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LOW DOSES OF TETRACYCLINE TRIGGER THE *E. COLI* GROWTH: A CASE OF HORMETIC RESPONSE

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☐ Hormesis is a biphasic dose-response relationship, occurring when low concentrations of toxic agents elicit apparent improvements. In this work, the ability of sub-inhibitory concentrations of Tetracycline to induce hormetic response in a model organism was investigated. To this aim a reference strain of *Escherichia coli*, MG1655, was exposed to six decreasing doses of Tetracycline (between 0.12 and 0.00375 µg/ml), much lower than the *Minimal Inhibitory Concentration* (4 µg/ml). An hormetic increase was observed at the intermediate concentrations (0.015-0.03 µg/ml) of the tested range. The *Colony Forming Unit* number, indeed, rose up to 141% and 121% as compared to the control. At the highest (0.12 µg/ml) and lowest (0.00375 µg/ml) concentrations a slight decrease in CFU number was found. Results demonstrated that, in *Escherichia coli,* low concentrations of Tetracycline bias the bacterial numerical increase through a hormetic response; the dose-response curve describing this numerical increase is an U-inverted curve. Furthermore, these data confirm that hormesis is common to many - if not all - living systems, including bacteria; they underline the relevance of a deepened knowledge of both the effects and the possible consequences of exposure to low doses of contaminants.

Keywords: *Hormesis; Antibiotic; Escherichia coli MG1655; Tetracycline; Biphasic dose-response; Bacterial growth*

INTRODUCTION

Hormesis is a biphasic dose-response relationship, regarded as an adaptive function. The hormetic response is characterized by a mild stimulation (30-60 %) of the considered function, the endpoint, at low concentrations, and by the inhibition of the same function at higher doses. Many phylogenetically distant organisms and biological systems exposed to a wide range of stimuli show a dose-dependent biphasic response (Stebbing 1998; Chapman 2001; Calabrese and Baldwin 2001, 2002; Calabrese 2008, 2012; Calabrese and Blain 2009). It has been demonstrated that antibiotics trigger this response in plants (Migliore *et al.* 2010a, 2010b). The hormetic curve can be either U- or inverted U-shaped depending on the endpoint. A U-shaped curve could be observed by plotting a detrimental effect (*i.e.* alterations of body functions, disease incidence or mortality). An inverted U-shaped curve could be found when

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physiological functions, as growth or survival rate, are considered (Calabrese and Baldwin, 2002). Four types of concentration-response curves have been identified (Townsend and Luckey 1960), the most frequently observed is the β-curve (an inverted U-shaped dose-response curve; True and Oglevee 1905; Calabrese and Baldwin 1993), with a single stimulatory peak immediately below the No Observed Effect Concentration (Stebbing 1982; Calabrese *et al.* 1999; Chapman 2000).

The hormesis definition does not take into account the output (beneficial or detrimental) of the observed stimulation, that must be inferred from the biological or ecological context (Calabrese and Baldwin 2002; Costantini *et al.* 2010). In an unpredictable variable environment, the hormetic process allows a single individual to overcome a stress condition of low/medium intensity. The final effect of the hormetic response cannot be foreseen.

Although the first historical trial demonstrating hormesis was performed on lower organisms (fungi; Southam and Ehrich 1943), few studies demonstrated the hormetic response in microorganisms. Marine and freshwater luminescent bacteria exposed to metals, showed a clear hormetic phenomenon in luminescence assays (Christofi *et al.* 2002; Shen *et al.* 2009; Deng *et al.* 2012). However, in pathogenic and commensal bacteria the occurrence of hormetic response induced by low doses of antibiotics has not been yet demonstrated.

Tetracycline is an antimicrobial drug commonly used either in human and veterinary clinic or in intensive farming. It is frequently found in both terrestrial and aquatic environmental compartments at very low concentrations, in the order of magnitude of ppb (Boxall *et al.* 2002, 2006; Boxall 2004; Sarmah *et al.* 2006; Brambilla *et al.* 2007; Migliore *et al.* 2007, 2010a; Wu *et al.* 2013).

Escherichia coli is a bacterial species belonging to *Enterobacteriaceae*, besides being the most widely studied prokaryotic model organism, it is a commensal inhabitant of the gastrointestinal tract in humans and warm-blooded organisms; it can also behave as an opportunistic pathogen, as well as one of the most frequently isolated ones (Berg 1996).

To the best of our knowledge, this work is the first attempt to investigate the possible hormetic response induced by low doses of Tetracycline in *E. coli*; to this aim, an *ad hoc* protocol has been set up to easily quantify this response.

MATERIALS AND METHODS

Bacterial strain: *Escherichia coli* MG1655, was employed to study the possible hormetic effect of sub-MIC concentrations of Tetracycline.

Minimal Inhibitory concentration: the MIC value was firstly experimentally assessed with a standard dilution method, in Mueller Hinton Broth. Sub-MIC doses were tested in advance to identify the concentration range

possibly inducing an hormetic effect.

   Experimental design:

a) Antibiotic dilutions. A 16 ml volume of Mueller Hinton Broth, containing 0.24 µg/ml Tetracycline was two-fold serially diluted in the same medium, to obtain six batches of culture medium: from 0.24 to 0.0075 µg/ml.

b) Microbial inoculum. A 1.2 ml volume of a fresh culture of the test strain, diluted to an A_{600} value of 0.14 (corresponding to the to 0.5 point of McFarland nephelometric standard) was added to 60 ml of Mueller Hinton Broth. From this suspension, 8 ml amounts were added to every antibiotic batch tube, so to obtain a final volume of 16 ml and Tetracycline concentrations ranging from 0.12 to 0.00375 µg/ml.

c) Incubation and sampling. The tubes were then incubated at 37 °C and sampled every 30 minutes, up to five hours. At each sampling time, the A_{600} value of the culture was determined, and serial tenfold dilutions were performed, plating 5 µl from each one on Trypticase Soy agar plates, in triplicate. At each sampling time, the best dilutions were chosen to obtain both appreciable and accurate CFU counts. The plates were incubated at 37 °C overnight before counting CFUs.

   These data, properly elaborated, give the CFU number in each culture at each sampling time. The results are the mean of six different experiments each performed in three replicates.

   One-way ANOVA test was utilized to evaluate differences in cell growths. Parametric hypotheses were tested.

RESULTS

The growth curve of *E. coli* MG1655 in the presence/absence of sub-MIC Tetracycline concentrations, in the range 0.00375 – 0.12 µg/ml, by far lower than the MIC value observed with this *E. coli* strain (4 µg/ml), was determined. The hormetic dose-response curve, namely the continuum between toxic effects and apparent improvement (*sensu* Stebbing 1997), was identified by studying the growth curve dynamics in the presence of these sub-MIC Tetracycline concentrations.

Control growth

During time from 0 to 3.5h, the bacterial density increased from about 1×10^6 to 3×10^7 CFU/ml, then the increase became very fast (logarithmic phase) reaching a density of about 1.5×10^8 CFU/ml after 4.5h; this last density was maintained until the end of the experiment (5h).

Cell growth under Tetracycline

The final (5h) CFU number at different Tetracycline concentrations is shown in Figure 1. The lowest concentration (0.00375 μg/ml) caused a slight decrease (92%) of the CFU number as compared to the control. At the next higher concentration, 0.0075 μg/ml, the slight decrease in CFU number disappeared and the bacterial counts started to increase (106% of the control). The increase was evident at 0.015 μg/ml (147% of the control, ANOVA, $F = 7.757$, $p < 0.02$) and at 0.03 μg/ml (121% of the control, ANOVA, $F = 1.06$, n.s.). At 0.06 μg/ml the growth increase attenuated (106% of the control) and at 0.12 μg/ml the CFU number dropped to the 86% of the control. The growth dynamics of the culture exposed to 0.015 μg/ml Tetracycline, as compared to the control one is shown in Figure 2, as the mean values of 6 experiments (3 replicates each; see also Table 1).

DISCUSSION

The present study shows how low Tetracycline concentrations are able to elicit, in *E. coli* MG1655, an hormetic dose-response curve, namely a continuum between population dynamic reduction (toxic effect) and growth promotion (improvement; *sensu* Stebbing 1997). Such response is induced by sub-MIC concentrations of Tetracycline, in a range well below the MIC value (4 μg/ml). The "apparent improvement" of the growth dynamics is replaced by the bacteriostatic effect of Tetracycline at the highest concentration tested.
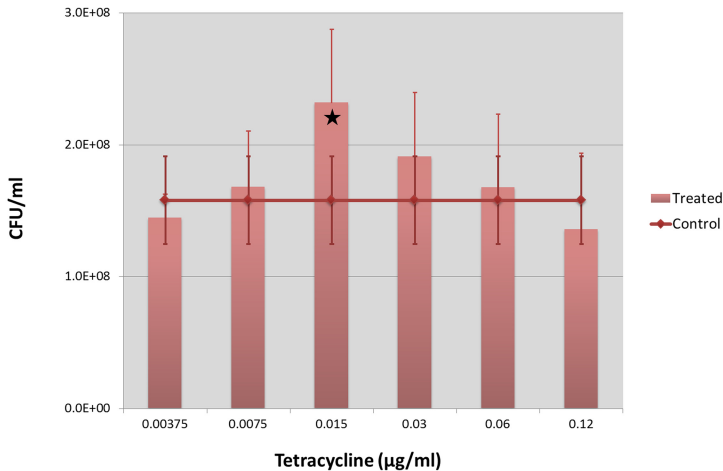

FIGURE 1. Hormetic response to low doses of Tetracycline on *Escherichia coli* MG1655. CFU number of *E. coli* MG1655 after 5 hours in cultures under different Tetracycline concentrations, much lower than the MIC. Mean values of 6 experiments (3 replicates each) and standard deviations are reported. Significant difference (ANOVA, $p < 0.02$) is indicated by a star.
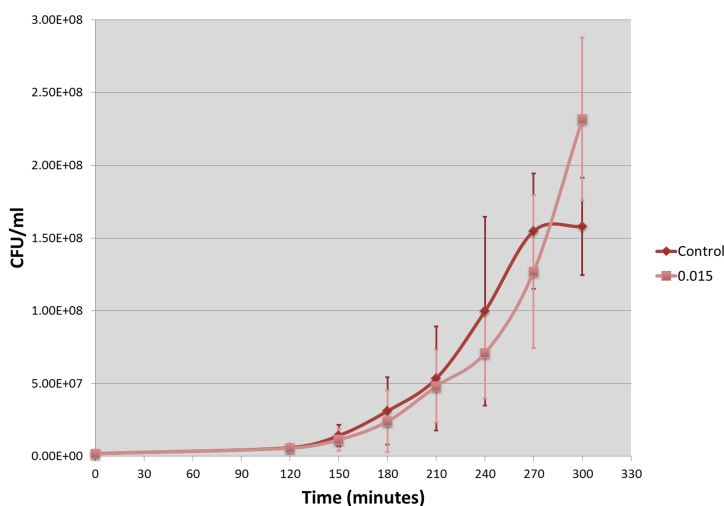
FIGURE 2. Comparison of the growth dynamics between control and Tetracycline exposed batches. Control growth results in a sigmoidal curve (red); 0.015 µg/ml Tetracycline exposed batch, showing the best increase, grows as an exponential curve (pink). Mean values of 6 experiments (3 replicates each) and standard deviations are reported.

TABLE 1. 1×10^8 CFU number of *E. coli* MG1655 after 5 hours in cultures under different Tetracycline concentrations (as µg/ml). Mean values of 3 replicates for each experiment are reported.

Experiment	Control	0.00375	0.0075	0.015	0.03	0.06	0.12
#1	1.49	1.50	2.05	2.29	1.91	1.84	1.20
#2	1.23	1.56	1.42	1.72	1.30	1.12	0.73
#3	1.39	1.20	1.81	3.00	1.97	2.20	2.40
#4	1.57	1.40	1.26	2.00	1.35	1.60	1.00
#5	1.61	1.31	1.27	1.91	1.14	0.96	1.30
#6	2.20	1.70	2.27	3.00	2.40	2.33	1.53

The hormetic curve has been obtained by exposing the bacterial cells to Tetracycline in the concentrations range 0.00375 – 0.12 µg/ml. The highest and statistically significant increase in cell count was found at 0.015 µg/ml, while the concentration of 0.12 µg/ml elicited only a slight bacteriostatic effect. The dose-response curve we found fit quite well with the theoretical model by Klonowski (2007), including the slight reduction above and below the hormesis concentration interval (A-B interval, in Figure 3). Concentrations of 0.0075 and 0.06 µg/ml are the A and B points of the model hormetic curve in the Figure 3: in that concentration interval, indeed, the hormetic increase has been found between 106% and 147% of the control.

The overall lack of literature concerning hormetic response in bacteria does not allow to speculate too much about the underlying mechanisms; experiments performed with the typical ribosome-inhibiting
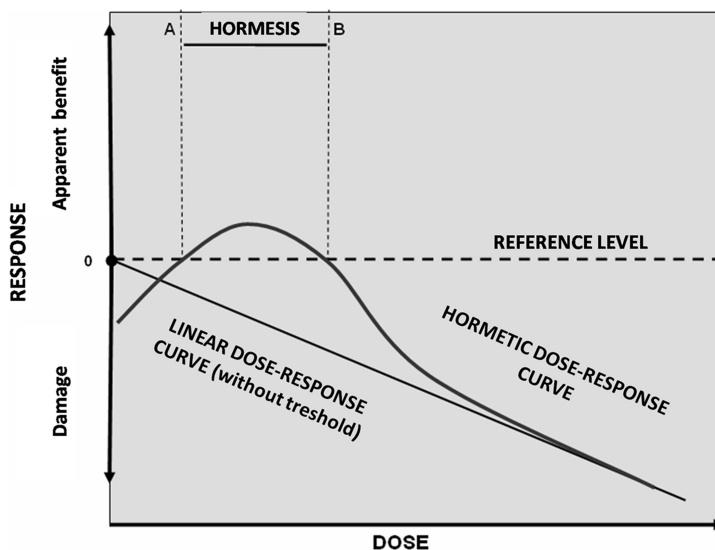
FIGURE 3. The hormetic dose-response and linear no-threshold relationships are shown. In the hormetic curve doses lower than A and higher than B cause toxic or other harmful effects, while, in the range A-B an apparent improvement is observed (by Klonowsky 2007; modified).

Tetracyclines, at doses by far higher than those causing the response we described, demonstrated that no morphological changes occur in *E. coli* cells (Oliva *et al.* 1992). The mechanism underlying this kind of hormetic response, that is a time-limited enhancing of the number of bacterial cells, recalls an r-strategy reaction - and, indeed, *E. coli* is an r-strategist species - global regulation systems could therefore be activated in the presence of many, even unrelated kind of stresses including these very low antibiotic doses.

Finding a clear hormetic response in microorganisms confirms that it is common to many - if not all - living systems. The sub-inhibitory doses of Tetracycline inducing the hormetic response are comparable to the concentrations commonly detected in antibiotic contaminated soils and waters (Sarmah *et al.* 2006; Brambilla *et al.* 2007; Migliore *et al.* 2007). The occurrence of the hormetic response at these concentrations opens new concerns about the effects of antibiotic environmental and food contamination. The recent discoveries about the multiple roles the gut-associated microbiota plays in shaping the fitness, the health and even the behaviour of its host (for a review see Clemente *et al.* 2012; Cryan and Dinan 2012), pose new questions about the bias that antibiotics could exert. Besides the well-known effects of therapeutic doses, a recurrent or constant very low dosage of food-introduced antibiotics, could elicit hormetic responses. The response can be of different extent in the different microbial populations, modifying the ratios within them. The long term periods effects of this should deserve to be studied.

Our results support the experimental evidences on the role of antibiotics as trigger of several responses in bacterial cells (Linares *et al.* 2006; Fajardo and Martinez 2008), other than the protection from competitors. On the basis of these results, further studies will be crucial, being this field of study still largely unexplored and being the effects and consequences of exposure to low doses, not yet considered.

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