Tyramine biosynthesis is transcriptionally induced at low pH and improves the fitness of *Enterococcus faecalis* in acidic environments

Marta Perez[a], Marina Calles-Enríquez[a], Ingolf Nes[b], Maria Cruz Martin[a], Maria Fernandez[a], Victor Ladero[a#], Miguel A. Alvarez[a]

[a]Instituto de Productos Lácteos de Asturias, IPLA-CSIC, Villaviciosa, Spain.
[b]Norwegian University of Life Sciences UMB, Ås, Norway.

Running title: Tyramine production enhances survival of *E. faecalis* at low pH

[#]Corresponding author: Victor Ladero

Mailing address: Instituto de Productos Lácteos de Asturias (IPLA-CSIC), Paseo Rio Linares s/n, 33300 Villaviciosa, Spain.

Phone: +34 985 89 21 31

Fax: +34 985 89 22 32

E-mail: ladero@ipla.csic.es

ABSTRACT

Enterococcus faecalis is a commensal bacterium of the human gut that requires the ability to pass through the stomach and therefore cope with low pH. *E. faecalis* has also been identified as one of the major tyramine producers in fermented food products, where they also encounter acidic environments. In the present work, we have constructed a non-tyramine producing mutant to study the role of the tyramine biosynthetic pathway, which converts tyrosine to tyramine via amino acid decarboxylation. Wild type strain showed a higher survival in a system that mimics gastrointestinal stress, indicating that the tyramine biosynthetic pathway has a role in acid resistance. Transcriptional analyses of the *E. faecalis* V583 tyrosine decarboxylase cluster showed that an acidic pH, together with substrate availability, induce its expression, and therefore the production of tyramine. The protective role of the tyramine pathway under acidic conditions appears to be exerted through the maintenance of the cytosolic pH. Tyramine production should be considered important in the adaptability of *E. faecalis* to acidic environments, such as fermented dairy foods, and to survive passage through the human gastrointestinal tract.

.

Keywords: *Enterococcus faecalis*, Tyramine, *tdc* cluster expression, gastrointestinal stress, internal pH.

Introduction

The ability of *Enterococcus faecalis* to tolerate wide ranges of pH, temperature and osmotic conditions allows it to colonize environments as different as water, soil, and foodstuffs especially fermented food products where it can be present in raw materials or contaminate them (Agudelo Higuita and Huycke 2014; Giraffa 2003; Lebreton *et al*. 2014). It is also a commensal of both human and animal gastrointestinal tracts (GIT). Some enterococcal strains, however, can also act as opportunistic pathogens, causing nosocomial infections such as endocarditis and bacteremia, usually following the colonization of the GIT (Agudelo Higuita and Huycke 2014; Paulsen *et al*. 2003; Ubeda *et al*. 2010). In fact, hospital-adapted, multi-antibiotic resistant enterococci have spread dramatically in recent decades; vancomycin-resistant (VRE) *E. faecalis* strains in particular can colonize healthy people and farm animals (Bonten *et al.* 2001), who along with certain foodstuffs (dairy and meat products) may act as VRE reservoirs (Giraffa 2003; Mathur and Singh 2005).

Little is known about the mechanisms used by VRE enterococci to colonize the human gut (Lebreton *et al*. 2014; Ubeda *et al.* 2010), although the intrinsic robustness of *E. faecalis* to different stresses may contribute towards its adaptability (Solheim *et al*. 2014). In lactic acid bacteria (LAB) and pathogens such as *Listeria monocytogenes* and *Escherichia coli*, amino acid decarboxylation is thought to provide an acid resistance system that helps them face the challenges of colonizing GIT environments (Castanie-Cornet and Foster 2001; Gahan and Hill 2014; Pessione 2012). Strains of enterococci of clinical, human and food origin can all decarboxylate the amino acid tyrosine to produce tyramine; indeed, the biosynthesis of tyramine is a general species trait of *E. faecalis* (Ladero *et al*. 2012).

Tyramine is a biogenic amine (BA) that can accumulate in foodstuffs via the action of microbial decarboxylases (Linares *et al.* 2011). The consumption of large amounts can cause toxicological effects including migraines and hypertension, and sometimes problems as serious as cerebral haemorrhages (EFSA 2011; Ladero *et al*. 2010a; Pessione 2012). These symptoms are together known as the "*cheese effect*" (Ladero *et al*. 2010a) since tyramine is one of the most commonly found and abundant BA in dairy products (Fernandez *et al*. 2007a; Linares *et al*. 2012a; Linares *et al*. 2011). Enterococci

are among the microorganisms responsible for tyramine biosynthesis in cheeses, constituting a serious food safety concern (Ladero *et al.* 2010b; Linares *et al.* 2012a). Tyramine is formed from tyrosine by the action of the enzyme tyrosine decarboxylase (TdcA). Tyramine is further secreted from the cell in exchange for tyrosine by the antiporter TyrP. The proteins involved in the tyramine pathway are encoded in the *tdc* cluster, which has been described in *E. faecalis* JH2-2 (Connil *et al*. 2002), *E. durans* IPLA655 (Ladero *et al*. 2013) and *E. faecium* RM58 (Marcobal *et al*. 2006a) among others. The *tdc* cluster has also been annotated in the genome sequence of other LAB (Linares *et al*. 2011), as well as in that of the clinically important VRE strain *E. faecalis* V583 (Paulsen *et al.* 2003). All the sequences share the same genetic organization, which comprises four genes (Fig. 1A): *tyrS*, an aminoacyl-tRNA synthetase-like gene; *tdcA*, which encodes the tyrosine decarboxylase; *tyrP*, which codes for the tyrosine/tyramine exchanger; and *nhaC-2*, which encodes an Na^+/H^+ antiporter, the involvement of which in the biosynthesis of tyramine remains unknown (Linares *et al*. 2011; Lucas *et al*. 2003).

Tyramine production in foodborne *E. durans* and *E. faecium* strains has been related to tolerance to low pH. The coupled reactions of decarboxylation and tyrosine/tyramine exchange have been proposed as a mechanism for adapting to acidic environments, as well as an indirect way of obtaining metabolic energy via proton motive force generation (Fernandez *et al*. 2007b; Marcobal *et al*. 2006b; Pereira *et al*. 2009). The possible roles of tyramine production in GIT resistance, immunomodulation and the adhesion of pathogens to enterocytes have all been examined (Fernandez de Palencia et *al*. 2011; Lyte 2004; Pereira *et al*. 2009). However, little is known about the regulation and physiological role of the tyramine production pathway in *E. faecalis.*

In this work, a *tdc* knockout mutant was constructed in order to characterize the *tdc* cluster of the tyramine-producing strain *E. faecalis* V583. A transcriptional study under different environmental conditions was performed, and the physiological role of tyramine production under stress conditions, including those encountered in GIT passage, was examined. Tyramine production via tyrosine decarboxylation is here suggested to provide a cytosolic pH maintenance mechanism that helps cope with acid stress.

Materials and methods

Strains, media and growth conditions

Escherichia coli Gene-Hogs (Invitrogen, Paisley, UK) was used as an intermediate host for the pAS222 cloning vector (Jonsson *et al*. 2009) and derived plasmid (pAS222 TDC, this work). The strain was cultured at 37ºC with aeration in Luria-Bertani medium (Green and Sambrook 2012) supplemented with 100 mg mL^{-1} of ampicillin (USB Corporation, Cleveland, OH) when necessary.

The wild-type *E. faecalis* V583 strain (hereafter referred to as 'wt') was used as a model strain since its genomic sequence was the first to become available for an *E. faecalis* strain and it is deposited in the American Type Culture Collection under the accession number ATCC 700802. The wt and the derived mutant *E. faecalis* V583 Δ*tdc* (hereafter referred to as 'Δ*tdc*') were grown routinely in M17 medium (Oxoid, Hampshire, United Kingdom) supplemented with 5 g L^{-1} glucose (Merck, Darmstadt, Germany) (GM17) at 37ºC under aerobic conditions with an initial inoculum of 0.1%.

When indicated, 10 mM tyrosine (Sigma-Aldrich, St. Louis, MO) was added (GM17+T). The latter medium was used to study the factors that affect the growth of the wt and Δ*tdc* strains by reducing the sugar concentration to 1 g L^{-1} glucose and/or the pH to 5 (initially pH 6.8) as indicated. Tyrosine consumption, and tyramine production were checked after 12 h of growth

To test the effect of tyrosine concentration on gene expression, wt cells were grown in 50 mL of chemically defined medium (CDM) (Poolman and Konings 1988) supplemented with different tyrosine concentrations at 37ºC for 4 h. To measure gene expression and tyramine production under controlled pH conditions, the wt strain was cultivated in a Six-Fors bioreactor (Infors AG, Bottmingen, Switzerland) in GM17 supplemented with tyrosine at a non-limiting concentration (15 mM, GM17+T15) for 6 h. The reactor was maintained at 37°C, 50 rpm stirring and with zero air input. The pH was maintained by automatically adding 2 N NaOH or 5 N HCl as needed.

All data are the means for at least three cultures independently grown under each condition.

DNA isolation

Total DNA was extracted from 2 ml of an overnight culture using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich), following the manufacturer's instructions. Plasmid extraction was performed following standard procedures (Green and Sambrook 2012).

PCR amplification and sequencing

PCR amplifications were performed in 25 µl reaction volumes with 1 µl of DNA as a template (typically 200 ng), 400 nM of each primer, 200 µM of dNTP (GE Healthcare, Little Chalfont, UK), the reaction buffer, and 1 U of Taq polymerase (Phusion High-Fidelity DNA Polymerase, Thermo Scientific, Madrid, Spain). All primers (Table 1) were designed based on the *E. faecalis* V583 genome sequence (GenBank accession number: AE016830) and synthesized by Macrogen (Seoul, Korea). Amplifications were performed using a MyCycler apparatus (Bio-Rad, Hercules, CA) under the following conditions: 94ºC for 5 min, 35 cycles of 94ºC for 30 s, 55ºC for 45 s, 72ºC for 1 min, and a final extension step at 72ºC for 5 min. The amplifications were analyzed by agarose gel electrophoresis; purification, when needed, was performed using the GenElute PCR Clean-Up Kit (Sigma-Aldrich). Sequencing of the PCR fragments were performed at Macrogen.

Construction of the *E. faecalis tdc* knockout mutant

An *E. faecalis* V583 non-tyramine-producing mutant, i.e., with a *tdc* cluster deletion from *tyrS* (793 nt from its start codon) to *nhaC-2* (691 nt from its start codon), was achieved by double-crossover homologous recombination with the cloning vector pAS222 following a previously described protocol (Jonsson *et al*. 2009). Briefly, the flanking fragments of the *tdc* cluster were amplified by splicing by overlap extension PCR (Horton *et al.* 1989), and two PCR reactions performed with primers T1 F, T2 R, and T3 F, T4 R (Table 1). The amplicons were purified, and a mix used as a template for PCR amplification with the outer primers T1 F and T4 R. The inner primer carrying

regions of homology for the fusion step was T3 F (Table 1). The PCR product was cloned into the *Sna*BI (Fermentas, Vilnius, Lithuania) site of pAS222 to generate pAS222 TDC, which was propagated in *E. coli* Gene-Hogs cells. pAS222 TDC was transformed into electrocompetent *E. faecalis* V583 cells obtained following a previously described protocol (Holo and Nes 1989) using 4% glycine in the growth medium. *E. faecalis* V583 cells harboring pAS222 TDC were grown in GM17 under previously described conditions (Biswas *et al*. 1993) in order to select bacteria showing evidence of double-crossover events. The deletion of *tdc* was checked by PCR amplification and further sequencing at Macrogen, using card F and ef0637 R primers (Table 1). The absence of tyramine biosynthesis was checked in the supernatant of overnight cultures in GM17+T as described below. A positive deletion mutant (*E. faecalis* V583 Δ*tdc*) was confirmed by both methods and selected for further analysis.

RNA isolation

E. faecalis cells were grown in the required medium for each experiment, as previously indicated. Adequate culture volumes (adjusted to a cell density of approximately OD_{600} = 2) were harvested by centrifugation in a refrigerated benchtop microcentrifuge (Eppendorf, Hamburg, Germany) running at maximum speed. Total RNA was extracted using TRI reagent (Sigma-Aldrich) as previously described (Linares *et al*. 2009). To eliminate any DNA contamination, 2 μg of total RNA samples were treated with 2U of DNAse I (Fermentas) for 2 h. Control PCR to ensure that no contaminant DNA remains was performed using specific primers to amplify *recA*. The total RNA concentration was determined in an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT).

Reverse transcription PCR (RT-PCR)

Total cDNA was synthesized from 0.5 μg of RNA using the reverse transcription (RT) iScript™ cDNA Synthesis kit (Bio-Rad), and 1 μL used as a template for PCR reactions involving 400 nM of each primer (Table 1), 200 μM of dNTP, the reaction buffer, and 1 U of Taq polymerase (DreamTaq, Fermentas). Five pairs of primers (Table 1) were used to amplify regions spanning the gene junctions.

196

197

198 Gene expression quantification by RT-qPCR

199 Gene expression analysis was performed by reverse transcription–quantitative real-time
200 PCR (RT-qPCR) in a 7500 Fast real-time PCR System (Applied Biosystems, Carlsbad,
201 CA) using SYBR Green PCR Master Mix (Applied Biosystems). Four-fold dilutions of
202 the cDNA samples were used as a template (4 μl) with 700 nM of each primer and
203 SYBR Green PCR Master Mix in a 20 μL final volume. Amplifications were performed
204 with specific primers (Table 1) based on internal sequences of the *tyrS* and *tdcA* genes
205 designed using Primer Express software (Applied Biosystems). Specific primers for
206 recombinase A (*recA)* and elongation factor thermo-unstable (*tufA)* genes were used as
207 internal controls to normalize the RNA concentration. The linearity and amplification
208 efficiency of the reactions were tested for each primer pair using six 10-fold serial
209 dilutions of total *E. faecalis* V583 DNA. A positive control with total *E. faecalis* V583
210 DNA was included for each run, and the resulting melting curves for the samples
211 compared with that of this positive control. A negative control with all the reaction
212 components except cDNA was included. Amplifications were performed using the
213 default cycling settings suggested by Applied Biosystems. The abundance of mRNA
214 species was calculated following the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen
215 (2001). The condition with the lowest level of expression was selected as the calibrator
216 for all experiments. RT-qPCR analysis was performed on RNA purified from at least
217 three independent cultures for each condition.

218

219 Determination of tyramine biosynthesis

220 Medium supernatants were recovered from centrifuged cultures from which RNA was
221 obtained, and filtered through 0.45 μm polytetrafluoroethylene (PTFE) filters (VWR,
222 Barcelona, Spain) for tyrosine and tyramine quantification by Ultra High Performance
223 Liquid Chromatography (UHPLC). The filtered supernatants were derivatized with
224 diethyl ethoxymethylenemalonate (Sigma-Aldrich) and further separated in a UPLC®
225 system (Waters, Milford, MA) using previously described column, solvent and gradient
226 conditions Redruello *et al*. (2013). Data were acquired and analyzed using Empower 2

software (Waters). The tyrosine and tyramine concentrations provided are the average of at least three independent cultures.

Gastrointestinal transit tolerance assay

Simulation of the digestion conditions influencing the survival of the microorganisms during their transit through the human GIT was performed as previously described Fernández de Palencia *et al.* (2008) with the following modifications. Cells of the wt and Δ*tdc* strains from late exponential phase cultures in GM17+T (approximately 10^{10} cfu ml^{-1}) were harvested and resuspended in the electrolyte solution supplemented with 10 mM tyrosine. After cell exposure to lysozyme, gastric (G) stress conditions were mimicked by treating cells with pepsin and a successively decreasing pH. Gastrointestinal (GI) stress analysis was simulated by exposure of the samples incubated at pH 5, 4.1 and 3 to bile salts and pancreatin at pH 8. Finally, to mimic colonic stress (Van den Abbeele *et al*. 2010), the GI pH 3 sample was adjusted to pH 7 and incubated overnight. Cell viability under each set of conditions was determined using the LIVE/DEAD® BacLight™ fluorescent stain (Molecular Probes, Leiden, The Netherlands) adhering to previously described conditions (Fernández de Palencia *et al*. 2008). The correlation between the green (live)/red (dead) bacteria fluorescent ratio (G/R) and viable cell numbers was previously established by plate counting. The values presented are the mean of three replicates from independent cultures, expressed as a percentage of the untreated control. Tyramine accumulation was also quantified by UHPLC as described above.

Measurement of intracellular pH

Cytosolic pH measurements were performed using carboxyfluorescein succinimidyl ester (cFSE, Sigma-Aldrich) (an internally conjugated fluorescence pH probe) following a previously described protocol (Sanchez *et al*. 2006) with slight modifications. The wt and Δ*tdc* strains were grown in GM17+T for 6 h. After collecting cells from 1 mL of culture and washing in CPK buffer (sodium citrate 50 mM, disodium phosphate 50 mM, potassium chloride 50 mM) at pH 7.0, they were resuspended in 1 mL of CPK buffer adjusted to different pH values and incubated at 30ºC for 30 min in the presence of the

cFSE probe. They were then washed again in CPK buffer and resuspended in 1 mL of the same plus 15 mM glucose at the pH required, and maintained for 15 min at 30ºC. The cells were then washed once again in CPK buffer at the pH required and resuspended in 100 μL of the same, also at the required pH. Finally, 100 μL of CPK buffer supplemented with 5 mM tyrosine (final concentration: 2.5 mM) were added to the treated cells, and 100 μL of CPK buffer without tyrosine to the control cells. Fluorescence intensities were measured for 10 min (intervals of 0.25 s) in a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Palo Alto, CA) with the excitation and emission values indicated by Breeuwer *et al.* (1996). Background fluorescence levels were assessed by measuring non-fluorescent control cells; these values were subtracted from the fluorescence results. The cytosolic pH values were determined from the ratio of the fluorescence signal at 440/490 nm taken from a calibration curve constructed using buffers at pH 4.5-8.0, after equilibrating the internal (pH_{in}) and external (pH_{out}) pH with 0.1% triton (Molenaar *et al*. 1991). The value given for each condition is the average of three independent replicates (each the mean of values obtained over 8 min of monitoring).

Statistical analysis

Means ± standard deviations were calculated from at least three independent replicates as indicated. Means were compared by the Student *t* test, or ANOVA and the Tukey *post hoc* test when indicated. Significance was set at $p<0.05$.

Results

Physiological role of the *tdc* cluster in *E. faecalis*

To study the physiological role of tyramine production in *E. faecalis*, a deletion mutant of the *tdc* cluster was obtained as indicated above. One clone - termed *E. faecalis* Δ*tdc* - was selected after checking for the deletion of the cluster by PCR using primers CardF and ef0637 R (Table 1). Analysis by UHPLC of the supernatants from overnight cultures of Δ*tdc* in GM17+T showed it to be unable to produce tyramine (data not shown).

To determine whether tyramine biosynthesis offers some advantage in terms of the growth of *E. faecalis* V583, the OD_{600} of wt and Δ*tdc* cultures in GM17+T were monitored (Fig. 2A). No differences were seen between the growth of both strains in these conditions. The influence of tyramine production was also examined under the stress condition of limited carbon source, monitoring the growth of the wt and Δ*tdc* strains in M17+T with 1 g L^{-1} glucose. Although both strains showed a reduction in the maximum OD_{600} reached (Fig. 2B) when grown with 5 g L^{-1} glucose (Fig. 2A), no difference was detected between the wt and Δ*tdc* strains. The significance of tyrosine decarboxylation under acid stress conditions was studied by comparing the growth of the wt and Δ*tdc* strains, adjusting the initial pH of the GM17+T medium to pH 5.0. Figure 2C shows that the tyramine-producing wt strain achieved a higher OD_{600} than the Δ*tdc* strain (1.5 *vs* 0.9), and showed a steeper exponential phase slope. Finally, an experiment combining a reduced carbon source and an acidic initial pH was performed. Cells were cultured in M17+T with 1 g L^{-1} glucose, adjusted to an initial pH of 5.0. The OD_{600} values recorded (Fig. 2D) were slightly lower than those obtained under acidic pH conditions (Fig. 2C). The OD_{600} returned by wt was twice that of Δ*tdc* (1.2 *vs* 0.6), showing that tyrosine decarboxylation enabled the wt strain to grow more quickly.

These results suggest that tyramine biosynthesis might play an important role in *E. faecalis* acid resistance by improving cell growth under acidic conditions, such as those encountered in GIT environments.

The tyrosine decarboxylation pathway improves survival under highly acidic gastric conditions

A gastric and gastrointestinal tolerance assay was performed for the wt and Δ*tdc* strains in the presence of tyrosine. Analysis of tyramine production in the wt strain (Fig. 3) showed it was able to produce tyramine under all the conditions assayed, with stronger production under the more acidic gastric conditions (pH 3.0, 2.1 and 1.8).

The viability of wt and Δ*tdc* cells under gastrointestinal stress was assessed using the LIVE/DEAD® BacLight™ fluorescent stain. Under G stress, the wt strain showed reduced viability (of around 10%) at pH 3.0, 2.1 and 1.8 compared to the untreated controls; at these pH values, greater tyramine production was detected (Fig. 3). The

Δ*tdc* cells showed reduced viability under all the conditions assayed, significantly so at pH 2.1 and 1.8 ($p<0.05$), at which approximately only 65% of the cells survived. The conditions under which tyramine production by strain wt was highest were those under which the survival of the Δ*tdc* mutant strain was poorest. Under GI and colonic stress conditions (exposure to proteolytic enzymes and bile salts), the survival of both populations was reduced to around 15%, with no difference observed between the strains, even though wt was still able to produce tyramine.

These results show that *E. faecalis* is probably able to survive GIT passage, and that tyramine biosynthesis, which has been shown to take place under these conditions, enhances cell survival (especially under G stress). Therefore, tyramine production may improve the fitness of *E. faecalis* under acidic conditions, potentially contributing towards *in situ* tyramine production and accumulation in the GIT. The influence of pH and tyrosine concentration on the regulation of *tdc* cluster transcription was therefore examined.

The catabolic genes *tdcA*, *tyrP* and *nhaC-2* are co-transcribed as a polycistronic mRNA

Before starting the transcriptional analysis of factors affecting *tdc* cluster expression, its transcriptional organization in *E. faecalis* V583 was examined. To determine whether the *tdc* cluster genes are co-transcribed, cDNA from total RNA of cultures grown in GM17+T was used in RT-PCR amplifications with five sets of primers (Table 1) designed to amplify the intergenic and flanking regions of the *tdc* cluster (Fig. 1A). As expected, no PCR products were obtained in RT-PCR amplifications of *tyrS* and *ef0632*, nor of the *nhaC-2* and *ef0636* intergenic regions (Fig. 1B) since these do not belong to the *tdc* cluster. Two amplification products were obtained (Fig. 1B), showing that *tdcA*, *tyrP* and *nhaC-2* are co-transcribed. No amplification was obtained for the *tyrS* and *tdcA* intergenic region, indicating that although *tyrS* belongs to the *tdc* cluster, it is not included in the catabolic operon. mRNA covering *tdcA*, *tyrP* and *nhaC-2* seemed to run from the putative *tdcA* promoter to the putative rho-independent terminator hairpin downstream of *nhaC-2* (ΔG=-11.5 kcal) (Figure S1). As indicated by the RT-PCR results, *tyrS* mRNA is individually transcribed in a monocistronic mRNA covering its own promoter to its putative rho-independent terminator hairpin (ΔG=-21.3 kcal) (Figure S1).

tyrS expression is repressed by high tyrosine concentrations

Initially, the influence of the amino acid substrate on *tyrS* expression was evaluated in CDM at different tyrosine concentrations (Fig. 4A). The highest concentration of tyrosine assayed was 5 mM; higher concentrations resulted in its precipitation. *tyrS* expression was quantified by RT-qPCR after 4 h of incubation. As shown in Figure 4A, *tyrS* was maximally transcribed in the absence of tyrosine (an inverse correlation was seen with tyrosine concentration). The expression of *tyrS* diminished progressively with the tyrosine concentration; at 5 mM tyrosine, minimum induction was observed. Analysis of the sequence upstream of *tyrS* showed strong homology with the structural features described for the *tyrS* leader region in *E. durans* (Linares *et al*. 2012b) (Figure S1). These results suggest that, in *E. faecalis*, a similar transcription antitermination mechanism mediated by tyrosine regulates *tyrS* transcriptional repression in the presence of tyrosine.

The expression of *tdcA* is enhanced by tyrosine

Since the *tdc* catabolic genes of *E. faecalis* V583 are co-transcribed in a polycistronic mRNA, only the expression of *tdcA* was studied. The same cDNA samples obtained for the aforementioned *tyrS* expression assay following 4 h of incubation with different tyrosine concentrations, were used to quantify *tdcA* expression. In contrast to that seen for *tyrS, tdcA* expression correlated positively with the tyrosine concentration until 0.5 mM tyrosine (Fig. 4A), after which no further induction was observed. At the same time point (after 4 h of incubation), tyramine production measured by UHPLC showed an increase as the tyrosine concentration increased (Fig. 4A). The concentrations of tyramine produced indicate that *E. faecalis* decarboxylates tyrosine efficiently, even at low concentrations of the substrate. This, plus the aforementioned result indicating *tyrS* to be maximally transcribed in the absence of tyrosine, meant that only the expression of *tdcA* under tyramine production conditions (substrate availability) was further studied.

Acidic pH increases *tdcA* expression and tyramine production

Results obtained by RT-qPCR analysis of *tdcA* expression (Fig. 4B) showed an approximate 10-fold up-regulation in the culture at pH 5.0 compared to that at pH 7.0 ($p<0.01$). Accordingly, tyramine production also reached its maximum under the acidic condition: 8.37 mM *vs* 4.38 mM at pH 7.0 ($p<0.05$). It is noteworthy that while the OD_{600} achieved at pH 7.0 was 2.04, the culture grown at pH 5.0 only reached an OD_{600} of 0.55 ($p<0.05$). These results highlight how an acidic pH can induce *tdcA* expression and tyramine biosynthesis in *E. faecalis*.

Tyramine biosynthesis counteracts acidification of the cytosol in acidic environments

Although the mechanism underlying the resistance to acid conferred by the production of tyramine remains unclear, several authors have indicated connections between decarboxylation reactions and the maintenance of pH homeostasis in acidic environments (Pereira *et al.* 2009; Romano *et al.* 2014). To confirm the function of tyrosine decarboxylation as a mechanism for neutralizing acidic conditions, cytosolic pH changes were monitored in the wt and Δ*tdc* strains at different pH (from 7.0 to 4.5) in the absence/presence of tyrosine (2.5 mM). Figure 5A indicates that the wt strain was able to maintain a neutral intracellular pH even at the lowest pH tested (pH_{out} 4.5) when in the presence of tyrosine. Compared to the control (tyrosine absence), the difference between the internal pH of the cells in the presence of tyrosine increased as the extracellular pH fell. This difference was significant even at pH_{out} 7.0. In contrast, the cytosolic pH of the Δ*tdc* strain fell as the extracellular pH decreased, both in the presence and absence of tyrosine (Fig. 5B), until eventually dropping below neutral at the lowest pH tested. These results indicate that the effect of the *E. faecalis tdc* cluster on pH homeostasis is greater at lower extracellular pH values, and that the production of tyramine counteracts the intracellular acidification produced by acidic pH challenge.

Discussion

Enterococci are LAB highly adapted to the GIT of human and animals and it is also an important member of fermented foods microbiota. Although usually commensals, they

411 have emerged as a cause of multidrug-resistant, nosocomial infections. Indeed, those
412 caused by VRE can be severe (Lebreton *et al*. 2014). Colonization of the GIT by VRE
413 has been indicated to significantly increase the risk of suffering a systemic enterococal
414 infection (Ubeda *et al*. 2010). Understanding colonization of both commensal and
415 opportunistic pathogen enterococci requires a better knowledge of the mechanisms by
416 which these bacteria cope with the acidic environment of the stomach. The
417 decarboxylation of amino acids has been indicated as a mechanism by which LAB and
418 human pathogenic bacteria can resist acidic conditions (Lund *et al*. 2014; Romano *et al*.
419 2014). Enterococci, such as *E. faecalis*, *E. faecium* and *E. durans* have been shown to
420 decarboxylate tyrosine to form tyramine, a toxic BA that can accumulate in food
421 (Ladero *et al*. 2012) – specially in cheese where enterococci are one of the main
422 responsible of tyramine accumulation (Ladero *et al.* 2010b). In fact, the capability to
423 decarboxylate tyramine could be an advantage for the microorganism against
424 acidification during the fermentation process. Therefore, the present work examined the
425 role of tyramine production by the strain *E. faecalis* V583 as a means of resisting the
426 effects of acid during GIT passage. The influence of environmental factors in the
427 transcriptional regulation of tyramine production was tested, and evidence is provided
428 that the tyramine biosynthetic pathway confers acid resistance by maintaining the
429 intracellular pH stable.

430 The physiological significance of tyramine production - which remains under discussion
431 - was studied by constructing a knockout deletion mutant of the *tdc* cluster of *E. faecalis*
432 V583. This mutant was unable to produce tyramine, confirming the involvement of the
433 *tdc* cluster in tyramine biosynthesis. The comparison of the growth fitness of wt and the
434 non-tyramine-producing Δ*tdc* in the presence of tyrosine and under different stress
435 conditions (carbon source limitation and/or acidic pH), showed that tyramine production
436 improved cell growth under acidic conditions. This indicates that tyramine biosynthesis
437 may help counteract acid stress (Fig. 2C, D). No significant advantage was observed for
438 either strain under conditions of sugar restriction (Fig. 2B). Previous comparative
439 proteomic studies of *E. faecalis* suggest that tyrosine decarboxylation does not compete
440 with other energy-supplying routes (Pessione *et al*. 2009). The present results are
441 therefore consistent with studies that suggest amino acid decarboxylation affords a
442 means of counteracting acid stress (Pereira *et al.* 2009; Trip *et al*. 2012) rather than it
443 being a mechanism for obtaining energy.

Tyramine biosynthesis in *E. faecalis* might, then, be considered an acid resistance mechanism that improves cell growth under acidic conditions. Microbes face the challenge of harsh acidic conditions in the GIT environment, and amino acid decarboxylation might play a role in their survival. The analysis of *E. faecalis* survival in an *in vitro* gastrointestinal model, and the production of tyramine under such conditions, was therefore tested. The results (Fig. 3) reveal that *E. faecalis* V583, like *E. durans* and *L. brevis* strains (Fernandez de Palencia *et al*. 2011; Russo *et al.* 2012), is able to produce tyramine when exposed to GI stress. Whereas some 50% of *E. durans* populations survive under G stress at pH 3.0 (Fernandez de Palencia *et al*. 2011), 85% of the present *E. faecalis* population survived. Similarly, when faced with highly acidic gastric conditions (pH 2.1 and pH 1.8), the survival of the wt and Δ*tdc* strains showed *E. faecalis* resistance to be enhanced by the presence of a functional tyramine biosynthetic pathway. This agrees with the finding that the tyramine producer *E. faecium* E17 conserves 91% of its viability in a medium buffered at pH 2.5 in the presence of tyrosine (Pereira *et al*. 2009). The resistance to acidic conditions improved by the tyramine pathway might explain why *E. faecalis,* followed by *E. faecium,* are likely the dominant enterococci in the human GIT (Nes *et al*. 2014). Altogether, these findings indicate that tyramine production should be considered an important characteristic that contributes to the colonization of the human GIT by opportunistic enterococci.

Since tyrosine decarboxylation improved *E. faecalis* fitness under acidic conditions, the effect of medium pH and substrate availability on the regulation of the *tdc* cluster transcription was examined. Different transcriptional organizations of the *tdc* cluster have been found in different strains. In *E. durans* IPLA655, *tdcA* and *tyrP* are elements of a single operon, while *tyrS* is transcribed independently (Linares *et al*. 2009). However, in *E. faecalis* JH2-2, the existence of a polycistronic mRNA covering *tyrS-tdcA-tyrP* has been described (Connil *et al.* 2002). Similarly, in *L. brevis* IOEB 9890, a polycistronic mRNA covering *tyrS, tdcA, tyrP* and *nhaC-2* has been indicated (Lucas *et al.* 2003). The present findings in *E. faecalis* V583 reveal a monocistronic mRNA covering *tyrS* and a polycistronic mRNA covering the operon formed by *tdcA-tyrP-nhaC-2* (Fig. 1A). The relative high abundance of the transcript *tdcA-tyrP* compared to the transcript *tyrP-nhaC-2* indicated *tdcA* and *tyrP* genes could be expressed from both a short (*tdcA-tryP*) and a long mRNA (*tdcA-tyrP-nhaC-2*), as the transcriptional

analysis of *L. brevis* IOEB 9890 *tdc* cluster has been suggested (Lucas *et al*. 2003). A potential weakest transcriptional terminator was found in the corresponding intergenic region (Fig. 1A, Figure S1) supporting this possibility. Thus, the expression analysis of each transcript - *tyrS* and *tdcA-tyrP-nhaC-2* – was performed separately by RT-qPCR. The expression of *tyrS*, which encodes a tyrosyl-tRNA synthetase-like enzyme, under different tyrosine concentrations revealed an inverse correlation between *tyrS* transcription level and tyrosine concentration, with the maximum expression seen in the absence of tyrosine (Fig. 4A). This agrees with other results published by our group (Linares *et al.* 2012b) that indicate *E. durans tyrS* to be repressed by tyrosine concentration. tRNA synthetase genes are strictly regulated - via a termination-antitermination system - by the corresponding amino acid. If its concentration is low, it does not become bound to the tRNA, thus ensuring amino acid availability to protein synthesis and growth. In the present work, the *tyrS* upstream region of *E. faecalis* showed the typical structural motifs (Figure S1) of a transcription antitermination system involving tyrosine (Grundy *et al.* 2002; Linares *et al.* 2012b), suggesting a similar mechanism may be involved in the regulation of *tyrS* expression in this specie. Tyrosine is a substrate amino acid for protein biosynthesis and *tyrS* could be a sensor of the intracellular tyrosine pool for use in the regulation of tyrosine decarboxylation (Fernandez *et al.* 2004; Linares *et al*. 2012b); the role of *tyrS* in the regulation of the tyramine operon, however, is unclear.

Several authors have shown that decarboxylation reactions depend on amino acid substrates being available (Calles-Enriquez *et al.* 2010; Coton *et al.* 2011; Linares *et al.* 2009). The effect of increasing the concentration of tyrosine on the *tdcA* expression profile was analyzed in *E. faecalis* V583, and showed it to be transcriptionally upregulated in response. An increase in tyramine production was therefore observed (Fig. 4A). This regulation by tyrosine has also been seen in the tyramine-producing *E. durans* IPLA655 and *Sporolactobacillus sp* 3PJ strains (Coton *et al.* 2011; Linares *et al.* 2012b). However, the relative induction levels observed were very low and saturation in the expression was observed at tyrosine concentrations above 0.5 mM. Nevertheless, *tdcA* up-regulation was very sensitive since even very low tyrosine levels (0.1 mM) were enough to increase it. Thus, the cells are able to decarboxylate tyrosine not only when it is in excess, as proposed in order to ensure protein biosynthesis (Linares *et al.* 2012b), but also at low substrate concentrations. The fact that *E. faecalis* is not

auxotrophic for tyrosine (it grew in CDM in the absence of tyrosine) might explain the functionality of the *tdc* operon even at low tyrosine concentrations.

The crucial induction factor in tyramine biosynthesis seems to be an acidic pH (Fernandez *et al*. 2007b; Linares *et al.* 2009; Marcobal *et al.* 2006b). The present results show a critical effect of low pH on the induction of *tdcA* and tyramine production in *E. faecalis* (Fig. 4B), confirming it to be a key factor in tyramine biosynthesis. The mechanism by which tyrosine decarboxylation exerts its role in acidic resistance remains unclear. Consistent with previous results in *E. faecium* (Pereira *et al.* 2009), the present data reveal tyramine production able to neutralize any acidification of the intracellular pH, the extent of tyrosine decarboxylation depending on external pH (Fig. 5A and 5B). It is noteworthy that, in the absence of tyrosine, the wt and Δ*tdc* strains were able to maintain their internal pH above 6.5, suggesting that other mechanisms are also active, such as F_0F_1 ATPase activity (Pereira *et al*. 2009). These results are consistent with those obtained by other authors (Romano *et al*. 2014; Trip *et al.* 2012; Wolken *et al.* 2006) who indicate amino acid decarboxylation pathways may be involved in cytoplasmic pH homeostasis through the alkalinizing effect of the decarboxylation reaction.

The present work provides evidence of a physiological role for tyramine biosynthesis in *E. faecalis*. It appears to be involved in resistance to acidic pH, since (i) the *tdc* cluster improves this bacterium's growth in acidic media, (ii) it enhances its survival under GIT conditions, especially at low pH, and (iii) the expression of *tdcA* is induced by acidic pH. The protective effect seems to be mediated via the maintenance of intracellular pH. The present results highlight the importance of the tyramine pathway of *E. faecalis* in survival under acidic conditions, such as those encountered in passage through the GIT, against which it showed resistance and the continued ability to produce tyramine. Thus, tyramine production might be considered an important characteristic that contributes towards adaptability and which aids in the colonization of the human digestive tract by commensal and opportunistic pathogen enterococci. The increase in tyramine production under acidic conditions might also have food safety implications since enterococci are the major tyramine producers in many cheeses, where acid pH conditions are found due to the fermentation process.

Acknowledgements

This work was funded by the Ministry of Economy and Competitiveness, Spain (AGL2013-45431-R) and the Spanish National Research Council, (CSIC201270E144). M.P. is beneficiary of a FPU fellowship from the Spanish Ministry of Education. We thank Pilar Fernández de Palencia and Paloma López for their help in the GIT survival experiments. The authors also thank Adrian Burton for language and editing assistance.

References

Agudelo Higuita N, Huycke M (2014) Enterococcal disease, epidemiology, and implications for treatment. In: Gilmore MS, Clewell DB, Ike Y, Shankar N (eds) Enterococci: from commensals to leading causes of drug resistant infection. Massachusetts Eye and Ear Infirmary, Boston

Biswas I, Gruss A, Ehrlich SD, Maguin E (1993) High-efficiency gene inactivation and replacement system for gram-positive bacteria. J Bacteriol 175(11):3628-35

Bonten MJ, Willems R, Weinstein RA (2001) Vancomycin-resistant enterococci: why are they here, and where do they come from? Lancet Infect Dis 1(5):314-25

Breeuwer P, Drocourt J, Rombouts FM, Abee T (1996) A novel method for continuous determination of the intracellular ph in bacteria with the internally conjugated fluorescent probe 5 (and 6-)-Carboxyfluorescein succinimidyl ester. Appl Environ Microbiol 62(1):178-83

Calles-Enriquez M, Eriksen BH, Andersen PS, Rattray FP, Johansen AH, Fernandez M, Ladero V, Alvarez MA (2010) Sequencing and transcriptional analysis of the *Streptococcus thermophilus* histamine biosynthesis gene cluster: factors that affect differential *hdcA* expression. Appl Environ Microbiol 76(18):6231-8 doi:10.1128/AEM.00827-10.

Castanie-Cornet MP, Foster JW (2001) *Escherichia coli* acid resistance: cAMP receptor protein and a 20 bp cis-acting sequence control pH and stationary phase expression of the *gadA* and *gadBC* glutamate decarboxylase genes. Microbiol 147:709-15

572 Connil N, Le Breton Y, Dousset X, Auffray Y, Rince A, Prevost H (2002) Identification
573 of the *Enterococcus faecalis* tyrosine decarboxylase operon involved in tyramine
574 production. Appl Environ Microbiol 68(7):3537-44
575 Coton M, Fernandez M, Trip H, Ladero V, Mulder NL, Lolkema JS, Alvarez MA,
576 Coton E (2011) Characterization of the tyramine-producing pathway in
577 *Sporolactobacillus* sp. P3J. Microbiol 157:1841-9 doi:10.1099/mic.0.046367-0
578 EFSA (2011) Scientific Opinion on risk based control of biogenic amine formation in
579 fermented foods. EFSA Panel on Biological Hazards (BIOHAZ). EFSA J
580 9(10):2393-2486
581 Fernandez de Palencia P, Fernandez M, Mohedano ML, Ladero V, Quevedo C, Alvarez
582 MA, Lopez P (2011) Role of tyramine synthesis by food-borne *Enterococcus*
583 *durans* in adaptation to the gastrointestinal tract environment. Appl Environ
584 Microbiol 77(2):699-702 doi:10.1128/AEM.01411-10.
585 Fernández de Palencia P, López P, Corbí A, Peláez C, Requena T (2008) Probiotic
586 strains: survival under simulated gastrointestinal conditions, in vitro adhesion to
587 Caco-2 cells and effect on cytokine secretion. Eur Food Res Technol
588 227(5):1475-1484 doi:10.1007/s00217-008-0870-6.
589 Fernandez M, Linares DM, Alvarez MA (2004) Sequencing of the tyrosine
590 decarboxylase cluster of *Lactococcus lactis* IPLA 655 and the development of a
591 PCR method for detecting tyrosine decarboxylating lactic acid bacteria. J Food
592 Protec 67(11):2521-9
593 Fernandez M, Linares DM, Del Rio B, Ladero V, Alvarez MA (2007a) HPLC
594 quantification of biogenic amines in cheeses: correlation with PCR-detection of
595 tyramine-producing microorganisms. J Dairy Res 74(3):276-82
596 doi:10.1017/S0022029907002488.
597 Fernandez M, Linares DM, Rodriguez A, Alvarez MA (2007b) Factors affecting
598 tyramine production in *Enterococcus durans* IPLA 655. Appl Microbiol
599 Biotechnol 73(6):1400-6 doi:10.1007/s00253-006-0596-y.
600 Gahan CG, Hill C (2014) *Listeria monocytogenes*: survival and adaptation in the
601 gastrointestinal tract. Front Cell Infect Microbiol 4:9 doi:
602 10.3389/fcimb.2014.00009.
603 Giraffa G (2003) Functionality of enterococci in dairy products. Int J Food Microbiol
604 88(2-3):215-22

605 Green MR, Sambrook J (2012) Molecular cloning: a laboratory manual, 4 edn. Cold
606 Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
607 Grundy FJ, Moir TR, Haldeman MT, Henkin TM (2002) Sequence requirements for
608 terminators and antiterminators in the T box transcription antitermination
609 system: disparity between conservation and functional requirements. Nuc Acid
610 Res 30(7):1646-55
611 Holo H, Nes IF (1989) High-frequency transformation, by electroporation, of
612 *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized
613 media. Appl Environ Microbiol 55(12):3119-23
614 Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR (1989) Engineering hybrid genes
615 without the use of restriction enzymes: gene splicing by overlap extension. Gene
616 77(1):61-8
617 Jonsson M, Saleihan Z, Nes IF, Holo H (2009) Construction and characterization of
618 three lactate dehydrogenase-negative *Enterococcus faecalis* V583 mutants. Appl
619 Environ Microbiol 75(14):4901-3
620 Ladero V, Calles-Enríquez M, Fernández M, Alvarez MA (2010a) Toxicological effects
621 of dietary biogenic amines. Curr Nutr Food Sci 6:145-156
622 Ladero V, Fernandez M, Calles-Enriquez M, Sanchez-Llana E, Canedo E, Martin MC,
623 Alvarez MA (2012) Is the production of the biogenic amines tyramine and
624 putrescine a species-level trait in enterococci? Food Microbiol 30(1):132-8 doi:
625 10.1016/j.fm.2011.12.016
626 Ladero V, Fernandez M, Cuesta I, Alvarez MA (2010b) Quantitative detection and
627 identification of tyramine-producing enterococci and lactobacilli in cheese by
628 multiplex qPCR. Food Microbiol 27(7):933-9 doi: 10.1016/j.fm.2010.05.026
629 Ladero V, Linares DM, Del Rio B, Fernandez M, Martin MC, Alvarez MA (2013) Draft
630 genome sequence of the tyramine producer *Enterococcus durans* strain IPLA
631 655. Genome announc 1:e00265-13 doi:10.1128/genomeA.00265-13.
632 Lebreton F, Willems R, Gilmore M (2014) *Enterococcus* diversity, origins in nature,
633 and gut colonization. In: Gilmore MS, Clewell DB, Ike Y, Shankar N (eds)
634 Enterococci: from commensals to leading causes of drug resistant infection.
635 Massachusetts Eye and Ear Infirmary, Boston
636 Linares DM, Del Rio B, Ladero V, Martinez N, Fernandez M, Martin MC, Alvarez MA
637 (2012a) Factors influencing biogenic amines accumulation in dairy products.
638 Front Microbiol 3:180 doi:10.3389/fmicb.2012.00180.

639 Linares DM, Fernandez M, Del-Rio B, Ladero V, Martin MC, Alvarez MA (2012b) The
640 tyrosyl-tRNA synthetase like gene located in the tyramine biosynthesis cluster
641 of *Enterococcus durans* is transcriptionally regulated by tyrosine concentration
642 and extracellular pH. BMC Microbiol 12:23 doi:10.1186/1471-2180-12-23.
643 Linares DM, Fernandez M, Martin MC, Alvarez MA (2009) Tyramine biosynthesis in
644 *Enterococcus durans* is transcriptionally regulated by the extracellular pH and
645 tyrosine concentration. Microbial Biotechnol 2(6):625-33 doi:10.1111/j.1751-
646 7915.2009.00117.x.
647 Linares DM, Martin MC, Ladero V, Alvarez MA, Fernandez M (2011) Biogenic amines
648 in dairy products. Crit Rev Food Sci Nutr 51(7):691-703
649 doi:10.1080/10408398.2011.582813.
650 Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-
651 time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25(4):402-
652 8 doi:10.1006/meth.2001.1262.
653 Lucas P, Landete J, Coton M, Coton E, Lonvaud-Funel A (2003) The tyrosine
654 decarboxylase operon of *Lactobacillus brevis* IOEB 9809: characterization and
655 conservation in tyramine-producing bacteria. FEMS Microbiol Lett 229(1):65-
656 71
657 Lund P, Tramonti A, De Biase D (2014) Coping with low pH: molecular strategies in
658 neutralophilic bacteria. FEMS Microbiol Rev 4(10):1574-6976 doi:
659 10.1111/1574-6976.12076
660 Lyte M (2004) The Biogenic amine tyramine modulates the adherence of Escherichia
661 *coli* O157 : H7 to intestinal mucosa. J Food Protec 67(5):878-883
662 Marcobal A, de las Rivas B, Munoz R (2006a) First genetic characterization of a
663 bacterial beta-phenylethylamine biosynthetic enzyme in *Enterococcus faecium*
664 RM58. FEMS Microbiol Lett 258(1):144-9 doi:10.1111/j.1574-
665 6968.2006.00206.x.
666 Marcobal A, Martin-Alvarez PJ, Moreno-Arribas MV, Munoz R (2006b) A
667 multifactorial design for studying factors influencing growth and tyramine
668 production of the lactic acid bacteria *Lactobacillus brevis* CECT 4669 and
669 *Enterococcus faecium* BIFI-58. Res Microbiol 157(5):417-24
670 doi:10.1016/j.resmic.2005.11.006.
671 Mathur S, Singh R (2005) Antibiotic resistance in food lactic acid bacteria-a review. Int
672 J Food Microbiol 105(3):281-95 doi: doi:10.1016/j.ijfoodmicro.2005.03.008.

673 Molenaar D, Abee T, Konings WN (1991) Continuous measurement of the cytoplasmic
674 pH in *Lactococcus lactis* with a fluorescent pH indicator. Biochim Biophys Acta
675 1115(1):75-83
676 Nes IF, Diep DB, Ike Y (2014) Enterococcal bacteriocins and antimicrobial proteins
677 that contribute to niche control. In: Gilmore MS, Clewell DB, Ike Y, Shankar N
678 (eds) Enterococci: from commensals to leading causes of drug resistant
679 infection. Massachusetts Eye and Ear Infirmary, Boston
680 Paulsen IT, Banerjei L, Myers GS, Nelson KE, Seshadri R, Read TD, Fouts DE, Eisen
681 JA, Gill SR, Heidelberg JF, Tettelin H, Dodson RJ, Umayam L, Brinkac L,
682 Beanan M, Daugherty S, DeBoy RT, Durkin S, Kolonay J, Madupu R, Nelson
683 W, Vamathevan J, Tran B, Upton J, Hansen T, Shetty J, Khouri H, Utterback T,
684 Radune D, Ketchum KA, Dougherty BA, Fraser CM (2003) Role of mobile
685 DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. Science
686 299(5615):2071-4
687 Pereira CI, Matos D, Romao MVS, Crespo MTB (2009) Dual role for the tyrosine
688 decarboxylation pathway in *Enterococcus faecium* E17: response to an acid
689 challenge and generation of a proton motive force. Appl Environ Microbiol
690 75(2):345-352 doi: 10.1128/AEM.01958-08
691 Pessione E (2012) Lactic acid bacteria contribution to gut microbiota complexity: lights
692 and shadows. Front Cell Infect Microbiol 2:86 doi: 10.3389/fcimb.2012.00086
693 Pessione E, Pessione A, Lamberti C, Coisson DJ, Riedel K, Mazzoli R, Bonetta S, Eberl
694 L, Giunta C (2009) First evidence of a membrane-bound, tyramine and beta-
695 phenylethylamine producing, tyrosine decarboxylase in *Enterococcus faecalis*: a
696 two-dimensional electrophoresis proteomic study. Proteomics 9(10):2695-710
697 doi: 10.1002/pmic.200800780
698 Poolman B, Konings WN (1988) Relation of growth of *Streptococcus lactis* and
699 *Streptococcus cremoris* to amino acid transport. J Bacteriol 170(2):700-7
700 Redruello B, Ladero V, Cuesta I, Alvarez-Buylla JR, Martin MC, Fernandez M,
701 Alvarez MA (2013) A fast, reliable, ultra high performance liquid
702 chromatography method for the simultaneous determination of amino acids,
703 biogenic amines and ammonium ions in cheese, using diethyl
704 ethoxymethylenemalonate as a derivatising agent. Food Chem 139(1-4):1029-35
705 doi:10.1016/j.foodchem.2013.01.071.

706 Romano A, Ladero V, Alvarez MA, Lucas PM (2014) Putrescine production via the
707 ornithine decarboxylation pathway improves the acid stress survival of
708 *Lactobacillus brevis* and is part of a horizontally transferred acid resistance
709 locus. Int J Food Microbiol 175:14-9 doi:10.1016/j.ijfoodmicro.2014.01.009
710 Russo P, Fernandez de Palencia P, Romano A, Fernandez M, Lucas P, Spano G, Lopez
711 P (2012) Biogenic amine production by the wine *Lactobacillus brevis* IOEB
712 9809 in systems that partially mimic the gastrointestinal tract stress. BMC
713 Microbiol 12:247 doi:10.1186/1471-2180-12-247
714 Sanchez B, de los Reyes-Gavilan CG, Margolles A (2006) The F1F0-ATPase of
715 *Bifidobacterium animalis* is involved in bile tolerance. Environ Microbiol
716 8(10):1825-33
717 Solheim M, La Rosa SL, Mathisen T, Snipen LG, Nes IF, Brede DA (2014)
718 Transcriptomic and functional analysis of NaCl-induced stress in *Enterococcus*
719 *faecalis*. PLoS One 9:e94571 doi: 10.1371/journal.pone.0094571
720 Trip H, Mulder NL, Lolkema JS (2012) Improved acid stress survival of *Lactococcus*
721 *lactis* expressing the histidine decarboxylation pathway of *Streptococcus*
722 *thermophilus* CHCC1524. J Biol Chem 287(14):11195-204
723 doi:10.1074/jbc.M111.330704
724 Ubeda C, Taur Y, Jenq RR, Equinda MJ, Son T, Samstein M, Viale A, Socci ND, van
725 den Brink MR, Kamboj M, Pamer EG (2010) Vancomycin-resistant
726 *Enterococcus* domination of intestinal microbiota is enabled by antibiotic
727 treatment in mice and precedes bloodstream invasion in humans. J Clin Invest
728 120(12):4332-41 doi: 10.1172/JCI43918
729 Van den Abbeele P, Grootaert C, Marzorati M, Possemiers S, Verstraete W, Gerard P,
730 Rabot S, Bruneau A, El Aidy S, Derrien M, Zoetendal E, Kleerebezem M, Smidt
731 H, Van de Wiele T (2010) Microbial community development in a dynamic gut
732 model is reproducible, colon region specific, and selective for *Bacteroidetes* and
733 *Clostridium* cluster IX. Appl Environ Microbiol 76(15):5237-46
734 doi:10.1128/AEM.00759-10
735 Wolken WA, Lucas PM, Lonvaud-Funel A, Lolkema JS (2006) The mechanism of the
736 tyrosine transporter TyrP supports a proton motive tyrosine decarboxylation
737 pathway in *Lactobacillus brevis*. J Bacteriol 188(6):2198-206
738 doi:10.1128/JB.188.6.2198-2206.2006

739

740

741 **FIGURE LEGENDS**

742 Figure 1.

743 Genetic organization and transcriptional analysis of the *tdc* cluster of *E. faecalis* V583.
744 (A) Diagram showing the genetic organization of the *tdc* cluster and flanking regions.
745 Putative promoters are indicated by broken arrows, and secondary structures and
746 transcription termination regions by lollipops. RT-PCR-targeted intergenic regions and
747 expected mRNA are indicated. (B) RT-PCR amplification of the intergenic regions:
748 fragment A (*ef0632-tyrS*), fragment B (*tyrS-tdcA*), fragment C (*tdcA-tyrP*), fragment D
749 (*tyrP-nhaC-2*) and fragment E (*nhaC-2-ef0636*). Negative controls (−) were performed
750 without reverse transcriptase, and positive controls (+) with chromosomal DNA. M,
751 Molecular weight markers (GeneRuler DNA ladder mix, Fermentas).

752

753

754 Figure 2.

755 Influence of different factors on the growth of *E. faecalis* V583 (continuous line) and *E.*
756 *faecalis* V583 Δ*tdc* (discontinuous line), in the presence of 10 mM tyrosine. (A) Effect
757 of tyramine biosynthesis on cells grown in GM17+T. (B) Influence of carbon source
758 depletion in cultures propagated in M17+T supplemented with glucose 1 g L^{-1}. (C)
759 Effect of acidic pH on cells cultured in GM17+T adjusted to an initial pH of 5.0. (D)
760 Influence of carbon source depletion and acidic pH on cultures grown in M17+T with
761 glucose 1 g L^{-1} and an initial pH adjusted to 5.0. The growth curves were monitored
762 over 12 h by measurement of the OD_{600}.

763

764

765 Figure 3.

766 Response of *E. faecalis* V583 and *E. faecalis* V583 Δ*tdc* in the gastrointestinal tolerance
767 assay. Survival (%) of the wt (gray bars) and Δ*tdc* (white bars) strains after gastric (G),

gastrointestinal (GI) and colonic stresses in the presence of 10 mM tyrosine. C, untreated cells (control). Survival was measured using the LIVE/DEAD® BacLight™ fluorescent stain. Values are expressed as a percentage of the control value (the 100% control values of the G/R ratio of untreated wt and Δ*tdc* strains were respectively 6.9 and 6.8, corresponding to $7.6x10^{10}$ and $7.5x10^{10}$ cfu mL^{-1}). Cells from cultures propagated with 10 mM tyrosine for 6 h were used. The asterisk indicates statistically significant difference ($p<0.05$; Student *t* test). The tyramine produced by the wt strain under each set of conditions is indicated.

Figure 4.

Quantification of gene expression measured by RT-qPCR, and tyramine production quantified by UHPLC. (A) Effect of different tyrosine concentrations on the expression of *tyrS* (white bars) and *tdcA* (gray bars), and on tyramine production, in *E. faecalis* V583 grown in CDM supplemented with 0, 0.1, 0.25, 0.5, 1, 1.5, 2.5 and 5 mM tyrosine. The lowest expression level for each gene was normalized to 1 and used as the reference condition. Bars with the same letter indicate statistically significant differences in relative expression with respect to the no-tyrosine condition (ANOVA and the Tukey *post hoc* test). (B) Influence of pH (5.0 *vs* 7.0) on *tdcA* transcription (gray bar) in *E. faecalis* V583 grown in GM17 + T15 for 6 h. The expression at pH 7.0 was normalized to 1 and used as the reference condition. The asterisk indicates statistically significant difference in relative induction ($p<0.01$). a and b indicate statistically significant differences ($p<0.05$) in tyramine production and OD_{600} respectively (Student t test).

Figure 5.

Variation in the intracellular pH (pH_{in}) at different extracellular pH (pH_{out}) (7, 6.5, 6, 5.5, 5 and 4.5) measured using a cFSE probe in resting cells of (A) *E. faecalis* V583 (continuous line) and (B) *E. faecalis* V583 *Δtdc* (discontinuous line), in the presence (black circles; 2.5 mM tyrosine;) and absence (white circles; control condition) of tyrosine. Cells from cultures propagated with 10 mM tyrosine for 6 h were used. Asterisks indicate statistically significant differences (*, $p<0.05$; **, $p<0.01$; Student *t* test).

800 **Table 1** Primers used in this study.

Primers	Function	Sequence (5´to 3´)	Reference
cardiolRT F	*ef0631-tyrS* RT-PCR	CTCCAGAAGTTGTTCGCGACAT	This work
tyrSRT R	*ef0631-tyrS* RT-PCR	CTGTAAGTTCTCTTAGTCCTTC	This work
tyrS3 F	*tyrS-tdcA* RT-PCR	TGCAGTCGATCCAACACAACATT	This work
tyrS4 R	*tyrS-tdcA* RT-PCR	TTGTAGCTCATTAAGTGAGCAAATTCATG	This work
tdcART F	*tdcA-tyrP* RT-PCR	GAATGGAACCGTGCAGGTAAAG	This work
tyrPRT R	*tdcA-tyrP* RT-PCR	GTTGAGGGCCACCTTCTTGAGGAAG	This work
tyrPRT 2F	*tyrP-nhaC-2* RT-PCR	GTGACTGATGCAGTCTTAGTTGC	This work
nhaC2RT R	*tyrP-nhaC-2* RT-PCR	CTGTCATCGCATTGTCGAATCC	This work
nhaC2RT F	*nhaC-2-ef0637* RT-PCR	CCCATTGCTTTGTCCCATTATCACCG	This work
ef0637 R	*nhaC-2-ef0637* RT-PCR and *tdc* deletion check	GATCCGCTTGTGAAGTTGTCGCTGCAG	(Ladero *et al.* 2012)
tdcV583q F	*tdcA* expression analysis	CTGCTGATATTATCGGTATCGGTT	This work
tdcV583q R	*tdcA* expression analysis	GTAGTTATGGTCAACTGGTACTGGG	This work
tyrSq F	*tyrS* expression analysis	AAACGTGAAGCACAAAGACGCT	This work
tyrSq R	*tyrS* expression analysis	TTTTGCGCTTCTTCTAATGCTG	This work
recA F	*recA* internal control	CAAGGCTTAGAGATTGCCGATG	This work
recA R	recA internal control	ACGAGGAACTAACGCAGCAAC	This work
EFV583-tuf F	*tuf* internal control	CAGGACATGCGGACTACGTTAA	This work
EFV583-tuf R	*tuf* internal control	TAGGACCATCAGCAGCAGAAAC	This work
T1 F	Δ*tdc* mutant construction	TCGATCCAACTGGAGATAGCATGCATA	This work
T2 R	Δ*tdc* mutant construction	AGTATTTGATGACATCACGATCAT	This work

T3 F	Δ*tdc* mutant construction	AACAATGTAATCGGTGAAATCCAGAATCCTA	This work
T4 R	Δ*tdc* mutant construction	GGATTCTGGATTTCACCGATTACATTGTT TGACGGTGATAATGGGACAAAGCAAT	This work
Card F	*tdc* deletion check	GATGATAGTGTCTTGGCTGCTTTAAAGG	(Ladero *et al*. 2012)

801 F, forward; R, reverse.

802

803

804

805

806

Figure 1

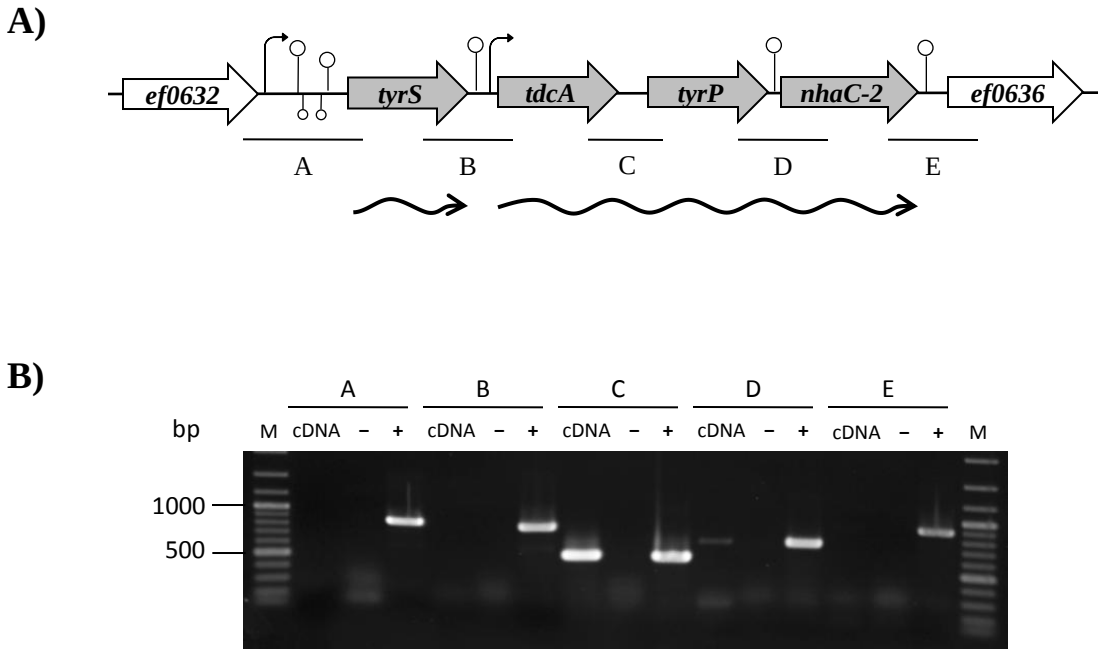


Figure 1.
Genetic organization and transcriptional analysis of the *tdc* cluster of *E. faecalis* V583. (A) Diagram showing the genetic organization of the *tdc* cluster and flanking regions. Putative promoters are indicated by broken arrows, and secondary structures and transcription termination regions by lollipops. RT-PCR-targeted intergenic regions and expected mRNA are indicated. (B) RT-PCR amplification of the intergenic regions: fragment A (*ef0632-tyrS*), fragment B (*tyrS-tdcA*), fragment C (*tdcA-tyrP*), fragment D (*tyrP-nhaC-2*) and fragment E (*nhaC-2-ef0636*). Negative controls (−) were performed without reverse transcriptase, and positive controls (+) with chromosomal DNA. M, Molecular weight markers (GeneRuler DNA ladder mix, Fermentas).

Figure 2

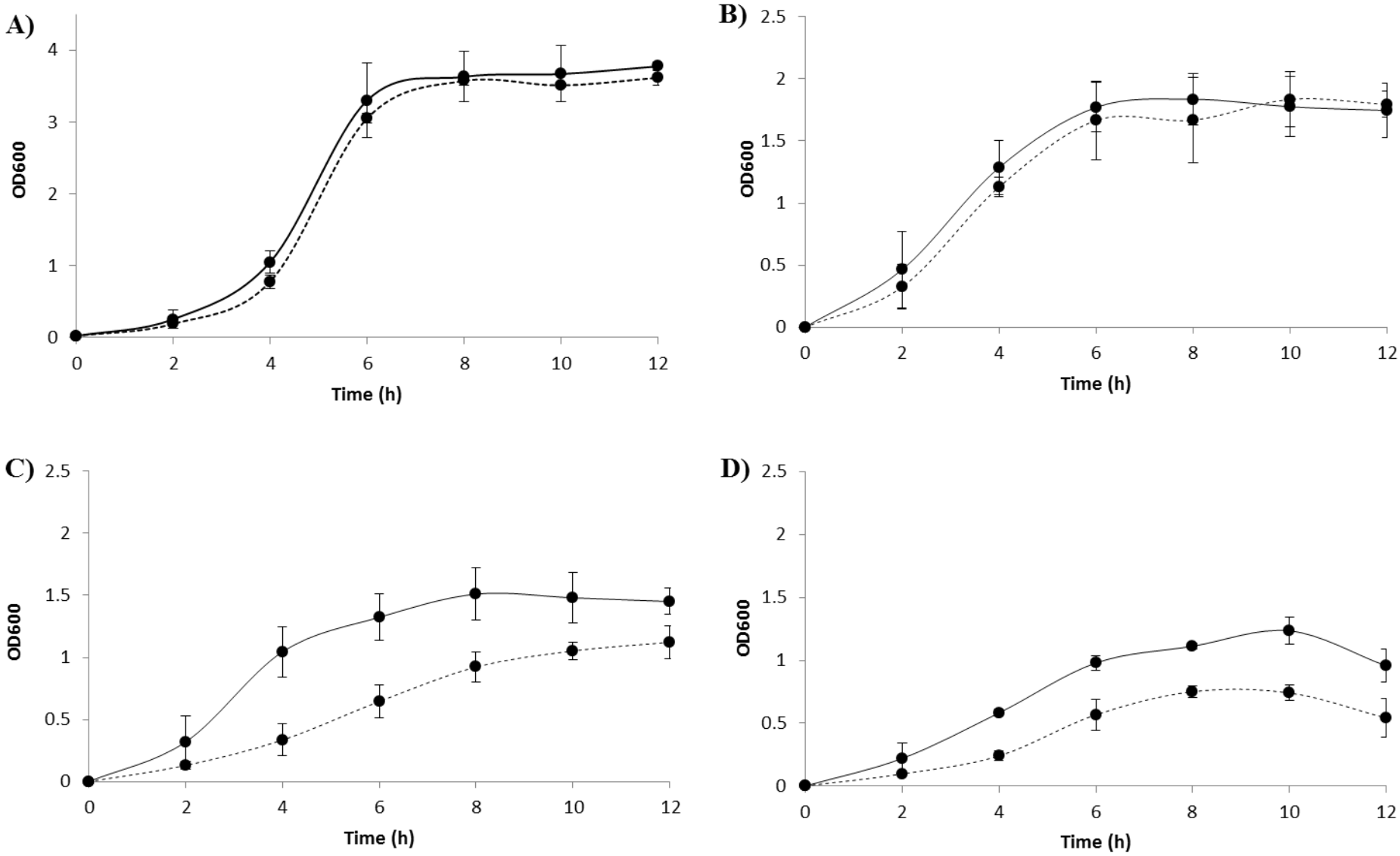
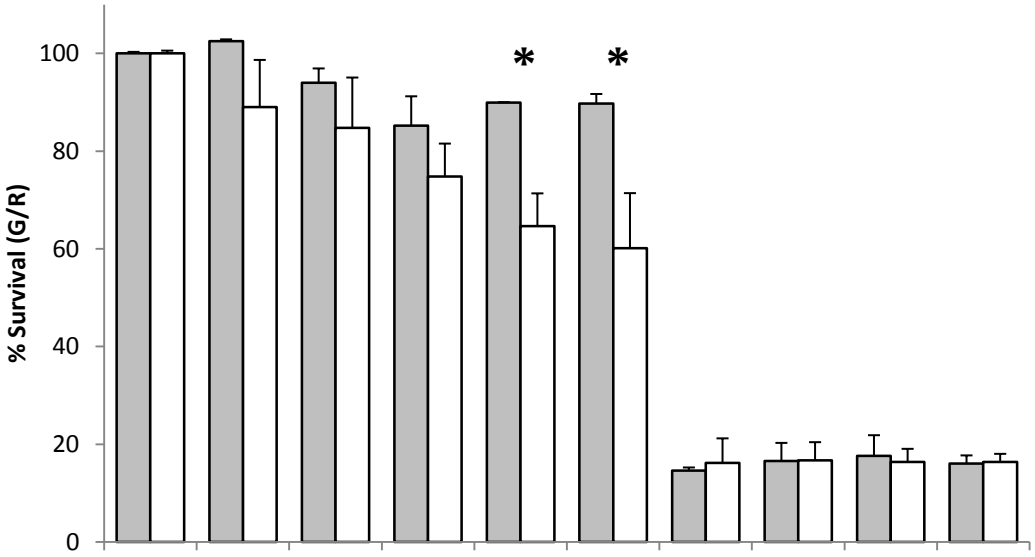


Figure 2.
Influence of different factors on the growth of *E. faecalis* V583 (continuous line) and *E. faecalis* V583 Δ*tdc* (discontinuous line), in the presence of 10 mM tyrosine. (A) Effect of tyramine biosynthesis on cells grown in GM17+T. (B) Influence of carbon source depletion in cultures propagated in M17+T supplemented with glucose 1 g L^{-1}. (C) Effect of acidic pH on cells cultured in GM17+T adjusted to an initial pH of 5.0. (D) Influence of carbon source depletion and acidic pH on cultures grown in M17+T with glucose 1 g L^{-1} and an initial pH adjusted to 5.0. The growth curves were monitored over 12 h by measurement of the OD_{600}.

Figure 3

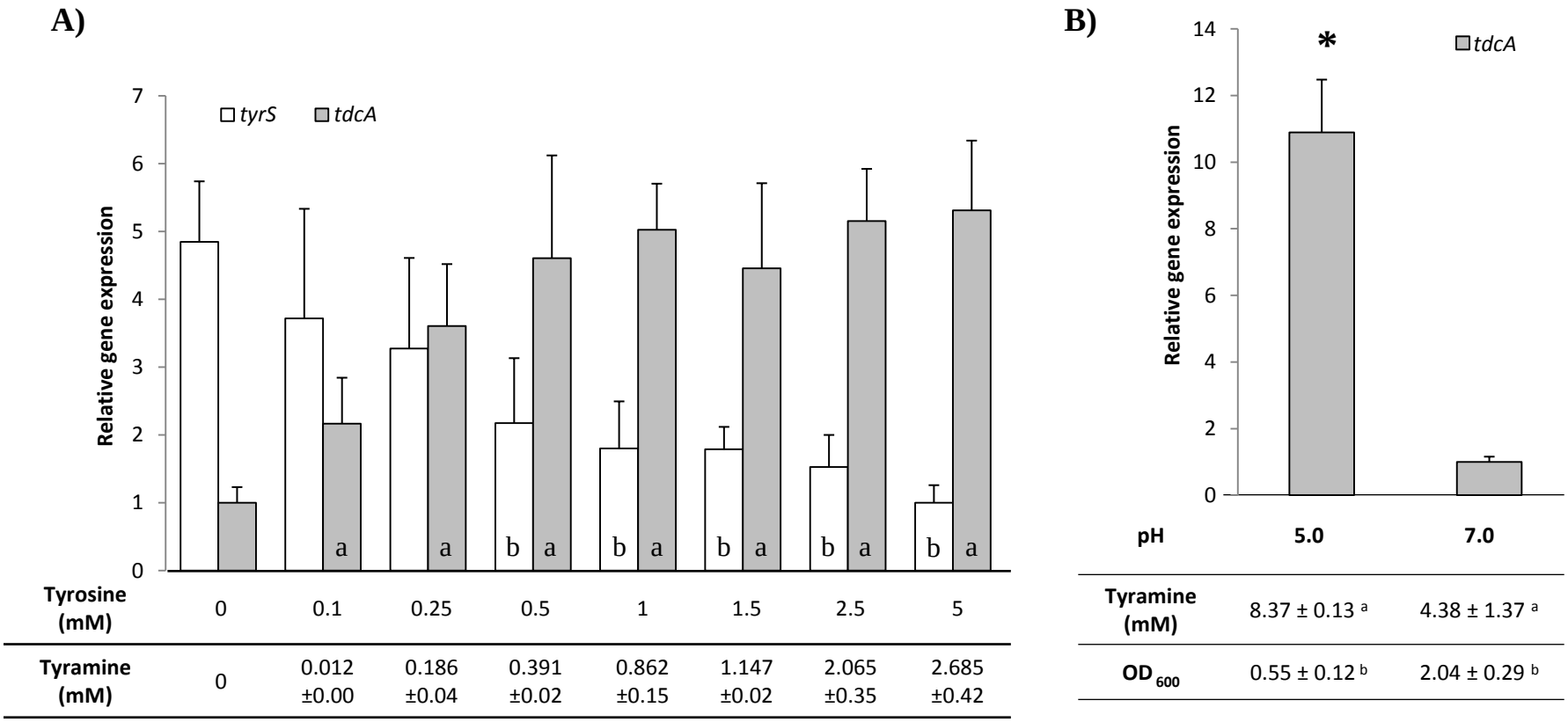


	C	G pH 5.0	G pH 4.1	G pH 3.0	G pH 2.1	G pH 1.8	GI pH 5.0	GI pH 4.1	GI pH 3.0	Colon pH 3.0
Tyramine (mM)	0.456 ± 0.09	0.645 ± 0.25	0.789 ± 0.34	0.896 ± 0.44	0.93 ± 0.42	0.941 ± 0.41	0.456 ± 0.16	0.599 ± 0.27	0.665 ± 0.32	0.674 ± 0.33

Figure 3.

Response of *E. faecalis* V583 and *E. faecalis* V583 Δ*tdc* in the gastrointestinal tolerance assay. Survival (%) of the wt (gray bars) and Δ*tdc* (white bars) strains after gastric (G), gastrointestinal (GI) and colonic stresses in the presence of 10 mM tyrosine. C, untreated cells (control). Survival was measured using the LIVE/DEAD® BacLight™ fluorescent stain. Values are expressed as a percentage of the control value (the 100% control values of the G/R ratio of untreated wt and Δ*tdc* strains were respectively 6.9 and 6.8, corresponding to 7.6×10^{10} and 7.5×10^{10} cfu mL^{-1}). Cells from cultures propagated with 10 mM tyrosine for 6 h were used. The asterisk indicates statistically significant difference ($p<0.05$; Student *t* test). The tyramine produced by the wt strain under each set of conditions is indicated.

Figure 4



Tyrosine (mM)	0	0.1	0.25	0.5	1	1.5	2.5	5
Tyramine (mM)	0	0.012 ±0.00	0.186 ±0.04	0.391 ±0.02	0.862 ±0.15	1.147 ±0.02	2.065 ±0.35	2.685 ±0.42

pH	5.0	7.0
Tyramine (mM)	8.37 ± 0.13 [a]	4.38 ± 1.37 [a]
OD_{600}	0.55 ± 0.12 [b]	2.04 ± 0.29 [b]

Figure 4. Quantification of gene expression measured by RT-qPCR, and tyramine production quantified by UHPLC. (A) Effect of different tyrosine concentrations on the expression of *tyrS* (white bars) and *tdcA* (gray bars), and on tyramine production, in *E. faecalis* V583 grown in CDM supplemented with 0, 0.1, 0.25, 0.5, 1, 1.5, 2.5 and 5 mM tyrosine. The lowest expression level for each gene was normalized to 1 and used as the reference condition. Bars with the same letter indicate statistically significant differences in relative expression with respect to the no-tyrosine condition (ANOVA and the Tukey *post hoc* test). (B) Influence of pH (5.0 *vs* 7.0) on *tdcA* transcription (gray bar) in *E. faecalis* V583 grown in GM17 + T15 for 6 h. The expression at pH 7.0 was normalized to 1 and used as the reference condition. The asterisk indicates statistically significant difference in relative induction ($p<0.01$). a and b indicate statistically significant differences ($p<0.05$) in tyramine production and OD_{600} respectively (Student t test).

Figure 5

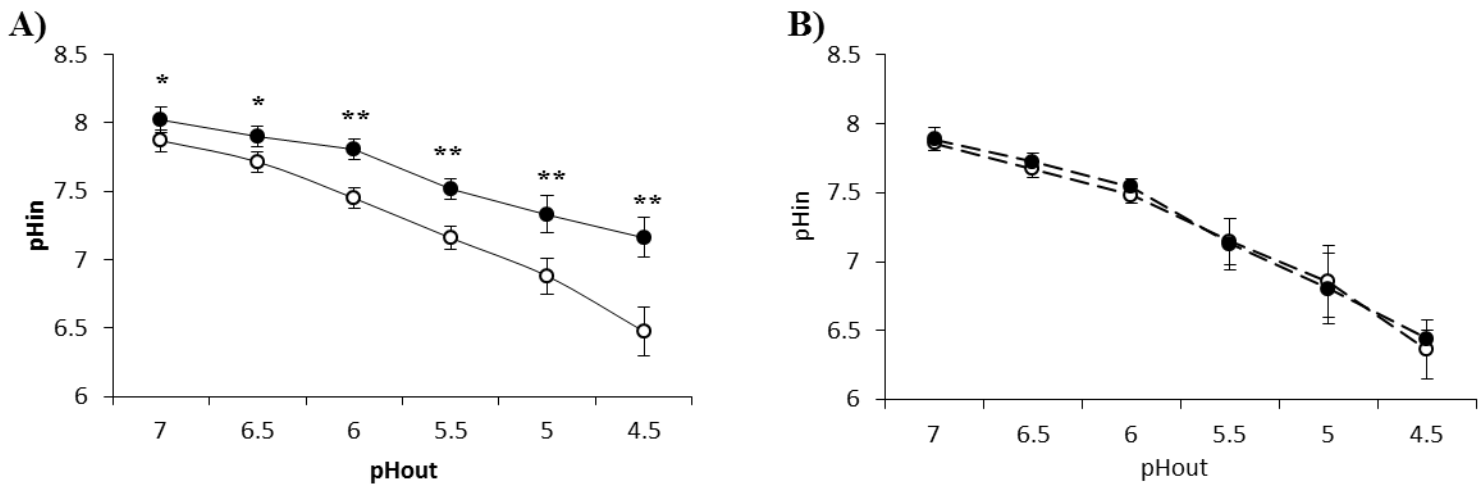


Figure 5.
Variation in the intracellular pH (pH_{in}) at different extracellular pH (pH_{out}) (7, 6.5, 6, 5.5, 5 and 4.5) measured using a cFSE probe in resting cells of (A) *E. faecalis* V583 (continuous line) and (B) *E. faecalis* V583 *Δtdc* (discontinuous line), in the presence (black circles; 2.5 mM tyrosine;) and absence (white circles; control condition) of tyrosine. Cells from cultures propagated with 10 mM tyrosine for 6 h were used. Asterisks indicate statistically significant differences (*, $p<0.05$; **, $p<0.01$; Student *t* test).