



## Pyruvate production through thiamine starvation

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(54) Title: PYRUVATE PRODUCTION THROUGH THIAMINE STARVATION

(57) Abstract: Provided are lactic acid bacteria which are deficient in thiamine and which are deficient in lactate dehydrogenase and/or lactate dehydrogenase activity, methods for producing such bacteria, and methods for producing pyruvate by culturing such bacteria in medium deficient in thiamine and under aeration.



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## PYRUVATE PRODUCTION THROUGH THIAMINE STARVATION

## FIELD OF THE INVENTION

The present invention relates to lactic acid bacteria deficient in lactate dehydrogenase and/or lactate dehydrogenase activity which become efficient pyruvate producers when starved for thiamine, and to methods of producing pyruvate by incubating or culturing such bacteria in thiamine-deficient media.

## BACKGROUND OF THE INVENTION

Pyruvate can be made by classical chemical synthesis or by microbial fermentation, where the latter approach is preferred, especially for sensitive applications such as use in food. In the 1990ies, Toray Industries in Japan acquired a range of patents (see, e.g., US 4,971,907; JPS6455185A (B2); and JPS6455186A (B2)) relating to fermentative production of pyruvate using the multi-vitamin auxotrophic yeast *Candida glabrata* (previously called *Torulopsis glabrata*), and *C. glabrata* continues to be one of the best pyruvate producers available. This yeast, however, is an opportunistic pathogen and not compatible with food fermentations.

*Lactococcus lactis* is a lactic acid bacterium commonly used in food fermentations. Normally, 95% of the sugar consumed by *L. lactis* is converted into lactic acid. However, the metabolic flux can be shifted to make these bacteria produce other products. An overview of glucose metabolism in *L. lactis* is shown in Fig. 1. Recently, *L. lactis* strain FS1076 was shown to be an efficient pyruvate producer (Suo *et al.*, Front. Bioeng. Biotechnol., 2021). The strain is a sub-strain of the strain MG1363, which has been genetically engineered to lack the four enzymes lactate dehydrogenase (LDH), phosphate acetyltransferase (PTA), alcohol dehydrogenase (ADH), and acetolactate synthase (ALS). As can be seen in Fig. 1, all four of these enzymes are involved in the conversion of pyruvate, directly or through the intermediate product Acetyl-CoA into other downstream products. Thus, when these enzymes are lacking, the pyruvate cannot be converted into other products (except Acetyl-CoA), which results in an accumulation of pyruvate in the FS1076 strain cell.

Though, although promising, several genetic modifications were needed to convert these bacteria into efficient pyruvate producers, which may be disadvantageous, both due to the amount of work involved in genetically modifying new bacterial strains to become efficient pyruvate producers, and due to the resistance in many countries against genetically modified organisms (GMO) in food products.

Thus, there is a need for a simpler and potentially non-GMO method for producing pyruvate and downstream products such as the commercially relevant product diacetyl (butter aroma).

#### SUMMARY OF THE INVENTION

5 It has been found by the present inventors that a *Lactococcus lactis* strain, which is deficient in lactate dehydrogenase (LDH), becomes an efficient producer of pyruvate when cultured under conditions where it is starved for thiamine and aerated.

10 Thus, the present invention provides a lactic acid bacterium which efficiently produces pyruvate when grown under certain conditions, and which only requires one genetic modification (causing deficient lactate dehydrogenase and/or lactate dehydrogenase activity). As will be described below, this genetic modification can be easily made using non-GMO methods, such as adaptive evolution and chemical mutagenesis. Therefore, this invention provides a simple and potentially non-GMO method for producing food-grade pyruvate in lactic acid bacteria.

15 So, in a first aspect the present invention relates to a lactic acid bacterium which is deficient in thiamine and which is deficient in lactate dehydrogenase and/or lactate dehydrogenase activity.

In some embodiments, the lactic acid bacterium is unable to produce thiamine.

In some embodiments, the lactic acid bacterium is unable to grow in medium lacking thiamine.

20 In some embodiments, the lactic acid bacterium belongs to any one of the following genera: *Lactococcus*, *Pediococcus*, *Enterococcus*, *Leuconostoc*, *Limosilactobacillus*, *Lactobacillus*, and *Streptococcus*.

In some embodiments, the lactic acid bacterium belongs to, or is derived from, any one of the following species: *Lactococcus lactis*, *Enterococcus faecium*, *Enterococcus casseliflavus*, *Leuconostoc mesenteroides*, *Lactococcus landensis*, *Limosilactobacillus reuteri*, and *Lactobacillus delbrueckii subsp. bulgaricus*.

25 In some embodiments, the lactic acid bacterium belongs to, or is derived from, any one of the following strains: *Lactococcus lactis subsp. biovar diacetylactis* RD1M5, *Enterococcus faecium* L117, *Enterococcus casseliflavus* L142, *Leuconostoc mesenteroides* JCFD003, *Lactococcus landensis* JCFD002, *L. landensis* JCFD006, *L. lactis subsp. cremoris* JCFD012, *L. lactis subsp. biovar diacetylactis* 170B, *L. lactis subsp. biovar diacetylactis* 171B, *L. lactis subsp. biovar*

*diacetylactis* 170A, *L. lactis* subsp. biovar *diacetylactis* SD96, *L. lactis* subsp. biovar *diacetylactis* JC0169A, *L. lactis* subsp. biovar *diacetylactis* JC0169B, *L. lactis* subsp. *lactis* SL195 (JC024), *L. lactis* subsp. *lactis* SL69 (JC023), *L. lactis* subsp. *lactis* JCFD025, *L. lactis* subsp. *lactis* JCFD027, *L. lactis* subsp. *lactis* JCFD026, *L. lactis* subsp. *lactis* 109, *L. lactis* subsp. *lactis* 110, *L. lactis* subsp. *lactis* 111, *L. lactis* subsp. *lactis* 116, *L. lactis* subsp. *lactis* 120, *L. lactis* subsp. *lactis* 118, *L. lactis* subsp. *lactis* 121, *L. lactis* subsp. *lactis* IO1, *L. lactis* subsp. *lactis* KF147, *Limosilactobacillus reuteri* CRL 1099, *L. reuteri* CRL 1100, *L. reuteri* CRL 1101, *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 958, and *L. lactis* subsp. *cremoris* CRL 985.

In a second aspect, the present invention relates to a composition comprising the lactic acid bacterium according to the first aspect, optionally wherein the lactic acid bacterium is in freeze-dried form, and optionally wherein the composition is thiamine-deficient.

In a third aspect, the present invention relates to a method of producing the lactic acid bacterium according to the first aspect, said method comprising:  
culturing a lactic acid bacterium deficient in lactate dehydrogenase and/or lactate dehydrogenase activity in a medium deficient in thiamine and comprising acetate, such as 1.5-150 mM acetate, preferably 15 mM acetate, at 25-35°C for at least 6 hours, optionally wherein the culture has a start cell density corresponding to an  $OD_{600nm} \leq 0.1$ , preferably an  $OD_{600nm} = 0.05$ .

In a fourth aspect, the present invention relates to a method of producing pyruvate, comprising:  
culturing the lactic acid bacterium according to the first aspect in medium deficient in thiamine, and under aeration, thereby obtaining pyruvate.

In some embodiments of the fourth aspect, the lactic acid bacterium is cultured in the presence of acetate, such as 1.5-150 mM acetate, preferably 15 mM acetate.

In some embodiments of the fourth aspect, the lactic acid bacterium is cultured in the presence of a source of oleate, such as in the presence of Tween 80, such as Tween 80 in a concentration of 0.01-1% (w/v), preferably Tween 80 in a concentration of 0.1% (v/v).

In some embodiments of the third or fourth aspect, the medium is thiamine-deficient milk.

In a fifth aspect, the present invention relates to use of the lactic acid bacterium according to the first aspect or the composition according to the second aspect for producing pyruvate.

In a sixth aspect, the present invention relates to a kit comprising a) a first lactic acid bacterium according to the first aspect or the composition according to the second aspect and b) a second lactic acid bacterium, or lysate or cell-free extract thereof, capable of producing one or more of  $\alpha$ -acetolactate, acetoin, and diacetyl from pyruvate, optionally wherein the first and the second lactic acid bacterium, or lysate or cell-free extract thereof, are in separate vials.

In a seventh aspect, the present invention relates to a method of producing one or more of  $\alpha$ -acetolactate, acetoin and diacetyl, comprising the steps of:

- a) culturing the lactic acid bacterium according to the first aspect or the composition according to the second aspect in a culture medium deficient in thiamine, and under aeration, thereby obtaining pyruvate, and
- b) contacting the pyruvate with a second lactic acid bacterium, or lysate or cell-free extract thereof, capable of producing one or more of  $\alpha$ -acetolactate, acetoin, and diacetyl from pyruvate.

#### LEGENDS TO THE FIGURES

Fig. 1: Overview of glucose and lactose metabolism in *Lactococcus lactis*. Abbreviations: PDHC, pyruvate dehydrogenase complex; PFL, pyruvate formate lyase; ALS,  $\alpha$ -acetolactate synthase; LDH, lactate dehydrogenase; ADH, alcohol dehydrogenase; PTA, phosphotransacetylase; ALD,  $\alpha$ -acetolactate decarboxylase; ACK, acetate kinase; DR, diacetyl reductase; Nox, NADH oxidase (Adapted from Ferreira and Mendes-Faia, 2020).

Fig. 2: Overview of glucose and lactose metabolism in *Lactococcus lactis* - with indication of principle of the present invention. Crosses indicate pathways that are blocked in *L. lactis* according to the invention, when starved for thiamine and aerated. Abbreviations: See Fig. 1 legend.

Fig. 3: Impact of initial cell density on pyruvate production by *L. lactis* RD1M5 cultured in SAN medium lacking thiamine. Conical flasks containing SAN medium devoid of thiamine were inoculated with RD1M5 cells to different initial cell densities as indicated and incubated with shaking. Metabolites were measured after 6 h and 16 h. Results are shown as mean  $\pm$  standard deviation for three replicates.

Fig. 4: Effect of incubation temperature on pyruvate production by *L. lactis* RD1M5 cultured in SAN medium lacking thiamine. Metabolites, cell growth ( $OD_{600nm}$ ), and pH, were measured at different time points in cultures incubated at the temperatures indicated. RD1M5 was cultured

in conical flasks with aeration (shaking) using an initial cell density corresponding to an optical density ( $OD_{600nm}$ ) of 0.5. Results are shown as mean  $\pm$  standard deviation for three replicates.

Fig. 5: Effect of acetate and Tween 80 on growth and pyruvate production by *L. lactis* strain RD1M5 in SAN medium lacking thiamine. Metabolites, cell growth ( $OD_{600nm}$ ), and pH were measured at different time points in cultures of RD1M5 incubated in different media, as indicated. SAN<sup>-</sup>: SAN medium without thiamine, SAN<sup>-</sup> + A: SAN medium without thiamine with 15 mM acetate, SAN<sup>-</sup> + A + T: SAN medium without thiamine with 15 mM acetate and with 0.1% Tween 80. RD1M5 cells were grown in conical flasks with aeration (shaking) at 30°C. The initial cell density was adjusted to a start  $OD_{600nm}=0.05$ . Results are shown as mean  $\pm$  standard deviation for three replicates.

Fig. 6: Pyruvate production by non-growing *L. lactis* RD1M5 cells in POM buffer containing 1% glucose. Conical flasks containing different cell densities of RD1M5 cells, as indicated, were incubated at 30°C with shaking. Metabolites were measured after 6 h and 16 h of incubation. Results are shown as mean  $\pm$  standard deviation for three replicates.

Fig. 7: Pyruvate production by *L. lactis* RD1M5 in milk treated to reduce its thiamine content. Conical flasks containing UHT milk, Florisil treated UHT milk, the latter supplemented with the vitamins present in SAN except thiamine, were supplemented with RD1M5 cells to an initial cell density of  $OD_{600nm}=0.05$  and incubated at 30°C with shaking. Metabolites were measured at different time points, as indicated. Untreated UHT milk contained 139.7 mM lactose and 19.3 mM citrate, whereas Florisil-treated UHT milk contained 116.3 mM lactose and 18.6 mM citrate. Results are shown as mean  $\pm$  standard deviation for three replicates.

## DETAILED DISCLOSURE OF THE INVENTION

### Definitions

By "lactic acid bacterium" is meant a bacterium belonging to the *Lactobacillales* order, which normally produces lactic acid as its main product of carbohydrate fermentation. Lactic acid bacteria are Gram-positive, acid-tolerant bacteria that are often used in food fermentations. Examples of lactic acid bacteria include, but are not limited to, bacteria belonging to the genera *Lactococcus*, *Pediococcus*, *Enterococcus*, *Leuconostoc*, *Limosilactobacillus*, *Lactobacillus*, and *Streptococcus*. An example of a species of lactic acid bacteria is *Lactococcus lactis*. A non-limiting list of species of lactic acid bacteria can be found at the National Center for Biotechnology Information (World-Wide Web (www) address

ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Tree&id=186826&lvl; accessed on 18 June 2023, hereby incorporated by reference in its entirety) and elsewhere herein.

Thiamine, also known as vitamin B1, belongs to the group of water-soluble vitamins. As a member of the vitamin B family, thiamine plays a crucial role in supporting various cell functions in living organisms (Tylicki et al., 2018). Thiamine pyrophosphate (TPP) is the active form of intracellular thiamine in *L. lactis* and serves as a coenzyme in numerous enzymatic reactions (Zhu et al., 2021). TPP forms a coordination bond with a metal ion, which helps anchor the cofactor's pyrophosphate moiety to a protein. This interaction occurs through a specific region of the protein structure (Brown et al., 1994). As such, "thiamine", as used herein, can mean thiamine in any form, including TPP, depending on the context.

By "deficient in thiamine", as used herein to describe a bacterium, is meant a bacterial cell which contains less than 500 nmol/g dry cell weight, such as 250 nmol/g dry cell weight, such as 100 nmol/g dry cell weight, such as 50 nmol/g dry cell weight, and such as 10 nmol/g dry cell weight, of thiamine. It may also mean a bacterial cell which contains undetectable levels of thiamine, or which does not contain any thiamine (i.e. which is "thiamine-free"), either due to an inability of the bacterium to produce thiamine, an impaired (such as reduced) ability of the bacterium to take up thiamine, or to it being or having been cultured in a medium which does not contain thiamine or contains very small amounts of thiamine. Preferably, the bacterial cell is deficient in thiamine due to a combination of the above. As such, a bacterium may be deficient in thiamine due to an inability of the bacterium to produce thiamine and it being cultured in a medium which does not contain thiamine or contains very small amounts of thiamine. Alternatively, a bacterium may be deficient in thiamine due to an inability of the bacterium to produce thiamine and a reduced ability to take up thiamine and it being cultured in a medium which only comprises very small amounts of thiamine. A bacterium may also be deficient in thiamine due to a reduced ability of the bacterium to produce thiamine and an inability to take up thiamine. By "undetectable levels of thiamine" is meant an amount of thiamine which is below the detection limit in an assay suitable for measuring thiamine levels, such as an assay described elsewhere herein.

By "deficient in thiamine", as used herein to describe a cell medium or a composition, is meant a medium or composition which contains less than 200 nM, such as less than 150 nM, such as less than 100 nM, such as less than 75 nM, such as less than 50 nM, such as less than 25 nM, and such as less than 10 nM, thiamine. It may also mean that the cell medium or composition contains undetectable levels of thiamine, or that it does not contain any thiamine (i.e. that it is "thiamine-free"). By "undetectable levels of thiamine" is meant an amount of thiamine which is below the detection limit in an assay suitable for measuring thiamine levels, such as an assay described elsewhere herein.

By "lactate dehydrogenase", as used herein, is meant all variants of the enzyme lactate dehydrogenase (LDH), which in *L. lactis*, for example, may be the protein encoded by the *ldh* gene (NCBI locus tag: H0A38\_RS02065). The term may also include any of the proteins encoded by the *ldhB* and *ldhX* genes in *L. lactis*, which have more than 30% sequence identity to the *ldh*-encoded enzyme (Makarova et al., 2006; Liu et al., 2020). By "lactate dehydrogenase activity", as used herein, is meant the enzymatic catalysis by the lactate dehydrogenase enzyme of the conversion of pyruvate to lactate and of NADH to NAD<sup>+</sup>.

That the bacterium is "deficient in lactate dehydrogenase", as used herein, can mean that the amount of LDH enzyme or functional LDH enzyme in the bacterium is reduced. In this context, "reduced" typically means that the amount of LDH protein or functional LDH protein present in the cell is reduced by at least 70%, such as at least 75%, such as at least 85%, such as at least 90%, such as at least 95%, as compared to a control. Typically, when the reduced expression is obtained by a genetic modification in the host cell, the control is the unmodified host cell, which means the strain from which the cell deficient in lactate dehydrogenase is derived but which does not comprise the mutation(s) causing the deficiency in lactate dehydrogenase. Methods for measuring mRNA or protein levels, such as qPCR and quantitative western blot, are generally known in the art. That the bacterium is "deficient in lactate dehydrogenase", as used herein, may also mean that the bacterium does not contain any LDH enzyme or any functional LDH enzyme, for example due to knockout of the gene encoding LDH or knockdown of its expression.

The term "knockdown", as used herein, refers to any of a range of techniques resulting in reduced expression of a gene in a host cell, such as introduction of a mutation in a promoter.

The term "knockout", as used herein, refers to any of a range of techniques resulting in abolished expression of a gene in a host cell, such as introduction of a mutation in, or deletion of, the gene. The term "deletion", as used herein, refers to a partial or complete removal of the coding sequence of a gene, which either results in abolished expression of that gene or in the expression of a non-functional gene product.

That the bacterium is "deficient in lactate dehydrogenase activity", as used herein, means that the bacterium can produce no more than 30 mM, such as no more than 25 mM, such as no more than 20 mM, such as no more than 15 mM, such as no more than 10 mM, and such as no more than 5 mM, lactate, when cultured in medium with 1% glucose, with a start OD<sub>600nm</sub>=10, for 16 hours at 30°C with 200 rpm shaking. These conditions were tested in Example 6 herein (results shown in Fig. 6(d)), where less than 25 mM lactate was produced.

The term "deficient in lactate dehydrogenase activity", as used herein, can also mean that the bacterium produces undetectable levels of lactate, or that it cannot produce any lactate, i.e., the lactate dehydrogenase activity may be reduced or abolished. The production of lactate may be measured using liquid chromatography. Such a method is described under "Analytical methods" in Example 1.

By "acetolactate decarboxylase", as used herein, is meant all variants of the enzyme acetolactate decarboxylase (ALD). In *L. lactis*, for example, ALD is the protein encoded by the gene *aldB* (NCBI locus tag: limg\_1275). By "acetolactate decarboxylase activity", as used herein, is meant the enzymatic catalysis by the acetolactate decarboxylase enzyme of the conversion of acetolactate to acetoin and CO<sub>2</sub>.

That a bacterial strain is "deficient in acetolactate decarboxylase", as used herein, can mean that the amount of ALD enzyme or functional ALD enzyme in the bacterium is reduced. In this context, "reduced" typically means that the amount of ALD protein present in the cell is reduced by at least 50%, such as at least 60%, such as at least 75%, such as at least 90%, such as at least 95%, as compared to a control. Typically, when the reduced expression is obtained by a genetic modification in the host cell, the control is the unmodified host cell, which means the strain from which the cell deficient in acetolactate decarboxylase is derived but which does not comprise the mutation(s) causing the deficiency in acetolactate decarboxylase. Methods for measuring mRNA or protein levels, such as qPCR and quantitative western blot, are generally known in the art. That the bacterium is "deficient in acetolactate decarboxylase", as used herein, may also mean that the bacterium does not contain any ALD enzyme or any functional ALD enzyme, for example due to knockout of the gene encoding ALD or knockdown of its expression.

That a bacterial strain is "deficient in acetolactate decarboxylase activity", as used herein, means that the concentration of pyruvate in the bacterium is at least 10%, preferably at least 50%, higher than the concentration of acetoin in the cell, i.e. that the acetoin:pyruvate ratio in the bacterium is at most 1:1.1, such as at most 1:1.2, such as at most 1:1.3, such as at most 1:1.4, and preferably no more than 1:1.5. Alternatively or additionally, it can mean that the bacterium produces undetectable levels of acetoin, or that it cannot produce any acetoin, i.e., the acetolactate decarboxylase activity may be reduced or abolished. The production of acetoin and pyruvate can be measured using liquid chromatography. Such a method is described under "Analytical methods" in Example 1.

By "culturing", as used herein, is meant that bacteria are incubated in a medium. The bacteria may grow during the incubation, or they may not grow. By "growth" is meant multiplication of cells. Bacterial growth can be assessed by determining if the OD<sub>600nm</sub> in the bacterial culture

increases over time by measuring it at different time points during incubation, as is exemplified in the Examples section herein.

"Sequence identity" is in the context of the present invention determined by comparing two optimally aligned sequences of equal length (e.g., DNA) according to the following formula:  
5 (N<sub>ref</sub> - N<sub>dif</sub>)·100/N<sub>ref</sub>, wherein N<sub>ref</sub> is the number of residues in one of the two sequences and N<sub>dif</sub> is the number of residues which are non-identical in the two sequences when they are aligned over their entire lengths and in the same direction. So, two sequences 5'-ATTCGGAA-3' and 5'-ATACGGGA-3' will provide the sequence identity 75% (N<sub>ref</sub>=8 and N<sub>dif</sub>=2).

#### *Specific embodiments of the invention*

10 It has been found by the present inventors that the *Lactococcus lactis* strain RD1M5, which is deficient in lactate dehydrogenase (LDH), becomes an efficient producer of pyruvate when cultured under conditions where it is starved for thiamine and aerated.

The enzyme pyruvate formate lyase (PFL) is oxygen sensitive and is rapidly inactivated in aerated cultures (Takahashi et al., 1987; Zelcbuch et al., 2016), and the enzymes pyruvate  
15 dehydrogenase complex (PDHc) and α-acetolactate synthase (ALS) use thiamine, also known as vitamin B1, as a co-factor, and depend on it to function (Nam et al., 2004). RD1M5 is a thiamine auxotroph, meaning that it cannot produce thiamine itself (see Table 2). Thus, when this strain is cultured in medium lacking thiamine, it is effectively starved for thiamine, and both PDHc and ALS are inactive. When it is at the same time cultured under aeration, PFL is  
20 also inactive, and since the strain is deficient in LDH, the result is that all pathways that catabolize pyruvate in this bacterial strain are inactive (see Fig. 2), thereby resulting in an accumulation of pyruvate (see the Examples herein).

Thus, as described above, the present invention provides a lactic acid bacterium which  
25 efficiently produces pyruvate when grown under conditions where it is thiamine-starved and aerated, and which advantageously only requires one genetic modification (causing deficient lactate dehydrogenase and/or lactate dehydrogenase activity). As described above, this genetic modification can be easily made using non-GMO methods, such as adaptive evolution and chemical mutagenesis. Thereby, the present invention provides a simple and potentially non-GMO method for producing food-grade pyruvate in lactic acid bacteria.

A thiamine- and lactate dehydrogenase/lactate dehydrogenase activity-deficient lactic acid bacterium

In a first aspect, the present invention relates to a lactic acid bacterium which is deficient in thiamine, and which is deficient in lactate dehydrogenase and/or lactate dehydrogenase activity.

In some embodiments, the lactic acid bacterium is unable to produce thiamine.

In some embodiments, the lactic acid bacterium is unable to grow in medium lacking thiamine.

In some embodiments, the lactic acid bacterium has an impaired uptake of thiamine.

In some embodiments, the lactic acid bacterium contains less than 500 nmol/g dry cell weight, such as 250 nmol/g dry cell weight, such as 100 nmol/g dry cell weight, such as 50 nmol/g dry cell weight, and such as 10 nmol/g dry cell weight, of thiamine.

Quantification of thiamine levels, either in cells or in cell medium, may be performed using an assay such as the assay described in Masuda et al., 2012 (hereby incorporated by reference herein in its entirety), in the section "Thiamine determination" on page 2062. In Masuda et al., the thiamine concentration is measured in cell culture supernatant and in cells, whereas, in the context of the present disclosure, the thiamine concentration may be measured using this method in cells and any type of cell medium.

Some species of lactic acid bacteria, including *L. lactis*, normally grow in SAN medium, whereas other species of lactic acid bacteria require other growth media. The exact composition of SAN medium can be seen in Table 1, and an experimental procedure that can be used to determine whether a given lactic acid bacterial strain, which can grow in SAN medium, can also grow in SAN medium without thiamine, is described in Examples 1 and 2. Lactic acid bacteria that are able to grow in standard thiamine-containing SAN medium and unable to grow in SAN medium lacking thiamine are considered to be unable to produce thiamine. For lactic acid bacteria that cannot grow in SAN medium, their ability to grow in thiamine-deficient medium can be tested using other growth media as appropriate.

As described in Example 2, using this method, the present inventors have identified several strains of lactic acid bacteria that are unable to produce thiamine. The tested bacterial strains were mainly isolated from sourdough, cheese and beans, and out of 28 strains tested, 27 were found to be thiamine auxotrophs (unable to produce thiamine; these are listed in Table 2).

If genetically altered to become deficient in lactate dehydrogenase and/or lactate dehydrogenase activity, all 27 of these strains identified as thiamine auxotrophs are expected to be suitable for use according to the present invention. Furthermore, other strains belonging to any one of the genera of the 27 strains are also expected to have a high likelihood of being thiamine auxotrophs. These genera include: *Lactococcus*, *Pediococcus*, *Enterococcus*, *Leuconostoc*, *Limosilactobacillus*, *Lactobacillus*, and *Streptococcus*. This can be tested for each strain using the method described in Example 2.

Thus, in some embodiments, the lactic acid bacterium belongs to any one of the following genera: *Lactococcus*, *Pediococcus*, *Enterococcus*, *Leuconostoc*, *Limosilactobacillus*, *Lactobacillus*, and *Streptococcus*.

In addition to the 27 strains identified as thiamine-auxotrophic herein and listed in Table 2 and below, the lactic acid bacteria strains *Limosilactobacillus reuteri* CRL 1099, *L. reuteri* CRL 1100, *L. reuteri* CRL 1101, *Lactobacillus delbrueckii subsp. bulgaricus* CRL 958, and *L. lactis subsp. cremoris* CRL 985 have previously been characterized as thiamine auxotrophic (Teran et al., 2021). These strains are likewise expected to be suitable for use according to the present invention, if genetically altered to become deficient in lactate dehydrogenase and/or lactate dehydrogenase activity.

Thus, in some embodiments, the lactic acid bacterium belongs to, or is derived from, any one of the following species: *Lactococcus lactis*, *Enterococcus faecium*, *Enterococcus casseliflavus*, *Leuconostoc mesenteroides*, *Lactococcus landensis*, *Limosilactobacillus reuteri*, and *Lactobacillus delbrueckii subsp. bulgaricus*.

In some embodiments, the lactic acid bacterium belongs to, or is derived from, any one of the following strains: *Lactococcus lactis subsp. biovar diacetylactis* RD1M5, *Enterococcus faecium* L117, *Enterococcus casseliflavus* L142, *Leuconostoc mesenteroides* JCFD003, *Lactococcus landensis* JCFD002, *L. landensis* JCFD006, *L. lactis subsp. cremoris* JCFD012, *L. lactis subsp. biovar diacetylactis* 170B, *L. lactis subsp. biovar diacetylactis* 171B, *L. lactis subsp. biovar diacetylactis* 170A, *L. lactis subsp. biovar diacetylactis* SD96, *L. lactis subsp. biovar diacetylactis* JC0169A, *L. lactis subsp. biovar diacetylactis* JC0169B, *L. lactis subsp. lactis* SL195 (JC024), *L. lactis subsp. lactis* SL69 (JC023), *L. lactis subsp. lactis* JCFD025, *L. lactis subsp. lactis* JCFD027, *L. lactis subsp. lactis* JCFD026, *L. lactis subsp. lactis* 109, *L. lactis subsp. lactis* 110, *L. lactis subsp. lactis* 111, *L. lactis subsp. lactis* 116, *L. lactis subsp. lactis* 120, *L. lactis subsp. lactis* 118, *L. lactis subsp. lactis* 121, *L. lactis subsp. lactis* IO1, *L. lactis subsp. lactis* KF147, *Limosilactobacillus reuteri* CRL 1099, *L. reuteri* CRL 1100, *L. reuteri* CRL 1101, *Lactobacillus delbrueckii subsp. bulgaricus* CRL 958, and *L. lactis subsp. cremoris* CRL 985.

In some embodiments, the lactic acid bacterium belongs to any one of the strains *L. lactis subsp. biovar diacetylactis* JC0169A and *L. lactis subsp. biovar diacetylactis* JC0169B.

In some embodiments, the lactic acid bacterium belongs to the strain *L. lactis subsp. biovar diacetylactis* RD1M5.

5 The strain *L. lactis subsp. biovar diacetylactis* RD1M5 (herein simply referred to as RD1M5) is a natural (non-GMO) dairy isolate (Liu et al., 2020) which is a natural thiamine auxotroph (see Example 2; Table 2) and lacks lactate dehydrogenase as a result of chemical mutagenesis and selection. In RD1M5, the *ldh* gene, encoding lactate dehydrogenase in *L. lactis*, has an eight-nucleotide insertion which causes a frameshift mutation. The result is decreased lactate  
10 formation by this strain (Liu et al., 2020).

In some embodiments, the lactic acid bacterium belongs to the strain *L. lactis subsp. biovar diacetylactis* RD1M5 or has at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, and such as at least 99%, sequence identity to this strain.

15 In a previous study, it was found that some lactic acid bacteria from nukazuke (a type of Japanese fermented food) produce thiamine, but at a very low level close to the detection limit, whereas others are thiamine auxotrophs (Masuda et al., 2012). An explanation for these low thiamine levels could be that a sufficient amount of thiamine is present in foods to support the growth of thiamine-requiring microorganisms, e.g. the thiamine content of milk, wheat bread  
20 and beans is 0.33 mg/L, 5.5 µg/g, and 5.6 µg/g, respectively (Demigne et al., 2005; Holmes et al., 1943; Lucia et al., 2000). This may have led to the loss of the ability of some lactic acid bacteria to synthesize thiamine during evolution. For this reason, and in line with the findings in Example 2 herein, there is a high chance that any given lactic acid bacterial strain, when tested for it, e.g. using the method described in Example 2, will prove to be a thiamine  
25 auxotroph.

For the purpose of the present invention, lactic acid bacteria that are not thiamine auxotrophs can be made thiamine auxotrophic using the non-GMO method described below, i.e. by random mutagenesis and selection.

30 Thus, a given lactic acid bacteria strain can be made thiamine auxotrophic, or impaired in its ability to take up thiamine, or deficient in lactate dehydrogenase and/or lactate dehydrogenase activity, or any combination of these, using genetic modifications such as targeted genome modifications or, preferably, using non-GMO methods such as random mutagenesis and selection, or any combination thereof.

Genetic alterations resulting in reduced or abolished activity of lactate dehydrogenase can include a mutation or deletion in the coding sequence of that protein which results in the expression of non-functional or less functional protein. Furthermore, genetic alterations resulting in reduced or abolished expression of the lactate dehydrogenase gene and/or reduced or abolished activity of lactate dehydrogenase, as used herein, may be indirect, meaning that they are not genetic alterations in the gene itself. Such genetic alterations may for example include the introduction of a nucleic acid sequence that reduces the expression of the lactate dehydrogenase gene, *e.g.*, a repressor that inhibits expression of the gene.

In some embodiments, the amount of LDH protein or functional LDH protein present in the cell is reduced by at least 70%, such as at least 75%, such as at least 80%, such as at least 85%, such as at least 90%, such as at least 95%, as compared to a control cell.

In *L. lactis*, for example, the enzyme lactate dehydrogenase (LDH), the activity of which is reduced or abolished, may be the protein encoded by the *ldh* gene (NCBI locus tag: H0A38\_RS02065). However, it may also be the protein encoded by the *ldhB* or *ldhX* genes.

The control cell to which the LDH amount or activity is to be compared is usually the unmodified host cell, which means the strain from which the cell deficient in lactate dehydrogenase and/or lactate dehydrogenase is derived but which does not comprise the mutation(s) causing the deficiency in lactate dehydrogenase (activity).

For the *L. lactis* strain RD1M5, the control cell may be the strain SD96 from which it is derived (Liu et al., 2020).

In some embodiments, the bacterium can produce no more than 30 mM, such as no more than 25 mM, such as no more than 20 mM, such as no more than 15 mM, such as no more than 10 mM, and such as no more than 5 mM, lactate, when cultured in medium with 1% glucose, with a start  $OD_{600nm}=10$ , for 16 hours at 30°C with 200 rpm shaking.

Standard recombinant DNA and molecular cloning techniques useful for carrying out embodiments of the present invention are well known in the art and are described by, *e.g.*, Sambrook, J., Fritsch, E. F., and Maniatis, T. (2012). *Molecular cloning: A laboratory manual*, 4th ed. Cold Spring Harbor Laboratory: Cold Spring Harbor, New York, and by Silhavy, T. J., Bannan, M. L., and Enquist, L. W. (1984). *Experiments with gene fusions*. Cold Spring Harbor Laboratory: Cold Spring Harbor, New York. Techniques for targeted genome editing, such as knockout of a target gene in a bacterial genome, include Clustered regularly interspaced short palindromic repeats (CRISPR)-based systems, such as CRISPR-Cas9.

In addition to targeted genome editing techniques, a particularly preferred method of the present invention, e.g. for reducing or abolishing the activity of lactate dehydrogenase, is random mutagenesis and selection. This method can also be used for creating thiamine auxotrophic mutants from a strain that produces thiamine or for creating mutants that have an impaired uptake of thiamine. An example of such a method is mutagenesis followed by a penicillin enrichment step and screening (or in short "penicillin enrichment"). In the following, the principle of this method will be described, using the generation of thiamine auxotrophic mutants as an example:

Exposure to the mutagen will result in random mutations in the genome, some of which occur in the thiamine biosynthesis genes. Mutations in thiamine biosynthesis genes will lead to loss of function/inability to synthesize thiamine. To enrich for mutants unable to synthesize thiamine, the library of mutated bacteria can be transferred to a defined medium without thiamine which contains penicillin, or another antibiotic that only kills actively growing cells ( $\beta$ -lactam antibiotics, cycloserine). Mutants unable to synthesize thiamine will not grow, and hence will survive this treatment. The survivors are plated on solid defined medium containing thiamine, which will allow auxotrophic mutants to grow, and then replica-plated onto solid medium without thiamine. Colonies only appearing on the first plate with thiamine, but not on the plate without thiamine, will be the thiamine auxotrophic mutants.

Penicillin enrichment can also be used to generate mutants that have an impaired ability to take up thiamine from the culture medium, for example using the following protocol:

Exposure to the mutagen will result in random mutations in the genome, some of which occur in genes involved in thiamine transport. Mutations in thiamine transport genes potentially can lead to loss of function/reduced ability to take up thiamine from the cell's surroundings. To enrich for mutants with an impaired uptake of thiamine, the library of mutated bacteria can be transferred to a defined medium containing "normal" levels of thiamine which also contains penicillin, or another antibiotic that only kills actively growing cells ( $\beta$ -lactam antibiotics, cycloserine). Mutants with an impaired ability to take up thiamine will not grow, and hence will survive this treatment. The survivors are plated on solid defined medium containing a "high" level of thiamine ("high" relative to the "normal" level), which will allow mutants with impaired thiamine uptake to grow, and then replica-plated onto solid medium with a "normal" level of thiamine. Colonies only appearing on the first plate with a "high" level of thiamine, but not on the plate with a "normal" level of thiamine, will be the mutants with impaired thiamine uptake.

A composition comprising a thiamine-deficient lactic acid bacterium

In a second aspect, the present invention relates to a composition comprising the lactic acid bacterium according to embodiments of the first aspect of the invention, optionally wherein the lactic acid bacterium is in freeze-dried form, and optionally wherein the composition is

5 thiamine-deficient.

The embodiments of the first aspect of the invention described above also apply to the second aspect of the invention.

In some embodiments, the composition comprises a cell medium or buffer. In further embodiments, the cell medium or buffer is thiamine-deficient.

10 In some embodiments, the composition comprises a lyophilization (freeze-drying) medium or components of a lyophilization medium.

The skilled person will know which cell medium, buffer, and/or lyophilization medium to use with a given strain of lactic acid bacteria.

15 When the composition comprising the lactic acid bacterium according to the invention is in freeze-dried form, it has the advantage that it can be stored for long periods of time and transported long distances.

A method of producing a thiamine-deficient lactic acid bacterium

In a third aspect, the present invention relates to a method of producing the lactic acid bacterium according to embodiments of the first aspect of the invention, said method

20 comprising:

culturing a lactic acid bacterium deficient in lactate dehydrogenase and/or lactate dehydrogenase activity in a medium deficient in thiamine and comprising acetate, such as 1.5-150 mM acetate, preferably 15 mM acetate, at 25-35°C for at least 6 hours, wherein the culture has a start cell density corresponding to an  $OD_{600nm} \leq 0.1$ , preferably an  $OD_{600nm} = 0.05$ .

25 The embodiments described above relating to the bacterium according to the first aspect of the invention also apply to the bacterium which is the product of the method according to the third aspect of the invention.

Thus, the following embodiments likewise apply to the lactic acid bacterium deficient in lactate dehydrogenase and/or lactate dehydrogenase activity which is cultivated according to the third aspect of the invention:

5 In some embodiments, the lactic acid bacterium deficient in lactate dehydrogenase and/or lactate dehydrogenase activity belongs to any one of the following genera: *Lactococcus*, *Pediococcus*, *Enterococcus*, *Leuconostoc*, *Limosilactobacillus*, *Lactobacillus*, and *Streptococcus*.

10 In some embodiments, the lactic acid bacterium belongs to, or is derived from, any one of the following species: *Lactococcus lactis*, *Enterococcus faecium*, *Enterococcus casseliflavus*, *Leuconostoc mesenteroides*, *Lactococcus landensis*, *Limosilactobacillus reuteri*, and *Lactobacillus delbrueckii subsp. bulgaricus*.

15 In some embodiments, the lactic acid bacterium deficient in lactate dehydrogenase and/or lactate dehydrogenase activity belongs to, or is derived from, any one of the following strains: *Lactococcus lactis subsp. biovar diacetylactis RD1M5*, *Enterococcus faecium L117*, *Enterococcus casseliflavus L142*, *Leuconostoc mesenteroides JCFD003*, *Lactococcus landensis JCFD002*, *L. landensis JCFD006*, *L. lactis subsp. cremoris JCFD012*, *L. lactis subsp. biovar diacetylactis 170B*, *L. lactis subsp. biovar diacetylactis 171B*, *L. lactis subsp. biovar diacetylactis 170A*, *L. lactis subsp. biovar diacetylactis SD96*, *L. lactis subsp. biovar diacetylactis JC0169A*, *L. lactis subsp. biovar diacetylactis JC0169B*, *L. lactis subsp. lactis SL195 (JC024)*, *L. lactis subsp. lactis SL69 (JC023)*, *L. lactis subsp. lactis JCFD025*, *L. lactis subsp. lactis JCFD027*, *L. lactis subsp. lactis JCFD026*, *L. lactis subsp. lactis 109*, *L. lactis subsp. lactis 110*, *L. lactis subsp. lactis 111*, *L. lactis subsp. lactis 116*, *L. lactis subsp. lactis 120*, *L. lactis subsp. lactis 118*, *L. lactis subsp. lactis 121*, *L. lactis subsp. lactis IO1*, *L. lactis subsp. lactis KF147*, *Limosilactobacillus reuteri CRL 1099*, *L. reuteri CRL 1100*, *L. reuteri CRL 1101*, *Lactobacillus delbrueckii subsp. bulgaricus CRL 958*, and *L. lactis subsp. cremoris CRL 985*.

25 In some embodiments, the lactic acid bacterium deficient in lactate dehydrogenase and/or lactate dehydrogenase activity belongs to any one of the strains *L. lactis subsp. biovar diacetylactis JC0169A* and *L. lactis subsp. biovar diacetylactis JC0169B*.

In some embodiments, the lactic acid bacterium deficient in lactate dehydrogenase and/or lactate dehydrogenase activity belongs to the strain *L. lactis subsp. biovar diacetylactis RD1M5*.

30 In some embodiments, the lactic acid bacterium deficient in lactate dehydrogenase and/or lactate dehydrogenase activity belongs to the strain *L. lactis subsp. biovar diacetylactis RD1M5* or has at least 90%, such as at least 91%, such as at least 92%, such as at least 93%, such

as at least 94%, such as at least 95%, such as at least 96%, such as at least 97%, such as at least 98%, and such as at least 99%, sequence identity to this strain.

In some embodiments, the lactic acid bacterium deficient in lactate dehydrogenase and/or lactate dehydrogenase activity is deficient in lactate dehydrogenase and/or lactate dehydrogenase activity as a result of some type of targeted genetic modification, whereas in  
5 other embodiments it is due to non-GMO methods, such as random mutagenesis and selection. Therefore, in some embodiments, the method is preceded by a step of generating and selecting a lactic acid bacterium deficient in lactate dehydrogenase and/or lactate dehydrogenase activity, optionally by a method of performing targeted genomic modifications and/or by a non-  
10 GMO method, such as random mutagenesis and selection.

In some embodiments, the lactic acid bacterium deficient in lactate dehydrogenase and/or lactate dehydrogenase activity is unable to produce thiamine (i.e., is thiamine auxotrophic) and/or has an impaired uptake of thiamine. In further embodiments, the inability to produce thiamine and/or the impaired thiamine uptake is a result of some type of targeted genetic  
15 modification, whereas in other embodiments it is due to non-GMO methods, such as random mutagenesis and selection.

Therefore, in some embodiments, the method is preceded by a step of generating and selecting a lactic acid bacterium, which is:

20 A) deficient in lactate dehydrogenase and/or lactate dehydrogenase activity, and  
B) unable to produce thiamine and/or impaired in its ability to take up thiamine;  
optionally by a method of performing targeted genomic modifications and/or by a non-GMO method, such as random mutagenesis and selection.

As such, in some embodiments, the characteristics under A), the characteristics under B), or the characteristics under both A) and B), have been generated and selected using targeted genomic modifications, whereas in other embodiments, the characteristics under A), the characteristics under B), or the characteristics under both A) and B), have been generated and selected using a non-GMO method, such as random mutagenesis and selection.  
25

In some embodiments, the medium contains less than 200 nM, such as less than 150 nM, such as less than 100 nM, such as less than 75 nM, such as less than 50 nM, such as less than 25 nM, and such as less than 10 nM, thiamine.  
30

Quantification of thiamine levels, either in cells or in cell medium, may be performed using an assay such as the assay described in Masuda et al., 2012 in the section "Thiamine determination" on page 2062. In Masuda et al., the thiamine concentration is measured in cell

culture supernatant and in cells, whereas, in the context of the present disclosure, the thiamine concentration may be measured using this method in cells and any type of cell medium.

The person skilled in the art will know which thiamine-deficient medium to choose for a given strain of lactic acid bacteria. An example of such a medium is SAN<sup>-thiamine+acetate</sup> medium.

- 5 In some embodiments, the medium is thiamine-deficient milk.

A method of producing pyruvate using a thiamine-deficient lactic acid bacterium

In a fourth aspect, the present invention relates to a method of producing pyruvate, comprising:

- 10 culturing the lactic acid bacterium according to embodiments of the first aspect of the invention in medium deficient in thiamine, and under aeration, thereby obtaining pyruvate.

The embodiments described above relating to the bacterium according to the first aspect of the invention also apply to the lactic acid bacterium which is cultivated according to the fourth aspect of the invention.

- 15 As described for the third aspect above, in some embodiments, the lactic acid bacterium deficient in lactate dehydrogenase and/or lactate dehydrogenase activity is deficient in lactate dehydrogenase and/or lactate dehydrogenase activity as a result of some type of targeted genetic modification, whereas in other embodiments it is due to non-GMO methods, such as random mutagenesis and selection. Therefore, in some embodiments, the method is preceded by a step of generating and selecting a lactic acid bacterium deficient in lactate dehydrogenase and/or lactate dehydrogenase activity, optionally by a method of performing targeted genomic modifications and/or by a non-GMO method, such as random mutagenesis and selection.
- 20

- In some embodiments, the lactic acid bacterium deficient in lactate dehydrogenase and/or lactate dehydrogenase activity is unable to produce thiamine (i.e., is thiamine auxotrophic) and/or has an impaired uptake of thiamine. In further embodiments, the inability to produce thiamine and/or the impaired thiamine uptake is a result of some type of targeted genetic modification, whereas in other embodiments it is due to non-GMO methods, such as random mutagenesis and selection.
- 25

- Therefore, in some embodiments, the method is preceded by a step of generating and selecting a lactic acid bacterium, which is:
- 30

A) deficient in lactate dehydrogenase and/or lactate dehydrogenase activity, and

B) unable to produce thiamine and/or impaired in its ability to take up thiamine; optionally by a method of performing targeted genomic modifications and/or by a non-GMO method, such as random mutagenesis and selection.

5 As such, in some embodiments, the characteristics under A), the characteristics under B), or the characteristics under both A) and B), have been generated and selected using targeted genomic modifications, whereas in other embodiments, the characteristics under A), the characteristics under B), or the characteristics under both A) and B), have been generated and selected using a non-GMO method, such as random mutagenesis and selection.

10 In some embodiments, the lactic acid bacterium is cultured in the presence of acetate, such as 1.5-150 mM acetate, preferably 15 mM acetate.

In some embodiments, the lactic acid bacterium grows during the cultivation, whereas in other embodiments the lactic acid bacterium is not growing during the cultivation.

15 In some embodiments, the lactic acid bacterium is cultured in the presence of a source of oleate, such as in the presence of Tween 80, such as Tween 80 in a concentration of 0.01-1% (v/v), preferably Tween 80 in a concentration of 0.1% (v/v).

In some embodiments, the lactic acid bacterium is cultivated at 25-35°C, such as at 25°C, such as at 26°C, such as at 27°C, such as at 28°C, such as at 29°C, such as at 30°C, such as at 31°C, such as at 32°C, such as at 33°C, such as at 34°C, and such as at 35°C, preferably at 30°C.

20 In some embodiments, the lactic acid bacterium is cultivated between 6 and 16 hours, such as for 6 hours, such as for 7 hours, such as for 8 hours, such as for 9 hours, such as for 10 hours, such as for 11 hours, such as for 12 hours, such as for 13 hours, such as for 14 hours, such as for 15 hours, and such as for 16 hours, preferably for 6 hours.

25 The person skilled in the art will know which thiamine-deficient medium to choose for a given strain of lactic acid bacteria. Examples of such media include SAN<sup>thiamine</sup> medium, and POM buffer with 1% glucose.

In preferred embodiments, the medium is thiamine-deficient milk.

Use of a thiamine-deficient lactic acid bacterium for producing pyruvate

In a fifth aspect, the present invention relates to use of the lactic acid bacterium according to the first aspect of the invention or the composition according to the second aspect of the invention for producing pyruvate.

5 A kit comprising a thiamine-deficient lactic acid bacterium and a second lactic acid bacterium

In a sixth aspect, the present invention relates to a kit comprising a) a first lactic acid bacterium according to the first aspect of the invention or the composition according to the second aspect of the invention and b) a second lactic acid bacterium, or lysate or cell-free extract thereof, capable of producing one or more of  $\alpha$ -acetolactate, acetoin, and diacetyl from pyruvate,  
10 optionally wherein the first and the second lactic acid bacterium, or lysate or cell-free extract thereof, are in separate vials.

In one embodiment, the second lactic acid bacterium, or lysate or cell-free extract thereof, is deficient in acetolactate decarboxylase and/or acetolactate decarboxylase activity.

In some embodiments, the amount of acetolactate decarboxylase (ALD) protein in the second  
15 lactic acid bacterium, or lysate or cell-free extract thereof, is reduced by at least 50%, such as at least 60%, such as at least 75%, such as at least 90%, such as at least 95%, as compared to a control.

Typically, when the reduced expression is obtained by a genetic modification in the host cell, the control is the unmodified host cell, which means the strain from which the cell deficient in  
20 acetolactate decarboxylase is derived but which does not comprise the mutation(s) causing the deficiency in acetolactate decarboxylase.

Lactic acid bacteria suitable for use as the second lactic acid bacterium according to the sixth aspect of the invention include *Streptococcus spp.* and *Lactococcus spp.* bacteria, such as bacteria selected from any one of the species: *Streptococcus agalactiae*, *S. bovis*, *S. casei*, *S. cremoris*, *S. durans*, *S. diacetylactis*, *S. faecalis*, *S. faecium*, *S. lactis*, *S. thermophilus*,  
25 *Lactococcus lactis ssp. lactis* (such as *L. lactis ssp. lactis* biovar. *diacetylactis* D1), and *L. lactis ssp. cremoris* (such as *L. lactis* subsp. *cremoris* MG1363).

Strains of all of these species have been reported as being able to produce products such as  $\alpha$ -acetolactate and/or diacetyl from pyruvate, e.g. wherein the strain is deficient in acetolactate  
30 decarboxylase and/or acetolactate decarboxylase activity (Godtfredsen *et al.*, Carlsberg Res.

Commun., 1983; Dorau et al., Microb. Cell Fact., 2019; Monnet et al., Journal of Dairy Science, 1994; EP0247646 B1 (Unilever NV, 1989); EP0483888 B1 (Unilever NV, 1995)). For example, the II-cat01 sub-strain of *L. lactis* subsp. *cremoris* MG1363 (MG1363  $\Delta$ ldhB  $\Delta$ ldhX  $\Delta$ pta  $\Delta$ adhE  $\Delta$ butBA  $\Delta$ aldB) is deficient in both acetolactate decarboxylase and acetolactate decarboxylase activity (Dorau et al., Microb. Cell Fact., 2019).

The kit according to the sixth aspect of the invention enables a method for producing one or more of  $\alpha$ -acetolactate, acetoin and diacetyl, as described below.

A method of producing one or more of  $\alpha$ -acetolactate, acetoin and diacetyl using a thiamine-deficient lactic acid bacterium and a second lactic acid bacterium

- 10 In a seventh aspect, the present invention relates to a method of producing one or more of  $\alpha$ -acetolactate, acetoin and diacetyl, comprising the steps of:
- a) culturing the lactic acid bacterium according to the first aspect of the invention or the composition according to the second aspect of the invention in a culture medium deficient in thiamine, and under aeration, thereby obtaining pyruvate, and
  - 15 b) contacting the pyruvate with a second lactic acid bacterium, or lysate or cell-free extract thereof, capable of producing one or more of  $\alpha$ -acetolactate, acetoin, and diacetyl from pyruvate.

The embodiments described for the second lactic acid bacterium, or lysate or cell-free extract thereof, according to the sixth aspect of the invention also apply to the second lactic acid bacterium, or lysate or cell-free extract thereof, according to the seventh aspect of the invention.

In embodiments where the second lactic acid bacterium, or lysate or cell-free extract thereof, is deficient in acetolactate decarboxylase activity, it may mean that the production of acetoin is so low that the concentration of  $\alpha$ -acetolactate in the cell medium or composition, after incubation or contact of the bacterium, or lysate or cell-free extract thereof, with the pyruvate produced by the first lactic acid bacterium, remains at least 10%, preferably at least 50%, higher than the concentration of acetoin, i.e. the acetoin: $\alpha$ -acetolactate ratio in the cell medium or composition is at least 1:1.1, such as at least 1:1.2, such as at least 1:1.3, such as at least 1:1.4, and preferably more than 1:1.5.

## EXAMPLE 1

*General methods*Reagents and growth medium

Glucose, lactose, yeast extract, sodium acetate, sodium chloride, tween 80, casein peptone,  
5 magnesium chloride, magnesium sulfate, potassium dihydrogen phosphate, dipotassium  
phosphate, potassium sulfate, potassium hydroxide, ammonium chloride, ascorbic acid, MOPS,  
adenosine, guanosine, thymidine, inosine, uridine, biotin, pyridoxal-HCl, folic acid, riboflavin,  
niacinamide pantothenate, L-alanine, L-glutamate, proline, L-arginine, glycine, L-lysine, L-  
10 phenylalanine, L-threonine, L-asparagine, L-glutamine, L-leucine, L-methionine, L-histidine,  
tyrosine, cysteine, tricine, ferrous sulfate, calcium chloride, magnesium chloride, manganese  
chloride, cobalt chloride, copper sulfate, zinc sulfate, boric acid and ammonium  
heptamolybdate were purchased from Sigma-Aldrich (Darmstadt, Germany). TTC (2,3,5-  
triphenyltetrazolium chloride), Florisil, L-isoleucine, peptone, and agar were purchased from  
Merck Millipore (Darmstadt, Germany). Cytidine was purchased from TCI Chemicals (Japan).

15 The POM buffer contained 1 mM MgCl<sub>2</sub>, 50 mM K<sub>2</sub>HPO<sub>4</sub>, and 50 mM KH<sub>2</sub>PO<sub>4</sub> to adjust the pH to  
7.4. For washing cells, 0.9% (w/v) NaCl was used. POM buffer and 0.9% NaCl solutions were  
sterilized by autoclavation for 15 min at 121°C.

Ultra-heat-treated (UHT) milk (15 g/L fat) was purchased from the local supermarket (Milsani,  
Albertslund, Denmark).

20 TTC plates were prepared as described previously, however, with some modifications (Monnet  
et al., 2000). Briefly, 15 g casein peptone, 5 g yeast extract, 0.5 g MgSO<sub>4</sub>, 15 agar, and 0.5 g  
ascorbic acid were dissolved in 890 mL water. The pH was adjusted to 7.0 and autoclaved for  
15 min at 121°C. Lactose with 10% (w/v) was also autoclaved in the same way. After cooling  
to 50°C, 10 mL filtered sterile TTC solution (10 g/L) and 100 mL lactose (100 g/L) were added.

25 The agar medium was swirled gently and poured into Petri dishes.

M17 was bought from oxoid (Thermo Fisher Scientific, USA) and supplemented with 1% lactose  
(LM17). Briefly, 37.25 g M17 powder was dissolved in 900 mL of distilled water and sterilized  
by autoclaving at 121°C for 15 minutes. After cooling to 50°C, 100 mL sterile lactose solution  
(10% w/v) was aseptically added to the autoclaved M17. In the slant medium, 15 g/L agar was  
30 added.

SAN medium (does not contain acetate) is a chemically defined minimal growth medium for cultivating *L. lactis* (Jensen and Hammer, 1993). Briefly, SAN<sup>-thiamine+acetate</sup> medium is based on SAN medium without thiamine and with 15 mM acetate added. SAN<sup>-thiamine+acetate+Tween 80</sup> is SAN<sup>-thiamine+acetate</sup> medium with 0.1% tween 80 added.

Table 1: Composition of SAN medium (adapted from Jensen and Hammer, 1993)

Constituent	Concentration in medium (mM)
NH <sub>4</sub> Cl	9.5
K <sub>2</sub> SO <sub>4</sub>	2.8×10 <sup>-1</sup>
KH <sub>2</sub> PO <sub>4</sub>	1.3
Glucose	55
MOPS	40
Tricine	4
NaCl	50
CaCl <sub>2</sub>	5×10 <sup>-4</sup>
MgCl <sub>2</sub>	5.2×10 <sup>-1</sup>
FeSO <sub>4</sub>	1×10 <sup>-2</sup>
(NH <sub>4</sub> ) <sub>6</sub> (MO <sub>7</sub> ) <sub>24</sub>	3×10 <sup>-6</sup>
H <sub>3</sub> BO <sub>3</sub>	4×10 <sup>-4</sup>
CoCl <sub>2</sub>	4×10 <sup>-5</sup>
CuSO <sub>4</sub>	1×10 <sup>-5</sup>
MnCl <sub>2</sub>	8×10 <sup>-5</sup>
ZnSO <sub>4</sub>	1×10 <sup>-5</sup>
L-Alanine	3.4
L-Arginine	1.1
L-Asparagine	8×10 <sup>-1</sup>
L-Cysteine	8×10 <sup>-1</sup>
L-Glutamate	2.1
L-Glutamine	7×10 <sup>-1</sup>
Glycine	2.7
L-Histidine	3×10 <sup>-1</sup>
L-Isoleucine	8×10 <sup>-1</sup>
L-Leucine	8×10 <sup>-1</sup>
L-Lysine-HCl	1.4
L-Methionine	7×10 <sup>-1</sup>
L-Phenylalanine	1.2
L-Proline	2.6
L-Serine	2.9
L-Threonine	1.7
L-Tryptophan	5×10 <sup>-1</sup>
L-Tyrosine	3×10 <sup>-1</sup>
L-Valine	9×10 <sup>-1</sup>

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Biotin	$4 \times 10^{-4}$
Pyridoxal-HCl	$1 \times 10^{-2}$
Folic acid	$2.3 \times 10^{-3}$
Riboflavin	$2.6 \times 10^{-3}$
Niacinamide	$8 \times 10^{-3}$
Thiamine-HCl	$3 \times 10^{-3}$
Pantothenate	$2 \times 10^{-3}$
<i>Adenosine</i>	$7.5 \times 10^{-5}$
<i>Guanosine</i>	$7 \times 10^{-5}$
<i>Cytidine</i>	$8.2 \times 10^{-5}$
<i>Thymidine</i>	$8.3 \times 10^{-5}$
<i>Inosine</i>	$7.5 \times 10^{-5}$
<i>Uridine</i>	$8.2 \times 10^{-5}$

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#### Lactic acid bacteria strains and cultivation

Lactic acid bacteria isolates from Technical University of Denmark's private culture collection, 28 in total, were used in the experiments described in Example 2 below. They are listed in  
5 Table 2, which shows the results of the experiments of Example 2.

*L. lactis* subsp. *lactis* biovar diacetylactis RD1M5 (RD1M5) is a strain of technological interest. This strain is an LDH-deficient strain obtained via adaptive evolution and chemical mutagenesis from *L. lactis* subsp. *lactis* biovar diacetylactis SD96 (Liu et al., 2020).

For cultivation, RD1M5 was streaked on a TTC plate for 24 h at 30°C. TTC is a water-soluble  
10 compound, which can be reduced to the red compound triphenyl formazan (TPF) if the pH is not too low. Lactate dehydrogenase deficient strains form red colonies on TTC plates as they are unable to form lactic acid and thus the pH in the vicinity of the colony is high enough for TTC to be reduced (Praveen-Kumar & Tarafdar, Biology and Fertility of Soils, 2003; Tanaka et al., Journal of Bioscience and Bioengineering, 2021). A single red colony of RD1M5 was  
15 inoculated into 50 mL LM17 (0.5% lactose), followed by culturing at 30°C with 200 rpm shaking for 10 h to prepare a pre-culture. For other strains, the frozen culture was spread on LM17 (0.5% lactose) agar plates for 24 h at 30°C, and then a single colony was inoculated into 50 mL LM17 (0.5% lactose) and incubated at 30°C without shaking for 10 h to prepare a pre-culture.

### Analytical methods

The pyruvate, glucose, acetate, lactate, citrate, and acetoin concentrations were determined utilizing an advanced high-performance liquid chromatography system incorporating an Aminex HPX-87H column (Bio-Rad, Hercules, USA) and a Shodex RI-101 refractive index detector (Showa Denko K.K., Tokyo, Japan). Moreover, pyruvate was quantified utilizing a UV detector set at a wavelength of 210 nm. The mobile phase, consisting of 5 mM H<sub>2</sub>SO<sub>4</sub>, was used at a flow rate of 0.5 mL/min, and the column oven temperature was consistently maintained at 60°C.

### EXAMPLE 2

#### 10 *Screening lactic acid bacteria for thiamine auxotrophy*

The purpose of this experiment was to identify thiamine-auxotrophic lactic acid bacteria strains, which may be suitable for use according to the present invention.

### Methods

A single colony from an LM17 agar plate was inoculated into liquid LM17 medium in a conical flask which subsequently was incubated at 30°C for 16 h. Cells were harvested by centrifugation at 10,000 rpm for 5 min, and the pellet washed three times with 0.9% NaCl solution, followed by resuspension in the same solution to attain the original volume. Each cell suspension was inoculated into 5 mL of SAN<sup>thiamine</sup> and SAN medium to an initial optical density (OD<sub>600nm</sub>) of 0.05 and incubated at 30°C for 24 h. After this, each culture was subcultured four times in the same medium for 24 h under the same conditions using a 1% inoculum during each transfer. Strains displaying good growth in SAN medium and no growth in SAN<sup>thiamine</sup> medium (observed by increased optical density), were defined as thiamine auxotrophs, i.e. unable to produce thiamine (Teran et al., 2021).

### Results

25 As shown in Table 1, different *L. lactis*, *Enterococcus* and *Leuconostoc* strains, mainly isolated from dairy, sourdough and beans, were screened as described above to determine if they were thiamine auxotrophs. Out of 28 tested strains, only one strain (*L. lactis subsp. lactis* 108) was

found to be able to synthesize thiamine to support its growth (it could grow in SAN<sup>-thiamine</sup> medium). All other tested strains, including RD1M5, were found to be thiamine auxotrophs.

Table 2: Growth of lactic acid bacterial strains in SAN<sup>-thiamine</sup> medium

	Microorganism	Source	Growth in SAN <sup>-thiamine</sup> medium
1	<i>Enterococcus faecium</i> L117	Cheese	-
2	<i>Enterococcus casseliflavus</i> L142	Cheese	-
3	<i>Leuconostoc mesenteroides</i> JCFD003	Cheese	-
4	<i>Lactococcus landensis</i> JCFD002	Cheese	-
5	<i>Lactococcus landensis</i> JCFD006	Cheese	-
6	<i>L. lactis</i> subsp. <i>cremoris</i> JCFD012	Cheese	-
7	<i>L. lactis</i> subsp. <i>biovar diacetylactis</i> 170B	Cheese	-
8	<i>L. lactis</i> subsp. <i>biovar diacetylactis</i> 171B	Cheese	-
9	<i>L. lactis</i> subsp. <i>biovar diacetylactis</i> 170A	Cheese	-
10	<i>L. lactis</i> subsp. <i>biovar diacetylactis</i> SD96	Cheese	-
11	<i>L. lactis</i> subsp. <i>biovar diacetylactis</i> RD1M5	Developed from SD96	-
12	<i>L. lactis</i> subsp. <i>biovar diacetylactis</i> JC0169A	Cheese	-
13	<i>L. lactis</i> subsp. <i>biovar diacetylactis</i> JC0169B	Cheese	-
14	<i>L. lactis</i> subsp. <i>lactis</i> SL195(JC024)	Cheese	-
15	<i>L. lactis</i> subsp. <i>lactis</i> SL69 (JC023)	Cheese	-
16	<i>L. lactis</i> subsp. <i>lactis</i> JCFD025	Cheese	-
17	<i>L. lactis</i> subsp. <i>lactis</i> JCFD027	Cheese	-
18	<i>L. lactis</i> subsp. <i>lactis</i> JCFD026	Cheese	-
19	<i>L. lactis</i> subsp. <i>lactis</i> 108	Sourdough	+
20	<i>L. lactis</i> subsp. <i>lactis</i> 109	Sourdough	-
21	<i>L. lactis</i> subsp. <i>lactis</i> 110	Sourdough	-
22	<i>L. lactis</i> subsp. <i>lactis</i> 111	Sourdough	-
23	<i>L. lactis</i> subsp. <i>lactis</i> 116	Sourdough	-
24	<i>L. lactis</i> subsp. <i>lactis</i> 120	Sourdough	-
25	<i>L. lactis</i> subsp. <i>lactis</i> 118	Sourdough	-
26	<i>L. lactis</i> subsp. <i>lactis</i> 121	Sourdough	-
27	<i>L. lactis</i> subsp. <i>lactis</i> IO1	Beans	-
28	<i>L. lactis</i> subsp. <i>lactis</i> KF147	Beans	-

## EXAMPLE 3

*Pyruvate production by RD1M5 in SAN medium without thiamine under aerated conditions*

The purpose of this experiment was to test whether the RD1M5 strain can produce pyruvate when cultured under conditions where it is starved for thiamine and aerated (due to the mechanisms described under *Summary of the invention* above).

Methods

RD1M5 was cultured in 50 mL LM17 medium overnight, and cells harvested by centrifugation at 10,000 RPM for 5 minutes. The pellet obtained was re-suspended in 0.9% NaCl and again harvested by centrifugation. This last step was repeated 3 times to remove traces of culture medium. Finally, the cells were re-suspended in SAN<sup>thiamine</sup> medium to a cell density corresponding to OD<sub>600nm</sub>=10. To measure absorbance, a V-1200 spectrophotometer (Radnor, Pennsylvania, USA) was used. This dense cell suspension was diluted in SAN<sup>thiamine</sup> medium to obtain 50 ml suspensions of cells with cell density corresponding to OD<sub>600nm</sub>-values of 0.05, 0.25, 0.5, 1, and 2, respectively. The samples were incubated at 30°C with 200 rpm shaking, and after 6 h and 16 h samples were acquired for HPLC analysis, and pH was determined concurrently.

Results

Production of pyruvate and other metabolites by RD1M5 in SAN<sup>thiamine</sup> medium was tested using different start cell densities and after 6 and 16 hours of culture. As is shown in Fig. 3(a), the bacteria successfully produced pyruvate. The highest pyruvate concentration obtained was 21.8 mM, after 16 hours of culturing. In this case, the initial cell density corresponded to OD<sub>600nm</sub>=0.25. In general, the pyruvate concentration, both measured after 6 h and 16 h, initially increased with increasing start cell density, peaking at OD<sub>600nm</sub>=0.5 for the 6 h measurement and at OD<sub>600nm</sub>=0.25 for the 16 h measurement, before decreasing at higher start cell densities. An explanation for this decrease could be that although the medium is devoid of thiamine, the cells still contain sufficient amounts of thiamine/TPP to maintain some PDHc and ALS activity. So, as more cells are added into the medium, there will be a carry-over of thiamine or TPP, resulting in a residual PDHc and PFL activity that slowly depletes the pyruvate accumulated. Fig. 3(c) and (e) also support this hypothesis. The higher the start cell density, the more acetoin and acetate was produced; both downstream products of PDHc and ALS enzymatic activity (see Fig. 1). Fig. 3(d) shows that the LDH-deficient strain could

surprisingly produce some lactate (at a maximum concentration of 8.5 mM). Like the pyruvate concentration, the lactate concentration starts to decrease when the start cell densities increase beyond a certain point; again, this may be explained by higher levels of thiamine/TPP in the medium due to more cells, whereby PDHc and ALS can keep some of their function and to some extent keep the NAD<sup>+</sup>/NADH balance.

The arguably best pyruvate production reached in our experiment was 21.4 mM after 6 h with a start cell density of OD<sub>600nm</sub>=0.5.

#### EXAMPLE 4

##### *Influence of incubation temperature on pyruvate production by RD1M5*

In Example 3 it was shown that the RD1M5 strain of *L. lactis* can produce pyruvate when it is starved for thiamine and aerated. The purpose of this experiment was to test the influence of incubation temperature on this process.

##### Methods

Suspensions (50 ml) of RD1M5 in SAN<sup>-thiamine</sup> medium adjusted to a cell density of 0.5 were prepared as described in Example 3. These were incubated at different temperatures: 25, 30, 35, 39, and 45°C with 200 rpm shaking, and samples were taken at various times for HPLC analysis. The pH and optical density (OD<sub>600nm</sub>) were measured concurrently.

##### Results

As is shown in Fig. 4(a), the highest pyruvate production was 23.9 mM at 30°C after 6 h, which decreased to 18.6 mM after 16 h. When the RD1M5 cells were incubated at 25°C, pyruvate production continued to increase until 16 h. Fig. 4(e) also shows that at 25°C the cells grew slower compared to cells grown at 30 and 35°C, but the cell density still reached its highest level, with an OD<sub>600nm</sub>=3.56. Our results also showed that at 45°C, the RD1M5 cellular metabolic activity that we monitored was inhibited. Nearly no pyruvate accumulated, and other metabolites were also consumed and generated slowly. As is shown in Fig. 4(f) and (g), at 45°C the cell density increased from OD<sub>600nm</sub>=0.5 to 0.9, and the pH dropped from 7.11 to 6.74.

## EXAMPLE 5

*Pyruvate production by growing cells & effect of acetate/Tween 80*

Acetyl-CoA is essential for fatty acid synthesis, which supports *L. lactis* growth. The acetyl-CoA can be synthesized from acetate by acetate kinase and phosphotransacetylase, which has been shown to increase the pool of intracellular acetyl-CoA (Nordkvist et al., 2003). Lactic acid bacteria are dependent on biotin for their growth. However, it has been documented that oleate or its derivative, such as Tween 80, can effectively substitute for biotin. Tween 80 is recognized for its ability to supply exogenous oleic acid, which can integrate into the membranes of lactic acid bacteria and stimulate their proliferation (Potter & Elvehjem, 1947; Station & Rouge, 1945). The purpose of this experiment was to test if it is possible to produce thiamine-deficient cells that can produce pyruvate by culturing RD1M5 cells in SAN<sup>-thiamine</sup> medium with addition of acetate and Tween 80.

Methods

Three media were used in this experiment: SAN<sup>-thiamine</sup> medium, SAN<sup>-thiamine+acetate</sup> medium with the additive of 15 mM sodium acetate, and SAN<sup>-thiamine+acetate+Tween 80</sup> medium containing 15 mM sodium acetate and 0.1% Tween 80. All of the chemically defined media were filtered with a 0.22 µm bottle-top filter (Thermo Scientific, America) to sterilize them. The start cell density was adjusted to OD<sub>600nm</sub>=0.05, and the cells were washed thrice with 0.9% NaCl. Then the cells were added to the three different compositions of SAN medium and incubated at 30°C with 200 rpm shaking. Samples were taken at various times to determine metabolites by HPLC. Also, the pH and cell density were measured.

Results

As shown in Fig. 5(f), SAN<sup>-thiamine</sup> medium could not support the growth of RD1M5. In contrast, RD1M5 grew in both SAN<sup>-thiamine+acetate</sup> and SAN<sup>-thiamine+acetate+Tween 80</sup> medium, where a cell density corresponding to an OD<sub>600nm</sub> of about 1.0 was obtained after 16 h. RD1M5 grew faster in the medium containing Tween 80 than in the SAN<sup>-thiamine+acetate</sup> medium. The results in Fig. 5(a), (b), (c), and (d) show that the RD1M5 cells grown in SAN<sup>-thiamine+acetate</sup> and SAN<sup>-thiamine+acetate+Tween 80</sup> medium produce pyruvate, lactate, and acetoin and consume glucose to a similar degree. A high yield of 31.1 mM pyruvate was obtained from a culture with a start cell density corresponding to an OD<sub>600nm</sub> of 0.05 after 16 h at 30°C (Fig. 5(a)). Thus, the results indicate that the Tween 80 does not increase the glycolysis process but that it increases cell growth.

## EXAMPLE 6

*Pyruvate production by non-growing thiamine-starved cells in POM buffer (1% glucose)*

The purpose of this experiment was to test if thiamine-starved RD1M5 cells can produce pyruvate in a different medium than SAN medium, i.e. POM buffer with 1% glucose.

5 Methods

RD1M5 cells were incubated in LM17 medium, growing at 30°C for 16 h. Cells were harvested by centrifugation, and washed three times with the same volume of 0.9% NaCl solution. To obtain thiamine-starved cells, the washed cells were added to SAN<sup>-thiamine+acetate</sup> medium adjusted to a start cell density corresponding to an OD<sub>600nm</sub> of 0.05 and grown for 16 h at 30°C with 200 rpm shaking, after which the cells were harvested by centrifugation and washed three times with 0.9% NaCl solution. The thiamine-starved cells were re-suspended in 0.9% NaCl solution. In 10 mL POM buffer (1% glucose), the resuspended cells were adjusted to an OD<sub>600nm</sub> of 0.5, 1, 3 and 10, respectively. After that, the cells were centrifuged, and the supernatant was removed. Different densities of cells were resuspended in 10 mL of POM buffer (1% glucose) in a 250 mL shaking flask, and the cells were incubated at 30°C with 200 rpm shaking. Metabolite analysis and pH measurement was conducted on samples collected at both 6 h and 16 h.

Results

As shown in Fig. 6(a), pyruvate can also be produced in non-growing thiamine-starved cells in POM buffer (1% glucose). The pyruvate production increased with increasing start cell density and with increasing culture time. With a start OD<sub>600nm</sub>=10 in POM buffer (1% glucose), the pyruvate production reached 35.9 mM, thus offering the potential for producing label-free (non-GMO) pyruvate. As is shown in Fig. 6(c), the production of the by-product acetoin also increased with increasing start cell density. This may be due to a limited extent of thiamine/TPP-dependent functioning of ALS. However, the acetoin production was still much lower than for the thiamine-enriched cells, as is shown in Fig. 3(c).

## EXAMPLE 7

*Pyruvate production by RD1M5 growing in thiamine-deficient milk*

The purpose of this experiment was to explore whether RD1M5 can produce pyruvate in milk.

Methods

5 It is desirable to be able to produce pyruvate in milk because milk is already allowed for human consumption, and potentially it would be possible to produce downstream products, including acetolactate, directly in the pyruvate-enriched milk. However, milk contains 0.47 µg/mL thiamine (Lalić et al., 2014), a barrier for production of pyruvate in milk by RD1M5. In order to remove thiamine from milk, we used Florisil (activated magnesium silicate) adsorption,  
10 which has been reported to be able to remove 95%-100% thiamine and 100% riboflavin from milk (Adamson, 1953). Florisil is commonly used as an adsorbent to remove impurities and unwanted compounds from food products. Its porous structure allows it to selectively adsorb substances such as pigments, pesticides, and other contaminants. According to the U.S. Food and Drug Administration, it is approved substances generally recognized as safe (see  
15 <https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=182.2437&SearchTerm=magnesium%20silicate>, accessed on 13 June 2023). To obtain thiamine-deficient milk, ultra-heat-treated (UHT) milk was treated by Florisil with some modifications (Adamson, 1953). Briefly, fifty grams of Florisil was suspended in 300 mL of 2% acetic acid for 5 min. After that, the Florisil particles are settled at the bottom of the beaker. The acetic acid solution  
20 is poured off, and the Florisil beads are washed 3 times using 300 mL water to remove extra acetic acid and Florisil dust. Filter paper was placed in a Buchner funnel, and the washed Florisil particles were placed in it and allowed to drain. To filter the UHT milk, the first 50 mL of milk was added to a Buchner funnel with the rate of filtration, and the filtered milk (still some water mixed with milk) was discarded. Then, 250 mL milk was continuously added to a Buchner  
25 funnel, and the filtered milk was collected as thiamine-deficient milk. After that, the treated milk was heated at 100°C for 30 min to sterilize the treated milk.

RD1M5 was cultured in LM17 medium, growing at 30°C for 10 h. The culture was centrifuged, and the cells were washed three times with the same volume of 0.9% NaCl solution. Then the washed cells were put in 10 mL treated UHT milk or UHT milk in a 250 mL shaking flask with  
30 a start OD<sub>600nm</sub> of 0.05 and incubated at 30°C for 24 h. Samples were taken after 6 h, 12 h, and 24 h, to determine metabolites by HPLC.

## Results

As is shown in Fig. 7(a), after 24 h, the RD1M5 in Florisil-treated UHT milk had accumulated 26.3 mM pyruvate, which is more than 4 times as much as that produced by RD1M5 in UHT milk. When a mixture of vitamins present in SAN medium were added to the treated milk, a higher production of 39.3 mM pyruvate could be achieved. Culturing RD1M5 in Florisil-treated milk also resulted in formation of acetoin, although less was formed than when RD1M5 was cultured in UHT milk (Fig. 7(d)). Formation of acetoin indicates that the thiamine was not 100% eliminated from the milk. Furthermore, when RD1M5 was cultured in Florisil-treated milk, 3 times as much lactate was formed as when RD1M5 was cultured in UHT milk (Fig. 7(e)), which may be explained by an influence on the redox balance of the low thiamine level that may force the cells to produce more lactate to keep the NADH/NAD<sup>+</sup>-balance, even though RD1M5 is an LDH-deficient strain.

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## CLAIMS

1. A lactic acid bacterium which is deficient in thiamine and which is deficient in lactate dehydrogenase and/or lactate dehydrogenase activity.
2. The lactic acid bacterium according to claim 1, wherein the lactic acid bacterium is unable  
5 to produce thiamine.
3. The lactic acid bacterium according to claim 1 or 2, wherein the lactic acid bacterium is unable to grow in medium lacking thiamine.
4. The lactic acid bacterium according to any one of claims 1-3, wherein the lactic acid bacterium belongs to any one of the following genera: *Lactococcus*, *Pediococcus*, *Enterococcus*,  
10 *Leuconostoc*, *Limosilactobacillus*, *Lactobacillus*, and *Streptococcus*.
5. The lactic acid bacterium according to any one of claims 1-4, wherein the lactic acid bacterium belongs to, or is derived from, any one of the following species: *Lactococcus lactis*, *Enterococcus faecium*, *Enterococcus casseliflavus*, *Leuconostoc mesenteroides*, *Lactococcus landensis*, *Limosilactobacillus reuteri*, and *Lactobacillus delbrueckii subsp. bulgaricus*.
- 15 6. The lactic acid bacterium according to any one of claims 1-5, wherein the lactic acid bacterium belongs to, or is derived from, any one of the following strains: *Lactococcus lactis subsp. biovar diacetylactis RD1M5*, *Enterococcus faecium L117*, *Enterococcus casseliflavus L142*, *Leuconostoc mesenteroides JCFD003*, *Lactococcus landensis JCFD002*, *L. landensis JCFD006*, *L. lactis subsp. cremoris JCFD012*, *L. lactis subsp. biovar diacetylactis 170B*, *L. lactis subsp. biovar diacetylactis 171B*, *L. lactis subsp. biovar diacetylactis 170A*, *L. lactis subsp. biovar diacetylactis SD96*, *L. lactis subsp. biovar diacetylactis JC0169A*, *L. lactis subsp. biovar diacetylactis JC0169B*, *L. lactis subsp. lactis SL195 (JC024)*, *L. lactis subsp. lactis SL69 (JC023)*, *L. lactis subsp. lactis JCFD025*, *L. lactis subsp. lactis JCFD027*, *L. lactis subsp. lactis JCFD026*, *L. lactis subsp. lactis 109*, *L. lactis subsp. lactis 110*, *L. lactis subsp. lactis 111*, *L. lactis subsp. lactis 116*, *L. lactis subsp. lactis 120*, *L. lactis subsp. lactis 118*, *L. lactis subsp. lactis 121*, *L. lactis subsp. lactis IO1*, *L. lactis subsp. lactis KF147*, *Limosilactobacillus reuteri CRL 1099*, *L. reuteri CRL 1100*, *L. reuteri CRL 1101*, *Lactobacillus delbrueckii subsp. bulgaricus CRL 958*, and *L. lactis subsp. cremoris CRL 985*.
- 25 7. A composition comprising the lactic acid bacterium according to any one of the preceding  
30 claims, optionally wherein the lactic acid bacterium is in freeze-dried form, and optionally wherein the composition is thiamine-deficient.

8. A method of producing the lactic acid bacterium according to any one of claims 1-6, said method comprising:

culturing a lactic acid bacterium deficient in lactate dehydrogenase and/or lactate dehydrogenase activity in a medium deficient in thiamine and comprising acetate, such as 1.5-150 mM acetate, preferably 15 mM acetate, at 25-35°C for at least 6 hours, optionally wherein the culture has a start cell density corresponding to an  $OD_{600nm} \leq 0.1$ , preferably an  $OD_{600nm} = 0.05$ .

9. A method of producing pyruvate, comprising:

culturing the lactic acid bacterium according to any one of claims 1-6 in medium deficient in thiamine, and under aeration, thereby obtaining pyruvate.

10. The method according to claim 9, wherein the lactic acid bacterium is cultured in the presence of acetate, such as 1.5-150 mM acetate, preferably 15 mM acetate.

11. The method according to claim 10, wherein the lactic acid bacterium is cultured in the presence of a source of oleate, such as in the presence of Tween 80, such as Tween 80 in a concentration of 0.01-1% (w/v), preferably Tween 80 in a concentration of 0.1% (v/v).

12. The method according to any one of claims 8-11, wherein the medium is thiamine-deficient milk.

13. Use of the lactic acid bacterium according to any one of claims 1-6 or the composition according to claim 7 for producing pyruvate.

14. A kit comprising a) a first lactic acid bacterium according to any one of claims 1-6 or the composition according to claim 7 and b) a second lactic acid bacterium, or lysate or cell-free extract thereof, capable of producing one or more of  $\alpha$ -acetolactate, acetoin, and diacetyl from pyruvate, optionally wherein the first and the second lactic acid bacterium, or lysate or cell-free extract thereof, are in separate vials.

15. A method of producing one or more of  $\alpha$ -acetolactate, acetoin and diacetyl, comprising the steps of:

a) culturing the lactic acid bacterium according to any one of claims 1-6 or the composition according to claim 7 in a culture medium deficient in thiamine, and under aeration, thereby obtaining pyruvate, and

b) contacting the pyruvate with a second lactic acid bacterium, or lysate or cell-free extract thereof, capable of producing one or more of  $\alpha$ -acetolactate, acetoin, and diacetyl from pyruvate.

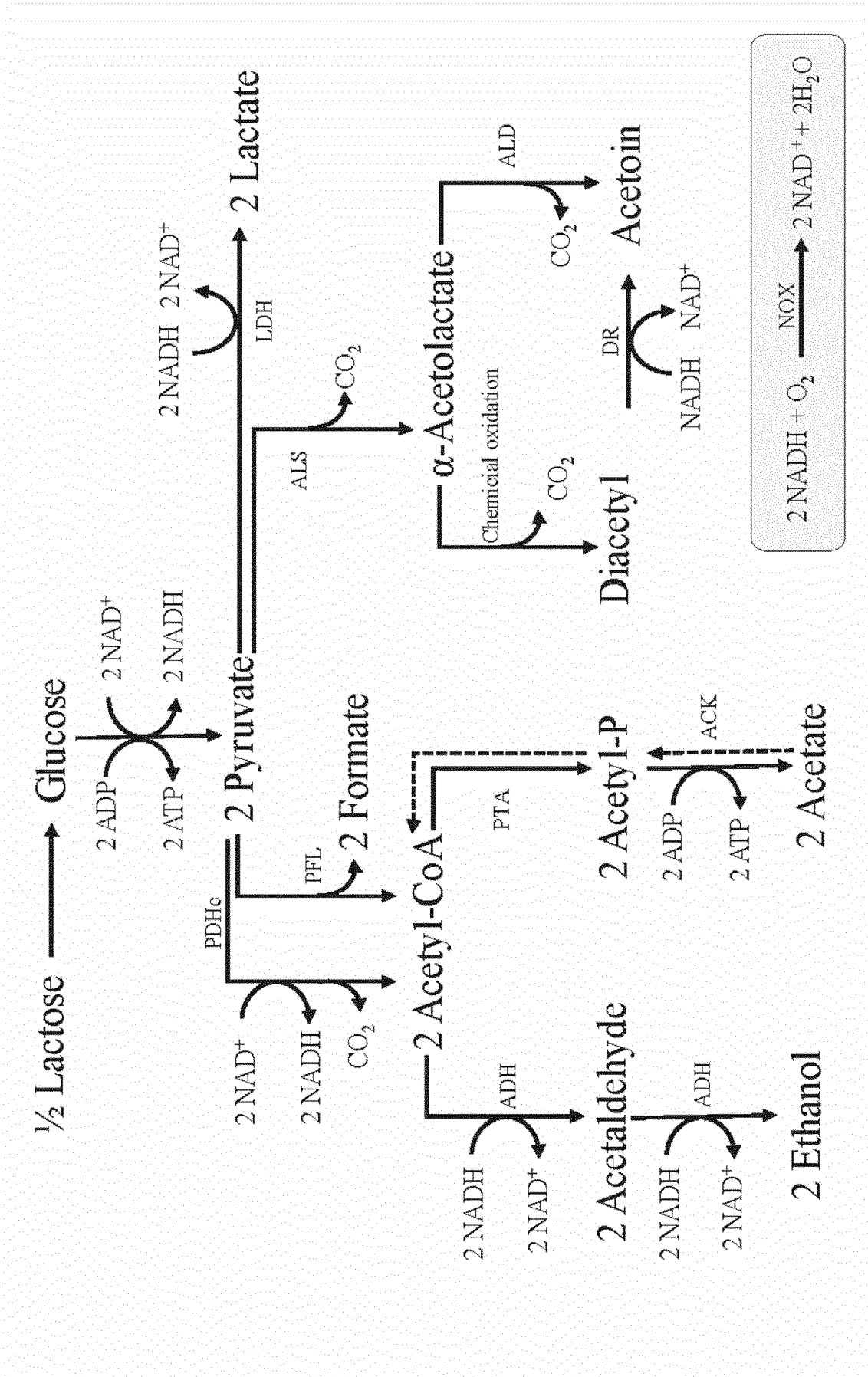


Fig. 1

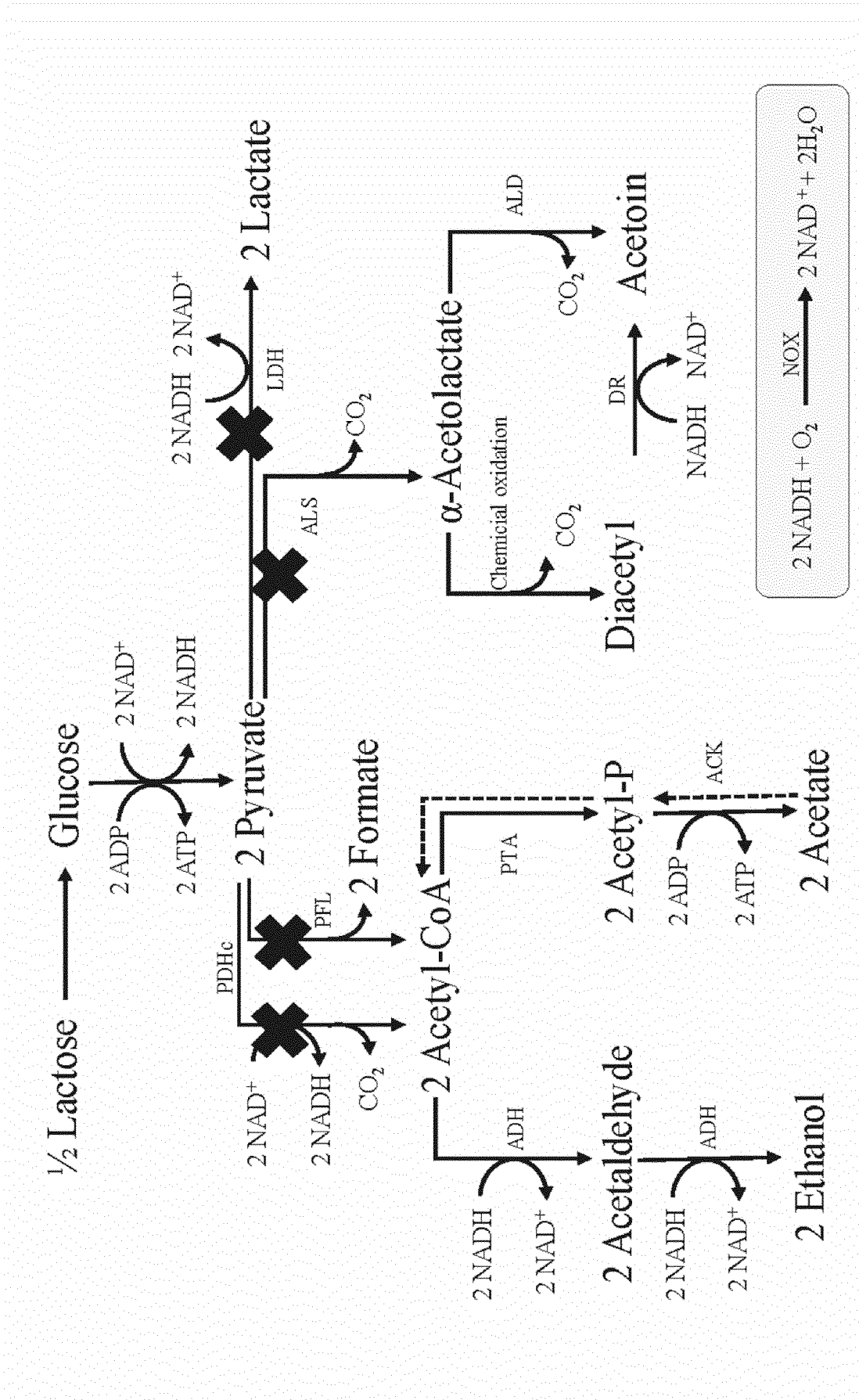


Fig. 2

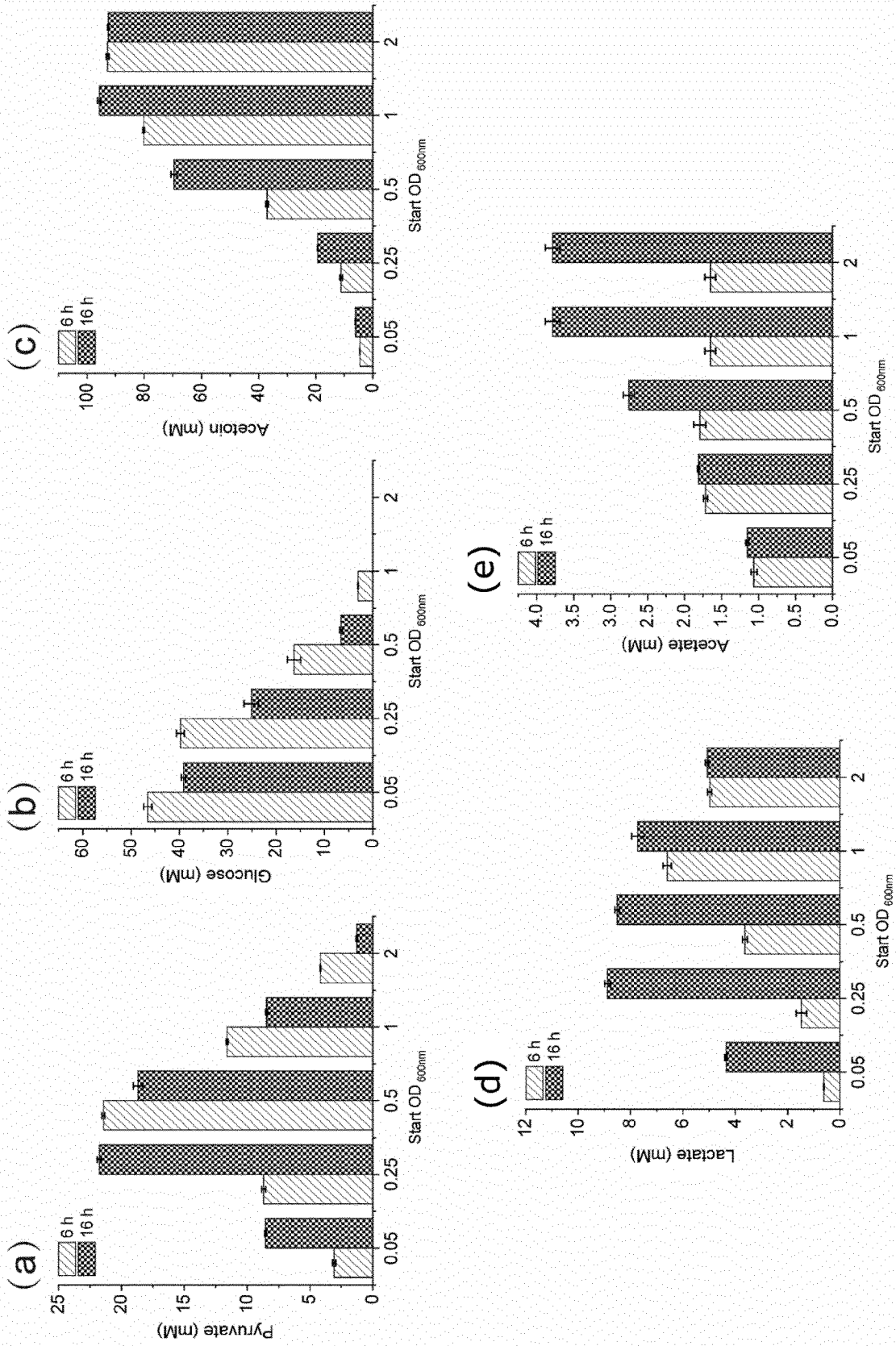


Fig. 3

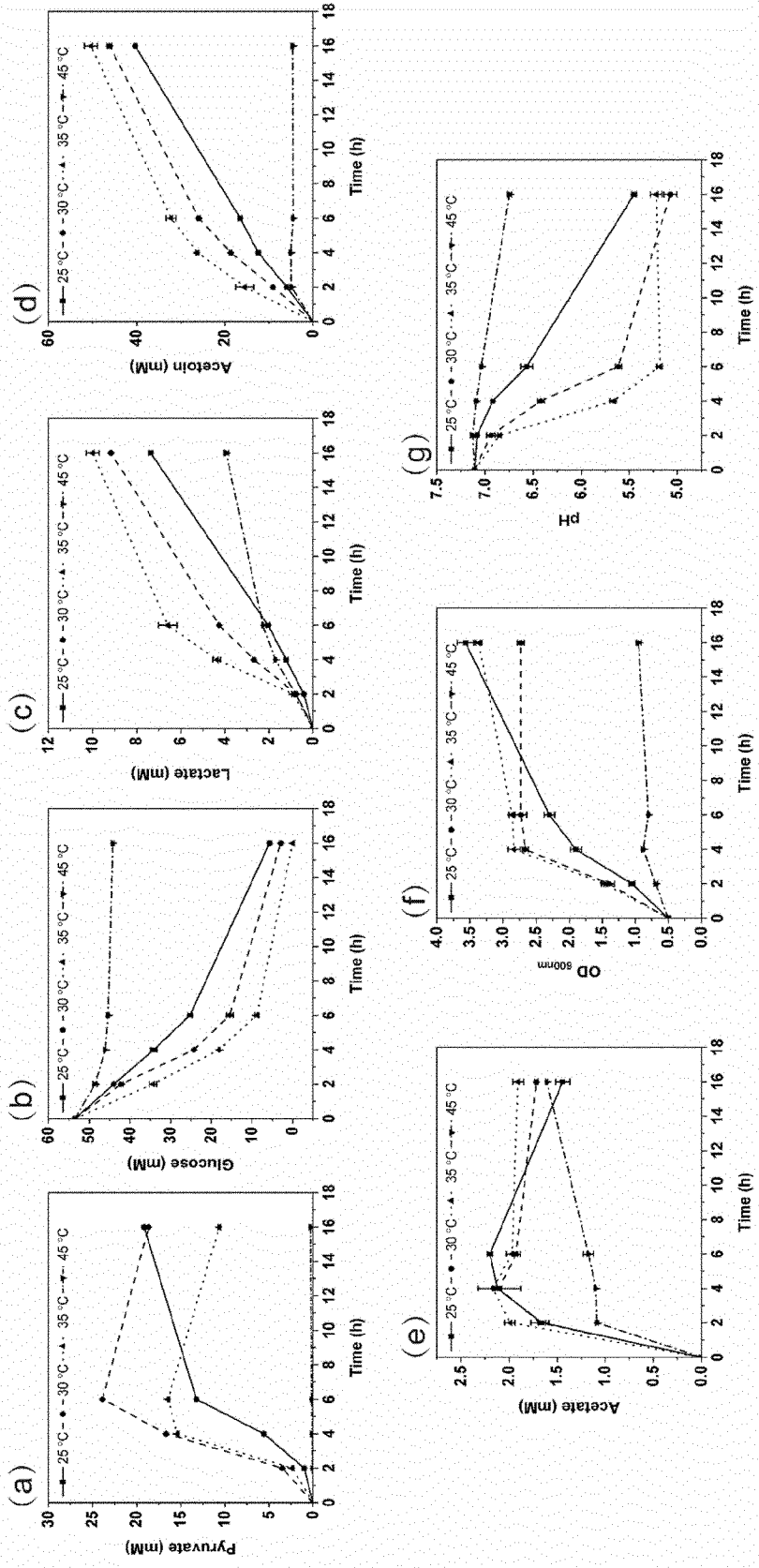


Fig. 4

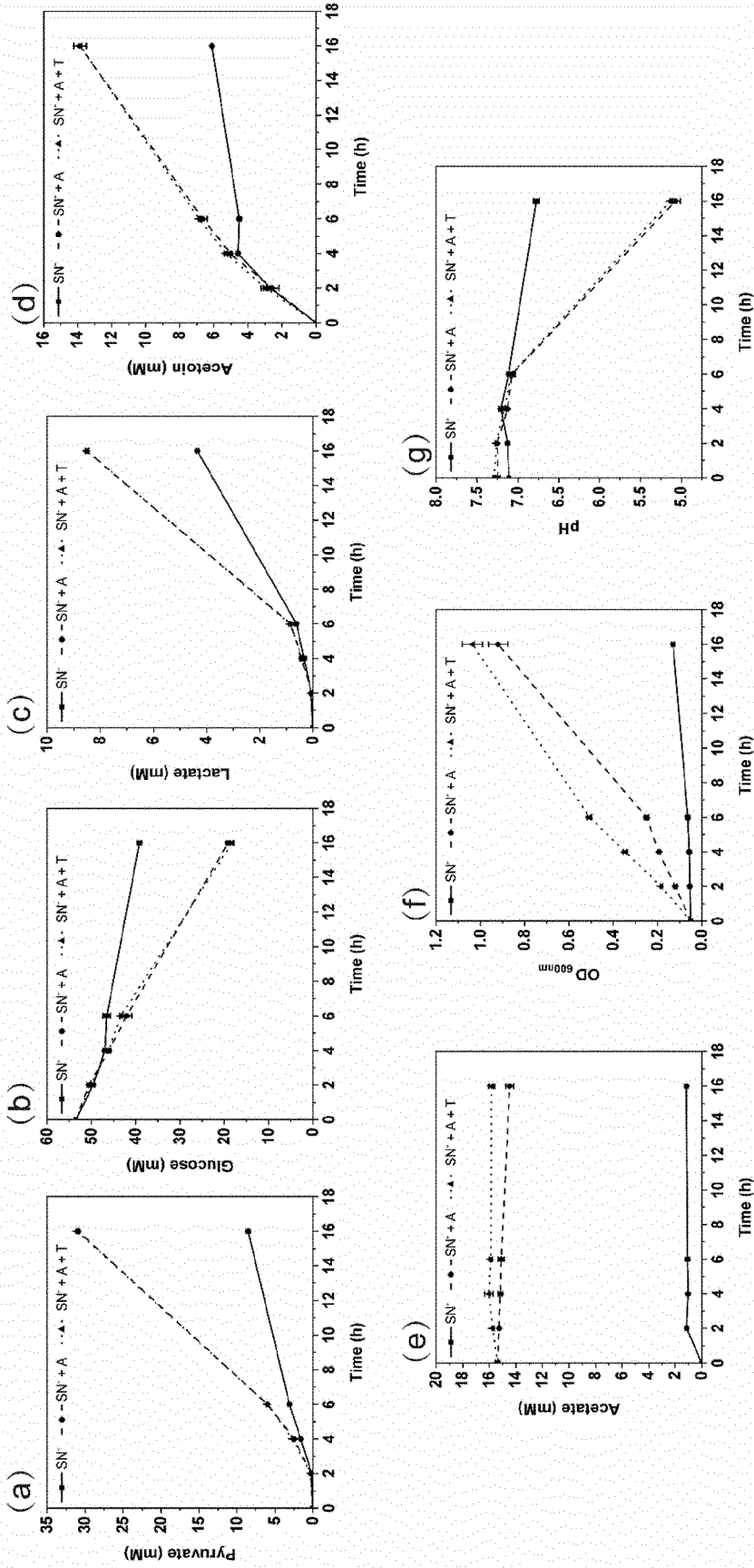


Fig. 5

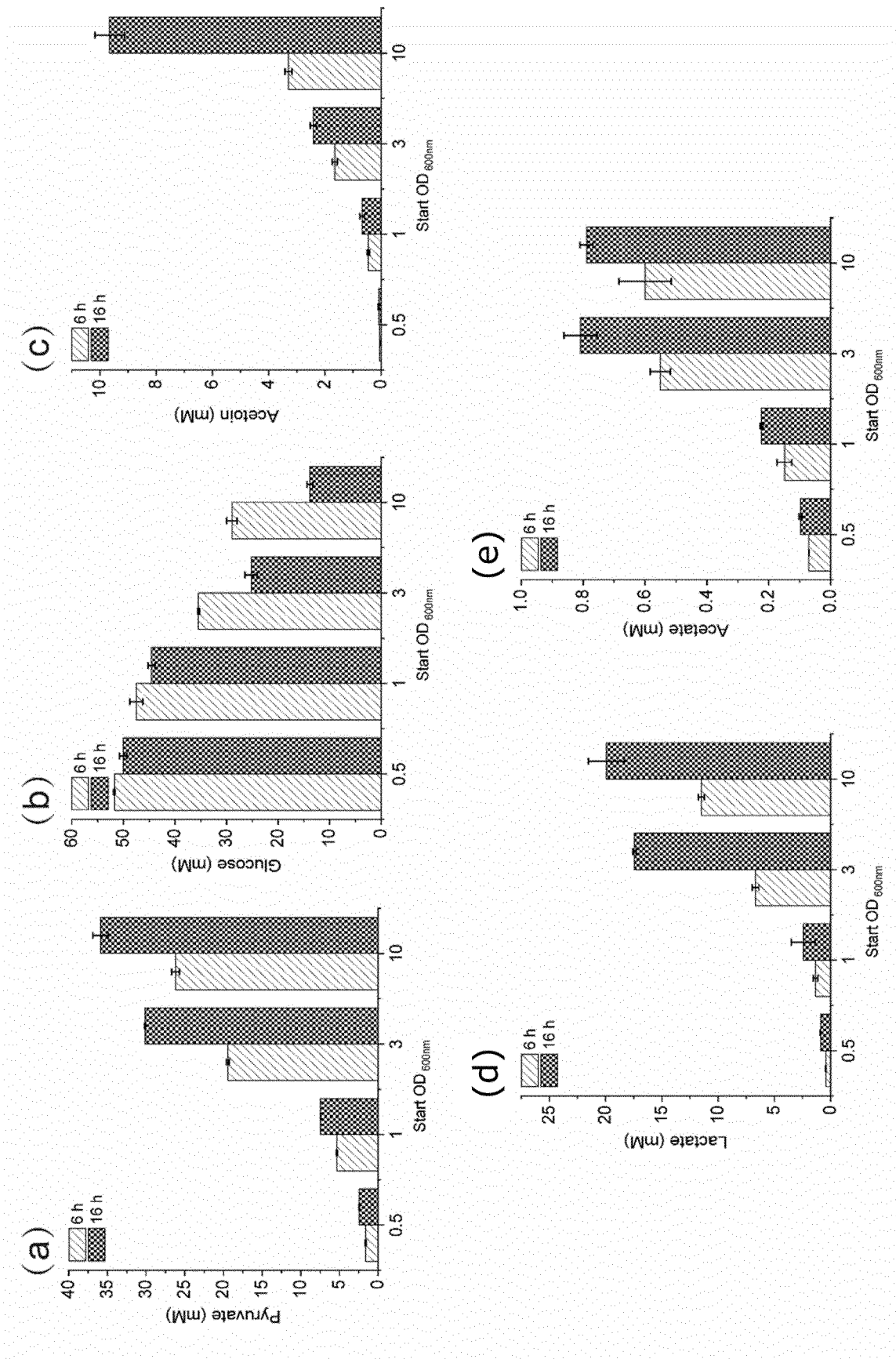


Fig. 6

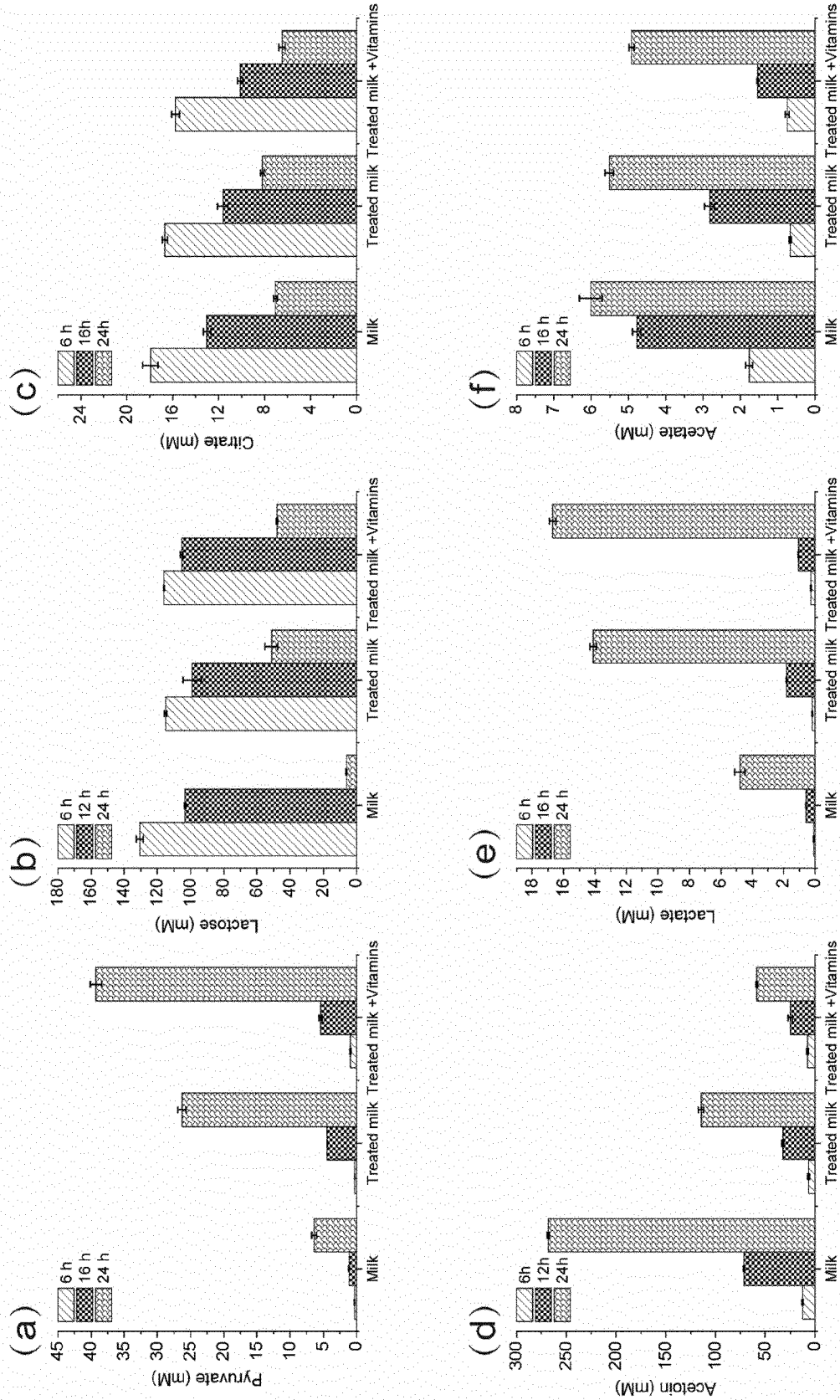


Fig. 7

# INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2024/075691

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> INV. C12N1 ADD. C12N1/20		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols) <b>C12N</b>		
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 practicable, search terms used) <b>EPO-Internal, WPI Data</b>		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
<b>X</b>	<b>WO 2017/060455 A1 (UNIV DANMARKS TEKNISKE [DK]) 13 April 2017 (2017-04-13)</b> page 7, line 25 - page 9, line 24 page 25, line 3 - page 29, line 11 -----	<b>1 - 15</b>
<b>X</b>	<b>WO 2016/097268 A1 (UNIV DANMARKS TEKNISKE [DK]) 23 June 2016 (2016-06-23)</b> page 10, line 5 - page 11, line 21 ----- - / - -	<b>1 - 15</b>
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C.		
<input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents :		
"A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "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	Date of mailing of the international search report	
<b>9 December 2024</b>	<b>07/01/2025</b>	
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer  <b>Stoyanov, Borislav</b>	

## INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2024/075691

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>LIU JIAN-MING ET AL: "From Waste to Taste-Efficient Production of the Butter Aroma Compound Acetoin from Low-Value Dairy Side Streams Using a Natural (Nonengineered) Lactococcus lactis Dairy Isolate",            JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY,            vol. 68, no. 21, 27 May 2020 (2020-05-27),            pages 5891-5899, XP093139537,            US            ISSN: 0021-8561, DOI:            10.1021/acs.jafc.0c00882</p>	1-7,15
A	<p>page 5895, right-hand column - page 5898,            right-hand column</p> <p>-----</p>	8-14
A	<p>TERAN MARÍA DEL MILAGRO ET AL:            "Thiamine-producing lactic acid bacteria and their potential use in the prevention of neurodegenerative diseases",            APPLIED MICROBIOLOGY AND BIOTECHNOLOGY,            vol. 105, no. 5,            6 February 2021 (2021-02-06), pages            2097-2107, XP037381050,            ISSN: 0175-7598, DOI:            10.1007/s00253-021-11148-7            cited in the application            table 1</p> <p>-----</p>	1-15

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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