

METHOD FOR IMPROVING THE FOAMING PROPERTY OF A LIQUID DAIRY-SUBSTITUTE PRODUCT

FIELD OF THE INVENTION

- 5 The invention relates to a method for improving the foaming property of a liquid dairy-substitute product, said method comprising a step of incorporating a casein composition comprising casein in the sole form of non-animal origin to a liquid dairy-substitute product.

BACKGROUND OF THE INVENTION

- 10 *The impact of dairy products on environment*

Environmental and ethical concerns related to food products of animal origin have raised over the last decades. The burden of animal breeding for a 7 billion population has become increasingly high. The environmental consequences are severe in terms of anthropogenic greenhouse gas (GHG) emissions, water consumption, pollution by effluents, land
15 occupation, deforestation, and others.

Animal farming contributes to climate change by emitting greenhouse gases such as carbon dioxide, methane, and nitrous oxide. These gases trap heat in the atmosphere and cause global warming. According to a report by the Food and Agriculture Organization of the United Nations (FAO), animal agriculture is responsible for about 14.5% of human-caused
20 greenhouse gas emissions, which is more than the emissions from all global transportation (Rotz (2017) Modeling greenhouse gas emissions from dairy farms. J Dairy Science, Volume 101, pages 6675–6690).

According to a report by the Food and Agriculture Organization (FAO) of the United Nations (FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS, *Greenhouse Gas
25 Emissions from the Dairy Sector. A Life Cycle Assessment*), the global dairy sector alone contributes about 4% of the total human-induced greenhouse gas emissions. This includes emissions from feed production, enteric fermentation (the digestive process of ruminant animals), manure management, processing and transportation of milk and dairy products. The main greenhouse gases emitted by the dairy sector are carbon dioxide (CO₂), methane
30 (CH₄), and nitrous oxide (N₂O). Methane is a particularly potent greenhouse gas, with a global warming potential 28 times higher than CO₂ over a 100-year period.

Global Pasture already represented in 2000 about 30 million km² (nearly the size of Africa), (Ramankutty, N., A. T. Evan, C. Monfreda, and J. A. Foley (2008), Farming the planet: 1.

Geographic distribution of global agricultural lands in the year 2000, *Global Biogeochem. Cycles*, 22, GB1003). Land use for dairy production can lead to deforestation, biodiversity loss, soil erosion, and desertification. It can also compete with other land uses, such as food crops, biofuels, or natural habitats.

- 5 Livestock industries also have a very strong impact on water consumption, (Sultana et al (2014). Comparison of water use in global milk production for different typical farms. *Agricultural Systems*, Volume 129, pages 9-21; Ercin and Aldaya (2012), The water footprint of soy milk and soy burger and equivalent animal products. *Ecological Indicators*, Volume 18, pages 392-402), and drain about 8% of global water supply, largely due to feed production
- 10 (Schlink et al (2010), Water requirements for livestock production : a global perspective; rev. Sci. tech. 29:603-619). A related environmental impact of dairy production is eutrophication, which is the enrichment of water bodies with excess nutrients, such as nitrogen and phosphorus. These nutrients can come from fertilizers used for feed crops, manure from animals, or wastewater from processing facilities. Eutrophication can also affect human
- 15 health by contaminating drinking water sources or causing harmful algal blooms.

- Animal farming also causes environmental problems such as deforestation, water pollution, soil degradation, and biodiversity loss. But in addition, animal welfare has become an increasingly important concern. The scale-up of meat and dairy production and processing has turned it into an intensive industrial process, which is increasingly perceived as ethically
- 20 not acceptable.

While the extensive use of food of animal origin is raising very serious environmental and ethical concerns, there is in the sale time a growing demand for food. According to estimates by the Food and Agriculture Organization (FAO), by 2050, food production, including meat, will have to increase by 70% to feed a world population of 10 billion.

25 *Food transition and the alternative protein*

A transition to a diet less rich in animal-based products can reduce the recourse to cattle breeding.

- Animal-free substitutes for meat and dairy are products that mimic the taste, texture, and nutritional value of animal products, but are made from plant-based or other non-animal
- 30 ingredients. They are often referred to as vegan or plant-based alternatives.

In the case dairy products, such alternative can also address other issues, related to health, such as lactose intolerances, allergies (Mousan and Kamat (2016) Cow's Milk Protein Allergy. *Clin Pediatr (Phila)*, Volume 55(11) pages 1054-63.; Manuyakorn and Tanpowpong (2018)

Cow milk protein allergy and other common food allergies and intolerances. *Paediatr Int Child Health*, Volume 39(1), pages 32-40), and others, such as a high content in saturated fatty acids, known to have a potential negative impact on health. (Micha and Mozaffarian (2010) Saturated fat and cardiometabolic risk factors, coronary heart disease, stroke, and diabetes: A fresh look at the evidence. *Lipids*, Volume 45, pages 893–905; Jakobsen et al (2009) Major types of dietary fat and risk of coronary heart disease: A pooled analysis of 11 cohort studies. *Am. J. Clin. Nutr*; Volume 89, pages 1425–1432; Nettleton et al (2017) Saturated fat consumption and risk of coronary heart disease and ischemic stroke: A science update. *Ann Nutr Metab* Volume 70, pages 26–33). Lactose intolerance is due to the lack of lactase, the enzyme that degrades lactose in the stomach and small intestine, and result in an accumulation of lactose in the colon, and its digestion by bacteria (Ugidos-Rodríguez et al (2018) Lactose malabsorption and intolerance: a review. *Food Funct*, Volume 9(8), pages 4056-4068). It is a very common feature in humans, is genetically determined, and results in the need for a dairy-free diet. In a dairy-substitute product, this composition could be modulated, to avoid lactose, and saturated fatty acids, and even, allergenic proteins.

However, plant-based dairy-substitute products have often proven to fall very short from really mimicking the conventional dairy products both in terms of organoleptic and nutritional properties, and notably in terms of protein composition.

Caseins

Indeed, replicating dairy texture, dairy foaming property, dairy taste, while preserving nutritional composition only with plant-based dairy-substitute products is a challenging task. For example, plant-based dairy-substitute products are by definition void of animal proteins, and therefore, do not contain caseins, milk's major protein, and a hallmark of most dairy products. Caseins represent more than 80% of cow's milk proteins, but bring also important functionalities such as curdling, melting and stretching. Mimicking both their nutritional input and texturing role is typically challenging.

The four major caseins, alpha-S1-, alpha-S2-, beta-, and kappa-caseins, represent more than 80% of cow's milk proteins, and nearly all of cheese proteins, since the other milk proteins (commonly named "whey proteins") such as beta-lactoglobulin, alpha-lactalbumin, bovine serum albumin, and immunoglobulins are removed in whey after curdling. The solubility of caseins highly depends on pH, temperature and salt concentration (Post et al. (2012) Effect of temperature and pH on the solubility of caseins: Environmental influences on the dissociation of caseins. *J. Dairy Sci.* Volume 95 : pages 1603–1616), but in milk, they are not found as soluble proteins, but organized in micelles, resulting in a colloidal structure. Casein

micelles are roughly spherical particles ranging from 50 to 600 nm in diameter, with an average diameter of around 200 nm (de Kruif (1998), Supra-aggregates of casein micelles as a prelude to coagulation J Dairy Sci, Volume 81 pages 3019-3028; de Kruif et al. (2012) Casein micelles and their internal structure. Advances in Colloid and Interface Science, Volume 171–172, pages 36-52). The evolution of this colloid into a curd is at the basis of cheese production (Gillis JC, Ayerbe A, Le fromage 4ème edition, Lavoisier - Technique Et Documentation 20 avril 2018). It is believed that κ -casein has a key role as a stabilizer of the micelle structure.

Caseins can be isolated from milk as sodium, calcium or potassium caseinates, by neutralization after acid-mediated or rennet-mediated coagulation (Sarode et al. (2016) Methods of Manufacture. In: Caballero, B., Finglas, P., and Toldrá, F. (eds.) The Encyclopedia of Food and Health vol. 1, pp. 676-682. Oxford: Academic Press) The different individual caseins (α -S1-, α -S2-, β -, or κ -caseins) can also be purified individually from bulk casein preparations, based on their different physico-chemical properties, using membrane filtration at low temperature (Murphy and Fox (1991) Fractionation of sodium caseinate by ultrafiltration. Food Chem. Volume 39 Pages 27–38; Ward and Bastian (1996) A method for isolating β -casein. J. Dairy Sci. Volume 79, pages 1332–1339; Huppertz et al. (2006). A method for the large-scale isolation of β -casein. Food Chem. Volume 99, pages 45–50; Lamothe et al. (2007). Short communication: Extraction of β -casein from goat milk. J. Dairy Sci. Volume 90, pages 5380–5382; O'Mahony, et al. (2007). Purification of β -casein from milk. US Pat. No. 0104847 A1) or selective precipitation (Law and Leaver (2007) Methods of extracting casein fractions from milk and caseinates and production of novel products. Hanna Research Institute, assignee. WO Pat. No. 03003847; Post et al. (2009). β -Casein as a bioactive precursor—Processing for purification. Aust. J. Dairy Technol. Volume 64, pages 84–88; Post and Hinrichs (2011) Large-scale isolation of food-grade β -casein. Milchwissenschaft, Volume 66, pages 361–364).

Precision fermentation

In order to more closely match dairy products compositions, with no recourse, or less recourse to animal-product, specific compounds can also be produced by precision fermentation. Production by fermentation is based on the growth of bacteria or fungi producing a compound of interest in a fermenter, usually followed by the recovery and purification of the compound of interest. The making of proteins by fermentation is a process that has been used in the food industry, with one of the first examples being recombinant chymosin, the first artificially produced enzyme to be registered and allowed by the US Food

and Drug Administration and represents today a large part (more 80% in the US today) of the rennet market. (Food Biotechnology in the United States: Science, Regulation, and Issues". U.S. Department of State. Retrieved 2006-08-14).

Several studies have described the production by fermentation in various microorganisms of milk's proteins, including caseins, whey proteins such as beta-lactoglobulins, and the use or potential use to make animal-free dairy substitutes, together with plant-based other ingredients:

- WO1991008675 describes the production of recombinant proteins for infant formula.
- WO2016029193 describes the general principle of using recombinant milk's protein in food applications, gives a formula for cream and milk.
- WO2018039632 describes the mixing of fermentation caseins and plant-based proteins.
- WO2020219596 describes the production of recombinant beta-lactoglobulin
- WO2020223700 describes the use of alpha-S1 and kappa casein, without beta casein, to make micelles, and curds for cheese and yoghourts applications.
- WO2022098835 describes the mixing caseins from different animals, and their use for making micelles, curds and cheese substitutes.
- WO2022098853 describes the mixing of alpha-S1 and kappa casein caseins to make micelles, one being recombinant, and their use for making micelles, curds and cheese substitutes
- WO2022038601 describes general methods to make micelles using recombinant caseins.
- WO2022239000 describes the production of beta lactoglobulin in *Pichia pastoris*, and products made thereof.
- WO2023133417 describes the mixing of recombinant caseins and lipids made by fermentation, wherein the lipid is not necessarily recombinant, and describes production processes for casein and lipids.
- WO2022058573 describes cheese substitutes made from recombinant casein.
- WO2022253816 describes a method to produce caseins by precision fermentation, and the use of these caseins for making cheese substitutes.

Specific problem to be solved

Dairy-substitute beverage, such as milk-substitutes (also known as plant-based milks or plant-based milk-substitutes), are designed to replace dairy beverage, such as milk, in both drinking and cooking applications. They are expected to have a low environmental impact, but also a good nutritional profile, and some are fortified with vitamins and minerals to match the nutritional content of dairy beverage, such as dairy milk.

There's a wide variety of milk-substitutes available, including almond milk, soy milk, rice milk, oat milk, and many others. Each has its own unique taste and nutritional profile. They also provide the opportunity to bypass the risks of lactose intolerance (by replacing lactose with other carbohydrates). These products had the highest market penetration among dairy substitutes, i.e., 15% of total milk beverages in 2022 (The Good Food Institute, 2022 State of the Industry Report, Plant-based meat, seafood, eggs, and dairy), in spite of strong differences observed between dairy milk and products such as oat milk for example.

However, getting closer to milk properties is the best way to secure further success. Foaming properties are an important aspect of dairy beverages, especially in the context of drinks like baristas, cappuccinos, lattes, and milkshakes.

In dairy milk, milk proteins stabilize the air bubbles in milk foams. The process of aeration in beverages, which is manifested as foam or bubbles, increases the sensory preference among consumers (Deotale et al. Characteristics of beverages and Its Relevance to Food Processing. Food Eng Rev 12, 229–250 (2020)). Over time, foam stability decreases as liquid flows out of the foam, causing air bubbles to come into close contact and fuse together. This leads to larger air bubbles which eventually collapse (Petrozzi, characterisation and visualisation of foam quality attributes such as foamability, foam stability and foam structure of coffee brews, whole uht milk and coffee-based beverages. African Journal of Food Science, Vol. 16(1) pp. 10-21, January 2022). Foaming in milk plays a significant role in the sensory properties of popular beverages. The foam enhances the flavor, texture, and visual appeal of milk-based beverages (Acharya, et al. Analyzing Milk Foam Using Machine Learning for Diverse Applications. Food Anal. Methods 15, 3365–3378 (2022)).

Milk's proteins and notably caseins, are known for their foaming and emulsifying properties (Mohanytu et al. Emulsifying and Foaming Properties of Acidic Caseins and Sodium Caseinate, Food Chemistry 28 (1988) 17-30 ; Gauding et al. Engineering of caseins and modulation of their structures and interactions, Biotechnology Advances 27 (2009) 1124–1131 ; Dickinson, Interfacial, Emulsifying and foaming properties of milk proteins, Advanced

Dairy Chemistry Volume 1: ProTeins. 3rd ed, Edited by P.F. Fox and P.L.H. McSweeney. Kluwer Academic/Plenum Publishers. 2003 ; Broyard and Gaucheron, Modifications of structures and functions of caseins: a scientific and technological challenge, Dairy Sci. & Technol. (2015) 95:831–862). However, both caseins and whey are missing in plant-based milk, often at the expense of foaming properties.

Proposed solution

We tested the impact of animal-free caseins made by microbial fermentation for their foaming impact on different liquid dairy-substitute products, such as plant-based milks.

Such caseins are made by production in microorganisms, instead of mammalian cells, and are therefore, either lacking mammalian post translational modifications (PTMs), either displaying fully different PTMs. In bacteria, PTMs are unfrequent, while in yeast they are frequent, but different from mammalian PTMs. Therefore, the physico-chemical properties of such caseins, and notably their tension-active profiles and foaming and emulsifying properties, are expected to be different as compared to caseins found in natural milk.

Milk's caseins are naturally phosphorylated, and important features of their global amphiphilic properties are linked to their phosphorylation. Phosphorylation and dephosphorylation can therefore impact their physical properties.

Milk's casein dephosphorylation was observed to decrease the emulsification properties of beta casein (McCarthy (2013). The physical characteristics and emulsification properties of partially dephosphorylated bovine β -casein. Food Chemistry 138:1304-11).

Milk's casein (over)phosphorylation can result in emulsifying properties enhancement (Broyard and Gaucheron (2015) Modifications of structures and functions of caseins: a scientific and technological challenge. Dairy Sci. & Technol. 95, 831–862) or decrease (Matheis et al. (1983) Phosphorylation of casein and lysozyme by phosphorus oxychloride. J Agric Food Chem 31:379–387; Van Hekken et al. (1996) Functional Properties of Chemically Phosphorylated Whole Casein, J Dairy Sci 79:1942-1949).

Milk's casein dephosphorylation was observed to decrease foam volume and stability (Van Hekken and Strange (1993) Functional Properties of Dephosphorylated Bovine whole Casein. J Dairy Sci 79:1942-1949) although another study claims an enhancement of foamability (Husband et al. (1997) A comparison of the functional and interfacial properties of β -casein and dephosphorylated β -casein. Journal of Colloid and Interface Science, Volume 195, Pages 77-85).

Milk's casein phosphorylation can result in foaming properties enhancement (Broyard and Gaucheron (2015) Modifications of structures and functions of caseins: a scientific and technological challenge. Dairy Sci. & Technol. 95, 831–862; Van Hekken et al. (1996) Functional Properties of Chemically Phosphorylated Whole Casein, J Dairy Sci 79:1942-1949).

- 5 In the view of such results, one could expect that animal-free caseins produced by fermentation in a bacterial cell, and therefore having low phosphorylation level (as compared with caseins produced in eukaryotic cells, such as milk's caseins), could have low foaming properties or at least unpredictable foaming properties.

- Surprisingly, we found that with plant-based milks having low foaming properties, or no
10 foaming properties, foaming could be significantly improved by adding a casein composition comprising casein in the sole form of non-animal origin made by fermentation in bacteria.

SUMMARY OF THE INVENTION

- There is a need for alternative natural food-additive for improving the foaming property of
15 liquid dairy-substitute products, such as plant-based edible compositions.

- We found that a small amount of non-animal casein made by precision fermentation was sufficient to improve the foaming property of liquid dairy-substitute products, such as milk-substitutes. For example, the foaming property of such casein could be used to achieve liquid dairy-like products, while adding a small amount can significantly improve the foaming
20 property, while bringing additional protein content.

- A first object of the present invention relates to the use of a casein composition comprising casein in the sole form of non-animal origin for improving the foaming property of a liquid dairy-substitute product, thereby obtaining a liquid dairy-like product, said liquid dairy-like product having an improved foaming property compared to the liquid dairy-substitute
25 product.

- A second object of the present invention relates to a method for improving the foaming property of a liquid dairy-substitute product, said method comprising a step of incorporating a casein composition comprising casein in the sole form of non-animal origin to a liquid dairy-substitute product thereby obtaining a liquid dairy-like product, said liquid dairy-like
30 product having an improved foaming property compared to the liquid dairy-substitute product.

A third object of the present invention relates to a method for preparing a liquid dairy-like product suitable for making a foamed dairy-like product, comprising the step of:

- a) providing a liquid dairy-substitute product;
- b) incorporating a casein composition comprising casein in the sole form of non-animal origin into the liquid dairy-substitute product, thereby obtaining a liquid dairy-like product suitable for making a foamed dairy-like product.

A fourth object of the present invention relates to a method for preparing a foamed dairy-like product, comprising the steps of:

- a) incorporating a casein composition comprising casein in the sole form of non-animal origin into a liquid dairy-substitute product, thereby obtaining a liquid dairy-like product;
- b) aerating the liquid dairy-like product thereby obtaining a foamed dairy-like product.

A fifth object of the present invention relates to a liquid dairy-like product suitable for making a foamed dairy-like product comprising:

- a) a liquid dairy-substitute product;
 - b) a casein composition comprising casein in the sole form of non-animal origin;
- wherein the liquid dairy-like product comprises proteins in the sole form of non-animal origin, wherein the liquid dairy-like product comprises from 0.1 % w/w to 10 % of casein of non-animal origin.

The following embodiments apply to each of the five objects of the invention.

- In some embodiments, the foamed dairy-like product is a foamed milk-like, a foamed drinking yogurt-like, a whipped yogurt-like, a whipped cream-like, a foamed smoothie or a foamed milkshake-like.

In some embodiments, the liquid dairy-substitute product is a liquid milk-substitute, a drinking yogurt-substitute, a liquid cream-substitute, a smoothie or a milkshake-substitute.

- In some embodiments, the liquid dairy-substitute product is a plant-based liquid dairy-substitute product, such as a plant milk, a drinking yogurt-substitute made from plant milk or a liquid cream-substitute made from plant milk.

In some embodiments, at least 90% of the casein of the casein composition is non-micellar casein.

In some embodiments, the liquid dairy-substitute product is at a temperature from 25°C to 100°C.

In some embodiments, the liquid dairy-substitute product is at a temperature from 50°C to 90°C, such as from 50°C to 80°C, for example at a temperature of about 60°C, about 70°C,
5 about 80°C or about 90°C.

In some embodiments, the foaming property is the volume increase after aerating and/or the foam stability.

In some embodiments, the casein of non-animal origin is substantially free of phosphorylation.

10 In some embodiments, the casein of non-animal origin is produced in bacteria, such as *E. Coli*.

In some embodiments, the liquid dairy-substitute product is not suitable for making a foamed dairy-substitute product having a volume increase after aerating over 45%, such as over 45% at 25°C and/or 60°C. Preferably the liquid dairy-substitute product is prepared
15 from coconut, rice, oat, or a combination thereof.

In some embodiments, the liquid dairy-like product comprises from 0.1 % to 10 % of casein of non-animal origin.

In some embodiments, the liquid dairy-like product comprises from 0.1 % to 5 % of casein of non-animal origin.

20 In some embodiments, the liquid dairy-substitute product is prepared from coconut, rice, oat, or a combination thereof.

In some embodiments, the casein of non-animal origin is one, two or three casein(s) selected from the group consisting of alpha-S1 casein, alpha-S2 casein, beta casein and kappa casein.

25 In some embodiments, the casein of non-animal origin is alpha-S1 casein, beta casein, a mixture of alpha-S1 casein and beta casein, a mixture of alpha-S1 casein and alpha-S2 casein, or a mixture of beta casein, alpha-S1 casein and alpha-S2 casein.

In some embodiments, the casein composition does not comprise at least one casein protein selected from the group consisting of alpha-S1 casein, alpha-S2 casein, beta casein and
30 kappa casein.

In some embodiments, the casein composition does not comprise kappa casein.

In some embodiments, the casein of non-animal origin is a mixture of alpha-S1 casein and beta casein.

In some embodiments, the casein of non-animal origin is beta casein.

5 In some embodiments, at least 50% of the casein of the casein composition is caseinate and/or acid casein, such as 1, 2, 3 or 4 caseinates selected from the group consisting of sodium caseinate, calcium caseinate, potassium caseinate and magnesium caseinate and/or acid casein.

10 In some embodiments, the foam of the foamed dairy-like product is stable for at least 10 minutes at room temperature, such as at least 50% of the volume of foam of the foamed dairy-like product is maintained after 10 minutes at room temperature.

In some embodiments, the liquid dairy-like product has protein content from 0.5 % to 40 %.

In some embodiments, the liquid dairy-like product has lipid content from 0 % to 50 %.

In some embodiments of the fourth object, the liquid dairy-substitute product is suitable for making a foamed dairy-like product at a temperature from 25°C to 100°C.

15 In some embodiments of the fourth object, the liquid dairy-substitute product is suitable for making a foamed dairy-like product at a temperature from 50°C to 90°C, such as from 50°C to 80°C, for example at a temperature of about 60°C, about 70°C, about 80°C or about 90°C.

20 In some embodiments of the fourth object, the casein of non-animal origin is a mixture of alpha-S1 casein and beta casein.

In some embodiments of the fourth object, the casein of non-animal origin is beta casein.

DETAILED DESCRIPTION

Definitions

25 In the context of the invention, the term "(w/w)" means weight/weight, on the total weight (mention is made when the quantities are evaluated on dry matter).

In the context of the invention, the term "liquid dairy product", also known as "dairy beverage", means liquid product made from (or containing) dairy milk. A liquid dairy product is preferably chosen from the group consisting in liquid milk, drinking yogurt, liquid cream, 30 smoothie, milkshake.

In the context of the invention, the term "dairy-substitute product" means a product designed to mimic the sensory characteristics and functional properties of a dairy product, while being made from ingredients of non-animal origin. Dairy-substitute products are formulated from non-animal sources, typically plant-based sources, to mimic taste, texture, and/or functionality of their dairy counterparts. Dairy-substitute product can be used in various culinary applications and serve as alternatives to traditional dairy product.

In the context of the invention, the term "milk-substitute" means a liquid that resembles dairy milk but being made from ingredients of non-animal origin. For example, a milk-substitute has one or more of the following: substantially similar (or similar) color, taste, nutritional content, and other quality as dairy milk. A milk-substitute is formulated from non-animal sources, typically plant-based sources. Typically, a milk-substitute includes its main ingredients (but water) from plant-based sources, and is commonly known as a "plant-based milk substitute". However, depending on the national/regional specific regulatory rules, a "plant-based milk-substitute" may be marketed as a "plant-based beverage" (e.g., almond-based beverage) or a "plant milk" (e.g., coconut milk). For example, in the European Union, "plant-based milk substitutes" for sales cannot be labeled as "milk", except for coconut milk which corresponds to a specific product. Examples of "plant-based milk-substitutes" according to the invention, which are marketed in Europe without using the term "milk", are disclosed in the Examples. In the present description, we will use the terms "milk-substitute", "plant-based milk" or "plant-milk", even if some national/regional specific regulatory rules prohibit the term "milk" for plant-based beverages, for the sake of simplicity.

The term "dairy milk" as used herein refers to a white fluid secreted by the mammary glands of female mammals. Dairy milk consists of an emulsion of fat in an aqueous solution comprising proteins (e.g., casein, albumin), sugars, inorganic salts, and other ingredients. Suitable mammals from which dairy milk can be obtained include but are not limited to cow, sheep, goat, buffalo, donkey, horse, camel, yak, water buffalo, human, and other mammals. Dairy milk obtained from cow typically contains around 3.5%-3.9% fat (whole cow milk). Fat levels can be reduced to standardized levels to obtain different grades of cow milk that comprise from 0% to about 75% by weight of the fat present in whole cow milk, including but not limited to 2% cow milk (cow milk comprising 2% by weight of fat), 1% cow milk (cow milk comprising 1% by weight of fat), and skim cow milk (cow milk comprising no fat).

In the context of the invention, the term "drinking yogurt-substitute" means a beverage that resembles drinking dairy yogurt but being made from ingredients of non-animal origin. For example, a drinking yogurt-substitute has one or more of the following: substantially similar

(or similar) color, taste, nutritional content, and other quality as drinking dairy yogurt. A drinking yogurt-substitute is formulated from non-animal sources, typically from plant-based sources, such as from plant-based milk-substitute, and eventually from plant-based oil. Typically, a drinking yogurt-substitute includes its main ingredients from plant-based sources,
5 such as from plant-based milk-substitute.

In the context of the invention, the term "dairy-like product" means a dairy-substitute product supplemented with a casein composition comprising casein in the sole form of non-animal origin. In the context of the invention, a dairy-like product has improved similarity in foaming property to a dairy product compared to a dairy-substitute product. The dairy-like
10 product may be a foamed milk-like, a foamed drinking yogurt-like, a whipped yogurt-like, a whipped cream-like, a foamed smoothie or a foamed milkshake-like.

In the context of the invention, the term "plant oil" or "plant-based oil" or "vegetable oil" means oil extracted from plant source.

In the context of the present invention, the term "casein" designates one or more casein
15 selected from the group consisting in alpha-S1-, alpha-S2-, beta-, or kappa-casein.

In the context of the present invention, the term "casein composition" designates a composition which contains casein. Such casein composition can be solid or liquid and contains at least one casein as defined above. Such solid casein composition is preferably a spray-dried casein composition. Such casein composition can result from the purification of
20 casein from the production of caseins by microbial cultures and subsequent purification. Such casein composition can comprise other compounds, notably calcium, other proteins, lipids, carbohydrates and others. A casein composition produced by a microbial culture (microbial fermentation) is a casein composition comprising casein in the sole form of non-animal origin.

25 In the context of the present invention, the term "of non-animal origin" means a compound or composition which has not been directly derived from an animal, produced from animal cells in culture, or isolated from animal products such as milk. Proteins produced in a microorganism by precision fermentation are thus "of non-animal origin" even though some products derived from animals, such as bacto peptone, or even waste from the dairy
30 industry, can be used in the fermentation medium of the microorganism. Therefore, in the context of the invention, a protein that is naturally produced in animals will be called of non-animal origin when it is produced in a microorganism (such as bacteria or yeast) or in non-

animal cells, such as in plant cells, even though its sequence or structure may be identical to the sequence or structure of the protein that would be isolated from animal.

In the context of the present invention, the term "animal-free" means a compound or composition which has not been derived from an animal, from animal cells in culture, or from
5 animal products such as milk, and whose production process does not involve any feedstock or additive of animal origin.

In the context of the present invention, the term "fermentation" designates the process used for the production of a compound of interest, comprising the steps of growing a cellular culture producing the compound of interest in a fermenter, and recovering the compound of
10 interest. In the case of microbial fermentation, bacteria or fungi (such as yeasts) are used for production.

In the context of the present invention, the term "recombinant" means that the organism or microorganism, is genetically modified so as to contain a nucleic acid molecule encoding at least a protein, as compared to a wild-type or non-modified organism or microorganism.

15 In the context of the present invention, the term "foamed" or "whipped" refers to a product which has been adapted, developed, or prepared to include air or gas bubbles (i.e., typically, although not solely, atmospheric air or nitrogen) dispersed homogeneously within, or just within a portion of, the product for a period of time.

In the context of the present invention, the term "aerating" or "foaming" consists in
20 dispersing and stabilizing a gas phase (i.e. typically, although not solely, nitrogen or atmospheric air) in the form of tiny bubbles within, or just within a portion of, a product matrix. Aerating can be performed, for example, by "frothing" or "whipping". Aerating processes can be performed batchwise or continuously. For instance, aerating may be performed by batch with standard kitchen mixers. Continuous aerating is generally
25 performed on an industrial scale, for instance using industrial equipment for aerating food products. The properties of the foamed product depend on the aerating operation, gas fraction, the bubble size distribution, as well as on the distribution of the ingredients between the bulk and the gas-product interface. Aeration requires mechanical energy to help create the bubbles. Regarding whipped cream, aeration may also require gas (e.g., N₂O)
30 under pressure to help create the bubbles. The incorporation of casein composition according to the present invention into a liquid dairy-substitute product improves the foaming property of said liquid dairy-substitute product and helps said liquid dairy-substitute

product to produce bubbles under mechanical aeration, improve stability and provide a number of other benefits which will be discussed throughout this specification.

In the context of the present invention, the term "foaming property" corresponds to the ability of a product to form and stabilize a gas phase (i.e. typically, although not solely, nitrogen or atmospheric air) in the form of tiny bubbles within the product matrix. The foaming property of a product can be evaluated by measuring various parameters including, for example, volume increase after aerating, overrun, or foam stability (Deotale et al. (2020), Foaming Characteristics of Beverages and Its Relevance to Food Processing; Food Engineering Reviews 12:229–250; Carrera Sanchez and Rodriguez Patino (2005) Interfacial, foaming and emulsifying characteristics of sodium caseinate as influenced by protein concentration in solution. Food Hydrocolloids :407–416; O'Chiu and Vardhanabhuti (2017) Utilizing whey protein isolate and polysaccharide complexes to stabilize aerated dairy gels. J. Dairy Sci. 100:3404–3412).

The term "overrun" means the volume increase of a product because the gas incorporated into said product. The overrun may be measured by comparing the weight of a given volume of a liquid product before aerating (e.g., before whipping) and the weight of the same volume of foam (i.e. obtained after aerating said liquid product) (Lim et al. (2008) High Hydrostatic Pressure modification of whey protein concentrate for Improved Functional Properties, Journal of Dairy Science Vol. 91 No. 4, 2008; O'Chiu and Vardhanabhuti (2017) Utilizing whey protein isolate and polysaccharide complexes to stabilize aerated dairy gels. Journal of Dairy Science Vol. 100 No. 5). Therefore, for a liquid product, the overrun may be calculated according to the following formula:

$$\% \text{ overrun} = (\text{weight of a volume } V \text{ of liquid} - \text{weight of the same volume } V \text{ of foam}) / \text{weight of the same volume } V \text{ of foam}) \times 100$$

The overrun may also be measured by comparing the volume of a given weight W of liquid product (i.e. before aerating) and the volume of the same weight W of foam obtained after aerating said liquid product (e.g., after whipping) (Raymundo et al 1998 Methode to evaluate foaming performance .Journal of Food Engineering 36:445-452), Therefore, for a liquid product, the overrun may also be calculated according to the following formula:

$$\% \text{ overrun} = (\text{Volume of foam} - \text{Volume of liquid}) / \text{Volume of liquid}) \times 100$$

"Volume of foam" is the volume of foam after aerating.

"Volume of liquid" is the volume of liquid before aerating.

However, these formulas do not appropriately reflect the amount of foam production when only a minor part of the total volume of said liquid product can be transformed into foam.

Therefore, in the context of the present invention, the foaming property of a liquid product is preferably evaluated using the parameter "volume increase after aerating" and/or "foam stability".

The term "volume increase after aerating" means the difference between the total volume of a liquid product after aerating (foam and liquid) and the total volume of the liquid product before aerating, normalized by the total volume of the liquid product before aerating:

$$\text{Volume increase after aerating (\%)} = ((\text{Total volume after aerating} - \text{Total volume before aerating}) / \text{Total volume before aerating}) \times 100$$

The volume increased after aerating can be measured by passing X mL of a liquid product, e.g., 100 mL of a liquid product, on a milk frother at Z°C, e.g., at a temperature from 20°C to 80°C, such as at 25°C or at 60°C. The total volume before and after aerating can be measured on a graduate cylinder. The volume increase after aerating can be measured as detailed in the Examples.

The term "foam stability" means the time that foam will maintain its initial volume as generated. The foam stability may be calculated as the % of the volume of foam maintained after a certain period of time. For example, the foam stability may be the % of the volume of foam maintained after Y minutes at Z°C, wherein 100% is the volume of foam as generated. In the example part, the foam stability may be the % of the initial volume of foam as generated maintained after 1 minute, 2 minutes, 5 minutes or 10 minutes at 25°C.

The foam stability can be the % of the volume of foam maintained after Y minutes, ex. 10 minutes, at Z°C, e.g., at a temperature from 20°C to 80°C, such as at 25°C or at 60°C, wherein 100% is the volume of foam as generated. The foam stability can be measured as detailed in the Examples.

The terms "glycosylation" and "glycosylated" as used herein refer to the attachment to proteins of glycan groups (i.e., monosaccharides, disaccharides, polysaccharides, linear glycans, branched glycans, glycans with galf residues, glycans with sulfate and/or phosphate residues via C-linkage, N-linkage, or O-linkage, or via glypiation or phosphoglycosylation. Non-limiting examples of such glycan groups include D-glucose, D-galactose, D-mannose, L-fucose, N-acetyl-D-galactose amine, N- acetyl-D-glucose amine, N- acetyl-D-neuraminic acid, galactofuranose, phosphodiesteres, acetylglucosamine, acetylgalactosamine, and sialic acid.

The terms "phosphorylation" and "phosphorylated" as used herein refer to the attachment to proteins of phosphate groups. Phosphorylation of caseins is an important posttranslational modification for casein from animal origin occurring after the synthesis of the polypeptide chains in the Golgi apparatus of the mammary epithelial cell under the action of protein
5 kinases. These kinases phosphorylate Ser or Thr by recognizing the tripeptide sequence Ser/Thr-X-Glu/SerP/Asp, where X represents any AA residue and P indicates phosphorylation.

Methods and use of the invention

A first object of the present invention relates to the use of a casein composition comprising
10 casein in the sole form of non-animal origin for improving the foaming property of a liquid dairy-substitute product, thereby obtaining a liquid dairy-like product, said liquid dairy-like product having an improved foaming property compared to the liquid dairy-substitute product.

In one embodiment, the present invention relates to the use of a casein composition
15 comprising casein in the sole form of non-animal origin for improving the foaming property of a dairy-substitute beverage, thereby obtaining a liquid dairy-like beverage, said dairy-like beverage having an improved foaming property compared to the liquid dairy-substitute product.

A second object of the present invention relates to a method for improving the foaming
20 property of a liquid dairy-substitute product, said method comprising a step of incorporating a casein composition comprising casein in the sole form of non-animal origin to a liquid dairy-substitute product thereby obtaining a liquid dairy-like product, said liquid dairy-like product having an improved foaming property compared to the liquid dairy-substitute product.

In one embodiment, the present invention relates to a method for improving the foaming
25 property of a dairy-substitute beverage, said method comprising a step of incorporating a casein composition comprising casein in the sole form of non-animal origin to a dairy-substitute beverage thereby obtaining a dairy-like beverage, said dairy-like beverage having an improved foaming property compared to the dairy-substitute beverage.

A third object of the present invention relates to a method for preparing a liquid dairy-like
30 product suitable for making a foamed dairy-like product, comprising the step of:

- a) providing a liquid dairy-substitute product;

b) incorporating a casein composition comprising casein in the sole form of non-animal origin into the liquid dairy-substitute product, thereby obtaining a liquid dairy-like product suitable for making a foamed dairy-like product.

In one embodiment, the present invention relates to a method for preparing a dairy-like beverage suitable for making a foamed dairy-like beverage, comprising the step of:

a) providing a dairy-substitute beverage product;

b) incorporating a casein composition comprising casein in the sole form of non-animal origin into a dairy-substitute beverage, thereby obtaining a dairy-like beverage suitable for making a foamed dairy-like beverage.

A fourth object of the present invention relates to a method for preparing a foamed dairy-like product, comprising the steps of:

a) incorporating a casein composition comprising casein in the sole form of non-animal origin into a liquid dairy-substitute product, thereby obtaining a liquid dairy-like product;

b) aerating the liquid dairy-like product thereby obtaining a foamed dairy-like product.

In one embodiment, the present invention relates to a method for preparing a foamed dairy-like beverage, comprising the steps of:

a) incorporating a casein composition comprising casein in the sole form of non-animal origin into a dairy-substitute beverage, thereby obtaining a dairy-like beverage;

b) aerating the dairy-like beverage thereby obtaining a foamed dairy-like beverage.

It is understood that the improved foaming property of the liquid dairy-like product, according to the first, second and third object of the present invention, results from the incorporation of the casein composition comprising casein in the sole form of non-animal origin to the first edible composition.

The following embodiments, unless otherwise specified, relates to the first, second, third and fourth objects of the invention.

Casein composition

In the context of the present invention, a casein composition comprising casein in the sole form of non-animal origin is used.

In some embodiments, the casein of non-animal origin represents more than 50% of the proteins of the casein composition comprising casein in the sole form of non-animal origin. In a preferred embodiment, the casein of non-animal origin represents at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% of the proteins of the casein composition. The amount of proteins can be measured by any method known in the art. The casein composition comprising casein in the sole form of non-animal origin may contain other components than casein, such as other proteins, carbohydrate and/or lipids. This is particularly true when said casein composition results from a culture of recombinant microorganisms expressing casein (microbial fermentation).

In some embodiments, casein of non-animal origin represents the major part of dry weight of the casein composition, for example casein of non-animal origin represents at least 25 % w/w of the casein composition, for example at least 50% w/w of the casein composition, such as at least 60% w/w, at least 70% w/w, at least 75% w/w, at least 80% w/w, at least 85% w/w, at least 90% w/w, or at least 91% w/w, at least 92% w/w, at least 93% w/w, at least 94% w/w, at least 95% w/w, at least 96% w/w, at least 97% w/w, at least 98% w/w, or at least 99% w/w of the casein composition. The percentage of casein in the casein composition corresponds to the mass of casein divided by the total dry mass of the casein composition.

In some embodiments, the casein composition is a spray-dried casein composition.

In some embodiments, the casein of non-animal origin is one, two or three casein(s) selected from the group consisting of alpha-S1 casein, alpha-S2 casein, beta casein and kappa casein.

In some embodiments, the casein of non-animal origin is alpha-S1 casein, beta casein, a mixture of alpha-S1 casein and beta casein, or a mixture of alpha-S1 casein and alpha-S2 casein, or a mixture of beta casein, alpha-S1 casein and alpha-S2 casein.

In some embodiments, the casein composition comprising casein in the sole form of non-animal origin does not comprise at least one casein protein selected from the group consisting in of alpha-S1 casein, alpha-S2 casein, beta casein and kappa casein.

In some embodiments, the casein composition comprising casein in the sole form of non-animal origin does not comprise kappa casein.

In some embodiments, the casein composition comprising casein in the sole form of non-animal origin contains at least one casein selected from the group consisting of alpha-S1 casein, alpha-S2 casein, beta casein and kappa casein.

5 In some embodiments, the casein composition comprising casein in the sole form of non-animal origin contains at least two caseins selected from the group consisting of alpha-S1 casein, alpha-S2 casein, beta casein and kappa casein.

In some embodiment, the casein composition comprising casein in the sole form of non-animal origin contains at least three caseins selected from the group consisting of alpha-S1 casein, alpha-S2 casein, beta casein and kappa casein.

10 In some embodiments, at least one casein of non-animal origin selected in the group consisting of alpha-S1 casein, alpha-S2 casein, beta casein and kappa casein is not present in the casein composition comprising casein in the sole form of non-animal origin. Preferably, kappa casein is not present in the casein composition comprising casein in the sole form of non-animal origin. In some embodiments, the casein composition comprising casein in the
15 sole form of non-animal origin contains only beta casein. In some other embodiments, the casein composition comprising casein in the sole form of non-animal origin contains only beta and alpha-S2 caseins. In some other embodiments, the casein composition comprising casein in the sole form of non-animal origin contains only beta and alpha-S1 caseins. In some other embodiments, the casein composition comprising casein in the sole form of non-animal origin contains only beta, alpha-S1 and alpha-S2 caseins.
20

In some embodiments, the casein composition comprising casein in the sole form of non-animal origin contains only beta casein and alpha-S1 casein, preferably with a ratio beta casein to alpha-S1 casein from 80:20 to 20:80, such as from 70:30 to 30:70, such as from 60:40 to 40:60, for example about 60:40 or about 50:50 or about 40:60. Surprisingly, the
25 inventors have shown that it is possible to improve the foaming property of a liquid dairy-substitute product using only beta casein and alpha-S1 casein.

According to the invention, the amino acid sequence of alpha-S1 casein may be SEQ ID NO: 2, and the amino acid sequence of beta casein may be SEQ ID NO: 4.

In some embodiments, at least 50% of the casein of the casein composition is beta casein,
30 such as at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or even 100% of the casein of the casein composition is beta casein. Surprisingly, the inventors have shown that it is possible to

improve the foaming property of a liquid dairy-substitute product using only beta casein, i.e., wherein 100% of the casein of the casein composition is beta casein.

Indeed, the inventors have shown that it is possible to improve the foaming property of a liquid dairy-substitute product, such as a dairy-substitute beverage, with casein of non-animal origin, without kappa casein, so that the inventors did not try to reconstitute micelle formation, or check whether the solution actually presents micelles.

The inventors have surprisingly shown that casein of non-animal origin without kappa casein, as defined in the present disclosure (e.g., alpha-S1 casein, beta casein, a mixture of alpha-S1 casein and beta casein, a mixture of alpha-S1 casein and alpha-S2 casein, or a mixture of beta casein, alpha-S1 casein and alpha-S2 casein), is able to improve the foaming property of a liquid dairy-substitute product without forming or being incorporated into micelles. This property is particularly surprising because the foaming property is also significantly increased when the foaming property is measured at a temperature above room temperature, such as at a temperature from 25°C to 100°C, such as from 50°C to 90°C, such as from 50°C to 80°C, for example at a temperature of about 60°C, about 70°C, about 80°C or about 90°C.

Therefore, in some embodiments, at least 50% of the casein of the casein composition is non-micellar casein such as at least 60%, such as at least 70%, such as at least 80%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% of the casein of the casein composition is non-micellar casein.

In some embodiments, at least 50% of the casein of the casein composition is micellar casein such as at least 60%, such as at least 70%, such as at least 80%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% of the casein of the casein composition is micellar casein.

In some embodiments, at least 50% of the casein of the casein composition is caseinate, such as at least 60%, such as at least 70%, such as at least 80%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% of the casein of the casein composition is caseinate. In a preferred embodiment, almost 100% of the casein of the casein composition is caseinate.

Caseinate may be 1, 2, 3 or 4 caseinates selected from the group consisting of sodium caseinate, calcium caseinate, potassium caseinate and magnesium caseinate.

In some embodiments, at least 50% of the casein of the casein composition is 1, 2, 3 or 4 caseinates selected from the group consisting of sodium caseinate, calcium caseinate, potassium caseinate, and magnesium caseinate, such as at least 60%, such as at least 70%,
5 such as at least 80%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% of the casein of the casein composition is 1, 2, 3 or 4 caseinates selected from the group consisting
10 of sodium caseinate, calcium caseinate, potassium caseinate and magnesium caseinate.

In some embodiments, at least 50% of the casein of the casein composition is sodium caseinate, such as at least 60%, such as at least 70%, such as at least 80%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least
15 98%, such as at least 99%, such as 100% of the casein of the casein composition is sodium caseinate.

In some embodiments, at least 50% of the casein of the casein composition is calcium caseinate, such as at least 60%, such as at least 70%, such as at least 80%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least
20 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% of the casein of the casein composition is calcium caseinate.

In some embodiments, at least 50% of the casein of the casein composition is potassium caseinate, such as at least 60%, such as at least 70%, such as at least 80%, such as at least
25 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% of the casein of the casein composition is potassium caseinate.

In some embodiments, at least 50% of the casein of the casein composition is magnesium caseinate, such as at least 60%, such as at least 70%, such as at least 80%, such as at least
30 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least

98%, such as at least 99%, such as 100% of the casein of the casein composition is magnesium caseinate.

In some embodiments, at least 50% of the casein of the casein composition is a mixture of 2, 3 or 4 caseinates selected from the group consisting of sodium caseinate, calcium caseinate, potassium caseinate and magnesium caseinate, such as at least 60%, such as at least 70%, such as at least 80%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% of the casein of the casein composition is a mixture of 2, 3 or 4 caseinates selected from the group consisting of sodium caseinate, calcium caseinate, potassium caseinate and magnesium caseinate, such as a mixture of sodium caseinate and calcium caseinate.

In some embodiments, at least 50% of the casein of the casein composition is acid casein, such as at least 60%, such as at least 70%, such as at least 80%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% of the casein of the casein composition is acid casein.

In some embodiments, at least 50% of the casein of the casein composition is a mixture of caseinates and acid casein, such as at least 60%, such as at least 70%, such as at least 80%, such as at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, such as at least 99%, such as 100% of the casein of the casein composition is a mixture of caseinates and acid casein. In these embodiments, the caseinate may be 1, 2, 3 or 4 caseinates selected from the group consisting of sodium caseinate, calcium caseinate, potassium caseinate and magnesium caseinate.

In some embodiments, the casein of the casein composition is a mixture of calcium caseinate and sodium caseinate. For example, the ratio calcium caseinate and sodium caseinate is from 80:20 to 20:80, such as from 70:30 to 30:70, such as from 60:40 to 40:60, for example about 60:40 or about 50:50 or about 40:60.

Casein of non-animal origin can be produced, by fermentation technologies. Several studies have described the production of milk's constitutive proteins or homologues by fermentation in various microorganisms (Goda et al (2000) Recombinant expression analysis of natural and synthetic bovine alpha-casein in *Escherichia coli*. Appl Microbiol Biotechnol, Volume 54, pages 671-676; Kim et al (1997) High-level expression of bovine beta-lactoglobulin in *Pichia*

- pastoris and characterization of its physical properties. Protein Eng, Volume10(11), pages 1339-45; Kim et al (2005) Production of human caseinomacropeptide in recombinant *Saccharomyces cerevisiae* and *Pichia Pastoris*, J Ind Microbiol. Biotechnology, Volume 32, pages 402-408; Choi BK and Jimenez-Flores R (2001) Expression and purification of Glycosylated Bovine b-casein (L70S/P71S) in *Pichia Pastoris*. J Agric Food Chem, Volume 49, pages 1761-1766; Viaene et al (1991) Efficient expression of bovine alpha-lactalbumin in *Saccharomyces cerevisiae*. Eur J Biochem, Volume 202(2), pages 471-7; US6,232,094; WO2013/148328; US2010/0223682; US5,942,274; WO2018039632 or plant cells (US20100119691, WO2022253816).
- 10 In some embodiments, the casein of non-animal origin from the casein composition comprising casein in the sole form of non-animal origin is produced by fermentation of a recombinant microorganism. For example, said casein is produced by fermentation of recombinant microorganism, such as fungus (yeast) or bacterium. In a preferred embodiment, said casein is produced by fermentation of a recombinant microorganism
- 15 chosen among *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium*, *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, *Pichia Pastoris*, or *Trichoderma reesei*, or in a microorganism chosen in the *Saccharomyces*, *Kluyveromyces*, *Pichia*, *Zygosaccharomyces*, *Candida*, or *Trichoderma* genus of fungi.

Such recombinant microorganism can be obtained by the introduction of a nucleic acid molecule encoding one or several caseins, alone or as part of a vector. The nucleic acid molecules can further comprise expression control sequences operably linked to the polynucleotide comprised in the nucleic acid molecule. The term "operatively linked" or "operably linked", as used throughout the present description, refers to a linkage between one or more expression control sequences and the coding region in the polynucleotide to be

20 expressed in such a way that expression is achieved under conditions compatible with the expression control sequence. Regulatory elements ensuring expression in fungi as well as in bacteria, are well known to those skilled in the art. They encompass promoters, enhancers, termination signals, targeting signals and the like. Promoters for use in connection with the nucleic acid molecule may be homologous or heterologous with regard to its origin and/or

25 with regard to the gene to be expressed. Suitable promoters are for instance promoters which lend themselves to constitutive expression. However, promoters which are only activated at a point in time determined by external influences can also be used. Artificial and/or chemically inducible promoters may be used in this context. For genetically modifying

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bacteria or fungi, the polynucleotides encoding one or several caseins can be introduced into plasmids

Thus, in accordance with the present invention, a recombinant organism or microorganism is produced by genetically modifying fungi or bacteria comprising introducing the above-
5 described polynucleotides, nucleic acid molecules or vectors into a fungus or bacterium. An overview of different expression systems is for instance contained in Baghban et al. Yeast Expression Systems: Overview and Recent Advances. Mol Biotechnol. 2019 May;61(5):365-384; Yang and Zhang. Engineering strategies for enhanced production of protein and bio-products in Pichia pastoris: A review. Biotechnol. Adv. 2018 Jan-Feb;36(1):182-195.; Mojzita
10 et al. Gene expression engineering in fungi. Curr. Opin. Biotechnol. 2019 Oct;59:141-149.; Gómez et al. Alternative Eukaryotic Expression Systems for the Production of Proteins and Protein Complexes. Adv Exp Med Biol. 2016;896:167-84.; Freudl. Signal peptides for recombinant protein secretion in bacterial expression systems. Microb Cell Fact. 2018 Mar 29;17(1):52.; Cui et al. Exploitation of Bacillus subtilis as a robust workhorse for production
15 of heterologous proteins and beyond. World J Microbiol Biotechnol. 2018 Sep 10;34(10):145.; Chen. Bacterial expression systems for recombinant protein production: E. coli and beyond. Biotechnol Adv. 2012 Sep-Oct;30(5):1102-7; Deckers et al, Genetically Modified Micro-Organisms for Industrial Food Enzyme Production: An Overview. Foods 2020, 9, 326. The host cell is cultured in nutrient media meeting the requirements of the particular
20 host cell used, in particular in respect of the pH value, temperature, salt concentration, aeration, antibiotics, vitamins, trace elements etc.

In a preferred embodiment, casein of non-animal origin is prepared as detailed in Example 1. Caseins are known in the art. One can cite, as illustrations, the caseins that are present in Uniprot or Genbank. Examples of such caseins are given in the table below.

Uniprot	casein type	origin
P02662	alpha-S1	<i>Bos taurus</i>
A0A3Q1MJE5	alpha-S1	<i>Bos taurus</i>
<u>A0A3Q1NG86</u>	alpha-S1	<i>Bos taurus</i>
P02663	alpha-S2	<i>Bos taurus</i>
P02666	beta	<i>Bos taurus</i>
P02668	kappa	<i>Bos taurus</i>
P18626	alpha-S1	<i>Capra hircus</i>
P33049	alpha S2	<i>Capra hircus</i>

P33048	beta	<i>Capra hircus</i>
P02670	kappa	<i>Capra hircus</i>
P04653	alpha-S1	<i>Ovis aries</i>
P04654	alpha-S2	<i>Ovis aries</i>
P11839	beta	<i>Ovis aries</i>
P02669	kappa	<i>Ovis aries</i>
O62823	alpha-S1	<i>Bubalus bubalis</i>
B6VPY3	alpha-S2	<i>Bubalus bubalis</i>
B7VGH4	beta	<i>Bubalus bubalis</i>
P11840	kappa	<i>Bubalus bubalis</i>

Table 1: examples of casein types from cow (*Bos taurus*), goat (*Capra hircus*), sheep (*Avis aries*) and buffalo (*Bubalus bubalis*).

Such casein usually includes a signal peptide, which is not present in the mature protein. It is possible to use, in the casein composition, fragments of casein proteins (in particular the mature caseins for which signal peptide is not present), or proteins that are homologous to natural caseins and having casein-like properties. Typically, caseins made by precision fermentation will have an additional methionine as first amino acid, a consequence of the expression of the protein from a classical expression system, wherein the translation initiation codon codes for a methionine. For example, alpha-S1 casein has the amino acid sequence SEQ ID NO: 2 and beta casein has the amino acid sequence SEQ ID NO: 4.

In some embodiments, the casein of non-animal origin from the casein composition comprising casein in the sole form of non-animal origin is:

- identical to one of the above sequences, or
- at least n % identical to one of these sequences, with n being an integer from 80 to 100, such as 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99.

AlphaS1 casein, alpha-S2 casein, beta casein and kappa casein of animal origin are highly phosphorylated milk proteins. In bovine milk, alpha-S1 casein has 2 common phosphorylation isoforms: alpha-S1 casein bearing 8 phosphorylations (α S1-CN-8P) and alpha-S1 casein bearing 9 phosphorylations (α S1-CN-9P); alpha-S2 casein is present with isoforms from 10P to 13P; beta casein is usually present with 5P and occasionally with 4P, and kappa casein is present with 1P to 2P and occasionally with 3P. Phosphorylation is one of the key factors responsible for the stabilization of calcium phosphate nanoclusters in casein micelles and the internal structure of the casein micelles.

In some embodiments, the casein of non-animal origin has non-native phosphorylation pattern. A "native phosphorylation pattern" means a phosphorylation pattern of casein from animal origin. For example, the casein of non-animal origin completely lacks or is substantially reduced in phosphorylation as compared to animal-derived casein. In a preferred embodiment, the casein of non-animal origin is substantially reduced in phosphorylation as compared to animal-derived casein, for example the casein of non-animal origin can be substantially free of phosphorylation. The term "substantially free of phosphorylation" means that the level of phosphorylation of casein of non-animal origin is at most 1% the level of phosphorylation of casein of animal origin (i.e., casein naturally occurring in animal milk). The level of phosphorylation can be measured by any method known in the art, such as by Western-Blot or by LC/MS.

In a preferred embodiment, the casein of non-animal origin is a mixture of alpha-S1 casein (e.g., having the amino acid sequence SEQ ID NO: 2) and beta casein (e.g., having the amino acid sequence SEQ ID NO: 4) which is substantially reduced in phosphorylation as compared to alpha-S1 casein and beta casein in bovine milk, preferably the mixture of alpha-S1 casein and beta casein of non-animal origin is substantially free of phosphorylation. In particular, the level of phosphorylation of said mixture of alpha-S1 casein and beta casein of non-animal origin is at most 1% of the level of phosphorylation of alpha-S1 casein and beta casein in bovine milk.

In another preferred embodiment, the casein of non-animal origin is beta casein (e.g., having the amino acid sequence SEQ ID NO: 4) which is substantially reduced in phosphorylation as compared to beta casein in bovine milk, preferably the beta casein of non-animal origin is substantially free of phosphorylation. In particular, the level of phosphorylation of said beta casein of non-animal origin is at most 1% of the level of phosphorylation of beta casein in bovine milk.

In some embodiments, the casein of non-animal origin has non-native glycosylation pattern. A "native glycosylation pattern" means a glycosylation pattern of casein from animal origin. For example, the casein of non-animal origin completely lacks or is substantially reduced in glycosylation as compared to animal-derived casein. In a preferred embodiment, the casein of non-animal origin is substantially reduced in glycosylation as compared to animal-derived casein, for example the casein of non-animal origin can be substantially free of glycosylation. The term "substantially free of glycosylation" means that the level of glycosylation of casein of non-animal origin is at most 1% the level of glycosylation of casein of animal origin (i.e.,

casein naturally occurring in animal milk). The level of glycosylation can be measured by any method known in the art, such as by LC/MS.

In a preferred embodiment, the casein of non-animal origin is substantially reduced in phosphorylation and glycosylation as compared to animal-derived casein, for example the
5 casein of non-animal origin can be substantially free of phosphorylation and substantially free of glycosylation.

It is known that dairy caseins, notably alpha-S1, alpha-S2 and beta caseins, are phosphorylated; in addition, kappa caseins are glycosylated (Walstra *et al.* (2006) Dairy Science and Technology. Taylor and Francis Group, Boca Ranton, USA; Martin *et al.*, (2003)
10 Non bovine caseins quantitative variability and molecular diversity. In: Fox PF and Mc Sweeney PLH. Advances in Dairy Chemistry – Proteins, Vol1, Springer, New-York, 227-310). Caseins phosphorylations play an important role in the interactions with calcium and in the structure of dairy casein micelles. Such post-translational modifications (PTMs) will be different in casein of non-animal origin, which are made in microorganisms. For example, in
15 bacteria, PTMs are much less frequent than in eukaryotic cells (Macek et al. (2019) Protein post-translational modifications in bacteria. Nat Rev Microbiol 17, 651–664). In non-animal eukaryotic cells (e.g., protists, fungi, plants), high levels of PTMs may be observed, but with different patterns as compared with mammalian cells.

It is also known that kappa caseins also play an important role in the formation of dairy
20 casein micelles. The fact that improving foaming property of a liquid dairy-substitute product can be achieved in the absence of conventional post-translational modifications and in the absence of kappa caseins is unexpected.

The fact that improving foaming property of a liquid dairy-substitute product can be achieved in the absence of post-translational modifications, or with low levels of post-translational
25 modifications and in the absence of kappa caseins was particularly unexpected.

Liquid dairy-substitute product

In the context of the present invention, a casein composition comprising casein in the sole form of non-animal origin is incorporated to a liquid dairy-substitute product.

30 In some embodiments, the liquid dairy-substitute product provided herein is a dairy-substitute beverage.

In some embodiments, the liquid dairy-substitute product provided herein is a liquid milk-substitute, a drinking yogurt-substitute, a liquid cream-substitute, a smoothie or a milkshake.

In some embodiments, the liquid dairy-substitute product provided herein is a substitute of dairy products commonly derived from dairy milk. Examples of products commonly derived from dairy milk include, but are not limited to, cooking milk, sweetened condensed milk, 5 flavored milk, yoghurt, smoothies, shakes, coffee whiteners, coffee creamers, infant formulas, weight loss beverages, nutritional supplemental beverages, clinical nutrition beverages, whipped cream and products suitable for preparing frozen confections (for example, ice cream, soft ice cream, frozen yoghurt, sundae, pudding, whipped topping) or combinations thereof. The liquid dairy-substitute product provided herein may be prepared 10 for human or animal consumption. The liquid dairy product analogs may be used for various purposes, including, but not limited to, feeding and delivery of active ingredients (for example, vitamins, minerals, nutrients, therapeutics).

In some embodiments, the liquid dairy-substitute product is a liquid milk-substitute or a 15 liquid dairy-substitute product made from a milk-substitute. Examples of a liquid dairy-substitute product made from milk-substitute include, but are not limited to, a drinking yogurt-substitute, a liquid cream-substitute, a smoothie or a milkshake.

The liquid dairy-substitute product has preferably a viscosity of less than 50 cP at 20°C, such as less than 40 cP at 20°C, such as less than 30 cP at 20°C, such as less than 20 cP at 20°C, 20 such as less than 10 cP at 20°C, such as less than 5 cP at 20°C, such as less than 4 cP at 20°C, such as less than 3 cP at 20°C. Viscosity is defined scientifically as a force multiplied by a time divided by an area. Thus its SI units are usually newton-seconds per square meter (N.s/m²), pascal-seconds (Pa.s) or centipoise (cP). The poise is often used with the metric prefix centi- because the viscosity of water at 20 °C (standard conditions for temperature and pressure) is almost exactly 1 centipoise. A centipoise is one hundredth of a poise, or one 25 millipascal-second (mPa.s) in SI units (1 cP = 10⁻³ Pa.s = 1 mPa.s). The viscosity may be determined, for example, using a viscometer or a rheometer. The viscosity values in the present description may be, for example, on shear rate of 100 s⁻¹ or on shear rate of 50 s⁻¹. (e.g., a viscosity of less than 50 cP at 20°C on shear rate of 100 s⁻¹).

30 In some embodiments, the milk-substitute is a plant milk (or plant-based milk). A plant milk may be prepared from almond, coconut, cashew, macadamia nut, walnut, hazelnut, pistachio, pecan, soy, oat, rice, hemp, pea, flax, quinoa, sunflower seed, banana, or a combination thereof. Said plant milks are commonly called almond milk, coconut milk, cashew milk, macadamia milk, walnut milk, hazelnut milk, pistachio milk, pecan milk, soy

milk, oat milk, rice milk, hemp milk, pea milk, flax milk, quinoa milk, sunflower seed milk, banana milk, or a combination thereof.

Methods for preparing plant milk are known in the art. In one example, the plant milk is almond milk, which can be made by grinding almonds in a blender with water, then straining
5 out the almond pulp with a strainer or cheesecloth. Almond milk can also be made by adding water to finely ground almonds, almond butter or cream. In another example, the plant milk is a coconut-based milk, which can be made by first grating the white inner flesh of a brown coconut and mixing the shredded coconut meat with a small amount of water in order to suspend the fat present in the grated meat. The grating process can be carried out manually
10 or by comminution, a process used to facilitate the grating.

The plant milk may also be a combination of one, two or more different types of plant-based milks produced from other plants sources mixed together.

The Applicant has surprisingly showed that a casein composition comprising casein in the sole form of non-animal origin as disclosed herein provides an impressive volume increase
15 after aerating to liquid dairy-substitute products having low or no volume increase after aerating, such as liquid dairy-substitute products prepared from coconut milk, rice milk, oat milk, or a combination thereof, for example plant coconut milk, rice milk, oat milk, or a combination thereof. Coconut milk, rice milk and oat milk have intrinsically low or no volume increase after aerating.

20 In some embodiments, the liquid dairy-substitute product has a volume increase after aerating below 46%, such as below 40%, such as below 35%, such as below 30%, such as below 25%, such as below 20%, such as below 15%, such as below 10%, such as below 9%, such as below 8%, such as below 7%, such as below 6%, such as below 5%, such as below 4%, such as below 3%, such as below 2%, such as below 1%.

25 In some embodiments, the liquid dairy-substitute product is not suitable for making a foamed dairy-substitute product having a volume increase after aerating over 10%, such as over 15%, such as over 20%, such as over 25%, such as over 30%, over 35%, such as over 40%, such as over 45%, such as over 50%, such as over 55%, such as over 60%, such as over 65%.

30 In a specific embodiment, the Applicant has surprisingly showed that a casein composition comprising casein in the sole form of non-animal origin as disclosed herein provides an impressive volume increase after aerating and foam stability to liquid dairy-substitutes products having low or no volume increase after aerating, especially at a temperature from

50°C to 90°C, such as 60°C. The liquid dairy-substitutes products having low or no volume increase after aerating may be, for example, prepared from coconut milk, rice milk, oat milk, or a combination thereof, in particular oat milk. In the Example part, the coconut milk, rice milk and oat milk had low or no volume increase after aerating (see Table 5).

- 5 The Applicant has also surprisingly showed that a casein composition comprising casein in the sole form of non-animal origin as disclosed herein provides an impressive foam stability to liquid dairy-substitutes products having high volume increase after aerating, especially at a temperature from 50°C to 90°C, such as 60°C. The liquid dairy-substitutes products having high volume increase after aerating may be, for example, prepared from almond milk,
- 10 cashew milk, hazelnut milk, soy milk, or a combination thereof, for example almond milk, cashew milk, hazelnut milk, soy milk, or a combination thereof. In the Example part, the almond milk, cashew milk, hazelnut milk and soy milk had high volume increase after aerating (see Table 5).

- In some embodiments, the liquid dairy-substitute product comprises lipids. Lipids play a role
- 15 in the organoleptic sensation. However, liquid dairy-substitute products with reduced fat content (low fat) or even with no fat have been produced during the last decades, in order to address health concerns. In the present invention, the lipids that are contained in the liquid dairy-substitute product are lipids of non-animal origin, such as lipids extracted from plant. Lipid content may be adjusted, in quantity and quality, in order to mimic the sensory
- 20 characteristics of a liquid dairy product.

- In some embodiments, the liquid dairy-substitute product has lipid content from 0 % to 50 %, such as from 0 % to 40 %, from 0 % to 30 %, from 0 % to 20 %, from 0 % to 10 %, from 1 % to 50 %, from 1 % to 40 %, from 1 % to 30 %, from 1 % to 30 %, from 1 % to 20 %, from 1 % to 10 %. Lipid content in a product corresponds to the mass of lipids in the
- 25 product divided by the total mass of the product. Lipid content of a food product may be determined by a variety of methods, including, but not limited to, AOAC International reference method AOAC 954.02.

- The lipid content of the liquid dairy-substitute product usually depends on the nature and amount of the ingredients used to prepare it, for example it depends on the nature and
- 30 amount of plant milk, and eventually of plant oil, used to prepare said liquid dairy-substitute product. For example:

- In liquid milk-substitute, fat content may vary from 0% in a totally fat-free product, to 30%. Fat content in a milk-substitute corresponds to the mass of lipids in the liquid milk-substitute divided by the total mass of the liquid milk-substitute.
- 5 - In drinking yogurt-substitute, fat content may vary from 0% in a totally fat-free product, to 20%. Fat content in a drinking yogurt-substitute corresponds to the mass of lipids in the drinking yogurt-substitute divided by the total mass of the drinking yogurt-substitute.
- 10 - In liquid cream-substitute, fat content may vary from 0% in a totally fat-free product, to 50%. Fat content in a liquid cream-substitute corresponds to the mass of lipids in the liquid cream-substitute divided by the total mass of the liquid cream-substitute.

In some embodiments, the liquid dairy-substitute product comprises plant oil. Plant oil brings lipids to the liquid dairy-substitute product. Examples of plant oils include, but are not limited to, sunflower oil, coconut oil, mustard oil, peanut oil, canola oil, corn oil, cottonseed oil, flax seed oil, olive oil, palm oil, rapeseed oil, safflower oil, sesame oil, soybean oil, almond oil, 15 beech nut oil, Brazil nut oil, cashew oil, hazelnut oil, macadamia nut oil, mongongo nut oil, pecan oil, pine nut oil, pistachio nut oil, walnut oil, avocado oil, grape oil, camelina oil, or a combination thereof.

In certain embodiments, the liquid dairy-substitute product may comprise plant butter, such as cocoa butter.

20 In some embodiments, the liquid dairy-substitute product comprises carbohydrates. Carbohydrates play a role in the organoleptic sensation. However, liquid dairy-substitute product with reduced carbohydrate content (low carbohydrate) or even with no carbohydrate have been produced during the last decades, in order to address health concerns. In the present invention, the carbohydrates that are contained in the liquid dairy-substitute product 25 are carbohydrate of non-animal origin, such as carbohydrate extracted from plant. The carbohydrate content can be any carbohydrate content commonly used in liquid dairy-substitute products since it is not an essential parameter of the present invention. Carbohydrate content may be adjusted, in quantity and quality, in order to mimic the sensory characteristics of a liquid dairy product.

30 In some embodiments, the liquid dairy-substitute product has carbohydrate content from 0 % to more than 50 %, such as from 0.1 % to 50 %, from 0.1 % to 40 %, from 0.1 % to 30 %, from 0.1 % to 20 %, from 0.1 % to 15 %, from 0.1 % to 10 %, from 0.1 % to 5 %, from 1 % to 5 %.

In some embodiments, the liquid dairy-substitute product has carbohydrate content from 0 % to more than 10 %, such as from 0 % to 9 %, from 0 % to 8 %, from 0 % to 7 %, from 0 % to 6 %, from 0 % to 5 %, from 0 % to 4 %, from 0 % to 3 %, from 0 % to 2 %, from 0 % to 1 %. Carbohydrate content in a product corresponds to the mass of carbohydrates in the product divided by the total mass of the product. Carbohydrate content of a product may be determined by a variety of methods, including, but not limited to, high performance liquid chromatography.

Examples of suitable carbohydrates include, but are not limited to, sucrose, glucose, fructose, mannose, steviolosides, or combinations thereof. In certain embodiments, examples of suitable carbohydrates may be selected from one or more of the following: sucrose, glucose and fructose. The liquid dairy-substitute product comprises carbohydrates of non-animal origin. Carbohydrates of non-animal origin may be glucose, saccharose or fructose. Glucose, saccharose and fructose can be obtained from plants.

In a preferred embodiment, the liquid dairy-substitute product does not comprise lactose.

In some embodiments, the liquid dairy-substitute product may also contain other ingredients of non-animal origin. The other ingredient content can be any ingredient commonly used in liquid dairy-substitute products since it is not an essential parameter of the present invention. Non limiting "other ingredients" include, but are not limited to:

- Antioxidants (e.g., rosemary, spearmint, ascorbic acid, sodium ascorbate, Maillard browning products [melanoidins], BHA, BHT, TBHQ, propyl gal late, tocopherols, vitamin A, vitamin E, carotenoids, flavonoids or combinations thereof. Antioxidant content may be between about 0.01 % and about 10%, between about 0.05% and about 5%, or between about 0.1 % and about 2% by weight.

- Vitamins (e.g., vitamin B 12, vitamin D, vitamin C, vitamin A, vitamin E, vitamin B, vitamin K, thiamine, riboflavin, pyridoxine, carotenoids (e.g., beta-carotene, zeaxanthin, lutein, lycopene), niacin, folic acid, pantothenic acid, biotin, choline, inositol) or combinations thereof.

- Emulsifiers (e.g., lecithin, carrageenan, cellulose gum, cellulose gel, starch, gum arabic, xanthan gum, mono- and diglycerides, propylene glycol monoesters, sodium stearoyl-2-lactylate, polysorbate 60, polysorbate 80, lecithin, hydroxylated lecithin, or combinations thereof. Emulsifier content may be between about 0.01 % and about 10%, between about 0.05% and about 5%, or between about 0.5% and about 2% by weight.

- Stabilizing agents (e.g., starches, gums [e.g., xanthan gum, bean gum, gear gum, gum arabic, gum ghatti, gum karaya, gum tragacanth, gellan gum], hydrocolloids [e.g., guar, acacia, locust bean gum, xanthan, gellan, carrageenan, cellulose, carboxymethyl cellulose, microcrystalline cellulose, methylcellulose hydroxypropyl methyl cellulose, hydroxypropyl cellulose, pectin, low methoxyl pectin, gelatin, agar, furcellaran, dextran, or combinations thereof. Stabilizing agent content may be between about 0.1 % and about 5%, between about 0.5% and about 3%, between about 0.7% and about 1.5% by weight. The stabilizing agent enhance physical properties by imparting viscosity or mouthfeel properties, stabilize and/or suspend insoluble materials and prevent separation or settling of ingredients.
- Preservatives (e.g., potassium sorbate, sorbic acid or combinations thereof).
- Buffering agents that prevent undesired creaming or precipitation upon addition of the milk-substitute into hot, acidic environments (e.g., when added to a hot beverage such as coffee; e.g., monophosphates, diphosphates, sodium mono- and bicarbonates, potassium mono- and bicarbonates, potassium phosphate, dipotassium phosphate, potassium hydrophosphate, sodium bicarbonate, sodium citrate, sodium phosphate, disodium phosphate, sodium hydrophosphate, sodium tripolyphosphate or combinations thereof).
- Minerals (e.g., chloride, sodium, calcium, iron, chromium, copper, iodine, zinc, magnesium, manganese, molybdenum, phosphorus, potassium, selenium, aluminum, soluble mineral salts, slightly soluble mineral salts, insoluble mineral salts, chelated minerals, mineral complexes, non- reactive minerals such as carbonate minerals, reduced minerals, ammonium or combinations thereof).
- Antimicrobial agents.
- Dietary fibers (e.g., oat fiber, barley fiber, rye fiber, or a combination thereof). Dietary fiber content may be between about 0% and about 40%, between about 1 % and about 20%, or between about 1.5% and about 5% by weight.
- Amino acids (e.g., essential amino acids, such as arginine, cysteine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine and/or valine, amino acid salts, amino acid chelates or combinations thereof).
- Salts (e.g., sodium citrate, sodium chloride, potassium citrate, potassium phosphate, dipotassium phosphate or combinations thereof. For example, salts are used to enhance flavor.
- pH-adjusting agents (e.g., organic pH adjusting agents, inorganic pH adjusting agents, food grade acids [e.g., acetic, lactic, hydrochloric, phosphoric, citric, tartaric, malic, glucono,

deltalactone, gluconic acid], basic pH adjusting agent [e.g., disodium diphosphate, potassium hydroxide] or combinations thereof).

- Binding agents (e.g., carrageenan, cellulose gum, cellulose gel, starch, maltodextrin, gum arabic, xanthan gum or combinations thereof; between about 0.01 % and about 10%,
5 between about 0.05% and about 5%, or between about 0.1 % and about 2% by weight).
- Prebiotics (e.g., fructooligosaccharides, galactooligosaccharides); and
- Biotics (e.g., Bifidobacterium spp., Clostridium spp., Bacteroides spp. Enterococcus faecalis, E. coli, Enterobacter cloacae, Klebsiella pneumoniae, Staphylococcus epidermidis, Staphylococcus haemolyticus, Lactoferrin or combinations thereof).
- 10 - Ferments. For example, the first edible composition, such as a dairy-substitute product, may contain bacteria as ferments that can be used for fermentation, such as any lactic acid bacteria from plant sources, e.g., *Lactobacillus plantarum*, *Lactobacillus fermentum* and *Lactobacillus brevis*, *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, *Bifidobacterium*, *Lactobacillus acidophilus*, or mixture thereof.
- 15 - Fruit preparation, such as, for example for smoothies, 50 to 80% by weight of fruit preparation on the weight of the product.
- Flavoring agents.

In some embodiments, the liquid dairy-like product does not contain gelatine.

- In some embodiments, the liquid dairy-like product does not contain foaming agents other
20 than the casein composition comprising casein in the sole form of non-animal origin.

Liquid dairy-like product

- In the context of the invention, a liquid dairy-like product is a liquid dairy-substitute product in which a casein composition comprising casein in the sole form of non-animal origin has
25 been added in order to improve the foaming property of the liquid dairy-substitute product. The liquid dairy-like product has improved foaming property compared to the liquid dairy-substitute product from which it is prepared.

In some embodiments, the liquid dairy-like product provided herein is a dairy-like beverage.

- In some embodiments, the foaming property is chosen from the volume increase after
30 aerating and/or the foam stability. Therefore, the liquid dairy-like product may have an improved volume increase after aerating compared to the liquid dairy-substitute product from

which it is prepared and/or an improved foam stability compared to the liquid dairy-substitute product from which it is prepared.

In the present invention, the parameter "volume increase after aerating" and/or the "foam stability", are the major criteria of the foaming property of a liquid dairy-substitute/like product.

Volume increase after aerating and/or foaming stability can be evaluated by simple volume measurements, as shown in the Examples.

In some embodiments, the liquid dairy-like product is suitable for making a foamed dairy-like product having a volume increase after aerating over 30%, such as over 35%, such as over 40%, such as over 45%, such as over 50%, such as over 55%, such as over 60%, such as over 65%, such as over 70%, such as over 75%, such as over 80%, such as over 85%, such as over 90%, such as over 95%, such as over 100%, such as over 110%, such as over 120%, such as over 130%, such as over 140%, such as over 150%, such as over 160%, such as over 170%, such as over 180%, such as over 190%, such as over 200%, such as from 50% to 200%, such as from 50% to 150%, such as from 100% to 200%, such as from 100% to 150%.

In some embodiments, about 100 mL of the liquid dairy-like product is suitable for making a foamed dairy-like product having a volume increase after aerating over 30%, such as over 35%, such as over 40%, such as over 45%, such as over 50%, such as over 55%, such as over 60%, such as over 65%, such as over 70%, such as over 75%, such as over 80%, such as over 85%, such as over 90%, such as over 95%, such as over 100%, such as over 110%, such as over 120%, such as over 130%, such as over 140%, such as over 150%, such as over 160%, such as over 170%, such as over 180%, such as over 190%, such as over 200%, such as from 50% to 200%, such as from 50% to 150%, such as from 100% to 200%, such as from 100% to 150%.

In some embodiments, the foam of the foamed dairy-like product is stable (i.e., the foam stability) for at least 1 minute, such as for 1 minute, such as for 2 minutes, such as for 5 minutes, such as for 10 minutes at room temperature (e.g., at 25°C). For example, at least 50% of the volume of foam of the foamed dairy-like product is maintained for 10 minutes at 25°C.

The foam stability can also be maintained at high temperature, such as a temperature over 50°C. In some embodiments, the foam of the foamed dairy-like product is stable (i.e. the foam stability) for at least 1 minute, such as for 1 minute, such as for 2 minutes, such as for

5 minutes, such as for 10 minutes at 60°C. For example, at least 50% of the volume of foam of the foamed dairy-like product is maintained for 10 minutes at 60°C.

In one embodiment, the liquid dairy-like product is suitable for the preparation of a foamed dairy-like product, for instance a foamed milk-like, a foamed drinking yogurt-like, a whipped yogurt-like, a whipped cream-like, a foamed smoothie or a foamed milkshake-like, preferably
5 in less than 5 minutes of whipping or frothing, starting from the liquid dairy-like product at an ambient temperature (i.e. from 15°C to 25°C).

In another embodiment, the liquid dairy-like product is suitable for the preparation of a foamed dairy-like product, for instance a foamed milk-like, a foamed drinking yogurt-like, a
10 whipped yogurt-like, a whipped cream-like, a foamed smoothie or a foamed milkshake-like, preferably in less than 5 minutes of whipping or frothing, starting from the liquid dairy-like product at a temperature from 25°C to 100°C, such as from 50°C to 90°C, such as from 50°C to 80°C, for example at a temperature of about 60°C, about 70°C, about 80°C or about 90°C.

15 In another embodiment, the liquid dairy-like product is suitable for the preparation of a foamed dairy-like product, for instance a foamed milk-like, a foamed drinking yogurt-like, a whipped yogurt-like, a whipped cream-like, a foamed smoothie or a foamed milkshake-like, preferably in less than 5 minutes of whipping or frothing, starting from the liquid dairy-like product at a temperature from 1°C to 15°C, such as from 1°C to 10°C, such as from 1°C to
20 5°C.

It was surprising for the inventors to achieve the preparation of a foamed dairy-like product at a large spectrum of temperatures while starting from a liquid dairy-like product, preferably in less than 5 minutes of whipping or frothing. In a short timespan of whipping or frothing, such as less than 5 minutes of whipping or frothing, the foamed dairy-like product may reach
25 a volume increase after aerating and/or a foam stability as disclosed above.

In some embodiments, the aeration step b) of the third object of the invention (e.g., the whipping or frothing step) lasts from 20 to 300 seconds, such as from 20 to 240 seconds, such as from 20 to 180 seconds, such as from 20 to 120 seconds.

In some embodiments, the foamed dairy-like product is further cooled down to and/or
30 maintained at a temperature from 1°C to 25°C, such as from 1°C to 15°C, such as from 1°C to 10°C, such as from 1°C to 5°C during the aeration step b). This embodiment is particularly interesting for preparing whipped yogurt-like and whipped cream-like.

In some embodiments, at least 30% v/v of the volume of the foamed dairy-like product is foam, such as at least 40% v/v, at least 50% v/v, at least 60% v/v, at least 70% v/v, at least 80% v/v, at least 90% v/v, such as from 30% v/v to 100% v/v, from 40% v/v to 100% v/v, from 50% v/v to 100% v/v, from 60% v/v to 100% v/v, from 70% v/v to 100% v/v, from 80% v/v to 100% v/v, from 90% v/v to 100% v/v, even about 100% v/v of the volume of the foamed dairy-like product is foam.

In some embodiments, the foamed dairy-like product has a ratio volume of foam to volume of liquid from 10:1 to 1:3, such as from 10:1 to 2:3, from 10:1 to 1:1, from 10:1 to 2:1, from 10:1 to 3:1, from 10:1 to 4:1, from 10:1 to 5:1, from 9:1 to 2:3, from 8:1 to 2:3, from 7:1 to 2:3, from 6:1 to 2:3, from 5:1 to 2:3, from 4:1 to 2:3, from 3:1 to 2:3, from 2:1 to 2:3.

In some embodiments, the liquid dairy-like product provided herein comprises from 0.1 % to 10 % of casein of non-animal origin, such as from 0.1 % to 9 %, such as from 0.1 % to 8 %, such as from 0.1 % to 7 %, such as from 0.1 % to 6 %, such as from 0.1 % to 5 %, such as from 0.1 % to 4 %, such as from 0.5 % to 10 %, such as from 0.5 % to 9 %, such as from 0.5 % to 8 %, such as from 0.5 % to 7 %, such as from 0.5 % to 6 %, such as from 0.5 % to 5 %, such as from 1 % to 10 %, such as from 1 % to 9 %, such as from 1 % to 8 %, such as from 1 % to 7 %, such as from 1 % to 6 %, such as from 1 % to 5 %, such as from 1 % to 4 %, such as from 2 % to 10 %, such as from 2 % to 9 %, such as from 2 % to 8 %, such as from 2 % to 7 %, such as from 2 % to 6 %, such as from 2 % to 5 %, such as from 2 % to 4 %, such as from 3 % to 10 %, such as from 3 % to 9 %, such as from 3 % to 8 %, such as from 3 % to 7 %, such as from 3 % to 6 %, such as from 3 % to 5 %, such as from 3 % to 4 % of casein of non-animal origin, such as about 1%, about 2%, about 3%, about 4%, about 5%, about 6%, about 7%, about 8%, about 9%, about 10% of casein of non-animal origin. For example, the liquid dairy-like product may comprise from 0.1% to 5% of casein of non-animal origin, such as from 0.5% to 5%, such as from 0.5% to 4%, such as from 0.5% to 3%, such as 0.5% to 2.5% of casein of non-animal origin. The inventors have surprisingly shown that a relatively low content of casein of non-animal origin is sufficient for improving the foaming property of a liquid dairy-substitute product. Casein content in a product corresponds to the mass of casein in the product divided by the total mass of the product.

It has to be understood that the liquid dairy-substitute product is free of casein, e.g., it does not even contain casein of non-animal origin. The casein in the liquid dairy-like product is therefore only brought by the casein composition added to the liquid dairy-substitute product.

from 0.1 % to 20 %, from 0.1 % to 15 %, from 0.1 % to 10 %, from 0.1 % to 5 %, from 1 % to 5 %.

In some embodiments, the liquid dairy-like product has carbohydrate content from 0 % to more than 10 %, such as from 0 % to 9 %, from 0 % to 8 %, from 0 % to 7 %, from 0 %
5 to 6 %, from 0 % to 5 %, from 0 % to 4 %, from 0 % to 3 %, from 0 % to 2 %, from 0 % to 1 %.

In some embodiments, the liquid dairy-like product provided herein may have similar, or substantially similar, colors as analogous dairy products, and/or similar, or substantially similar color to the liquid dairy-substitute product from which it is prepared. The color may
10 be determined, for example, using a colorimeter or spectrophotometer.

In some embodiments, the liquid dairy-like product provided herein may have similar, or substantially similar, flavors as analogous dairy products, and/or similar, substantially similar or superior flavor to the liquid dairy-substitute product from which it is prepared. The flavor can be evaluated, for example, by various methods, including but not limited to by blind
15 tasting performed by human testers or human sensory experts.

In some embodiments, the liquid dairy-like product provided herein may also contain other ingredients corresponding to the "other ingredients" contained in the liquid dairy-substitute product. Therefore, the similar, colors as analogous dairy products, and/or similar may also contain the "other ingredients" detailed in the section "Liquid dairy-substitute product"
20 above.

The liquid dairy-like product provided herein may be used as a base for production of other dairy product analogs with certain nutritional profiles. For example, such dairy product analogs may be milk analogs, yogurt analogs, cheese analogs, cream analogs, whipped cream analogs, ice-cream analogs, milkshake analogues, or smoothie analogues.

25 In some embodiments, the liquid dairy-like product has a pH from pH 6 to pH 7.5, such as pH 6.7.

In some embodiments, the liquid dairy-like product does not contain gelatine.

In some embodiments, the liquid dairy-like product does not contain foaming agents other than the casein composition comprising casein in the sole form of non-animal origin.

Liquid dairy-like product suitable for making a foamed dairy-like product

In a fifth object of the present invention relates to a liquid dairy-like product suitable for making a foamed dairy-like product comprising:

- a) a liquid dairy-substitute product;
- 5 b) a casein composition comprising casein in the sole form of non-animal origin, wherein at least 90% of the casein of the casein composition is non-micellar casein;

wherein the liquid dairy-like product comprises proteins in the sole form of non-animal origin, wherein the liquid dairy-like product comprises from 0.1 % w/w to 10 % of casein of non-
10 animal origin.

In some embodiments, the liquid dairy-like product comprises from 0.1 % to 10 % of casein of non-animal origin, such as from 0.1 % to 9 %, such as from 0.1 % to 8 %, such as from 0.1 % to 7 %, such as from 0.1 % to 6 %, such as from 0.1 % to 5 %, such as from 0.1 % to 4 %, such as from 0.5 % to 10 %, such as from 0.5 % to 9 %, such as from 0.5 % to 8
15 %, such as from 0.5 % to 7 %, such as from 0.5 % to 6 %, such as from 0.5 % to 5 %, such as from 1 % to 10 %, such as from 1 % to 9 %, such as from 1 % to 8 %, such as from 1 % to 7 %, such as from 1 % to 6 %, such as from 1 % to 5 %, such as from 1 % to 4 %, such as from 2 % to 10 %, such as from 2 % to 9 %, such as from 2 % to 8 %, such as from 2 % to 7 %, such as from 2 % to 6 %, such as from 2 % to 5 %, such as from 2 %
20 to 4 %, such as from 3 % to 10 %, such as from 3 % to 9 %, such as from 3 % to 8 %, such as from 3 % to 7 %, such as from 3 % to 6 %, such as from 3 % to 5 %, such as from 3 % to 4 % of casein of non-animal origin, such as about 1%, about 2%, about 3%, about 4%, about 5%, about 6%, about 7%, about 8%, about 9%, about 10% of casein of non-animal origin. For example, the liquid dairy-like product may comprise from 0.1% to 5% of
25 casein of non-animal origin, such as from 0.5% to 5%, such as from 0.5% to 4%, such as from 0.5% to 3%, such as 0.5% to 2.5% of casein of non-animal origin. The inventors have surprisingly shown that a relatively low content of casein of non-animal origin is sufficient for improving the foaming property of a liquid dairy-substitute product.

The specific embodiments detailed in the section "Liquid dairy-like product" above apply to
30 the fourth object of the invention. For example:

In some embodiments, the liquid dairy-like product has a pH from pH 6 to pH 7.5, such as pH 6.7.

In some specific embodiments, the casein of the casein composition is calcium caseinate or sodium caseinate.

In some specific embodiments, the liquid dairy-substitute product is suitable for making a foamed dairy-like product at a temperature from 25°C to 100°C.

- 5 In some specific embodiments, the liquid dairy-substitute product is suitable for making a foamed dairy-like product at a temperature from 50°C to 90°C, such as from 50°C to 80°C, for example at a temperature of about 60°C, about 70°C, about 80°C or about 90°C.

In some specific embodiments, the casein of non-animal origin is a mixture of alpha-S1 casein and beta casein.

- 10 In some specific embodiments, the casein of non-animal origin is beta casein.

DESCRIPTION OF THE FIGURES

Figure 1: shows the volume of a milk-like before and after aerating. Initial volume before foaming (left) and volume after aerating (right); the volume of liquid and foam are measured after aerating.

- 5 **Figure 2:** shows the ratio foam to liquid of a milk-like before and after aerating at 25°C. Black bars: volume of foam; dotted bars: volume of liquid; 0: no caseins, MP: sodium caseinates from bovine milk, Armor Proteins, PF: sodium caseinates made by precision fermentation, as in Example 1, Pea: Pea proteins. Top: t=0 after aerating; bottom: t=10 min after aerating.
- 10 **Figure 3:** shows the ratio foam to liquid of a milk-like before and after aerating at 60°C. Black bars: volume of foam; dotted bars: volume of liquid; 0: no caseins, MP: sodium caseinates from bovine milk, Armor Proteins, PF: sodium caseinates made by precision fermentation, as in Example 1, Pea: Pea proteins. Top: t=0 after aerating; bottom: t=10 min after aerating.

EXAMPLES

Example 1: Synthesis of recombinant casein

Synthetic genes coding for alpha-S1 and beta casein (related to natural genes P02662, and P02666, respectively), were modified, in order to remove the signal peptide, and the sequence of the new synthetic open reading frames (SEQ ID NO: 2 and SEQ ID NO: 4) are shown in Table 2, last column. They were cloned together into pET25b+, in a same operon under the control of the T7 promoter of pET25b+, with (i) both synthetic genes being under the control of a T7 ribosome binding site and (ii) the gene beta casein gene being proximal to the promoter and the alpha-S1 distal to the promoter. The resulting plasmid, capable of co-expressing the two proteins, was transformed into BL21(DE3) strains (Novagene). Individual transformed clones were isolated, and for each synthetic gene, one clone was used to inoculate LB medium.

Name and Uniprot reference of protein precursor	sequence of casein precursor	sequence of recombinant casein
Alpha-S1 casein P02662	MKLLILTCLVAVAL ARPKHPIKHQGLPQEVLNENLLRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAMEDIKQMEAESISSSEIIVPNSVEQKHIQKEDVPSERYLGYLEQLLRLKKYKVPQLEIVPNSAEERLHSMKEGIHAQQKEPMIGVNQELAYFYPELFRQFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGSENSEKTTMPLW (SEQ ID NO: 1)	MR PKHPIKHQGLPQEVLNENLLRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAMEDIKQMEAESISSSEIIVPNSVEQKHIQKEDVPSERYLGYLEQLLRLKKYKVPQLEIVPNSAEERLHSMKEGIHAQQKEPMIGVNQELAYFYPELFRQFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGSENSEKTTMPLW (SEQ ID NO: 2)
Beta casein P02666	MKVLILACLVALALA RELEELNVPGEIVESLSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVYPFGPIPNSLPQNIPPLTQTPVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFTESQSLTLTDVENLHLPLLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV (SEQ ID NO: 3)	M RELEELNVPGEIVESLSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVYPFGPIPNSLPQNIPPLTQTPVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFTESQSLTLTDVENLHLPLLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV (SEQ ID NO: 4)

Table 2: Sequences of natural casein (precursors) and of the related recombinant proteins. In natural casein, signal peptide is indicated in bold. In recombinant casein, the methionine resulting from cloning into the pET expression system is indicated in bold.

A typical casein production and purification experiment is described below.

5 ***Cell cultivation***

In order to produce a batch of alpha-S1 and beta caseins, the resulting strain was cultivated as follow (3500 L fermentation scale).

Five concentrated stocks of the strain of each 1 mL, stored in LB with 10 % DMSO at -80°C, were thawed and used to inoculate four or five precultures of a volume of 1 L each, respectively (0.1 % v/v inoculation rate), in a revivification medium Y15 composed of yeast extract (15 g/L) and NaCl (5 g/L), and supplemented with 100 µg/mL of ampicillin. The five precultures were grown in 4 L or 5 L erlenmeyers at 30°C, for 9 hours, at 170 rpm (orbital diameter: 25 mm). After 9 hours, optical densities (600 nm) in the range of 2.5-3 were achieved.

5 L of the first preculture were used to inoculate a volume of 250 L (2 % v/v inoculation rate), in an optimized culture medium, supplemented with 100 µg/mL of ampicillin and with 15 g/L of glucose. The culture was stirred in a 350 L bioreactor at 37°C for 10 hours. Stirring, aeration and pressure were adjusted to maintain pO₂ at 15%. pH was regulated to 7.2, with the automatic addition of a solution of ammonia (20.5 % v/v) and a solution of phosphoric acid (5 mol/L). After 10 hours, an optical density in the range of 15-17 was obtained.

160 L of this culture were used to inoculate a fermenter with an initial volume of 2700 L (6 % v/v inoculation rate), in an optimized culture medium, supplemented with 100 µg/mL of ampicillin and 1 g/L of glucose. The culture was stirred in a 3500 L bioreactor at 37°C, until optical density (600nm) reached 20, then at 35.4°C until the end of the run. After 30 minutes, glucose was added regularly in the bioreactor, using a concentrated 400 g/L glucose solution, and the feed was adjusted to sustain growth and production, without glucose accumulation in the medium. Ampicillin was added in the bioreactor each 9 hours, to maintain a concentration of 100 µg/mL, and antifoam (5% stock solution, Struktol J673A) was added when necessary. Stirring, aeration and pressure were adjusted to maintain pO₂ at 15%. pH was regulated to 7.2, with the automatic addition of a solution of ammonia (20.5 % v/v) and a solution of phosphoric acid (5 mol/L). IPTG was added at a final concentration of 0.2 mM when optical density reached 40±5, and the culture was stopped after about 24

hours, optical densities (600 nm) in the range of 80 could be achieved. This resulted in around 3.5 tons of culture broth with 4.4 % of dry biomass.

Downstream processing

The culture medium was concentrated using a self-cleaning disk-stack centrifuge (GEA, Easy-scale, SSE20 or SC15 model, centrifugal force: 17000-20000 G, back pressure : 5-5.5 bars, temperature < 20 °C).

The concentrated biomass was then washed two times with osmosis water, in order to remove all compounds from the fermentation medium (salts, sugars, nutrients...). The concentrated biomass was mixed with water, until obtaining a homogeneous suspension. The suspension was then separated by centrifugation, using a self-cleaning disk-stack centrifuge (GEA, Easy-scale or SC15 model, centrifugal force: 17000-20000 G, back pressure: 4-5 bars, temperature < 20 °C). Concentrated cells were obtained after separation, and washing water was eliminated. This washing procedure was repeated twice, according to a similar procedure, to finally obtain a pellet of concentrated washed cells.

Washed concentrated bacteria were then resuspended in osmosis water, until having a homogeneous suspension, and the pH was adjusted to 8.5 with NaOH 30 % w/v (Brenntag, 144562). The resulting suspension was heated during 1 hour at 95°C, in a 2 m³ double-jacket tank, allowing efficient bacterial lysis and thus, *E. coli* strain deactivation, *E. coli* proteins precipitation and caseins solubilization. After 1 hour, the pH of the suspension was 7.82; it was re-adjusted to 8.5 with NaOH 30 % w/v. After cooling, a dense suspension was obtained.

The suspension was then separated by centrifugation, using a self-cleaning disk-stack centrifuge (GEA, Easy-scale model, centrifugal force: 17000 G, back pressure : 5 bars, temperature < 20 °C), with the aim of eliminating all the insoluble particles (cellular debris, *E. coli* precipitated proteins), and recovering purified caseins in the soluble fraction. After separation, the supernatant containing caseins and a heavy phase were obtained. The heavy phase was washed with osmosis water and resulting suspension was separated by centrifugation in the same experimental conditions than those described for the first separation. The supernatant was collected.

To improve their quality and reduce the quantity of insoluble particles inside, both supernatants were filtered on a 0.8 µm ceramic mineral membrane (Pall).

The pH of the permeate was then adjusted to 4.6 with a 20 % v/v solution of lactic acid (Corbion, 1001013890), at room temperature, in an agitated double-jacket tank from VMI, in

order to reach the isoelectric point of caseins, and consequently, lead to their precipitation. The resulting suspension was concentrated by centrifugation using a self-cleaning disk-stack centrifuge (GEA, Easy-scale model, centrifugal force: 17000 G, bowl rotation speed: 12000 rpm, bowl filling : 80%-90% of the capacity, back pressure : 5 bars, temperature < 20 °C).

- 5 After separation, concentrated acidic caseins were collected, while the light phase was discarded. Acidic caseins were then washed with osmosis water until obtaining a homogeneous suspension, which was then concentrated by centrifugation, using a self-cleaning disk-stack centrifuge (GEA, Easy-scale model, centrifugal force : 17000 G, back pressure : 5 bars, temperature < 20 °C). The pH of the suspension before separation was
10 measured and adjusted to 4.6 with lactic acid if it was necessary, to avoid caseins loss in the washing water.

Acid caseins

- Concentrated acidic washed caseins were obtained after separation, and washing water was eliminated. This washing procedure was repeated twice, according to a similar
15 procedure, to finally obtain a pellet of concentrated white and odorless washed acidic caseins.

Calcium caseinates production

- A suspension of calcium hydroxide (1 mol.L^{-1}) was added slowly to the suspension of acidic caseins to adjust the pH to 7, at room temperature, in order to obtain a suspension of
20 calcium caseinates.

Sodium caseinates production

- A solution of sodium hydroxide (1 mol.L^{-1}) was added slowly to the suspension of acidic caseins to adjust the pH to 7, at room temperature, in order to obtain a suspension of sodium caseinates.

- 25 The suspensions of acidic caseins, calcium caseinates or sodium caseinates were dried using a multi-stage spray-dryer (Sicca Dania, direct configuration, inlet temperature: 80-85°C, outlet temperature: 180-185°C, dual-fluid buses: 1-3.5 bars), to obtain a powder, with a dry matter from 94 % to 97 %.

- Caseins content could be estimated by the analysis of SDS PAGE gels, and the
30 comparison of samples with protein standards (Purified alpha and beta caseins from milk (Sigma)). Sodium and calcium caseinate powders contained about 80% of recombinant casein (and about 90% of total proteins), with a ratio of about 50% (48% to 52%, depending on experiment), similar to the initial composition in the cell before treatment,

showing that in the condition we used, there was no bias regarding the purification process of α s1 casein vs. β casein.

Further analysis was conducted for other compounds by various methods. Analysis of a calcium caseinate sample is described in Table 3.

Compound	% (w/w)
Fat	4.26
Including saturated fat	1.35
Carbohydrates	ND
Proteins (total)	87.38
Calcium	1.07
Sodium Chloride	0.59
Polyphenols	0.96
Moisture (water)	4.57

5 **Table 3 :** Composition of a calcium caseinate sample. Only detected compounds accounting for more than 0.1 % are accounted for

Example 2: Foaming effect of a mixture of alpha-S1 casein and beta casein produced by precision fermentation in milk-substitutes (spray-dried; casein purity: 80% w/w)

We tested the impact of the caseinates from precision fermentation on the foaming of plant-based milk-substitutes made from almond, cashew, hazelnut, soy, coconut, rice and oat. Products from different brands were tested for some of them.

3% of sodium caseinates from precision fermentation (w/w), made as described in example 1 (spray dry, 80% caseins. Calcium caseinate composition is indicated in Table 3, and sodium caseinate composition is considered to be the same, but for salts since sodium hydroxide instead of calcium hydroxide, was used to adjust pH to 7), were mixed with each type of plant-based milk-substitute, whose composition is described in Table 4. Some of these products contain additives such as emulsifiers or stabilizers, which may have an impact on foaming properties. In the mixture, the casein content is 2.4% (w/w). The mixture was stirred for 15 minutes at 750 rpm at room temperature. 98 mL of each solution were passed on a milk frother (Burnnove) at 25°C or 60°C. The foam height was measured before and

after aerating on a graduated cylinder (Figure 1). The same measure was taken after 10 minutes at room temperature to analyze foam stability.

In order to compare the foaming effect with other protein sources, the same procedure was realized with sodium caseinates from bovine milk (Armor Protéines, France), with pea protein
5 (Nutralys S85F 2.0, Roquette, France, (84% proteins, dry weight)) and without added proteins.

Results are summarized on Figure 2 (25°C) and Figure 3 (60°C), and in Table 5.

At 25°C, milk-substitutes made from almond, cashew, hazelnut and soy were foaming naturally, and no significant difference was observed with the addition of natural or
10 recombinant caseins. In contrast, coconut-based milk-substitute as well as milk-substitutes from rice or oat were not naturally foaming, or barely foaming; but foaming could be observed in the presence of natural or recombinant caseins. In such cases, the foaming impact of caseins was far superior to the foaming impact of pea proteins, which brought only slight foaming effect in rice- and oat-based products, and lower or similar effect in coconut-
15 based products.

Foaming stability, as measured by the decrease after 10 minutes, was in general positively related to the foaming formation efficacy. However, these two parameters were not always correlated: Strong instability (V foam 10min <20%) was observed with milk and pea proteins in Alpro's Hazelnut-based product, and with precision fermentation proteins in Bjorg's Rice-
20 based and in Alpro's Oat-based products, all these combinations having yet at least 90% of volume increase after aerating. In opposite, good stability (V foam 10min >65%) was observed with Pea protein in Bjorg's Rice-based product, and in the absence of added proteins in Bjorg's Oat-based product, whereas these two combinations had low volume increase after aerating (6% and 8%, respectively).

At 60°C, milk-substitutes from almond, cashew, and soy were foaming naturally. No
25 significant difference was observed with the addition of natural or recombinant caseins. Milk-substitutes from hazelnut had less foaming power, which could be rescued by the addition of caseins from cow's milk (Bjorg) or recombinant (Alpro and Bjorg). In contrast, coconut-based milk-substitute as well as milk-substitutes from rice or oat were not naturally foaming. In
30 these products, foaming could be achieved in the presence of recombinant casein. Caseins from milk were efficient only in Bjorg milk-substitutes from coconut and rice. Pea proteins

were efficient only in Alpro and Bjorg coconut-based milk-substitutes. Stability, as monitored by foaming after 10 minutes (cooling at room temperature), was in general positively correlated with foaming. However, a strong foam instability (V foam 10 min = 7%) was observed with caseins from milk in Alpro almond-based milk-substitute, having good (170%) volume increase after aerating.

Base	Brand	Composition
Almond	Alpro	Water, almond (2.3%), tricalcium phosphate, table sea salt, stabilizers (locust bean seed flour, gellan gum), emulsifier (lecithins (sunflower)), vitamins (B2, B12, E, D2).
Almond	Bjorg	Almond milk (water, almonds 2.8%), unrefined cane sugar, rice starch, sea salt
Cashew	Bjorg	Water, cashews 4%, rice starch, sea salt, stabilizers: guar gum, xanthan gum
Hazelnut	Alpro	Water, sugar, hazelnut (2.5%,) tricalcium phosphate, table sea salt, stabilizers (locust bean seed flour, gellan gum), emulsifier (lecithins (sunflower)), vitamins (riboflavin (B2), B12, E, D2)
Hazelnut	Bjorg	Water, hazelnuts 6.5%, cane sugar, corn maltodextrin, sea salt, thickener: guar gum, xanthan gum
Soy	Alpro	Soy base (water, shelled non-GMO soybeans (8%)), sugar, acidity regulators (potassium phosphates), calcium carbonate, flavouring, table salt, stabilizer (gellan gum), vitamins (B2, B12, D2)
Soy	Bjorg	Tonyu (water, soybeans 8%)
Coconut	Alpro	Water, coconut milk (5.3%) (coconut cream, water), rice (3.3%), tricalcium phosphate, stabilizers (guar gum, gellan gum, xanthan gum), sea salt, vitamins (B12, D2), flavourings
Coconut	Bjorg	Water, coconut 5.4%, unrefined cane sugar, stabilizers: guar gum, xanthan gum; Natural coconut flavour, sea salt
Rice	Bjorg	Water, rice (12%), sunflower oil, sea salt
Oat	Bjorg	Water, oats 11%, cold-pressed sunflower oil, sea salt
Oat	Oatly	Water, oats 10%, rapeseed oil, calcium carbonate, calcium phosphates, salt, vitamins (D2, riboflavin, B12), potassium iodide

Table 4: Composition of tested plant-based milks

Milk substitute	Brand	Protein type	V liquid (ml) 25°C	V foam (ml) 25°C	Volume increase 25°C	V foam 10min 25°C	V liquid (ml) 60°C	V foam (ml) 60°C	Volume increase 60°C	V foam 10min 60°C
Almond	Alpro	0	2	212	118%	80%	5	235	145%	61%
Almond	Alpro	MP	0	220	124%	91%	2	263	170%	7%
Almond	Alpro	PF	0	198	102%	87%	0	265	170%	79%
Almond	Alpro	Pea	10	188	102%	80%	2	246	153%	72%
Almond	Bjorg	0	12	202	118%	73%	10	220	135%	65%
Almond	Bjorg	MP	0	236	141%	76%	0	270	176%	61%
Almond	Bjorg	PF	0	218	122%	91%	0	265	170%	75%
Almond	Bjorg	Pea	10	188	102%	70%	0	250	155%	67%
Cashew	Bjorg	0	0	184	88%	91%	2	220	127%	65%
Cashew	Bjorg	MP	0	188	92%	84%	0	240	145%	53%
Cashew	Bjorg	PF	0	168	71%	90%	0	218	122%	79%
Cashew	Bjorg	Pea	0	174	78%	82%	0	216	120%	80%
Hazelnut	Alpro	0	10	210	124%	62%	90	70	63%	71%
Hazelnut	Alpro	MP	0	230	135%	11%	98	0	0%	NA
Hazelnut	Alpro	PF	0	216	120%	88%	0	270	176%	74%
Hazelnut	Alpro	Pea	20	204	129%	6%	60	140	104%	50%
Hazelnut	Bjorg	0	0	236	141%	75%	60	164	129%	55%
Hazelnut	Bjorg	MP	0	230	135%	83%	2	263	170%	56%
Hazelnut	Bjorg	PF	0	220	124%	88%	0	265	170%	68%
Hazelnut	Bjorg	Pea	10	202	116%	75%	60	140	104%	14%
Soy	Alpro	0	2	226	133%	83%	0	270	176%	76%
Soy	Alpro	MP	0	208	112%	86%	0	270	176%	70%
Soy	Alpro	PF	0	194	98%	94%	0	254	159%	81%
Soy	Alpro	Pea	0	190	94%	82%	0	248	153%	81%
Soy	Bjorg	0	0	224	129%	86%	0	280	186%	74%
Soy	Bjorg	MP	0	200	104%	79%	0	255	160%	72%
Soy	Bjorg	PF	0	190	94%	78%	0	242	147%	79%
Soy	Bjorg	Pea	0	194	98%	91%	0	232	137%	84%

Milk substitute	Brand	Protein type	V liquid (ml) 25°C	V foam (ml) 25°C	Volume increase 25°C	V foam 10min 25°C	V liquid (ml) 60°C	V foam (ml) 60°C	Volume increase 60°C	V foam 10min 60°C
Coconut	Alpro	0	96	16	16%	0%	98	0	0%	NA
Coconut	Alpro	MP	0	196	100%	77%	98	0	0%	NA
Coconut	Alpro	PF	0	180	84%	84%	0	244	149%	82%
Coconut	Alpro	Pea	0	180	84%	80%	0	186	90%	53%
Coconut	Bjorg	0	98	0	0%	NA	98	0	0%	NA
Coconut	Bjorg	MP	0	206	110%	81%	0	260	165%	39%
Coconut	Bjorg	PF	0	194	98%	90%	0	252	157%	74%
Coconut	Bjorg	Pea	30	142	76%	66%	10	214	129%	63%
Rice	Bjorg	0	98	0	0%	NA	98	0	0%	NA
Rice	Bjorg	MP	40	112	55%	45%	0	265	170%	50%
Rice	Bjorg	PF	10	196	110%	19%	10	250	165%	51%
Rice	Bjorg	Pea	92	12	6%	67%	98	0	0%	NA
Oat	Bjorg	0	94	12	8%	100%	98	0	0%	NA
Oat	Bjorg	MP	0	238	143%	71%	98	0	0%	NA
Oat	Bjorg	PF	0	234	139%	73%	10	212	127%	44%
Oat	Bjorg	Pea	80	60	43%	20%	98	0	0%	NA
Oat	Oatly	0	84	58	45%	83%	98	0	0%	NA
Oat	Oatly	MP	4	202	110%	58%	98	0	0%	NA
Oat	Oatly	PF	0	204	108%	70%	10	200	114%	47%
Oat	Oatly	Pea	80	54	37%	15%	98	0	0%	NA

Table 5: V liquid: volume of liquid, after aerating of 98mL (V initial=Vi) of the product; V foam: volume of foam immediately after aerating of 98mL of the product. The volume

increase after aerating is calculated according to the formula: Volume increase after aerating (%) = (V foam + V liquid -Vi)/Vi x 100. Volume of foam is also measured 10 minutes after aerating (V foam 10 min), and expressed here as a percentage of V foam. Measurements are made for aerating at 25°C and 60°C. 0: no caseins, MP: sodium caseinate from bovine milk, 5 Armor Proteins, PF: sodium caseinate made by precision fermentation, as in Example 1, Pea: Pea proteins.

Example 3: Foaming effect of a beta casein composition produced by precision fermentation in milk-substitutes (spray-dried; casein purity: 80% w/w)

- 10 Beta casein was produced and purified as described in Example 1, except that no Alpha S1 casein was expressed, produced and purified (i.e., SEQ ID NO: 2 was not cloned into the pET25b+ vector).
- The foaming effect of a beta casein composition, made by precision fermentation was performed as described in Example 2, excepting that only beta casein was incorporated into
- 15 the recipe (no Alpha S1 casein was incorporated into the recipe).
- The results are presented in the following tables.

Milk Substitute	Brand	Protein type	% of casein (w/w)	Initial Volume (ml) 25°C	Total Volume (ml) 25°C	Volume liquid (ml) 25°C	Volume foam (ml) 25°C	% increase volume after aerating (%) 25°C	% increase volume 10 min after aerating (%) 25°C
Just water	NA	Precision Fermentation Beta casein	3%	98	260	20	240	165%	13%
Coconut	Alpro	Precision Fermentation Beta casein	3%	98	210	0	210	114%	78%

Milk Substituted	Brand	Protein type	% of casein (w/w)	Initial Volume (ml) 25°C	Total Volume (ml) 60°C	Volume liquid (ml) 60°C	Volume foam (%) 60°C	% increase volume after aerating (%) 60°C	% increase volume 10 min after aerating (%) 60°C
Just water	NA	Precision Fermentation Beta casein	3%	98	255	0	255	160%	11%
Coconut	Alpro	Precision Fermentation Beta casein	3%	98	252	10	242	157%	61%

CLAIMS

1. Use of a casein composition comprising casein in the sole form of non-animal origin for improving the foaming property of a liquid dairy-substitute product, thereby obtaining a liquid dairy-like product, said liquid dairy-like product having an improved foaming property compared to the liquid dairy-substitute product.
2. A method for improving the foaming property of a liquid dairy-substitute product, said method comprising a step of incorporating a casein composition comprising casein in the sole form of non-animal origin to a liquid dairy-substitute product thereby obtaining a liquid dairy-like product, said liquid dairy-like product having an improved foaming property compared to the liquid dairy-substitute product.
3. A method for preparing a liquid dairy-like product suitable for making a foamed dairy-like product, comprising the step of:
 - a) providing a liquid dairy-substitute product;
 - b) incorporating a casein composition comprising casein in the sole form of non-animal origin into the liquid dairy-substitute product, thereby obtaining a liquid dairy-like product suitable for making a foamed dairy-like product.
4. A method for preparing a foamed dairy-like product, comprising the steps of:
 - a) incorporating a casein composition comprising casein in the sole form of non-animal origin into a liquid dairy-substitute product, thereby obtaining a liquid dairy-like product;
 - b) aerating the liquid dairy-like product thereby obtaining a foamed dairy-like product.
5. The method according to any of claim 3 or 4, wherein the foamed dairy-like product is a foamed milk-like, a whipped yogurt-like, a whipped cream-like, a foamed smoothie or a foamed milkshake-like.
6. The method or use according to any of any of the preceding claims, wherein the liquid dairy-substitute product is a liquid milk-substitute, a drinking yogurt-substitute, a liquid cream-substitute, a smoothie or a milkshake-substitute.
7. The method or use according to any of any of the preceding claims, wherein at least 90% of the casein of the casein composition is non-micellar casein.

8. The method or use according to any of any of the preceding claims, wherein the liquid dairy-substitute product is at a temperature from 25°C to 100°C.

9. The method or use according to any of any of the preceding claims, wherein the liquid dairy-substitute product is at a temperature from 50°C to 90°C, such as from 50°C to 80°C, for example at a temperature of about 60°C, about 70°C, about 80°C or about 90°C.

10. The method or use according to any of the preceding claims, wherein the foaming property is the volume increase after aerating and/or the foam stability.

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11. The method or use according to any of the preceding claims, wherein the liquid dairy-like product comprises from 0.1 % to 10 % of casein of non-animal origin.

12. The method or use according to any of the preceding claims, wherein the liquid dairy-like product comprises from 0.1 % to 5 % of casein of non-animal origin.

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13. The method or use according to any of the preceding claims, wherein the casein of non-animal origin is substantially free of phosphorylation.

14. The method or use according to any of the preceding claims, wherein the casein of non-animal origin is produced in bacteria, such as *E. Coli*.

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15. The method or use according to any of the preceding claims, wherein the liquid dairy-substitute product is not suitable for making a foamed dairy-substitute product having a volume increase after aerating over 45%, such as over 45% at 25°C and/or 60°C.

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16. The method or use according to any of the preceding claims, wherein the liquid dairy-like product comprises from 0.1 % to 10 % of casein of non-animal origin.

17. The method or use according to any of the preceding claims, wherein the liquid dairy-like product comprises from 0.1 % to 5 % of casein of non-animal origin.

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18. The method or use according to any of the preceding claims, wherein the liquid dairy-substitute product is prepared from coconut, rice, oat, or a combination thereof.

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19. The method or use according to any of the preceding claims, wherein the casein of non-animal origin is one, two or three casein(s) selected from the group consisting of alpha-S1 casein, alpha-S2 casein, beta casein and kappa casein.

5 **20.** The method or use according to any of the preceding claims, wherein the casein of non-animal origin is alpha-S1 casein, beta casein, a mixture of alpha-S1 casein and beta casein, a mixture of alpha-S1 casein and alpha-S2 casein, or a mixture of beta casein, alpha-S1 casein and alpha-S2 casein.

10 **21.** The method or use according to any of the preceding claims, wherein the casein composition does not comprise at least one casein protein selected from the group consisting of alpha-S1 casein, alpha-S2 casein, beta casein and kappa casein.

22. The method or use according to any of the preceding claims, wherein the casein
15 composition does not comprise kappa casein.

23. The method or use according to any of the preceding claims, wherein the casein of non-animal origin is a mixture of alpha-S1 casein and beta casein.

20 **24.** The method or use according to any of the preceding claims, wherein the casein of non-animal origin is beta casein.

25. The method or use according to any of the preceding claims, wherein at least 50% of the casein of the casein composition is caseinate, such as 1, 2, 3 or 4 caseinates selected
25 from the group consisting of sodium caseinate, calcium caseinate, potassium caseinate and magnesium caseinate.

26. The method or use according to any of the preceding claims, wherein the foam of the foamed dairy-like product is stable for at least 10 minutes at room temperature, such as at
30 least 50% of the volume of foam of the foamed dairy-like product is maintained after 10 minutes at room temperature.

27. The method or use according to any of the preceding claims, wherein (i) the liquid dairy-like product has protein content from 0.5 % to 40 % and/or (ii) the liquid dairy-like
35 product has lipid content from 0 % to 50 %.

28. A liquid dairy-like product suitable for making a foamed dairy-like product comprising:
a) a liquid dairy-substitute product;

b) a casein composition comprising casein in the sole form of non-animal origin,
wherein at least 90% of the casein of the casein composition is non-micellar casein;

5 wherein the liquid dairy-like product comprises proteins in the sole form of non-animal origin,

wherein the liquid dairy-like product comprises from 0.1 % w/w to 10 % of casein of non-animal origin.

10 **29.** The product according to claim 28, wherein the liquid dairy-substitute product is suitable for making a foamed dairy-like product at a temperature from 25°C to 100°C.

30. The product according to claim 28 or 29, wherein the liquid dairy-substitute product is suitable for making a foamed dairy-like product at a temperature from 50°C to 90°C, such as
15 from 50°C to 80°C, for example at a temperature of about 60°C, about 70°C, about 80°C or about 90°C.

31. The product according to any of claims 28 to 30, wherein the casein of non-animal origin is a mixture of alpha-S1 casein and beta casein.

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32. The product according to any of claims 28 to 31, wherein the casein of non-animal origin is beta casein.