(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 June 2013 (27.06.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/093049 A2

(51) International Patent Classification:
A23C 19/032 (2006.01) *A23L 1/22* (2006.01)
A23C 19/064 (2006.01) *C12N 9/88* (2006.01)
A23L 1/29 (2006.01)

(21) International Application Number:
PCT/EP2012/076744

(22) International Filing Date:
21 December 2012 (21.12.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PA 2011 01005 23 December 2011 (23.12.2011) DK

(71) Applicant: **CHR. HANSEN A/S** [DK/DK]; Boege Alle 10-12, DK-2970 Hoersholm (DK).

(72) Inventors: **MOELLER, Kirsten Kastberg;** Soelvgade 13, 5. mf., DK-1307 Copenhagen K (DK). **RATTRAY, Fergal;** Kjaerstrupvej 31, st., DK-2500 Valby (DK).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available):* AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available):* ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

[Continued on next page]

(54) Title: METHOD FOR MAKING CHEESE

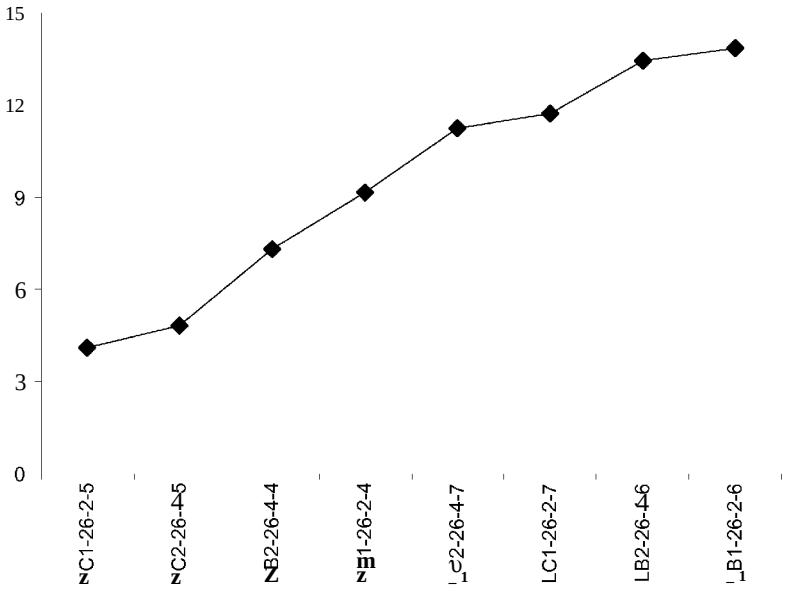

Fig 1

(57) Abstract: The present invention relates to a method for producing cheese with good flavor, especially low-salt cheese.

WO 2013/093049 A2

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description (Rules 13bis.4(d)(i) and 48.2(a)(viii))*

METHOD FOR MAKING CHEESE

FIELD OF INVENTION

The present invention relates to a method for producing cheese with good flavor, especially low-salt cheese.

BACKGROUND OF INVENTION

Cheese is consumed globally in large and increasing quantities (IDF 2010) and contributes to dietary sodium almost exclusively in the form of NaCl (salt, unless otherwise stated), which is traditionally added in varying amounts dependent on the cheese variety (Guinee and Fox 2004). Hence, in view of an efficient sodium reduction strategy, cheese, apart from constituting a direct and often daily source of sodium, plays an important role in defining the level of salty taste generally accepted by the human perception (taste adaptation) (Walsh 2007). Sodium reduction of significant magnitudes, however, represents a great challenge to the cheese industry since the level of salting is tightly balanced to take advantage of multiple direct and indirect functions of salt in cheese. Particularly in mature cheeses, salt has a critical influence on microbial and enzyme activities needed for a series of biochemical ripening events to proceed in a desired direction.

Cheddar cheese represents a widely consumed international cheese type, a fairly high source of sodium (typically in the range of 1.6 to 2.0% w/w salt (Guinee et al. 2008)) and consequently a priority product for sodium reduction. It is therefore not surprising that numerous previous investigations attempted to restore the quality of low-salt Cheddar cheese. In particular, a series of cheese trials were conducted in the 1980's evaluating with varying degrees of success the partial substitution of NaCl by KCl and other chloride salts (Fitzgerald and Buckley 1985; Lindsay et al. 1982; Reddy and Marth 1993).

However, solutions based on mere salt substitution are not acceptable to the customers, and therefore an objective of the present invention is to provide other solutions to producing low-salt cheeses with a sensory quality matching the corresponding cheeses with a normal salt content (around 1.6 to 1.9%) (Table 1).

Table 1: NaCl levels of various cheese types

Cheese	Normal salt (% (w/w))		Low salt (% (w/w))	
	lower limit (including)	upper limit	lower limit	upper limit (excluding)
Cheddar	1.5	1.9	0.7	1.5
Emmental	0.7	1.5	0.3	0.7
Danbo	1.4	1.9	0.7	1.4
Parmesan	2.5	4.0	1.2	2.5
Feta	3.0	4.0	1.5	3.0
Blue cheese	3.5	4.5	1.7	3.5
Camembert	2.5	3.3	1.2	2.5
Gouda	1.5	2.0	0.7	1.5
Edam	1.5	2.0	0.7	1.5
Mozzarella	0.7	1.5	0.3	0.7

SUMMARY OF INVENTION

The present inventors have surprisingly found out that it is possible to obtain low-salt cheeses of high flavor intensity when a glutamate decarboxylase (GAD)-negative starter plus a GAD-negative *Lactobacillus {Lb.) helveticus* adjunct culture is used. By combining these strains it was possible to significantly boost the level of glutamate in the low-salt cheese and thereby increase the sensory quality. The GAD-negative starter culture ensures that no glutamate is converted to γ-amino butyric acid (GABA) and $C0_2$. As glutamate imparts a flavor-enhancing effect and compensates for lack of flavor commonly found in low-salt cheese, then it is important that it accumulates to as high concentrations as possible during ripening. Thus, increased accumulation can be achieved by using a GAD-negative starter culture. Glutamate levels were boosted even further in the cheese when a *Lb. helveticus* adjunct culture was also used. The *Lb. helveticus* adjunct was also GAD-negative, but was further characterized by having a high lytic and peptidolytic activity, and thus releasing high levels of glutamate into the cheese. Thus, it is contemplated that both the starter culture and the *Lb. helveticus* adjunct culture should be GAD-negative in order to get the optimal (highest) flavor intensity.

A further improvement in the sensory quality of low-salt cheese could be achieved with the use of camel chymosin as coagulant. Normal coagulants such as bovine chymosin, microbially-derived coagulants (from *Mucor miehei)* and plant-derived coagulants are problematic in low-salt cheese. The increased water activity in low-salt cheese results in unbalanced ripening due to the high general (unspecific) proteolyic activity of bovine chymosin, microbially-derived coagulants (from *Mucor miehei)* and plant-derived coagulants. This commonly results in the accumulation of undesirable bitter-tasting peptides derived from the hydrolysis of the caseins during ripening. Surprisingly, the inventors were able to use camel chymosin as coagulant in

order to reduce the problem of bitterness in low-salt cheeses. Camel chymosin has a much lower general proteolytic activity than that of bovine chymosin, microbially-derived coagulants or plant-derived coagulants, and thus, it was possible to produce a low-salt cheese with markedly lower rates of bitter-tasting peptide formation during ripening. Levels of bitter-tasting peptides were reduced even further in the cheese when camel chymosin was used in combination with the *Lb. helveticus* adjunct due to aforementioned high lytic and peptidolytic activity.

Thus, the best possible low-salt cheese is produced by combining these two concepts: (i) using a GAD-negative starter culture and a GAD-negative *Lb. helveticus* adjunct culture for boosting desirable flavor intensity and (ii) using camel chymosin as coagulant and a *Lb. helveticus* adjunct for the reduction of undesirable, bitter off-taste.

In accordance with the surprising findings, the present invention in its broadest scope relates to a method for producing a cheese, comprising the following steps:

b) inoculating a milk substrate with a lactic acid bacteria (LAB) starter culture which is GAD-negative;

c) adding a coagulant to the milk substrate;

d) allowing the resulting product from steps b) and c) to coagulate; and

e) using the coagulum from step d) to produce cheese.

Also, the invention relates to a cheese which is obtainable by the method of the invention; to a lactic acid bacterial strain, which is GAD-negative and to a LAB culture, which is GAD-negative (and which comprises more than two different strains).

DETAILED DISCLOSURE

In a first aspect, the present invention relates to a method for producing a cheese, comprising the following steps:

a) optionally providing a milk substrate;

b) inoculating the milk substrate with a lactic acid bacteria (LAB) starter culture, which is Glu decarboxylation (GAD)-negative;

c) adding a coagulant to the milk substrate;

d) allowing the resulting product from steps b) and c) to coagulate;

e) using the coagulum from step d) to produce cheese;

f) optionally storing the cheese.

The following methods are parts of the invention:

- A method for producing a cheese, comprising the following steps:
  b) inoculating a milk substrate with a LAB starter culture, which is GAD-negative;
  c) adding a camel chymosin to the milk substrate;

d) allowing the resulting product from steps b) and c) to coagulate;
e) using the coagulum from step d) to produce cheese;
f) optionally storing the cheese.

- A method for producing a cheese, comprising the following steps:
b) inoculating a milk substrate with a LAB starter culture, which is GAD-negative and a *Lactobacillus {Lb.) heiveticus* strain (such as a strain having a high lytic and peptidolytic activity), which is GAD-negative;
c) adding a camel chymosin to the milk substrate;
d) allowing the resulting product from steps b) and c) to coagulate;
e) using the coagulum from step d) to produce cheese;
f) optionally storing the cheese.

- A method for producing a cheese, comprising the following steps:
b) inoculating a milk substrate with a LAB starter culture, which is GAD-negative;
c) adding a camel chymosin to the milk substrate;
d) allowing the resulting product from steps b) and c) to coagulate;
e) using the coagulum from step d) to produce low-salt cheese;
f) optionally storing the cheese.

- A method for producing a cheese, comprising the following steps:
b) inoculating a milk substrate with a LAB starter culture, which is GAD-negative and a *Lb. heiveticus* strain (such as a strain having a high lytic and peptidolytic activity or a strain having proteolytic activity), which is GAD-negative;
c) adding a camel chymosin to the milk substrate;
d) allowing the resulting product from steps b) and c) to coagulate;
e) using the coagulum from step d) to produce low-salt cheese;
f) optionally storing the cheese.

- A method for producing a cheese, comprising the following steps:
b) inoculating a milk substrate with a LAB starter culture, which is GAD-negative and a *Lb. heiveticus* strain (such as a strain having a high lytic and peptidolytic activity or a strain having proteolytic activity), which is GAD-negative;
c) adding a coagulant to the milk substrate;
d) allowing the resulting product from steps b) and c) to coagulate;
e) using the coagulum from step d) to produce low-salt cheese;
f) optionally storing the cheese.

- A method for producing a cheese, comprising the following steps:
b) inoculating a milk substrate with a LAB starter culture, which is GAD-negative;

c) adding a camel chymosin to the milk substrate;
d) allowing the resulting product from steps b) and c) to coagulate;
e) using the coagulum from step d) to produce low-salt Cheddar cheese;
f) optionally storing the cheese.

- A method for producing a cheese, comprising the following steps:
b) inoculating a milk substrate with a LAB starter culture, which is GAD-negative and a *Lb. helveticus* strain (such as a strain having a high lytic and peptidolytic activity or a strain having proteolytic activity), which is GAD-negative;
c) adding a camel chymosin to the milk substrate;
d) allowing the resulting product from steps b) and c) to coagulate;
e) using the coagulum from step d) to produce low-salt Cheddar cheese;
f) optionally storing the cheese.

A method for producing a cheese, comprising the following steps:
b) inoculating a milk substrate with a LAB starter culture, which is GAD-negative and a *Lb. helveticus* strain (such as a strain having a high lytic and peptidolytic activity or a strain having proteolytic activity), which is GAD-negative;
c) adding a coagulant to the milk substrate;
d) allowing the resulting product from steps b) and c) to coagulate;
e) using the coagulum from step d) to produce low-salt Cheddar cheese;
f) optionally storing the cheese.

It should be understood the step e) in the methods of the invention comprises further cheese-making procedures. The exact procedure to be used depends on the cheese to be made. Normally the procedure includes one or more of: cutting the coagulum, draining off the whey from the coagulum (curd), scalding (heating) the curd, adding salt to the curd, molding, and ripening (storing), see e.g. Kosikowski and Mistry (1997).

The starter culture may comprise one or more LAB strains, such as from 2 to 20 strains, from 3 to 20 strains or from 4 to 20 strains. It presently preferred that the starter culture contains more than 1 strain, preferably more than 2 different strains, such as more than 3, more than 4, more than 5 or more than 6 different strains.

In a presently preferred embodiment, the starter culture comprises only GAD-negative strains, preferably no GAD-positive strains are used for inoculation of the milk substrate.

Further interesting embodiments of the methods of the invention are:

- The method of the invention, wherein the total GAD activity results in that below 0.05 mM (such as below 0.04 or below 0.03 mM) GABA is produced following incubation in a glutamate-containing medium at 35°C for 24 hours.

- The method of the invention, wherein the total GAD activity results in that below 0.05 mM GABA (such as below 0.04 or below 0.03 mM) is produced following incubation in a glutamate-containing medium at 35°C for 48 hours.

- The method of the invention, wherein the total GAD activity results in that below 0.05 mM GABA (such as below 0.04 or below 0.03 mM) is produced following incubation in a glutamate containing medium at 35°C for 72 hours.

- The method of the invention, wherein the glutamate-containing medium is milk (e.g. reconstituted skimmed milk or boiled reconstituted skimmed milk).

- The method of the invention, wherein the step c) is performed before, during and/or after step b).

- The method of the invention, wherein the starter culture comprises LAB selected from the group consisting of Lactobacillales which includes *Lactococcus* spp., *Streptococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pseudoleuconostoc* spp., *Pediococcus* spp., *Brevibacterium* spp., *Bifidobacterium* spp., *Enterococcus* spp. and *Propionibacterium* spp.

- The method of the invention, wherein the starter culture comprises a strain selected from the group consisting of: CHCC 4356 deposited as DSM 25485, CHCC 4427 deposited as DSM 25486, CHCC 5188 deposited as DSM 25487, CHCC 1916 deposited as DSM 21405 and IG227 deposited as DSM 14797. It is presently preferred that the strain is a component of a LAB starter culture comprising 2 to 20 different strains. It is presently also preferred that the strain constitutes 5 to 95%, such as 20 to 95%, of the starter culture, as measured in cell forming units (CFU).

- The method of the invention, wherein the milk substrate is inoculated with 10E5 to 10E12 CFU/mL of the starter culture.

- The method of the invention, which comprises the further step of inoculating the milk substrate with a *Lb. helveticus* strain (such as a strain having a high lytic and peptidolytic activity or a strain having proteolytic activity), said strain is GAD-negative. It should be understood that only one starter culture (which may contain several different strains) is added to the milk substrate, optionally together with one adjunct culture or strain (such as a *Lb. helveticus* strain). Preferably, no further cultures are added. Preferably, the adjunct culture is also GAD negative, and most preferably the adjunct culture does only comprise GAD-negative strains.

- The method of the invention, wherein the milk substrate is inoculated with 10E5 to 10E12 CFU/mL of the strain.

- The method of the invention, wherein the resulting product of step b) has a GABA content below 0.05 mM, such as below 0.04 mM, below 0.03 mM or below 0.02 mM.

- The method of the invention, wherein the resulting product of step c) has a GABA content below 0.05 mM, such as below 0.04 mM, below 0.03 mM or below 0.02 mM.

- The method of the invention, wherein the resulting product of step d) has a GABA content below 0.05 mM, such as below 0.04 mM, below 0.03 mM or below 0.02 mM.

- The method of the invention, wherein the resulting product of step e) has a GABA content below 0.05 mM, such as below 0.04 mM, below 0.03 mM or below 0.02 mM.

- The method of the invention, wherein the resulting product of step e) has a GABA content below 0.20 mmol/kg, such as below 0.10 mmol/kg, below 0.05 mmol/kg, below 0.04 mmol/kg, below 0.03 mmol/kg or below 0.02 mmol/kg.

- The method of the invention, wherein the resulting product of step f) (after storage for 5 months) has a GABA content below 0.20 mmol/kg, such as below 0.10 mmol/kg, below 0.05 mmol/kg, below 0.04 mmol/kg, below 0.03 mmol/kg or below 0.02 mmol/kg.

- the method of the invention, wherein the resulting product of step f) (after storage for 9 months) has a GABA content below 0.20 mmol/kg, such as below 0.10 mmol/kg, below 0.05 mmol/kg, below 0.04 mmol/kg, below 0.03 mmol/kg or below 0.02 mmol/kg.

- The method of the invention, wherein the cheese is selected from the group consisting of Cheddar, Parmesan, Mozzarella, Emmental, Danbo, Gouda, Edam, Feta-type, blue cheeses, Camembert and Brie.

- The method of the invention, wherein the cheese is a Cheddar cheese.

- The method of the invention, wherein the cheese is a low-salt cheese, such as a cheese which contains NaCl in a concentration in the range from 0.2% to 1.7% (w/w).

- The method of the invention, wherein the cheese contains NaCl in a concentration in the range from 0.5% to 1.3%, such as from 0.6% to 1.1%.

- The method of the invention, wherein the cheese has a moisture content in the range from 25% to 70%, such as from 25 to 60%.

- The method of the invention, wherein the coagulant is an aspartic protease, such as chymosin or rennet.

- The method of the invention, wherein the coagulant is selected from the group consisting of bovine chymosin (such as CHY-MAX®).

- The method of the invention, wherein the coagulant is selected from the group consisting of camel chymosin (such as CHY-MAX® M).

- The method of the invention, wherein the storing is performed for a period of 1 to 24 months at a temperature in the range of 5 to 20°C.

- The method of the invention, which comprises a packaging step (before, during or after step f))-

In a further aspect, the present invention relates to a cheese, which is obtainable by the method of the invention, such as a Cheddar cheese. Preferred embodiments of this aspect are:

- A cheese which has a GABA content below 0.20 mmol/kg, such as below 0.10 mmol/kg, below 0.05 mmol/kg, below 0.04 mmol/kg, below 0.03 mmol/kg or below 0.02 mmol/kg;

- A cheese which after storage (ripening/maturation) for 5 months has a GABA content below 0.20 mmol/kg, such as below 0.10 mmol/kg, below 0.05 mmol/kg, below 0.04 mmol/kg, below 0.03 mmol/kg or below 0.02 mmol/kg; or
- A cheese which after storage (ripening/maturation) for 9 months has a GABA content below 0.20 mmol/kg, such as below 0.10 mmol/kg, below 0.05 mmol/kg, below 0.04 mmol/kg, below 0.03 mmol/kg or below 0.02 mmol/kg. It is preferred that the cheeses are low-salt cheese, such as low-salt Cheddar cheeses. Normally cheddar cheeses are ripened at a temperation in the range of 8 to 14 degrees C. Presently most preferred is a low-salt Cheddar that has a GABA content below 0.20 mmol/kg if ripened for 5 months at a temperature of 8C.

In another aspect, the present invention relates to a LAB strain, which is GAD-negative. An interesting strain is a strain selected from the group consisting of CHCC 4356 deposited as DSM 25485, CHCC 4427 deposited as DSM 25486, CHCC 5188 deposited as DSM 25487, CHCC 1916 deposited as DSM 21405 and IG227 deposited as DSM 14797; or a mutant or a variant thereof, especially a mutant of any of these strains, which is GAD-negative.

In a fourth aspect, the present invention relates to a LAB culture, which is GAD-negative. An interesting embodiment is a culture, which contains 2 to 20 different LAB strains, such as 2 to 20 or 3 to 20 different LAB strains. Presently preferred is a culture, which contains
1) a bacterial strain selected from the group consisting of CHCC 4356 deposited as DSM 25485, CHCC 4427 deposited as DSM 25486, CHCC 5188 deposited as DSM 25487, CHCC 1916 deposited as DSM 21405 and IG227 deposited as DSM 14797; mutants or variants thereof and
2) a LAB strain, which is GAD-negative and which is different from the strain in 1).
In this aspect, the LAB culture can contain 1, 2, 3, 4, 5 or more additional strains, which are different from the strains in 1) and 2).

In a further aspect, the present invention relates to the use of a strain of the invention in a process for the manufacture of cheese, especially a low-salt cheese

In a still further aspect, the present invention relates to the use of a LAB culture of the invention in a process for the manufacture of cheese.

In yet an aspect, the present invention relates to the use of camel chymosin, e.g. CHY-MAX® M, in a process for manufacture of low-salt cheese, esp. Cheddar cheese.

DEFINITIONS

In the present context, the term "glutamate (Glu) decarboxylation negative" ("GAD-negative") and the term "glutamate decarboxylase negative"refers to strains of bacteria, especially LAB,

which are unable to convert the free amino acid, glutamate into γ-amino butyric acid (GABA) and $C0_2$. Preferred GAD-negative strains produce GABA to a level of less than 0.05 mM when incubated within the range of 15 to 55°C in a glutamate-containing medium. The incubation period may be up to 72 hours, but shorter or longer periods of incubation are also possible. A typical glutamate-containing medium may be milk with or without glutamate supplementation. In cases, where no glutamate supplementation is used, then the natural level of glutamate in the medium serves as the source of glutamate. In addition, glutamate may be released from various protein and peptide components of the medium during incubation. Other media such as MRS, M17 and Brain Heat Infusion medium (BHI) may also be used for the incubation of the bacteria.

More specifically, a strain (or a culture consisting of one or more strains) is considered to be GAD-negative if the strain or culture produces less than 0.05 mM GABA in milk, as determined according to the following assay:

Reconstituted skim milk (9.5%, boiled) is inoculated with 10E8 cell forming units of the strain/culture per ml_ of milk and allowed to stand at 35°C for 24 hours. After the 24 hours the pH has dropped below 5.0, and the content of GABA is measured. If the concentration of GABA is lower than 0.05 mM, the strain/culture is considered to be glutamate decarboxylase negative. Preferred strains produce less than 0.04 or less than 0.03 mM GABA in the above assay. Other preferred strains produce less than 0.05, less than 0.04 or less than 0.03 mM GABA in an assay as above, after the pH has dropped below 5.0, but when the milk has been incubated for 48 or 72 hours (instead of 24 hours).

Strains (or cultures) of bacteria that are able to produce GABA to a level of greater than or equal to 0.05 mM are in the present context described as "glutamate decarboxylation positive" ("GAD-positive") or "glutamate decarboxylase positive".

As used herein, the term "lactic acid bacteria" (LAB) designates gram-positive, microaerophilic or anaerobic bacteria, which ferment sugars with the production of acids including lactic acid as the predominantly produced acid, acetic acid and propionic acid. The industrially most useful lactic acid bacteria are found within the order "Lactobacillales" which includes *Lactococcus* spp., *Streptococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pseudoleuconostoc* spp., *Pediococcus* spp., *Brevibacterium* spp., *Enterococcus* spp. and *Propionibacterium* spp. Additionally, lactic acid producing bacteria belonging to the group of the strict anaerobic bacteria, bifidobacteria, i.e. *Bifidobacterium* spp., are generally included in the group of LAB. These are frequently used as food cultures alone or in combination with other lactic acid bacteria.

By the term "cheese" is understood any cheese, including hard, semi-hard and soft cheeses, such as cheeses of the following types: Cheddar, Parmesan, Mozzarella, Emmentaler, Danbo,

Gouda, Edam, Feta-type, blue cheeses, Camembert and Brie. The person skilled in the art knows how to convert the coagulum into cheese, methods can be found in the literature, see e.g. Kosikowski and Mistry (1997). The definition of the term "low-salt" in connection with cheese depends on the cheese type and should normally be understood as cheese, which contains NaCl within a concentration range of 0.2 to 1.7% (w/w) (including the range of 0.2 to 1.6%). More specifically, the term low-salt in the present context can also be read from Table 1 and Table 2. From Table 2 it should be understood that a low-salt cheese of Cheddar type preferably contains an amount of salt in the range 0.1 up to 1.5% (w/w), more preferred in the range 0.2 to 1.3% (w/w) and most preferred in the range 0.3 to 1.1% (w/w).

Table 2: Definition of low-salt related to specific cheese types (% (w/w))

Cheese	Preferred Salt range		More Preferred salt range		Most preferred salt range	
	lower limit (including)	upper limit (excluding)	lower limit	upper limit	lower limit	upper limit
Cheddar	0.1	1.5	0.2	1.3	0.3	1.1
Parmesan	0.1	2.5	0.2	2.0	0.3	2.0
Mozzarella	0.1	0.7	0.2	0.6	0.2	0.5
Emmental	0.1	0.7	0.2	0.6	0.2	0.5
Danbo	0.1	1.4	0.2	1.2	0.3	1.0
Gouda	0.1	1.5	0.2	1.3	0.3	1.2
Edam	0.1	1.5	0.2	1.3	0.3	1.2
Feta-type	0.1	3.0	0.2	2.5	0.3	2.0
Blue cheese	0.1	3.5	0.2	3.3	0.3	3.0
Camembert	0.1	2.5	0.2	2.2	0.3	2.0

In the present context, the term "milk substrate" may be any raw and/or processed milk material that can be subjected to fermentation according to the method of the invention. Thus, useful milk substrates include, but are not limited to, solutions/suspensions of any milk or milk-like products comprising protein, such as whole or low-fat milk, skimmed milk, buttermilk, reconstituted skimmed milk, condensed milk, dried milk, whey, whey permeate, lactose, mother liquid from crystallization of lactose, whey protein concentrate, or cream. Obviously, the milk substrate may originate from any mammal, e.g. being substantially pure mammalian milk, or reconstituted milk. Preferably, at least part of the protein in the milk substrate is proteins naturally occurring in milk, such as casein or whey protein. However, part of the protein may be proteins which are not naturally occurring in milk. Prior to fermentation, the milk substrate may be homogenized and pasteurized according to methods known in the art.

The term "milk" is to be understood as the lacteal secretion obtained by milking any mammal, such as cows, sheep, goats, buffaloes or camels. In a preferred embodiment, the milk is cow's milk. The term milk also comprises soy milk. Optionally, the milk is acidified, e.g. by addition of an acid (such as citric, acetic or lactic acid), or mixed, e.g. with water or cream. The milk may be raw or processed, e.g. by filtering, sterilizing, pasteurizing, homogenizing etc., or it may be reconstituted dried milk. An important example of "bovine milk" according to the present invention is pasteurized cow's milk. It is understood that the milk may be acidified, mixed or processed before, during and/or after the inoculation with bacteria. The term "providing a milk substrate" includes pretreatment of the milk (substrate), such as the processing steps described above.

By the term "camel chymosin" should be understood a chymosin obtainable by the method of EP1334182B1, incl. the chymosin derived from *Camelus dromedarius.* CHY-MAX® M is presently preferred.

"Fermentation" in the methods of the present invention means the conversion of carbohydrates into alcohols or acids through the action of a microorganism. Preferably, fermentation in the methods of the invention comprises conversion of lactose to lactic acid.

LAB, including bacteria of the species Lactobacillus sp. and Streptococcus thermophilus, are normally supplied to the dairy industry either as frozen or freeze-dried cultures for bulk starter propagation or as so-called "Direct Vat Set" (DVS) cultures, intended for direct inoculation into a fermentation vessel or vat for the production of a dairy product, such as a fermented milk product. Such cultures are in general referred to as "starter cultures" or "starters".

In the present context, the term "packaging" (a suitable amount of) the fermented milk in a suitable package relates to the final packaging of the fermented milk to obtain a product that can be ingested by e.g. a person or a group of persons. A suitable package may thus be a bottle or similar, and a suitable amount may be e.g. 10 mL to 5000 mL, but it is presently preferred that the amount in a package is from 50 mL to 1000 mL.

In the present context, the term "mutant" should be understood as a strain derived from a strain of the invention by means of e.g. genetic engineering, radiation and/or chemical treatment. It is preferred that the mutant is a functionally equivalent mutant, e.g. a mutant that has substantially the same, or improved, properties (e.g. regarding glutamate decarboxylase activity (an interesting mutant has decreased activity compared to mother strain), flavor, acidification speed, and/or phage robustness) as the mother strain. Such a mutant is a part of the present invention. Especially, the term "mutant" refers to a strain obtained by subjecting a strain of the invention to any conventionally used mutagenization treatment including treatment with a chemical mutagen such as ethane methane sulphonate

(EMS) or N-methyl-N'-nitro-N-nitroguanidine (NTG), UV light or to a spontaneously occurring mutant. A mutant may have been subjected to several mutagenization treatments (a single treatment should be understood one mutagenization step followed by a screening/selection step), but it is presently preferred that no more than 20, or no more than 10, or no more than 5, treatments are carried out. In a presently preferred mutant, less that 5%, or less than 1% or even less than 0.1% of the nucleotides in the bacterial genome have been shifted with another nucleotide, or deleted, compared to the mother strain.

In the present context, the term "variant" should be understood as a strain, which is functionally equivalent to a strain of the invention, e.g. having substantially the same, or improved, properties (e.g. regarding glutamate decarboxylase activity (an interesting variant has decreased activity compared to mother strain), flavor potential, acidification speed and/or phage robustness). Such variants, which may be identified using appropriate screening techniques, are a part of the present invention.

The use of the terms "a", "an", "the" and similar referents in the context of describing the invention (especially in the context of the following claims) is to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising", "having", "including" and "containing" are to be construed as open-ended terms (i.e. meaning "including, but not limited to,"), unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it was individually recited herein. All methods described herein can be performed in any suitable order, unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g. "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention, unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

EXAMPLES

Example 1:

Screening for GAD-negative strains

Strains were screened based on their inability to decarboxylate glutamate by measuring the levels of γ-amino butyric acid (GABA) and glutamate following growth in milk. Two g of frozen culture pellets was dispersed into 200 g of double boiled 9.5% reconstituted skimmed milk. A 0.01% final inoculation rate was prepared by taking 2 g of this dispersion and inoculating into

200 g of double boiled 9.5% reconstituted skimmed milk, previously spiked with 1 mM glutamate (spiking was performed by the addition of 2 ml_ of a 100 mM glutamate solution to 200 g of 9.5% reconstituted skimmed milk). The cultures were incubated at 30°C for *Lc. lactis* and 37 C for *Lb. helveticus* for 72 hours. After incubation 1.0 g of fermented skim milk was mixed with 1.0 ml_ of 4% (w/v) TCA and the samples were mixed, and left stand at room temperature for 30 min. The samples were then centrifuged at 15,000 x g for 20 min at 4°C, and 1 ml_ of supernatant was transferred into a fresh eppendorf, and re-centrifuged at 15,000 x g for 10 min at 4°C. The final supernatant was stored at -50°C until analysis by GC-MS. Free amino acids were determined according to the method of Glastrup and Houlberg (2009).

Using this screening method it was possible to select strains CHCC 1916, IG227, CHCC 4356, CHCC 4427 and CHCC 5188, which clearly exhibited a glutamate decarboxylase negative phenotype. Glutamate decarboxylase negative strains were characterized by their inability to produce GABA in the presence of glutamate (Table 2). Consequently, any of these strains alone or in combination could be used as a starter culture in order to ensure that glutamate conversion to GABA is limited and/or prevented. For the formulation of a phage robust culture, then a combination of GAD-negative strains is preferable. Thus, a phage robust culture with GAD-negative properties was formulated by mixing strains of CHCC 1916, IG227, CHCC 4427 and CHCC 5188.

For cheese application experiments (Example 3), the GAD-negative strains were grown in a milk-based medium, harvested, concentrated and frozen in order to produce a DVS culture. In addition to producing a frozen DVS culture, a freeze-dried culture is also possible. Production of the culture in DVS format is for purposes of convenience during cheese application experiments, but the essential characteristic (i.e. absence of GAD activity) of the culture remains whether it is concentrated or not.

Table 3: Screening of glutamate decarboxylase-negative strains

Strain or culture	Glutamate (mM)	γ-amino butyric acid (mM)	GAD reaction
CHCC 1916	1.6	<LOQ	Negative
IG227	1.6	<LOQ	Negative
CHCC 4356	2.0	<LOQ	Negative
CHCC 4360	1.4	<LOQ	Negative
CHCC 4427	1.6	<LOQ	Negative
CHCC 5188	1.4	<LOQ	Negative
R-604	0.0	0.5	Positive
LH-32	4.8	<LOQ	Negative

LOQ (limit of quantification) = 0.01 mM

For purposes of comparison, the commercial starter culture F-DVS R-604 (Chr. Hansen) was included in the screening experiment. This culture exhibited a GAD-positive phenotype and is consequently unsuitable for cheese trials, where high levels of glutamate are required. Other commercial starter cultures with GAD activity include: F-DVS R-603 (Chr. Hansen), F-DVS R-607 (Chr. Hansen), F-DVS R-608 (Chr. Hansen), CHOOZIT MA 11 (DuPont), CHOOZIT MA 14 (DuPont), CHOOZIT MA 16 (DuPont) and CHOOZIT MA 19 (DuPont).

The screening method was also used in order to select a *Lb. heiveticus* adjunct culture (F-DVS LH-32) with no GAD activity. In addition to this property, the screening revealed that the *Lb. heiveticus* strain was able to increase the level of glutamate in the milk. Milk was spiked with 1 mM glutamate at the start of the fermentation and after incubation for 72 hours, the level had increase to 4.8 mM. This *Lb. heiveticus* culture is commercially available from Chr. Hansen as F-DVS LH-32. Other similar commercial cultures include: F-DVS LHB01 (Chr. Hansen), F-DVS LHB02 (Chr. Hansen), CHOOZIT FLAV 25, (DuPont), CHOOZIT FLAV 54 (DuPont), CHOOZIT HelvA (DuPont), CHOOZIT HelvB (DuPont), CHOOZIT LH 11 (DuPont) and L100 (CSK Food Enrichment).

Example 2:

Production of normal-salt and low-salt Cheddar cheeses with reduced bitterness

All cheeses were manufactured in duplicate. A normal level of salt was defined as 1.8% (w/w) and low-salt cheese was defined as 0.8% (w/w).

The trial was set-up according to Table 4, wherein the starter culture is the GAD-positive F-DVS R-604 (Chr. Hansen).

Table 4: Salt and coagulant variables

	Cheese ID	Salt level	Coagulant
1	NB1-26-2-4 NB2-26-4-4	Normal (1.8% (w/w))	Bovine chymosin
2	NC1-26-2-5 NC2-26-4-5	Normal (1.8% (w/w))	Camel chymosin
3	LB1-26-2-6 LB2-26-4-6	Low (0.8% (w/w))	Bovine chymosin
4	LC1-26-2-7 LC2-26-4-7	Low (0.8% (w/w))	Camel chymosin

1. Normal-salt cheese made with bovine chymosin as coagulant (NB1-26-2-4, NB2-26-4-4)
2. Normal-salt cheese made with camel chymosin as coagulant (NCl-26-2-5, NC2-26-4-5)
3. Low-salt cheese made with bovine chymosin as coagulant (LB1-26-2-6, LB2-26-4-6)
4. Low-salt cheese made with camel chymosin as coagulant (LCl-26-2-7, LC2-26-4-7)

Cheddar cheese-making trials were undertaken on at the Application and Technology Centre, Chr. Hansen A/S, Horsholm, Denmark. Open vats of cheese on a 150 L scale were manufactured. Fresh, pasteurised (74°C, 15 s) bovine milk (3.40% fat, 3.25% protein) was delivered on the morning of production from Aria Foods Amba (Christiansfeld Mejericenter, Denmark). The milk was analysed by MilkoScan™ (FOSS, Hillerod, Denmark) and standardised to a protein:fat ratio of 0.9 and heated to 31-32°C for cheese-making. Frozen DVS starter (R-604) was added at a rate of 0.012% (w/w). The milk was coagulated by the addition of CHY-EXTRA® (bovine chymosin) at a rate of 0.0061% w/w (600 IMCU-mL^{-1}; Chr. Hansen A/S) or of CHY-MAX® M (camel chymosin) at a rate of 0.0029% (w/w) (1000 IMCU-mL^{-1}; Chr. Hansen A/S). The curd grains were heated to target temperature and cooked at this temperature for variable lengths of time (Table 5).

Table 5: Settings of variable technological parameters of cheese-making protocols used for manufacture of full fat Cheddar cheese of variable final salt content and equal final moisture content (37.0 to 38.0% (w/w))

Salt level	Target salt (% (w/w))	Curd grain cutting size (mm^3)	Cooking (°C/min)	Cheddaring[a] ($\#_{layers}$/min)	Chip size (L x W x T) (cm)	Salting rate (% (w/w))
Low	0.8	125	40.5/45	1/25-3/15-3/15-4/15-6/15-6/15	4.0 x 1.8 x 2.0	0.95
Normal	1.8	1000	37.0/5	1/20-2/25-3/30-3/15-4/15-6/10	1.7 x 1.1 x 3.0	3.70

Abbreviations: L, length; T, thickness; W, width

[a] All turning steps are tabled with the number of curd blocks per pile and the time interval of each turn.

The milled curds were dry-salted in a warm vessel by manually mixing approximately half the amount of salt thoroughly into the curd followed by a few minutes rest before mixing in the remaining salt; except for the lowest rate of salting which was added all at once (Table 5). The salted curds were mixed thoroughly every 5 min over a mellowing period of 20 min to obtain a uniform distribution of the salt; whey drainage during mellowing was confined by a plastic lining of the vessel. Following molding, the curds were pre-pressed at 0.20 MPa for 15 min and then at 0.57 MPa for 17 hours. After pressing, each cheese was cut into 4 equal blocks, which were vacuum packed separately and ripened at 9°C until analysis.

Cheese compositional analysis

The NaCl content was determined by automated potentiometric endpoint titration of Cl^- with $AgNO_3$ (DL50, Mettler-Toledo A/S, Glostrup, Denmark. Moisture was analyzed gravimetrically by drying to a constant weight at 102°C. Fat and protein were determined by near infrared reflectance spectroscopy using a Cheddar cheese calibration model (FoodScan™ Lab, FOSS). The pH was measured potentiometrically (pHC2002, Radiometer Analytical SAS, Lyon, France) in a paste prepared by macerating 10 g of grated cheese in 10 mL of deionised water. Duplicate samples were prepared for the above analyses.

Peptide analysis

The pH 4.6-soluble peptides were analyzed by reversed phase (RP) HPLC electro spray ionization mass spectrometry. Thawed, grated cheese (10.0 g) was dispersed in 40 mL of 0.5 mol-L^{-1} Na_3 citrate by stirring in a 45°C water bath for 1 h. After cooling to room temperature, the cheese slurries were diluted to 5% (w/v) with water and stirred for 5 min. Aliquots of the cheese slurries were cooled to 12 °C and adjusted to pH 4.5 by adding 1 mol-L^{-1} cold HCl with

vigorous mixing. Total volumes of 20 ml_ were centrifuged (2,000 x g, 30 min, 5°C), and the supernatants were filtered (0.20 μηη) and stored at -20°C until analysis. Thawed samples were centrifuged (13,000 x g, 3 min, ambient) before injection (30 μI) onto the RP-HPLC (1100 Series, Agilent Technologies ApS) column (Zorbax 300 SB-C18, 2.1 x 150 mm, 5 μηη, Agilent Technologies ApS). Separation of the peptides was performed at 40 °C using binary gradient elution by mixing 0.1% (v/v) TFA (for spectroscopy; Merck Chemicals, Darmstadt, Germany) in water (eluent A) with 0.1% (v/v) TFA in acetonitrile:water (90:10 (v/v)) (eluent B) (acetonitrile was Rathburn HPLC Grade S, Mikrolab Aarhus A/S, Hojbjerg, Denmark). A linear gradient was generated by increasing the concentration of eluent B from 2 to 55% over 60 min, after which the column was purged with 100% B for 6 min and re-equilibrated at 2% B for 10 min. The flow rate was 0.25 mL-min^{-1}, and detection was at 210 and 280 nm. The instrument was interfaced with Bruker Daltonik MSD Trap Control software, Ver. 5.3 and ChemStation for LC 3D systems, Rev. B.01.03-SR2 (Agilent Technologies ApS) and data were processed in Bruker Daltoniks DataAnalysis software, Ver. 3.3 (Agilent Technologies ApS). The area of selected peaks ($\mathbf{A_{210\,nm}}$) was calculated by integration in ChemStation software, Rev. B.01.03 (Agilent Technologies ApS) and used for peak-wise quantitative comparison of the underlying peptide(s) across cheeses.

Free amino acid analysis

Free amino acid determination of the cheeses was performed by RP-HPLC with o-phthaldialdehyde and fluorenylmethyl chloroformate pre-column derivitisation according to the method of Butikofer and Ardo (1999).

Sensory Analysis

Descriptive sensory analysis was performed in collaboration with the research group of Sensory Science, Department of Food Science, University of Copenhagen. The sensory panel consisted of 10 external persons (7 females, 3 males, aged between 22 and 39 years) previously tested, selected and trained according to IS022935-1:IDF99-1 (2009). Four training sessions of 2 hours guided by a panel leader took place on 4 consecutive days and served to further develop a pre-drafted vocabulary until agreement was reached upon a "best shared language" describing the sensory properties experienced within the set of experimental cheeses. Qualitative and quantitative calibration of the panel was achieved through fine-tuning of descriptor and scale definitions, use of reference materials, standardization of evaluation procedures and evaluation of sub-sets of the experimental cheeses. In the following week, 4 evaluation sessions were undertaken on 4 consecutive days. Samples of rind-free cheese (9 x 1.5 x 1.5 cm of which 1.5 cm were cut off) were freshly cut on the day of use, distributed and blinded in transparent plastic beakers with lids and tempered to 14°C during 2 hours prior to presentation to the panel (IS022935-2:IDF99-2 2009). The normal-salt cheese was selected for serving (blinded) to the panel as an additional "warm-up sample" at the start of each evaluation session, which took place in test rooms complying with IS08589 (2007). All

descriptors were scored on a horizontal 15 cm unstructured line scale anchored at both ends with appropriate extremes and data were collected by FIZZ software, Ver. 2.46B (Biosystemes, Couternon, France). Panelists were provided with unsalted and unflavored crisp bread, peeled cucumber slices and tap water for palate cleansing. All 8 different samples were served and evaluated one by one in randomized order in all 4 sessions (Latin square design, FIZZ, Ver. 2.46B, Biosystemes).

Characteristics of normal-salt and low-salt cheeses

One of the major defects associated with the production of low-salt cheese is that a serious flavor defect known as "bitterness" develops. Bitterness is due to the accumulation of mainly small to medium-sized, hydrophobic peptides in the cheese above a certain threshold. The accumulation of these peptides is often due to excessive activity of the coagulant in the low-salt environment. The coagulants used to manufacture such cheese have typically been bovine chymosin or acid proteinases from moulds such as *Mucor miehei.* However, recently, it has been shown that camel chymosin can be used to produce cheese. Camel chymosin has a lower general proteolytic activity than bovine chymosin.

After ripening for 9 months at 9 °C the cheeses were assessed by a trained sensory panel. Cheeses manufactured with low salt and bovine chymosin (LB1-26-2-6, LB2-26-4-6) had the highest bitterness score (Figure 1). This was further illustrated by the highest concentration of hydrophobic peptides in these cheeses (retention time 47-50 min, Figure 2). More specifically, higher concentrations of small to medium-sized, hydrophobic peptides (retention time 35-45 min, Figure 2) were present in the low-salt cheeses with bovine chymosin (LB1-26-2-6, LB2-26-4-6). Surprisingly, it was found that when camel chymosin was used in the manufacture of low-salt cheese (LCl-26-2-7, LC2-26-4-7), the bitterness score was significantly lower than that of the low-salt cheeses manufactured with bovine chymosin (LB1-26-2-6, LB2-26-4-6). This finding is also supported by the chemical analysis of the cheeses which showed lower concentrations of (small to medium-sized) hydrophobic peptides in the low-salt cheeses manufactured with camel chymosin (LCl-26-2-7, LC2-26-4-7).

For normal-salt cheeses, a similar trend regarding the effect of camel chymosin was found. The bitterness score of and level of (small to medium-sized) hydrophobic peptides in the cheeses manufactured with camel chymosin (NCl-26-2-5, NC2-26-4-5) were significantly lower than the corresponding normal-salt cheeses manufactured with bovine chymosin (NB1-26-2-4, NB2-26-4-4). In conclusion, the use of camel chymosin results in lower levels of (small to medium-sized) hydrophobic peptides in, and hence, lower bitterness intensity of both low- and normal-salt cheeses.

Example 3:

Production of low-salt Cheddar cheese with reduced bitterness and increased flavor intensity

All cheeses were manufactured in duplicate. A low-salt cheese was defined as 0.8% (w/w).

The trial was set-up according to Table 6.

Table 6: Starter culture, adjunct culture and coagulant variables

	Cheese ID	**Starter culture**	**Adjunct culture**	**Coagulant**
1	R1-40-4-4 R2-40-4-6	GAD-positive (F-DVS R-604, Chr. Hansen)	none	Bovine chymosin
2	B1-42-1-4 B2-42-5-5	GAD-negative (F-DVS)	none	Bovine chymosin
3	C1-42-1-5 C2-42-5-4	GAD-negative (F-DVS)	none	Camel chymosin
4	BH1-42-1-6 BH2-42-5-7	GAD-negative (F-DVS)	*Lb. helveticus* (F-DVS LH-32, CHr. Hansen)	Bovine chymosin
5	CH1-42-1-7 CH2-42-5-6	GAD-negative (F-DVS)	*Lb. helveticus* (F-DVS LH-32, Chr. Hansen)	Camel chymosin

1. GAD-positive starter culture and bovine chymosin as coagulant (Rl-40-4, R2-40-4-6)
2. GAD-negative starter culture and bovine chymosin as coagulant (Bl-42-1-4, B2-42-5-5)
3. GAD-negative starter culture and camel chymosin as coagulant (Cl-42-1-5, C2-42-5-4)
4. GAD-negative starter, *Lb. helveticus* adjunct culture and bovine chymosin as coagulant (BH1-42-1-6, BH2-42-5-7)
5. GAD-negative starter, *Lb. helveticus* adjunct culture and camel chymosin as coagulant (CHl-42-1-7, CH2-42-5-6)

Cheeses were manufactured as described previously (Example 2). F-DVS R-604 was added at a rate of 0.012% (w/w). F-DVS GAD-negative starter culture was added at a rate of 0.02% (w/w). F-DVS LH-32 *Lb. helveticus* adjunct culture was added at a rate of 0.0013% (w/w).

Cheese compositional analysis, pH 4.6-soluble peptide analysis, free amino acid analysis and sensory analysis were performed as described above (Example 2).

Characteristics of low-salt cheese with reduced bitterness and increased flavor intensity

Low-salt cheeses manufactured with the GAD-positive starter (F-DVS R-604) plus bovine chymosin (Rl-40-4-4, R2-40-4-6) had the highest bitterness score (Figure 3) and the highest levels of hydrophobic peptides (retention time 47-50 min, Figure 5). More specifically, higher concentrations of small to medium-sized, hydrophobic peptides (retention time 35-45 min, Figure 2) were present in the low-salt cheeses with bovine chymosin (LB1-26-2-6, LB2-26-4-6). Low-salt cheeses produced with the GAD-negative starter plus bovine chymosin (Bl-42-1-4, B2-42-5-5) were similar to Rl-40-4-4, R2-40-4-6 in terms of having a high bitterness score (Figure 3) and high levels of (small to medium-sized) hydrophobic peptides (retention time 47-50 min, Figure 5). However, low-salt cheeses produced with the GAD-negative starter plus *Lb. helveticus* adjunct culture plus camel chymosin (CHl-42-1-7, CH2-42-5-6) had a significantly lower bitterness score (Figure 3) and lower levels of (small to medium-sized) hydrophobic peptides (retention time 47-50 min, Figure 5). The low-salt cheeses produced with the GAD-negative starter plus *Lb. helveticus* adjunct culture plus bovine chymosin (BH1-42-1-6, BH2-42-5-7) were also found to have a low bitterness score (Figure 3) and low levels of (small to medium-sized) hydrophobic peptides (retention time 47-50 min, Figure 5), but not as low as the low-salt cheeses, where the bovine chymosin was replaced with camel chymosin (CHl-42-1-7, CH2-42-5-6).

Taken in their totality, these results surprisingly show that low-salt cheese with reduced bitterness can be produced by using a GAD-negative starter culture plus a *Lb. helveticus* adjunct culture. A further reduction in bitterness can be achieved if camel chymosin is used instead of bovine chymosin.

The low-salt cheeses were also assessed for their flavor intensity (umami) by a trained sensory panel. The low-salt cheeses with the highest flavor intensity (Figure 4) were produced using the GAD-negative starter plus *the Lb. helveticus* adjunct culture (BH1-42-1-6, BH2-42-5-7, CHl-42-1-7, CH2-42-5-6). Indeed, cheeses with highest flavor intensity scores (BH1-42-1-6, BH2-42-5-7, CHl-42-1-7, CH2-42-5-6) also had the highest levels of glutamate (28.6 ± 0.9 mmol/kg, after 5 months ripening) (Table 7). In contrast, low-salt cheeses (Rl-40-4-4, R2-40-4-6) manufactured with the GAD-positive starter without *Lb. helveticus* adjunct culture were characterised by significantly lower flavor intensities (Figure 4) and glutamate levels (1.2 ± 0.1 mmol/kg, after 5 months ripening) (Table 7). Low-salt cheeses manufactured with the GAD-negative starter and without *Lb. helveticus* adjunct culture (Bl-42-1-4, B2-42-5-5, Cl-42-1-5, C2-42-5-4) had higher levels of glutamate (4.2 ± 0.2 mmol/kg) than cheeses (Rl-40-4-4, R2-40-4-6) made with the GAD-positive (F-DVS R-604) starter (Table 7).

Taken in their totality, these results surprisingly show that low-salt cheese with increased flavor intensity (umami) can be produced by using a GAD-negative starter culture plus a *Lb. helveticus* adjunct culture.

Table 7: Glutamate levels in cheeses produced with a GAD-negative starter culture, a GAD-negative *Lb. helveticus* adjunct culture and camel chymosin as coagulant

Cheese	Glutamate (mmol/kg)		γ-amino butyric acid (mmol/kg)	
	Day 1	Month 5	Day 1	Month 5
R1-40-4-4	1.83	1.31	<LOQ	3.62
R2-40-4-6	1.74	1.12	<LOQ	3.89
B1-42-1-4	0.70	3.98	<LOQ	<LOQ
B2-42-5-5	0.82	4.41	<LOQ	<LOQ
C1-42-1-5	0.72	4.18	<LOQ	<LOQ
C2-42-5-4	0.85	4.32	<LOQ	<LOQ
BH1-42-1-6	1.79	28.4	<LOQ	<LOQ
BH2-42-5-7	1.65	27.8	<LOQ	<LOQ
CH1-42-1-7	1.73	28.3	<LOQ	<LOQ
CH2-42-5-6	1.74	29.9	<LOQ	<LOQ

LOQ (limit of quantificatoion) = 0.2 mmol/kg

Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention, unless otherwise indicated herein or otherwise clearly contradicted by context.

LEGENDS TO DRAWING

Figure 1 depicts the bitterness score of cheeses produced with a low (L) (0.8% (w/w)) or a normal (N) (1.8% (w/w)) salt content and bovine (B) or camel (C) chymosin. After 9 months ripening.

Figure 2 is RP-HPLC chromatograms of cheeses produced with a low (L) (0.8% (w/w)) or a normal (N) (1.8% (w/w)) salt content and bovine (B) or camel (C) chymosin. After 9 months ripening.

Figure 3 depicts the bitterness score of cheeses produced with a GAD-negative or GAD-positive (R) starter culture, *Lb. heiveticus* (H) or no adjunct culture and bovine (B) or camel (C) chymosin as coagulant. After 5 months ripening.

Figure 4 depicts the umami (flavor intensity) score of cheeses produced with a GAD-negative or GAD-positive (R) starter culture, *Lb. heiveticus* (H) or no adjunct culture and bovine (B) or camel (C) chymosin as coagulant. After 5 months ripening.

Figure 5 is RP-HPLC chromatograms of cheeses produced with a GAD-negative or GAD-positive (R) starter culture, *Lb. heiveticus* (H) or no adjunct culture and bovine (B) or camel (C) chymosin as coagulant. After 5 months ripening.

DEPOSITS AND EXPERT SOLUTION

The following strains were deposited at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstr. 7B, D-38124 Braunschweig (DSM):
CHCC 4356 deposited as DSM 25485 on 14th December 2011,
CHCC 4427 deposited as DSM 25486 on 14th December 2011,
CHCC 5188 deposited as DSM 25487 on 14th December 2011,
CHCC 1916 deposited as DSM 21405 on 23rd April 2008 and
IG227 deposited as DSM 14797 on 5th February 2002.

The deposits were made according to the Budapest treaty on the international recognition of the deposit of microorganisms for the purposes of patent procedure.

The Applicant requests that a sample of the deposited microorganism should be made available only to an expert approved by the Applicant.

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All references cited in this patent document are hereby incorporated herein in their entirety by reference.

CLAIMS

1. A method for producing a cheese, comprising the following steps:

a) providing a milk substrate;

b) inoculating the milk substrate with a lactic acid bacteria (LAB) starter culture which is Glu decarboxylation (GAD) negative;

c) adding a coagulant to the milk substrate;

d) allowing the resulting product from steps b) and c) to coagulate;

e) using the coagulum from step d) to produce cheese;

f) optionally storing the cheese.

2. The method of the preceding claim, wherein the starter culture comprises one or more LAB strains, such as from 2 to 20 strains, from 3 to 20 strains or from 4 to 20 strains.

3. The method of any preceding claim, wherein the starter culture comprises only GAD-negative strains, i.e. no GAD-positive strains are used for inoculation of the milk substrate.

4. The method of any preceding claims, wherein the total GAD activity results in that below 0.05 mM (such as below 0.04 or below 0.03 mM) GABA is produced following incubation in a glutamate containing medium at 35°C for 24 hours.

5. The method of any preceding claims, wherein the total GAD activity results in that below 0.05 mM GABA (such as below 0.04 or below 0.03 mM) is produced following incubation in a glutamate-containing medium at 35°C for 48 hours.

6. The method of any preceding claims, wherein the total GAD activity results in that below 0.05 mM GABA (such as below 0.04 or below 0.03 mM) is produced following incubation in a glutamate-containing medium at 35°C for 72 hours.

7. The method of the preceding claim, wherein the glutamate containing medium is milk (e.g. reconstituted skimmed milk or boiled reconstituted skimmed milk).

8. The method of any preceding claim, wherein the step c) is performed before, during and/or after step b).

9. The method of any preceding claims, wherein the starter culture comprises LAB selected from the group consisting of Lactobacillales" which includes *Lactococcus* spp., *Streptococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pseudoleuconostoc* spp., *Pediococcus* spp., *Brevibacterium* spp., *Bifidobacterium* spp., *Enterococcus* spp. and *Propionibacterium* spp.

10. The method of any preceding claim, wherein the starter culture comprises a strain selected from the group consisting of: CHCC 4356 deposited as DSM 25485, CHCC 4427 deposited as DSM 25486, CHCC 5188 deposited as DSM 25487, CHCC 1916 deposited as DSM 21405 and IG227 deposited as DSM 14797.

11. The method of the preceding claim, wherein the strain is a component of a LAB starter culture comprising 2 to 20 different strains.

12. The method of the preceding claim, wherein the strain constitutes 20 to 95% of the starter culture, measured in CFU.

13. The method of any preceding claim, wherein the milk substrate is inoculated with 10E5 to 10E12 CFU/mL of the starter culture.

14. The method of any preceding claim, which comprises the further step of inoculating the milk substrate with *Lb. helveticus* strain (such as a strain having a high lytic and peptidolytic activity or a strain having proteolytic activity), said strain is GAD-negative.

15. The method of the preceding claims, wherein the milk substrate is inoculated with 10E5 to 10E12 CFU/mL of the *Lb. helveticus* strain.

16. The method of any preceding claim, wherein the resulting product of step b) has a GABA content below 0.05 mM, such as below 0.04 mM, below 0.03 mM or below 0.02 mM.

17. The method of any preceding claim, wherein the resulting product of step c) has a GABA content below 0.05 mM, such as below 0.04 mM, below 0.03 mM or below 0.02 mM.

18. The method of any preceding claim, wherein the resulting product of step d) has a GABA content below 0.05 mM, such as below 0.04 mM, below 0.03 mM or below 0.02 mM.

19. The method of any preceding claim, wherein the resulting product of step e) has a GABA content below 0.05 mM, such as below 0.04 mM, below 0.03 mM or below 0.02 mM.

20. The method of any preceding claim, wherein the resulting product of step e) has a GABA content below 0.20 mmol/kg, such as below 0.10 mmol/kg, below 0.05 mmol/kg, below 0.04 mmol/kg, below 0.03 mmol/kg or below 0.02 mmol/kg.

21. The method of any preceding claim, wherein the resulting product of step f) (after storage for 5 months) has a GABA content below 0.20 mmol/kg, such as below 0.10 mmol/kg, below 0.05 mmol/kg, below 0.04 mmol/kg, below 0.03 mmol/kg or below 0.02 mmol/kg.

22. The method of any preceding claim, wherein the resulting product of step f) (after storage for 9 months) has a GABA content below 0.20 mmol/kg, such as below 0.10 mmol/kg, below 0.05 mmol/kg, below 0.04 mmol/kg, below 0.03 mmol/kg or below 0.02 mmol/kg.

23. The method of any preceding claim, wherein the cheese is a selected from the group consisting of Cheddar, Parmesan, Mozzarella, Emmental, Danbo, Gouda, Edam, Feta-type, blue cheeses, Camembert and Brie.

24. The method of any preceding claim, wherein the cheese is a Cheddar cheese.

25. The method of any preceding claim, wherein the cheese is a low-salt cheese, such as a cheese which contains NaCl in a concentration in the range from 0.2% to 1.7% (w/w) (including the range from 0.2 to 1.6% (w/w)).

26. The method of any preceding claim, wherein the cheese contains NaCl in a concentration in the range from 0.5% to 1.3%, such as from 0.6% to 1.1%.

27. The method of any preceding claim, wherein the cheese has a moisture content in the range from 25% to 70%, such as from 25 to 60%.

28. The method of any preceding claim, wherein the coagulant is an aspartic protease, such as chymosin or rennet.

29. The method of any preceding claim, wherein the coagulant is selected from the group consisting of bovine chymosin (such as CHY-MAX®).

30. The method of any preceding claim, wherein the coagulant is selected from the group consisting of camel chymosin (such as CHY-MAX® M).

31. The method of any preceding claim, wherein the storing is performed for a period of 1 to 24 months at a temperature in the range of 5 to 20°C.

32. The method of any preceding claim, which comprises a packaging step (before, during or after step f)).

33. A method for producing a cheese, comprising the following steps:

b) inoculating a milk substrate with a LAB starter culture, which is GAD-negative;

c) adding a camel chymosin to the milk substrate;

d) allowing the resulting product from steps b) and c) to coagulate;

e) using the coagulum from step d) to produce cheese;
f) optionally storing the cheese.

34. A method for producing a cheese, comprising the following steps:
b) inoculating a milk substrate with a LAB starter culture, which is GAD-negative and a *Lb. heiveticus* strain (such as a strain having a high lytic and peptidolytic activity or a strain having proteolytic activity), which is GAD-negative;
c) adding a camel chymosin to the milk substrate;
d) allowing the resulting product from steps b) and c) to coagulate;
e) using the coagulum from step d) to produce cheese;
f) optionally storing the cheese.

35. A method for producing a cheese, comprising the following steps:
b) inoculating a milk substrate with a LAB starter culture, which is GAD-negative;
c) adding a camel chymosin to the milk substrate;
d) allowing the resulting product from steps b) and c) to coagulate;
e) using the coagulum from step d) to produce low-salt cheese;
f) optionally storing the cheese.

36. A method for producing a cheese, comprising the following steps:
b) inoculating a milk substrate with a LAB starter culture, which is GAD-negative and a *Lb. heiveticus* strain (such as strain having a high lytic and peptidolytic activity or a strain having proteolytic activity), which is GAD-negative;
c) adding a camel chymosin to the milk substrate;
d) allowing the resulting product from steps b) and c) to coagulate;
e) using the coagulum from step d) to produce low-salt cheese;
f) optionally storing the cheese.

37. A method for producing a cheese, comprising the following steps:
b) inoculating a milk substrate with a LAB starter culture, which is GAD-negative and a *Lb. heiveticus* strain (such as a strain having a high lytic and peptidolytic activity or a strain having proteolytic activity), which is GAD-negative;
c) adding a coagulant to the milk substrate;
d) allowing the resulting product from steps b) and c) to coagulate;
e) using the coagulum from step d) to produce low-salt cheese;
f) optionally storing the cheese.

38. A method for producing a cheese, comprising the following steps:
b) inoculating a milk substrate with a LAB starter culture, which is GAD-negative;
c) adding a camel chymosin to the milk substrate;

d) allowing the resulting product from steps b) and c) to coagulate;
e) using the coagulum from step d) to produce low-salt Cheddar cheese;
f) optionally storing the cheese.

39. A method for producing a cheese, comprising the following steps:
b) inoculating a milk substrate with a LAB starter culture, which is GAD-negative and a *Lb. heiveticus* strain (such as a strain having a high lytic and peptidolytic activity or a strain having proteolytic activity), which is GAD-negative;
c) adding a camel chymosin to the milk substrate;
d) allowing the resulting product from steps b) and c) to coagulate;
e) using the coagulum from step d) to produce low-salt Cheddar cheese;
f) optionally storing the cheese.

40. A method for producing a cheese, comprising the following steps:
b) inoculating a milk substrate with a LAB starter culture, which is GAD-negative and a *Lb. heiveticus* strain (such as a strain having a high lytic and peptidolytic activity or a strain having proteolytic activity), which is GAD-negative;
c) adding a coagulant to the milk substrate;
d) allowing the resulting product from steps b) and c) to coagulate;
e) using the coagulum from step d) to produce low-salt Cheddar cheese;
f) optionally storing the cheese.

41. The method of any preceding claim, wherein the starter culture contains more than 1 strain, preferably more than 2 different strains, such as more than 3, more than 4, more than 5 or more than 6 different strains.

42. A cheese which is obtainable by the method of any preceding claims.

43. The cheese of the preceding claim, which is a low-salt cheese.

44. The cheese of any preceding claims, which is a Cheddar cheese.

45. A LAB strain, which is GAD-negative.

46. A bacterial strain selected from the group consisting of: CHCC 4356 deposited as DSM 25485, CHCC 4427 deposited as DSM 25486, CHCC 5188 deposited as DSM 25487, CHCC 1916 deposited as DSM 21405 and IG227 deposited as DSM 14797; and mutants or variants thereof.

47. A bacterial strain selected from the group consisting of: CHCC 4356 deposited as DSM 25485, CHCC 4427 deposited as DSM 25486, CHCC 5188 deposited as DSM 25487, CHCC 1916 deposited as DSM 21405 and IG227 deposited as DSM 14797; and mutants thereof, which are GAD-negative.

48. A LAB culture which is GAD-negative.

49. The culture of the preceding claims, which contains 2 to 20 different LAB strains, such as 2 to 20 or 3 to 20 different LAB strains.

50. The culture of any preceding claims, which contains more than 1 strain, preferably more than 2 different strains, such as more than 3, more than 4, more than 5 or more than 6 different strains.

51. The culture of any preceding claim, which contains
1) a bacterial strain selected from the group consisting of: CHCC 4356 deposited as DSM 25485, CHCC 4427 deposited as DSM 25486, CHCC 5188 deposited as DSM 25487, CHCC 1916 deposited as DSM 21405 and IG227 deposited as DSM 14797; and mutants or variants thereof, and
2) a LAB strain, which is GAD-negative and which is different from the strain in 1).

52. Use of a strain of any preceding claim in a process for manufacture of cheese.

53. Use of a culture of any preceding claim in a process for manufacture of cheese.

54. The use of any preceding claim, wherein the cheese is a low-salt cheese.

55. Use of camel chymosin, e.g. CHY-MAX® M, in a process for manufacture of low-salt cheese.

56. The use of any preceding claim, wherein the cheese is Cheddar cheese.

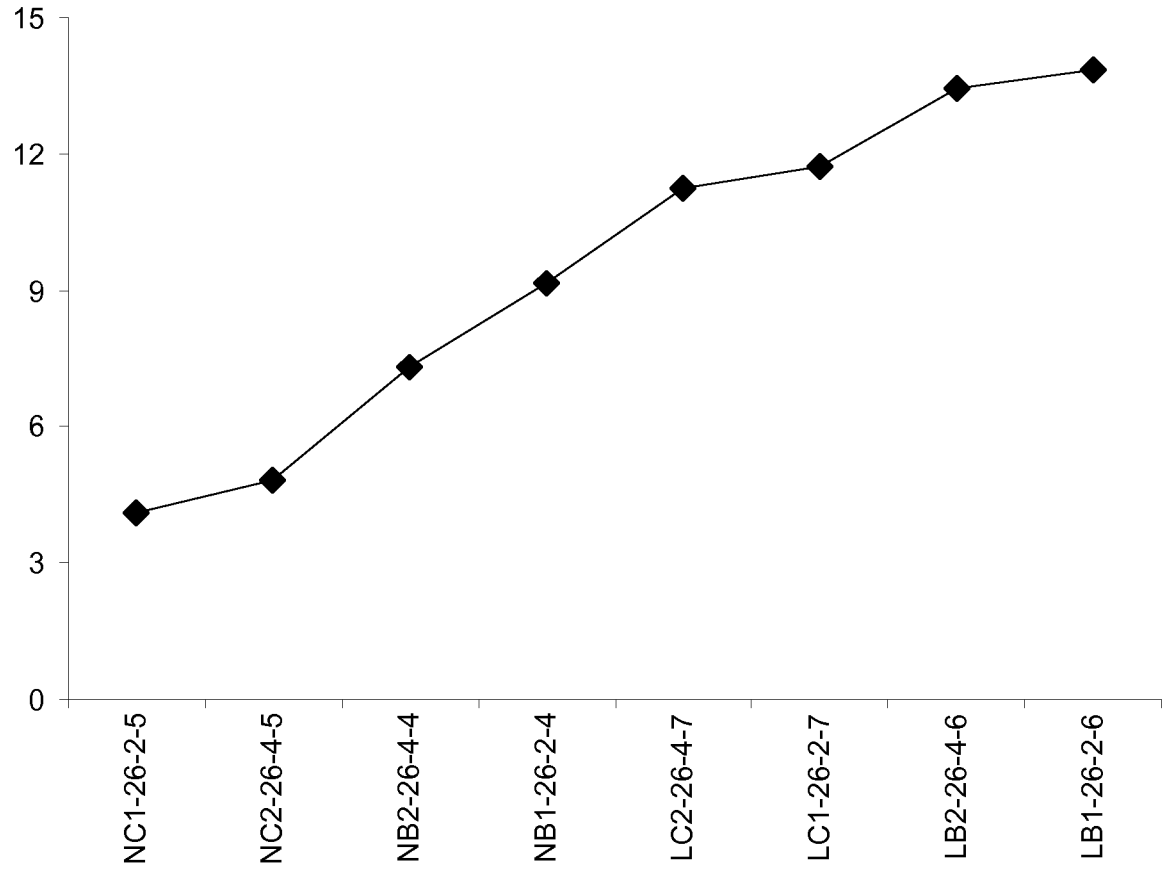
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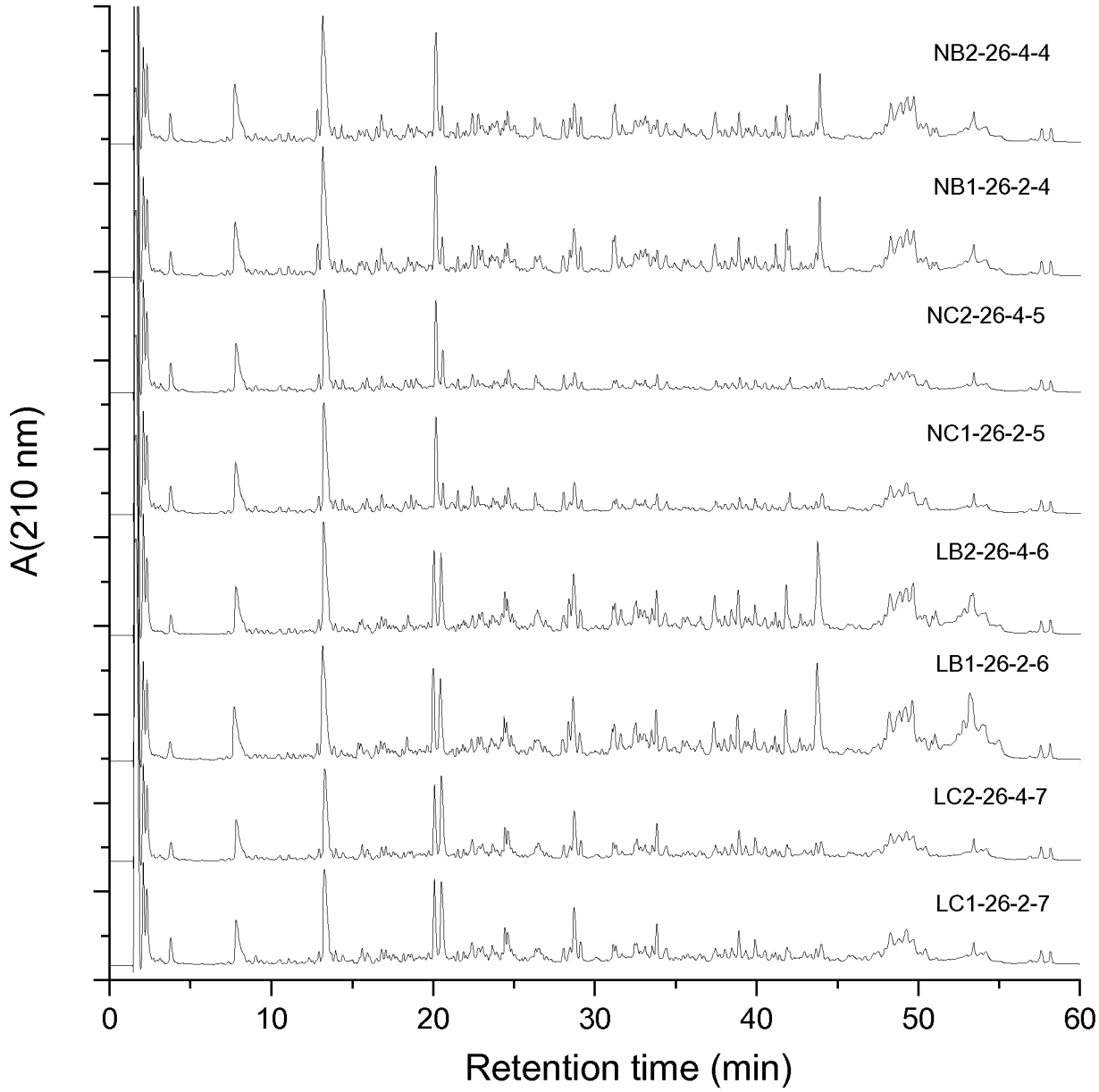
Fig 1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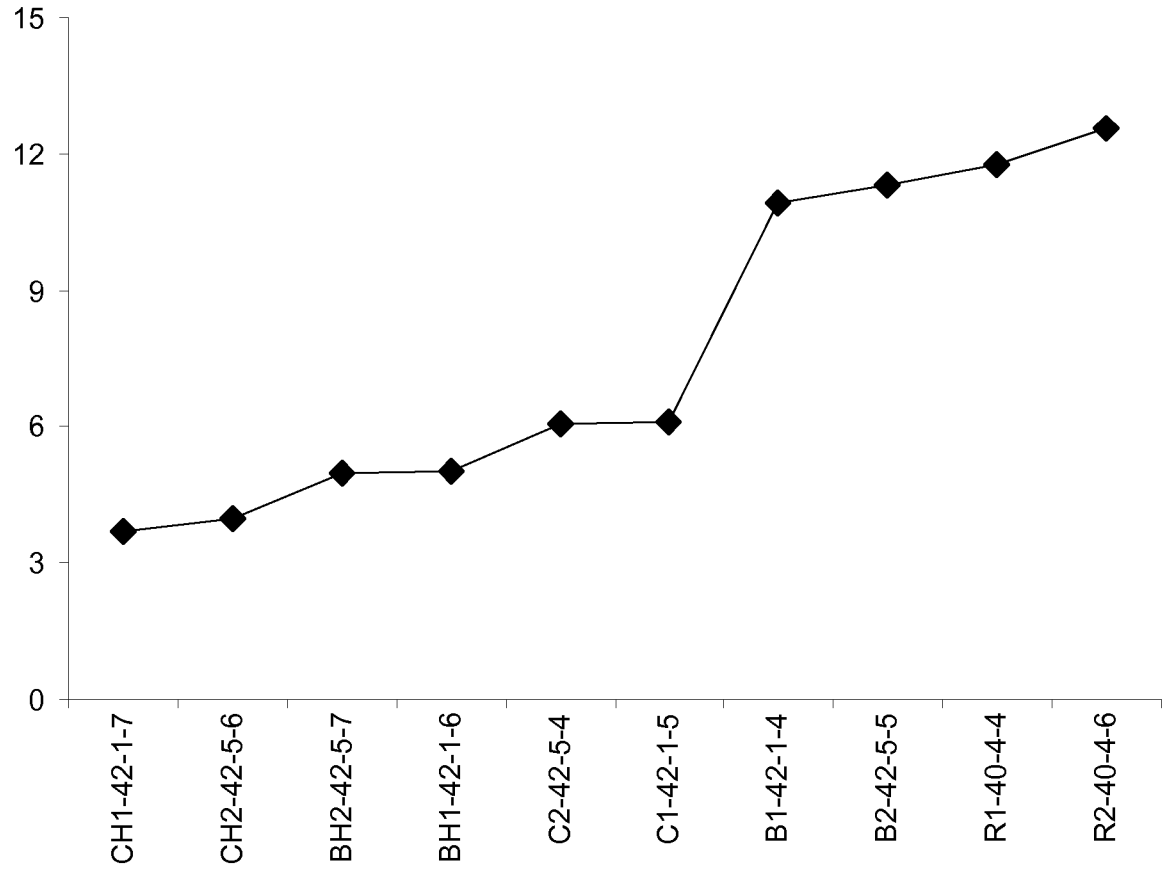
20
30
40
50
60
Retention time (min)

Fig 2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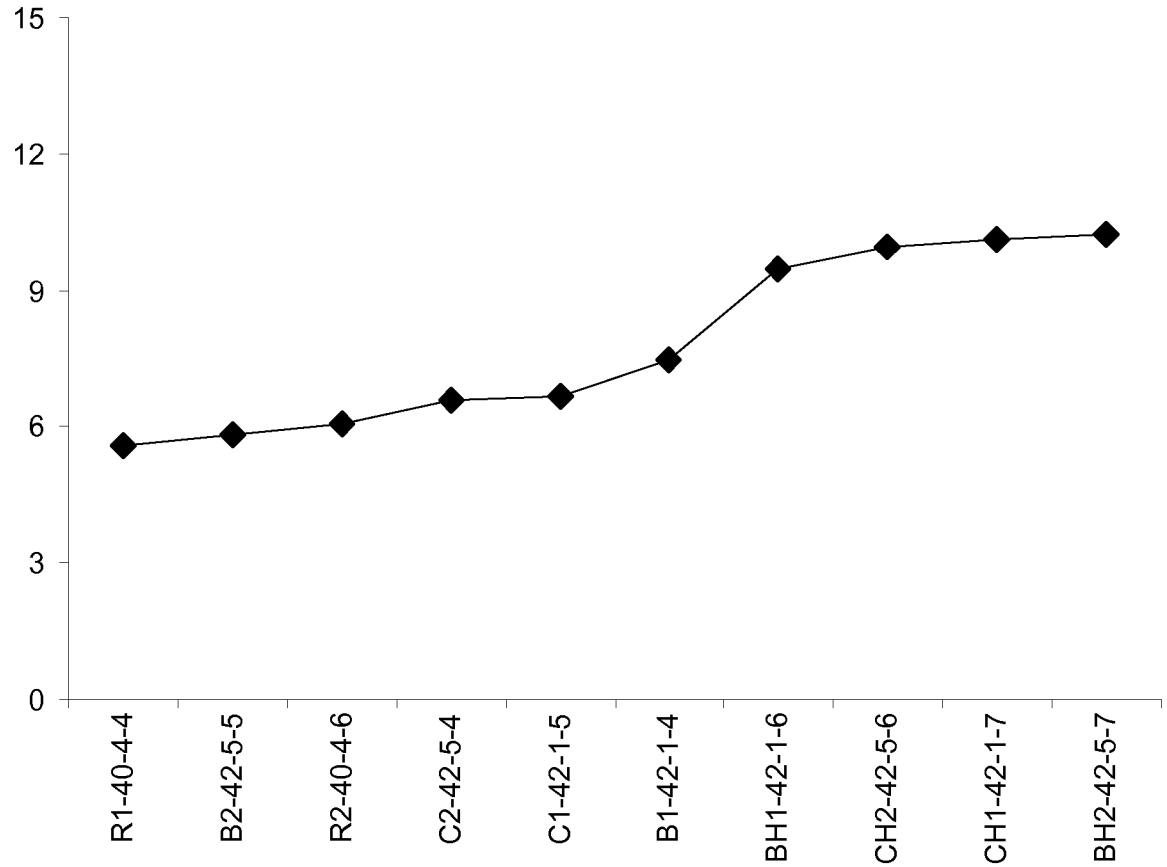
R2-40-4-6

Fig 3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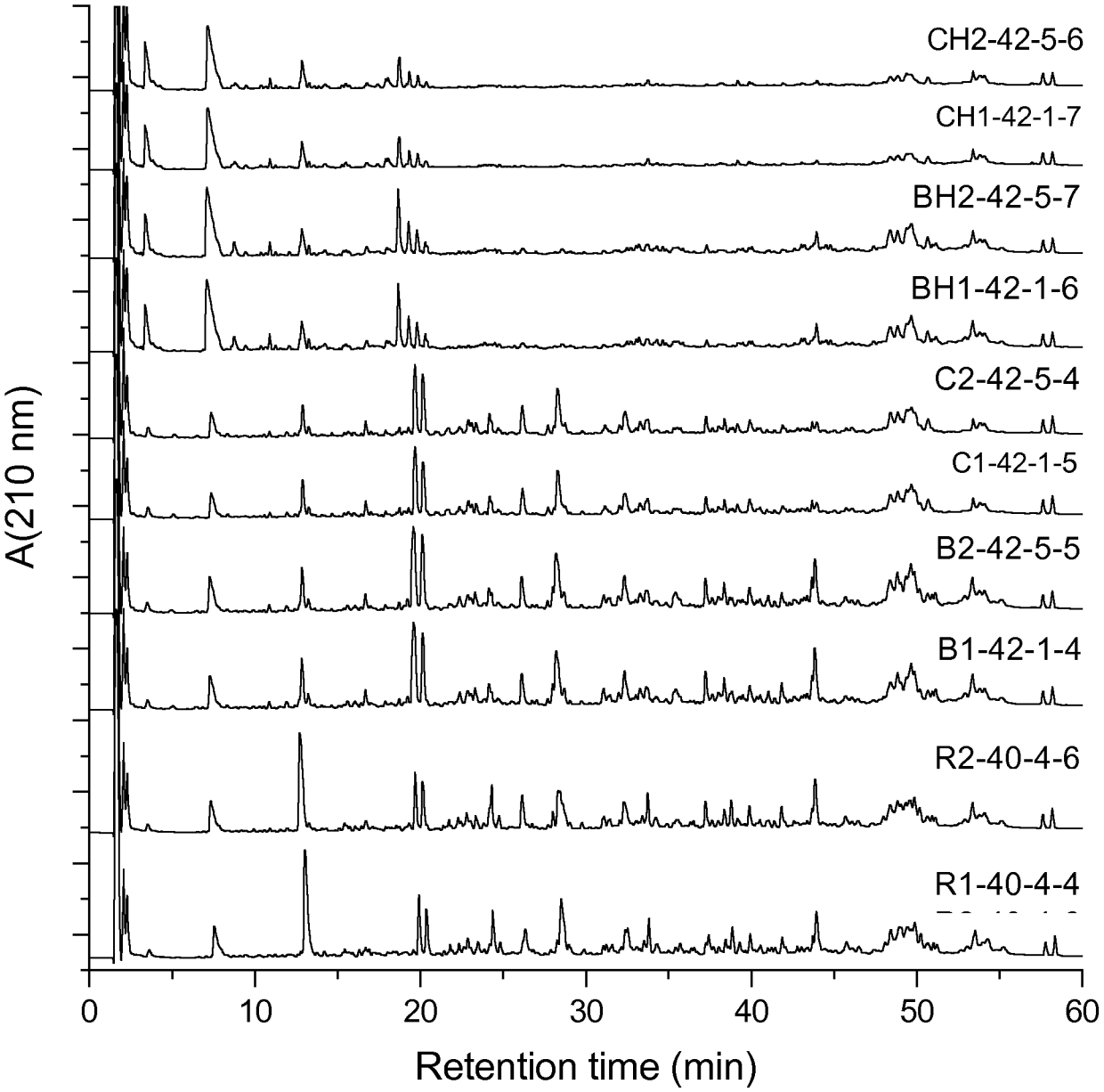
BH2-42-5-7

Fig 4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BH1-42-1-6
C2-42-5-4
C1-42-1-5
B2-42-5-5
B1-42-1-4
R2-40-4-6
R1-40-4-4
A(210 nm)
0
10
20
30
40
50
60
Retention time (min)

Fig 5