

**New Bacteria and Consortia for the reduction of ammonia
and/or methane emission in manure**

This invention is related to a new bacterium, a
5 consortium comprising this bacterium and the use of the
consortium for reduction of ammonia and/or methane emission
in manure.

Manure is organic matter and can be used as organic
fertilizer in agriculture. Manure contributes to the
10 fertility of the soil by adding organic matter and
nutrients, such as nitrogen, that are trapped by bacteria in
the soil. Manure contains nitrogen (N) in inorganic and
organic forms. Organic N is not available for crop growth
until it is mineralized to ammonium (NH_4^+). Ammonium N is
15 fairly building and available for plant uptake, but a
portion is immobilized by microbial biomass and nitrifying
bacteria convert NH_4^+ to nitrate (NO_3^-) which is subject to
loss by leaching or denitrification and subsequent loss to
the atmosphere. Volatile ammonia (NH_3) in manure is
20 transformed from NH_4^+ and can be lost to the atmosphere
after land application. Nitrogen lost to the atmosphere is
not available for crop production. In addition, ammonia
(NH_3) emission from livestock production causes undesirable
environmental effects. Besides the undesirable effect of
25 NH_3 , manure also comprises methane (CH_4). Methane is a
greenhouse gas and it is generally known that the emission
of CH_2 in the atmosphere should be reduced.

There is thus a need for a product that can be added to
manure which can improve the quality of manure by providing
30 an increase amount of nutrients, such as nitrogen, available
to crops.

There is a need for a product that can be added to manure which can provide an improved fertilizing quality to crop plants.

In addition there is a need for a product that can be
5 added to manure which helps to reduce the ammonia and/or methane emission in manure.

It is an object of this invention, amongst other objects, to provide a consortium of micro-organisms which can be added to manure, for improving the quality of manure.

10 Yet, it is an other object of this invention to provide a consortium of micro-organisms which can process the manure so that ammonia and/or methane emission is reduced.

Yet, it is an other object of this invention to provide a consortium of micro-organisms that can be used for
15 improving manure so that the nitrogen availability to plants increases.

It is an other object of this invention, to provide a bacterium, which forms part of the consortium that can provide the above objects.

20 This object, amongst other objects, is met, at least partially, if not completely by the bacterium or consortium as claimed in the annex.

Especially, this object is met at least partially, if not completely by a bacterium comprising a partial 16S rDNA
25 nucleic acid sequence having more than 99.6% sequence identity to the sequences presented as SEQ ID NO:1, or the complement thereof. The inventor surprisingly found a new bacterium that resides in a consortium. The bacterium in the consortium and the consortium provide an improved quality to
30 manure in a way that less ammonia and/or methane is emitted from manure, when compared with manure where this consortium is not added.

The partial 16S rDNA can be found using the 16S rDNA sequencing method as described in Hall, L., Doerr, K.A., Wohlfiel, S.L., Roberts, G.D., 2003. Evaluation of the MicroSeq system for identification of mycobacteria by 16S ribosomal DNA sequencing and its integration into a routine clinical mycobacteriology laboratory, J. Clin. Microbiol. 4, 1447-1453, of which the reference is incorporated in its entirety. The PCR was performed on bacterial suspension with the following primers (sequences in 5' to 3' direction)

10 16S500F (tggagagtttgatcctggctcag) and 16S500R (taccgcggctgctggcac).

Sequence identity, as used herein, is defined as the number of identical consecutive aligned nucleotides, or amino acids, over the full length of the present sequences divided by the number of nucleotides, or amino acids, of the full length of the present sequences and multiplied by 100%. For example, a sequence with 80% identity to SEQ ID No. 1 comprises over the total length of 550 amino acids of SEQ ID No. 1 440 identical aligned consecutive amino acids, i.e.,

20 $440/550 * 100\% = 80\%$.

The invention is related to a newly found bacterium. A phylogenetic tree has been measured using the UPGMA algorithm (Unweighted Pair Group Method with Arithmetic Means) provided by the program of Bionumerics (from Applied Maths) measuring the PCT ribotype band sizes. It was found that the bacterium having a 16S rDNA sequence comprising SEQ ID NO:1 belongs to the genus *Bacteriodetes*. In one embodiment, the invention is related to a bacterium wherein the bacterium is a *bacteriodetes sp.*

30 In another embodiment the invention is related to a bacterium wherein the bacterium is as is deposited at CBS under the deposit no CBS 134116as deposited at CBS (Centraal

Bureau voor Schimmelcultures on 27 December 2012 under the Budapest Treaty), wherein CBS 134116 is a bacteriodetes.

In yet another embodiment, the bacterium has a sequence identity which is at least 85, 86, 87, 88, 89, 90, 91, 92,
5 93, 94, 95, 96, 97, 98, 99, 100 % identical with the sequence as is presented as SEQ ID NO:1.

In one aspect, the invention is related to a consortium comprising a bacterium as described above. The consortium may further comprise yeast of the genus *Candida*, preferably
10 *Candida boidinii* C. Ramírez and/or *Candida ethanolica* Rybárová, Stros & Kocková-Kratochvílová, and/ or may further comprise the bacteria *Lactobacillus rhamnosus/casei*, *Acetobacter pasteurianus/lovaiensus* and/ or *Rhodococcus facians/yunnanensis*.

15 The consortium may further comprise other lactic acid bacteria and other acetic acid bacteria.

In one embodiment, the consortium improves manure and reduces the emission of ammonia and/or methane compared with manure where no consortium according to this invention is
20 added. With manure is understood excrements of animals, and can be in a semi liquid state, such as slurry.

In another embodiment, the invention is related to a consortium as is deposited at the CBS, and has the deposit number CBS134115 as deposited at Centraal Bureau for
25 Schimmelcultures.

In another embodiment, the consortium according to the invention further comprises a saccharide, preferably a monosaccharide and/or disaccharide. It is thought that the saccharide provides an environment for the bacteria and
30 yeast in the consortium, which provides a composition of the bacteria and yeasts with a ratio in a way that in manure the production of ammonia and/or methane decreases. It is thought that the consortium provides an environment in

manure, which is not favourable for ammonia producing bacteria.

In yet another embodiment, the saccharide is derived from cane source or beat source and is preferably a cane
5 molasses.

In another aspect, the invention is related to the use of SEQ ID NO:1 for detecting or identifying a bacterium having the nucleic acid sequence or at least 99.6% sequence identity with SEQ ID NO:1.

10 In another aspect, the invention is related to a consortium of micro-organisms that comprises micro-organisms that provide reduction of the emission of ammonia and/or methane (NH₃) in manure, as can be found in a consortium as is deposited at CBS under the deposit no of CBS 134115.

15 In another aspect, the invention is related to the use of a bacterium according to the invention or a consortium according to the invention, for the reduction of nitrogen in manure. The use can, for example, be performed by spraying a solution comprising the bacterium or the consortium
20 according to the invention on manure. This can, for example, be performed using a method and product as described in the patent application NL2009019 of which the reference is incorporated in its entirety.

The manure is preferably animal manure, coming from
25 e.g. pigs, cows, poultry or horses.

In another aspect the invention is related to a method for reducing the emission of ammonia and/or methane in manure comprising adding the bacterium or the consortium according to the invention to manure and incubating said
30 bacterium or consortium for a sufficient time allowing to reduce the formation of ammonia and/or methane in the manure.

Another aspect according to the invention is the use of manure according to the invention as an organic fertilizer. The consortium or the bacterium in the consortium according to the invention contribute to a reduced emission of ammonia. It is thought that more nitrogen is in a phase that can be used by plants, such as crop plants. It is surprisingly found that the consortium and the bacterium according to the invention can be used for improving manure and that beside the reduction of ammonia is obtained, also the fertilizing properties of the manure according to the invention is improved.

The advantages and embodiments as described for several aspects in the invention can be valid for the other aspects according to the invention.

The present invention will be further described in detail in the following example of preferred embodiments of the invention.

Figure 1

Dendrogram of Cluster analysis based on pairwise similarities of the new found bacterium Bacteroidetes phylum (figure 1) (CBS 134116). The numbers above show the amount of similarity measured using the similarity-based clustering using the method Unweighted pair-grouping (UPGMA)

Example 1

Test of ammonia emission in manure of a pig farm

In two livestock buildings for pigs, measurement of ammonia emission have been performed. In one building, the device as described in patent application NL2009019 has been used to treat the slurry coming from the pigs. The

consortium as deposited at CBS under no. CBS 134115, has been sprayed on the slurry excreted by the pigs in building 1. Building 2 comprised slurry that was not treated with the consortium.

- 5 In each building 80 pigs were kept and the measurement was performed over a period of 12 weeks. Each day a composition comprising the consortium is sprayed in building 1 during 12 weeks. NH₃ emission has been measured during 24 hours using the norm NEN 2826 (NEN 2826, 1999:
- 10 Luchtkwaliteit. Uitworp door stationaire puntbronnen. Monsterneming en bepaling van het gehalte aan gasvormig ammoniak) by taking samples using a gas washing bottle comprising an absorption liquid. The ammonia measurements were performed by performing an absorption method and wet
- 15 chemical analysis.

- The samples were taken during several cycles of weeks of the pigs and during several months of the year. Table 1 shows the results of the emission of ammonia. The manure that was treated with the consortium as deposited at
- 20 CBS under no. CBS 134115 showed an average decrease of 35% ammonia emission in the manure compared with the manure from building 2. The measurements were taken in several seasons (summer, winter and autumn) and also on different growing stages of the pigs (12 weeks in total, and each period of
- 25 two weeks counts for 1 stage).

In the setup of the measurement, the amount of animals, the ages and the weight of the animals were in both animal buildings similar.

Table 1

Emission of ammonia per year in kg/animal			
Stage	Piggery 1	Piggery 2 (control)	% reduction
4	6,2	11,3	45
2	3,5	5,5	35
5	4,3	7,2	39
1	4,2	5,8	26
5	4,7	6,2	25
3	2,9	4,8	38
Average	6,8	4,3	35

Example 2**Test of methane emission in manure for a pig form**

A similar setup as in example 1 was performed. The methane was measured by taking a sample and using the lung method according to the norm NEN-EN, during 24h.

There was an average methane reduction of 19% found in two different farms.

Table 2 shows the results in one farm of the methane measurement in piggery 1, where the consortium mixture was added to the manure, compared with piggery 2 (control), where no consortium mixture was added.

Table 2			
	Piggery 1	Piggery 2	Stage
Methane ppm	14,8	17,8	1 (summer)
Methane emission/animal/year (kg/animal/year)	1,0	1,6	1 (summer)
Methane ppm	37,0	52,6	2 (winter)
Methane emission/animal/year (kg/animal/year)	1,9	3,9	2 (winter)
Methane ppm	32	60,6	3 (spring)
Methane emission/animal/year (kg/animal/year)	2,1	4,3	3 (spring)

SEQUENCE LISTING

```

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5  <120> New bacteria for improving manure
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10 <170> BiSSAP 1.2
    <210> 1
    <211> 498
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    <221> source
20 <222> 1..498
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        /mol_type="unassigned DNA"
    <400> 1
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    atgtagcaat acatttgatg gcgaccggca aacgggtgcg gaacacgtac acaacottcc      120
    tataagtggg gaatagccca gagaaatttg gattaatacc ccgtaacata acgatgtggc      180
30 atcacattgt tattatagct tcggcgctta ttgatgggtg tgcggctgat tagatagttg      240
    gcggggtaac ggcccaccaa gtctacgac agtagctgat gtgagagcat gatcagccac      300
35 acggggcactg agacacgggc ccgactccta cgggaggcag cagtaaggaa tattgggtcaa      360
    tggacgcaag tctgaaccag ccatgccgcg tgaaggatta aggtcctctg gattgtaaac      420
    ttctttttatc tgggacgaaa aaaggcgatt cttcgtcact tgacgggtacc agatgaataa      480
40 gcaccggcta actccgtg                                     498

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Conclusies

1. Bacterie omvattende een partieel 16S rDNA
nucleïnezuursequentie met meer dan 99,6% sequentie
5 identiteit met de sequentie zoals voorgesteld in SEQ ID NO:
1, of het complement hiervan.

2. Bacterie volgens conclusie 1, waarbij de bacterie
een *bacterioidetes* sp. is.
10

3. Bacterie volgens conclusie 1 of 2, waarbij de
bacterie is zoals gedeponeerd bij het CBS met het deposit nr
CBS 134116.

15 4. Bacterie volgens conclusie 1-3 met de sequentie
zoals voorgesteld in SEQ ID NO: 1, of een complement
hervan.

5. Consortium van micro-organismen voor het verbeteren
20 van mest omvattende een bacterie volgens één der conclusies
1-4.

6. Consortium volgens conclusie 5, waarbij het
consortium verder een saccharide omvat, bij voorkeur een
25 monosaccharide en/of dissaccharide.

7. Consortium volgens conclusie 5 of 6, waarbij de
saccharide afgeleid is van een suikerrietbron of bietbron en
is bij voorkeur een suikerrietmolasses.
30

8. Gebruik van SEQ ID NO: 1 voor het bepalen of
identificeren van een bacterie met nucleïnezuursequentie of

ten minste 95% sequentie identiteit met de sequentie zoals voorgesteld in SEQ ID NO: 1.

5 9. Een consortium van micro-organismen die micro-organismen omvat die de emissie van ammoniak en/of methaan verminderen zoals gevonden kan worden in een consortium zoals gedeponeerd onder CBS met het deposit nr CBS 134115.

10 10. Consortium van micro-organismen volgens conclusie 9, omvattende het consortium zoals gedeponeerd bij CBS onder het deposit nr CBS 134115.

15 11. Gebruik van een bacterie volgens conclusie 1 tot 4 of een consortium volgens conclusie 5-10 voor de vermindering van ammonia en/of methaan emissie in mest.

12. Gebruik volgens conclusie 10, waarbij de mest een dierlijke mest is.

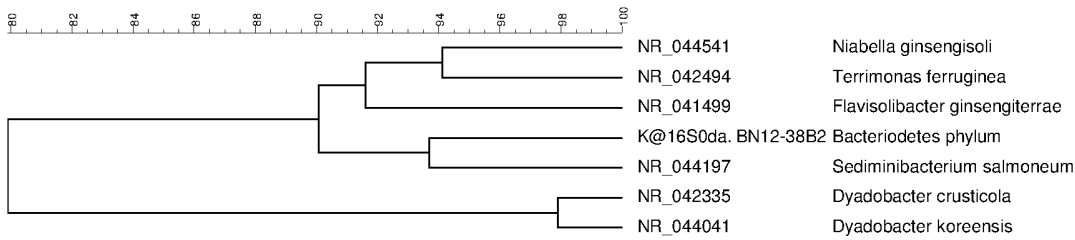
20 13. Mest omvattende een bacterie volgens conclusie 1 tot 4 of een consortium volgens conclusies 5 tot 10.

25 14. Werkwijze voor het reduceren van ammoniak en/of methaan in mest omvattende de stappen voor het toevoegen van een bacterie volgens conclusie 1-5 of een consortium volgens conclusie 6-10 aan mest en incuberen van deze bacterie in consortium gedurende een voldoende tijd om de reductie van de vorming van ammoniak en/of methaan in mest toe te laten.

30 15. Gebruik van mest volgens conclusie 13 als een organische meststof.

Figures

Figure 1



SAMENWERKINGSVERDRAG (PCT)

RAPPORT BETREFFENDE NIEUWHEIDSONDERZOEK VAN INTERNATIONAAL TYPE

IDENTIFICATIE VAN DE NATIONALE AANVRAGE	KENMERK VAN DE AANVRAGER OF VAN DE GEMACHTIGDE
	3Q/2NJ69/KM/3
Nederlands aanvraag nr.	Indieningsdatum
2010074	31-12-2012
	Ingeroepen voorrangsdatum
Aanvrager (Naam)	
Rinagro B.V.	
Datum van het verzoek voor een onderzoek van internationaal type	Door de Instantie voor Internationaal Onderzoek aan het verzoek voor een onderzoek van internationaal type toegekend nr.
09-03-2013	SN 59693
I. CLASSIFICATIE VAN HET ONDERWERP (bij toepassing van verschillende classificaties, alle classificatiesymbolen opgeven)	
Volgens de internationale classificatie (IPC)	
C12N1/20 C02F3/34	C12R1/01 A01C3/00
C12R2/225	
II. ONDERZOCHE GEBIEDEN VAN DE TECHNIEK	
Onderzochte minimumdocumentatie	
Classificatiesysteem	Classificatiesymbolen
IPC	C12N C12R C02F A01C
Onderzochte andere documentatie dan de minimum documentatie, voor zover dergelijke documenten in de onderzochte gebieden zijn opgenomen	
III. ☐	GEEN ONDERZOEK MOGELIJK VOOR BEPAALDE CONCLUSIES (opmerkingen op aanvullingsblad)
IV. ☐	GEBREK AAN EENHEID VAN UITVINDING (opmerkingen op aanvullingsblad)

**ONDERZOEKSRAPPORT BETREFFENDE HET
RESULTAAT VAN HET ONDERZOEK NAAR DE STAND
VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE**

Nummer van het verzoek om een onderzoek naar
de stand van de techniek
NL 2010074

A. CLASSIFICATIE VAN HET ONDERWERP

INV. C12N1/20 C12R1/01 C12R1/225 C02F3/34 A01C3/00
ADD.

Volgens de Internationale Classificatie van octrooien (IPC) of zowel volgens de nationale classificatie als volgens de IPC.

B. ONDERZOCHETE GEBIEDEN VAN DE TECHNIEK

Onderzochte minimum documentatie (classificatie gevolgd door classificatiesymbolen)

C12N C12R C02F A01C

Onderzochte andere documentatie dan de minimum documentatie, voor dergelijke documenten, voor zover dergelijke documenten in de onderzochte gebieden zijn opgenomen

Tijdens het onderzoek geraadpleegde elektronische gegevensbestanden (naam van de gegevensbestanden en, waar uitvoerbaar, gebruikte trefwoorden)

EP0-Internal, BIOSIS, Sequence Search, FSTA, WPI Data

C. VAN BELANG GEACHTE DOCUMENTEN

Categorie °	Geöciteerde documenten, eventueel met aanduiding van speciaal van belang zijnde passages	Van belang voor conclusie nr.
X	DATABASE EMBL [Online]	1,2
Y	14 juni 2005 (2005-06-14), "Uncultured Bacteroidetes bacterium clone NMT sF40 16S ribosomal RNA gene, partial sequence.", XP002703676, gevonden in EBI accession no. EM STD:DQ066616 Database accession no. DQ066616 * sequentie * ----- -/--	11-15

☒ Verdere documenten worden vermeld in het vervolg van vak C.

☒ Leden van dezelfde octrooifamilie zijn vermeld in een bijlage

° Speciale categorieën van aangehaalde documenten

A niet tot de categorie X of Y behorende literatuur die de stand van de techniek beschrijft

D in de octrooiaanvraag vermeld

E eerdere octrooi(aanvraag), gepubliceerd op of na de indieningsdatum, waarin dezelfde uitvinding wordt beschreven

L om andere redenen vermelde literatuur

O niet-schriftelijke stand van de techniek

P tussen de voorrangsdatum en de indieningsdatum gepubliceerde literatuur

T na de indieningsdatum of de voorrangsdatum gepubliceerde literatuur die niet bezwarend is voor de octrooiaanvraag, maar wordt vermeld ter verheldering van de theorie of het principe dat ten grondslag ligt aan de uitvinding

X de conclusie wordt als niet nieuw of niet inventief beschouwd ten opzichte van deze literatuur

Y de conclusie wordt als niet inventief beschouwd ten opzichte van de combinatie van deze literatuur met andere geöciteerde literatuur van dezelfde categorie, waarbij de combinatie voor de vakman voor de hand liggend wordt geacht

Z lid van dezelfde octrooifamilie of overeenkomstige octrooipublicatie

Datum waarop het onderzoek naar de stand van de techniek van internationaal type werd voltooid

18 juli 2013

Verzenddatum van het rapport van het onderzoek naar de stand van de techniek van internationaal type

Naam en adres van de instantie

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Fax: (+31-70) 340-3016

De bevoegde ambtenaar

van de Kamp, Mart

**ONDERZOEKSRAPPORT BETREFFENDE HET
RESULTAAT VAN HET ONDERZOEK NAAR DE STAND
VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE**

Nummer van het verzoek om een onderzoek naar
de stand van de techniek
NL 2010074

C.(Vervolg). VAN BELANG GEACHTE DOCUMENTEN

Categorie °	Geciteerde documenten, eventueel met aanduiding van speciaal van belang zijnde passages	Van belang voor conclusie nr.
X	DATABASE EMBL [Online] 2 juni 2010 (2010-06-02), "Lactobacillus rari culture-collection IMAU:80584 16S ribosomal RNA gene, partial sequence.", XP002703677, gevonden in EBI accession no. EM_STD:HM058778 Database accession no. HM058778	1,2
Y	* sequentie *	11-15
X	----- QIUHUA BAO ET AL: "Isolation and identification of cultivable lactic acid bacteria in traditional yak milk products of Gansu Province in China", THE JOURNAL OF GENERAL AND APPLIED MICROBIOLOGY, deel 58, nr. 2, 2012, bladzijden 95-105, XP055071835, ISSN: 0022-1260, DOI: 10.2323/jgam.58.95	1,2,5-7
Y	* het gehele document *	11-15
	* tabel 3 *	
Y	----- US 6 025 187 A (PENAUD JEAN [FR]) 15 februari 2000 (2000-02-15) * het gehele document *	11-15
Y	----- WO 2007/072935 A1 (KOU SAIHATSU [JP]) 28 juni 2007 (2007-06-28) * het gehele document *	11-15
A	----- CHADWICK ET AL: "Emissions of ammonia, nitrous oxide and methane from cattle manure heaps: effect of compaction and covering", ATMOSPHERIC ENVIRONMENT, PERGAMON, GB, deel 39, nr. 4, februari 2005 (2005-02), bladzijden 787-799, XP027740824, ISSN: 1352-2310 [gevonden op 2005-02-01] * het gehele document *	11-15
A	----- MELSE ET AL: "Evaluation of Four Farm-scale Systems for the Treatment of Liquid Pig Manure", BIOSYSTEMS ENGINEERING, ACADEMIC PRESS, UK, deel 92, nr. 1, september 2005 (2005-09), bladzijden 47-57, XP005012595, ISSN: 1537-5110, DOI: 10.1016/J.BIOSYSTEMSENG.2005.05.004 * het gehele document *	11-15

**ONDERZOEKSRAPPORT BETREFFENDE HET
RESULTAAT VAN HET ONDERZOEK NAAR DE STAND
VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE**

Informatie over leden van dezelfde octrooifamilie

Nummer van het verzoek om een onderzoek naar
de stand van de techniek

NL 2010074

In het rapport genoemd octrooi geschrift	Datum van publicatie	Overeenkomend(e) geschrift(en)	Datum van publicatie
US 6025187	A	15-02-2000	AT 228491 T 15-12-2002
			CA 2166846 A1 10-07-1996
			DE 69624954 D1 09-01-2003
			DE 69624954 T2 26-02-2004
			DK 720974 T3 24-03-2003
			EP 0720974 A1 10-07-1996
			ES 2185752 T3 01-05-2003
			FR 2729156 A1 12-07-1996
			PT 720974 E 31-03-2003
			US 6025187 A 15-02-2000

WO 2007072935	A1	28-06-2007	JP 2009201354 A 10-09-2009
			WO 2007072935 A1 28-06-2007



OCTROOICENTRUM NEDERLAND

WRITTEN OPINION

File No. SN59693	Filing date (<i>day/month/year</i>) 31.12.2012	Priority date (<i>day/month/year</i>)	Application No. NL2010074
International Patent Classification (IPC) INV. C12N1/20 C12R1/01 C12R1/225 C02F3/34 A01C3/00			
Applicant Rinagro B.V.			

This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the application
- ☒ Box No. VIII Certain observations on the application

	Examiner van de Kamp, Mart
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WRITTEN OPINION

Application number

NL2010074

Box No. I Basis of this opinion

1. This opinion has been established on the basis of the latest set of claims filed before the start of the search.
2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the application and necessary to the claimed invention, this opinion has been established on the basis of:
 - a. type of material:
 - ☒ a sequence listing
 - ☐ table(s) related to the sequence listing
 - b. format of material:
 - ☐ on paper
 - ☒ in electronic form
 - c. time of filing/furnishing:
 - ☒ contained in the application as filed.
 - ☒ filed together with the application in electronic form.
 - ☐ furnished subsequently for the purposes of search.
3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
4. Additional comments:

Box No. V Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty	Yes: Claims	3, 4, 8, 10-15
	No: Claims	1, 2, 5-7, 9
Inventive step	Yes: Claims	8, 10
	No: Claims	1-7, 9, 11-15
Industrial applicability	Yes: Claims	1-15
	No: Claims	

2. Citations and explanations

see separate sheet

WRITTEN OPINION

Application number
NL2010074

Box No. VIII Certain observations on the application

see separate sheet

1 **Re Item V**

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1.1 Reference is made to the following documents:

- D1** DATABASE EMBL [Online] 14 juni 2005 (2005-06-14),
"Uncultured Bacteroidetes bacterium clone NMT sF40 16S
ribosomal RNA gene, partial sequence.", gevonden in EBI
accession no. EM_STD:DQ066616 Database accession no.
DQ066616
- D2** DATABASE EMBL [Online] 2 juni 2010 (2010-06-02),
"Lactobacillus rapi culture-collection IMAU:80584 16S ribosomal
RNA gene, partial sequence.", gevonden in EBI accession no.
EM_STD:HM058778 Database accession no. HM058778
- D3** QIUHUA BAO ET AL: "Isolation and identification of cultivable
lactic acid bacteria in traditional yak milk products of Gansu
Province in China", THE JOURNAL OF GENERAL AND APPLIED
MICROBIOLOGY, deel 58, nr. 2, 1 januari 2012 (2012-01-01),
bladzijden 95-105, XP055071835, ISSN: 0022-1260, DOI:
10.2323/jgam.58.95
- D4** US 6 025 187 A (PENAUD JEAN [FR]) 15 februari 2000
(2000-02-15)
- D5** WO 2007/072935 A1 (KOU SAIHATSU [JP]) 28 juni 2007
(2007-06-28)

1.2 The present application does not meet the criteria of patentability, because
the subject-matter of **claims 1, 2, 5-7** is not new in view of **D1** and **D2**.

D1 discloses the 16S rDNA sequence of a *Bacteroidetes* bacterium. SEQ ID
NO:1 displays 99.6% nt seq identity in 498-nt overlap (1-498:17-514) with said
sequence. **D1** anticipates the subject-matter of **claims 1, 2**. When it would be
argued that **D1** does not disclose the bacterium but only its 16S rDNA
sequence, it is submitted that the skilled person is enabled to isolate said
bacterium using the sequence disclosed in **D1** without inventive skill.

D2 discloses the 16S rDNA sequence of a *Lactobacillus rapi* bacterium. SEQ ID NO:2 displays 94.8% nt seq identity in 504-nt overlap (3-504:7-510) with said sequence. **D3** discloses the corresponding bacterium as well as compositions containing said bacterium as well as other bacteria. **D3** anticipates the subject-matter of **claims 1, 2, 5-7**. Note that the consortium according to **claim 5** is a 'consortium (...) voor het verbeteren van mest etc.' which is interpreted as a consortium (...) suitable for improving the quality of manure etc.

- 1.3 The subject-matter of **claims 3, 4, 8** is new.
- 1.4 Regarding **claim 9**, its subject-matter refers to a consortium of microorganisms comprising microorganisms that can be found in the consortium deposited as CBS 134115. Said subject-matter lacks clarity, as the consortium of CBS 134115 not only contains the *Bacteroidetes* and *Lactobacillus* strains deposited as CBS 134116 and CBS134117 respectively, but also other microorganisms that have not been disclosed in detail, such as yeasts, further lactic acid bacteria and acetic acid bacteria (description page 4 lines 10-20). This lack of clarity prohibits the assessment of novelty of the subject-matter of **claim 9**.
- 1.5 The subject-matter of **claim 10** is new.
- 1.6 The subject-matter of **claims 11-15** is new. However, assessment of novelty of the subject-matter of **claims 11-15** is problematic insofar as reference is made to a consortium according to **claim 9**, in view of lack of clarity thereof (*vide supra*, 1.4).

Claims 11-14 further contain small errors:

- **Claim 11** refers to a consortium according to **claims 5-10**. **Claim 8** does not relate to a consortium, hence the reference in **claim 11** to **claim 8** is erroneous.
- **Claim 12** refers to a use according to **claim 10**. Probably a use according to **claim 11** is meant.

- **Claim 13** refers to a consortium according to **claims 5-10**. **Claim 8** does not relate to a consortium, hence the reference in **claim 13** to **claim 8** is erroneous.

- **Claim 14** refers to a bacterium according to **claims 1-5**. **Claim 5** does not relate to a bacterium but to a consortium, probably a bacterium according to **claim 1-4** is meant. **Claim 14** further refers to a consortium according to **claims 6-10**. This is probably meant to be a consortium according to **claims 5-10** as in **claims 11, 13**. Again, it is noted that **claim 8** does not relate to a consortium.

- 1.7 Taking **D4** or **D5** as closest prior art, the subject-matter of **claim 3** differs in that it concerns a different bacterium. The problem underlying **claim 3** may be summarised as the provision of a further bacterium for reduction of ammonia and/or methane in manure to improve the quality of said manure. The solution would be a bacterium according to **claim 3**. However, the application as filed contains proof of an effect (reduction of ammonia, methane in manure, *cf.* Example 1, 2) only for a consortium containing said bacteria deposited as CBS 134115, not for bacteria deposited as CBS 134116 resp. CBS 134117 separated from said consortium. Hence, the problem is not considered to be solved for bacteria deposited as CBS 134116 resp. CBS 134117. Hence, the subject-matter of **claim 3** is not considered to involve an inventive step, unless evidence would be filed for the separated strains deposited as CBS 134116 resp. CBS 134117.
- 1.8 What is said for **claim 3** applies, *mutatis mutandis*, also to the subject-matter of **claim 4** which is hence not considered to involve an inventive step too. There is a further problem with **claim 4**, in that there is no technical and causal link between the presence of a 16S rDNA sequence according to SEQ ID NO:1 or 2 and the effect of reduction of ammonia, methane in manure that is shown for a consortium deposited as CBS 134115.
- 1.9 The subject-matter of **claim 8** is considered to involve an inventive step, as it provides a non-obvious solution to the problem of providing a further method for detection or identification of a bacterium using 16S rDNA technology, as it is using a novel and non-obvious SEQ ID NO (1 or 2)

1.10 Taking **D4** or **D5** as closest prior art, the subject-matter of **claim 10** differs in that it concerns a novel consortium. The problem underlying **claim 10** may be summarised as the provision of a further consortium for reduction of ammonia and/or methane in manure to improve the quality of said manure. The solution is the consortium according to **claim 10** deposited as CBS 134115. In view of the effect that is disclosed for the use of said consortium in examples 1, 2, involvement of inventive step is acknowledged for **claim 10**.

1.11 Taking **D4** or **D5** as closest prior art, the subject-matter of **claim 11** differs in that it concerns a different bacterium. The problem underlying **claim 11** may be summarised as the provision of the use of a further bacterium or consortium for reduction of ammonia and/or methane in manure to improve the quality of said manure. The solution is related to the use of a bacterium according to any of **claims 1 to 4** or of a consortium according to any of **claims 5-10**.

First, **claim 8** does not relate to a consortium, hence the reference in **claim 11** to **claim 8** is erroneous.

Second, the problem is not solved for every bacterium or consortium according to **claims 1, 2, 5-7**, in view of the lack of novelty of **claims 1, 2, 5-7** (*vide supra*, 1.2). There is no indication that the bacteria disclosed in **D1, D2** are suitable for reduction of ammonia, methane in manure.

Third, in the absence of a proven effect for the bacteria according to **claim 3** (*vide supra*, 1.7) the problem is not solved for the bacteria of **claim 3**.

Fourth, what is said for **claim 3** applies also to **claim 4**, *mutatis mutandis* (*vide supra*, 1.8). Cf. what is said in 1.8 concerning the lack of a technical, causal relationship between a 16S rDNA sequence and the effect of reduction of ammonia, methane in manure.

Fifth, due to problems with clarity which prohibited assessment of novelty of the subject-matter of **claim 9** (*vide supra*, 1.4), assessment of involvement of inventive step is prohibited too. It is added, that it is likely that the problem is not solved over the breadth of **claim 9**.

Sixth, involvement of inventive step can be acknowledged for the subject-matter of **claim 11** insofar as the consortium according to **claim 10** is concerned, in line of what is said regarding **claim 10** above (1.10).

Inventive step is also acknowledgeable for dependent **claim 12** insofar as the consortium according to **claim 10** is concerned.

- 1.12 What is said for involvement of inventive step of **claim 11** applies, *mutatis mutandis*, to **claims 12-15** too, namely that inventive step can only be acknowledged insofar as the consortium according to **claim 10** is concerned. For other subject-matter in **claims 12-15**, inventive step is not acknowledged.

2 **Re Item VIII**

Certain observations on the application

- 2.1 **Claim 9** is not clear. *Vide supra*, 1.4.
- 2.2 **Claims 11-14** contain erroneous references. *Vide supra*, 1.6.