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

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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 (OD$_{600}$) 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 ($10^5$ cfu/ml each), and the putrescine producing *L. lactis* CECT 8666 ($10^6$ cfu/ml) were directly inoculated into sterilized bottles containing 200 ml of commercial pasteurized milk; the mould *P. roqueforti* ($10^3$ cfu/ml) and CaCl$_2$ (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 ∆∆Ct 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 ± 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 OD$_{600}$ 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

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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. *p<0.05, **p<0.001.
8 Tables

Table 1. Microorganisms and primers used in this work.

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<td><em>Penicillium roqueforti</em></td>
<td>1AM8 Proteolytic mould from Cabrales cheese</td>
<td>(Florez et al., 2007)</td>
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<td><em>L. lactis</em> subsp. <em>cremoris</em></td>
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*CECT: Colección Española de Cultivos Tipo*
Figure 1
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