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(54) Title: FERMENTED SOY-BASED FOOD PRODUCT

(57) Abstract: A process for fermenting a substrate comprising soy protein and a carbohydrate using both thermophilic lactic acid bacteria as well as mesophilic lactic acid bacteria.



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FERMENTED SOY-BASED FOOD PRODUCT

Field of the Invention

- 5 The present invention relates to the field of fermented (or cultured) soy-containing products, especially fermented soy-based beverages.

Background of the invention

- 10 Consumers have become more knowledgeable about protein and its role in a healthy diet. This new understanding has had a profound effect, stimulating consumer interest and demand for healthier beverages that are fortified with protein. Because beverages are a convenient way to incorporate protein into the diet, manufacturers continue to formulate new products in an effort to make protein more accessible to a wider group of consumers.
- 15 The two most popular beverage proteins are milk protein and soy protein, and their various isolate derivatives. According to the U.S. Food and Drug Administration, the consumption of food products rich in soy protein can reduce cholesterol, enhance athletic performance, and even aid in the battle against diabetes. In addition, interest for milk replacement by soy protein has increased in view of, on the one hand, issues
- 20 in relation to over-sensitivity and/or intolerance towards milk constituents experienced by growing numbers of consumers, a desire for products suitable for (strict) vegetarians and, on the other hand, elevated milk protein prices and supply issues that some manufacturers are experiencing relative to this commodity. Soy proteins have been proposed to replace milk proteins, either partially or totally, depending on the system,
- 25 and dairy-like products have been developed based entirely on soy protein. In view of soy protein's documented health benefits it is desirable to incorporate substantial quantities of soy protein in beverages. However, incorporation of soy protein into e.g. beverages presents several challenges. The incorporation of soy protein in beverages is known to impart a noticeable aftertaste and a distinctive "beany"
- 30 taste. Different types of processes to isolate soy protein have been proposed which aim to remove these undesirable (off-) flavour-notes. Unfortunately, however, it is almost impossible to completely remove the typical soy "off-notes" from sources of soy protein such as soy concentrates and soy isolates. In addition, in most product applications the intensity of soy off-notes increases during processing and storage,

probably as a result of the formation of off-flavour compounds from precursor molecules.

US 3,364,034 describes a method for removing characteristic flavour and/or odour from vegetable protein materials to provide a substantially bland product, comprising:

- 5 inoculating said protein materials with bacteria selected from the group consisting of *Lactobacillus lactis*, *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, *Leuconostoc citrovorum*, *Pediococcus cerevisiae*, *Pseudomonas ovalis*, *Pseudomonae fragi*, *Aerobacter aerogenes*, *Streptococcus lactis*, incubating for 16-144 hours under conditions conducive to bacterial growth; and terminating said bacterial growth after
- 10 said material is rendered substantially bland.

US 4,664,919 describes a process for producing yogurt-like food, comprising fermenting soy milk with *Streptococcus sojilactis*. It is observed in the US patent that the yogurt-like food so obtained does not have peculiar 'green' smell of soy milk and that it has a good taste. It is further stated that the aforementioned *Streptococcus* strain

15 is capable of removing the 'green' smell of soybeans and that the amount of diacetyl and acetone formed is larger than that of other lactic acid bacteria. Data provided about the *Streptococcus sojilactis* show that the micro-organism is capable of growing at temperatures in the range of 30-40 °C, but not at 20 °C or less or at temperatures of 45 °C or higher.

- 20 US 6,599,543 relates to process for preparing a fermented soybean milk comprising: contacting dehulled and dehypocotyl whole soybeans with warm or hot water; removing warm or hot water-soluble component from the soybeans; pulverizing the soybeans to make a slurry; removing insoluble component from the slurry to make a soybean milk, inoculating a lactic acid bacterium of the genus *Bifidobacterium*, *Lactobacillus*
- 25 *bulgaricus* and one strain selected from the group consisting of *Lactobacillus acidophilus* and *Lactobacillus casei* into the soybean milk, adding one or more saccharides which can be utilized by the lactic acid bacterium to the soybean milk, and fermenting the soybean milk to produce the fermented soybean milk. The removal of hypocotyl from the soybeans as taught by US 6,599,543 is laborious and costly.

- 30 EP-A 0 386 817 describes a process of preparing a fermented soymilk, which comprises the steps of:

- a) inoculating soymilk with an exocellular polysaccharide-forming lactic acid bacterium;
- b) incubating the inoculated soymilk; and
- 35 c) recovering the fermented product.

Example 1 of the European patent application describes how 100 ml of soybean milk containing 0.5 wt.% of a added lactose was fermented with a culture of exocellular polysaccharide-forming lactic acid bacteria (*Streptococcus cremoris*) for 15 hours at 25 °C, after which the pH had dropped to 4.6. After fermentation, a product was obtained with a highly viscous consistency and practically no beany flavour.

EP-A 1 145 648 describes a method of preparing a lactic acid fermented soy proteinaceous food ingredient with reduced levels of flatulence inducing oligosaccharides and conserved levels of isoflavones, said method comprising:

- a) providing an aqueous crude soy material containing soy protein, flatulence-inducing oligosaccharides, and isoflavones; and
- b) simultaneously treating the aqueous crude soy material with (1) a source of glycosidase activity that is effective to hydrolyze the flatulence-inducing oligosaccharides to fermentable saccharides and (2) a lactic acid producing culture that ferments the fermentable saccharides.

Example 2 describes how a 30% solids slurry of defatted soy flour in water was simultaneously inoculated with about 5 percent of a lactic acid culture (mixture of *Lactococcus lactis* and *Streptococcus cremoris*) and about 0.01 percent of α -galactosidase enzyme. The inoculated slurry was held at 35 °C for 4 hours, during which time the pH dropped from 6.6 to 5.3.

JP-A-2004-261003 describes a method of preparing fermented soybean milk by fermenting soybean milk and by adding palatinose (isomaltulose) before, during or after fermentation. In the Japanese application it is observed that the grass smell inherent to soybean milk can be reduced to a certain extent by fermenting soybean milk with a combination of a lactic acid bacterium and a bifidobacterium, but that satisfactory results cannot always be established, in particular due to the taste and smell of acetic acid, which is metabolic product of the fermentation. Palatinose is incorporated in the fermented soybean milk to suppress the unpleasant taste, fermentation smell, harshness and acetic acid smell generated in the course of lactic acid fermentation or acetic acid fermentation. The examples of the Japanese patent application describe the preparation of fermented drink yogurts from a soybean milk to which isomaltulose and sucrose are added prior and/or after fermentation. In the examples a starter culture is used that comprises *Lactobacillus delbrueckii* subsp. *Bulgaricus* and *Streptococcus thermophilus*. The drink yogurts disclosed in the Japanese application are extremely sweet as they contain more than 8 wt.% of added disaccharide (isomaltulose and/or sucrose).

Murti TW et al., Journal of Food Science (1993), 58(1), pages 153-157 describe experiments in which soymilk and cow milk were inoculated with *Streptococci*, *Lactobacilli* in the absence or presence of *Bifidobacteria* and wherein the concentration of a number of volatile compounds was monitored during fermentation. The results show that during fermentation of soy milk at 42 °C the concentration of n-hexanal was decreased from 2.31 ppm to 0.55 ppm (no *Bifidobacteria*) or 0.45 ppm (with *Bifidobacteria*) after 4 hours. The lactic acid content after 4 hours of fermentation was at least 0.4 wt.%.

EP 1145648 discloses that low cost soy proteinaceous food ingredients that have reduced levels of those oligosaccharides that lead to abdominal discomfort. Such can be achieved by fermenting soy flour with a dairy culture comprising *Lactobacillus helveticus* and *Lactobacillus casei*, or a dairy culture comprising *Lactobacillus casei* and *Streptococcus cremoris*.

There is a desire for a soy protein-containing food product which is processed such that it has a reduced amount of undesired flavour components (compared to products not so processed), and preferably such process should be able to deliver both products (depending on the conditions) that can have a dairy-like flavour character or a more bland flavour character. Such more bland character would allow processing into various other products. Preferably, such should be achievable with a limited number of different ingredients, to allow for convenient processing. So the aim is that the taste profile can be steered towards a desired one, be it bland or dairy or something in between.

Summary of the Invention

It has now been found that the above objective can be achieved, at least in part, by a process for modifying the flavour of a soy protein containing substrate, said method comprising the steps of:

- providing a pasteurised or sterilised aqueous liquid comprising 0.5-15% by weight of dissolved soy protein and at least 0.1% by weight of carbohydrates, said soy protein being derived from soybeans that have not been dehypocotylized,
- inoculating said soy protein-containing liquid with bacteria selected from mesophilic lactic acid bacteria (LAB) selected from the group consisting of *Lactococcus lactis* (incl. subspecies *lactis*, *cremoris*, and biovar *diacetylactis*),

- Lactobacillus brevis*, *Lactobacillus fermentum*, *Lactobacillus sake*, *Lactobacillus sanfranciscensis*, *Leuconostoc pseudomesenteroides*, *L. acidophilus*, *L. reuteri*, *L. rhamnosus*, in an amount of between 10^5 to 10^9 CfU / ml substrate, and inoculating said soy-protein-containing liquid with thermophilic lactic acid bacteria (LAB) in an amount of between 10^5 to 10^9 CfU / ml substrate,
- 5 - fermenting the inoculated aqueous liquid by incubation at a temperature of 20 to 45 °C for 0.5-24 hours,

and wherein:

- 10 ○ inoculation with said mesophilic LAB and thermophilic LAB is done simultaneously, or
- inoculation with thermophilic LAB is carried out first and subsequently the inoculation with said mesophilic LAB is carried out in a period of less than 1 hour between both inoculations or
- 15 ○ inoculation with said mesophilic LAB is carried out first and subsequently the inoculation with thermophilic LAB is carried out in a period of less than 0.4 hours between both inoculations,

wherein the bacteria of said mesophilic LAB and the bacteria of the thermophilic LAB are inoculated in a ratio CfU of mesophilic LAB to thermophilic LAB of between 10:1 and 1:100, preferably 1:1 to 1:40.

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Detailed Description of the Invention

The present method employs soy protein derived from soybeans that have not been dehypocotylized (as such avoids additional process steps). Accordingly, the aforementioned soy isolate, soy concentrate and/or soy flour are derived from

25 optionally dehulled soybeans that still comprise the hypocotyl. Most preferably the latter sources of soy protein sources are derived from dehulled soybeans that still comprise the hypocotyls. The term "dehypocotylized soybeans" as used herein refers to soybeans from which the hypocotyl has been removed. Hypocotyl is a botanical term for a part of a germinating seedling of a seed plant. As the plant embryo grows at

30 germination, it sends out a shoot called a radicle that becomes the primary root and penetrates down into the soil. After emergence of the radicle, the hypocotyl emerges and lifts the growing tip above the ground, bearing the embryonic leaves (called cotyledons) and the plumule that gives rise to the first true leaves. The hypocotyl is the primary organ of extension of the young plant and develops into the stem.

Unlike the process taught by US 6,599,543, the present method does not require that the soy protein in the substrate is derived from soybeans from which the hypocotyl has been removed. This is a distinct advantage as it allows sources of soy bean that may be perceived as more natural than dehypocotylized soybeans, and also it may lead to higher levels of certain beneficial components in the soy protein material, such as e.g. isoflavones.

Fermentation of soy protein containing substrates with thermophilic lactic acid bacteria may be known to provide beneficial flavour components to such soy protein preparations, which, when untreated, suffer from off-flavours. The presence of such positive "dairy" and "yoghurt" notes may mask soy off flavours to some extent, but there is a desire for improvement, also as such thermophilic bacteria have a limited ability to reduce the level of components which are present which are responsible to part of the off-flavour. Surprisingly, it was found that undesired soy off notes are more efficiently removed when a mesophilic culture is added to a thermophilic culture mix.

Additionally, it was surprisingly found that, if one ensures that sufficient thermophilic lactic acid bacteria are present, the positive effects of the thermophilic lactic acid bacteria are not completely suppressed by the mesophilic lactic acid bacteria, as mesophilic lactic acid bacteria are also known to use as part of their metabolism some of the products produced by the thermophilic lactic acid bacteria. It was also surprisingly found that depending on the reaction conditions (e.g. fermentation time) one could produce a product having a more dairy character in taste, or a more bland character, and for both of which the level of off-flavour components is reduced. This brings the benefit of being able to produce a variety of products with different taste profiles, whilst only two different lactic acid bacteria strains are used, which is definitely an advantage from a processing point of view, as it gives flexibility in manufacturing with a limited number of bacteria strains which one needs to keep.

In the process according to the present invention the mesophilic LAB is selected from the group consisting of *Lactococcus lactis* (incl. subspecies *lactis*, *cremoris*, and biovar *diacetylactis*), *Lactobacillus brevis*, *Lactobacillus fermentum*, *Lactobacillus sake*, *Lactobacillus sanfranciscensis*, *Leuconostoc pseudomesenteroides*, *L. acidophilus*, *L. rhamnosus*, *L. reuteri*. These were found to give a reduction on one or more of the off-flavour components, whilst they still could be combined with thermophilic lactic acid

bacteria that generate positive flavours, without too much of a detrimental effect on production of positive flavour components by these thermophilic components.

"Mesophilic" herein is to be understood as having a temperature optimum for growth of between 25 and 37°C.

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In the process of the invention, it was found that the combination works well when the thermophilic LAB is selected from the group consisting of *Streptococcus thermophilus*, *Lactobacillus delbruecki*, *Lactobacillus delbruecki* subsp *bulgaricus*, *Lactobacillus delbruecki* subsp *lactis*, *L. helveticus*. "Thermophilic" herein is to be understood as

10 cultures having a temperature optimum for growth of above 40°C and below 50°C, preferably 40-46°C, more preferably 41-45°C.

The term "lactic acid bacteria" as used herein refers to acid tolerant, non-sporulating, non-respiring rod-shaped Gram positive bacilli or cocci that produce lactic acid as the

15 major metabolic endproduct of carbohydrate fermentation. As used herein, the term "lactic acid bacterium" does not encompass *Bifidobacterium*.

In the process according to the present invention, the bacteria of the mesophilic LAB and the bacteria of the thermophilic LAB are inoculated in a ratio Cfu of mesophilic LAB

20 to thermophilic LAB of between 10:1 and 1:100, preferably 1:1 to 1:40, and more preferably between 1:1 and 1:20. The ratio is e.g. depending on the fermentation time and therefore the specific ratio can vary for short fermentation e.g. 3h (leading to a neutral pH product) and long fermentation e.g 8 and 24h (for acidic end product).

25 The actual fermentation itself is preferably carried out at a temperature of 25 to 40°C, as at such temperature both will grow and the desired flavour effects are achieved.

The duration of the fermentation herein typically lies in the range of 1-12 hours, more preferably of 2-10 hours. According to another preferred embodiment the fermentation

30 time does not exceed 14 hours, even more preferably it does not exceed 10 hours and most preferably it does not exceed 8 hours.

It is beneficial for the fermentation that at least some carbohydrates, preferably simple sugars such as monosaccharides and disaccharides, are present. Often such materials

35 will already be part of the soy protein preparation, or they may need to be added as

- such. In this connection, it is preferred that upon the start of the fermentation the substrate which is inoculated comprises at least 0.1% by weight of carbohydrates, and preferably comprises between 0.1% and 10%, and more preferably between 0.2% and 5% by weight of carbohydrates. Preferred carbohydrates are in this connection
- 5 glucose, fructose, galactose, sucrose, raffinose, stachyose, lactose and combinations thereof. However, as the present method enables the preparation of a pleasant tasting beverage without using high amounts of sweetener to mask soy off-flavour notes. Thus, advantageously less than 6 wt.% of disaccharides are added before, during or after fermentation. According to a particularly preferred embodiment, the fermented
- 10 product in the sealed container contains less than 5 wt.% of disaccharides, most preferably less than 4 wt.% of disaccharides. According to yet another preferred embodiment, the packaged fermented product contains less than 8 wt.% of mono- and/or disaccharides, most preferably less than 5 wt.% of mono- and/or disaccharides.
- 15 As to the level of soy protein present in the substrate to be inoculated: it is believed that the process works well for wide ranges of soy protein concentration. Most commercial products for human consumption will contain between 1 and 5% protein, but the process may well be carried out at lower or higher protein concentrations. In the latter case, the fermentation may be done on a concentrated substrate, which is diluted in
- 20 one or other form prior to consumption. Such processes on concentrated soy protein may be more economical. In this connection, it is preferred that the liquid which is inoculated comprises soy protein in an amount of 0.1 to 10% by weight, and preferably such is from 0.2 to 5% by weight.
- 25 "Soy protein content", as used herein, refers to the total amount of soy protein and soy protein derived peptides contained in the fermented product. The fermented product may be based on soy protein, on soy protein hydrolysate or combinations thereof. As will be understood by the skilled person (enzymatic) hydrolysis of peptide bonds may occur during fermentation.
- 30 The substrate that is fermented in the present method, i.e. the aqueous liquid containing 0.5-15 wt.% of dissolved soy protein is preferably prepared from a soy protein source selected from the group consisting of soy isolate, soy concentrate, soy flour and combinations thereof. According to a particularly preferred embodiment, the latter soy protein sources were obtained from soy beans exhibiting very low
- 35 lipoxigenase activity, e.g. a lipoxigenase activity of less than 15 kU/mg. Even more

preferably the soy protein source is derived from soy beans exhibiting a lipoxygenase activity of less than 10 kU/mg, most preferably of less than 8 kU/mg. Likewise, it is advantageous to prepare the present aqueous liquid from a soy protein source having a lipoxygenase activity of less than 5 kU per gram of soy protein. Most preferably the soy protein source has a lipoxygenase activity of less than 1 kU per gram of soy protein.

Lipoxygenase activity is suitably determined spectrophotometrically by using a dye-coupling assay specific for hydroperoxides generated from linoleic acid, as fully described by Anthon and Barrett (*J. Agric. Food Chem.* **2001**, 49, 32-37).

The present method employs soy protein derived from soybeans that have not been dehypocotylized (as such avoids additional process steps). Accordingly, the aforementioned soy isolate, soy concentrate and/or soy flour are derived from optionally dehulled soybeans that still comprise the hypocotyl. Most preferably the latter sources of soy protein sources are derived from dehulled soybeans that still comprise the hypocotyls. The term "dehypocotylized soybeans" as used herein refers to soybeans from which the hypocotyl has been removed. Hypocotyl is a botanical term for a part of a germinating seedling of a seed plant. As the plant embryo grows at germination, it sends out a shoot called a radicle that becomes the primary root and penetrates down into the soil. After emergence of the radicle, the hypocotyl emerges and lifts the growing tip above the ground, bearing the embryonic leaves (called cotyledons) and the plumule that gives rise to the first true leaves. The hypocotyl is the primary organ of extension of the young plant and develops into the stem.

The present method is advantageously employed to produce a fermented aqueous liquid that can be used as a base for the production of a beverage. In case the product so produced is a beverage, it has a relatively low viscosity, e.g. a viscosity at a temperature of 7 °C of less than 50 mPa.s at 100 s⁻¹, most preferably of less than 25 mPa.s at 100 s⁻¹. A viscosity of 50 mPa.s at 100 s⁻¹ means that the product is 50 times more viscous than water and about 25 times more viscous than milk. Viscosity can suitably be measured with the help of a rheometer AR1000 (TA Instruments, Etten-Leur, the Netherlands), using a 40-mm diameter, 2% angle cone measuring system. A steady-state shear process should be used, increasing shear rate from 0.01 to 250/s. The measuring temperature is 7 °C and only the data point at 100 s⁻¹ is used.

C₅-C₉ n-alkanals and (*E*)-2-hexenal are flavour molecules that are believed to be largely responsible for the 'beany' off-flavour often found in soy based products. The inventors have discovered that it is possible to remove typical soy-related off-notes in a very effective way using the process as set out herein.

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Components such as diacetyl and acetaldehyde, when present, may impart a more dairy character of the soy protein containing product. So a fermentation which produces one or preferably both of these compounds, along removal with above "beany" off-flavour molecules, can impart an improved flavour profile with a dairy

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character, whereas a fermentation which does not or only in a low amount produces one or preferably both of diacetyl and acetaldehyde, along removal with above "beany" off-flavour molecules, can impart an improved flavour profile with a more bland character. In this way, the flavour profile can be improved but also steered towards a more bland or a more dairy character.

15

By using the process according to the present invention, one can achieve during fermentation the following changes in concentrations of flavour compounds:

- the concentration of n-hexanal decreases by at least 40%, preferably by at least 50%;
- at least two of n-pentanal concentration, n-heptanal concentration, and n-nonanal concentration decrease by at least 30%

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when compared to such levels in the soy protein material prior to fermentation according to this invention. Likewise, during fermentation (*E*)-2-hexenal concentration decreases by at least 20%, preferably by at least 30%. It was found that by achieving such reductions, the products were perceived as having much lower levels of off tastes which are usually associated with soy protein material.

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The process according to the present invention preferably further comprises a pasteurising or sterilising step of the so-fermented aqueous composition, depending upon the further desired use. Also, the fermented product obtained by the current process is preferably filled into containers, which containers are subsequently sealed. Optionally, an edible acid is added to the fermented product prior to the filling into the containers so as to adjust the pH to less than 4.5 if an acid product is desired, and wherein the fermented product is not subjected to pasteurisation or sterilisation prior, during or after the filling into the containers.

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If so desired, an edible acid is added to the fermented product before the fermentation has reached the point at which product inhibition prevents further production of lactic acid by lactic acid bacteria.

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The process according to the present invention is especially suited to prepare a beverage comprising soy protein, but the process may also be employed to prepare set products, such as set yoghurts.

10 The invention is further illustrated by means of the following non-limiting examples:

EXAMPLES

Analytical methods

15 *Analysis of off-flavour volatiles and diacetyl by Solid Phase Micro Extraction (SPME) followed by GC-MS*

2 g of sample was put in a 20 ml headspace vial and sealed with an airtight cap.

Samples were analyzed by means of solid phase micro extraction. Fiber used:

Carboxen/PDMS 85 µm ex. Supelco. Analyses were carried out on an Agilent GC/MS

20 (MS: LECO Pegasus IV TOF; 15 scans/s; m/z: 29-250 at 1700 V), equipped with a Gerstel CIS-4 injector and a Gerstel MPS-2 autosampler with SPME option. Column: VF-5; 50m * 0.2 mm * 0.33 µm.

GC program:

- 25
- 40°C(2 min) –(3°/min)->160°C(0 min)-(20°/min)->250°C (2 min)
 - Gas: Helium
 - Flow: 1 ml/min, constant flow

SPME Sampling time: 35 min at 40°C

30 Desorption: 40 minutes at 170°C

Split-less time: 2 min.

Quantitative analysis of acetaldehyde

1g of sample was put in a closed 150 ml glass headspace vial. All samples were analyzed in triplicate. External calibration levels were constructed by adding

acetaldehyde to 1 g of soy base prepared according to example 1, at levels of 0, 0.2, 0.5, 2 and 5 ppm.

Calibration lines were constructed by plotting the peak areas of ion 45 (for acetaldehyde) against the amount added. These calibration lines were then used to determine the amount of acetaldehyde in the fermented soy samples.

Analyses were carried out on the Ionicon Analytik PTR-MS (Proton Transfer Reaction-Mass Spectrometry).

After 1 hour equilibrium time, the headspace was drawn at 33 ml.min⁻¹ into the heated transfer line, of which 20 ml.min⁻¹ entered the ionisation source, for 0.5 minutes. The drift pressure (2.2mbar), voltage (600V) and temperature (70°C) were set to operate the ionisation condition at E/N of 140Td.

The ion mass and dwell time to monitor the acetaldehyde over the samples were:

- Ion monitored on PTR-MS: 45
- Dwell time: 500 ms

Sensory analysis

The fermented products were evaluated/smelled by an expert tasting panel (n=10), with a ranking test on soy and yoghurt intensity.

Lipoxygenase activity

Lipoxygenase activity was determined spectrophotometrically by using a dye-coupling assay specific for hydroperoxides generated from linoleic acid, as fully described by Anthon and Barrett (*J. Agric. Food Chem.* **2001**, 49, 32-37).

Enzyme extracts were obtained by homogenising 100 mg of defatted soybean grindings in 20 ml of 100 mM pH 6.0 Na₂HPO₄ buffer + 1% w/v NaCl then centrifuging at 15,000 rpm at 4°C for 30 minutes and filtering the supernatant through a 0.2 µm filter.

To 500 µl of a solution of 10 mM 3-(dimethylamino)benzoic acid in 100 mM pH 6.0 Na₂HPO₄ was added 20 µl of 27 mM linoleic acid dispersed in 1.4% w/v Tween 20, and 10 µl of enzyme extract. After 5 minutes, 500 µl of a solution containing 0.2 mM 3-methyl-2-benzothiazolinone hydrazone and 0.1 mg/ml bovine haemoglobin was added. After a further 5 minutes, 500 µl of 1% w/v sodium lauryl sulphate was added to terminate the reaction. The absorbance at 598 nm was then measured. The above actions can be carried out in a single 4.5 ml 1 cm path cuvette.

A standard curve was produced by reacting dilutions of soybean lipoxygenase from Sigma-Aldrich, of certificated activity. This was used to calculate the activities of the extracts from the soybeans. Blank readings were subtracted, obtained using denatured enzyme extracts heated at 95°C for 30 minutes. One unit of activity is the increase of
5 0.001 absorbance units at 598 nm per minute from the hydroperoxidation of linoleic acid.

Viable counts

The number of live bacteria in the cultures was determined by plate counting
10 appropriate dilutions of samples containing *Lactobacillus brevis*, *Lactobacillus sanfranciscensis* on MRS agar and anaerobic incubation for 3 days at 30°C. *Lactococcus lactis* (FD-0013) and *Streptococcus thermophilus* (YF-L01) mixed cultures were enumerated using M17 agar and aerobic incubation for 3 days at 30°C (FD-0013) or 37°C (YF-L01). Numbers are expressed as Colony forming units per ml of product
15 (Cfu/ml).

Strains

Lactobacillus brevis Lb20 (CBS122084, deposited under the Budapest treaty at Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands)
20 *Streptococcus thermophilus* YF-L01 DF (commercial culture of available under this name from Chr Hansen, Denmark)
Lactobacillus sanfranciscensis ATCC27651 (freely available from ATCC)
complex *Lactococcus lactis* culture FD-0013 (commercially available under code FD-0013 from Chr Hansen, Denmark)
25 *Lactobacillus delbrueckii* subsp. *lactis* Lb05-14 (CBS109270, deposited under the Budapest treaty at Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands)

Example 1

Combinations of *Streptococcus thermophilus* and *Lactobacillus brevis*

30 A: raw material

A soy base was prepared by dissolving 5.5% Sunopta SSFR powder (resulting in a protein concentration of 2.5% protein) and 0.5% sucrose in water. The mix was preserved by a heat treatment for 12 seconds at 120°C, packed aseptically and cooled for further storage at 5°C.

B: fermentation

A preculture of *Lactobacillus brevis* Lb20 (CBS122084, deposited at Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands), was prepared by overnight cultivation in MRS broth at 30°C. A two-fold concentrated culture of this was obtained by washing and re-suspending the cells in a Peptone Physiological salt solution. Soy base as prepared under A was heated to 30°C and inoculated with 1% of the concentrated cell preparation of *L. brevis* Lb20 so-obtained.

The so-obtained soy-protein preparation containing Lb20 was further inoculated with YF-L01 DF within 5 minutes and incubated at 30°C for up to 24 hours. YF-L01 DF (commercial culture of *Streptococcus thermophilus* available under this name from Chr Hansen, Denmark) was provided as frozen pellets and dosed at 0.02% in said soy base.

As a control, one sample of soy protein (same concentration as under A above) was inoculated with YF-L01 DF only and also incubated at 30°C for up to 24 hours.

After inoculation with YF-L01 DF only or with combination of YF-L01 DF and *L. brevis* Lb20 the pH of the soy base was measured at regular intervals. The viable count (Colony forming units per ml, Cfu/ml) of the cultures was measured after inoculation (t=0), after 3 and 8 hours by plating appropriate dilutions on MRS agar and incubation at 30°C for 2 days (for the quantification of Lactobacilli). Streptococci were quantified by plating samples on M17 agar containing 0.5% glucose and incubation at 37°C for 2 days. (Table 1.1 and 1.2).

Table 1.1 Viable counts of soy base fermented with *L. brevis* and YF-L01

Inoculation			Cfu/ml MRS			Cfu/ml M17		
culture 1 / MESOPH.		Culture 2 / THERMO.	0h	3h	8h	0h	3h	8h
<i>L.brevis</i>	&	YF-L01 DF	2.35E +07	3.75E +07	7.30E +07	1.97E +07	1.20E +08	1.67E +08
None	&	YF-L01 DF				1.97E +07	6.55E +07	7.80E +07

Table 1.2 Acidification of soy base fermented with *L. brevis* and YF-L01

Inoculation			pH			
culture 1 / MESOPHILE		Culture 2 / THERMO.	0h	3h	8h	24h
<i>L.brevis</i>	&	YF-L01 DF	6.6	5.79	4.9	4.57
None	&	YF-L01 DF	6.61	5.85	4.94	4.5

Soy base was inoculated with *L. brevis* at about 2×10^7 CfU/ml and *S. thermophilus* at about 2×10^7 CfU/ml. Therefore, the ratio of combined cultures was 1:1 for the combination of *L. brevis* and *S. thermophilus*.

The pH measurements show that YF-L01 alone acidified the soy base to pH 6.0 in less than 3h and products had a pH of about 4.5 after 24h at 30°C. Mixed cultures of *L. brevis* and YF-L01 achieved acidification to pH 6.0 within 3h and products had a pH of 4.6 after 24 h. Viable counts of the thermophilic culture (*S. thermophilus* on M17 agar) increased to a two-fold higher level in the combination when compared to the mixture. 300 ml portions of products harvested after 3, 8, or 24 h fermentation were pasteurised by incubation at 85°C for 30 minutes in a water bath and subsequently evaluated for volatiles and smell.

C: volatiles

An aliquot of the sample fermented with the combined culture of *L. brevis* Lb20 and YF-L01 and an aliquot of the sample fermented with YF-L01 only were subjected to SPME followed by GC-MS analysis. It was found that during fermentation with the combined culture the peak areas for pentanal, hexanal, heptanal, nonanal, (*E*)-2-hexenal, (*E*)-2-nonenal, (*E,E*)-2,4-Heptadienal, (*E,E*)-2,4-decadienal and 2-Heptanone, which all are identified as contributing to off-flavours, had decreased considerably as shown in

Table 1.3.

Table 1.3 Soy off-flavours in soy products fermented with *L. brevis* and YF-L01

	Decrease in peak area (%)after 3h compared to fermentation with YF-L01 only	Decrease in peak area (%)after 8h compared to fermentation with YF-L01 only	Decrease in peak area (%)after 24h compared to fermentation with YF-L01 only
(<i>E,E</i>)-2,4-Decadienal	6%	29%	63%
(<i>E,E</i>)-2,4-Heptadienal,	3%	43%	70%
(<i>E</i>)-2-Hexenal	45%	44%	30%
(<i>E</i>)2-Nonenal	31%	41%	48%
Heptanal	15%	29%	10%
2-Heptanone	61%	69%	73%
Hexanal	16%	15%	1%
Nonanal	15%	39%	47%
pentanal	29%	40%	47%

- 5 The analyses further showed that during fermentation with a combination of *L. brevis* Lb20 and YF-L01 the concentration of diacetyl and acetaldehyde (quantified by PTR-MS) is changed as shown in table 1.4.

Table 1.4 Dairy flavours in soy products fermented with *L. brevis* and YF-L01

	Decrease in peak area (%) or ppm compared to fermentation with YF-L01 only, after 3h fermentation	Decrease in peak area (%) or ppm compared to fermentation with YF-L01 only, after 8h fermentation	Decrease in peak area (%) or ppm compared to fermentation with YF-L01 only, after 24h fermentation
Diacetyl	6%	29%	76%
Acetaldehyde	0.5 ppm	1.5 ppm	1.7 ppm

Thus, the volatile analysis shows that combined fermentation for a period of 3h of a soy protein containing a preparation with *L.brevis* Lb20 (a mesophilic culture) and *S. thermophilus* YF-L01 DF (a thermophilic culture) result in a lower amount of those compounds contributing to soy off-flavours and a similar amount of components contributing to a positive flavour impression than by fermentation with a thermophilic organism alone. In contrast prolonged fermentation for 8 and 24 hours with a mixture of mesophilic and thermophilic culture leads to reduction in off-flavour compounds but also to reduced levels of positive aroma compounds.

By fermentation with a thermophile microorganism only, a fairly strong dairy character is obtained, due to production of e.g. diacetyl and acetaldehyde. Using jointly a mesophilic and thermophilic micro organism in the fermentation (according to the invention, when used e.g. in the ratio as in example 1) gives lower amounts of diacetyl and acetaldehyde than with a thermophile microorganism alone, but also gives removal of off flavours. After short fermentation times (e.g. 3 hours) the dairy character is quite prominent due to higher levels of diacetyl and acetaldehyde, whereas after 8 hours the taste character is less intense dairy. By adjusting e.g. the ratio between the meso and thermophilic microorganisms and the fermentation time, the taste profile can be steered towards a desired one, be it bland or dairy or something in between.

D: sensory

A higher intensity of yoghurt odour was observed for the soybase inoculated with the combination of cultures than the one inoculated with the *S. thermophilus* only, after 3 hours fermentation time. After 8 hours fermentation, the soybase inoculated with a combination of cultures seemed to be perceived as less intense in yoghurt odour than the one inoculated with the *S. thermophilus* only. The sensory description is in line with the volatile data described above.

Example 2

Combinations of *Streptococcus thermophilus* and *Lactobacillus sanfranciscensis*

A: raw material

A soy base was prepared by dissolving 5.5% Sunopta SSFR powder (resulting in a protein concentration of 2.5% protein) and 0.5% sucrose in water. The mix was

preserved by a heat treatment for 12 seconds at 120°C, packed aseptically and cooled for further storage at 5°C.

B: fermentation

- 5 A preculture of *Lactobacillus sanfranciscensis* ATCC27651 (freely available) was prepared by overnight cultivation in MRS broth at 30°C. Cells were washed in a Peptone Physiological salt solution and concentrated twofold.

Soy base as prepared under A was heated to 30°C and inoculated with 1% of the concentrated cell preparation of *L. sanfranciscensis*.

10

The so-obtained soy-protein preparation containing *L. sanfranciscensis* was further inoculated with YF-L01 DF within 5 minutes and incubated at 30°C for up to 24 hours. YF-L01 DF (*Streptococcus thermophilus* culture from Chr Hansen, Denmark) was provided as frozen pellets and dosed at 0.02% in said soy base.

15

As a control, one sample of soy protein (same concentration as under A above) was inoculated with YF-L01 DF only and also incubated at 30°C for up to 24 hours.

20

After inoculation with single cultures or with combinations of both cultures the pH of the soy base was measured at regular intervals. The viable count (Colony forming units per ml, Cfu/ml) of the cultures was determined after inoculation (t=0), after 3 and 8 hours by plating appropriate dilutions on MRS agar and incubation at 30°C for 2 days (for the quantification of Lactobacilli). Streptococci were quantified by plating samples on M17 agar containing 0.5% glucose and incubation at 37°C for 2 days (Tables 2.1 and 2.2).

25

Table 2.1 Viable counts of soy base fermented with *L. sanfranciscensis* and YF-L01

Inoculation			Cfu/ml MRS			Cfu/ml M17		
culture 1 / MESOPHILE		Culture 2 / THERMO.	0h	3h	8h	0h	3h	8h
None	&	YF-L01 DF				1.97E +07	6.55E +07	7.80E +07
<i>L.sanfranciscensis</i>	&	YF-L01 DF	1.02E +07	3.70E +08	1.33E +09	1.97E +07	5.15E +08	1.51E +09

Table 2.2 Acidification of soy base fermented with *L. sanfranciscensis* and YF-L01

Inoculation			pH			
culture 1 / MESOPHILE		Culture 2 / THERMO.	0h	3h	8h	24h
None	&	YF-L01 DF	6.61	5.85	4.94	4.5
<i>L.sanfranciscensis</i>	&	YF-L01 DF	6.53	5.6	4.73	4.45

Soy base was inoculated with cultures at about 1×10^7 CfU/ml for *L. sanfranciscensis* and about 2×10^7 CfU/ml for *S. thermophilus*, resulting in a 1:2 ratio in the combination of *L. sanfranciscensis* and *S. thermophilus*.

The pH measurements show that YF-L01 alone acidified the soy base to pH 6.0 in less than 3h and products had a pH of about 4.5 after 24h at 30°C. Also mixed mesophilic and thermophilic cultures achieved acidification to pH 6.0 within 3h and products had a pH of about 4.5 in 24 h. Viable counts of the thermophilic culture (*S. thermophilus* on M17 agar) increased about fourfold in 8 h. In the combination with *L. sanfranciscensis* an increase of about 75 fold of *S. thermophilus* was found after 24h.

300 ml portions of products harvested after 3, 8, or 24 h fermentation were pasteurised by incubation at 85°C for 30 minutes in a water bath and subsequently evaluated for volatiles and smell.

C: volatiles

An aliquot of the sample fermented with the combined culture of *L. sanfranciscensis* and YF-L01 and an aliquot of the sample fermented with YF-L01 only were subjected to SPME followed by GC-MS analysis. It was found that during fermentation with the combined culture the peak areas for pentanal, hexanal, heptanal, (*E*)-2-hexenal, (*E*)-2-nonenal, (*E,E*)-2,4-Heptadienal, (*E,E*)-2,4-decadienal and 2-Heptanone, which all are identified as contributing to off-flavours, had decreased considerably as shown in Table 2.3.

Table 2.3 Soy off-flavours in soy products fermented with *L. sanfranciscensis* and YF-L01

	Decrease in peak area (%) after 8h compared to fermentation with YF-L01 only	Decrease in peak area (%) after 24h compared to fermentation with YF-L01 only
(<i>E,E</i>)-2,4-Decadienal	57%	38%
(<i>E,E</i>)-2,4-Heptadienal	49%	55%
(<i>E</i>)-2-Hexenal	37%	31%
(<i>E</i>)-2-Nonenal	6%	41%
Heptanal	13%	8%
2-Heptanone	29%	66%
Hexanal	16%	21%
Nonanal	0%	42%
Pentanal	24%	38%

5 The analyses further showed that during fermentation with a combination of *L. sanfranciscensis* and YF-L01 the concentration of diacetyl and acetaldehyde (quantified by PTR-MS) is changed as shown in table 2.4.

Table 2.4 Dairy flavours in soy products fermented with *L. sanfranciscensis* and YF-L01

	Decrease in peak area (%) or ppm compared to fermentation with YF-L01 only, after 8h ferm.	Decrease in peak area (%) or ppm compared to fermentation with YF-L01 only, after 24h fermentation
Diacetyl	33%	96%
Acetaldehyde	1.5 ppm	1.7 ppm

10

Thus the volatile analysis shows that combined fermentation for a period of 8 and 24 hours of a soy protein containing a preparation with *L. sanfranciscensis* (a mesophilic culture) and *S. thermophilus* YF-L01 DF (a thermophilic culture) result in a lower

amount of those compounds contributing to soy off-flavours but also to reduced levels of positive aroma compounds.

D: sensory

- 5 After 8 hours fermentation, the soybase inoculated with a combination of cultures seemed to be perceived as less intense in yoghurt odour than the one inoculated with the *S. thermophilus* only. The sensory description is in line with the volatile data described above.

10 **Example 3**

Combinations of *Streptococcus thermophilus* and *Lactococcus lactis*

A: raw material

- A soy base was prepared by dissolving 5.5% Sunopta SSFR powder (resulting in a protein concentration of 2.5% protein) and 0.5% sucrose in water. The mix was
15 preserved by a heat treatment for 12 seconds at 120°C, packed aseptically and cooled for further storage at 5°C.

B: fermentation

- YF-L01 DF (*Streptococcus thermophilus* culture from Chr Hansen, Denmark) and FD-
20 0013 (complex *Lactococcus lactis* culture, commercially available from Chr Hansen, Denmark) were provided as frozen pellets and dosed as such at 0.02% (weight) in a soy base as prepared under A (which had been heated to 30°C), within 5 minutes of each other and was incubated at 30°C for up to 24 hours.

- 25 As a control, one sample of soy protein (same concentration as under A above) was inoculated with YF-L01 DF only and was also incubated at 30°C for up to 24 hours.

- After inoculation with a single culture or with combinations of cultures the pH of the soy base was measured at regular intervals. The viable count (Colony forming units per ml, Cfu/ml) of the cultures was determined after inoculation (t=0) by plating appropriate
30 dilutions on M17 agar containing 0.5% glucose and incubation at 37°C for 2 days for the quantification of Lactococci and/or Streptococci. (Tables 3.1 and 3.2).

Table 3.1 Viable counts of soy base fermented with *L. lactis* and YF-L01

Inoculation			Cfu/ml M17
culture 1 / MESOPHILE		Culture 2 / THERMO.	0h
None	&	YF-L01 DF	1.97E+07
FD-0013	&	YF-L01 DF	3.10E+07

Table 3.2 Acidification of soy base fermented with *L. lactis* and YF-L01

Inoculation			pH			
culture 1 / MESOPHILE		Culture 2 / THERMO.	0h	3h	8h	24h
None	&	YF-L01 DF	6.61	5.85	4.94	4.5
FD-0013	&	YF-L01 DF	6.56	5.66	4.84	4.47

- 5 Soy base was inoculated with cultures at about 1×10^7 Cfu/ml for *L. lactis* and about 2×10^7 Cfu/ml for *S. thermophilus*, resulting in a ratio of approximately 1:2 for the combined fermentation.

The pH measurements show that the thermophilic culture alone acidified the soy base to pH 6.0 in less than 3h and products had a pH of about 4.5 after 24h at 30°C. Also

- 10 mixed mesophilic and thermophilic cultures achieved acidification to pH 6.0 within 3h and products had a pH of about 4.5 in 24 h.

300 ml portions of products harvested after 3, 8, or 24 h fermentation were pasteurised by incubation at 85°C for 30 minutes in a water bath and subsequently evaluated for volatiles and smell.

15

C: volatiles

An aliquot of the sample fermented with the combined culture of *L. lactis* and YF-L01 and an aliquot of the sample fermented with YF-L01 only were subjected to SPME followed by GC-MS analysis. It was found that during fermentation with the combined

20 culture the peak areas for pentanal, hexanal, heptanal, (*E*)-2-hexenal, (*E*)-2-nonenal, (*E,E*)-2,4-Heptadienal, (*E,E*)-2,4-decadienal and 2-Heptanone, which all are identified as contributing to off-flavours, had decreased considerably as shown in Table 3.3.

Table 3.3 Soy off-flavours in soy products fermented with *L. lactis* and YF-L01

	Changes* in peak area (%) after 3h compared to fermentation with YF-L01 only	Changes* in peak area (%) after 8h compared to fermentation with YF-L01 only	Changes* in peak area (%) after 24h compared to fermentation with YF-L01 only
(<i>E,E</i>)-2,4-Decadienal	39%	-44%	-23%
(<i>E,E</i>)-2,4-Heptadienal	41%	-33%	-26%
(<i>E</i>)-2-Hexenal	-20%	-28%	-22%
(<i>E</i>)-2-Nonenal	-6%	-16%	0%
Heptanal	6%	-59%	-46%
2-Heptanone	6%	-10%	-2%
Hexanal	-7%	-42%	-50%
Nonanal	-17%	17%	-65%
Pentanal	5%	-45%	-24%

* negative number denote decrease, positive numbers increase in peak area

- The analyses further showed that during fermentation with a combination of *L. lactis* and YF-L01 the concentration of diacetyl and acetaldehyde (quantified by PTR-MS) is changed as shown in table 3.4.

Table 3.4 Dairy flavours in soy products fermented with *L. lactis* and YF-L01

	Changes* in peak area (%) or ppm compared to fermentation with YF-L01 only, after 3h fermentation	Changes* in peak area (%) or ppm compared to fermentation with YF-L01 only, after 8h fermentation	Changes* in peak area (%) or ppm compared to fermentation with YF-L01 only, after 24h fermentation
Diacetyl	52%	10%	-28%
Acetaldehyde	0.5 ppm	0.4 ppm	0.5 ppm

* negative number denote decrease, positive numbers increase in peak area

Thus the volatile analysis shows that combined fermentation for a period of 3h and 8h of a soy protein containing a preparation with *L. lactis* (a mesophilic culture) and *S. thermophilus* YF-L01 DF (a thermophilic culture) result in a lower amount of many of soy off-flavours and a similar or even increased amount of components contributing to a positive dairy flavour impression than by fermentation with the thermophilic organism alone. In contrast prolonged fermentation for 24 hours with a mixture of mesophilic and thermophilic culture leads to reduction in off-flavour compounds but also to reduced levels of positive aroma compounds.

D: sensory

A higher intensity of yoghurt odour was observed for the soybase inoculated with the combination of cultures than the one inoculated with the *S. thermophilus* only, after 3 hours and 8 hours fermentation time. The sensory description is in line with the volatile data described above.

Example 4

Combinations of *Lactobacillus delbrueckii* and *Lactobacillus brevis*

A: raw material

A soy base was prepared by dissolving 5.5% Sunopta SSFR powder (resulting in a protein concentration of 2.5% protein) and 0.5% sucrose in water. The mix was preserved by a heat treatment for 12 seconds at 120°C, packed aseptically and cooled for further storage at 5°C.

B: fermentation

A preculture of *Lactobacillus brevis* Lb20 (CBS122084, deposited under the Budapest treaty at Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands), was prepared by overnight cultivation in MRS broth at 30°C. A two-fold concentrated culture of this was obtained by washing and re-suspending the cells in water and the OD₆₀₀ of this was measured. A cell suspension of 8-fold higher OD₆₀₀ was obtained by concentrating the cells by centrifugation and resuspension in a smaller volume.

A preculture of *Lactobacillus delbrueckii* subsp. *lactis* Lb05-14 (CBS109270, deposited under the Budapest treaty at Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands), was prepared by overnight cultivation in MRS broth at 37°C. Cells were

concentrated or diluted to the same OD₆₀₀ of *Lactobacillus brevis* Lb20 with water. A cell suspension with a 50-fold higher OD₆₀₀ was obtained by centrifugation of the cells and resuspending in a smaller volume.

Soy base as prepared under A was inoculated with 1% of the concentrated cell preparation of *L. brevis* Lb20 so-obtained. The so-obtained soy-protein preparation containing Lb20 was further inoculated with 1% of the concentrated cell preparation of Lb05-14 within 5 minutes.

Similarly, a combination of cultures was made by inoculating soy base with 1% of Lb20 of 8-fold OD₆₀₀ and 1% of Lb05-14. Another combination of cultures was made by inoculating soy base with 1% of Lb20 and 1% of Lb05-14 with 50-fold OD₆₀₀.

As a control, one sample of soy protein (same concentration as under A above) was inoculated with 2% of Lb20 only. A second control was prepared by inoculation with 2% of Lb05-14 only.

After inoculation with Lb20 only or with Lb05-14 only or with different combinations of *L. delbreuckii* Lb05-14 and *L. brevis* Lb20 the pH of the soy base was measured at regular intervals. The viable count (Colony forming units per ml, Cfu/ml) of the cultures was measured after inoculation (t=0), after 5 and 24 hours by plating appropriate dilutions on MRS agar and incubation at 45°C for 2 hours and subsequently at 43°C 3~4 days (for the quantification of *L. delbreuckii*). *L. brevis* was quantified by plating samples on MRS agar containing 1 mg/l Vancomycin and incubation at 30°C for 3~4 days. (Table 4.1 and 4.2).

Table 4.1 Viable counts of soy base fermented with *L. brevis* and *L. delbreuckii*

Inoculation			Cfu/ml MRS, 43°C			Cfu/ml MRS Vm, 30°C		
culture 1 / MESOPH.		Culture 2 / THERMOPH.	0h	5h	24h	0h	5h	24h
<i>L.brevis</i> 1	&	<i>L. delbrueckii</i> 1	2.09E+07	5.15E+07	7.80E+07	1.42E+07	9.30E+07	4.25E+08
<i>L.brevis</i> 8	&	<i>L. delbrueckii</i> 1	2.09E+07	5.50E+07	1.08E+08	1.07E+08	1.89E+08	6.10E+08
<i>L.brevis</i> 1	&	<i>L. delbrueckii</i> 50	4.19E+08	7.10E+08	6.50E+08	1.42E+07	2.70E+07	1.10E+08
<i>L.brevis</i> 2	&	None	0	0	0	2.84E+07	1.22E+08	1.58E+08
None	&	<i>L. delbrueckii</i> 2	4.17E+07	5.75E+07	5.60E+07	0	0	0

Table 4.2 Acidification of soy base fermented with *L. brevis* and *L. delbrueckii*

Inoculation			pH			
culture 1 / MESOPHILE		Culture 2 / THERMOPHILE.	0h	3h	6h	24h
<i>L.brevis</i> 1	&	<i>L. delbrueckii</i> 1	6.78	6.21	5.60	4.08
<i>L.brevis</i> 8	&	<i>L. delbrueckii</i> 1	6.78	5.81	5.40	4.10
<i>L.brevis</i> 1	&	<i>L. delbrueckii</i> 50	6.78	5.81	5.46	3.98
<i>L.brevis</i> 2	&	None	6.78	6.19	5.88	5.20
None	&	<i>L. delbrueckii</i> 2	6.78	6.28	6.02	5.30

5

Soy base was inoculated with *L. brevis* at about 1.4×10^7 CfU/ml and *L. delbrueckii* at about 2.1×10^7 CfU/ml in the first combination. Therefore, the ratio of combined cultures was 1:1.5 for the combination of *L. brevis* and *L. delbrueckii*. For the second combination soy base was inoculated with *L. brevis* at about 1.1×10^8 CfU/ml and *L. delbrueckii* at about 2.1×10^7 CfU/ml. Therefore, the ratio of combined cultures was 4.5:1. In the third combination soy base was inoculated with *L. brevis* at about 1.4×10^7 CfU/ml and *L. delbrueckii* at about 4.2×10^8 CfU/ml. Therefore, the ratio of combined cultures was 1:30.

10

The pH measurements show that Lb20 alone and Lb05-14 alone acidified the soy base to pH 6.0 in about 6h and products had a pH of about 5.7 after 24h at 30°C. Mixed cultures of Lb20 and Lb05-14 achieved acidification to pH 6.0 within 4h and products had a pH of about 4.0 after 24 h. Viable counts of the thermophilic culture (*L. delbrueckii* on MRS agar) increased slightly over 5hr in the single culture and increased to a 1.5 to 5-fold higher level over 24 hr in the mixed cultures. Viable counts of the mesophilic culture (*L. brevis* on MRS Vm agar) increased 5.5-fold over 24h in the single culture and increased 6 to 30 fold in the combined cultures. 500 ml portions of products harvested after 24 h fermentation were pasteurised by incubation at 85°C for 30 minutes in a water bath and subsequently evaluated for smell.

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25 C: sensory

A higher intensity of dairy / yoghurt odour was observed for the soybase inoculated with the combination of cultures than the ones inoculated with the *L. delbrueckii* only or

the *L.brevis* only, after 24 hours fermentation time, with a highest intensity for the 1:1 ratio. The soy odour character was significantly lower in inoculated samples with *L. delbrueckii* only, *L.brevis* only and the combination *L. delbrueckii* : *L.brevis* in a 1:1 ratio.

- 5 Optimal ratio in this selected range was thus the 1:1 ratio between *L. delbrueckii* and *L.brevis*, with a significantly reduced soy odour and generated yoghurt character.

PCT

0-1	Form PCT/RO/134 (SAFE) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)	
0-1-1	Prepared Using	PCT Online Filing Version 3.5.000.219 MT/FOP 20020701/0.20.5.9
0-2	International Application No.	
0-3	Applicant's or agent's file reference	F8074 (V) \GLW

1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	13
1-2	line	18-26
1-3	Identification of deposit	
1-3-1	Name of depositary institution	CBS Centraalbureau voor Schimmelcultures
1-3-2	Address of depositary institution	Uppsalalaan 8, NL-3584 CT Utrecht, The Netherlands / P.O. Box 85167, NL-3508 AD Utrecht, The Netherlands
1-3-3	Date of deposit	26 January 2001 (26.01.2001)
1-3-4	Accession Number	CBS 109270
1-5	Designated States for Which Indications are Made	All designations
2	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
2-1	page	13
2-2	line	18-26
2-3	Identification of deposit	
2-3-1	Name of depositary institution	CBS Centraalbureau voor Schimmelcultures
2-3-2	Address of depositary institution	Uppsalalaan 8, NL-3584 CT Utrecht, The Netherlands / P.O. Box 85167, NL-3508 AD Utrecht, The Netherlands
2-3-3	Date of deposit	22 November 2007 (22.11.2007)
2-3-4	Accession Number	CBS 122084
2-5	Designated States for Which Indications are Made	All designations

FOR RECEIVING OFFICE USE ONLY

0-4	This form was received with the international application: (yes or no)	Yes
0-4-1	Authorized officer	Krista Delimon

FOR INTERNATIONAL BUREAU USE ONLY

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0-5-1	Authorized officer	

Claims

1. A process for modifying the flavour of a soy protein containing substrate, said method comprising the steps of:
 - providing a pasteurised or sterilised aqueous liquid comprising 0.5-15% by weight of dissolved soy protein and at least 0.1% by weight of carbohydrates, said soy protein being derived from soybeans that have not been dehypocotylized,
 - inoculating said soy protein-containing liquid with bacteria selected from mesophilic lactic acid bacteria (LAB) selected from the group consisting of *Lactococcus lactis* (incl. subspecies *lactis*, *cremoris*, and biovar *diacetylactis*), *Lactobacillus brevis*, *Lactobacillus fermentum*, *Lactobacillus sake*, *Lactobacillus sanfranciscensis*, *Leuconostoc pseudomesenteroides*, *L. acidophilus*, *L. reuteri*, *L. rhamnosus*, in an amount of between 10^5 to 10^9 Cfu / ml substrate, and inoculating said soy-protein-containing liquid with thermophilic lactic acid bacteria (LAB) in an amount of between 10^5 to 10^9 Cfu / ml substrate,
 - fermenting the inoculated aqueous liquid by incubation at a temperature of 20 to 45 °C for 0.5-24 hours,
 - and wherein:
 - o inoculation with said mesophilic LAB and thermophilic LAB is done simultaneously, or
 - o inoculation with thermophilic LAB is carried out first and subsequently the inoculation with said mesophilic LAB is carried out in a period of less than 1 hour between both inoculations or
 - o inoculation with said mesophilic LAB is carried out first and subsequently the inoculation with thermophilic LAB is carried out in a period of less than 0.4 hours between both inoculations,
- wherein the bacteria of the mesophilic LAB and the bacteria of the thermophilic LAB are inoculated in a ratio Cfu of mesophilic LAB to thermophilic LAB of between 10:1 and 1:100, preferably 1:1 to 1:40.

2. Process according to claim 1, wherein the thermophilic LAB is selected from the group consisting of *Streptococcus thermophilus*, *Lactobacillus delbrueckii*, *Lactobacillus delbrueckii* subsp *bulgaricus*, *Lactobacillus delbrueckii* subsp *lactis*, *L. helveticus*.
3. Process according to claim 1 or 2, wherein the mesophilic cultures are cultures having a temperature optimum for growth of between 25 and 37°C.
4. Process according to any of the preceeding claims, wherein the thermophilic cultures are cultures having a temperature optimum for growth of above 40°C and below 50°C, preferably 40-46°C, more preferably 41-45°C.
5. Process according to any of the preceding claims, wherein the pasteurised or sterilised aqueous liquid which is inoculated comprises soy protein in an amount of between 1 and 10% by weight, preferably between 1 and 8% by weight.
6. Process according to any of the preceding claims, wherein the pasteurised or sterilised aqueous liquid which is inoculated comprises carbohydrates in an amount of 0.1 to 10% by weight, preferably 0.2 to 5% by weight.
7. Process according to any of the preceding claims, wherein the carbohydrates comprise mono- and/or di-saccharide.
8. Process according to any of the preceding claims, wherein in total less than 6% disaccharides by weight of the fermented product are added before, during or after fermentation.
9. Process according to any of the preceding claims, wherein the fermentation is carried out at a temperature of 25 to 40°C.

10. Process according to any of the preceding claims, wherein during fermentation the following changes in concentrations of flavour compounds occur:
 - concentration of n-hexanal decreases by at least 40%, preferably by at least 50%;
 - at least two of n-pentanal concentration, n-heptanal concentration and n-nonanal concentration decrease by at least 30%.
11. Process according to any of the preceding claims, wherein during fermentation (*E*)-2-hexenal concentration decreases by at least 20%, preferably by at least 30%.
12. Process according to any of the preceding claims, wherein the process comprises a further step comprising pasteurising or sterilising the so-fermented aqueous composition.
13. Process according to any one of the preceding claims, comprising filling the fermented product into containers and subsequently sealing the filled containers, wherein edible acid is added to the fermented product prior to the filling into the containers so as to adjust the pH to less than 4.5 and optionally the fermented product is not subjected to pasteurisation or sterilisation prior, during or after the filling into the containers.
14. Process according to claim 13, wherein the edible acid is added to the fermented product before the fermentation has reached the point at which product inhibition prevents further production of lactic acid by lactic acid bacteria.
15. Process according to any one of the preceding claims, wherein the aqueous liquid containing 0.5-15 wt.% of dissolved soy protein is prepared from a soy protein source selected from the group consisting of soy isolate, soy concentrate, soy flour and combinations thereof, said soy protein source being derived from soy beans exhibiting a lipoxxygenase activity of less than 15 kU/mg, more preferably of less than 10 kU/mg.

16. Process according to any of the preceding claims, wherein the substrate is a liquid, and the resulting product is a beverage.

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2010/056271

A. CLASSIFICATION OF SUBJECT MATTER

INV. A23J1/14 A23L1/015 A23L2/66
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A23J A23L

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EP0-Internal, BIOSIS, FSTA, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 599 543 B1 (YOKOYAMA HITOSHI [JP] ET AL) 29 July 2003 (2003-07-29) cited in the application	1-16
Y	column 6, lines 34-39 column 1, lines 58-63 column 2, lines 15-57 column 3, lines 1-32 examples 1-2	6-9, 16
Y	EP 0 386 817 A1 (UNILEVER NV [NL]; UNILEVER PLC [GB]) 12 September 1990 (1990-09-12) cited in the application page 2, lines 13-54 examples 1,3 ----- -/--	6-9, 16



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International application No

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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A,P	WO 2009/065722 A1 (UNILEVER NV [NL]; UNILEVER PLC [GB]; UNILEVER HINDUSTAN [IN]; BATENBUR) 28 May 2009 (2009-05-28) page 6, lines 8-31 page 8, lines 4-10 page 12, line 21 - page 13, line 5 -----	1-16
A,P	WO 2009/065724 A1 (UNILEVER NV [NL]; UNILEVER PLC [GB]; UNILEVER HINDUSTAN [IN]; BECKMANN) 28 May 2009 (2009-05-28) page 6, line 26 - page 7, line 31 page 11, lines 9-20 -----	1-16

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Information on patent family members

International application No

PCT/EP2010/056271

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