Salmonella typhimurium survival in soil and manure assayed using classic and DNA/mRNA based methods - the role of predation and soil temperature.

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INTRODUCTION

The usual practice of addition of animal manure to ground can provide opportunity for *Salmonella* contamination of soils. In this study the survival of tetracycline resistant *Salmonella typhimurium* in soils has been examined by three different techniques: cultivation-based method, direct quantification of mRNA and DNA-based qPCR. Although the presence of *Salmonella* in environmental samples has historically been detected by cultivation-based methods, molecular methods have become important techniques for faster and more-specific detection. DNA-based quantitative PCR without previous pre-enrichment broth may detect damaged or stressed bacteria but inactive or non viable bacteria as well. Alternatively, the mRNA direct quantification method allows quantification of expressing genes of viable cells, however this method in environmental samples is challenging due to the low stability of mRNA. The capacity of these 3 techniques to detect the survival of *S. typhimurium* has been evaluated under 3 different factors: temperature, type of soil and manure treatment. Furthermore, the role of predation has been evaluated in this assay to relate the survival of *Salmonella* with the estimated most probable number of protozoa presents in different soil samples at different conditions.

MATERIAL AND METHODS

Soils and soils inoculation:
Agricultural top soil and soil from horizon B was collected from ?????. 30 g. of each soil was stored in closed glass flasks. Each sample was inoculated with 1 ml of tetraciclyne resistant *Salmonella thyphimurium* DSM544 to yield 10^8 cfu/g soil.

Manure treatment.
Some of the agricultural top soil samples were amended with row cow's manure. The manure was applied to 30 g. of soil corresponding to a normal agricultural practice of 3 kg/m^2. 1ml. of *S. thyphimurium* resuspended in manure was added.

Temperature exposure:
The samples were incubated at 5, 15 or 25 ºC

Salmonella detection techniques:

Cultivation based methods: 0.5 g. of soil was plated in 25 µg/ml tetracycline agar Müller plates.

DNA was extracted from 0.5 g. **of Horizon B** soil samples using the *MOBIO* soil kit → DNA → **Quantification of *invA* DNA** by real time PCR

DNA/mRNA was extracted from 0.5 g. of **agricultural top soil** samples by Griffiths method (2000) modified by Nicolaisen (2008) → DNA/mRNA → DNAse treatment → Reverse transcriptase → **Quantification of *invA* cDNA** by real time PCR

Protozoa counting

Dilutions of soil were incubated in modified Neff's amoeba saline (Page, F.C., 1988) at 15 ºC. MPN was estimated following the method described by Briones & Reichardt (1999) and Halvorson & Ziegler (1933).

CONCLUSIONS

•The quantification of *Salmonella typhimurium* with traditonal culture and with molecular methods in soil samples showed superior values at 5 ºC.

•Direct quantification of *invA* mRNA was achieved in agricultural top soil with or without manure tratment. Faster degradation of mRNA at high temperature was observed.

•The addition of manure to agricultural top soil produced a disminution of the levels of CFU g^{-1} soil. Alternatively, the *invA* DNA/mRNA copy number presented similar values in top soil no treated with manure.

•Protozoa most probable number was superior in soils amended with manure; a correlation with low levels of CFU/g^{-1} in this soil was determined.

• Horizon B presented the lowest levels of protozoa number at the end of the assay, as well as the highest level of *S. typhimurium* survival refering to plate counting results.

RESULTS AND DISCUSSION

Levels of *S. typhimurium* assayed by plate counting

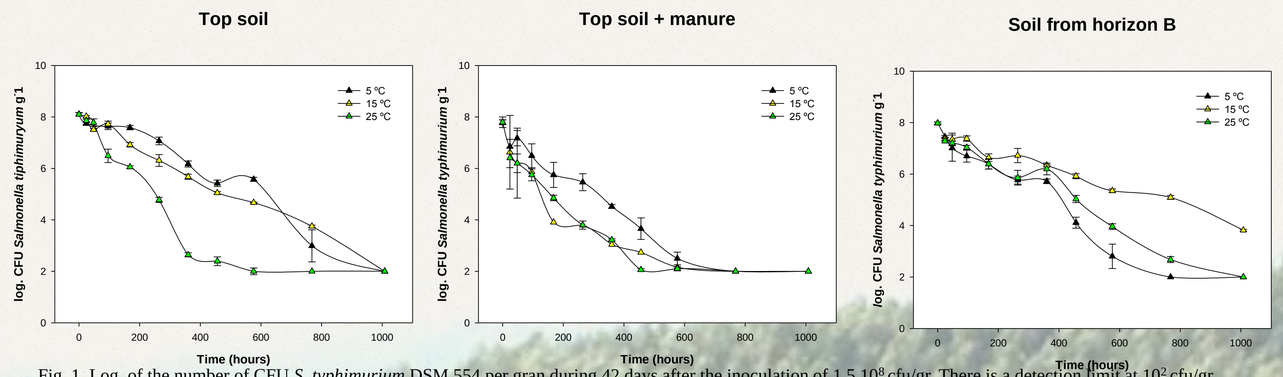

Fig. 1. Log. of the number of CFU *S. typhimurium* DSM 554 per gran during 42 days after the inoculation of 1,5 10^8 cfu/gr. There is a detection limit at 10^2 cfu/gr.

S. typhimurium assayed by plate counting showed a lower survival at 25 ºC in top soil. Significant difference ($p<0.001$ one way ANOVA) between the levels of *S. typhimurium* at 25 ºC degrees and 5º C were found at the 96 hour time point after inoculation. Addition of manure reduced *S. typhimurium* levels in soil samples, this reduction can be observed in samples at 5 and 15 ºC. There is no significance difference when both type of samples are incubated at 25 ªC.

In soil from horizon B the difference in the levels of *S. typhimurium* between the temperatures, started to be significant ($p<0,05$) at 11 days from the beginning of the assay, showing superior survival at 15 ºC

Quantification of *invA* DNA & mRNA

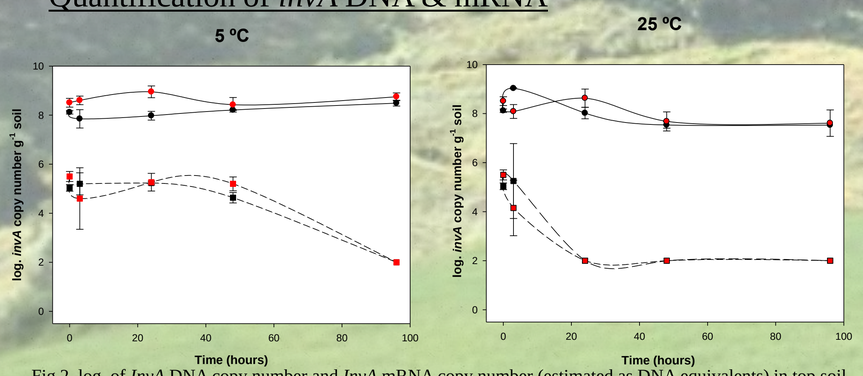

Fig 2. log. of *InvA* DNA copy number and *InvA* mRNA copy number (estimated as DNA equivalents) in top soil with or without amended with manure. There is a detection limit at 10^2 *InvA* copy number g^{-1}soil.

•5 ºC: *invA* mRNA was recovered 48 h after the inoculation of *S. typhimurium.*

•25 ºC: *invA* mRNA was recovered 3 h. after the inoculation of *S typhimurium.*

•The relative amount of mRNA in the samples was below 1% of the DNA detected.

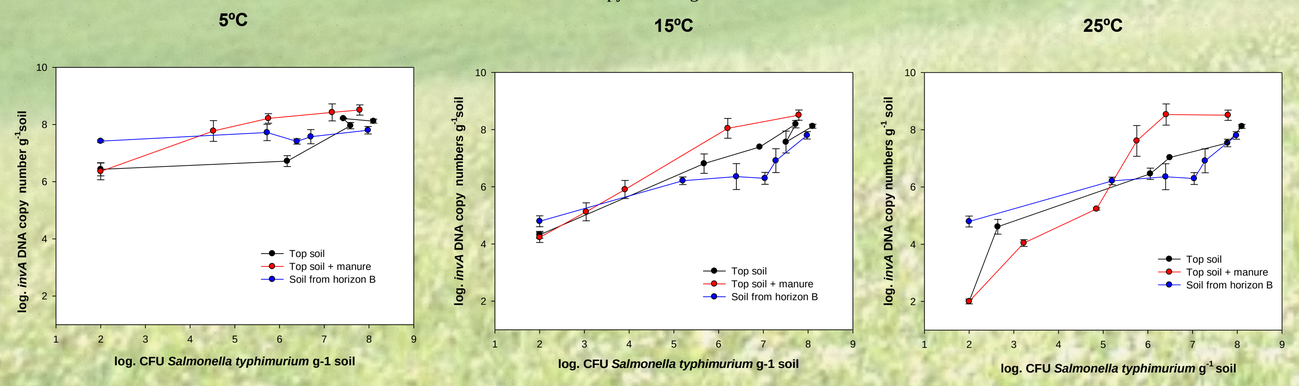

Fig. 3. Comparison between CFU counts and Real-Time PCR for *S. typhimurium*.

•15 ºC and 25 ºC: Significant relation between the direct DNA and the plating based enumeraction (Pearson coefficient > 0.5) was showed in all the samples.

•5 ºC: There were no relation between the levels of *invA* DNA and the number of *S. typhimurium* CFU/g^{-1} soil. The DNA quantification values were very high even when *S. typhimurium* was no detected by plate counting.

•Manure treatment: *invA* DNA/CFU g^{-1} ratio was superior in soil amended with manure, specially at low temperatures. The manure treatment might induce the VBNC (viable but non culturable) state of the cells.

Protozoa counting

Protozoa counting results were higher in soil amended with manure. During the first 24 days of the assay, samples at low temperaures presented a negative correlation with the results of plate counting. (Pearson coefficient < -0.5)

Temperatures →	0 days			24 days			42 days		
	5	15	25	5	15	25	5	15	25
Top soil	4.01 ±0.27[a]	4.01 ±0.27[a]	4.01 ±0.27[a]	4.51 ±0.33[a]	5.10 ±0.28[a]	6.11 ±0.18[a]	5.14 ±0.18[a]	6.02 ±0.38[a]	4.23 ±0.28[a]
Soil from horizonB	3.31 ±0.26[a]	3.31 ±0.26[a]	3.3 ±0.26[a]	3.81 ±0,13[a]	4.51 ±0.09[a]	4.62 ±0.38[a]	3.51 ±0.28[a]	3.03 ±0.48[a]	5.52 ±0.09[a]
Top soil +manure	4.61 ±0.53[a]	4.61 ±0.53[a]	4.61 ±0.53[a]	6.51 ±0.43[a]	6.81 ±0.30[a]	5.03 ±0.48[a]	4.31 ±0.08[a]	4.36 ±0.18[a]	4.51 ±0.38[a]

Table 1: Log. MPN protozoa counting. [a]: Mean standar error.

Horizon soil B presented the lowest levels of protozoa. The high levels of CFU/g detected in this soil may explain the *Salmonella typhimurium* predation by protozoa.