Putrescine production by *Lactococcus lactis* subsp. *cremoris* CECT 8666 is reduced by NaCl via a decrease in bacterial growth and the repression of the genes involved in putrescine production

Beatriz del Rio*, Begoña Redruello, Victor Ladero, Maria Fernandez, Maria Cruz Martin and Miguel A. Alvarez

Instituto de Productos Lácteos de Asturias, IPLA-CSIC, Paseo Rio Linares s/n, 33300 Villaviciosa, Spain.

E-mail addresses:

beadelrio@ipla.csic.es (B. del Rio)

bredruel@ipla.csic.es (B. Redruello)

ladero@ipla.csic.es (V. ladero)

mfernandez@ipla.csic.es (M. Fernandez)

mcm@ipla.csic.es (M.C. Martin)

maag@ipla.csic.es (M.A. Alvarez)

*Corresponding author

Mailing address: Instituto de Productos Lácteos de Asturias (IPLA-CSIC)

Paseo Rio Linares s/n, 33300 Villaviciosa, Spain.

Tel.: +34 985 89 21 31

Fax: +34 985 89 22 33

E-mail: beadelrio@ipla.csic.es

Abstract

The reduction of NaCl in food is a public health priority; high NaCl intakes have been associated with serious health problems. However, it is reported that reducing the NaCl content of cheeses may lead to an increase in the content of biogenic amines (BAs). The present work examines the effect of NaCl on the accumulation of putrescine (one of the BAs often detected at high concentration in cheese) in experimental Cabrales-like cheeses containing *Lactococcus lactis* subsp. *cremoris* CECT 8666, a dairy strain that catabolises agmatine to putrescine via the agmatine deiminase (AGDI) pathway. The genes responsible for this pathway are grouped in the AGDI cluster. This comprises a regulatory gene (*aguR*) (transcribed independently), followed by the catabolic genes that together form an operon (*aguBDAC*). Reducing the NaCl concentration of the cheese led to increased putrescine accumulation. In contrast, increasing the NaCl concentration of both pH-uncontrolled and pH-controlled (pH 6) cultures of *L. lactis* subsp. *cremoris* CECT 8666 significantly inhibited its growth and the production of putrescine. Such production appeared to be inhibited via a reduction in the transcription of the *aguBDAC* operon; no effect on the transcription of *aguR* was recorded. The present results suggest that low-sodium cheeses are at risk of accumulating higher concentrations of putrescine.

Keywords: *Lactococcus lactis*; biogenic amines; putrescine; agmatine deiminase pathway; AGDI cluster; NaCl

1 Introduction

Cheeses are fermented food products that may accumulate high concentrations of biogenic amines (BAs) such as tyramine, histamine, putrescine and cadaverine (Alvarez and Moreno-Arribas, 2014; EFSA, 2011; Linares et al., 2011; Redruello et al., 2013). BAs are biologically active nitrogenous organic bases largely produced via the enzymatic decarboxylation of their precursor amino acids through the action of food spoilage microorganisms (Alvarez and Moreno-Arribas, 2014; Linares et al., 2011). The consumption of foods with high BA contents may cause intoxication symptoms including respiratory distress, headache, and hyper- or hypotension, among others (Broadley, 2010; Ladero et al., 2010; Stratta and Badino, 2012). BAs are therefore considered indicators of low food quality and low acceptability (Shalaby, 1996).

Many factors affect the BA content of cheeses, including the presence of microorganisms with the required decarboxylase activity, and technological factors (pasteurization, the ripening process followed, the temperature and duration of ripening, and NaCl content) (Linares et al., 2011, 2012). NaCl is an important ingredient in cheese-making, contributing to product flavour and texture, and influencing microbial growth (Guinee, 2004; Linares et al., 2012). It has been proposed to reduce the growth of BA producers (Andic et al., 2010; Chander et al., 1989; Gardini et al., 2001; Sumner et al., 1990) and inhibit the decarboxylase activity responsible for BA biosynthesis (Chander et al., 1989). BAs are therefore less likely to accumulate in products with high NaCl contents (Andic et al., 2010; Loizzo et al., 2013).

The NaCl concentration of cheese may be as high as 6% (w/w) depending on the variety (Guinee, 2004). The World Health Organization recommends sodium intake be reduced to avoid its undesirable effects on human health, such as hypertension and the consequently increased risk of cardiovascular and kidney disease. The dairy industry has reacted to this advice by introducing low- and reduced-salt cheeses. A number of studies have investigated the effect of NaCl reduction on the growth of microbial pathogens during cheese manufacture (Leong et al., 2014; Shrestha et al., 2011) and on the sensory properties of the final product (Drake et al., 2011; Ganesan et al., 2014; Moller et al., 2013). However, the reduction of NaCl in cheese could lead to the increased accumulation of BAs (Loizzo et al., 2013).

The aim of the present work was to determine whether or not NaCl concentration influences the accumulation, in cheese, of putrescine (one of the most common BAs in cheese along with tyramine and histamine) (Fernandez et al., 2007; Linares et al., 2011; Spano et al., 2010). To this end, experiments were performed using a Cabrales-like mini cheese model developed by our group (Herrero-Fresno et al., 2012) and the *Lactococcus lactis* subsp. *cremoris* strain CECT 8666. Cabrales is a famed traditional blue cheese manufactured in the mountainous area of the “Picos de Europa” (Principality of Asturias, Northern Spain); due to its characteristics –made with raw milk, blue-veined, high proteolytic activity, and long ripening period– it can accumulate high BA concentrations (Redruello et al., 2013). Cabrales cheese has a NaCl content of approximately 2.3% (w/w), moisture of 40% and a water activity (a_w) of 0.92

(Florez et al., 2006). The *L. lactis* CECT 8666 strain, which was originally isolated from an artisanal cheese, has been extensively characterized as a model of lactococcal putrescine production (del Rio et al., 2015a, 2015b; Ladero et al., 2011; Linares et al., 2013, 2015). The strain produces this BA through the catabolism of agmatine - a decarboxylated derivative of arginine (Simon and Stalon, 1982) - via the agmatine deiminase (AGDI) pathway. The cluster of genes responsible for this pathway includes the regulatory gene *aguR*, followed by the catabolic genes *aguB*, *aguD*, *aguA* and *aguC* (del Rio et al., 2015b; Ladero et al., 2011) which are co-transcribed as a polycistronic mRNA (operon *aguBDAC*) (Linares et al., 2015). *aguR* encodes AguR, a transmembrane one-component signal transduction protein that senses the extracellular agmatine concentration and triggers the transcription of *aguBDAC*, and thus the biosynthesis of putrescine (Linares et al., 2015). Since NaCl was found to have an effect on the accumulation of putrescine in the chosen cheese model, experiments were performed to see whether it influenced the growth of *L. lactis* CECT 8666 and the expression of its AGDI cluster. Since the growth of *L. lactis* results in acidification of the culture medium, and since the pH affects putrescine production (del Rio et al., 2016), experiments were performed in both pH-uncontrolled and pH-controlled cultures.

2 Materials and Methods

2.1 Microorganisms and culture conditions

Table 1 shows the microorganisms used in the present study. *L. lactis* subsp. *cremoris* CECT 8666, a putrescine-producing strain, was previously isolated from Genestoso cheese, a Spanish artisanal cheese made from raw milk without the addition of commercial starter cultures (Fernandez et al. 2011; Ladero et al. 2011). This and all other *L. lactis* strains were grown in M17 (Oxoid, UK) supplemented with 1% galactose (GalM17) at 32°C without aeration. The species and subspecies of all the lactococcal strains used in this work had been previously determined by molecular methods (Fernandez et al. 2011). Galactose instead of glucose was used as carbon source, because it does not exerts carbon catabolite repression on the agmatine deiminase pathway of *L. lactis* subsp. *cremoris* CECT 8666 (Linares et al., 2013). The mould *Penicillium roqueforti* was propagated on malt extract agar (MEA, Oxoid) slants. Where indicated, the growth medium was supplemented with agmatine (Sigma-Aldrich, Barcelona, Spain). For all fermentation assays, overnight cultures of *L. lactis* strains were used as inocula (1% v/v). Each fermentation experiment was performed using the same stock culture medium, and the same overnight culture as inocula to ensure identical cfu/ml, so that the only difference between the cultures was the NaCl concentration.

pH-uncontrolled fermentations of *L. lactis* subsp. *cremoris* CECT 8666 were performed in 30 ml of GalM17 supplemented with 20 mM agmatine. Sampling (2 ml) was performed every hour for 12 h. The pH of the samples was measured using a CRISON miCropH 2001 pH-meter (Crison Instruments, S.A., Barcelona, Spain). pH-controlled fermentations involving *L. lactis* subsp. *cremoris* CECT 8666 were performed in a Six-Fors® bioreactor (Infors AG,

Bottmingen, Switzerland) containing 300 ml of GalM17 supplemented with 1 mM agmatine. This agmatine concentration is enough to ensure the transcriptional activity of the catabolic genes of the AGDI cluster in *L. lactis* (Linares et al., 2013). The reactor was maintained at 32°C, stirring at 50 rpm and with zero air input. A fixed pH of 6 was maintained by the automatic addition of 1 N NaOH or 1 N HCl as needed. Samples (2 ml) were collected each hour. Microbial growth was examined in all cultures by measuring absorbance at 600 nm (OD₆₀₀) using a spectrophotometer (Eppendorf, NY, USA). Sample supernatants were obtained by centrifugation (2000 g for 15 min) and putrescine concentration determined by ultra-high performance liquid chromatography (UHPLC) following the protocol of Redruello et al. (2013). Three biological replicates were performed for each experiment.

2.2 *Cabrales-like mini cheeses*

Table 1 shows the lactococcal and mould strains used to make Cabrales-like mini cheeses following the model described by Herrero-Fresno et al. (2012), with some modifications. Overnight cultures of the non-putrescine-producing strains used as starters *L. lactis* SK11, 1AA23, 3AA11 and LC44 (10⁵ cfu/ml each), and the putrescine producing *L. lactis* CECT 8666 (10⁶ cfu/ml) were directly inoculated into sterilized bottles containing 200 ml of commercial pasteurized milk; the mould *P. roqueforti* (10³ cfu/ml) and CaCl₂ (0.02 % w/v) were also added to the bottles. The mixtures were then left at 30°C for 45 min to initiate the growth of the cultures. After this time, liquid rennet extract of bovine origin (Naturen™ Extra 1000 NB, Christian Hansen, Hoersholm,

Denmark) was added (0.05 IMCU/ml). The bottles were inverted three times and left to coagulate under supervision in a water bath at 30°C for approximately 1 h. After coagulation, the bottles were left for 30 min more at 30°C to achieve the appropriate curd consistency. The curd was then cut and inverted for 20 min to promote draining. The bottles were then centrifuged at 220 g for 10 min at room temperature and the whey discarded. All steps were performed under sterile conditions. The products were weighed and dry-salted to generate cheeses with 0%, 1%, 2%, 3%, 4%, 5% or 6% NaCl (w/w). At this point, agmatine was added to a final non-limiting concentration of 20 mM. The mini-cheeses were kept into screw-capped sterile jars placed in a ripening chamber at 15°C for 120 days. The jars were kept closed throughout the ripening period to preserve the sterile conditions and moisture. Samples were collected at days 0, 7, 15, 30, 60, 90 and 120 days. Putrescine accumulated in the samples was extracted and analyzed as explained below. Three different mini-cheese elaborations were performed.

2.3 Extraction and quantification of putrescine from cheese samples

The putrescine accumulated in the experimental cheeses was extracted following the protocol described by Krause et al. (1995) with some modifications. One gram of cheese was homogenized with 10 ml of 0.1 M HCl containing 0.2% 3,3'-thiodipropionic acid (TDPA) (Fluka, Madrid, Spain) using an Ultra-Turrax T50 homogenizer (OMNI International, CT, USA) for 2 min at 20,000 rpm. This mixture was kept in an ultrasonic bath for 30 min and centrifuged at 5000 g for 20 min. The top fat layer was removed and the

supernatant collected and filtered through a 0.45 µm membrane. Supernatant samples (100 µl) were then deproteinated using trichloroacetic acid (TCA) added to a final concentration of 12%. The mixture was then incubated at 4°C for 30 min and centrifuged at 13,000 g for 15 min. These final supernatants were collected and brought to pH 7 via the addition of NaOH to avoid the interference of the acid pH on the derivatisation process. The putrescine concentration was then analyzed by UHPLC as described above.

2.4 RNA extraction

L. lactis CECT 8666 bacteria were grown at pH 6 in a Six-Fors® bioreactor containing 300 ml of GalM17 supplemented with 1 mM agmatine (a concentration sufficient to activate the transcription of the AGDI genes [Linares et al., 2015]) and 0%, 1%, 2% or 3% NaCl. Cells were harvested by centrifugation after 8 h of growth. Total RNA was extracted following the protocol described in del Rio et al. (2015a).

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

cDNA was 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 quantitative PCR (qPCR) using an ABI Prims Fast 7500 sequence detection system (Applied Biosystems, Carlsbad, CA). Reactions were performed as previously described (Linares et

al., 2009) in 25 µl reaction volumes, which included the primers and Power SYBR® Green PCR Master Mix (which contains ROX as a passive reference) (Applied Biosystems). Cycling was performed under the Applied Biosystems default settings. Amplifications were performed with the previously described specific primers (Table 1): the primer pair qAguR-F/qAguR-R and the primer pair qPTC-F/ anqPTC-R were used to amplify *aguR* and *aguB* (the first gene of the AGDI cluster, representative of the operon *aguBDAC*) respectively (Linares et al., 2013). The primer pairs qtufF/qtufR (Linares et al., 2013) and rpoA-F/rpoA-R (Taibi et al., 2011) were used to amplify the thermo-unstable elongation factor (*tuf*) and the RNA polymerase alpha-subunit (*rpoA*) genes respectively, which were used as references. Samples with no DNA were included in each run as negative controls. Relative gene expression was calculated using the $\Delta\Delta C_t$ comparative method (Livak and Schmittgen, 2001). For each condition, RT-qPCR analysis was performed on RNA samples purified from three different cultures.

2.6 Statistical analysis

Means $\pm$ standard deviations were calculated from three independent replicates. The Student *t* test was used to examine differences between groups. Significance was set at $p < 0.05$.

3 Results

3.1 NaCl reduced the accumulation of putrescine in experimental Cabrales-like cheeses

Experimental cheeses were made with *L. lactis* strains previously reported to be non-putrescine producers (Ladero et al., 2011) as starters, but containing *L. lactis* subsp. *cremoris* CECT 8666, a well-known dairy putrescine producer (Table 1). Seven different cheeses were made with increasing concentrations of NaCl (0%, 1%, 2%, 3%, 4%, 5% or 6% NaCl). Putrescine accumulation was determined after 7, 15, 30, 60, 90 and 120 days of ripening (Fig. 1). As shown, the cheeses made with NaCl concentrations of <3% NaCl showed no differences in putrescine accumulation (approximately 15 mM) after 120 days. In contrast, higher NaCl concentrations led to proportional reductions in putrescine accumulation - cheeses made with 4%, 5% and 6% NaCl reached 8.5 mM, 4.6 mM and 0.1 mM putrescine, respectively, after 120 days of ripening.

3.2 NaCl inhibits the growth of L. lactis CECT 8666 and reduces putrescine production

The effect of NaCl on *L. lactis* CECT 8666 growth and putrescine production was then examined. The strain was grown in GalM17 supplemented with 0%, 1%, 2%, 3%, 4%, 5% or 6% of NaCl in the presence of 20 mM agmatine for 12 h. The change in the pH of the medium was also recorded. Cultures with 5% or 6% NaCl showed no growth after 12 h of fermentation (data not shown) and were excluded from further analysis.

Putrescine biosynthesis increased with reducing NaCl concentration (Fig. 2A). After 12 h of fermentation, a gradual reduction in this accumulation was observed with increasing NaCl concentration (0-2%). No putrescine was produced in cultures grown at 3% or 4% NaCl.

Bacterial growth decreased with increasing NaCl (Fig. 2B), revealing a possible association between growth and putrescine production. However, the culture grew to an approximate OD₆₀₀ of 2 in a medium supplemented with 3% NaCl, with no accumulation of putrescine. Since the pH of the medium clearly correlated with bacterial growth, it was lower in cultures with lower NaCl concentrations (Fig. 2C).

3.3 Effect of NaCl on putrescine production and the growth of L. lactis CECT 8666 in pH-controlled cultures

The transcriptional activity of the *aguBDAC* operon, and consequently putrescine production, is known to be influenced by the pH of the medium (del Rio et al., 2016). To eliminate this effect, the influence of NaCl on putrescine production and on the transcriptional activity of the AGDI cluster was examined in pH-controlled cultures.

L. lactis CECT 8666 cells were grown in a bioreactor at pH 6 in GalM17 medium supplemented with either 0%, 1%, 2%, 3% or 4% NaCl in the presence of 1 mM agmatine for 12 h. Bacterial growth decreased with increasing NaCl

concentration (Fig. 3A). Cultures grown in 4% NaCl showed very little growth after 12 h of fermentation (data not shown) and were therefore excluded from further analysis. A delay in putrescine production was observed in cultures grown with 1% NaCl compared to cultures grown with 0% NaCl, which correlated with a delay on bacterial growth (Fig. 3B), similar to that recorded for uncontrolled-pH cultures (Fig. 2A). No putrescine was produced in cultures grown with either 2% or 3% NaCl after 12 h of fermentation (Fig. 3B), despite growth to an OD₆₀₀ of about 2 and 1 respectively.

3.4 NaCl inhibits transcription of the catabolic operon aguBDAC without affecting the expression of the regulatory gene aguR

The expression profiles of both *aguR* and *aguB* were analyzed by RT-qPCR using RNA from the pH-controlled cultures (described above). Comparative analysis showed the transcription of the *aguR* gene not to be regulated by the concentration of NaCl in the culture medium (Fig. 4A). In contrast, the transcription of the *aguBDAC* operon was significantly inhibited by increasing concentrations of NaCl; the greatest transcriptional activity was observed at 0% NaCl (500 times the expression at 3% NaCl), becoming progressively reduced at 1% and 2% NaCl (Fig. 4B).

4 Discussion

NaCl has an important influence on the flavour and taste of cheese, and on the growth and metabolism of desirable bacteria, including starter lactic acid

bacteria (LAB) and non-starter LAB that add flavour during aging (McMahon et al., 2014). NaCl can also improve food safety by preventing the growth of pathogens (Guinee, 2004; Taormina, 2010). Reducing the salt content of cheese may allow the growth of unwanted bacteria, such as BA-producing bacteria, during ripening. The present study reveals the influence of NaCl on the accumulation of putrescine in experimental Cabrales-like cheeses, along with its effect on the growth of the putrescine-producing strain *L. Lactis* CECT 8666, and on the transcriptional regulation of its AGDI cluster.

The present results show that increasing the salt content of the experimental cheeses - inoculated with the putrescine-producing *L. lactis* strain - leads to significant reductions in putrescine accumulation, even over short ripening periods (7 days) (Fig. 1). Although *L. lactis* has been awarded Qualified Presumption of Safety status, some dairy strains can produce putrescine (Ladero et al., 2011). Indeed, *L. lactis* is one of the main putrescine producers in cheese (Ladero et al., 2012), and the presence of putrescine-producing *L. lactis* in starter cultures, or in the natural microflora of the milk used for cheese manufacturing, could lead to the accumulation of this BA. The present results thus show that reducing the NaCl content of cheeses in an attempt to improve their health-associated qualities could lead to an increase in putrescine accumulation. This might not only reduce their quality but also their safety.

The reduced putrescine accumulation recorded for cheeses with high NaCl concentrations might be a consequence of the reduced growth of the putrescine-producing strains, as suggested for other BAs (Andic et al., 2010;

Chander et al., 1989; Gardini et al., 2001; Sumner et al., 1990). Indeed, an increase in the NaCl content of the culture medium significantly reduced the growth of *L. lactis* CECT 8666 in both pH-uncontrolled and pH-controlled cultures (Fig. 2B and Fig. 3A respectively). This inhibitory effect is well described in the literature (Simsek et al., 2009; Uguen et al., 1999), although the level of osmotic tolerance is strain-dependent (O'Callaghan and Condon, 2000; Troller and Stinson, 1981). Some cultures, however, showed growth after 12 h of fermentation with NaCl, yet unexpectedly did not accumulate putrescine (e.g., see results for pH-uncontrolled cultures grown at 3% NaCl [Fig. 2] and pH-controlled cultures grown at 2% or 3% NaCl [Fig. 3]). These results suggest that other mechanisms are involved, so tests were performed to determine whether NaCl might influence the transcriptional regulation of the putrescine biosynthesis via the AGDI pathway. Transcriptional analysis of the AGDI cluster in *L. lactis* CECT 8666 cultures grown with increasing concentrations of NaCl revealed the latter to inhibit the transcription of the *aguBDAC* catabolic operon (Fig. 4B). The regulation of putrescine biosynthesis in *L. lactis* CECT 8666 has been extensively studied (del Rio et al., 2015b; del Rio et al., 2016; Ladero et al., 2011; Linares et al., 2013, 2015). It should be remembered that AguR - encoded by the first gene of the AGDI cluster- is a transmembrane protein, which functions as a one-component signal transduction system that senses the agmatine concentration of the medium and accordingly regulates the transcription of the *aguBDAC* operon (Linares et al., 2015; Linares et al., submitted). The expression of *aguR* is generally very low and would seem to be constitutive since it is not affected by the concentration of agmatine (Linares et al., 2015), glucose (Linares et al., 2013) or pH of the culture medium (del Rio et

al., 2016). In the present work, the expression of *aguR* was not affected by the NaCl concentration of the culture medium. This result reinforces the idea that *aguR* is constitutively expressed, ensuring the presence of AguR on the cell surface where it can act as an agmatine sensor. Previous results strongly suggest that the presence of agmatine would cause the dimerization of AguR that would bind through its C-terminal LuxR_C-like domain to the *aguB* promoter inducing the transcription of the catabolic genes and therefore putrescine production (Linares et al., 2015). The extracellular NaCl might interfere on the sensing of agmatine by AguR and/or affect the subsequent structural arrangements including the dimerization and the binding to the *aguB* promoter.

To the best of our knowledge this is the first study to assess the effect of NaCl on the transcriptional activity of the AGDI cluster, and its influence on putrescine production via this pathway. In fact, very few reports exist concerning the effect of NaCl on the transcriptional activity associated with any BA production pathway. However, those that do exist indicate - unlike in the present work - NaCl to positively regulate the expression of such genes, e.g., the *tdcA* gene of *Streptococcus thermophilus* which codes for tyrosine decarboxylase (La Gioia et al., 2011), and the *hdcA* gene of *S. thermophilus*, which codes for histidine decarboxylase (Rossi et al., 2011). In these studies, the indirect involvement of these decarboxylative pathways in supplying the energy necessary to counteract the osmotic stress induced by NaCl is postulated. The AGDI pathway has a physiological role in enhancing the growth of *L. lactis* (del Rio et al., 2015b), but there is no evidence that it plays any important function in the osmoprotection of *L. lactis* against increased environmental NaCl.

Together, the results of the present study suggest that low NaCl concentrations in cheese would allow for better growth of potential putrescine-producing microorganisms, while allowing the transcriptional activation of the catabolic genes of the AGDI cluster, the main pathway for putrescine biosynthesis in cheeses. The overall effect would be the increased production and accumulation of putrescine, bringing into question the supposed benefit to health of consuming low sodium cheese.

5 Acknowledgements

This work was funded by the Spanish Ministry of Economy and Competitiveness (AGL2013-45431-R) and by the GRUPIN14-137 project, which is co-financed by the Plan for Science, Technology and Innovation of the Principality of Asturias 2013-2017 and the European Regional Development Funds. The authors thank Adrian Burton for language and editing assistance.

6 References

- Alvarez, M.A., Moreno-Arribas, V., 2014. The problem of biogenic amines in fermented foods and the use of potential biogenic amine-degrading microorganisms as a solution. *Trends in Food Science & Technology* 39, 146-155.
- Andic, S., Genccelep, H., Kose, S., 2010. Determination of Biogenic Amines in Herby Cheese. *International Journal of Food Properties* 13, 1300-1314.

- Broadley, K.J., 2010. The vascular effects of trace amines and amphetamines. *Pharmacology & Therapeutics* 125, 363-375.
- Chander, H., Batish, V.K., Babu, S., Singh, R.S., 1989. Factors Affecting Amine Production by a Selected Strain of *Lactobacillus bulgaricus*. *Journal of Food Science* 54, 940-942.
- del Rio, B., Ladero, V., Redruello, B., Linares, D.M., Fernandez, M., Martin, M.C., Alvarez, M.A., 2015a. Lactose-mediated carbon catabolite repression of putrescine production in dairy *Lactococcus lactis* is strain dependent. *Food Microbiology* 48, 163-170.
- del Rio, B., Linares, D.M., Ladero, V., Redruello, B., Fernandez, M., Martin, M.C., Alvarez, M.A., 2015b. Putrescine production via the agmatine deiminase pathway increases the growth of *Lactococcus lactis* and causes the alkalization of the culture medium. *Applied Microbiology and Biotechnology* 99, 897-905.
- del Rio, B., Linares, D., Ladero, V., Redruello, B., Fernández, M., Martin, M.C. and Alvarez, M.A. 2016. Putrescine biosynthesis in *Lactococcus lactis* is transcriptionally activated at acidic pH and counteracts acidification of the cytosol. *International Journal of Food Microbiology*. Submitted
- Drake, S.L., Lopetcharat, K., Drake, M.A., 2011. Salty taste in dairy foods: can we reduce the salt? *Journal of Dairy Science* 94, 636-645.
- EFSA, 2011. Scientific opinion on risk based control of biogenic amine formation in fermented foods. Panel on Biological Hazards (BIOHAZ). *EFSA Journal* 9, 2393-2486.
- Fernandez, E., Alegria, A., Delgado, S., Martin, M.C., Mayo, B., 2011. Comparative phenotypic and molecular genetic profiling of wild

- Lactococcus lactis* subsp. *lactis* strains of the *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* genotypes, isolated from starter-free cheeses made of raw milk. *Applied and Environmental Microbiology* 77, 5324-5335.
- Fernandez, M., Linares, D.M., del Rio, B., Ladero, V., Alvarez, M.A., 2007. HPLC quantification of biogenic amines in cheeses: correlation with PCR-detection of tyramine-producing microorganisms. *Journal of Dairy Research* 74, 276-282.
- Florez, A.B., Lopez-Diaz, T.M., Alvarez-Martin, P., Mayo, B., 2006. Microbial characterisation of the traditional Spanish blue-veined Cabrales cheese: identification of dominant lactic acid bacteria. *European Food Research and Technology* 223, 503-508.
- Florez, A.B., Alvarez-Martin, P., Lopez-Diaz, T.M., Mayo, B., 2007. Morphotypic and molecular identification of filamentous fungi from Spanish blue-veined Cabrales cheese, and typing of *Penicillium roqueforti* and *Geotrichum candidum* isolates. *International Dairy Journal* 17, 7.
- Ganesan, B., Brown, K., Irish, D.A., Brothersen, C., McMahon, D.J., 2014. Manufacture and sensory analysis of reduced- and low-sodium Cheddar and Mozzarella cheeses. *Journal of Dairy Science* 97, 1970-1982.
- Gardini, F., Martuscelli, M., Caruso, M.C., Galgano, F., Crudele, M.A., Favati, F., Guerzoni, M.E., Suzzi, G., 2001. Effects of pH, temperature and NaCl concentration on the growth kinetics, proteolytic activity and biogenic amine production of *Enterococcus faecalis*. *International Journal of Food Microbiology* 64, 105-117.
- Guinee, T.P., 2004. Salting and the role of salt in cheese. *International Journal of Dairy Technology* 57, 99-109.

- Herrero-Fresno, A., Martinez, N., Sanchez-Llana, E., Diaz, M., Fernandez, M., Martin, M.C., Ladero, V., Alvarez, M.A., 2012. *Lactobacillus casei* strains isolated from cheese reduce biogenic amine accumulation in an experimental model. *International Journal of Food Microbiology* 157, 297-304.
- Krause, I., Bockhardt, A., Neckermann, H., Henle, T., Klostermeyer, H., 1995. Simultaneous determination of amino acids and biogenic amines by reversed-phase high-performance liquid chromatography of the dansyl derivatives. *Journal of Chromatography A* 715, 13.
- La Gioia, F., Rizzotti, L., Rossi, F., Gardini, F., Tabanelli, G., Torriani, S., 2011. Identification of a tyrosine decarboxylase gene (*tdcA*) in *Streptococcus thermophilus* 1TT45 and analysis of its expression and tyramine production in milk. *Appl Environ Microbiol* 77, 1140-1144.
- Ladero, V., Calles-Enríquez, M., Fernández, M., Alvarez, M.A., 2010. Toxicological effects of dietary biogenic amines. *Current Nutrition and Food Science* 6, 145-156.
- Ladero, V., Cañedo, E., Perez, M., Martin, M.C., Fernandez, M. and Alvarez, M.A., 2012. Multiplex qPCR for the detection and quantification of putrescine-producing lactic acid bacteria in dairy products. *Food Control* 27, 307-313.
- Ladero, V., Rattray, F.P., Mayo, B., Martin, M.C., Fernandez, M., Alvarez, M.A., 2011. Sequencing and transcriptional analysis of the biosynthesis gene cluster of putrescine-producing *Lactococcus lactis*. *Applied and Environmental Microbiology* 77, 6409-6418.

- Leong, W.M., Geier, R., Engstrom, S., Ingham, S., Ingham, B., Smukowski, M., 2014. Growth of *Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli* O157:H7, and *Staphylococcus aureus* on cheese during extended storage at 25 degrees C. *Journal of Food Protection* 77, 1275-1288.
- Linares, D.M., Alvarez-Sieiro, P., del Rio, B., Ladero, V., Redruello, B., Martin, M.C., Fernandez, M. and Alvarez, M.A. A new method for controlling inducible gene expression in *Lactococcus lactis* - the agmatine-controlled expression system - and its use in the heterologous production of *Myxococcus xanthus* prolyl-endopeptidase. *Microbial Cell Factories*. Submitted.
- Linares, D.M., del Rio, B., Ladero, V., Martinez, N., Fernandez, M., Martin, M.C., Alvarez, M.A., 2012. Factors influencing biogenic amines accumulation in dairy products. *Frontiers in Microbiology* 3, 180.
- Linares, D.M., del Rio, B., Ladero, V., Redruello, B., Martin, M.C., Fernandez, M., Alvarez, M.A., 2013. The putrescine biosynthesis pathway in *Lactococcus lactis* is transcriptionally regulated by carbon catabolic repression, mediated by CcpA. *International Journal of Food Microbiology* 165, 43-50.
- Linares, D.M., del Rio, B., Redruello, B., Ladero, V., de Jong, M.M., Kuipers, O.P., Fernandez, M., Alvarez, M.A., 2015. AguR, a transmembrane transcription activator of the putrescine biosynthesis operon in *Lactococcus lactis*, acts in response to the agmatine concentration. *Applied and Environmental Microbiology* 81, 6145-6157.
- Linares, D.M., Fernandez, M., Martin, M.C., Alvarez, M.A., 2009. Tyramine biosynthesis in *Enterococcus durans* is transcriptionally regulated by the

- extracellular pH and tyrosine concentration. *Microbial Biotechnology* 2, 625-633.
- Linares, D.M., Martin, M.C., Ladero, V., Alvarez, M.A., Fernandez, M., 2011. Biogenic amines in dairy products. *Critical Reviews in Food Science and Nutrition* 51, 691-703.
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402-408.
- Loizzo, M.R., Menichini, F., Picci, N., Puoci, F., Spizzirri, U.G., Restuccia, D., 2013. Technological aspects and analytical determination of biogenic amines in cheese. *Trends in Food Science and Technology* 30, 38-55.
- McMahon, D.J., Oberg, C.J., Drake, M.A., Farkye, N., Moyes, L.V., Arnold, M.R., Ganesan, B., Steele, J., Broadbent, J.R., 2014. Effect of sodium, potassium, magnesium, and calcium salt cations on pH, proteolysis, organic acids, and microbial populations during storage of full-fat Cheddar cheese. *Journal of Dairy Science* 97, 4780-4798.
- Moller, K.K., Rattray, F.P., Bredie, W.L., Hoier, E., Ardo, Y., 2013. Physicochemical and sensory characterization of Cheddar cheese with variable NaCl levels and equal moisture content. *Journal of Dairy Science* 96, 1953-1971.
- O'Callaghan, J., Condon, S., 2000. Growth of *Lactococcus lactis* strains at low water activity: correlation with the ability to accumulate glycine betaine. *International Journal of Food Microbiology* 55, 127-131.
- Redruello, B., Ladero, V., Cuesta, I., Alvarez-Buylla, J.R., Martin, M.C., Fernandez, M., Alvarez, M.A., 2013. A fast, reliable, ultra high

performance liquid chromatography method for the simultaneous determination of amino acids, biogenic amines and ammonium ions in cheese, using diethyl ethoxymethylenemalonate as a derivatising agent. Food Chemistry 139, 1029-1035.

Rossi, F., Gardini, F., Rizzotti, L., La Gioia, F., Tabanelli, G., Torriani, S., 2011. Quantitative analysis of histidine decarboxylase gene (hdcA) transcription and histamine production by *Streptococcus thermophilus* PRI60 under conditions relevant to cheese making. Applied and Environmental Microbiology 77, 2817-2822.

Shalaby, A.R., 1996. Significance of biogenic amines to food safety and human health. Food Research International 29, 675-690.

Shrestha, S., Grieder, J.A., McMahon, D.J., Nummer, B.A., 2011. Survival of *Listeria monocytogenes* introduced as a post-aging contaminant during storage of low-salt Cheddar cheese at 4, 10, and 21 degrees C. Journal of Dairy Science 94, 4329-4335.

Simon, J.P., Stalon, V., 1982. Enzymes of agmatine degradation and the control of their synthesis in *Streptococcus faecalis*. Journal of Bacteriology 152, 676-681.

Simsek, O., Buzrul, S., Akkoc, N., Alpas, H., Akcelik, M., 2009. Stress response kinetics of two nisin producer strains of *Lactococcus lactis* spp. *lactis*. Applied Biochemistry and Biotechnology 158, 387-397.

Spano, G., Russo, P., Lonvaud-Funel, A., Lucas, P., Alexandre, H., Grandvalet, C., Coton, E., Coton, M., Barnavon, L., Bach, B., Rattray, F., Bunte, A., Magni, C., Ladero, V., Alvarez, M., Fernandez, M., Lopez, P., de Palencia, P.F., Corbi, A., Trip, H., Lolkema, J.S., 2010. Biogenic amines in

fermented foods. European Journal of Clinical Nutrition 64 Suppl 3, S95-100.

Stratta, P., Badino, G., 2012. Scombroid poisoning. Canadian Medical Association journal 184, 674.

Sumner, S.S., Roche, F., Taylor, S.L., 1990. Factors controlling histamine production in Swiss cheese inoculated with *Lactobacillus buchneri*. Journal of Dairy Science 73, 3050-3058.

Taibi, A., Dabour, N., Lamoureux, M., Roy, D., LaPointe, G., 2011. Comparative transcriptome analysis of *Lactococcus lactis* subsp. *cremoris* strains under conditions simulating Cheddar cheese manufacture. International Journal of Food Microbiology 146, 263-275.

Taormina, P.J., 2010. Implications of salt and sodium reduction on microbial food safety. Critical Reviews in Food Science and Nutrition 50, 209-227.

Troller, J.A., Stinson, J.V., 1981. Moisture Requirements for Growth and Metabolite Production by Lactic Acid Bacteria. Applied and Environmental Microbiology 42, 682-687.

Uguen, P., Hamelin, J., Le Pennec, J.-P., Blanco, C., 1999. Influence of Osmolarity and the Presence of an Osmoprotectant on *Lactococcus lactis* Growth and Bacteriocin Production. Applied and Environmental Microbiology 65, 291-293.

7 Figure legends

Figure 1. Effect of NaCl on putrescine accumulation in the experimental Cabrales-like cheeses. Cheeses were made using seven different concentrations of NaCl ranging from 0% to 6%. They were then ripened for 4 months, and sampling performed on days 7, 15, 30, 60, 90 and 120. The data represent one representative experiment of triplicates.

Figure 2. Effect of NaCl on *L. lactis* CECT 8666 putrescine production (A), growth (B) and pH (C). Cells were grown in GalM17 with 20 mM agmatine and either 0%, 1%, 2%, 3% or 4% NaCl.

Figure 3. Effect of NaCl on *L. lactis* CECT 8666 growth (A) and putrescine production (B) when cells were grown in a bioreactor at pH 6 in GalM17 with 1 mM agmatine and 0%, 1%, 2% or 3% NaCl.

Figure 4. Influence of NaCl on the expression of *aguR* (A) and *aguBDAC* (B) as determined by RT-qPCR. *L. lactis* CETC 8666 cells were grown in a bioreactor at pH 6 in GalM17 with 1 mM agmatine and 0%, 1%, 2% or 3% NaCl. Total RNA was extracted from samples collected at the end of the exponential phase and the cDNA synthesised. The expression was calculated relative to the transcript level detected in samples grown with 3% NaCl. Data were normalized to the total RNA content using *tufA* as a reference gene. The data represent the mean of three different RNA extractions; vertical bars represent standard deviation.

8 Tables

Table 1. Microorganisms and primers used in this work.

Strain/Primers	Relevant Features/Sequence (5' to 3')	Reference or source
Strains		
<i>Penicillium roqueforti</i> 1AM8	Proteolytic mould from Cabrales cheese	(Florez et al., 2007)
<i>L. lactis</i> subsp. <i>cremoris</i> CECT 8666	Putrescine producer, AGDI cluster	(Fernandez et al., 2011), CECT ^a
<i>L. lactis</i> subsp. <i>cremoris</i> SK11	Non putrescine-producing starter	(Ladero et al., 2011)
<i>L. lactis</i> subsp. <i>cremoris</i> 1AA23	Non putrescine-producing starter	(Ladero et al., 2011)
<i>L. lactis</i> subsp. <i>cremoris</i> 3AA11	Non putrescine-producing starter	(Ladero et al., 2011)
<i>L. lactis</i> subsp. <i>cremoris</i> LC44	Non putrescine-producing starter	(Ladero et al., 2011)
Primers		
qAguR-F	CTATCGACAGGTTAAGCAAAGCAGTT	(Linares et al., 2013)
qAguR-R	TCCAAAGATGATGGCCATTATGC	(Linares et al., 2013)
qPTC-F	ACTTGGTGGACATGAAACAATAGAAGAT	(Linares et al., 2013)
qPTC-R	GTCAACACGTGCCATTATGATATCG	(Linares et al., 2013)
qtufF	TCTTCATCATCAACAAGGTCTGCTT	(Linares et al., 2013)
qtufR	GAACACATCTTGCTTTCACGTCAA	(Linares et al., 2013)
rpoA-F	CACGGGCAGGTTCAACTTG	(Taibi et al., 2011)
rpoA-R	TTCCGGCTGACGAAAATAAAG	(Taibi et al., 2011)

^aCECT: Colección Española de Cultivos Tipo

Figure 1

Figure 2

Figure 3

Figure 4