Genetic and Functional Analysis of Biogenic Amine Production Capacity Among Starter and Non-Starter Lactic Acid Bacteria Isolated from Artisanal Cheeses

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Short title: BA production capacity among dairy LAB

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Abstract

This work reports the capacity of 137 strains of starter and non-starter LAB belonging to nine species of the genera *Lactobacillus*, *Lactococcus*, *Streptococcus* and *Leuconostoc* (all isolated from artisanal cheeses) to produce histamine, tyramine, putrescine and β-phenylethylamine, the biogenic amines (BA) most commonly found in dairy products. Production assays were performed in liquid media supplemented with the appropriate precursor amino acid; culture supernatants were then tested for BA by (U)HPLC. In addition, the presence of key genes involved in the biosynthetic pathways of the target BA, including the production of putrescine via the agmatine deiminase pathway, was assessed by PCR. Twenty strains were shown to have genes involved in the synthesis of BA; these belonged to the species *Lactobacillus brevis* (4), *Lactobacillus curvatus* (3), *Lactococcus lactis* (11) and *Streptococcus thermophilus* (2). With the exception of the two *S. thermophilus* strains, all those possessing genes involved in BA production synthesized the corresponding compound. Remarkably, all the putrescine-producing strains used the agmatine deiminase pathway. Four *Lb. brevis* and two *Lb. curvatus* strains were found able to produce both tyramine and putrescine. There is increasing interest in the use of autochthonous LAB strains in starter and adjunct cultures for producing dairy products with 'particular geographic indication' status. Such strains should not produce BA; the present results show that BA-production capacity should be checked by (U)HPLC and PCR.

Key words: Biogenic amines, tyramine, putrescine, tyrosine decarboxylase, agmatine deiminase.

Introduction

Lactic acid bacteria (LAB) play an essential role in the production of fermented dairy products, with *Lactococcus lactis* and *Streptococcus thermophilus* being the species most commonly used as primary fermentation starters [1]. Their major function is the rapid production of lactic acid from lactose, resulting in a lowering of the pH.

The so-called non-starter lactic acid bacteria (NSLAB) participate in the development of the final organoleptic properties of fermented dairy products [2]. NSLAB may be present in the milk itself, be part of the flora of dairy facilities, or be added to fermentations as adjunct cultures [3]. These bacteria are frequently facultative, heterofermentative lactobacilli belonging to the species *Lactobacillus casei/paracasei*, *Lactobacillus plantarum* or *Lactobacillus curvatus* [4,5]. *Leuconostoc* may be involved in the development of aroma components [6]. There is increasing interest in the characterization and use of NSLAB from artisanal products for use in tailored cultures to be employed in the manufacture of dairy products with 'protected geographic indication' (PGI) status. Their use would help maintain their typical organoleptic characteristics [6-9].

The long and safe history of the use of LAB in dairy products has resulted in the assignment of Qualified Presumption of Safety (QPS) status (awarded by the European Food Safety Authority [EFSA]) to the majority of LAB. However, some properties and enzymatic activities can generate undesirable flavours [10] or even toxic compounds such as biogenic amines (BA) [11], the presence of which should be avoided in dairy products.

BA are low molecular weight nitrogenous compounds formed by the decarboxylation of certain amino acids that may be present in foods. The consumption of foods with high

BA concentrations may cause intoxications manifested as headache, nausea or vomiting, alterations in blood pressure, rashes, etc. [12]. Cheese is the fermented food most commonly associated with BA poisoning; indeed, the term *cheese reaction* was coined to refer to it [13]. Tyramine, putrescine and histamine are the most commonly encountered and abundant BA; in cheese [11,14,15]. Certainly, cheese provides an ideal matrix for the production and accumulation of BA since the amino acid substrates required are made easily available by casein proteolysis, and the low pH favours decarboxylase gene transcription and enzyme activity [11]. Further, cheese naturally contains milk-derived Gram positive LAB, generally of the genera *Lactobacillus* and *Enterococcus*, which possess decarboxylating activity [11,16]. BA-producing strains have also been described among the species most commonly used as dairy starters, such as *Lactococcus lactis, Streptococcus thermophilus* and *Lactobacillus delbrueckii* [17-19]. BA producers may also enter dairy products via contamination [20,21].

The selection of starter strains with no BA-producing capacity would be a good starting point for reducing BA accumulation in dairy products [22]. Different methods have been devised for assessing the capacity of LAB to produce BA, including the use of differential media and pH indicators [23]. Unfortunately, the strong acidification of the medium occasioned by harmless LAB can result in false negatives. Moreover, these methods target the presence of amino acid decarboxylases and do not test the presence of deimination routes involved in the production of some BA such as putrescine [11]. Analytical methods that directly detect BA compounds in culture supernatants after incubation with an amino acid precursor have also been commonly used [24,25]. However, culture-independent methods based on PCR techniques, aimed to detect the genetic determinants involved in the synthesis of BA, are now regarded as the most suitable for screening collections of isolates [26]. Agreement between the results

obtained by analytical and molecular methods strengthens the case for the use of the latter [27,28].

In the present work, U(H)PLC and PCR methods were used to examine the capacity of 137 LAB strains (four genera, nine species), isolated from artisanal cheeses, and all with potential for use in dairy starter or adjunct cultures designed for the production of artisanal cheeses with PGI status, to produce histamine, tyramine, putrescine and β-phenylethylamine.

2. Materials and Methods

Bacterial strains

One hundred and thirty seven strains isolated from different artisanal cheeses [29,30], identified by comparison of partial 16S rRNA gene sequences, and belonging to four different genera - *Lactococcus, Streptococcus, Leuconostoc,* and *Lactobacillus* - were assessed for their capacity to produce BA (Table 1). *Lactococcus lactis*, *Streptococcus thermophilus* and *Leuconostoc mesenteroides* strains were grown statically in M17 (Oxoid) supplemented with 0.5% glucose and 0.5% lactose (w/v) at either 30ºC (*Lc. lactis, Le. mesenteroides*) or 37ºC (*S. thermophilus* strains). All *Lactobacillus* strains, which belonged to six species (Table 1), were grown statically in MRS (Oxoid) at 30ºC, except those belonging to *Lb. delbrueckii* which were grown at 37ºC.

In vivo BA-production capacity

BA production was assessed in triplicate in culture supernatants of the LAB strains grown for 24 h in 10 ml M17 or MRS broth supplemented with 1 mM tyrosine (M17/MRS-T), 1 mM histidine (M17/MRS-H), 1 mM ornithine (M17/MRS-O) or 1 mM agmatine (M17/MRS-A). Both ornithine and agmatine are precursors of putrescine,

although via different pathways. Tyramine, histamine and putrescine detection was performed as previously described [31] after the centrifugation of the cultures (2000 x g for 15 min) and filtering of the supernatant through a 0.2 μm pore diameter membrane (Pall, USA), followed by derivatization of 100 μl with diethyl ethoxymethylene malonate. Derivatized samples were analyzed by (U)HPLC in a Waters H-Class Acquity UPLC apparatus with a UV detector (Waters, USA) controlled by Empower 2.0 software (Waters), under the conditions described by Redruello et al. [32].

Detection of BA-producing genes

The presence of the tyrosine decarboxylase gene *tdcA*, the histidine decarboxylase gene *hdcA*, the ornithine decarboxylase gene *odc*, and the *aguA* and *aguD* genes from the agmatine deiminase cluster (AgdI), was checked by PCR using the primer pairs P2-for and P1-rev [33], JV16HC and JV17HC [34], ODC3 and ODC16 [35], and Seq1 and Seq2 [17], respectively. The PCR conditions were those described in [33-35,17], respectively and were performed in a MyCyclerTM thermal cycler (Bio-Rad, Spain) using DreamTaq polymerase (Fermentas, Lithuania). Total DNA from the strains was obtained as previously described [36] and used as a template in PCR. Total DNA from the tyramine- and putrescine-producing strain *Enterococcus faecalis* V583 [27], from the ODC+ strain *Lactobacillus saerimneri* 30A [37], and from the histamine-producer *Lactobacillus buchneri* B301 [38], were used to provide positive controls.

PCR products were separated in 0.8% (w/v) agarose gels in 1XTAE buffer, and visualized after staining with ethidium bromide using a GelDoc 2000 system (Bio-Rad, Hercules, USA). The Gene Ruler DNA ladder mix (Fermentas, Lithuania) was used as molecular weight marker.

Compliance with Ethical Requirements

This study does not involve animal or human subjects.

Results and Discussion

The selection of starter strains with no BA-producing capacity is an important step towards reducing the presence of these toxins in dairy products [22]. In this work, 137 LAB strains, previously isolated from artisanal cheeses made from raw milk, were evaluated for their BA-producing capability.

Twenty (14.69%) of the 137 examined strains were found to possess genes involved in BA production, including 4 strains of *Lb. brevis*, 3 of *Lb. curvatus*, 11 of *Lc. lactis* (8 belonging to *Lc. lactis* subsp. *lactis* and 3 to *Lc. lactis* subsp. *cremoris*), and 2 of *S. thermophilus* (Table 1 and Fig. 1).

Eighteen (13.1%) of the tested strains showed the capacity to produce at least one BA in a supplemented medium. These corresponded to all the strains in which the presence of genes involved in BA production were detected by PCR, except for the two *S. thermophilus* strains (see below). Six strains (4.4%), four *Lb. brevis* and two *Lb. curvatus* strains, produced both tyramine and putrescine.

Similar percentages of BA-producing strains have been reported by other authors [19,39]. During their analysis of dairy isolates, Bunkova et al. [19] found 20% of the strains tested to produce tyramine and to be positive for the *tdcA* gene. In some studies, higher percentages of BA-producers have been reported [39,40], but in most of these investigations strains of *Enterococcus* were analyzed. The capacity to produce BA is widespread among enterococci and has been shown as a species-specific trait in some enterococcal species [27,41]; thus increasing the occurrence of BA producing strains. It was, therefore decided not to include enterococcal strains in the present work.

All of the strains that gave a positive PCR result for the presence of genes involved in BA production were able to synthesize the corresponding BA (Table 1), except for two strains of *S. thermophilus*. Both of these strains possessed the histidine decarboxylase gene *hdcA*, but no histamine was found in the culture supernatant after 24 or even after 48 h of culture in M17-H. This might be due to a non-functionality of the HDC cluster or because the conditions assayed were not optimal for histamine production in these strains since the production of BA can be affected by different cultures conditions [11]. Certainly, some authors report that all *S. thermophilus* strains with the capacity to produce histamine from histidine produce small amounts of histamine in broth but not in milk etc. [18,42]. In any event, the present work highlights a good correlation between the results of molecular and functional analysis of BA-producing capacity. All the BA-producing strains returned positive PCR results, indicating that this culture-independent technique is suitable for assessing this property in potential LAB starter strains [28].

Even though two *S. thermophilus* strains were negative for the *in vivo* production of BA, their possession of genes involved in BA production must be seen as a risk. These genes could be horizontally transferred to other LAB present in the culture or dairy product [43-45], conferring the ability to produce histamine upon them.

Of the 137 strains tested, seven produced tyramine from tyrosine in broth, and were positive for *tdcA* in PCR tests (Table 1). All these strains belonged to *Lb. brevis* or *Lb. curvatus*. Tyramine-producing strains of these species have been isolated from cheeses by other authors [44,46,47]. In *Lb. brevis*, tyramine production has been described as a strain level trait - perhaps horizontally acquired [44,48]. For *Lb. curvatus* there are insufficient data to confirm whether it is a species- or strain-dependent trait. The majority of *Lb. curvatus* strains isolated from meat, however, were have been reported

196 tyramine producers [49-51]. All the present tyramine producers, independent of their
197 species, were 'strong tyramine producers' (Table 2). *Lb. curvatus* strains have been
198 described as strong tyramine producers by other authors [47,47], showing high
199 conversion rates in broth media supplemented with tyrosine. *Lb. brevis* has also been
200 described as a strong tyramine producer, although different media and conditions were
201 assayed and variation in tyramine production capacity was observed [52].
202 None of the tested strains was able to produce β-phenylethylamine under the present
203 assay conditions. No specific phenylalanine decarboxylases have been described, but
204 several authors have reported that certain tyrosine decarboxylases can use phenylalanine
205 as an alternative substrate, converting it into β-phenylethylamine [53]. In the present
206 work, only the *E. faecalis* positive control was able to produce β-phenylethylamine in
207 medium supplemented with tyrosine (data not shown).
208 Putrescine is produced from arginine via a decarboxylation and a deimination reaction
209 [11,54]. However, the order of these reactions can differ, and, depending on that order,
210 two different pathways are recognized: (i) the ornithine decarboxylation pathway
211 (ODC) (in which arginine is first deiminated to form ornithine, which is then
212 decarboxylated to form putrescine), and (ii) the agmatine deimination pathway (AGDI)
213 (in which arginine is first decarboxylated to form agmatine, which is then deiminated to
214 form putrescine) [11,54]. To distinguish which pathway was being used, the tested
215 strains were grown in media supplemented with ornithine or agmatine. No strain
216 produced putrescine from ornithine. Although the ODC pathway has been described in
217 several LAB strains, including strains of *Lb. brevis* [25,43], it is not a pathway
218 commonly used by dairy bacteria [11,54,55]. Thus, the lack of strains with ODC
219 pathway capacity among those tested in the present work was expected. Seventeen
220 strains of the 137 examined were, however, able to produce putrescine from agmatine

(Table 1). Putrescine is the most commonly found BA in dairy products [14]. It is not surprising, therefore, that the largest number of BA-producing strains detected should be putrescine producers. It is important to highlight that all the putrescine producers detected in the present survey have the AGDI and not the ODC pathway. Although the prevalence of the AGDI pathway in dairy strains has been previously suggested [11], a test to determine the presence of the AGDI pathway is not usually done. In fact, as far as we are aware, this is the first study to include screening for the AGI pathway when testing for BA-producing capacity in dairy LAB.

The production of putrescine via the AGDI pathway has, however, been described in *Lb. brevis* of non-dairy origin by other authors [48,56]. All the present strains of *Lb. brevis* shown to be putrescine producers were also tyramine producers. It has been suggested that, in this species, the AGDI genetic determinants are linked to those of the TDC pathway, producing a *locus* of acid resistance mechanisms probably acquired by horizontal gene transfer [48,43]. Two of the three *Lb. curvatus* strains tested produced putrescine from agmatine and also returned positive PCR results (Table 1), both strains were also able to produce tyramine. As in *Lb. brevis*, these two BA-producing capacities have been related to acid resistance. The corresponding genes have been described as lying adjacent to one another in the chromosome of some dairy isolates of *Lb. curvatus* [43].

Among the *Lc. lactis* strains tested, i.e., of both subspecies *lactis* and *cremoris*, 11 were shown to produce putrescine from agmatine. Such a capacity had already been reported for some *Lc. lactis* strains [17], and putrescine-producing *Lc. lactis* can be found in large numbers in cheeses with high putrescine concentrations [55]. Not all the *Lc. lactis* strains tested were able to produce putrescine, although the capacity to produce it from agmatine has been described as a species level trait [17]. Traditionally, BA-producing

246 pathways have been thought horizontally acquired [44,48]. The present *Lc. lactis* strains
247 unable to synthesize putrescine may have lost this capacity during their use in the dairy
248 environment: putrescine would negatively affect acidification and/or final flavour and
249 such putrescine-producing strains would have been avoided [17]. The loss of this
250 capacity seems to have occurred in two ways: (i) via the loss of the AGDI pathway
251 genes, as has been shown for strains of *Lc. lactis* subsp. *cremoris*, and (ii) the
252 inactivation of the cluster by an insertion element (IS) in *Lc. lactis* subsp. *lactis* strains
253 [17]. To differentiate between putrescine and non-putrescine producers, a specific PCR
254 test is available [17] in which non-putrescine-producing *Lc. lactis* subsp. *cremoris*
255 strains do not render a PCR band, while *Lc. lactis* subsp. *lactis* non-putrescine-
256 producing strains do, although the amplification product is 1 kb larger than expected
257 due to the presence of an IS element. In the present work, none of the non-putrescine-
258 producing strains of *Lc. lactis* subsp. *cremoris* was associated with any positive
259 amplification, while those of *Lc. lactis* subsp. *lactis* rendered the expected enlarged
260 amplicon (Fig. 1).
261 Variation in the efficiency of putrescine production was observed among the producing
262 strains of *Lc. lactis*; this allowed their classification as 'strong" or 'medium putrescine
263 producers' (Table 2). Variation in the capacity to produce putrescine from agmatine has
264 been described before among *Lc. lactis* subsp. *cremoris* strains [31]. In the present
265 work, however, the greatest variation was observed among the *Lc. lactis* subsp. *lactis*
266 producers (Table 2).
267 One of the most effective measures for reducing the presence of BA in dairy products is
268 the use of starter cultures that have been properly tested and selected as non-BA-
269 producers [22]. The present results show that both culture-dependent and culture-
270 independent methods are appropriate for ruling out BA-producing strains for use as

starters and adjunct cultures. The culture-independent methods based on PCR testing not only detected BA producers but also non-producer strains that possessed genes involved in BA production; these pose a risk since they might be spread by horizontal gene transfer.

There is increasing interest in the use of autochthonous LAB strains in starter and adjunct cultures for producing dairy products with PGI status. [8,57]. Strains intended for use in their manufacture should be systematically monitored for BA production capacity to avoid the accumulation of these toxins and thus produce safer dairy products.

Conclusions

This work shows that some of the strains belonging to the species most frequently used in the manufacture of dairy products can produce BA, highlighting the importance of adequately selecting indigenous strains for inclusion in starter and adjunct cultures. The prevalence of putrescine-producing strains (which use the AGDI pathway) is noteworthy. The literature contains little on this, even though putrescine is one of the commonest BA in dairy products and the AGDI pathway is the main route of its synthesis. Tests for the presence of the AGDI pathway should be included when examining the BA production capability in dairy strains. As shown in this work, the capacity to produce BA can be tested by either chromatographic or molecular methods, although PCR testing affords many advantages.

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Conflict of interest

None.

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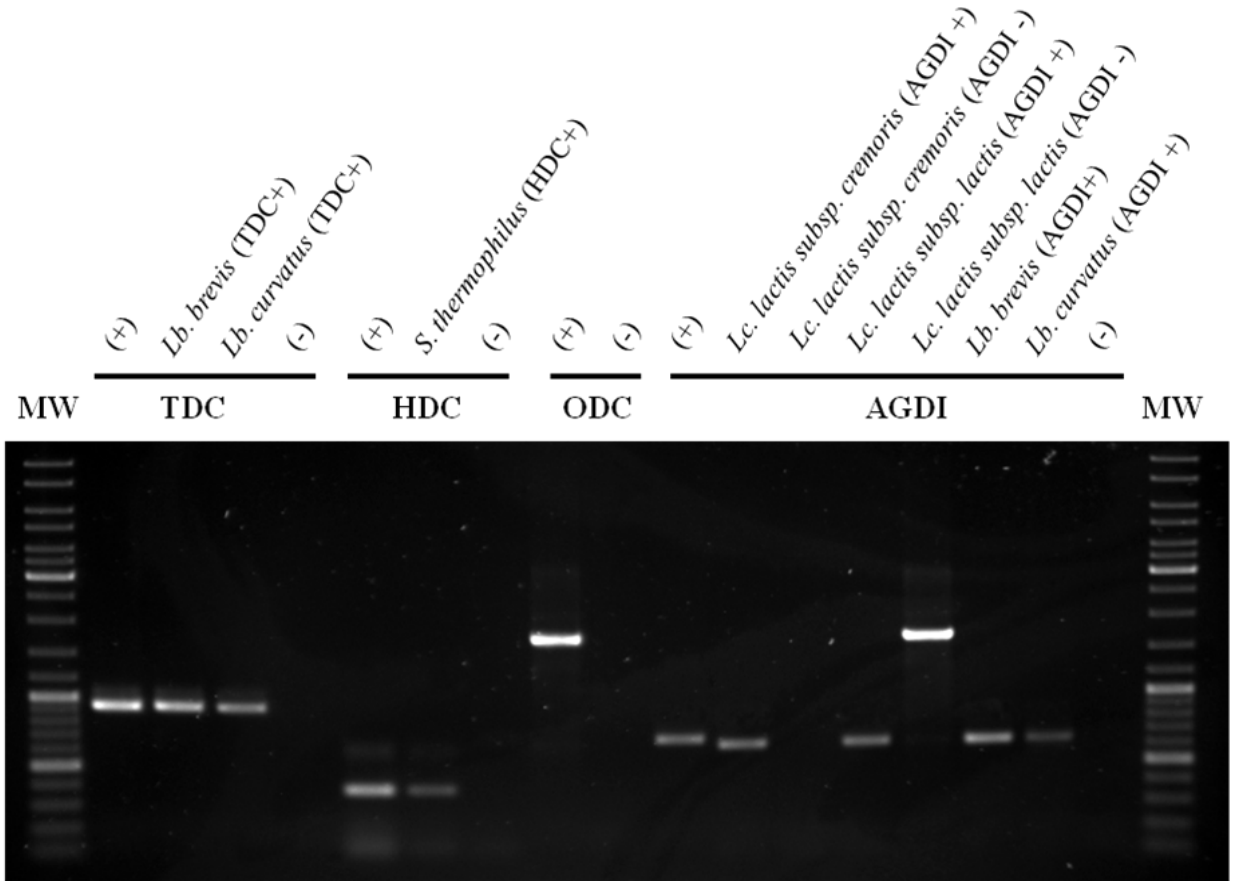

Fig 1: Results of PCR tests for the presence of genes involved in BA production (*tdcA*, *hdcA*, *odc* and *aguD-AguA*). A representative of each positive species is shown. For *Lc. lactis* subsp. *lactis* and *cremoris*, a representative of the negative strains is also shown (see text for details). For each BA cluster, the negative (-) and positive (+) controls (*E. faecalis* V583 for TDC and AGDI, *Lb. buchneri* B301 for HDC and *Lb. saerimneri* 30A for ODC) are indicated. MW: Molecular weight standard Gene Ruler (Fermentas). TDC: Tyramine-producing cluster; HDC: Histamine-producing cluster; ODC: Putrescine-producing cluster (via the ornithine decarboxylase pathway); AGDI: putrescine producing cluster (via the agmatine deiminase pathway).

Table 1: BA-producing strains among the LAB tested. Number of strains with the capacity to produce tyramine (Tym), β-phenylethylamine (β-phe), histamine (Him) or putrescine (Put) in supplemented broth, as determined by (U)HPLC, and the presence of the corresponding genes, as shown by PCR. N: number of strains tested. ODC: ornithine decarboxylase pathway. AGDI: agmatine deiminase pathway.

Species	**N**	**Tym**		**β-phe**	**Him**		**Put (ODC)**		**Put (AGDI)**	
		PCR	**(U)HPLC**	**(U)HPLC**	**PCR**	**(U)HPLC**	**PCR**	**(U)HPLC**	**PCR**	**(U)HPLC**
Lactobacillus brevis	4	4	4	0	0	0	0	0	4	4
Lactobacillus casei	12	0	0	0	0	0	0	0	0	0
Lactobacillus curvatus	3	3	3	0	0	0	0	0	2	2
Lactobacillus delbrueckii	9	0	0	0	0	0	0	0	0	0
Lactobacillus fermentum	10	0	0	0	0	0	0	0	0	0
Lactobacillus plantarum	19	0	0	0	0	0	0	0	0	0
Lactococcus lactis subsp. *lactis*	16	0	0	0	0	0	0	0	8	8
Lactococcus lactis subsp. *cremoris*	7	0	0	0	0	0	0	0	3	3
Leuconostoc mesenteroides	14	0	0	0	0	0	0	0	0	0
Streptococcus thermophilus	43	0	0	0	2	0	0	0	0	0
Total	**137**	**7**	**7**	**0**	**2**	**0**	**0**	**0**	**17**	**17**

Table 2: Classification of tyramine- and putrescine-producing strains based on their production capacity. The strains were classified as 'strong' (more than 90% of the substrate present [1 mM tyrosine or agmatine] converted after 24 h of incubation), 'medium' (between 40 and 90% converted) or 'poor' (<40% converted) producers. N: number of strains tested. Tym: tyramine. Put: putrescine. AGDI: agmatine deiminase pathway. CR: conversion rate.

Species	**N**	**Tym**			**Put (AGDI)**		
		CR>90	**90>CR>40**	**CR<40**	**CR>90**	**90>CR>40**	**CR<40**
Lactobacillus brevis	4	4	0	0	1	2	1
Lactobacillus curvatus	3	3	0	0	2	0	0
Lactococcus lactis subsp. *lactis*	8	0	0	0	5	3	0
Lactococcus lactis subsp. *cremoris*	3	0	0	0	0	3	0
Total	18	7	0	0	8	8	1