The role of tyramine synthesis by food-borne *Enterococcus durans* in the adaptation to the gastrointestinal tract environment

Pilar Fernández de Palencia[1], Maria Fernández[2], Maria Luz Mohedano[1], Victor Ladero[2], Cristina Quevedo[1], Miguel A. Alvarez[2] and Paloma López[1*]

[1]Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9. 28040 Madrid, Spain.

[1]Instituto de Productos Lácteos de Asturias, CSIC, Carretera de Infiesto s/n, 33300 Villaviciosa, Asturias, Spain.

*Corresponding author. Mailing address: Centro de Investigaciones Biológicas, Ramiro de Maeztu 9, 28040 Madrid, Spain. Tel: 34-918373112. Fax: 34-915360432. e-mail: plg@cib.csic.es.

Key words: Biogenic amine, tyramine, *Enterococcus durans,* gastrointestinal stress, immunomodulation, cheese

Short title: The tyramine-producer *Enterococcus durans* 655 in the gastrointestinal tract environment

Abstract

Biogenic amines in food constitute a human health risk. We here report that tyramine producing *Enterococcus durans* IPLA655 (from cheese) was able to produce tyramine under conditions simulating transit through the gastrointestinal tract. Activation of the tyramine biosynthetic pathway contributed to binding and immunomodulation of enterocytes.

Biogenic amines (BA) are formed by the decarboxylation of amino acids, and are involved in important biological functions in the human body, such as nervous transmission, gastric acid secretion, immune response, cell growth and differentiation. Alterations of physiological concentrations of BA have been correlated with several disorders such as allergy, Parkinson's syndrome and migraine (15). The levels of BA in the body are in most cases the sum of endogenous synthesis and exogenous contribution. However, in the case of tyramine the source is only exogenous, mainly by the ingestion of foodstuffs in which BA have accumulated by the action of decarboxylating bacteria, though the synthesis by the gastrointestinal tract (GIT) microbiota should not be dismissed. The consumption of food containing high concentrations of tyramine, can induce adverse reactions such as nausea, headaches, or blood pressure alterations, especially in combination with the use of monoamine oxidase inhibitors as antidepressants (8). The contribution of GIT microbiota to polyamine biosynthesis, a particular type of BA, has been quantified (13); however there is no information regarding the contribution of indogenous microbiota to the biosynthesis of tyramine, or the role of food-borne BA-producing microorganisms, once they reach the GIT.

Certain species of *Enterococcus* and *Lactobacillus* are the main organisms responsible for tyramine accumulation in fermented foods (10). Tyramine producing enterococci have been isolated from human faeces (7). In order to contribute to the BA pool a food-borne tyramine producing strain must survive passing through the GIT and produce tyramine under such conditions. The contribution will be enhanced if the microorganism is able to persist in the intestine. In order to test these possibilities, *Enterococcus durans* IPLA655, a strain isolated from cheese that is able to synthesize tyramine via tyrosine decarboxylation (3), was selected for this study.

To monitor its survival and tyramine production capability under GIT conditions, a model system, previously validated with lactic acid bacteria (LAB) and *Bifidobacteria* strains from food origin, was used (2, 4). This model simulates the normal physiological conditions of the GIT, including the presence of lysozyme (saliva) and gastric (G) stress provoked by pepsin at gradually lower pH values (pH from 5.0 to 1.8). After G stress at pH 5.0 or pH 4.1 the small intestine stress was also assayed (presence of bile salts and pancreatin at pH 6.5 (GI). Bacteria were grown to early stationary phase in ESTY medium (Pronadisa, Madrid, Spain) -which contains a tyrosine basal concentration of about 26 μM- supplemented with 0.5% glucose in the absence or presence of 10 mM tyrosine and after sedimentation and resuspension in fresh medium exposed to the various stresses.

Tyramine production under these conditions was quantified by reverse phase-HPLC (RP-HPLC) (6), which revealed that the bacterium in the presence of 10 mM tyrosine, was able to produce tyramine under the assayed conditions (Fig. 1). Maximum production was observed after G stress at pHs 5.0 and 4.1, at which approximately $2x10^8$ cfu ml^{-1} (viable plate counting on ESTY solid medium) were able to synthesize and release to the culture supernatant a high concentration of tyramine (729 $\pm$ 25 μM)

during the 20 min incubation time. The higher production observed at pH 5.0 in *E. durans* could correlate with the detection at this pH of the maximum transcription levels of the *tyrP* and *tdcA* genes encoding respectively the tyrosine/tyramine antiporter and the tyrosine decarboxylase, which catalyzes the synthesis of tyramine from tyrosine (9); pH 5.0 is also close to the reported optimal pH (pH 5.4) of tyrosine decarboxylase (11). Interestingly, significant concentrations of tyramine (270 μM) were also observed in the samples exposed to pH 1.8, even though only $8.6x10^1$ cfu ml^{-1} were detected at the end of the assay, indicating that, under gastric conditions, the tyrosine decarboxylase could catalyze tyramine biosynthesis either in non-viable cells and/or in culture supernatants.

In order to understand the role of tyramine biosynthesis in cell survival under GIT conditions, the assays were performed in the presence or absence of 10 mM tyrosine. In addition, a knock-out strain was constructed by replacing the *tdcA* gene by the chloramphenicol resistance gene using pMN20-CM, a suicide pUC19-derived plasmid harbouring the 5´and 3´ flanking regions of *E. durans* IPLA655 *tdcA* gene. This plasmid was introduced by electroporation and the double-crossover mutant genotype was confirmed by PCR and Southern hybridization (data not shown). The inability of *E. durans* Δ*tdcA* to produce tyramine was confirmed by RP-HPLC (data not shown).

The survival of the wild-type and mutant strains under GIT stress conditions was assessed by viable plate counting (Fig. 2) after growth to early stationary phase in ESTY medium plus 0.5% glucose either in the presence or absence of tyrosine. Approximately 50% of both bacterial populations were able to survive under G stress at pH 3.0, either in the presence or absence of tyrosine; and in most of the analyzed conditions no differences were detected between the two strains. We detected a significant increase of cell survival only for the mutant strain under GI stress at pH 5.0. Possibly this was due to utilization of the tyrosine for protein synthesis, since we

detected a marked reduction of the tyrosine levels (from 10 mM to 306 μM) without concomitant tyramine production in supernatant samples of the mutant strain (data not shown). In addition, under G stress at pH 2.1 a marked increase of cell survival from $6.46x10^4$ to $2.69x10^6$ cfu ml^{-1} was detected in presence of tyrosine in the wild-type and not in the mutant strain. This accords with the finding that the tyramine biosynthetic pathway conserves the cell viability of *Enterococcus faecium* E17 in a medium buffered at pH 2.5 (14). However, the GIT challenge involves not only acidic stress, but also exposure to lysozyme, proteolytic enzymes and bile salts and the *Enterococcus* genus seems to be well adapted to intestinal conditions (5). This could explain the lack of a clear effect of the tyrosine decarboxylation at other pHs, besides 2.1, for *E. durans* IPLA655.

In any case, the results revealed that the tyramine producing *E. durans* IPLA655 is able to survive and to produce tyramine during the passage through the GIT and therefore may contribute to the tyramine content in the host. This contribution would be greater if such strains were able to colonize the gut and continue to synthesize tyramine. The ability for gut colonization is related to the capacity to adhere to the intestinal epithelium. The difficulty of studying bacterial adhesion *in vivo*, has led to the development of *in vitro* model systems that are based on adhesion to tissue culture cell lines such as Caco-2 cells, which, when differentiated, mimic small intestine mature enterocytes. Bacteria were exposed to the cells using the conditions previously described (4) and their adhesion was assessed by plate counting. In addition, interaction of the strains with the Caco-2 cells was visualized by phase-contrast and fluorescence microscopy (Supplemental material) since both the *E. durans* wild-type, and the mutant strains had been transformed with the plasmid pMV158GFP, which encodes the green fluorescence protein (GFP) (12). The ability to bind to Caco-2 cells was analyzed in the

presence or absence of 10 mM tyrosine. For the adhesion assay $1.25x10^5$ epithelial cells were exposed to $1.25x10^7$ bacteria in the presence of 1 ml of DMEM medium (Invitrogen, Barcelona, Spain) for 1 h at 37ºC under a 5% CO_2 atmosphere as previously described (4). Interestingly, when tyrosine was present in the adhesion assay, a significant increase (approximately three-fold) in the adherence of *E. durans* IPLA655 to Caco-2 cells was observed (Fig. 3). In contrast, the presence of tyrosine did not affect the binding of the mutant strain. RP-HPLC analysis of the supernatants from the adhesion samples revealed that in the presence of 10 mM tyrosine the dairy strain was able to synthesize tyramine ($1.4\pm0.2x10^7$ bacteria produced 141 ± 15 nmol of tyramine in one hour). Supplementing the assay with 140 µM tyramine did not affect the binding of either strain to Caco-2 cells (Fig. 3). These results suggest that activation of the tyramine biosynthetic pathway, rather than the actual production of tyramine, could be involved in this enhancement of the adhesion.

The production of the pro-inflammatory TNF-α by Caco-2 cells ($1.25x10^5$ cells) after eight hours exposure to the *E. durans* strains ($1.25x10^8$ cfu) was quantified as previously described in sample supernatants (2) in the presence or absence of 10 mM tyrosine. In the absence of tyrosine the presence of either strain did not significantly affected the levels of the cytokine produced and secreted by the Caco-2 cells (Table 1). In the control samples lacking bacteria, the presence of tyrosine resulted in a two-fold decrease of the TNF-α levels, which was accompanied by a consumption of 83.5% of the tyrosine (Table 1). Significantly lower levels of this cytokine (8 % and 3.8 %) were detected in the presence of the wild-type strain compared with the mutant and the control, when tyrosine was included in the assay (Table 1). The production of tyramine was confirmed in the wild-type strain samples, reaching a concentration of 3.12 ±19 mM, in the presence of 10 mM tyrosine. The lack of a cytotoxic effect due to tyramine

and bacteria was confirmed using the Cell proliferation kit XTT (Roche Diagnostic, Mannheim, Germany) (data not shown). Moreover, similar levels of tyrosine (approximately 4.3 mM) were detected in samples exposed to both strains indicating that difference in cytokine levels provoked by the bacteria were not due to differences in tyrosine availability for the Caco-2 cells. Therefore, the reduction in the synthesis of TNF-α by the wild-type strain could be associated with the tyramine biosynthetic pathway.

The overall results indicate that *E. durans* IPLA655, a tyramine producing strain present in cheese, can survive in the intestinal environment and synthesize tyramine in the colon using this as a survival and colonization mechanism, by enhancing the adhesion to the intestinal epithelium and reducing the type Th1 activation of the immune system. Unfortunately for the host organism, these increased levels of tyramine could provoke adverse reactions, especially in those individuals with a reduced detoxification system (1). These results offer further evidence of the importance of eliminating the presence of BA producing strains in order to manufacture safer foods.

AKNOWLEDGEMENTS

We thank to Dr Stephen Elson for critical reading of the manuscript and Isabel Cuesta for her technical assistance. This work was supported by the European Union grant FP7-2008-FOOD-211441 BIAMFOOD and the Ministry of Education and Science, Spain (AGL2009-12998-C03-01 and AGL2010-01024).

REFERENCES

1. Bodmer, S., C. Imark, and M. Kneubühl. 1999. Biogenic amines in foods: histamine and food processing. Inflam. Research. **48:** 296-300.

2. Fernández de Palencia, P., M. L. Werning, E. Sierra-Filardi, M. T. Dueñas, A. Irastorza, A. L. Corbí, and P. López. 2009. Probiotic properties of the 2-substituted (1,3)-β-D-glucan producing *Pedioccus parvulus* 2.6. Appl. Environ. Microbiol. **75:** 4887-4891.

3. Fernández M., D. M. Linares, A. Rodríguez, and M. A. Alvarez. 2007. Factors affecting tyramine production in *Enterococcus durans* IPLA655. Appl. Microbiol. Biotechnol. **73:** 1400-1406.

4. Garai-Ibabe, G., M.T. Dueñas, , A. Irastorza, E. Sierra-Filardi, , M.L. Werning, P. López, A.L. Corbí, and P. Fernández de Palencia. 2010. Naturally occurring 2-substituted (1,3)-β-D-glucan producing *Lactobacillus suebicus* and *Pediococcus parvulus* strains with potential utility in the production of functional foods. Bioresource Technology. **101:** 9254–9263.

5. Klein, G. 2003. Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. Int J. Food Microbiol. **88:** 123-131.

6. Krause, I., A. Bockhardt, H. Neckermann, T. Henle, and H. Klostermeyer. 1995. Simultaneous determination of amino acids and biogenic amines by reversed-phase high

1 performance liquid chromatography of the dabsyl derivatives. J. Chromatography A.
2 **715:** 67–79.
3 **7. Ladero V, M. Fernández, and M. A. Alvarez.** 2009. Isolation and identification of
4 tyramine-producing enterococci from human fecal samples. Can. J. Microbiol. **55:** 215-
5 218.
6 **8. Ladero V., M. Calles-Enríquez, M. Fernández, and M. A. Alvarez**. 2010.
7 Toxicological effects of dietary biogenic amines. Curr. Nutr. Food Sci. **6:** 145-156.
8 **9. Linares, D. M., M. Fernández, M. C. Martín, and M. A. Alvarez.** 2009. Tyramine
9 biosynthesis in *Enterococcus durans* is transcriptionally regulated by the extracellular
10 pH and tyrosine concentration. Microbial Biotech**. 2:** 625-633.
11 **10. Linares, D. M., M. C. Martín, V. Ladero, M. A. Alvarez, and M. Fernández.**
12 2010. Biogenic Amines in Dairy Products. Crit. Rev. Food Sci. In Press.
13 **11. Moreno-Arribas, M. V., and A. Lonvaud-Funel.** 2001. Purification and
14 characterization of tyrosine decarboxylase of *Lactobacillus brevis.* FEMS Microbiol.
15 Lett. **195**:103–107.
16 **12. Nieto, C., and M. Espinosa.** 2003. Construction of the mobilizable plasmid
17 pMV158GFP, a derivative of pMV158 that carries the gene encoding the green
18 fluorescent protein. Plasmid. **49:** 281-285.
19 **13. Noack, J., B. Kleessen, and M. Blaut.** 1999. Contribution of the intestinal
20 microflora to polyamine formation in the gut. Cost 917. Biogenically active amines in
21 food vol III. European Commission.
22 **14. Pereira, C. I. Matos, D. M. V. San Romão, M. V. and M. T. Barreto Crespo.**
23 2009. Dual Role for the tyrosine decarboxylation pathway in *Enterococcus faecium*
24 E17: response to an acid challenge and generation of a proton motive force. Appl.
25 Environ. Microbiol. **75**: 345 **–352.**

15. Premont R. T., R. R. Gainetdinov, and M. G. Caron. 2001. Following the trace of elusive amines. Proc. Natl. Acad. Sci. U S A. **98:** 9474-9475.

LEGEND TO THE FIGURES

Figure 1. Tyramine produced (grey bars) by *E. durans* 655 under GIT stress. The number of cells in these cultures is expressed as log cfu ml^{-1} (white bars). Each value is the mean of three independent experiments. The experiments were performed in the presence of 10 mM tyrosine.

Figure 2. Cell survival after gastric (G) and gastrointestinal (GI) stresses. *E. durans* IPLA655 and IPLA655 *ΔtdcA* strains were subjected to various G or GI stress as described in the text, in the presence (grey bars) or absence (white bars) of 10 mM tyrosine. Each value is the mean of three independent experiments. Differences of survival in the presence or absence of tyrosine was tested by two tail t-Student test. **P<0.01.

Figure 3. Adhesion of *E. durans* IPLA655 and IPLA655 *ΔtdcA* strains to Caco-2 cells. Adhesion levels are expressed as percentages of the total number of bacteria (adhered plus unadhered) detected after exposure to Caco-2 cells for 1 h in the presence of either 10 mM tyrosine (grey bars) or 140 μM tyramine (black bars) or in the absence of both compounds (white bars). Each adhesion assay was conducted in triplicate. Each value is the mean of three independent experiments for which three independent determinations were performed. Differences of adhesion in the presence or absence of tyrosine was tested by two tail t-Student test. **P<0.01.

Table 1. Immunomodulation of Caco-2 cells by *E. durans* strains

Bacteria	TNF-α (pg ml^{-1})	Tyrosine (μM)	Tyramine (μM)
wt without tyrosine	289.28 ±39.22	15.4± 0.5	10.1± 2.1
wt with tyrosine	9.33 ±1.04	4,196± 78	3,116 ±189
Δ*tdcA* without tyrosine	558.5 ±83.75	20.03 ±1.3	< 0.05
Δ*tdcA* with tyrosine	116 ±13.75	4,512 ±365	< 0.05
None without tyrosine	489.06 ±76.94	nd	< 0.05
None with tyrosine	247.25 ±54.17	1,654.13±87.12	< 0.05

TNF-α produced by Caco-2 cells after 8 h incubation in response to wild-type or mutant strains in the presence or absence of tyrosine or tyramine. The tyramine and tyrosine concentrations in the cell supernatants were also quantified by RP-HPLC. Each determination was performed in triplicate and the mean value and standard deviation are indicated.

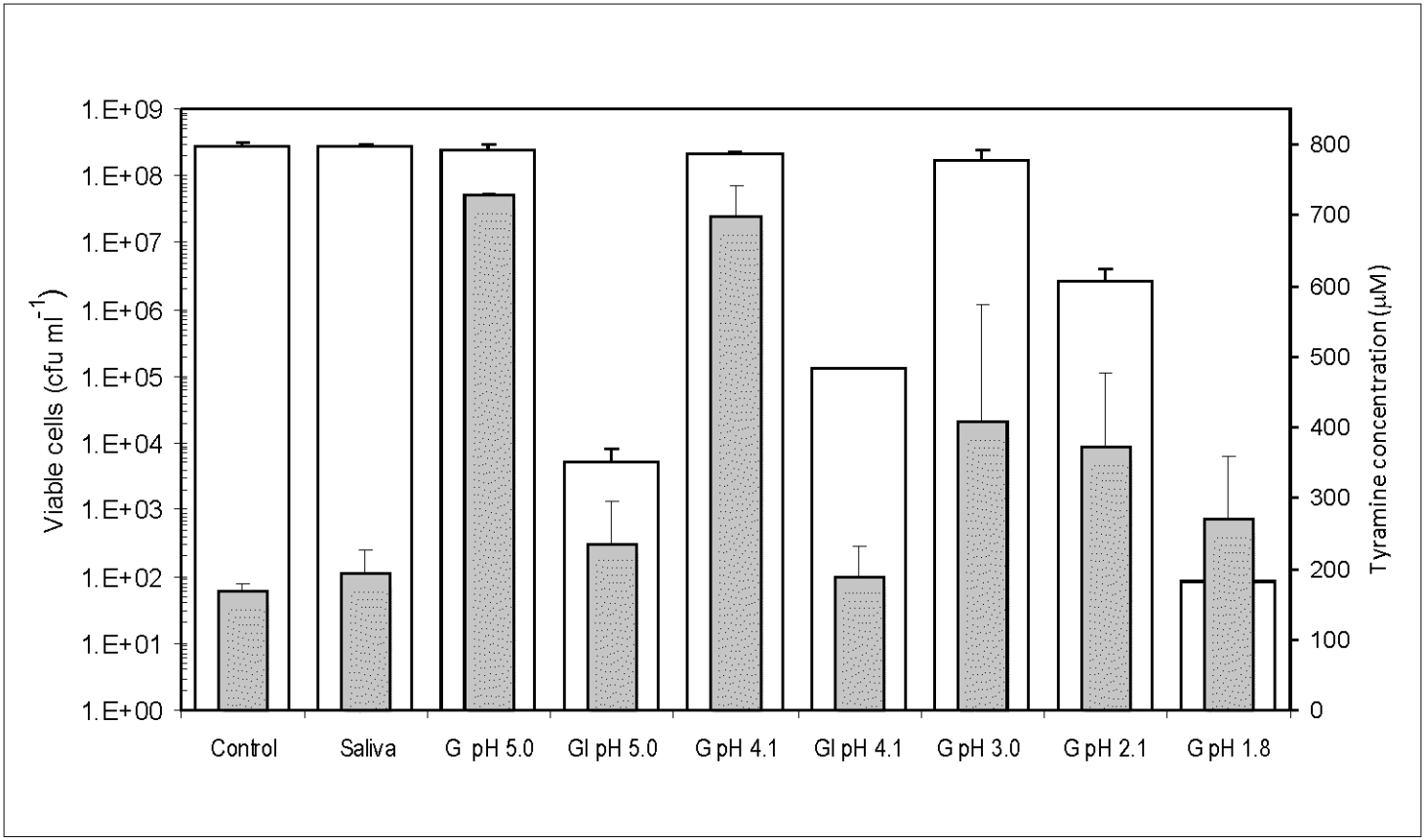

1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
Viable cells (cfu ml-1)
800
700
600
500
400
300
200
100
0
Tyramine concentration (µM)
Control
Saliva
G pH 5.0
Gl pH 5.0
G pH 4.1
Gl pH 4.1
G pH 3.0
G pH 2.1
G pH 1.8

Figure 1

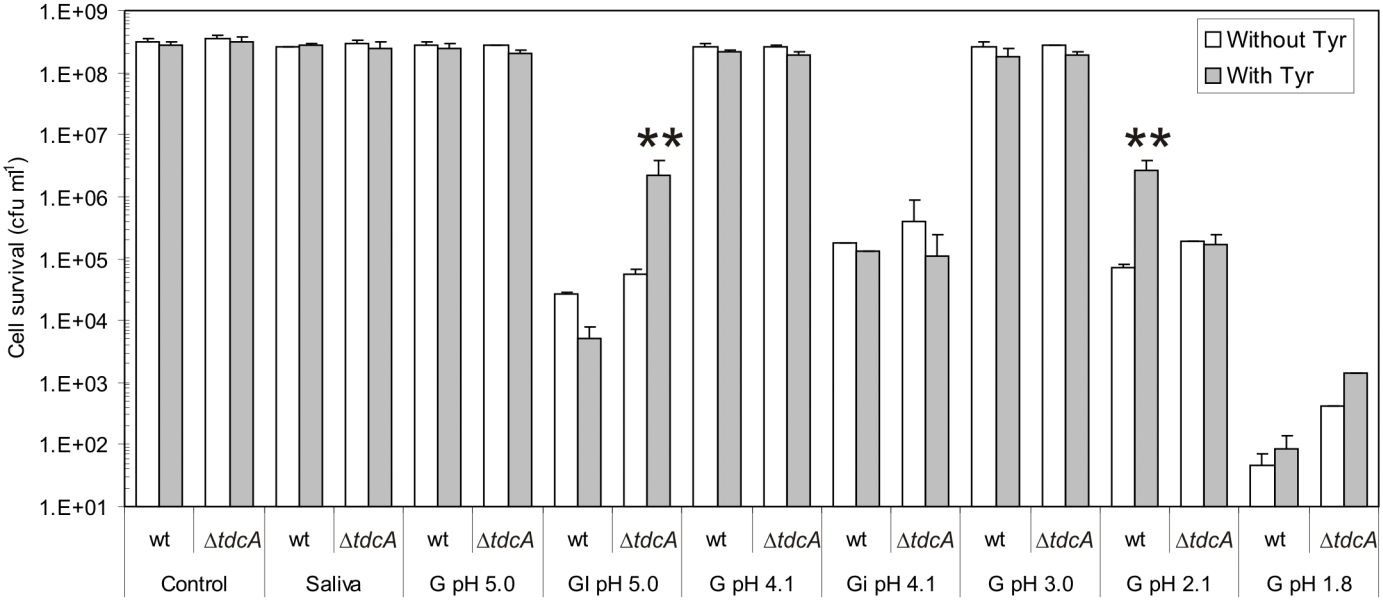

Cell survival (cfu ml-1)
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
Without Tyr
With Tyr
**
**
wt
ΔtdcA
Control
Saliva
G pH 5.0
GI pH 5.0
G pH 4.1
Gi pH 4.1
G pH 3.0
G pH 2.1
G pH 1.8

Figure 2

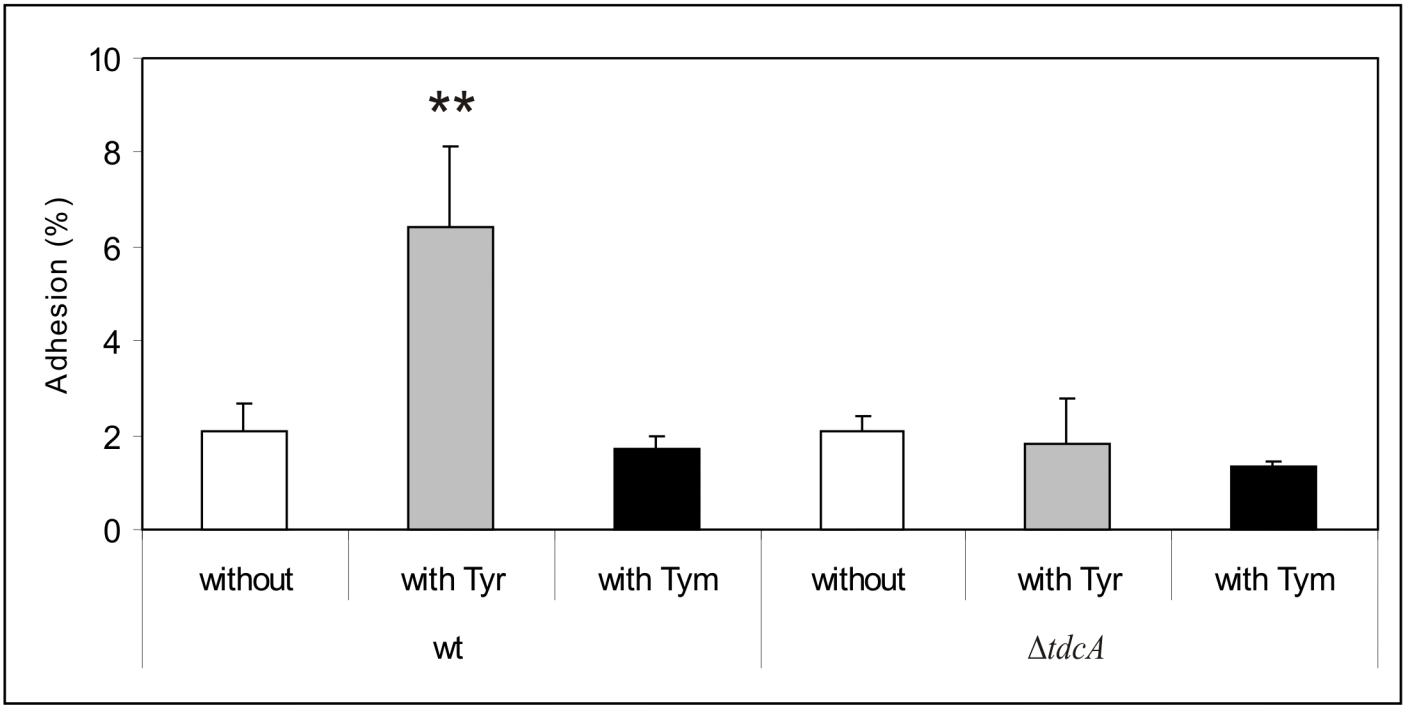
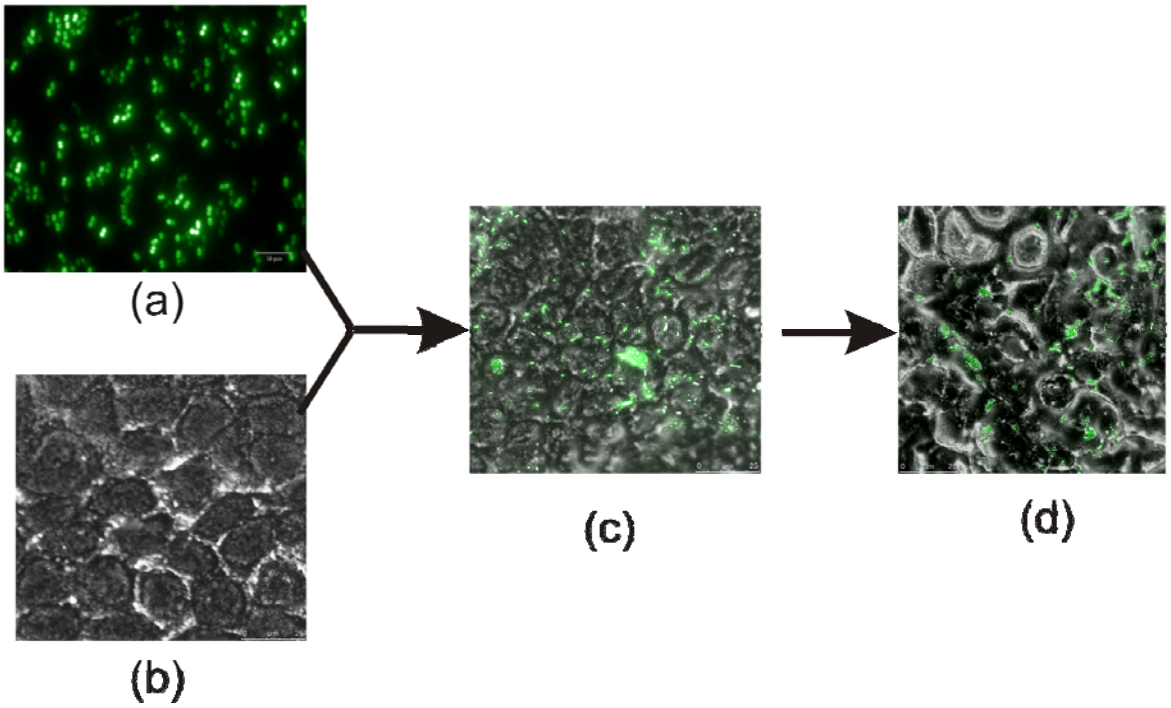

10
8
6
4
2
0
Adhesion (%)
**
without
with Tyr
with Tym
without
with Tyr
with Tym
wt
ΔtdcA

Figure 3



Detection of binding of *Enterococcus durans* to Caco-2 cells by Confocal laser scanning microscopy. Caco-2 cells (b) were exposed to *E. durans* 655[pMV158GFP] (a) for 1 h at 37ºC under CO_2 atmosphere. After the incubation time unbound bacteria were removed by washing three times with PBS at pH 7.1. Samples were observed prior to (c) or after washing (d) by a confocal laser scanning microscope (CLSM), Leica TCS-SP2-AOBS model (Leica Microsystems GmbH, Wetzlar, Germany) with a x100 magnification objective and numerical aperture of 1.6. Confocal illumination was provided by Argon laser (488 nm laser excitation) and with a long pass 520– 565 nm filter (for green emission). Image analysis was performed using FRET and FRAP software (Leica Microsystems GmbH, Wetzlar, Germany).