Lactose-mediated carbon catabolite repression of putrescine production in dairy

*Lactococcus lactis* is strain dependent

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Abbreviations

CCR: carbon catabolite repression
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* 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 *Streptococcus mutans* (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 (Cocaing-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\textsuperscript{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\textsubscript{600}) using a spectrophotometer (Eppendorf, NY). The pH of the medium was monitored using a CRISON miCropH 2001 pH-meter (Crisom Instruments S.A., Barcelona, Spain).
2.2. Analytical chromatography methods

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

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

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

For all RT-qPCR experiments, \( L. \ lactis \) was grown in a Six-Fors® bioreactor (Infors AG, Bottmingen, Switzerland) containing 300 ml of M17 supplemented with 5 mM agmatine 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 65°C 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 △△Ct 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
consumption. After 10 h of fermentation, the strain accumulated 19 mM, 14 mM and 17 mM putrescine when the M17 medium was supplemented with 0.5% glucose (Fig. 1A), 0.5% lactose (Fig. 1C) or 0.5% maltose (Fig. 1E) respectively. However, when the medium was supplemented with 1% of either glucose or lactose, a drastic reduction in putrescine production was observed (Fig. 1B and 1D); indeed, compared to the amount accumulated in the presence of 0.5% glucose (19 mM putrescine), a reduction of 88% was seen (2.1 mM putrescine). Similarly, compared to the amount of putrescine accumulated in the presence of 0.5% lactose (14 mM), in the presence of 1% lactose an 84% reduction was seen (2.2 mM putrescine). However, the presence of 1% maltose in the culture medium did not inhibit putrescine biosynthesis (Fig. 1F); 18.1 mM putrescine were produced, similar to that obtained with 0.5% maltose supplementation (17 mM putrescine) (Fig. 1E). These results confirm that both glucose and lactose repress putrescine production in *L. lactis* subsp. *lactis* T3/33.

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

Figure 2 shows the effect of the carbon source (M17 supplemented with 0.5% or 1% glucose, lactose or maltose) on strain T3/33 growth in the presence and absence of 20 mM agmatine, and on the pH of the medium. Growth in M17 with 0.5% glucose, lactose or maltose plus 20 mM agmatine was clearly better than when agmatine was absent (Fig. 2A, 2C and 2E respectively). After 10 h, the cultures grown in medium without agmatine reached OD<sub>600</sub>=3.5 (glucose), OD<sub>600</sub>=3.1 (lactose) and OD<sub>600</sub>=3.3 (maltose), while those supplemented with 20 mM agmatine reached OD<sub>600</sub>=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 \textit{aguB} - the first gene of the \textit{aguBDAC} operon (Linares et al., 2013) - was analyzed by RT-qPCR as representative of the whole \textit{aguBDAC} mRNA. To avoid the possible effect of pH on the expression of the \textit{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 \textit{aguB} (using \textit{rpoA} as a reference gene). Comparative analysis showed the transcription of the \textit{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 \textit{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 \textit{L. lactis}

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

4. Discussion

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

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

Interestingly, increased bacterial growth was observed under the culture conditions that allowed putrescine production to rise to above 15 mM (i.e., in the presence of agmatine, and with either maltose or low concentrations of glucose or lactose) compared to those in which putrescine production was weaker (in the presence of agmatine but with high concentrations of glucose or lactose, or the absence of agmatine) (Fig. 2). This might be 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' TGAAADCGTTCCA 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
patterns were observed when the medium was supplemented with either high or low maltose concentrations (Fig. 1).

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

Since milk has a 4% of lactose (Shakeel-Ur-Rehman et al., 2004), to use as starters *L. 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.
Table 1. Primers used for gene expression quantification by RT-qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>aguB(^a)</td>
<td>aguB-F</td>
<td>CCGAAAATCGCTTGACTTCAA</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td>aguB-R</td>
<td>TCAGCATAATCAGACATCAACCAA</td>
<td>This work</td>
</tr>
<tr>
<td>rpoA(^b)</td>
<td>rpoA-F</td>
<td>CACGGGCAGGTCAACTTG</td>
<td>(Taibi et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>rpoA-R</td>
<td>TTCCGGCTGACGAAAATAAAG</td>
<td>(Taibi et al., 2011)</td>
</tr>
<tr>
<td>tufA(^b)</td>
<td>qtufF</td>
<td>TCTTCATCATCAAAAGGTCTGCTT</td>
<td>(Linares et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>qtufR</td>
<td>GAACACATCTTGTTCACGTCAA</td>
<td>(Linares et al., 2013)</td>
</tr>
</tbody>
</table>

\(^a\) Target gene

\(^b\) Reference gene
Table 2. Putrescine accumulation by *L. lactis* strains grown in M17+A supplemented with different sugars.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Putrescine (mM) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5% glucose</td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. <em>lactis</em> T3/33</td>
<td>6.17 ± 0.32</td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. <em>lactis</em> T1/48</td>
<td>4.17 ± 0.08</td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. <em>lactis</em> L39</td>
<td>2.66 ± 0.7</td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. <em>lactis</em> 1AA17</td>
<td>15.97 ± 0.34</td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. <em>lactis</em> 1AA59</td>
<td>10.36 ± 4.26</td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. <em>lactis</em> 2BA40</td>
<td>10.84 ± 0.25</td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. <em>lactis</em> 1A38</td>
<td>2.36 ± 0.12</td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. <em>cremoris</em> GE2-14</td>
<td>6.26 ± 0.24</td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. <em>cremoris</em> 3AA23</td>
<td>9.85 ± 2.04</td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. <em>cremoris</em> 2A22</td>
<td>6.04 ± 0.22</td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. <em>cremoris</em> 3AA9</td>
<td>5.48 ± 0.48</td>
</tr>
</tbody>
</table>

SD: standard deviation
Figure 1
Figure 2
Figure 3
Figure 4