Mitochondria are membrane-bound cell organelles that function as power generators of the eukaryotic cell, converting oxygen and nutrients into adenosine triphosphate (ATP). Mitochondria also store calcium for cell signalling activities, generate heat and mediate cell growth and death. Mitochondria are unlike other cellular organelles in that they have two distinct membranes and a unique genome. Furthermore, they reproduce by binary fission.

Mitophagy: The selective degradation of dysfunctional or superfluous mitochondria by autophagy.

- 25 **Mixed producer:** An individual who produces urolithin B and/or isourolithin A in addition to urolithin A as final urolithins.

Non-producer: An individual who does not produce any of the final metabolites urolithin A, urolithin B or isourolithin A, or only does so in low amounts ($< 5 \mu\text{g}/\text{mg}$ creatinine in urine, as defined herein).

Pericarp: The outer layer of a ripe fruit.

Pomace: The solid remains of fruits after pressing for juice or oil.

Punicoside: The term punicosides refers to the punicalagins and punicalins in pomegranate fruit, including punicalagin A and B, punicalin A and B and punicalin isomers.

Prebiotics: Dietary ingredients, usually oligosaccharides, that provide a health benefit to the host mediated
5 by the modulation of the human gut microbiota.

Skeletal muscle: Skeletal muscles are the most common of the three types of muscles in the body. Most skeletal muscles are attached to bones by tendons, and they produce all the movements of body parts in relation to each other. Skeletal muscles are under voluntary control of the somatic nervous system.

UA-producer: an individual who only produces urolithin A as a final urolithin.

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DETAILED DESCRIPTION OF THE INVENTION

At least three types of urolithin producers or metabotypes have been documented in a number of clinical trials conducted by several research groups. Firstly, there are individuals who are described as not producing
15 the final urolithin metabolites or even any kind of urolithins, or only traces thereof. These individuals are often referred to as “nonproducers”. According to different clinical trials, the group of non-producers accounts for 5-30% of a population (Tomas-Barberan et al. 2014; Li et al. 2015). Reports of absence of excreted urolithin by individuals in this group should however be understood as an analytical issue rather than a *de facto* proof that these individuals do not produce urolithins at all (Cerdá et al. 2004; Nuñez-Sánchez
20 et al. 2014; Truchado et al. 2012). This was pragmatically addressed in an intervention study (Li et al. 2015) in which participants with a baseline urolithin A content in urine of up to 2.9 µg/mg creatinine were classified as nonproducers.

Therefore, as mentioned above, “non-producers” are defined herein as individuals - who upon ingesting food sources containing ellagitannins and/or ellagic acid – produce the final urolithin A, urolithin B and isouroulothin
25 A in low and presumably physiologically insignificant amounts, as there seems to be no scientific rationale for claiming a complete absence of these metabolites in this group of individuals. Based on the observations by Li et al. (2015), we define non-producers as individuals with a maximum amount of final urolithins in the urine of <5 µg/mg creatinine.

Secondly, there is a predominating group of individuals who only produce urolithin A as the final metabolite.
30 This group of exclusive urolithin A-producers, UA-producers, accounts for 25-80% of a population. Finally, a

more heterogeneous group exists consisting of individuals who produce urolithin A in addition to urolithin B and/or isourolithin A. This group of mixed producers makes up 10-50% of a given population. Other metabolotypes may exist, since the majority of the metabolotype studies have been conducted in a Spanish population which may not be representative for all cultures and ethnic groups.

- 5 The straightforward interpretation of the occurrence of these groups is that non-producers lack one or more bacteria species or bacteria groups necessary for the efficient production of final urolithins or urolithins altogether or alternatively that these bacteria are only present at insufficiently low levels. Genetic polymorphisms or differences in the composition or functionality of the gut microbiota will also contribute to the different response of individuals to intake of ellagitannin-rich foods. In this regard, a highly variable
- 10 metabolism between individuals has been described for some phenolic compounds besides ellagitannins, such as lignans and procyanidins (Ávila-Gálvez et al. 2020).

In other words, non-producers are presumably a rather heterogeneous group with various underlying causes for their insignificant production of final urolithins or urolithins altogether. The UA-producers seems to be a more homogenous group with a gut microbiota comprising all the necessary bacteria species for urolithin A

15 production, however with variations between different populations. Finally, the group of mixed producers probably comprises at least three sub-metabotypes: production of urolithin A + isourolithin A, production of urolithin A + urolithin B and production of urolithin A + isourolithin A + urolithin B. To our knowledge, no attempt to characterize these different sub-metabotypes has been done until now.

Obviously, the non-producers are not able to benefit from the positive health effects of urolithins or only to

20 a very limited extent. It thus seems evident that activation of an urolithin production in these individuals would be an advantage to their general health through an improved mitochondrial function. We thus find it reasonable to expect that non-producers would benefit from changing their metabolotype towards a urolithin producing metabolotype.

Only a few studies deal with conversions between metabolotypes. The metabolotype of healthy, lactating

25 women was determined four times during the first year after having given birth (Cortés-Martín et al. 2019). It turned out that 20% of the women from the mixed producer group changed metabolotype to become UA-producers for unknown reasons. Another clinical study reports the conversion of non-producers to urolithin producers among healthy overweight-obese individuals. Conversions in three out of six non-producers were observed upon intake of pomegranate extract. The circumstances of these conversions seem somewhat

30 unclear, since the group of converted non-producers differed at baseline from obligate non-producers in their significantly higher level of *Gordonibacter*. Furthermore, the study did not report the actual urolithin production for the converted non-producers (González-Sarrias et al. 2017).

The inventors have now found that by administering a composition which comprises a preparation of the fruit of *Punica granatum*, such as a pomace extract, and one or more human milk oligosaccharides (HMOs), individuals identified as non-producers may change their metabotype to UA-producer or mixed producer, more consistently and with a significantly higher resulting urolithin production than when a corresponding amount of pomegranate extract is administrated alone.

In a first aspect, the present invention thus provides a composition comprising

- a. a preparation of the fruit of *Punica granatum*, and
- b. one or more human milk oligosaccharides (HMOs).

In an embodiment, the HMO is selected from neutral HMOs or acidic HMOs. The neutral HMO can be one or more fucosylated HMOs or one or more non-fucosylated HMOs.

Following the administration of the composition according to the first aspect, an amount equal to or higher than 5 µg/mg creatinine of one or more of the final urolithin metabolites in the urine renders a previous non-producer a UA-producer or a mixed producer.

The preparation of the fruit of *Punica granatum* is obtainable by methods known in the art. In some embodiments, the preparation of the fruit of *Punica granatum* is obtainable from the pericarp of *Punica granatum*. The preparation may be an extract of the fruit of *Punica granatum* obtained from the pomace of *Punica granatum*, i.e. by extracting the remains of squeezed fruits. The extract is preferably a dry extract, such as a powder or a granulate. Other methods of preparing an extract of the fruit of *Punica granatum* well known in the art, are also within the scope of the invention.

Specific embodiments relate to extracts of the fruit of *Punica granatum* comprising at least 40% polyphenols. The extract of the fruit of *Punica granatum* according to the present invention preferably further comprises at least 30% punicosides and/or at least 20% punicalagins.

The composition according to the first aspect of the present invention may be such that the ratio (w/w) between the preparation of the fruit of *Punica granatum* and the one or more human milk oligosaccharides (HMOs) is comprised in the range of 5:1 – 1:10.

In one embodiment, the composition according to the first aspect comprises a preparation, such as an extract, of the fruit of *Punica granatum*, one or more HMOs selected from neutral HMOs or acidic HMOs. The neutral HMO can be one or more fucosylated HMOs or one or more non-fucosylated HMOs.

In a second aspect, the present invention provides a composition according to the first aspect for use in the treatment of a human subject identified as being a non-producer, said identification comprising the following steps:

- 5 a) Orally administering to the human subject a standardized dose of ellagitannins for three consecutive days,
- b) Obtaining a urine sample from the human subject 72-88 hours after the human subject has ingested the first standardized dose of ellagitannins,
- c) Analyzing the urine sample for the presence and levels of urolithin A, urolithin B and isourolithin A,
- 10 d) Comparing the level of urolithin A, urolithin B and isourolithin A with metabotype reference levels for UA-producers and mixed producers, respectively;
- e) Determining whether the subject is a UA-producer, mixed producer or non-producer, based on the comparison of the subject levels with the reference levels,

15 wherein the presence of urolithin A, urolithin B and isourolithin A in a total amount less than 5µg/mg creatinine in urine classifies the subject as a non-producer.

In a third aspect, the invention also relates to a method of treatment of a human subject identified as being a non-producer, said method comprising:

- a) Orally administering to the human subject a standardized dose of ellagitannins for three consecutive days,
- 20 b) Obtaining a urine sample from the human subject 72-88 hours after the human subject has ingested the first standardized dose of ellagitannins,
- c) Analyzing the urine sample for the presence and levels of urolithin A, urolithin B and isourolithin A,
- d) Comparing the level of urolithin A, urolithin B and isourolithin A with metabotype reference levels for UA-producers and mixed producers;
- 25 e) Determining whether the subject is a UA-producer, mixed producer or non-producer, based on the comparison of the subject levels with the reference levels,
- f) administering a therapeutically effective dose of a composition according to the first aspect comprising a preparation of the fruit of *Punica granatum* together with one or more human milk oligosaccharides (HMOs), to any human subject identified as being a non-producer; and
- 30 g) optionally monitoring said identified subject for the production of one or more final urolithins over a period of at least 30 days, such as 30 days, 60 days or preferably 90 days.

In a preferred embodiment of the second and third aspect, the treatment of individuals identified as non-producers involves administering a daily dose of the composition according to the first aspect containing between 500 – 10000 mg HMOs/day and 500 – 5000 mg of a preparation of the fruit of *Punica granatum*/day.

5 Human milk oligosaccharides (HMOs) are a group of complex sugars, or oligosaccharides, that are present at high concentrations in human milk. HMOs serve as metabolic substrates for select gut microbes, thus contributing to the development of the infant gut microbiota. In other words, HMOs function as prebiotic substances.

10 HMOs have been found to shape the infant's gut microbiota by selectively stimulating the growth of specific bacteria, especially bifidobacteria. These bifidogenic effects of HMOs are structure-specific and may vary depending on the HMO composition in the milk of different individuals.

The structural diversity represented by HMOs can be broadly divided into fucosylated (neutral HMOs), sialylated (acidic HMOs) and non-fucosylated neutral structures. Approximately 200 different HMOs have been identified so far. The building blocks of human milk oligosaccharides are the five monosaccharides D-
15 glucose, D-galactose, N-acetylglucosamine, L-Fucose, and sialic acid. Lactose forms the reducing end of milk oligosaccharides. The amount and composition of HMOs produced in human milk are highly variable between women.

Apart from having prebiotic effects, HMOs also act as soluble decoy receptors that block the attachment of viral, bacterial or protozoan pathogens to epithelial cell surface sugars. This function as an anti-adhesive may
20 help prevent infectious diseases in the gut and also in the respiratory and urinary tracts.

Supplementation of 2'-fucosyllactose (2'FL) and lacto-N-neotetraose (LNnT) at daily doses up to 20 g to healthy adults modified the gut microbiota with the primary impact being substantial increases in relative abundance of Actinobacteria and *Bifidobacterium* in particular and a reduction in relative abundance of Firmicutes and Proteobacteria. This modulation occurred within 1- 2 weeks (Elison et al. 2016).

25 In another embodiment, the composition according to the first aspect comprises one or more HMOs selected from 2-fucosyllactose (2'-FL), 3-fucosyllactose (3-FL), difucosyllactose (DFL), lacto-N-tetraose (LNT), lacto-N-neotetraose (LNnT), 3-sialyllactose (3'-SL), 6-sialyllactose (6'-SL), lacto-N-fucopentaose 1 (LNFP-1) or a mixture thereof.

Preferably, the one or more HMOs comprises, consists of or essentially consists of 2'-FL and at least one of
30 LNnT and LNT; at least one of 2'-FL and DFL and at least one of LNnT and LNT (e.g. 2'-FL, DFL and at least one

of LNnT and LNT); 2'-FL and 6'-SL; DFL and 6'-SL; 2'-FL, DFL and 6'-SL; 2'-FL, 6'-SL and at least one of LNnT and LNT; or 2'-FL, DFL, 6'-SL and at least one of LNnT and LNT.

In some embodiments the composition according to the first aspect is administered to a human subject who is a non-producer as discussed hereinabove. In some embodiments, administration of a composition according to the first aspect of the invention to a human subject of the non-producer group results over time in an alteration of the gut microbiota of said human subject, which is capable of improved catabolization of ellagitannins and/or ellagic acid into one or more of the final urolithins.

Specific embodiments relate to the use of a composition as disclosed herein for enhancing urolithin production by administration to a human subject for a duration of at least 30 days, such as at least 50 days, such as at least 100 days, such as at least 150 days. Preferably, the composition is administered at least until the subject produces one or more final urolithins.

The present composition may be formulated in a formulation, preferably an ingestible formulation, such as a medicament, a dietary supplement, a food additive or a medical food or a food for special medical purpose. The formulation may comprise other ingredients, such as, but not limited to, an excipient, a coating agent, a flavouring agent, one or more herbal extracts or preparations, one or more nutrients, such as one or more vitamins and/or minerals. The composition may be formulated as a tablet, a pill, a capsule, a powder or a granulate. Other formulations suited for the purpose will be obvious to the skilled man and are also envisioned. The composition may be formulated as distinct formulations, wherein the first device comprises the preparation of the fruit of *Punica granatum* and the second device comprises the one or more HMOs. Alternatively, the composition may be formulated as a single formulation.

By analysing the level of urolithins produced by human gut microbiota, the inventors have found a synergistic effect of an HMO blend + a preparation of the fruit of *Punica granatum* compared with administering an HMO blend or a preparation of the fruit of *Punica granatum* individually regarding the final urolithins, urolithin A and iso-urolithin A, and the intermediary urolithin, urolithin C (see Example 5).

In a fourth aspect, the present invention thus also relates to a method for increasing the production of urolithins, in particular final urolithins, in the human gut, said method comprising administering a composition according to the first aspect as described above to a human subject, in particular a middle-aged or elderly (50+) human subject. The composition may be administered as an ingestible medicament, as a medical device, a dietary supplement, a food additive or a medical food or a food for special medical purpose. Preferably, the one or more HMOs in the composition is at a dosage of between 500 mg and 10000 mg

HMOs/day, such as at least 500 mg HMOs/day, such as at least 750 mg HMOs/day, such as at least 1000 mg HMOs/day, such as at least 2000 mg HMOs/day, such as at least 5000 mg HMOs/day.

In an embodiment, the method for increasing the production of urolithins in a human subject according to the fourth aspect comprises administering a composition according to the first aspect which contains
5 between 500 mg and 10000 mg HMOs/day, such as at least 500 mg HMOs/day, such as at least 750 mg HMOs/day, such as at least 1000 mg HMOs/day, such as at least 2000 mg/day such as at least 5000 mg HMOs/day.

In another embodiment, the method for increasing the production of urolithins in a human subject according to the fourth aspect comprises administering a composition according to the first aspect which contains at
10 least 75 mg punicalagins/day, such as between 75 mg/day and 1500 mg/day, such as between 100 mg/day and 1000 mg/day, preferably at least 300 mg/day.

In another embodiment, the method for increasing the production of urolithins in a human subject according to the fourth aspect leads to an increased production of urolithins when compared with the achievable level of urolithins produced following ingesting one of the individual components of the composition according to
15 the first aspect.

In a preferred embodiment, the present invention relates to a method for increasing the production of one or more of urolithin A, urolithin B and isourolithin A in the gut of a human subject, said method comprising administering a composition according to the first aspect as described above to a human subject, in particular a middle-aged or elderly (50+) human subject.

20

The preparation of the fruit of *Punica granatum* comprises preferably at least 40% polyphenols, at least 30% punicosides and at least 20% punicalagins. In some embodiments, the composition is administered to a human subject at a dosage of at least 75 mg punicalagins/day, such as between 75 mg/day and 1500 mg/day, such as between 100 mg/day and 1000 mg/day, preferably at least 300 mg/day. In some embodiments, the
25 dosage of punicosides is at least 100 mg/day, such as between 100 mg/day and 2000 mg/day, such as between 100 mg/day and 1200 mg/day, preferably at least 400 mg/day. The dosage of polyphenols is at least 125 mg/day, such as between 125 mg/day and 2500 mg/day, such as between 250 mg/day and 1500 mg/day, preferably at least 500 mg/day.

In a preferred embodiment the composition of the present invention may be formulated as a kit comprising
1) tablets containing pomegranate extract, 2) a powder or granulate combination of the two HMOs 2'-
Fucosyllactose (2'-FL) and Lacto-N-neotetraose (LNnT) and 3) instructions for use.

In another embodiment the composition of the present invention may be formulated as tablets comprising
5 a mixture of pomegranate extract, 2'-Fucosyllactose (2'-FL) and Lacto-N-neotetraose (LNnT).

In another embodiment the composition of the present invention may be formulated as a kit comprising
1) tablets containing pomegranate extract, 2) a powder or granulate of one or more HMOs selected from 2'-
FL, 3-FL, DFL, LNT, LNnT, 3'-SL, 6'-SL, LNFP-1 or a mixture thereof, and 3) instructions for use.

In another embodiment the composition of the present invention may be formulated as tablets comprising
10 a mixture of pomegranate extract and one or more HMOs selected from 2'-FL, 3-FL, DFL, LNT, LNnT, 3'-SL, 6'-
SL, LNFP-1 or a mixture thereof.

In other embodiments of the present invention, the pomegranate extract may be replaced by other natural
sources of ellagitannins and/or ellagic acid, such as walnut, raspberry, strawberry and blackberry.

EXAMPLES**Example 1**

Formulation of a kit comprising a pomegranate extract and a combination of HMOs

- 5 A kit consisting of a pomegranate extract and a combination of the two HMOs 2'-Fucosyllactose (2'-FL) and Lacto-N-neotetraose (LNnT) was developed. The kit consisted of a tablet formulation and a powder formulation.

A dry ethanolic extract of *P. granatum* was used for the tablet formulation. The ethanolic extract was based on pomegranate fruit pomace (Monteloeder S.L., Elche, Alicante, Spain). The content of punicalagins was
10 min. 30%. The daily dose of the pomegranate extract was 1000 mg/4 tablets.

The following tablet excipients were added to the formulation: microcrystalline cellulose, cross-linked sodium carboxymethyl cellulose, sodium carboxymethyl cellulose, silicon dioxide, magnesium salts of vegetable fatty acids, hydroxypropyl methyl cellulose, polyethylene glycol.

- As the other active ingredient, a blend of the two HMOs 2'-Fucosyllactose and Lacto-N-neotetraose (Glycom
15 A/S, Hørsholm, Denmark) was used for a powder formulation. The mass ratio between the two HMOs was 2:1 (2'-FL/LNnT). The daily dose of the 2'-FL/LNnT-blend was 5000 mg/stick pack. The following excipient was added to the formulation: magnesium stearate (0.25 mass %).

Example 2

- 20 *Diagnosis of non-producers by urine sample*

Non-producers, i.e. individuals who are not capable of producing final urolithins in a total amount higher than 5 µg/mg creatinine in urine, obviously do not benefit from the health benefits related to stimulation and improvement of mitochondrial function conferred by these urolithins. Non-producers are diagnosed by the following method:

- 25 A daily dose of pomegranate extract (2 g/8 tablets) containing 30% punicalagins is consumed by the individual for three consecutive days. In the morning of the fourth day, a urine sample is collected and the urolithin content is analysed by LC-MS/MS analysis.

This allows the classification of the individual according to capacity to metabolize ellagitannins into final urolithins. If the total amount of final urolithins is less than 5 µg/mg creatinine in urine, the individual is

classified as a non-producer. The exclusive presence of intermediary urolithins does not change the status of non-producer.

Example 3

Determination of beneficial HMOs for final urolithin production by a gastrointestinal simulation model

- 5 The impact of pomegranate extract combined with one or more HMOs on gut microbiome was investigated in the M-TripleSHIME™ *in vitro* gastrointestinal model (Prodigest). The typical reactor setup of the M-TripleSHIME™ consists of a succession of four reactors simulating the different parts of the human gastrointestinal tract. The first two reactors are of the fill-and-draw principle to simulate different steps in food uptake and digestion, with peristaltic pumps adding a defined amount of SHIME feed (140 ml 3x/day) and pancreatic and bile liquid (60 ml 3x/day), respectively, to the stomach and small intestine compartment
10 and emptying the respective reactors after specified intervals. The last two compartments are continuously stirred reactors with constant volume and pH control. The retention time and pH of the different vessels are chosen to resemble *in vivo* conditions in the different parts of the colon. The proximal colon is set to pH 5.4-5.6 and retention time= 12 h, and the distal colon is set to pH 6.0-6.5 and retention time= 20 h.
- 15 Pomegranate extract with a minimum content of 30% punicalagins and a mixture of 2'-Fucosyllactose and Lacto-N-neotetraose (2'-FL and LNnT) in 2:1 mass ratio is added to the SHIME feed in a concentration that equals 5 grams per day of each ingredient.

- Upon inoculation with fecal microbiota from a non-producer individual, these reactors simulate the proximal, transverse and distal colon. After a two-week adaptation of the microbial communities in the different
20 regions of the colon, a representative microbial community is established in the three colon compartments, which differs both in composition and functionality in the different colon regions.

Further, porcine mucin is included in the reactors simulating the colon to take into account the colonisation of the mucous layer. Thus, the M-TripleSHIME™ permits culturing both the luminal and mucous-associated microbial community over periods of several weeks.

- 25 The M-TripleSHIME™ is run in four stages:

1. Stabilization: After inoculation of the reactors with a fresh fecal sample provided by a healthy non-producer, a two-week stabilization period allows the microbial community to differentiate in the different reactors depending on the local environmental conditions.

- During this period, the basic nutritional matrix is provided to support the maximum diversity of the gut
30 microbiota originally present in the fecal inoculum.

2. Control: During this two-week period, a standard nutrient matrix is dosed into the model for a period of 14 days. The baseline microbial community composition and activity in the different reactors is determined by analysis of samples and is used as a reference.

3. Treatment – part 1: The SHIME system is operated under normal conditions for 3 weeks, but with the standard nutrient matrix supplemented with pomegranate extract (min. 30% punicalagins) and the HMOs. The HMOs tested is a 2:1 mixture of 2'-FL and LNnT.

4. Treatment – part 2: The SHIME system is operated under normal conditions for 3 weeks, but with the standard nutrient matrix supplemented with pomegranate extract (min. 30% punicalagins) only.

5. Washout: During this two-week period, the SHIME system is run again with the standard nutrient matrix only.

Sample of the liquids in each reactor are collected regularly and are analysed for the composition of the produced urolithins and the composition of the resident microbial community using 16S rRNA sequencing.

The results from the fermentation system show that final urolithins, especially urolithin A, are produced upon 3 weeks treatment with pomegranate extract and an HMO mixture of 2'-FL and LNnT (2:1). Furthermore, this effect was maintained during the subsequent 3 weeks' treatment with pomegranate extract only, suggesting that a balance had been reached in the gut microbiota towards final urolithin production.

The profiling of the microbial community showed that during the first treatment period the abundance of *Akkermansia* increased in the distal part of the colon regions. Practically no such change occurred in the proximal part. The effect was maintained in the second treatment period.

This *in vitro* study shows that feeding the M-TripleSHIME™ with pomegranate extract and the HMO-blend 2'-FL and LNnT (2:1) activated the production of final urolithins in a gut microbiota originating from a non-producer. Furthermore, the effect was maintained during the 3 weeks with pomegranate extract added as the sole supplement to the nutrient matrix. Thus, the gut microbiota of a non-producer may be altered towards the gut microbiota of a urolithin producer by treatment with pomegranate extract and an HMO-blend of 2'-FL and LNnT.

Example 4

Treatment of non-producers with a combination of an ellagitannin-rich food source and an HMO blend

The following intervention study was designed to investigate whether a combination of an ellagitannin-rich food source and an HMO blend was able to activate the production of one or more final urolithins by the gut microbiota in non-producers.

This clinical study was a two-part study. In the first part of the study, the urolithin metabotype of a group of potential study participants were determined. Only non-producers were recruited to the second part of the study. In the second part of the study, the effect of 3 weeks' daily consumption of pomegranate extract on urolithin production was determined for each study participant, followed by a comparison of

- 1) the effect of continued daily consumption of pomegranate extract on urolithin production, with
- 2) the effect of daily consumption of a combination of pomegranate extract plus an HMO blend on urolithin production.

The dose and composition of the HMO blend was based on a preliminary *in vitro* study.

Part 1 Metabotype identification

Sixty healthy volunteers (30 males and 30 females) were recruited for this metabotype identification study. Exclusion criteria were: a history of gastrointestinal disease or any chronic disease, following a weight-reducing diet, ongoing treatment with dietary supplements, intake up antibiotics, prebiotics, probiotics or medication up to 8 weeks before the study, pregnancy or lactation. All of the subjects filled out a questionnaire about their dietary habits prior to participation in the study. The intake of ellagitannin-containing sources such as berries (strawberry, raspberry, blackberry etc. and derived foodstuffs such as jam), pomegranates, chocolate, nuts and wine were forbidden for 1 week before the study and during the study (part 1).

A daily dose of pomegranate extract (2 g/8 tablets) containing 40% punicalagins was consumed by the study participant for three consecutive days. In the morning of the fourth day, a urine sample was collected and the urolithin content was analyzed by LC-MS/MS analysis.

The study participants were categorized as UA-producers, mixed producers or non-producers.

Only non-producers were recruited to the second part of the study.

Part 2 Determination of the effect of pomegranate extract vs. pomegranate extract + HMO blend on urolithin production in non-producers

The non-producers from part 1 were randomized to the following two treatments:

1. Three weeks' treatment with pomegranate extract whereupon urolithin production was tested by a urine
5 test, followed by another three weeks' treatment with pomegranate extract (PE+PE).
2. Three weeks' treatment with pomegranate extract whereupon urolithin production was tested by a urine
test, followed by three weeks' treatment with pomegranate extract and an HMO blend of 2'-FL and LNnT
(PE+HMO).

A dry ethanolic extract of *P. granatum* was used for a tablet formulation. The ethanolic extract was based on
10 pomegranate fruit pomace (Monteloeder S.L., Elche, Alicante, Spain). The content of punicalagins was 40%.
The daily dose of pomegranate extract was 1000 mg/4 tablets.

A blend of the two HMOs 2'-Fucosyllactose and Lacto-N-neotetraose (2'-FL and LNnT) in the mass ratio 2:1
was administered as a powder. The daily dose of the blend was 5000 mg.

After 3 weeks' and 6 weeks' treatment, a daily dose of pomegranate extract (2 g/8 tablets) containing 40%
15 punicalagins was consumed by the study participant for three consecutive days. In the morning of the fourth
day, a urine sample was collected and the urolithin content was analyzed by LC-MS/MS analysis.

In week 3 and 6 of the study period (i.e. the week preceding pomegranate extract administration and urine
collection), the intake of ellagitannin-containing sources such as berries (strawberry, raspberry, blackberry
etc. and derived foodstuffs such as jam), pomegranates, chocolate, nuts and wine were forbidden.

20 Final urolithins were measured in urine samples for some of the participants after 6 weeks' treatment.
Significantly more non-producers had become producers in the PE+HMO group compared with the PE+PE
group ($P < 0.05$). Thus, the hypothesis that the combination of pomegranate extract plus HMO activates the
production of final urolithins in non-producers, to a higher degree than pomegranate extract alone, was
substantiated.

25 No side effects were reported during the study.

Example 5

Stimulation of urolithin production in vitro with a combination of an ellagitannin-rich food source and an HMO blend

The following study was designed to assess the impact of an HMO blend, a pomegranate extract (PG) and the combination of both on the urolithin production by the gut microbiota of nine randomly selected human donors in a short-term *in vitro* simulation. The company ProDigest's (<https://www.prodigest.eu/en>) short-term colonic simulation model was used to study the interaction between test products and the gut microbiome.

All donors were between 50 and 70 years old, healthy and with no history of antibiotic use 6 months before the experiment. Fecal material was collected, and fecal suspensions prepared and mixed with a cryoprotectant. The obtained suspensions were aliquoted, flash frozen and then preserved at -80°C. Just before the experiment, fecal samples were defrosted and immediately added to the reactors.

The reactors were incubated for 48 hours (37°C, shaking at 90 rpm, anaerobic conditions) with a sugar-depleted nutritional medium containing basal nutrients of the colon. Samples from the reactors were collected at 0h (baseline), after 24 h and after 48h (follow-up). Each fecal sample was used for 4 different treatments:

- Control treatment (blank): only nutritional medium was added to the reactor.
- Human milk oligosaccharide (HMO) treatment: 3 g/l of an HMO blend was added to the reactor at baseline.
- Pomegranate extract (PG) treatment: 1 g/l was added to the reactor at baseline.
- Pomegranate extract + HMO (PG + HMO) treatment: 1g/l of PG and 3 g/l of an HMO blend was added to the reactor at baseline.

Incubations were performed in single repetition, resulting in 36 independent incubations.

The pomegranate extract was an ethanolic extract based on pomegranate fruit pomace (Monteloeder S.L., Elche, Alicante, Spain). The content of punicalagins was approx. 40%.

The HMO blend was a powder consisting of 2'-fucosyllactose (2'FL) and lacto-N-neotetraose (LNnT) in the mass ratio 4:1 (2'FL:LNnT). The HMO blend was provided by Glycom A/S, Hørsholm, Denmark.

The concentration of the final urolithins urolithin A, isourolithin A and urolithin B was measured at 0h, 24h and 48h. Furthermore, the concentration of the intermediary urolithins urolithin C and urolithin D was measured at 0h, 24h and 48h.

A synergistic effect of HMO+PG treatment compared with HMO treatment or PG treatment, respectively, was seen for urolithin A production in donors 1, 2 and 3 and to a lesser extent in donors 6 and 8 (Figure 2).

A synergistic effect of HMO+PG treatment compared with HMO treatment or PG treatment, respectively, was seen for isourolithin A production in donors 1 and 5 and to a lesser extent in donors 6 and 8 (Figure 3).

- 5 A synergistic effect of HMO+PG treatment compared with HMO treatment or PG treatment, respectively, was seen for urolithin C production in donors 5 and 7 and to a lesser extent in donor 1 (Figure 4).

A synergistic effect of HMO+PG treatment was not observed for urolithin D production.

Urolithin B was not detected in any of the samples. This may be explained by the late synthesis of this compound in the metabolic pathway of final urolithins (Figure 1).

- 10 Thus, overall the combination of HMO+PG treatment improved urolithin production in a synergistic manner in fecal sample incubations originating from 7 out of 9 randomly assigned donors (*i.e.* all donors but donors 4 and 9). The effect was most pronounced for the production of urolithin A. The average data for urolithin A production across the 9 donors are presented in Figure 5.

- It is noted that any stimulating effect on non-producers (possibly donors 7 and 9) cannot be expected during
15 a 48h *in vitro* study, since adequate amounts of relevant bacteria for the catabolization of ellagitannins/ellagic acid would probably need weeks to proliferate to a sufficient level.

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