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Transcriptome analysis shows activation of the arginine deiminase pathway in *Lactococcus lactis* as a response to ethanol stress

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ABSTRACT

This paper describes the molecular response of *Lactococcus lactis* NZ9700 to ethanol. This strain is a well-known nisin producer and a lactic acid bacteria (LAB) model strain. Global transcriptome profiling using DNA microarrays demonstrated a bacterial adaptive response to the presence of 2% ethanol in the culture broth and differential expression of 67 genes. The highest up-regulation was detected for those genes involved in arginine degradation through the arginine deiminase (ADI) pathway (20–40 fold up-regulation). The metabolic responses to ethanol of wild type *L. lactis* strains were studied and compared to those of regulator-deletion mutants MGΔargR and MGΔahrC. The results showed that in the presence of 2% ethanol those strains with an active ADI pathway reached higher growth rates when arginine was available in the culture broth than in absence of arginine. In a chemically defined medium strains with an active ADI pathway consumed arginine and produced ornithine in the presence of 2% ethanol, hence corroborating that arginine catabolism is involved in the bacterial response to ethanol. This is the first study of the *L. lactis* response to ethanol stress to demonstrate the relevance of arginine catabolism for bacterial adaptation and survival in an ethanol containing medium.

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1. Introduction

Lactic acid bacteria (LAB) play an essential role in the process of fermentation of numerous foods and beverages (Bourdichon et al., 2012) giving rise to dairy products, meat and cereal-based foods (Kabak and Dobson, 2011), fermented vegetables (Hurtado et al., 2012; Settanni and Corsetti, 2008) and wine (Matthews et al., 2004; Mills et al., 2005). *Lactococcus lactis* has been associated with food production and preservation for centuries and it is by far the best studied of the food-related LAB, which is largely due to its major industrial importance as a starter in the manufacture of cheese. Its main activity during milk fermentation is the conversion of lactose to lactic acid, which results in the lowering of the pH in the product. Moreover, the capacity for lactate and bacteriocin production of *L. lactis* is beneficial for food preservation. During these food- and beverage related industrial processes, LAB can be exposed to a number of environmental stresses, among which low and high temperatures, oxidative stress, high osmotic pressure, acidity, nutrient starvation and the presence of

ethanol are included. Growth performance and robustness to withstand environmental stresses are key properties for good starters. Bacterial mechanisms of stress resistance are based on bacterial adaptive responses and cross protection to those external factors. Advances in the genome, transcriptome and proteome research of *L. lactis* have turned this economically important LAB also into a widely used Gram-positive model organism (Pinto et al., 2011). *L. lactis* stress responses have been studied over the last years (Papadimitriou et al., 2016) and reports can be found on the response of *L. lactis* to osmotic stress (Sanders et al., 1998; Zhang et al., 2010), oxidative stress (Larsen et al., 2016; Miyoshi et al., 2003; Sheng et al., 2016), to both oxidative and acidic conditions (Cretenet et al., 2011), to acid stress (Budin-Verneuil et al., 2007; Carvalho et al., 2011; Carvalho et al., 2013; Hartke et al., 1996; Rallu et al., 1996; Sanders et al., 1995; Zhang et al., 2007), to heat- (Kim and Batt, 1993) and cold- (Panoff et al., 1994; Wouters et al., 2001) shocks, to starvation (Dressaire et al., 2011; Price et al., 2012) and to the presence of antibiotics (Dorrian et al., 2011). Cross-protective responses and interactive pathways have been demonstrated in a number of such responses of *L. lactis* to oxidative stress (Dijkstra et al., 2014; Duwat et al., 2000), osmotic, acid and thermal stress (Abdullah-al-Mahin et al.,

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2010; Van de Guchte et al., 2002; Zhang et al., 2014). Cross-protection induced by the expression of an adaptive response to one stress agent can be advantageous for bacterial tolerance to subsequent stress conditions; it increases the fitness of a bacterial culture to harsh conditions and will allow an optimal performance of a fermentative process carried out by this culture. Ethanol is a well-known antimicrobial agent, and tolerance to ethanol may be considered an indicator of bacterial robustness and might become a criterion for starter selection.

Arginine, a non-essential amino acid in *L. lactis*, can be synthesized de novo from glutamate in eight enzymatic steps, and is completely degraded into ornithine, ammonium and carbon dioxide via the arginine deiminase pathway (ADI pathway), which takes place in three enzymatic steps catalysed by the enzymes arginine deiminase (ArcA), ornithine carbamoyltransferase (ArcB) and carbamate kinase (ArcC). Arginine metabolism in *L. lactis* has been shown to be regulated by two transcriptional regulators named ArgR and AhrC (Larsen et al., 2004); both transcriptional regulators are required for repression of arginine biosynthesis in presence of the amino acid, and AhrC is an anti-repressor required to activate the ADI pathway of arginine degradation (Larsen et al., 2005).

This study aimed to identify the global adaptive response of *L. lactis* during growth in the presence of ethanol, which is a notorious stress factor for bacterial growth. Additionally, the bacteriocin nisin produced by some *L. lactis* strains had been previously reported to exert an inhibitory effect upon LAB strains isolated from wines and responsible for wine spoilage (Rojo-Bezares et al., 2007). The putative usage of a nisin-producer for wine preservation was an additional issue of interest for our study. Under these oenological conditions, ethanol exposure of wine LAB strains is a continuous and concentration-increasing exposure. We chose the model strain *L. lactis* subsp. *cremoris* NZ9700, which is a well-known nisin producer, its full genome had been sequenced and had been extensively studied (de Ruyter et al., 1996; Mu et al., 2015), nevertheless, no reports can be found on its response to ethanol. In this work we studied the molecular response of *L. lactis* NZ9700 to 2% ethanol exposure by whole-genome transcription profiling. To confirm and extend the obtained results, we then studied the arginine metabolism of the plasmid-free model strain *L. lactis* subsp. *cremoris* MG1363 and its single deletion mutants MGΔargR and MGΔahrC, whose ADI pathways of arginine degradation are either expressed or repressed.

2. Materials and methods

2.1. Bacterial strains and media

L. lactis strains used in this study are listed in Table 1. *L. lactis* was grown at 30 °C in M17 broth (Terzaghi and Sandine, 1975) with 0.5% glucose as the carbon source (GM17). A chemically defined medium (CDM) was prepared as described by Larsen et al. (2004); CDM buffer containing 15 free amino acids (CDM15) was prepared as previously described (Larsen et al., 2004). Arginine (Merck-VWR, Llinars del Vallès, Spain) stock solution was made in distilled water; pH was set to 7.0 with HCl. Growth and cell density were determined by measurement of the optical density at 600 nm (OD₆₀₀) of the culture using a spectrophotometer (Ultraspec 2000, Pharmacia Biotech, Cambridge, UK).

Table 1
Bacterial strains.

Strains	Descriptions	Characteristics	Source of reference
NZ9700	<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	Nisin producer	Kuipers et al., 1993
MG1363	<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	Plasmid free strain	Gasson, 1983
MGΔargR	Deletion mutant <i>argR</i> of <i>L. lactis</i> subsp. <i>cremoris</i> MG1363	Active ADI pathway	Larsen et al., 2004
MGΔahrC	Deletion mutant <i>ahrC</i> of <i>L. lactis</i> subsp. <i>cremoris</i> MG1363	ADI pathway not expressed	Larsen et al., 2004

2.2. Transcriptome analysis using *L. lactis* DNA microarrays

RNA was isolated from cells grown to mid-exponential (OD₆₀₀ = 0.4) and stationary phase (OD₆₀₀ = 1) in GM17 with 0% and 2% ethanol. Cells were harvested by centrifugation at 12,000 × g for 2 min at 4 °C. Supernatants were discarded and cell pellets were immediately frozen in liquid nitrogen and stored at −80 °C. Pellets were resuspended in 400 μl of T₁₀E₁ buffer (10 mM Tris-HCl pH 8.0, 1 mM Na₂-EDTA), and 50 μl 10% SDS (w/v), 500 μl phenol/chloroform: isoamyl alcohol (24/24:1) (Sigma-Aldrich Chemie, Zwijndrecht, Netherlands), 500 mg glass beads (50–105 μm of diameter, Fischer Scientific BV, Den Bosch, the Netherlands), and 175 μl Macaloid suspension (Bentone MA, Elementis Specialities Inc., Hightstown, NJ) was added. The Macaloid suspension was made as follows: 2 g macaloid was boiled for 5 min in 100 ml T₁₀E₁, cooled to room temperature, sonicated by bursts until a gel was formed, centrifuged and resuspended in 50 ml T₁₀E₁. Cells were disrupted by shaking twice for 45 s in a Biospec Mini-bead Beater-8 (Biospec, OK, USA). The cell lysate was cleared by centrifugation and 500 μl supernatant was extracted with 500 μl chloroform:isoamyl alcohol (24:1). Total RNA was isolated from the water phase using the High Pure RNA Isolation Kit (Roche Applied Science, Mannheim, Germany) according the manufacturer's instructions. All reagents used for RNA work were treated with diethylpyrrocarbonate (DEPC) (Sigma-Aldrich, St. Louis, MO). RNA quantity was determined spectrophotometrically and RNA quality was verified on an Agilent Bioanalyzer 2100 using RNA 6000 LabChips (Agilent Technologies Netherlands BV, Amstelveen, the Netherlands). 20 μg total RNA was used for the synthesis of aminoallyl-dUTP-labelled copy DNA (cDNA) using SuperScript III Reverse Transcriptase (Life Technologies, Carlsbad, California, US). Aminoallyl-dUTPs-containing cDNA was subsequently labelled using CyDye-NHS-esters Cy3 and Cy5 (Amersham Biosciences Europe GmbH). Labelled DNA was purified using NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, GmbH & Co. KG, Germany). Hybridisation (16 h at 45 °C) of Cy-labelled cDNA was performed in Ambion Slidehyb 1 hybridisation buffer (Ambion Europe Ltd., Huntington, UK) on full-genome *L. lactis* NZ9000 DNA Microarray slides (Kuipers et al., 2002) supplemented with probes for the nisin biosynthesis-cluster genes. Slides were scanned using a GenePix Autoloader 4200AL scanner (Molecular Devices Corporation, Sunnyvale, CA).

2.3. DNA microarray data analysis

Slide images were analyzed using ArrayPro 4.5 (Media Cybernetics Inc., Silver Spring, MD) and the data processed and normalized using MicroPrep software (van Hijum et al., 2003) and following standard routines provided by GENOME2D software available at <http://genome2d.molgenrug.nl/index.php/analysis-pipeline>. For each DNA microarray experiment, at least three independent biological replicates and two technical replicates (dye-swaps) were performed to discard possible differences due to variations in Cy3/Cy5 hybridisation. Expression ratios were calculated and a gene was considered differentially expressed when a *p* value of at least < 0.05 was obtained and the expression fold-change was at least > 1.8.

Fig. 1. Growth curves and nisin production of *L. lactis* NZ9700 in GM17 with 0%, 2%, 4% and 6% (vol/vol) ethanol.

2.4. Nisin production

The production of nisin by *L. lactis* NZ9700 grown in presence of ethanol was determined by calculating the minimal inhibitory concentration (MIC) in the microtiter dilution assay (Rojo-Bezares et al., 2006) of cell free supernatants. For these experiments fresh inocula of *L. lactis* NZ9700 (10^5 cells/ml) were incubated for up to 30 h at 25 °C in 100 ml of culture broth containing 0%, 2%, 4%, 6%, 8% and 10% (vol/vol) ethanol (Panreac, S.A., Barcelona, Spain) in GM17 with its concentration adjusted to account for the addition of ethanol. Bacterial growth was monitored by OD_{600} . Aliquots were taken at different incubation times and centrifuged at $12,000 \times g$ for 5 min at 4 °C. Cell free supernatants were boiled in a water bath for 10 min. Samples were tested for their antimicrobial activity by the microtiter dilution method, using serial double dilutions against the indicator strain *Pediococcus pentosaceus* FBB63 and incubation in microtiter plates at 30 °C for 48 h following the method described by Rojo-Bezares et al. (2006) to determine the corresponding MIC values. Positive and negative controls were included in all the assays.

2.5. Ethanol stress

To confirm and further investigate the results of ethanol resistance revealed by the transcriptome analysis, the wild type NZ9700 and MG1363 *L. lactis* subsp. *cremoris* strains and the mutant strains MGΔargR and MGΔahrC were grown at 25 °C with the following concentrations of ethanol (Panreac, S.A., Barcelona, Spain) in the culture broth: 2%, 4%, 6% and 10% (vol/vol). Growth and cell density were determined by measurement of the OD_{600} of the culture. All experiments were carried out in triplicates. Culture conditions in the CDM for strain growth were: either absence or presence of arginine (Merck, Darmstadt, Germany) (2.5 mg/ml) and either absence or presence of ethanol (Panreac, Barcelona, Spain) (2% vol/vol) in the culture broth. Strains were incubated at 25 °C without agitation. We determined growth curves by measuring OD_{600} . Samples were taken at the initial moment of inoculation and after 10 h incubation, which corresponded to the stationary growth phase. These samples were stored at −80 °C until HPLC processing.

2.6. HPLC analysis

Cell free supernatants obtained at stationary phase ($OD_{600} = 1$) of growth in CDM (10 h incubation) containing either 0.25 g/l arginine or no arginine, with either 0% or 2% ethanol as described above, were analyzed by HPLC. Samples were centrifuged at $12,000 \times g$ for 5 min at 4 °C and were boiled in a water bath for 10 min. Analyses were performed on a modular Agilent 1200 Series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with one G1311A quaternary pump, an on-line G1322A degasser, a G1316A column oven, a G2913A automatic injector and a G1315B photodiodearray detector (DAD) controlled by the Chemstation Agilent software. Chromatographic separation was performed in an ACE HPLC column (5 C18-HL) particle size 5 mm (250 mm, 4.6 mm) following the method described by Gómez-Alonso et al. (2007) with the derivatisation reagent diethyl ethoxymethylenemalonate (Sigma-Aldrich Chemie, Steinheim, Germany). Amino acids were identified on the basis of the aminoenone derivative retention times of the corresponding standards (Merck, Darmstadt, Germany) and quantified using the internal standard method. Statistical analysis of data was performed using SPSS 12 statistical software (SPSS Inc., Chicago, IL). Analysis of variance (ANOVA) was applied for the HPLC data, which showed normal distribution and homogeneous variances. IBM-SPSS Statistics 19.0 software for Windows (IBM-SPSS Inc., Chicago, IL, USA) was used for data processing.

3. Results

3.1. *L. lactis* NZ9700 growth and nisin production in the presence of ethanol

To determine the effect of ethanol on cell growth and nisin production of our model strain *L. lactis* NZ9700, it was incubated in GM17 containing from 0% to 10% (vol/vol) ethanol as indicated in Methods section, and the results of cell growth and nisin activity are shown in Fig. 1. *L. lactis* NZ9700 was not able to grow in the presence of 8% and 10% ethanol. Remarkably, it was able to grow in the presence of 2% ethanol with a growth rate of about half of that of control samples without ethanol in the culture broth. The strain still produced nisin, but only 25% when compared to control conditions in the absence of

Fig. 2. The ADI pathway genes up-regulated in presence of 2% ethanol.

ethanol, as shown in Fig. 1. Culture broth pH values were lower (pH 4.88) for cultures in the absence of ethanol after 24 h incubation, than for cultures containing 2%–6% ethanol (pH 5.08–5.15), which correlated with the higher cell density of ethanol-free cultures. Given these results, we chose 2% ethanol in the culture broth for studying the *L. lactis* NZ9700 transcriptome response with DNA microarrays.

3.2. Transcriptome profile of *L. lactis* NZ9700 grown in the presence of 2% ethanol

Results of the transcriptome analysis of *L. lactis* NZ9700 grown in GM17 containing 2% ethanol are shown in the Supplementary material. Comparison of transcriptomes of *L. lactis* NZ9700 grown in the presence of 2% ethanol with those of *L. lactis* NZ9700 grown under reference ethanol-free conditions showed differential expression of 67 genes in the mid-exponential growth phase. This response to ethanol involved up-regulation of the expression of the following genes:

- genes of the deiminase pathway of arginine degradation (ADI pathway) (up to 42.7 fold activation) (Fig. 2)
- genes of the alcohol dehydrogenase pathway (up to 4.9 fold activation)
- genes of ABC-type multidrug resistance transporters (up to 4.3 fold activation)
- genes involved in sugar transport and metabolism (up to 4.0 fold activation)

- genes encoding stress proteins: *cspC* and *cspD* (up to 2.0 fold activation)
- gene involved in synthesis of fatty acids (1.9 fold activation).

The up-regulation of expression of the majority of genes was maintained till the stationary phase of bacterial growth. Additionally, in the stationary phase up-regulation of cell wall protein-encoding genes was detected and the expression of two cold shock protein genes increased even more (Table S1 of the Supplementary material).

In contrast, down-regulation of the expression of genes during the mid-exponential growth phase (Table S2 of the Supplementary material) included the following genes:

- genes involved in pyrimidine and purine biosynthesis (down 16.4-fold),
- iron transport genes (down 4.2-fold)
- genes of the nisin operon: *nisB*, *nisC*, *nisE*, *nisF*, *nisG*, *nisI*, *nisK*, *nisP* and *nisT* (down 4.3-fold).

According to these results, the strongest response to ethanol was the activation of expression of genes encoding proteins of the ADI pathway for arginine degradation shown in Fig. 2. In the section below the results of the metabolic study carried out with wild type NZ9700 and MG1363 *L. lactis* strains and the deletion mutants MGΔ*argR* and MGΔ*ahrC* are shown. Mutant MGΔ*argR* is the deletion mutant of strain MG1363 that lacks the transcriptional regulator ArgR, which represses the expression of the ADI genes, and in combination with arginine and the transcriptional regulator AhrC completely represses arginine biosynthetic pathways (Larsen et al., 2008). MGΔ*ahrC* is the deletion mutant of the transcriptional regulator AhrC, the anti-repressor that allows expression of the ADI pathway genes in presence of arginine (Larsen et al., 2004).

Fig. 3. Growth curves of *L. lactis* strains in CDM in presence of 0% and 2% ethanol, with (2.5 mg/ml) and without arginine in the culture broth.

Fig. 4. Arginine and ornithine concentrations in the bacterial culture broth:

3.3. Effect of ethanol on arginine metabolism

Fig. 3 shows the growth curves of strains *L. lactis* NZ9700, MG1363, MGΔargR, and MGΔahrC when grown in the chemically defined medium in the presence of 0% and 2% ethanol, w/o arginine (2.5 mg/ml) in the culture broth. Fig. 3A and B show that both wild type strains NZ9700 and MG1363 grew at slower rates when grown in 2% ethanol

in the absence of arginine when compared to conditions of presence of arginine in the culture broth, which indicates that arginine availability positively contributed to bacterial growth in the ethanol containing broth. In contrast, *L. lactis* mutant MGΔargR, expressing both fully active ADI pathway and arginine biosynthetic pathway, showed higher growth rate during growth in 2% ethanol in the absence of arginine (Fig. 3C) than the wild type. Finally, Fig. 3D shows the growth curves of *L. lactis* mutant MGΔahrC, which does not express its ADI pathway. This mutant, in the presence of ethanol, showed reduced growth rates either with arginine (2.5 mg/ml) or without arginine, as it was not able to degrade this amino acid for obtaining energy; its growth rates in the presence of 2% ethanol were half of the values reached in the absence of ethanol (Fig. 3D).

Fig. 4 shows arginine and ornithine concentrations in the bacterial culture supernatants at stationary growth phase after 10 h incubation of the *L. lactis* strains in the presence of either 0% or 2% ethanol, and either 0 mg/ml or 2.5 mg/ml arginine in the chemically defined culture broth. It is shown that ornithine was produced (> 20 mg/l in the culture supernatant) only when arginine was available in the culture broth and only in those samples of strains expressing an active ADI pathway (wild type MG1363 and mutant MGΔargR, Fig. 4A and B respectively), and the highest production of ornithine (122.1 mg/l) was shown for mutant MGΔargR, with an active ADI pathway and no repression of its arginine biosynthetic pathways, growing in the presence of 2% ethanol and arginine (Fig. 4B). Thus, the deletion mutant MGΔahrC (Fig. 4C), not expressing the ADI pathway for arginine degradation, was unable to generate ornithine (< 20 mg/l threshold limit). Moreover, it is shown that more arginine was consumed in those samples grown in the presence of 2% ethanol than in the corresponding samples grown in absence of ethanol.

4. Discussion

We have studied the molecular responses of *L. lactis* subsp. *cremoris* to the stress due to the presence of ethanol in the bacterial growth medium. Ethanol is known to be a potent antimicrobial agent and to act at the lipid-water interface, altering the stability and integrity of bacterial cell membranes (Weber and de Bont, 1996). The effect of ethanol on microorganisms related to industrial alcoholic fermentations, such as *Saccharomyces cerevisiae*, *Oenococcus oeni* or *Clostridium thermocellum* (Cafaro et al., 2014; Ma and Liu, 2010; Voigt et al., 2013; Yang et al., 2012), and on some model microorganisms (Chong et al., 2013; Seydlová et al., 2012) has been reported before. *L. lactis* subsp. *cremoris* NZ9700 is a strain obtained from a fermented dairy starter and a well-known nisin producer, which is distributed in laboratories and collections around the world and has become a prototype for genetic and physiological studies in LAB. Nevertheless, this is to our knowledge the first transcriptome and metabolic study of the response of *L. lactis* to ethanol, which is an antimicrobial agent that may well be considered a good indicator of bacterial robustness.

Our transcriptome analyses identified down-regulation of genes related to purine and pyrimidine biosynthesis, which implies the inhibition of bacterial growth and is in accordance with the observed reduced growth of *L. lactis* in presence of ethanol (Fig. 1). These results were as expected for a potent bactericidal agent such as ethanol. Down-regulation was also observed for genes related to nisin production, which is encoded by the nisin operon *nisABTCIP* that contains the structural gene *nisA* and the necessary biosynthetic and immunity genes for its expression (Kuipers et al., 1993), and two contiguous operons, i.e. *nisRK*, encoding regulation by a two-component regulatory system, and *nisFEG*, also involved in immunity. Our results showed that with the exception of the structural gene *nisA* and the regulatory *nisR*, all the other nisin-related genes were down regulated at the mid-exponential growth phase in presence of ethanol. This down-regulation at the transcriptional level was corroborated by the results of Fig. 1 that shows a progressive decrease of nisin production as ethanol concentration in

Fig. 5. Scheme of the metabolic pathways of arginine in *L. lactis*.

the culture broth increased. It should be taken into account that nisin production implies a fitness cost, and its inhibition at transcriptional level could favor cell survival and adaption to harsh conditions. In contrast to our results, some *L. lactis* engineered strains with enhanced nisin production showed increased tolerance to low pHs during fermentation (Zhang et al., 2016).

Regarding the activation of genes related to cold shock stress (*cspA*, *cspC* and *cspD*), the proteins encoded by these genes act as chaperons to protect peptide synthesis from cold and a variety of other stress factors (Wouters et al., 2001; Yu et al., 2009), and similarly *Lactobacillus plantarum* (Van Bokhorst-van de Veen et al., 2011) was also reported to down regulate the transcriptional factor *ctsR* in the presence of 8% ethanol and thus to activate expression of genes coding for chaperon proteins. *Oenococcus oeni*, a LAB of relevance in wine making, was also reported to utilize the *ctsR* gene product to regulate stress responses through the major molecular chaperones, which include Hsp, Csp (Grandvalet et al., 2005) and the small heat shock protein Lo18 (Maitre et al., 2014), which seems to improve tolerance not only to heat, but also to acid conditions when expressed in *L. lactis* (Weidmann et al., 2016).

Our transcriptome analyses also revealed that *L. lactis* subsp. *cremoris* requires energy in the presence of ethanol and activated pathways for sugar transport and metabolism, as well as ABC-type multidrug pumps and alcohol dehydrogenase activities to expel and degrade the toxic agent ethanol. Similarly, *O. oeni* (Bourdineaud et al., 2004) was shown to over-express the ABC-transporter gene *omrA* as response to and protection from ethanol, and *L. plantarum* (Van Bokhorst-van de Veen et al., 2011) was reported to transcriptionally activate its citrate metabolism (*citCDEF* operon) and utilize citrate from the medium to obtain energy to overcome ethanol stress. Similarly to our results, a study by NMR of *L. lactis* subsp. *cremoris* MG1363 (Carvalho et al., 2013) also reported activation of glucose uptake by a cellobiose-specific PTS system and genes coding for glycolytic enzymes as a response to acid stress. Up-regulation of transcription of ABC transporter permeases and cellobiose-transport PTS genes has been associated with strain robustness under heat and oxidative stress (Dijkstra et al., 2014) and in this regard, our results show the same type of response, suggesting that ethanol tolerance could also be considered a robustness characteristic for *L. lactis* strains.

Our results showed that the transcriptional response of *L. lactis* subsp. *cremoris* to ethanol also includes activation of genes related to fatty acid synthesis at the stationary growth phase. The *L. plantarum* transcriptional response to ethanol (Van Bokhorst-van de Veen et al., 2011) was also reported to differentially express genes encoding cell-surface lipoproteins and teichoic acid biosynthesis enzymes. These results imply changes in the bacteria cell wall and membrane, which are obviously needed to overcome the effects of ethanol at the lipid-water interface.

The core response to ethanol of *L. lactis* subsp. *cremoris* was transcriptional up-regulation (up to 42.7 fold) of the *arcABC1C2TD1D2* operon (Fig. 2), encoding proteins of the ADI pathway for arginine degradation (Fig. 5). The final products of arginine degradation through this pathway are ornithine, ammonia and CO₂. This pathway renders one molecule of ATP (Fig. 5) and consumes two protons, which contributes to the internal pH homeostasis and opposes external acid stress. Activation of this pathway has been shown to be a major protection mechanism of LAB species against acidic environments such as the oral cavity (Curran et al., 1995), lactic fermentation processes (Pessione et al., 2010), wine (Arena and Manca de Nadra, 2005), and salt- and temperature stresses (Vrancken et al., 2009). Our results of transcriptional activation of *L. lactis* ADI pathway for arginine consumption in presence of ethanol, corroborated by the metabolic study with the wild type and mutant strains, indicated that the ADI pathway provides useful energy for cell survival and tolerance to ethanol. Although the pleiotropic transcriptional regulator CodY has been described as the overall regulator of nitrogen metabolism in *L. lactis* (Guédon et al., 2005), in the presence of glucose the *arc* operon is actually regulated by the carbon catabolite control protein (CcpA), which represses *arc* expression and constitutes a link between regulation of carbon metabolism and regulation of nitrogen metabolism in *L. lactis* MG1363 (Zomer et al., 2007). As glucose is being consumed, both transcriptional factors ArgR and AhrC take the lead and regulate *arc* expression. When the *L. lactis* strains of our study were grown in chemically defined culture broth, the presence of glucose initially could have triggered repression of the ADI pathway through CcpA. Our results show that when glucose is partially consumed (middle of exponential growth phase and stationary phase) and in the presence of arginine in the culture broth, the transcriptional regulators ArgR and AhrC exert their action and the ADI

pathway of arginine degradation contributes to bacterial growth in the presence of ethanol in those strains expressing the *arc* operon. Fig. 3A and B show that at the end of the exponential growth phase (8–10 h culture) and in the presence of 2% ethanol, samples with active ADI pathways and with available arginine showed the largest differences in cell population when compared with control cultures without arginine. This mechanism of resistance through the ADI pathway has been described at the proteomic level for *L. lactis* subsp. *cremoris* against oxidative stress (H_2O_2), to which this species is subjected during cheese manufacturing (Rochat et al., 2012). Other studies related to the production of cheese flavour also reported arginine degradation as the source of energy for *L. lactis* subsp. *lactis* to survive under starvation stress when cells were deprived of sugar (Brandsma et al., 2012), and more recently it has been suggested that the capability of *L. lactis* to persist in a viable not culturable state could be due to the fact that *L. lactis* cells could switch from glycolysis to nitrogen catabolism for obtaining the required energy to survive (Ruggirello et al., 2016).

In the wine industry LAB play an important role in the secondary fermentation that takes place after alcoholic fermentation, named the malolactic fermentation (MLF) that is a requisite for the sensory properties of premium red wines. Wine heterofermentative LAB are able to degrade arginine, and do so through the ADI pathway (Liu and Pilone, 1998). Although many LAB species are present in the initial grape must, *O. oeni* becomes the dominant species and finally triggers and conducts MLF, and this is mainly due to the excellent adaptation of this species to the aggressive ecological medium that wine is for bacterial growth. *O. oeni* possesses the *arc* genes (Araque et al., 2009) and therefore, arginine would stimulate *O. oeni* growth and resistance in wine, which was reported by Bourdineaud (2006). Nevertheless, some other studies reported that arginine does not stimulate growth of *O. oeni* in wine under standard MLF conditions (Terrade and Mira de Orduña, 2009). Our results support that arginine and glucose enhance bacterial growth and resistance to ethanol by utilizing the sugar and the ADI pathway for arginine degradation to obtain energy and overcome the stress due to the presence of ethanol in their growth medium.

Summarizing, our study provides deeper insight into the stress tolerance mechanisms against ethanol that *L. lactis*, and other ADI pathway-possessing LAB strains of industrial relevance utilize when they become exposed to ethanol.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ijfoodmicro.2017.05.017>.

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