Lactose-mediated carbon catabolite repression of putrescine production in dairy *Lactococcus lactis* is strain dependent

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Abbreviations

CCR: carbon catabolite repression

AGDI: agmatine deiminase pathway

BA: biogenic amine

PTS: phosphotransferase systems

Abstract

Lactococcus lactis is the lactic acid bacterial (LAB) species most widely used as a primary starter in the dairy industry. However, several strains of *L. lactis* produce the biogenic amine putrescine via the agmatine deiminase (AGDI) pathway. We previously reported the putrescine biosynthesis pathway in *L. lactis* subsp. *cremoris* GE2-14 to be regulated by carbon catabolic repression (CCR) via glucose but not lactose (Linares et al., 2013). The present study shows that both these sugars repress putrescine biosynthesis in *L. lactis* subsp. *lactis* T3/33, a strain isolated from a Spanish artisanal cheese. Furthermore, we demonstrated that both glucose and lactose repressed the transcriptional activity of the *aguBDAC* catabolic genes of the AGDI route. Finally, a screening performed in putrescine-producing dairy *L. lactis* strains determined that putrescine biosynthesis was repressed by lactose in all the *L. lactis* subsp. *lactis* strains tested, but in only one *L. lactis* subsp. *cremoris* strain. Given the obvious importance of the lactose-repression in cheese putrescine accumulation, it is advisable to consider the diversity of *L. lactis* in this sense and characterize consequently the starter cultures to select the safest strains.

Keywords: *Lactococcus lactis*; biogenic amines; putrescine; AGDI pathway; CCR; lactose.

1. Introduction

Lactococcus lactis is a lactic acid bacterial (LAB) species widely used in the dairy industry as a main component of starter cultures for cheese making. *L. lactis* is involved in the enzymatic degradation of casein, the acidification of the milk through the formation of lactic acid from lactose, and in conferring flavor and texture characteristics to final product (Kuipers, 2001; Smit et al., 2005). However, strains of both *L. lactis lactis* and *L. lactis cremoris* have recently been shown to produce the undesirable biogenic amine (BA) putrescine (Ladero et al., 2012b; Ladero et al., 2011; Linares et al., 2013). In fact, *L. lactis* is one of the main putrescine producers in cheese (Ladero et al., 2012).

Putrescine is a nitrogenous metabolite that, in dairy products, is mainly synthesized by the enzymatic deimination of agmatine (Linares et al., 2012), a cationic compound derived from the decarboxylation of the amino acid arginine (Simon and Stalon, 1982). In fermented dairy products, particularly cheese, putrescine is commonly found with other BAs, such as histamine, tyramine and cadaverine (Bunkova et al., 2010; Fernandez et al., 2007; Linares et al., 2011; Spano et al., 2010). The presence of putrescine in food confers an undesirable flavor, and at high concentrations it can have toxicological effects, e,g., increased cardiac output, tachycardia, hypotension, and even carcinogenic effects (Ladero et al., 2010). However, in bacteria such as *Enterococcus faecalis*, putrescine helps protect against oxidative and osmotic stress (Llacer et al., 2007). In addition, it seems to contribute towards an increased tolerance towards other

stresses such as acid and heat stress, as described for the buccal cavity-dwelling bacterium *Streptrococcus mutants* (Griswold et al., 2006).

In *L. lactis* (including both *L. lactis* subsp*. lactis* and *L. lactis* subsp. *cremoris*), agmatine is catabolized to putrescine in the agmatine deiminase (AGDI) pathway (Ladero et al., 2011; Linares et al., 2013). The AGDI cluster of *L. lactis* comprises a putative regulatory gene (*aguR*) followed by the *aguB*, *aguD*, *aguA* and *aguC* catabolic genes which are co-transcribed into a single polycistronic RNA (Ladero et al., 2011; Linares et al., 2013). External agmatine enters the cell in exchange for internal putrescine via the action of the agmatine/putrescine antiporter AguD (encoded by *aguD*). It is then deiminated to N-carbamoyl putrescine by agmatine deiminase (AguA, encoded by *aguA*), which in turn is phosphorolyzed by putrescine transcarbamylase (AguB, encoded by *aguB*), to render putrescine and carbamoyl phosphate. This last metabolite is used as a substrate by carbamate kinase (AguC, encoded by *aguC*) for ADP phosphorylation with the generation of one molecule of ATP and one ammonium ion.

A recent analysis of the influence of glucose on putrescine production in *L. lactis* subsp. *cremoris* GE2-14 (Linares et al., 2013) has shown that the AGDI pathway is subject to carbon catabolite repression (CCR) by glucose. CCR is a regulatory mechanism used by bacteria to regulate the metabolism of carbon and other energy sources (Zomer et al., 2007). In Gram-positive bacteria, CCR is mediated by the catabolite control protein CcpA (Warner and Lolkema, 2003) which binds to *cis*-acting catabolite responsive

elements (*cre* sites) to repress the transcriptional activity of CcpA-regulated genes (Seidel et al., 2005).

In *L. lactis*, sugars can enter the cell via: i) phosphotransferase systems (PTS), which are involved in both the transport and phosphorylation of sugar at the expenses of phosphoenolpyruvate, and ii) non-PTS systems (Cocaign-Bousquet et al., 2002; Neves et al., 2005). Glucose intake occurs via the mannose-PTS system (Neves et al., 2005), the glucose-PTS system (Thompson and Saier, 1981) and via the action of a permease (Thompson et al., 1985), lactose enters either via a lactose-PTS system (PTS^{Lac}) (de Vos et al., 1990; Postma et al., 1993) or a permease (de Vos and Vaughan, 1994), and maltose is transported by a non-PTS ATP-dependent permease (Law et al., 1995). Therefore, in *L. lactis*, glucose and lactose may be considered PTS-sugars, and maltose a non-PTS sugar.

In *L. lactis* subsp. *cremoris* GE2-14, high glucose concentrations transcriptionally repress putrescine biosynthesis via the mediation of CcpA (Linares et al., 2013). However, high concentrations of other sugars - including lactose - do not impair putrescine production.

The present results confirm the repressor effect of glucose on putrescine production in *L. lactis* subsp. *lactis* T3/33. The effect of lactose (PTS-sugar) and maltose (non-PTS-sugar) on putrescine biosynthesis is also reported, as is the effect of all three sugars on bacterial growth and on the pH of the culture medium in the presence of agmatine. The kinetics of sugar consumption are examined, and the effect of glucose, lactose and

maltose on the transcriptional activity of the AGDI pathway catabolic genes recorded. Finally, several putrescine-producing *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* strains are screened for putrescine biosynthesis repression by these three sugars.

2. Material and methods

2.1. Bacterial strains and culture conditions

The *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* strains used in this study are the following: *L. lactis* subsp. *lactis* T3/33, T1/48, L39, 1AA17, 1AA59, 2BA40 and 1A38; *L. lactis* subsp. *cremoris* GE2-14, 3AA23, 2A22 and 3AA9. These strains were originally isolated from traditional cheeses and confirmed to produce putrescine from agmatine via the ADGI pathway (Ladero et al., 2011). The strains were grown in M17 (Oxoid, UK), supplemented with the indicated amounts (0.5% or 1%) of glucose (GM17), lactose (LM17) or maltose (MM17), at 32ºC without aeration. Where indicated, the media was supplemented with 20 mM agmatine (M17+A) (Sigma-Aldrich, St. Louis, MO). For all fermentation assays, an overnight culture of the corresponding *L. lactis* strain was used as an inoculum (1% v/v).

Batch fermentations were performed in 30 ml of media. Sampling (2 ml) was carried out each hour for 10 h. During this time, microbial growth was monitored by measuring the optical density of the culture at 600 nm (OD_{600}) using a spectrophotometer (Eppendorf, NY). The pH of the medium was monitored using a CRISON miCropH 2001 pH-meter (Crison Instruments S.A., Barcelona, Spain).

128 *2.2. Analytical chromatography methods*

129 Cultures were centrifuged at 8000 *g*. The putrescine, agmatine and ammonia
130 concentrations in the supernatants were analyzed by ultra high performance liquid
131 chromatography (UHPLC) using a Waters H-Class ACQUITY UPLC™ apparatus
132 controlled by Empower 2.0 software and employing a UV-detection method based on
133 derivatization with diethyl ethoxymethylene malonate (Sigma-Aldrich) (Redruello et al.,
134 2013).

135 Sugars were determined by high performance liquid chromatography (HPLC) using a
136 chromatographic system composed of an Alliance 2695 module injector (Waters,
137 Milford, MA, USA) and a 2414 Differential Refractometer detector (Waters), all
138 controlled with Empower software (Waters). Supernatants (20 µl) were isocratically
139 separated in a 300 x 7.8 mm (7 µm) ICSep ION-300 ion-exchange column with a ICSep
140 ICE-GC-801 20 x 4.0 mm Guard Cartridge (Transgenomic, San Jose, CA) at a flow rate
141 of 0.4 ml/min at 65°C. Sulfuric acid (8.5 mN) was used as the mobile phase. The sugars
142 were analyzed using a 2414 Refractometer (Waters).

143

144 *2.3. Quantification of gene expression by reverse transcription quantitative PCR (RT-*
145 *qPCR)*

146 For all RT-qPCR experiments, *L. lactis* was grown in a Six-Fors® bioreactor (Infors AG,
147 Bottmingen, Switzerland) containing 300 ml of M17 supplemented with 5 mM agmatine
148 and 2% glucose, lactose or maltose at a fixed pH (pH 5, maintained by the automatic

addition of 1 N NaOH or 1 N HCl as needed). The reactor was maintained at 32°C, stirring at 50 rpm and with zero air input. Total RNA was extracted from 2 ml of cultures collected at the end of the exponential phase of growth. The cells were harvested by centrifugation, resuspended in 1 ml of TRI Reagent® (Sigma-Aldrich) and mechanically disrupted with 50 μm-diameter glass beads (Sigma-Aldrich) in a bead beater (FastPrep-24 system, MP Biomedicals, Illkirch, France). The samples were shaken three times for 30 s at power setting 6. During the shaking intervals the cells were kept on ice for 1 min. RNA samples (2 μg of total RNA) were treated with 2 U of DNase (Fermentas, Vilnius, Lithuania) for 30 min at 37°C to eliminate any contaminating DNA. The reaction was stopped by adding 3 μl of 25 mM EDTA at 65ºC for 10 min. The absence of contaminating DNA was checked by quantitative real-time PCR (qPCR) using the corresponding RNA as a template, Power SYBR® Green PCR Master Mix (Applied Biosystems, UK), and a primer pair for amplifying the *rpoA* reference gene (Taibi et al., 2011), following the protocol described below for qPCR amplification. cDNA was then synthesized from DNase-treated RNA samples using the iScript™ cDNA Synthesis Kit (Bio-Rad, Barcelona, Spain) following the manufacturer's recommendations. cDNA samples were analyzed by qPCR using the primers listed in Table 1. The design of the primer pair used to amplify the *aguB* gene was based on the nucleotide sequence of the *aguB* gene of *L. lactis* subsp. *lactis* available in databases (Ladero et al., 2011); this was performed using Primer Express Software v.3.0 (Applied Biosystems). The primer pairs used to amplify *tufA* gene have been previously described (Linares et al., 2013). The linearity and amplification efficiency of the reactions were tested for each primer pair using five measuring points over a 10-fold dilution series of *L. lactis* subsp. *lactis*

T3/33 genomic DNA (obtained using Kirby lytic mix following the protocol described by Hopwood [1985]). All reactions, which included the template, 900 nM of each primer, and Power SYBR® Green PCR Master Mix containing ROX as a passive reference, were performed in 25 μl volumes. Amplification and detection were performed using an ABI Prism Fast 7500 sequence detection system (Applied Biosystems). The PCR reaction was initiated by pre-incubation at 50°C for 2 min. This was followed by denaturation at 95°C for 10 min, and then 40 cycles of denaturation at 95°C for 15 s, plus annealing at 60°C for 60 s. Threshold cycle (Ct) values were calculated automatically using 7500 Software v.2.0.4 (Applied Biosystems). No-template samples were included in each run as negative controls. Relative gene expression was calculated using the ΔΔCt comparative method as previously described (Livak and Schmittgen, 2001). Statistical comparisons were made using Student *t* test; significance was set at $p < 0.05$.

3. Results

3.1. Inhibitory effect of glucose and lactose on putrescine production

A strain previously identified as a putrescine producer - *L. lactis* subsp. *lactis* T3/33 - (Ladero et al., 2011) was used to determine the effect of glucose, lactose and maltose on putrescine biosynthesis. Cells were cultivated for 10 h in M17+A supplemented with 0.5% or 1% of the corresponding sugar. Putrescine, agmatine and ammonium were determined over time by UHPLC. Figure 1 shows the results obtained. In all cases, putrescine biosynthesis and ammonia production correlated directly with agmatine

194 consumption. After 10 h of fermentation, the strain accumulated 19 mM, 14 mM and 17
195 mM putrescine when the M17 medium was supplemented with 0.5% glucose (Fig. 1A),
196 0.5% lactose (Fig. 1C) or 0.5% maltose (Fig. 1E) respectively. However, when the
197 medium was supplemented with 1% of either glucose or lactose, a drastic reduction in
198 putrescine production was observed (Fig. 1B and 1D); indeed, compared to the amount
199 accumulated in the presence of 0.5% glucose (19 mM putrescine), a reduction of 88%
200 was seen (2.1 mM putrescine). Similarly, compared to the amount of putrescine
201 accumulated in the presence of 0.5% lactose (14 mM), in the presence of 1% lactose an
202 84% reduction was seen (2.2 mM putrescine). However, the presence of 1% maltose in
203 the culture medium did not inhibit putrescine biosynthesis (Fig. 1F); 18.1 mM putrescine
204 were produced, similar to that obtained with 0.5% maltose supplementation (17 mM
205 putrescine) (Fig. 1E). These results confirm that both glucose and lactose repress
206 putrescine production in *L. lactis* subsp. *lactis* T3/33.

207

208 *3.2. Effect of carbon source and agmatine on bacterial growth and pH*

209 Figure 2 shows the effect of the carbon source (M17 supplemented with 0.5% or 1%
210 glucose, lactose or maltose) on strain T3/33 growth in the presence and absence of 20
211 mM agmatine, and on the pH of the medium. Growth in M17 with 0.5% glucose, lactose
212 or maltose plus 20 mM agmatine was clearly better than when agmatine was absent
213 (Fig. 2A, 2C and 2E respectively). After 10 h, the cultures grown in medium without
214 agmatine reached OD_{600}=3.5 (glucose), OD_{600}=3.1 (lactose) and OD_{600}=3.3 (maltose),
215 while those supplemented with 20 mM agmatine reached OD_{600}=4.7 (glucose),

OD_{600}=4.4 (lactose) and OD_{600}=4.6 (maltose). However, increasing the glucose or lactose concentration of the medium to 1% (Fig. 2B and 2D respectively) impaired the growth increment made possible by agmatine supplementation (Fig. 2A and 2C, respectively). Thus, cultures with either 1% glucose or lactose reached an OD_{600} of approximately 3.5, regardless of agmatine presence/absence. In contrast, the cultures with 0.5% maltose and 1% maltose showed similar final OD values in the presence of agmatine (Fig. 2E and 2F respectively).

The growth of strain T3/33 led to the acidification of the medium from pH 6.9 to approximately pH 5.2 after 6 h of fermentation in cultures supplemented with 0.5% glucose or 0.5% lactose (Fig. 2A and 2C respectively). This was maintained until the end of fermentation. Cultures supplemented with 0.5% maltose needed 10 h of fermentation to reach pH 5.2 (Fig. 2E). When these cultures were supplemented with 20 mM agmatine, the medium became similarly acidified, but later it progressively alkalinized up to pH 6. When glucose or lactose was increased to 1%, the addition of 20 mM agmatine did not lead to this alkalization (Fig. 2B and 2D). When the maltose concentration was increased to 1%, the addition of 20 mM agmatine made the pH rise, although less so compared to that seen with 0.5% maltose cultures (Fig. 2F).

3.3. Kinetics of sugar consumption

Since the type and the concentration of the sugar present in the culture medium affected putrescine biosynthesis, the kinetics of sugar consumption were analyzed in

cultures grown in M17 medium supplemented with 0.5% or 1% of glucose, lactose or maltose, both in the presence and absence of 20 mM agmatine. Incubations proceeded for 10 h at 32°C. Sugar consumption was monitored in supernatants by HPLC (Fig. 3). Cultures grown in 0.5% glucose fully consumed the sugar after 6 h of fermentation (Fig. 3A), very similarly to the cultures grown in 0.5% lactose (Fig. 3B). However, in cultures supplemented with 0.5% maltose, the sugar consumption rate was much lower than for lactose and glucose, lasting until 9 h of fermentation (Fig. 3C). Cultures supplemented with either 1% glucose, lactose or maltose did not completely consume the sugar after 10 h of fermentation (Fig. 3A, 3B and 3C respectively). The sugar consumption rate changed after 6 h of fermentation; beyond this point, in the presence of glucose or lactose, the consumption graph showed a less steep slope (Fig. 3A and 3B). No differences were observed in the kinetics of sugar consumption between cultures with or without 20 mM agmatine when the cells were growing in 0.5% or 1% of either glucose or lactose (Fig. 3A and Fig. 3B). Cells grown in the medium supplemented with 1% maltose, however, appeared to consume the sugar slightly more quickly when in the presence of 20 mM agmatine than when without it (Fig. 3C). Thus, after 10 h of fermentation, only 0.16% of maltose remained unconsumed in the culture supplemented with agmatine, while 0.38% maltose remained in that without agmatine.

3.4. Glucose and lactose inhibit the transcription of the AGDI cluster catabolic genes

Having observed the inhibitory effect of glucose and lactose on putrescine production in *L. lactis* subsp. *lactis* T3/33, the influence of the carbon source on the transcriptional

activity of the catabolic genes of the AGDI cluster was further analyzed. The expression profile of *aguB* - the first gene of the *aguBDAC* operon (Linares et al., 2013) - was analyzed by RT-qPCR as representative of the whole *aguBDAC* mRNA. To avoid the possible effect of pH on the expression of the *aguBDAC* genes, the analysis was performed in a bioreactor at a fixed pH of 5. The culture medium was supplemented with 5 mM agmatine (experiments performed at our laboratory have indicated this to be sufficient for the complete transcriptional activation of the AGDI genes [data not shown]). The medium was also supplemented with 2% glucose, lactose or maltose. Figure 4 shows the relative expression of *aguB* (using *rpoA* as a reference gene). Comparative analysis showed the transcription of the *aguBDAC* operon to be significantly inhibited by the presence of glucose or lactose (5-fold; $p<0.05$) compared to cultures grown with maltose. Similar results were obtained when *tufA* was used as the reference gene (data not shown). These results clearly indicate repression of the catabolic genes of the AGDI cluster at the transcriptional level by the presence of high concentrations of either lactose or glucose.

3.5. The lactose repression of putrescine biosynthesis is strain-dependent in L. lactis

The effect of glucose, lactose and maltose on putrescine production in other *L. lactis* subsp. *lactis* and *L. lactis* subps. *cremoris* strains (see Material and Methods section) was also examined. All the strains selected use the AGDI pathway and are putrescine producers (Ladero et al., 2011). The capacity of these strains to produce and accumulate putrescine in M17+A supplemented with either 0.5% glucose or 1% of

282 glucose, lactose or maltose, was determined by UHPLC analysis of culture
283 supernatants (Table 2). All the strains showed CCR by glucose since putrescine
284 accumulation in the presence of 1% glucose was much smaller than in the presence of
285 0.5% glucose, a concentration that is not repressive towards putrescine production
286 (Linares et al., 2013). When 1% lactose was used as the carbon source, two different
287 responses were observed. All the assayed *L. lactis* subsp. *lactis* strains showed lactose
288 repression of putrescine biosynthesis. However, in all the assayed *L. lactis* subsp.
289 *cremoris* strains but one (i.e., *L. lactis* subsp. *cremoris* 3A23), lactose had no repressive
290 effect on putrescine production. When the strains were grown with 1% maltose,
291 putrescine accumulated in all the cultures at higher concentrations than those observed
292 in cultures grown in the presence of 0.5% glucose. This clearly indicates that maltose
293 exerts no CCR on putrescine biosynthesis in the strains tested.

294

295 **4. Discussion**

296 *L. lactis* is of great economic importance - it is used as a starter to ferment
297 approximately 100 million tons of milk every year (Mierau and Kleerebezem, 2005).
298 Nonetheless, and despite having been awarded qualified presumption of safety (QPS)
299 status by the European Food Safety Authority (EFSA), some strains have been shown
300 to produce putrescine. In fact, *L. lactis* is one the main producers of putrescine in
301 cheese (Ladero et al, 2012a). Putrescine-producing *L. lactis* strains in starter cultures
302 could have negative repercussions on consumer health. At the very least, putrescine

303 could cause economic losses by negatively effecting the taste and aroma of milk
304 products.

305 Ladero et al. (2011) reported the existence of *L. lactis* dairy isolates capable of
306 producing putrescine via the deimination of agmatine in the AGDI pathway. Later,
307 Linares et al. (2013) characterized the genetic organization of this pathway in the dairy
308 isolate *L. lactis* subsp. *cremoris* GE2-14, and determined the repression exerted by
309 glucose on the transcriptional activity of the AGDI cluster (with the ensuing inhibition of
310 putrescine production). In that study, glucose was the only carbon source tested that
311 was able to repress putrescine biosynthesis. The present study examined the effect of
312 glucose, lactose (PTS-sugars) and maltose (non-PTS sugar) on putrescine biosynthesis
313 in *L. lactis* subsp. *lactis* T3/33, and confirms the suppressor effect of high
314 concentrations of glucose on putrescine production. In media with 1% glucose,
315 putrescine production was drastically reduced (88% compared to media with 0.5%
316 glucose; Fig. 1B). Similarly, compared to media with 0.5% lactose, the presence of 1%
317 lactose reduced the production of putrescine by more than 80% (Fig. 1D). The non-
318 repressor effect of maltose previously described (Linares et al., 2013) is confirmed.

319 Interestingly, increased bacterial growth was observed under the culture conditions that
320 allowed putrescine production to rise to above 15 mM (i.e., in the presence of agmatine,
321 and with either maltose or low concentrations of glucose or lactose) compared to those
322 in which putrescine production was weaker (in the presence of agmatine but with high
323 concentrations of glucose or lactose, or the absence of agmatine) (Fig. 2). This might be
324 explained in that the production of putrescine from agmatine generates one molecule of

ATP. Thus, under better putrescine-production conditions, there would be more energy for growth. The positive effect of putrescine production on bacterial growth has also been reported in other bacteria that use the AGDI pathway, such as *Enterococcus faecalis* V583 (Suarez et al., 2013). However, in yet others, such as *Streptococcus mutants*, no such effect is seen (Griswold et al., 2006).

The catabolism of glucose and lactose appears to be closely connected to putrescine production, since putrescine biosynthesis was repressed under conditions in which glucose or lactose remain available in the culture medium. The presence of maltose does not, however, seem to affect putrescine production. The fact that glucose and lactose act as repressors of putrescine biosynthesis in strain T3/33 suggests that both sugars behave mainly as PTS-sugars that repress the AGDI pathway. Glucose has a similar repressive effect in *L. lactis* subsp. *cremoris* GE2-14 (Linares et al., 2013) and *Streptococcus mutants* UA159 (Griswold et al., 2006). Certainly, the analysis of the *aguB* promoter region of the *L. lactis* AGDI cluster (which controls the expression of the genes) sequences available in databases revealed the presence of a *cre* site consensus sequence (5' TGAAADCGTTCCCA 3'; D: A, T or G) similar to that found in *L. lactis* subsp. *cremoris* GE2-14 (Linares et al., 2013). The results of the transcriptional analysis of the AGDI cluster of strain T3/33 confirm the repression of the transcriptional activity of the *aguBDAC* operon by high concentrations of glucose and lactose: transcription fell 5-fold compared to that recorded in the presence of high concentrations of the non-repressor sugar maltose (Fig. 4). Since maltose is a non-PTS sugar, CCR of the AGDI pathway mediated by CcpA should not be expected. Indeed, and unlike the observed repressor effect of glucose and lactose, similar putrescine accumulation

348 patterns were observed when the medium was supplemented with either high or low
349 maltose concentrations (Fig. 1).

350 The different repression patterns seen in *L. lactis* subsp. *cremoris* GE214 and *L. lactis*
351 subsp. *lactis* T3/33 suggested the effect of the carbon source on putrescine production
352 in other *L. lactis* using the AGDI pathway. Seven *L. lactis* subsp. *lactis* and four *L. lactis*
353 subsp. *cremoris* strains were tested, and all showed the repression of putrescine
354 biosynthesis by glucose but not by maltose (as expected given the results of Linares et
355 al. [2013]). Interestingly, different responses were found to high concentrations of
356 lactose: putrescine production was impaired in all *L. lactis* subsp. *lactis* strains tested,
357 as well as in *L. lactis* subsp. *cremoris* 3AA23. No repression was seen, however, in *L.*
358 *lactis* subsp. *cremoris* GE2-14, 2A22 or 3AA9. The non-repressing effect of lactose on
359 putrescine production was earlier described by Linares et al. (2013) who reported an
360 absence of lactose repression (even at concentrations of 4%) on putrescine production
361 in *L. lactis* subsp. *cremoris* GE2-14. However, the results of the present work suggest
362 that, in addition to glucose, lactose might also be involved in the CCR of the AGDI
363 pathway in some *L. lactis lactis* and *cremoris* strains. The present results indicate a
364 consistency of glucose-mediated repression of the AGDI pathway in *L. lactis*, the
365 absence of maltose-mediated repression, and more diversity with respect to lactose-
366 mediated repression. A comprehensive study with a larger number of *L. lactis* strains is
367 needed.

368 Since milk has a 4% of lactose (Shakeel-Ur-Rehman et al., 2004), to use as starters *L.*
369 *lactis* strains in which putrescine biosynthesis is repressed by lactose could prevent

putrescine accumulation in cheese. However, there are cheeses in which the population of *L. lactis* remains dominant even during their maturation (Florez and Mayo, 2006), when lactose has already been consumed (Portnoi et al., 2009). Therefore, in such cases it would be safer to use *L. lactis* strains that do not carry the AGDI cluster.

In summary, the dairy industry needs to bear in mind the diversity of *L. lactis* in terms of putrescine production when developing starter cultures. In some strains, putrescine biosynthesis suffers CCR by lactose, but in others this sugar has no inhibitory effect on putrescine production at all, even at high concentrations. *L. lactis* strains need to be well characterized and selected if dairy products are not to accumulate putrescine.

5. Conclusions

This work shows that the production of the biogenic amine putrescine by dairy *Lactococcus lactis* subsp*. lactis* T3-33 via the AGDI pathway is subject to carbon catabolite repression by lactose and glucose; the increase of growth and alkalinization of the medium associated to putrescine production are also inhibited by glucose and lactose. Further, we have determined that lactose and glucose inhibit the *L. lactis* AGDI route at the transcriptional level. Finally, the analysis of several putrescine-producing *L. lactis* strains isolated from cheese have revealed that carbon catabolite repression of putrescine biosynthesis by lactose -the available sugar in dairy fermentations- is strain-dependent in *L. lactis.*

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Figure Legends

Figure 1. Putrescine production, agmatine catabolism and ammonia production in *L. lactis* subsp*. lactis* T3/33*.* Cells were grown in M17 supplemented with 20 mM agmatine and different concentrations of sugar: (A) 0.5% glucose, (B) 1% glucose, (C) 0.5% lactose, (D) 1% lactose, (E) 0.5% maltose and (F) 1% maltose. 2 ml samples were collected each hour. Supernatants were analyzed by UHPLC to determine the concentrations of putrescine, agmatine and ammonia in the extracellular medium. A representative experiment is shown.

Figure 2. Effect of putrescine production on cell growth and pH. Cells were grown for 10 h in M17 supplemented with different concentrations of sugar in the presence or absence of 20 mM agmatine. Bacterial growth was determined by measuring the absorbance of the culture at 600 nm (OD_{600}) (solid lines); the pH was also monitored (dashed lines). (A) 0.5% glucose, (B) 1% glucose, (C) 0.5% lactose, (D) 1% lactose, (E) 0.5% maltose and (F) 1% maltose. GM17: M17 supplemented with glucose, GM17+A: GM17 supplemented with 20 mM agmatine, LM17: M17 supplemented with lactose, LM17+A: LM17 supplemented with 20 mM agmatine, MM17: M17 supplemented with maltose, MM17+A: MM17 supplemented with 20 mM agmatine. A representative experiment is shown.

Figure 3. Kinetics of sugar consumption. Cells were grown for 10 h in M17 supplemented with different concentrations of sugar (0.5% or 1%) in the presence or

absence of 20 mM agmatine. The sugar concentration of the medium was determined in supernatants by HPLC: (A) glucose, (B) lactose and (C) maltose. GM17: M17 supplemented with glucose, GM17+A: GM17 supplemented with 20 mM agmatine, LM17: M17 supplemented with lactose, LM17+A: LM17 supplemented with 20 mM agmatine, MM17: M17 supplemented with maltose, MM17+A: MM17 supplemented with 20 mM agmatine. A representative experiment is shown.

Figure 4. Effect of carbon source on the expression of the catabolic genes of the AGDI cluster, as evaluated by RT-qPCR. Cells were grown in a bioreactor at a fixed pH of 5 in M17 supplemented with 2% glucose (GM17), lactose (LM17) or maltose (MM17) in the presence of 5 mM agmatine. Samples were collected at the end of the exponential phase; total RNA was then extracted and cDNA synthetized. The expression of the *aguB* gene was assessed by RT-qPCR and calculated relative to the transcript level detected in samples grown in glucose. Data were normalized to the total RNA content using *rpoA* as a reference gene. The data represent the mean of three different RNA extractions; vertical bars represent standard deviations. *$p<0.05$.

553 Table 1. Primers used for gene expression quantification by RT-qPCR.

Gene	Primer	Sequence (5'-3')	Reference
aguB[a]	aguB-F	CCGAAAATCGCTTGACTTCAA	This work
	aguB-R	TCAGCATAATCAGACATCAACCAA	This work
rpoA[b]	rpoA-F	CACGGGCAGGTTCAACTTG	(Taibi et al., 2011)
	rpoA-R	TTCCGGCTGACGAAAATAAAG	(Taibi et al., 2011)
tufA[b]	qtufF	TCTTCATCATCAACAAGGTCTGCTT	(Linares et al., 2013)
	qtufR	GAACACATCTTGCTTTCACGTCAA	(Linares et al., 2013)

554 [a] Target gene

555 [b] Reference gene

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Table 2. Putrescine accumulation by *L. lactis* strains grown in M17+A supplemented with different sugars.

Strain	Putrescine (mM) ± SD			
	0.5% glucose	1% glucose	1% lactose	1% maltose
L. lactis subsp. *lactis* T3/33	6.17 ± 0.32	3.62 ± 0.33	3.91 ± 0.35	7.20 ± 0.63
L. lactis subsp. *lactis* T1/48	4.17 ± 0.08	0.16 ± 0.02	0.16 ± 0.01	14.73 ± 0.25
L. lactis subsp. *lactis* L39	2.66 ± 0.7	0.07 ± 0.01	0.11 ± 0.04	3.65 ± 0.3
L. lactis subsp. *lactis* 1AA17	15.97 ± 0.34	2.24 ± 0.15	1.84 ± 1.60	13.24 ± 0.32
L. lactis subsp. *lactis* 1AA59	10.36 ± 4.26	4.31 ± 0.09	6.36 ± 0.42	14.93 ± 0.47
L. lactis subsp. *lactis* 2BA40	10.84 ± 0.25	0.67 ± 0.06	2.16 ± 0.17	11.99 ± 0.85
L. lactis subsp. *lactis* 1A38	2.36 ± 0.12	0.05 ± 0.04	0.08 ± 0.01	8.38 ± 3.38
L. lactis subsp. *cremoris* GE2-14	6.26 ± 0.24	4.67 ± 1.17	9.25 ± 0.44	10.60 ± 5.13
L. lactis subsp. *cremoris* 3AA23	9.85 ± 2.04	3.31 ± 0.12	5.95 ± 0.19	14.01 ± 0.04
L. lactis subsp. *cremoris* 2A22	6.04 ± 0.22	2.67 ± 0.14	5.95 ± 0.19	3.86 ± 0.64
L. lactis subsp. *cremoris* 3AA9	5.48 ± 0.48	3.75 ± 0.18	5.67 ± 0.4	5.6 ± 2.7

SD: standard deviation

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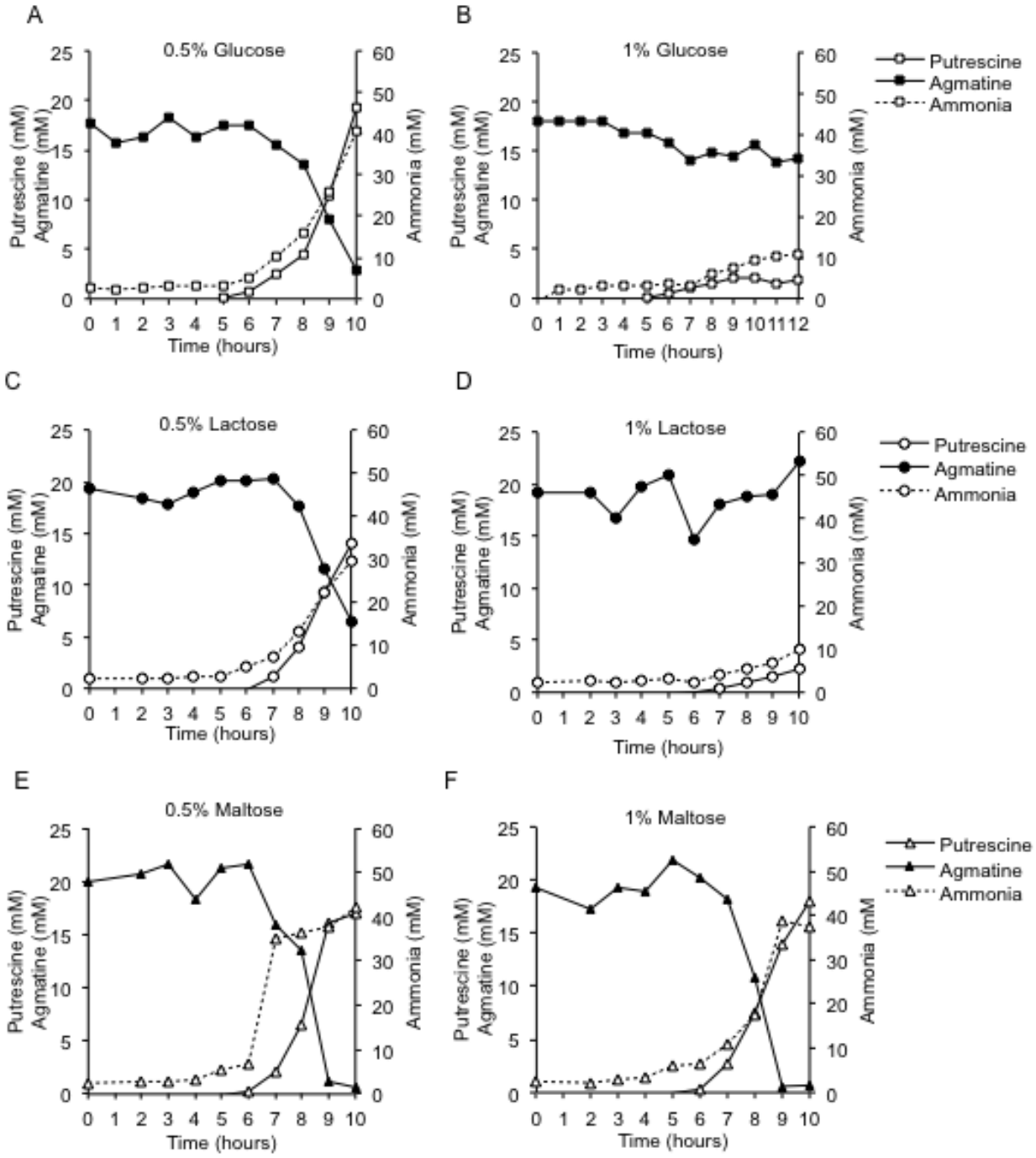

Figure 1

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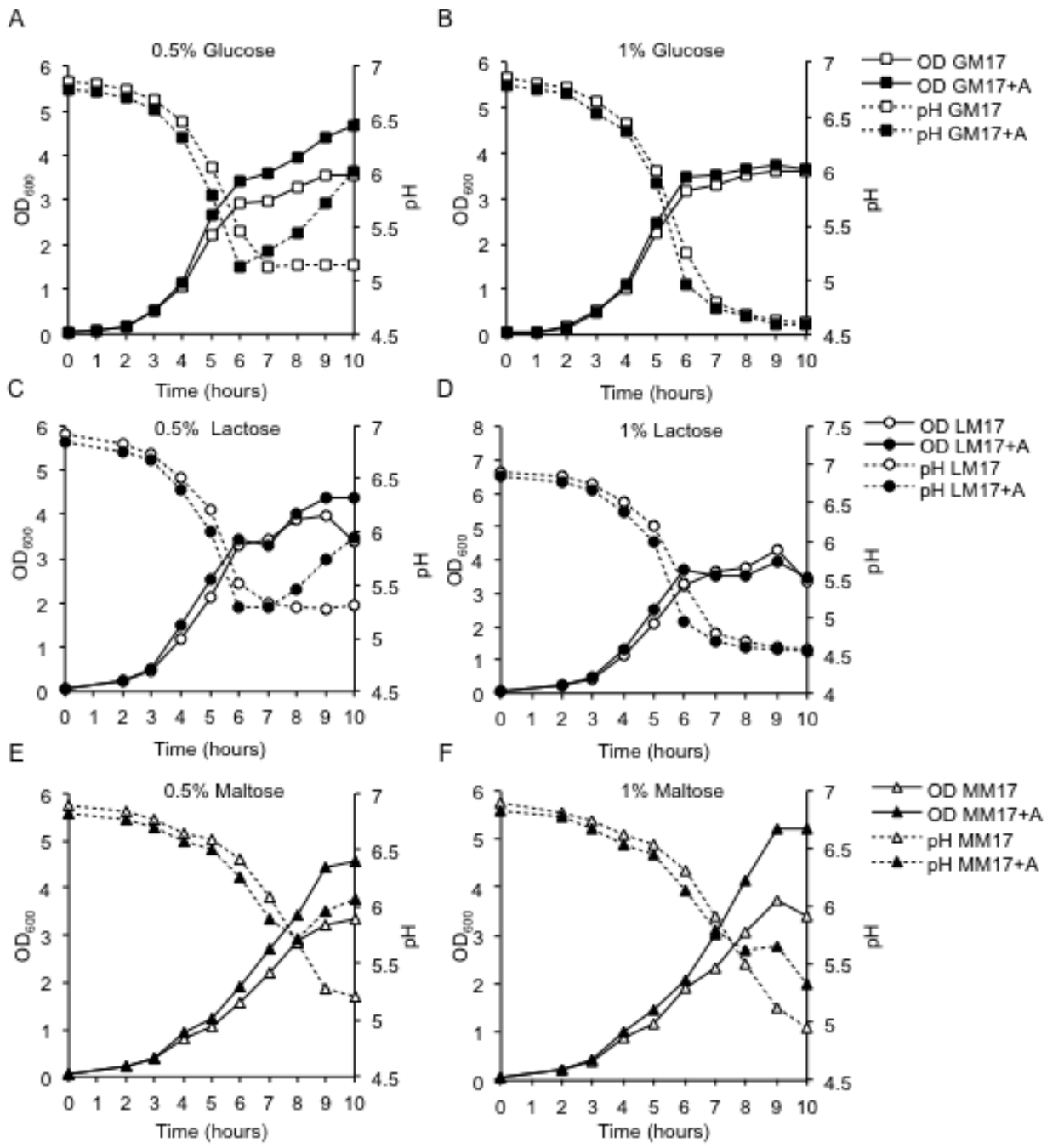

Figure 2

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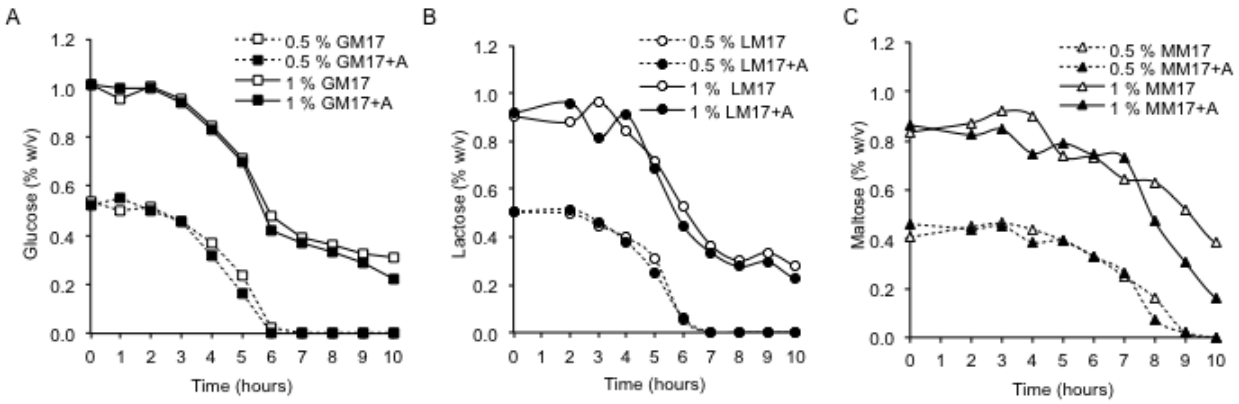

Figure 3

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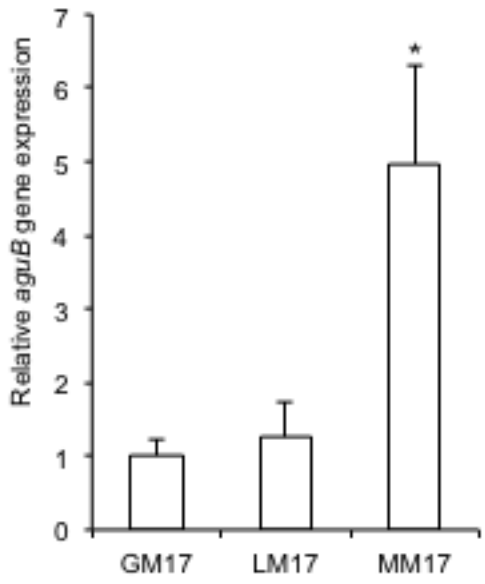

Figure 4