

1 Putrescine production via the agmatine deiminase pathway increases the growth of
2 *Lactococcus lactis* and causes the alkalinization of the culture medium

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18

1 **Abstract**

2

3 *Lactococcus lactis* is the most important starter culture organism used in the dairy
4 industry. Although *L. lactis* species have been awarded Qualified Presumption of
5 Safety status by the European Food Safety Authority, and Generally Regarded as
6 Safe status by the US Food and Drug Administration, some strains can produce
7 the biogenic amine putrescine. One such strain is *L. lactis* subsp. *cremoris* CECT
8 8666 (formerly *L. lactis* subsp. *cremoris* GE2-14), which was isolated from
9 Genestoso cheese. This strain catabolizes agmatine to putrescine via the agmatine
10 deiminase (AGDI) pathway, which involves the production of ATP and two
11 ammonium ions. The present work shows that the availability of agmatine and its
12 metabolization to putrescine allows for greater bacterial growth (in a biphasic
13 pattern) and causes the alkalinization of the culture medium in a dose-dependent
14 manner. The construction of a mutant lacking the AGDI cluster (*L. lactis* CECT
15 8666 $\Delta agdi$) confirmed the latter's direct role in putrescine production, growth and
16 medium alkalinization. Alkalinization did not affect the putrescine production
17 pattern and was not essential for increased bacterial growth.

18

19 Keywords: *Lactococcus lactis*; biogenic amines; putrescine; agmatine deiminase;
20 AGDI cluster; bacterial growth

21

1 **Introduction**

2

3 The ability of *Lactococcus lactis* - a lactic acid bacterium (LAB) - to rapidly ferment
4 lactose, its notable proteolytic activity and its production of flavor molecules
5 (Beresford et al. 2001; Kelly et al. 2010; Kuipers, 2001), have made it one of the
6 most commonly used primary starters in the manufacture of cheese, fermented
7 milk, sour cream and buttermilk. *L. lactis* has been granted Qualified Presumption
8 of Safety status by the European Food Safety Authority, and Generally Regarded
9 as Safe status by the US Food and Drug Administration.

10

11 However, our group has characterized several *L. lactis* subsp. *lactis* and *L. lactis*
12 subsp. *cremoris* strains from dairy products that produce the biogenic amine (BA)
13 putrescine (Ladero et al. 2011b). Indeed, putrescine-producing *L. lactis* has been
14 detected in high concentrations in a number of cheeses (Ladero et al. 2012a).
15 Along with tyramine and histamine, putrescine is one of the most common BAs in
16 dairy products (Fernandez et al. 2007). Its presence in fermented foods confers
17 undesirable flavors (Ladero et al. 2012c) and it can have toxic effects when
18 ingested, such as increased cardiac output, tachycardia and hypotension. It can
19 also potentiate the effects of other BAs (Ladero et al. 2010; Romano et al. 2012). It
20 may even have a role in promoting malignancy: it is known to be involved in
21 growth-related processes and has been suggested to increase intestinal and
22 colonic tumorigenesis and neoplasm proliferation (Gerner and Meyskens, 2004;
23 Ignatenko et al. 2006; Ladero et al. 2010; Seiler et al. 1998).

1
2 In *L. lactis*, putrescine is produced through the catabolism of agmatine, a
3 decarboxylated derivative of arginine (Simon and Stalon, 1982), via the agmatine
4 deiminase (AGDI) pathway (Ladero et al. 2011b) (Fig. 1a). This metabolic pathway
5 involves the sequential action of three enzymes: agmatine deiminase (AguA),
6 putrescine carbamoyltransferase (AguB) and carbamate kinase (AguC). Agmatine
7 is deiminated by AguA, rendering one ammonium ion and N-carbamoyl putrescine,
8 which is phosphorylated by AguB to produce putrescine and carbamoyl phosphate.
9 This last product is used as a substrate for ADP phosphorylation by AguC, a
10 reaction that renders one molecule of ATP and another ammonium ion. Putrescine
11 is then exchanged with external agmatine by the agmatine/putrescine antiporter
12 (AguD). The gene cluster organization for agmatine catabolism in *L. lactis* (Fig. 1b)
13 seems to be unique among LAB, the potential regulatory gene *aguR* being located
14 upstream of the catabolic genes and orientated in the same direction (Ladero et al.
15 2011b). The catabolic genes are organized as follows: *aguB* (encoding AguB),
16 *aguD* (encoding AguD), *aguA* (encoding AguA) and *aguC* (encoding AguC).
17 Transcriptional analysis of the AGDI cluster of *L. lactis* subsp. *lactis* (Ladero et al.
18 2011b) and *L. lactis* subsp. *cremoris* CECT 8666 (formerly *L. lactis* subsp. *cremoris*
19 GE2-14) (Linares et al. 2013) has shown that the catabolic genes together form the
20 *aguBDAC* operon, and that they are transcribed as a single polycistronic mRNA.

21

22 Although the genetics and transcriptional regulation of putrescine production have
23 been characterized in *L. lactis* (Ladero et al. 2011b; Linares et al. 2013) the

1 physiological role of putrescine production in this organism has not been studied.
2 In the present work, *L. lactis* subsp. *cremoris* CECT 8666, isolated from an
3 artisanal cheese and previously selected as a model putrescine-producing strain
4 (Linares et al. 2013), was used to assess the effect of putrescine production on
5 bacterial growth and on the alkalization of the culture medium.

6

7 **Material and Methods**

8

9 *Bacterial strains and culture conditions*

10

11 Table1 shows the bacterial strains used. *L. lactis* subsp. *cremoris* GE2-14, the
12 putrescine-producing strain used in this study, was previously isolated from
13 Genestoso cheese, a Spanish artisanal cheese made from raw milk without the
14 addition of commercial starter cultures (Fernandez et al. 2011; Ladero et al.
15 2011b). *L. lactis* subsp. *cremoris* GE2-14 was deposited in the Colección Española
16 de Cultivos Tipo (CECT) with the accession No CECT 8666. *L. lactis* CECT 8666
17 and the derivative agmatine deiminase mutant (*L. lactis* CECT 8666 $\Delta agdI$), were
18 grown in M17 (Oxoid, UK) supplemented with 0.5% (w/v) glucose (GM17) (unless
19 otherwise indicated) at 32°C without aeration. Where indicated, the medium was
20 supplemented with 20 mM of agmatine (GM17+A) (Sigma-Aldrich, St. Louis, MO).
21 For all fermentation assays, overnight cultures of *L. lactis* strains were used (1%
22 v/v inoculum).

23

1 pH-uncontrolled fermentations were performed in 30 ml of GM17 or GM17+A.
2 Sampling (2 ml) was performed every hour for 12 h. The pH of the samples was
3 measured using a CRISON miCropH 2001 pH-meter (Crison Instruments S.A.,
4 Barcelona, Spain). For pH-controlled fermentations, cells were grown in a Six-
5 Fors[®] bioreactor (Infors AG, Bottmingen, Switzerland) containing 300 ml of
6 GM17+A. The reactor was maintained at 32°C, stirring at 50 rpm, and with zero air
7 input. The fixed pH of 5.5 was maintained by the automatic addition of 1 N NaOH
8 or 1 N HCl, as needed. 2 ml samples were collected each hour. Microbial growth
9 was examined in all cultures by measuring absorbance at 600 nm (OD₆₀₀) using a
10 spectrophotometer (Eppendorf, NY, USA). Putrescine, agmatine and ammonium
11 ion concentrations were analyzed as explained below.

12

13 *Putrescine, agmatine and ammonium ions: analysis by ultra-high performance*
14 *liquid chromatography*

15

16 Sample supernatants were obtained by centrifugation (2000 x g for 15 min).
17 Putrescine, agmatine and ammonium ions were analyzed by ultra-high
18 performance liquid chromatography (UHPLC) using a Waters H-Class ACQUITY
19 UPLC[®] apparatus controlled by Empower 2.0 software and employing a UV-
20 detection method based on derivatization with diethyl ethoxymethylene malonate
21 (Sigma-Aldrich), following the protocol of (Redruello et al. 2013).

22

23 *DNA manipulation procedures*

1

2 *L. lactis* total DNA was extracted using Kirby lytic mix according to Hopwood et al.
3 (1985). Plasmid DNA was prepared from *Escherichia coli* by the alkaline lysis
4 method (Green et al. 2012). Restriction endonuclease digestions, alkaline
5 phosphatase treatments, ligations and other DNA manipulations were performed
6 according to standard procedures (Green et al. 2012). The DNA modification
7 enzymes were from commercial sources and used following the supplier's
8 recommendations. Electrotransformation of *E. coli* was achieved in a Bio-Rad
9 pulser using protocols provided by the supplier; *L. lactis* was electrotransformed as
10 described by de Vos et al. (1989). PCR amplifications were performed in a
11 MyCycler™ thermal cycler (Bio-Rad, Spain) using Phusion High-Fidelity DNA
12 polymerase (Thermo Scientific, Spain) according to the manufacturer's protocol. All
13 DNA fragments amplified were checked by nucleotide sequencing at Macrogen
14 Inc. (Seoul, Republic of Korea).

15

16 *Construction of the L. lactis* CECT 8666 $\Delta agdi$ mutant

17

18 The *L. lactis* CECT 8666 $\Delta agdi$ mutant lacking the AGDI cluster (GenBank
19 Accession No HG317493.1) was constructed by homologous recombination, using
20 the selection/counter-selection vector pCS1966 (Solem et al. 2008). Table 1 shows
21 the primers and plasmids used to generate the AGDI knock-out. The primers were
22 designed to include the following restriction recognition sites: SpeI in primer KO-
23 214AguR-AF2, PstI in KO-214AguR-AR, PstI in CKPstF, and XhoI in NoxXho. A

1 826 bp PCR fragment containing a 610 bp fragment of the *ycaC* gene (the gene
2 upstream from *aguR*, GenBank Accession No HG317493.1), the intergenic region
3 between *ycaC* and *aguR*, and the encoding sequence of the five first amino acids
4 of the *aguR* gene of *L. lactis* CECT 8666, was amplified using primers KO-
5 214AguR-AF2 and KO-214AguR-AR. The amplification conditions were: 1 min of
6 initial denaturation at 98°C, followed by 35 amplification cycles (denaturation for 30
7 s at 98°C, annealing for 30 s at 55°C, and extension for 1 min at 72°C), and a final
8 extension step of 10 min at 72°C. The resulting fragment was digested with the
9 restriction enzymes *SpeI* and *PstI* and cloned into the pCS1966 vector, rendering
10 the plasmid pIPLA1269. A second 889 bp PCR fragment containing the last 260 bp
11 of the *aguC* gene plus the last 427 bp of the *yrfB* gene (the gene downstream of
12 *aguC*, GenBank Accession No HG317493.1) and the intergenic region of *aguC* and
13 *yrfB*, were PCR amplified using primers CKPstF and NoxXho. The amplification
14 conditions were: 1 min of initial denaturation at 98°C, followed by 35 amplification
15 cycles (denaturation for 30 s at 98°C, annealing for 30 s at 55°C, and extension for
16 1 min at 72°C), and a final extension step for 10 min at 72°C. The resulting
17 fragment was digested with *PstI* and *XhoI* and cloned into the plasmid pIPLA1269,
18 rendering the plasmid pIPLA1292. Plasmid pIPLA1292 was then transformed into
19 *L. lactis* CECT 8666 electrocompetent cells and mutant bacteria lacking the AGDI
20 cluster were selected following the method of Solem et al. (2008). *L. lactis* CECT
21 8666 $\Delta agdi$ mutants were confirmed by nucleotide sequence analysis of the
22 amplicon obtained using the primers KO-214AguR-AF2 and NoxXho, which

1 rendered the expected 1715 bp fragment instead of the 7112 bp fragment
2 corresponding to the wild type strain (data not shown).

3

4 **Results**

5

6 *Putrescine production, bacterial growth and pH*

7

8 The production of putrescine was monitored over the growth curve. Figure 2A
9 shows that the *L. lactis* CECT 8666 grown in GM17+A accumulated 20 mM of
10 putrescine 10 h after inoculation (during the stationary phase) (Fig. 2b). Putrescine
11 production was first detected after 6 h of fermentation and it was continuously
12 produced over the next 4 h until the agmatine was used up. Figure 2A shows that
13 putrescine production strictly followed agmatine consumption. Concomitant
14 production of ammonium ions was also observed (Fig. 2a).

15

16 A typical growth curve was recorded over 10 h for the culture grown in GM17, with
17 an exponential growth phase starting after 2 h of inoculation and lasting until 5 h of
18 fermentation (OD_{600} 3.6). At this point the culture entered the stationary phase, in
19 which it remained until the end of fermentation (Fig. 2b). However, the culture
20 grown in GM17+A shown a biphasic growth curve typical of diauxic growth. The
21 first exponential growth phase lasted until 5 h of fermentation, as described above
22 for the GM17-grown cultures, but the stationary growth phase lasted only 1 h
23 before a second growth phase set in. This lasted until 10 h of fermentation. At the

1 end of this second growth phase in the GM17+A-grown cultures, the OD₆₀₀ was
2 much higher (4.2) than that recorded for the GM17 cultures (3.2) (Fig. 2b).

3
4 The change in the pH of the culture media was also monitored (Fig. 2b). The
5 growth of *L. lactis* CECT 8666 in both GM17 and GM17+A induced an acidification
6 of the medium from pH 6.9 to pH 5.8 by 5 h post-inoculation. However, the pH of
7 the culture grown in GM17 remained at pH 5.8 until the end of fermentation, while
8 the pH of the GM17+A medium alkalinized, reaching a final pH of 6.5. The
9 alkalinization of the medium in the culture supplemented with agmatine paralleled
10 the production of ammonium ions, a result of the catabolism of agmatine to
11 putrescine (Fig. 2a).

12 13 *Effect of agmatine concentration on bacterial growth and pH*

14
15 To confirm the correlation between putrescine production and both the
16 enhancement of growth and the alkalinization of the culture medium, *L. lactis*
17 CECT 8666 was grown in GM17 supplemented with increasing amounts of
18 agmatine (0, 1, 5, 10 and 20 mM). Figure 3 shows the accumulation of putrescine,
19 the production of ammonium ions, agmatine consumption, microbial growth (OD₆₀₀)
20 and the pH of the culture medium over time. Putrescine accumulation (Fig. 3a) and
21 ammonium ion production (Fig. 3b) depended on the starting concentration of the
22 agmatine in the medium (Fig. 3c). As expected from the stoichiometry of the
23 reaction (Fig. 1a), the ammonium ion concentration was twice that of the

1 putrescine. Interestingly, putrescine was first detected after 6 h of incubation,
2 independent of the agmatine concentration. Agmatine was consumed as
3 putrescine was produced (Fig. 3c). Agmatine concentrations of 1 and 5 mM were
4 used up by 8 h; concentrations of 10 and 20 mM lasted 2 h more. After 10 h of
5 culture almost all the agmatine had been catabolized to putrescine, independent of
6 the initial agmatine concentration.

7
8 Differences in bacterial growth were observed depending on the agmatine
9 concentration (Fig. 3d). Cultures supplemented with 20 mM agmatine showed the
10 second growth phase described above and an increase in the final OD_{600}
11 compared to cultures without agmatine ($OD_{600}=4.48$ and $OD_{600}=3.48$ respectively).
12 Cultures supplemented with 10 mM agmatine showed a slight increase in the
13 second stationary phase OD_{600} compared to those with no added agmatine
14 ($OD_{600}=4.09$ and $OD_{600}=3.48$ respectively), although in cultures supplemented with
15 20 mM agmatine, the second growth phase was not as clear. In contrast, cultures
16 supplemented with 1 and 5 mM agmatine showed bacterial growth similar to that
17 seen for those with no added agmatine. Alkalinization of the medium was also
18 clear in the cultures supplemented with agmatine above 5 mM (Fig. 3e). The initial
19 pH of the culture medium (pH 6.9) acidified to pH 5.4 by 6 h of fermentation. After
20 this time, the pH of the cultures supplemented with agmatine (5 mM and above)
21 became alkalinized at higher agmatine concentration and in a manner proportional
22 to the ammonium ion production (Fig. 3e). Agmatine concentrations below 5 mM

1 had no significant effect on the pH, which remained at pH 5.4, as for cultures
2 grown without agmatine.

3

4 *Role of the AGDI cluster in putrescine production, bacterial growth and pH*

5

6 To confirm the involvement of the AGDI cluster in the catabolism of agmatine to
7 putrescine, and its effect on bacterial growth and changes in pH, an *L. lactis* CECT
8 8666 $\Delta agdi$ mutant lacking the AGDI cluster was constructed (see Material and
9 Methods section 2.4). Both, wild type (wt) and $\Delta agdi$ mutant strains were grown in
10 GM17 and GM17+A for 10 h and samples collected every hour to determine
11 putrescine production, microbial growth and the pH of the culture medium (Fig. 4).

12 As expected, the deletion of the AGDI cluster completely abolished the catabolism
13 of agmatine to putrescine, whereas 10 mM putrescine was accumulated in the wt
14 culture (Fig. 4a). In addition, the deletion of the AGDI cluster resulted in the
15 absence of the second growth phase when cells were grown in GM17+A
16 ($OD_{600}=3.59$ compared to $OD_{600}=4.41$ for wt cultures) (Fig. 4b). Moreover, medium
17 alkalization was only detectable in wt cultures grown in GM17+A (Fig. 4c). The
18 $\Delta agdi$ mutant culture did not alkalinize the culture medium, and the pH (5.52)
19 reached after 7 h was maintained over the rest of the fermentation (Fig. 4c), as
20 seen for the wt and $\Delta agdi$ cultures grown in GM17.

21

22 *The alkalization caused by the AGDI pathway has no effect on bacterial growth*

23

1 The effect of culture medium alkalization due to putrescine production in
2 GM17+A, and its involvement in the regulation of putrescine production, was also
3 examined. For this, wt and $\Delta agdi$ mutant cultures were grown independently in a
4 Six-Fors[®] bioreactor containing 300 ml of GM17+A. No control of pH was imposed
5 until it reached 5.6 (after 5 and 6 h of fermentation, for the wt and $\Delta agdi$ cultures
6 respectively); it was then fixed by the bioreactor at 5.6. Putrescine production,
7 bacterial growth and pH were monitored for 12 h (Fig. 5). The putrescine
8 production pattern for the wt (Fig. 5b) was similar to that of cultures grown with no
9 pH control (Fig. 2a, 3a and 4a), in which putrescine production started after 6 h of
10 fermentation and an accumulation of 12 mM putrescine was recovered by 12 h. As
11 expected, no putrescine production was detected in the $\Delta agdi$ mutant cultures.
12 Further, the absence of alkalization did not prevent the appearance of a second
13 growth phase in the wt culture after 12 h of fermentation (Fig. 5c). These results
14 suggest that culture medium alkalization is not essential for the increase in
15 growth observed in wt cultures grown in GM17 supplemented with 20 mM
16 agmatine.

17

18 **Discussion**

19

20 Putrescine is a BA frequently found in fermented products (Garcia-Villar et al.
21 2009; Ladero et al. 2011a) and one of the most abundant in dairy products
22 (Fernandez et al. 2007; Ladero et al. 2010), in which LAB species such as
23 *Enterococcus faecalis*, *Enterococcus hirae*, *Lactobacillus brevis* and *Lactobacillus*

1 *curvatus* have all been identified as producers (Ladero et al. 2012b; Ladero et al.
2 2011b). Recently, our group reported that *L. lactis* subsp. *lactis* and *L. lactis* subsp.
3 *cremoris* were also putrescine producers in dairy products, and that in some cases
4 putrescine concentrations could reach high concentrations (Ladero et al. 2011b;
5 Ladero et al. 2012b).

6
7 Until now, the physiological role of putrescine production in *L. lactis* had not been
8 studied. The present work reports the direct involvement of the AGDI pathway in
9 enhancing the growth of *L. lactis* CECT 8666 when grown in a medium
10 supplemented with agmatine concentrations higher than the usually found in milk
11 and dairy products (Galgano et al. 2012). In fact, cultures grown in medium
12 supplemented with agmatine concentrations above 5 mM underwent a second
13 growth phase, while cultures with no agmatine remained in the stationary phase
14 (Fig. 2b and 3d). This effect was completely abolished in the $\Delta agdi$ mutant, which
15 lacks the AGDI cluster (Fig. 4b), suggesting a direct role for the AGDI pathway in
16 enhancing bacterial growth in the presence of agmatine. It would seem clear that
17 the catabolism of agmatine via this pathway enables *L. lactis* to reach higher cell
18 densities in fermentative media. This effect of agmatine catabolism on bacterial
19 growth has also been observed in *E. faecalis*, the presence of 10 mM agmatine
20 increasing growth by 60% (Suarez et al. 2013). However, this is not a general
21 effect in all microorganisms that use the AGDI pathway. For example, in
22 *Streptococcus mutants* putrescine production enhances acid tolerance and

1 contributes to the competitive fitness of the organism at low pH, but does not
2 increase its growth (Griswold et al. 2006).

3

4 Unlike *Pseudomonas aeruginosa* (Chou et al. 2008), in which putrescine is
5 catabolized as a source of energy, *L. lactis* CECT 8666 was unable to further
6 metabolize putrescine and it was fully exported to the extracellular medium. This
7 suggests that the effect of its presence on cell growth is not due to the putrescine
8 alone, but a consequence of other products of agmatine catabolism, i.e., ATP and
9 ammonium ions. According to the stoichiometry of the AGDI pathway in *L. lactis*,
10 one mole of agmatine renders one mole of putrescine, 1 mole of ATP, and 2 moles
11 of ammonium ions (Ladero et al. 2011b) (Fig. 1a), which causes the alkalinization
12 of the medium. However, this increase in the pH of the culture medium does not
13 seem to be responsible for the increase in growth in the presence of agmatine;
14 growth was similar (biphasic) whether the pH was fixed at pH 5.6 (Fig. 5) or left
15 uncontrolled (Figs. 2b, 3d, 4b), despite the putrescine production was faster and
16 the final growth higher in cultures with no pH control. The ATP generated during
17 the catabolism of agmatine results in additional energy that would help to improve
18 the growth and competitiveness of the bacteria. This is reminiscent of the arginine
19 deiminase pathway (ADI) in *L. lactis*, which catabolizes arginine into ornithine and
20 improves the survival of the bacteria by supplying them with additional energy
21 (Larsen et al. 2004).

22

1 Two interesting effects were observed when the cells were grown in the presence
2 of agmatine. Independent of the initial amount of agmatine present in the culture,
3 putrescine production was not observed until the beginning of the stationary phase.
4 (Fig. 2a and Fig. 3a). Indeed, in those cultures with an agmatine concentration of
5 over 5 mM, a second growth phase was observed (Fig. 2b and Fig. 3d). These
6 effects were not observed with the mutant strain (Fig. 4b), indicating the
7 involvement of the AGDI cluster. The biphasic growth curve observed in *L. lactis*
8 CECT 8666 when grown in the presence of agmatine is typical of the diauxic
9 growth that bacteria show when they grow in the presence of two alternative
10 sugars (Cai et al. 2012; Okada et al. 1981; Solopova et al. 2014). The most-
11 preferred sugar, generally glucose, is metabolized during a first growth cycle until it
12 is exhausted. The cells then enter a stationary phase before starting to metabolize
13 the less-preferred carbon source, whereupon they enter a second growth cycle.
14 This would seem to be the pattern followed for agmatine utilization in *L. lactis*
15 subsp. *cremoris* CECT 8666. In fact, recent studies have shown that agmatine
16 utilization in this strain is subjected to catabolite repression (Linares et al. 2013).
17 The regulatory mechanism seems to involve the negative regulation of the
18 transcription of the *aguBDAC* operon via carbon catabolite protein A (CcpA), which
19 exerts its effect by binding to a *cre* site downstream of the -10 region of the
20 *aguBDCA* promoter (Linares et al. 2013). This would explain the delay seen in
21 putrescine production, which would be impaired until the culture leaves the
22 stationary phase and enters the second growth phase during which agmatine is
23 used, and putrescine, ATP and ammonium ions are produced.

1

2 Traditionally, the production of BA was thought to be involved with acid stress
3 resistance in prokaryotes (Linares et al. 2011; Spano et al. 2010); indeed, such a
4 role for the AGDI pathway has been suggested in *S. mutants* (Griswold et al. 2006;
5 Griswold et al. 2009), *L. brevis* (Lucas et al. 2007) and *E. faecalis* (Llacer et al.
6 2007). These microorganisms seem to use the ammonium ions generated by the
7 ADGI pathway to neutralize any increase in acidity. In *L. lactis* subsp. *cremoris*
8 CECT 8666, ammonium ions were produced and accumulated in the extracellular
9 medium as result of agmatine deamination, and an agmatine dose-dependent
10 alkalization of the medium was recorded in those cultures supplemented with
11 concentrations of over 5 mM (Fig. 3). At first sight, the alkalization of the medium
12 would seem to agree with the idea that BA biosynthesis in lactic acid bacteria
13 represents a system for resisting acid stress in fermentative environments
14 (Bearson et al. 1997; Wolken et al. 2006). However, putrescine production was not
15 essential for the enhancement of growth observed following the first stationary
16 phase; the artificial maintenance of the pH at pH 5.6, did not impair the growth
17 promoted by agmatine (Fig. 5).

18

19 Together, the present results indicate that, in *L. lactis* subsp. *cremoris* CECT 8666,
20 agmatine provides an alternative source of energy via the AGDI pathway, allowing
21 for renewed growth after the culture enters the stationary phase following the
22 depletion of more preferred carbon sources. This reiterates the need to select
23 starter strains that do not produce BAs. Apart from the obvious food safety issue

1 surrounding the presence of putrescine in dairy products, and the fact that it can
2 affect their organoleptic characteristics, the presence of putrescine-producing *L.*
3 *lactis* strains could adversely affect the process of acidification during fermentation.
4 Further, in the presence of agmatine, putrescine-producing *L. lactis* strains could
5 have a selective advantage over the *L. lactis* strains with no AGDI pathway activity.

6

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8

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13

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Table 1. Bacterial strains, primers and plasmids used in the present study.

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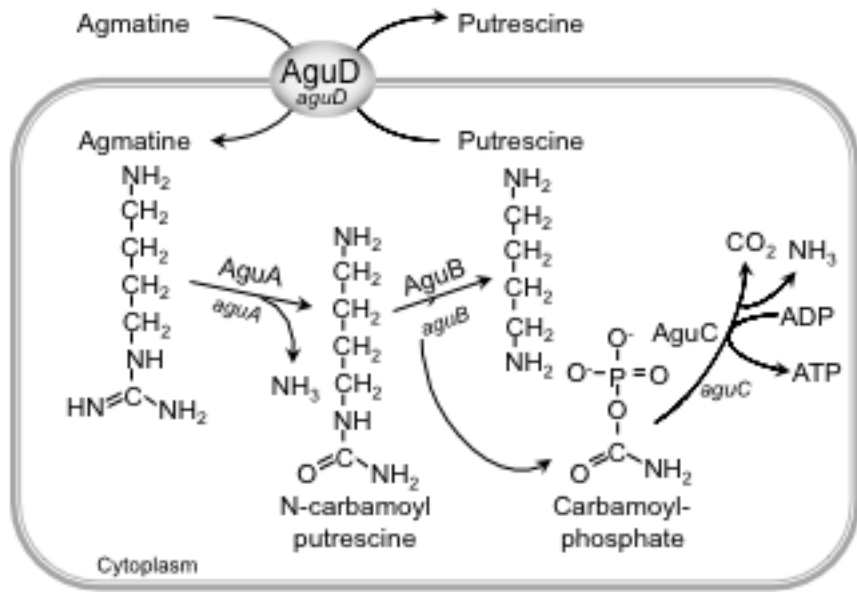
Material	Relevant Features	Reference, source
Bacteria		
<i>L. lactis</i> subsp. <i>cremoris</i> CECT 8666*	AGDI cluster, putrescine producer	(Fernandez et al. 2011), CECT
<i>L. lactis</i> CECT 8666 Δ <i>agdi</i>	CECT 8666 knock-out for AGDI cluster	This study
Primers		
KO-214AguR-AF2	5'-CACATG <u>ACTAG</u> TTTAGAACCTAGAAACCCAGAAAC-3'	This study
KO-214AguR-AR	5'-AACTGCAGATTTAACATCATCGGATTAGACCTAC-3'	This study
CKPstF	5'-AACTGCAGCTGCGGCAAATTGGCAGAGCTTG-3'	This study
NoxXho	5'-CATCCGCTCGAGCCCTAATTTCTGAAGTCATTAATATAAAC-3'	This study
Plasmids		
pCS1966	Selection/counterselection vector	(Solem et al. 2008)
pPLA1269	pCS1966 bearing a 826 bp fragment of CECT 8666 <i>ycaC-aguR</i> genes	This study
pPLA1292	pCS1966 bearing a 1728 bp fragment of CECT 8666 <i>ycaC-aguR</i> (826 bp) and <i>aguC-vrfB</i> (889 bp) genes	This study

*formerly *L. lactis* subsp. *cremoris* GE2-14

Restriction sites are underlined

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(a)



(b)

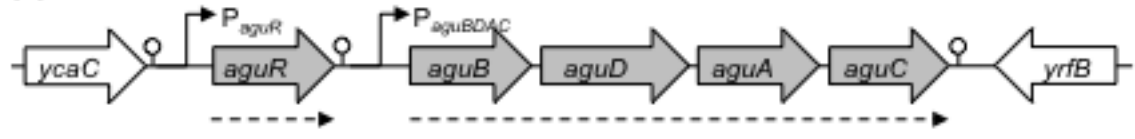


Fig. 1

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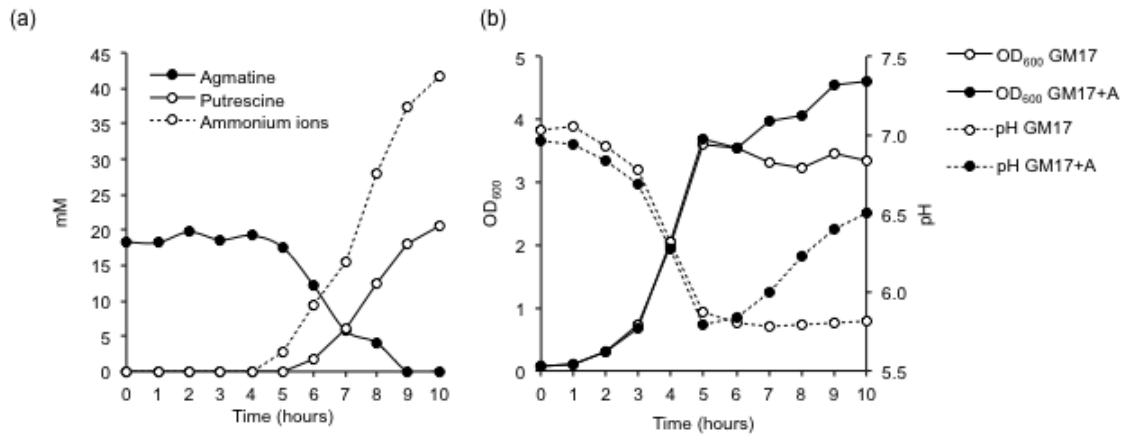


Fig. 2

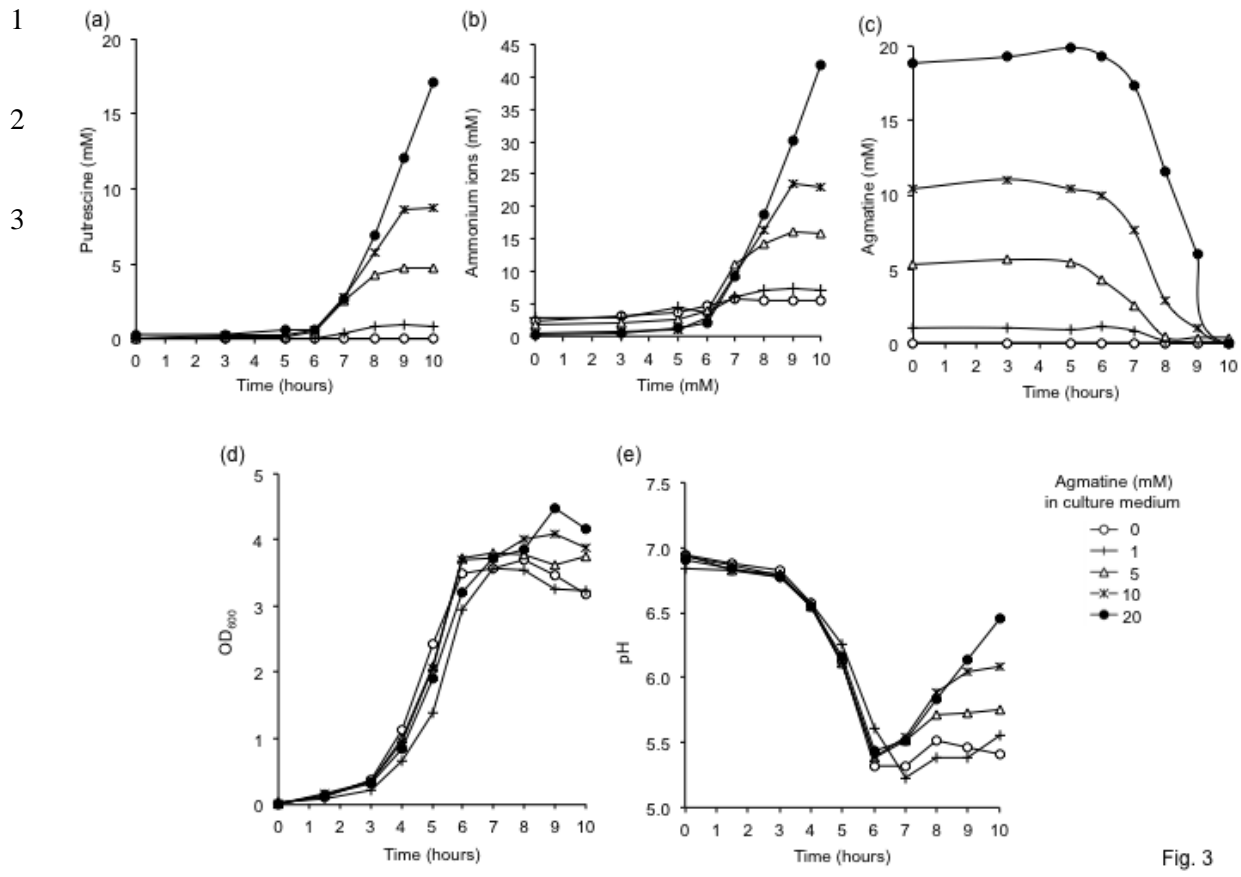


Fig. 3

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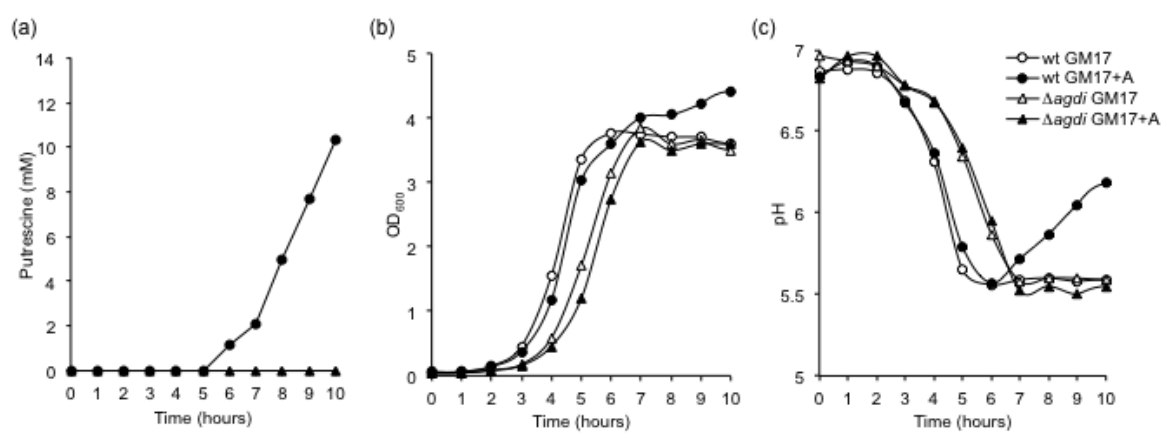


Fig. 4

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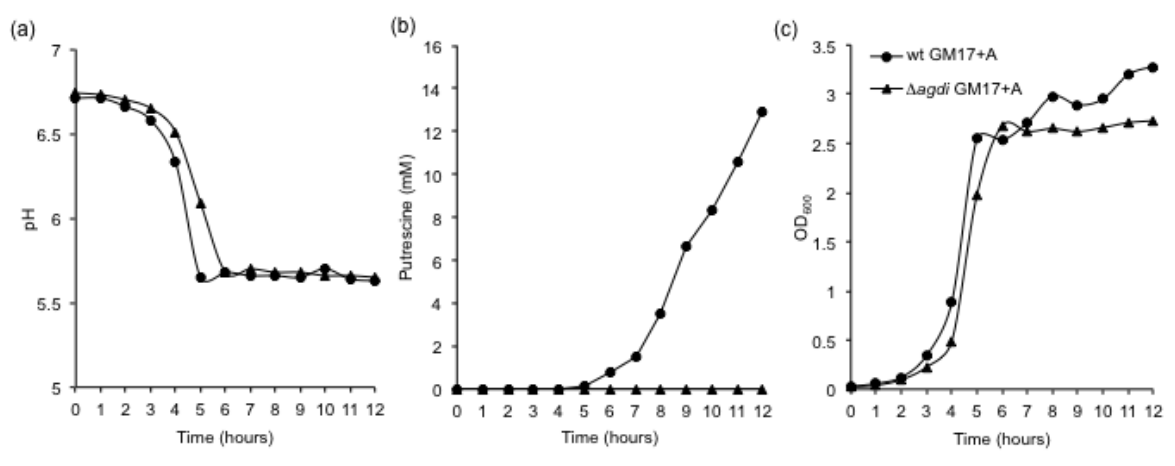


Fig. 5