

Salmonella persistence in soil depends on reciprocal interactions with indigenous microorganisms

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Summary

Fresh fruits and vegetables have numerous benefits to human health. Unfortunately, their consumption is increasingly associated with food-borne diseases, *Salmonella enterica* being their most frequent cause in Europe. Agricultural soils were postulated as reservoir of human pathogens, contributing to the contamination of crops during the growing period. Since the competition with the indigenous soil microbiota for colonization sites plays a major role in the success of invading species, we hypothesized that reduced diversity will enhance the chance of *Salmonella* to successfully establish in agricultural environments. We demonstrated that the abundance of *Salmonella* drastically decreased in soil with highly diverse indigenous prokaryotic community, while in soil with reduced prokaryotic diversity, *Salmonella* persisted for a long period. Furthermore, in communities with low diversity, *Salmonella* had an impact on the abundance of other taxa. The high physiological plasticity allows *Salmonella* to use agricultural soils as alternative habitat which might provide a

route of animal/human infections. In addition, adjusted transcriptional profile with amino acid biosynthesis and the glyoxylate cycle most prominently regulated, suggests an adaptation to the soil environment. Our results underline the importance of the maintenance of diverse soil microbiome as a part of strategy aiming at reduced risk of food-borne salmonellosis outbreaks.

Introduction

Salmonella was the most frequent cause of food-related disease in Europe in 2016 (EFSA and ECDC, 2017). Contamination of crop plants in the field can take place via aerial and/or below-ground plant parts (Koukkidis and Freestone, 2018). Several reports showed that human pathogenic bacteria persist in different ecological niches, including soil or organic fertilizer, for extended periods (Islam *et al.*, 2004; Barak and Liang, 2008; Brennan *et al.*, 2014; Jechalke *et al.*, 2019). There is reasonable body of evidence suggesting that *Salmonella enterica* adapts to different environments such as manure or soil (Semenov *et al.*, 2010; Fornefeld *et al.*, 2017). The fate of *Escherichia coli* and *Salmonella* in soil and soil-related environments was investigated in several studies (Ongeng *et al.*, 2014; Mallon *et al.*, 2015; Fornefeld *et al.*, 2017; Wang *et al.*, 2018). Furthermore, a number of studies have shown that the survival and success of an invader in a new (soil) environment depends on various biotic and abiotic factors. For example, the survival of *Listeria monocytogenes* in soil depends on the acidity and the present microbiota (Locatelli *et al.*, 2013). The soil type, root exudates and organic fertilizer might also influence the persistence of pathogens in soil (Berg and Smalla, 2009; Locatelli *et al.*, 2013; Schlaeppli *et al.*, 2014; Jechalke *et al.*, 2019). However, the relationship between the biotic diversity and biotic resistance towards invading organisms, is not fully understood; it seems that environments with high biological diversity have narrower niches and/or might have a higher chance to harbour natural antagonists and competitors (Chapin III *et al.*, 2000; Loreau and Hector, 2001; Mallon *et al.*, 2015; Li *et al.*, 2018). Accordingly, persistence of *E. coli* O157:H7 in soil was strongly correlated with a low microbial diversity and

Received 23 July, 2019; revised 27 February, 2020; accepted 1 March, 2020. *For correspondence. E-mail adam.schikora@julius-kuehn.de

a high amount of available resources (Westphal *et al.*, 2011; van Elsas *et al.*, 2012). Nonetheless, a possible failure of an invader cannot lead to the conclusion that the resident microbial diversity remains unchanged. Recently, Mallon *et al.* (2018) observed shifts in microbial communities after unsuccessful invasions of *E. coli*.

In this study, we investigated the invasion and persistence of *S. enterica* in agricultural soil. Moreover, we assessed the influences of reduction in microbial diversity as well as inoculation of *S. enterica* on the structure of soil prokaryotic community. We hypothesized that higher initial diversity of indigenous soil bacteria would have a negative impact on the invasion and persistence of *Salmonella* in agricultural soil(s). Furthermore, we hypothesized that inoculation of *Salmonella* into soil with lower diversity would lead to an enhanced persistence. Finally, we wondered if *Salmonella* inoculation would shift the prokaryotic community composition. To test those hypotheses, we used an agricultural soil, diluvial sand (DS) soil and three *S. enterica* strains. We used *S. Senftenberg* as well as the *S. Typhimurium* strains 14028s and its derivative LT2, which were isolated from basil, poultry and a common lab strain respectively. The survival of the *Salmonella* strains was followed by cultivation-dependent and cultivation-independent methods. The diversity of prokaryotic communities in DS soil (untreated DS soil) and DS soil with reduced community (autoclaved DS soil) was analysed by sequencing of 16S rRNA gene amplicons from total community DNA. To evaluate a possible response to the soil environment with high and low microbial diversity we assessed the transcriptome of *S. enterica* Typhimurium 14028s, exposed to mineral medium and resuspended in untreated and autoclaved DS soil using a newly designed experimental approach.

Results

Salmonella persists better in DS soil with reduced microbial diversity

The persistence of *Salmonella* was assessed in autoclaved diluvial sand (DS) soil after inoculation with *S. enterica* serovar Typhimurium strain 14028s (*S. Typhimurium* 14028s), *S. enterica* serovar Typhimurium LT2 DSM 18522 (*S. Typhimurium* LT2) or *S. enterica* serovar Senftenberg (*S. Senftenberg*) in three independent experiments (Fig. 1A–C). In parallel, *Salmonella* strains were inoculated into untreated DS soil, which served as a control. The initial level of inoculation was about 10^6 , 10^7 and 10^6 colony-forming units/g soil dry weight (CFU (g dw)⁻¹) in the first, second and third experimental repetition, respectively. CFU counts revealed that in the untreated DS soil all tested strains declined steadily and were below the quantification limit (10^2 CFU/g soil dry weight (dw)) 63 days

post-inoculation (dpi) for *S. Typhimurium* LT2 as well as *S. Senftenberg*, and after 77 days for *S. Typhimurium* 14028s (Fig. 1; blue lines). In contrast, in the autoclaved DS soil, *Salmonella* CFU counts increased initially by one to two orders of magnitude to 10^8 CFU (g dw)⁻¹ and even 10^9 CFU (g dw)⁻¹ (Fig. 1; red lines). After the initial increase, the abundance of *Salmonella* decreased over the experimental period. However, the *Salmonella* CFU counts in autoclaved DS soil never dropped below the detection limit (10^1 CFU (g dw)⁻¹) and only in the first experiment, *S. Senftenberg* CFU counts were below the quantification limit at 161 dpi (Fig. 1; red diamonds). These results, reproducible in all three experiments, suggest that the persistence of *Salmonella* in autoclaved DS soil was enhanced compared with the untreated soil. Furthermore, we did not observe significant differences between the persistence of different strains (Fig. 1, Supporting Information Tables S1A and S2). The CFU counts of culturable, mostly fast-growing bacteria, were stable throughout the experiments in untreated soil and increased for more than one order of magnitude right after autoclaving of the soil (Fig. 1; dashed lines, circles). Despite the differences in the number of *Salmonella* cells inoculated to the soil between the repetitions ($\sim 10^6$ – 10^7 CFU (g dw)⁻¹), the influence of autoclaving was significant (Fig. 1).

To further corroborate those observations, we used a cultivation-independent approach, based on quantitative-PCR (qPCR), targeting the *Salmonella*-specific *invA* gene. This approach allows to detect also viable but non-culturable (VBNC) bacteria. The copy number of the *invA* gene/g dw soil followed the same pattern as observed for the CFU counts (Supporting Information Fig. S1). The amounts of quantified *S. Typhimurium* 14028s and *S. Typhimurium* LT2 CFU decreased by factor 1000 in untreated DS soil, and the *invA* copy number/g dw was between 0.4 and 0.6 log units higher than the CFU (g dw)⁻¹ for all tested *Salmonella* strains after 7 days ($p < 0.05$, Student's *t*-test). Using this culture-independent method, *invA* was detectable, even in situation when due to low colony numbers on the agar plates, the CFU counts were not quantifiable, for example, in untreated DS soil 91 days after inoculation. In autoclaved DS soil, only 10-fold reduction was observed between day 7 and day 91 for all three strains, and the numbers were not different for *S. Typhimurium* 14028s and LT2 while the *invA* copy numbers for *S. Senftenberg* were 0.7 and 1.3 log units higher than the CFU counts after 7 and 91 days respectively. (Supporting Information Fig. S1).

Taken together, we observed that autoclaving the soil increased the ability of *Salmonella* to persist in the soil environment. The higher *invA* copy numbers compared with the CFU data might result from either dead cells or a VBNC state of *Salmonella*. *S. Typhimurium* 14028s

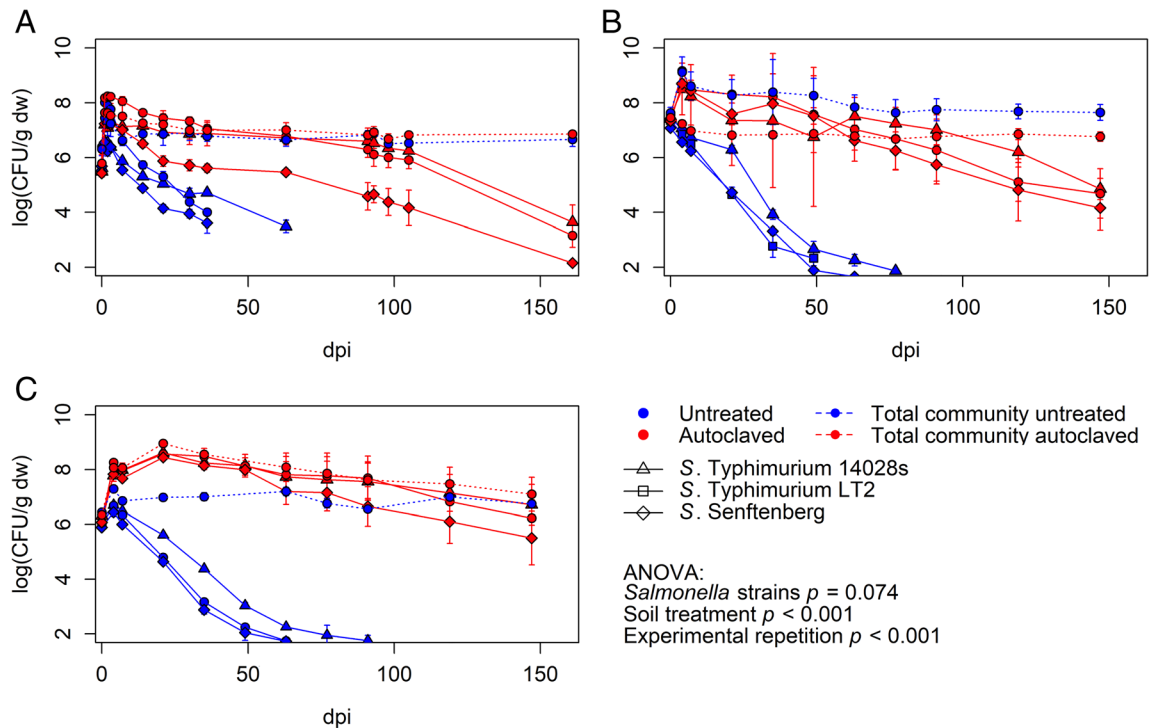


Fig. 1. Persistence of *Salmonella* in soil. Number of colony-forming units (CFU) (g dw)⁻¹ was calculated in untreated DS soil (blue lines) and autoclaved DS soil (red lines) in three independent experiments (A–C). XLD medium supplemented with rifampicin was used for *Salmonella* CFU counts (solid lines) and R2A medium for total culturable community CFU counts (dashed lines, circles). The *Salmonella enterica* serovar Typhimurium strain 14028s (*S. Typhimurium* 14028s, triangles), *Salmonella enterica* serovar Typhimurium strain LT2 DSM 18522 (*S. Typhimurium* LT2, rectangles) and *Salmonella enterica* serovar Senftenberg (*S. Senftenberg*, diamonds) were inoculated on day 0. The *Salmonella* CFU counts are further plotted for days post-inoculation (dpi). Error bars represent the standard deviation ($n = 4$). The influencing factors were assessed with ANOVA and the ‘multcomb’ package, $p < 0.05$ was considered significant. Slopes of linear regressions were analysed by a generalized linear model using the mixed procedure (Supporting Information Table S1).

showed the best persistence in soil in all three experiments and treatments (Fig. 1, Supporting Information Tables S1A and S2). Based on this, *S. Typhimurium* 14028s was selected for the following experiments.

Diversity influences the ability of *Salmonella* to persist in soil

In order to further substantiate the results, we asked the question how differences in the diversity level of microbial communities influence the persistence of *Salmonella* in soil? To this end, we used a modified experimental setup in which *S. Typhimurium* 14028s was confronted with none, 10% or 100% of the microbial community extracted from untreated DS soil, however, with the same bacterial abundance (assessed on R2A medium and adjusted to 10⁶ CFU (g dw)⁻¹). Similar to previous observations (Fig. 1), CFU counts of *Salmonella* decreased drastically in untreated DS soil to 10² CFU (g dw)⁻¹ within 91 days. In autoclaved DS soil, the CFU counts for *Salmonella*, after an initial increase of 2 orders of magnitudes, stabilized at approximately 10⁶ CFU (g dw)⁻¹ (Fig. 2). However, inoculation with 10% of the native DS microbial

community had moderate impact on *Salmonella* persistence (Fig. 2 and Supporting Information Table S1B), and resulted in a decrease of *Salmonella* CFU counts from 10⁸ CFU (g dw)⁻¹ after the initial increase of 2 orders of magnitude, to 10⁵ CFU (g dw)⁻¹. Whereas after an inoculation with 100% of the native DS soil microbial community, the CFU counts for *Salmonella* dropped, after the initial increase, to 10² CFU (g dw)⁻¹, very similar to the untreated DS soil (Fig. 2). The CFU counts of culturable, mostly fast-growing bacteria were, after an initial increase, stable throughout the experiments (Supporting Information Fig. S2 and Table S1B).

These observations imply that the level of microbial diversity is an influencing factor in the persistence of *S. Typhimurium* 14028s in the soil environment.

Presence of *Salmonella* does not influence the prokaryotic diversity in untreated DS soil

The prokaryotic community composition of untreated DS soil inoculated with *S. Typhimurium* 14028s (10⁶ CFU (g dw)⁻¹) was analysed and compared with untreated DS soil using 16S rRNA gene amplicon sequencing. We

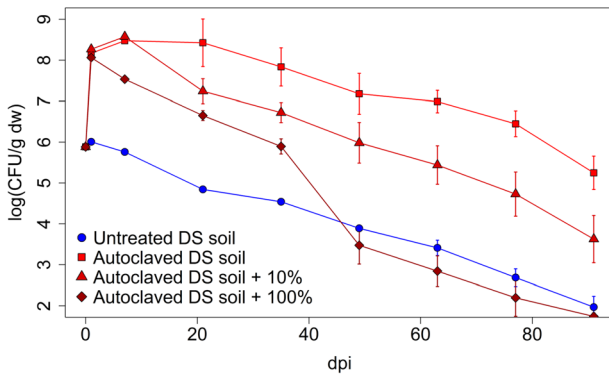


Fig. 2. Influence of the diversity on the persistence of *Salmonella* in soil. Number of colony-forming units (CFU) (g dw)⁻¹ was calculated in untreated DS soil (blue lines, circles) and autoclaved DS soil (red lines), using XLD medium supplemented with rifampicin. The autoclaved soil was supplemented with no (rectangles), 10% (triangles) or 100% (diamonds) of the indigenous soil community diversity at the same bacterial abundance. The *Salmonella enterica* serovar Typhimurium strain 14028s was inoculated on day 0. The *Salmonella* CFU counts are plotted for days post-inoculation (dpi). Error bars represent the standard deviation ($n = 4$). Slopes of linear regressions were analysed by a generalized linear model using the mixed procedure (Supporting Information Table S1).

harvested samples before *Salmonella* inoculation (day 0), on day 7 (*Salmonella* successfully persisted in the soil) and on day 91 (*Salmonella* detected only in autoclaved DS soil). Between 5716 and 114,843 sequence reads were obtained per sample and assigned to 2390 OTUs. The prokaryotic phyla on day 0 represent the initial composition of the prokaryotic community in untreated DS soil (Fig. 3A and Supporting Information Table S3), this composition changed during the experiment, while the inoculation of *Salmonella* had no impact on the community composition and diversity (Figs 4, 5 and Supporting Information Table S4). Nonetheless, some taxonomic groups responded to the presence of *Salmonella*, though without influencing the most abundant groups in these communities (Fig. 3B).

These results revealed that the presence of *S. Typhimurium* 14028s in untreated DS soil had no impact on the overall prokaryotic community structure.

Salmonella has the ability to influence prokaryotic communities with reduced diversity

Furthermore, we tested if findings observed in untreated DS soil hold true for the autoclaved DS soil. Indeed, even if the number of sequence reads obtained directly after autoclaving was not sufficient for further analyses (data not shown), the soil prokaryotic diversity was significantly reduced also 7 and 91 days after autoclaving, if compared with the untreated DS soil (Figs 4, 5 and Supporting Information Table S4). We also observed

drastic changes in prokaryotic community composition (Fig. 3 and Supporting Information Table S3). Most importantly, the presence of *Salmonella* reduced the prokaryotic diversity in autoclaved soil, as revealed by the alpha-diversities and ANOSIM (Figs 4, 5 and Supporting Information Table S4). This difference was more pronounced at 7 dpi (Fig. 4 and Supporting Information Table S4). An additional analysis of the diversity using only OTUs not assigned to *Salmonella* led to the same result, indicating that the influence is independent of the abundance of *Salmonella* itself (Supporting Information Fig. S3 and Table S4). Taken together, the effect of *Salmonella* on the diversity of the community was visible only in autoclaved DS soil (Figs 4 and 5).

Because of the differences in the prokaryotic diversity between the treatments, an ANOVA with post-hoc Tukey was performed with the aim to identify the influencing factors. In contrast to the inoculation with *Salmonella* and the time after inoculation, autoclaving had the strongest impact on the alpha-diversity (Fig. 4). Nevertheless, the time passed after inoculation had an influence on the species richness in both sample types, with and without *S. Typhimurium* 14028s in autoclaved DS soil, but not in untreated DS soil. The Pielou's evenness was influenced only in samples with *S. Typhimurium* 14028s (Supporting Information Table S2). Additional diversity analysis indicated higher variations between the replicates of samples with autoclaved DS soil (Fig. 5). While the untreated DS soil samples with and without *Salmonella* clustered together, a clear distinction between the autoclaved DS soil with *Salmonella* or without *Salmonella* was visible. This difference was more pronounced at 7 dpi, suggesting that the influence of *Salmonella* abundance on the diversity decreased over time (Fig. 5).

When analysing the taxonomic composition of autoclaved DS soil, we observed that *Proteobacteria* was the major phylum one week after the inoculation with *Salmonella* (Fig. 3 and Supporting Information Table S3). The high abundance of *Proteobacteria* could not be explained by the introduced *Salmonella* alone (see also below). In contrast, *Firmicutes* was the most abundant phylum in samples without *Salmonella*. Here, most of the OTUs belonged to the genus *Bacillus* (Fig. 3 and Supporting Information Table S3). Interestingly, the abundance of the genus *Tumebacillus* was increased in autoclaved DS soil as well as the genera *Arthrobacter* and *Streptomyces* from *Actinobacteria*. In contrast, the genus *Adhaeribacter* of the phylum *Bacteroidetes* was present in very high relative abundance in the autoclaved DS soil samples with *Salmonella* (Fig. 3B). Taken together, the presence of *Salmonella* had a significant impact on the

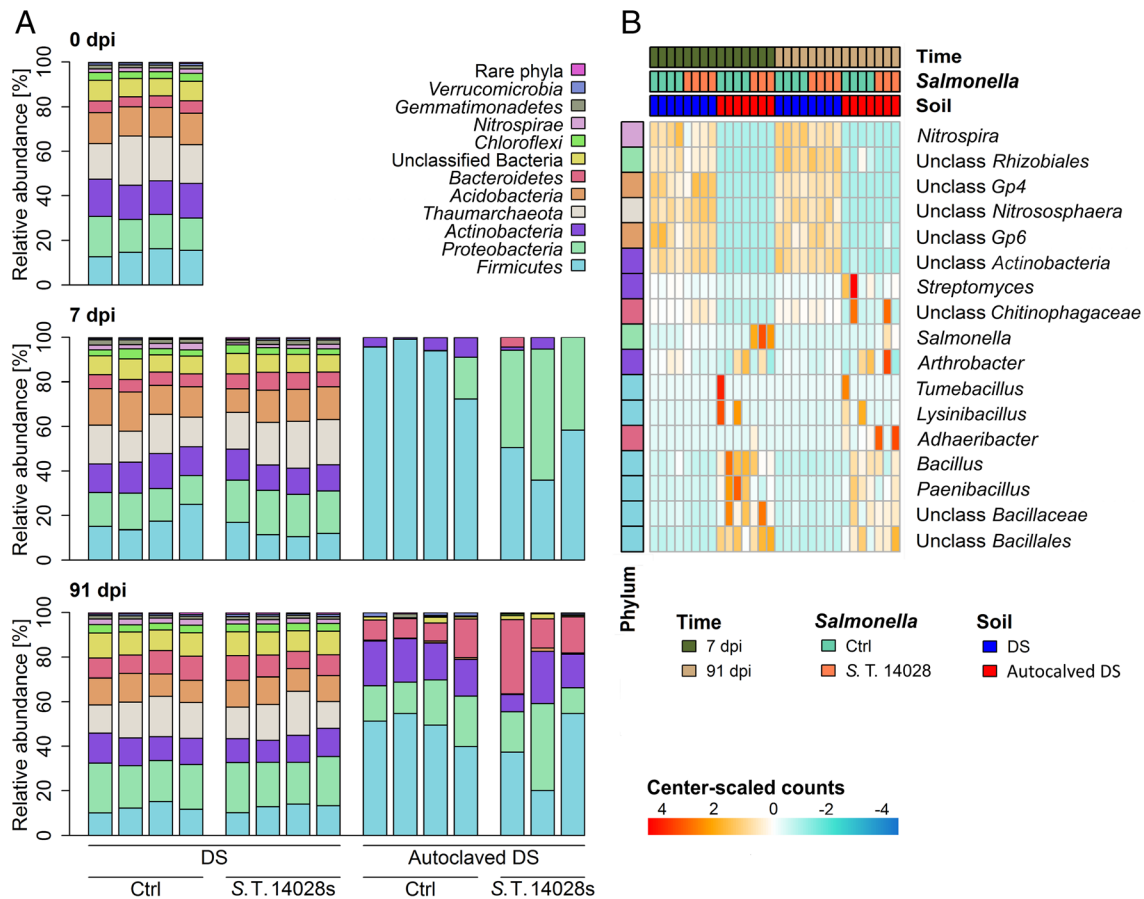


Fig. 3. Taxonomic distribution of microbial phyla and treatment responding groups in untreated and autoclaved DS soil. Diluvial sand soil was used as untreated soil (DS) or autoclaved (Autoclaved DS) and inoculated with *Salmonella enterica* serovar Typhimurium strain 14028s (S. T. 14028s) on day 0. $MgCl_2$ (10 mM) was added in mock controls (Ctrl). Total DNA was sampled immediately before inoculation or 7 and 91 days post-inoculation (dpi). The relative abundance was calculated as percentage of 16S rRNA gene sequences belonging to a particular phylum in each sample (A). The abundance of significantly responding taxonomic groups (with a p -value of < 0.05) was represented using a colour-code (centre-scaled counts). Negative binomial regression and generalized linear model fit and a likelihood ratio test post-hoc false discovery rate multiple correction tests were used. The colour code shows the relative abundance of each taxa (B). Each experimental variant was represented by at least three replicates.

composition of the prokaryotic community in previously autoclaved DS soil.

Salmonella responds to the soil environment and changes its transcriptional profile

The persistence in soil motivated us to address the question on how *Salmonella* responds to the soil environment. We performed a transcriptome analysis of *S. Typhimurium* 14028s exposed for 24 h to the mineral medium (mock control) and untreated DS soil or autoclaved DS soil suspensions. The analysis revealed that the expression of 447 genes was higher in soil suspensions compared with mineral medium of which 84 were upregulated exclusively in untreated DS soil suspension, 80 in autoclaved DS soil suspension and 283 in response to both soil suspensions (Fig. 6). The

expression of 136 genes was downregulated in samples exposed to soil suspensions compared with samples exposed to mineral medium, of which 34 were downregulated in samples exposed to DS soil suspension, 8 in samples exposed to autoclaved DS soil suspension and 94 in both soil suspensions (Fig. 6). Among the upregulated genes, in samples exposed to both types of soil suspension compared with the mineral medium, genes assigned to translation and peptide biosynthesis were highly enriched (Supporting Information Table S5). The difference between samples exposed to autoclaved and untreated DS soil was also striking. In particular, the enrichment of genes related to gene ontology (GO) terms associated with the biosynthesis of amino acids was prominent. Genes assigned to GO terms connected to the metabolism of glutamine and alpha-amino acids were enriched in response to

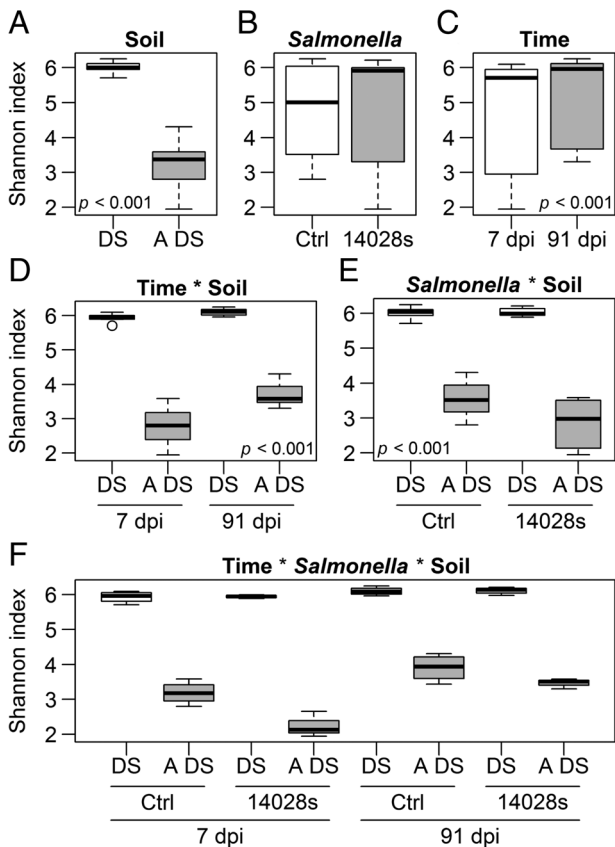


Fig. 4. Effects of soil treatment, *Salmonella* inoculation and time on the alpha diversity. Diversity in the prokaryotic community is represented by the Shannon index. The effects of autoclaving the soil (A), the absence or presence of *Salmonella* (B), time (C), soil type independent of time (D), soil independent of *Salmonella* (E) and soil type independent of *Salmonella* and time (F) were calculated. The significance was tested using one-way ANOVA with post-hoc Tukey. Boxes represent the interquartile range between the first and the third quartile and the lines mark the median. Abbreviation: DS, untreated DS soil; A DS, autoclaved DS soil; Ctrl, MgCl₂ control; 14028s, soil inoculated with *S. Typhimurium* 14028s).

untreated DS soil suspension (Fig. 6 and Supporting Information Table S5). In contrast, *Salmonella* exposed to suspension of autoclaved DS soil strongly upregulated the expression of genes assigned to GO terms representing synthesis of leucine, valine and isoleucine (Fig. 6 and Supporting Information Table S5). Very remarkable was the increased expression of genes linked to the glyoxylate cycle in *S. Typhimurium* 14028s exposed to autoclaved DS soil suspension, a variation of the tricarboxylic acid cycle (Fig. 6 and Supporting Information Table S5). No enrichment of genes related to specific GO terms was found for down-regulated genes (Supporting Information Table S5).

In summary, the transcriptional pattern of *S. Typhimurium* 14028s indicated a distinct response to different soil environments.

Preadaptation to the soil environment allows a better persistence in soil

Changes in the transcriptome observed in response to the soil-related environment further encouraged us to ask if soil pre-adapted bacteria exhibit different physiological properties. To answer this question, *Salmonella* was pre-adapted to autoclaved DS soil for 180 days and thereafter soil with the pre-adapted *Salmonella* was used to inoculate untreated DS soil in different ratios. *Salmonella* grown in Luria Bertani (LB) medium and inoculated into untreated DS soil was used as control. Similar to previous observations (Fig. 1), the CFU counts of LB-originated *Salmonella* declined from initial 10⁴ to 10² CFU (g dw)⁻¹ within 35 days. If compared with this treatment, bacteria which were previously pre-adapted to soil showed a better persistence in untreated DS soil. Regardless of the used inoculation ratios, *Salmonella* persisted at 10³–10⁴ CFU (g dw)⁻¹ over the experimental period of 35 days (Fig. 7). In summary, these results support the notion that *Salmonella* is able to change its physiology in order to better persist in this environment and to establish in soil.

Discussion

Despite the fact that cases of fresh produce-related salmonellosis are continuously increasing in Europe (EFSA and ECDC, 2017), very little is known about the ecology of *S. enterica* in agricultural systems. Our study showed that *S. Typhimurium* 14028s, LT2, as well as *S. Senftenberg* were able to persist in agricultural soil with reduced diversity for several months. The analysis of factors that influence the survival of *Salmonella* is crucial to understand its persistence in the soil environment. We designed here a soil inoculation experiment with defined and stable humidity, temperature and pre-growth conditions in order to study an additional factor, namely, the microbial diversity in soil. The diversity of the soil microbiota has been demonstrated to influence the survival of *E. coli* via its suppressive or supportive abilities (van Elsas *et al.*, 2012; Ma *et al.*, 2013; Mallon *et al.*, 2015). We hypothesized that severe reduction of microbial diversity will improve the invasion and persistence of *S. enterica* in soil.

This severe reduction of the microbial diversity was achieved by autoclaving, whereby some microorganisms survived and repopulated the soil. The physical and chemical characteristics of soil are altered by autoclaving of the soil (Berns *et al.*, 2008). However, the influence of the bacterial community was clear. We observed a long-lasting enhanced persistence of *Salmonella* in autoclaved DS soil compared with untreated soil in all three experiments. The notion of a reduced prokaryotic community

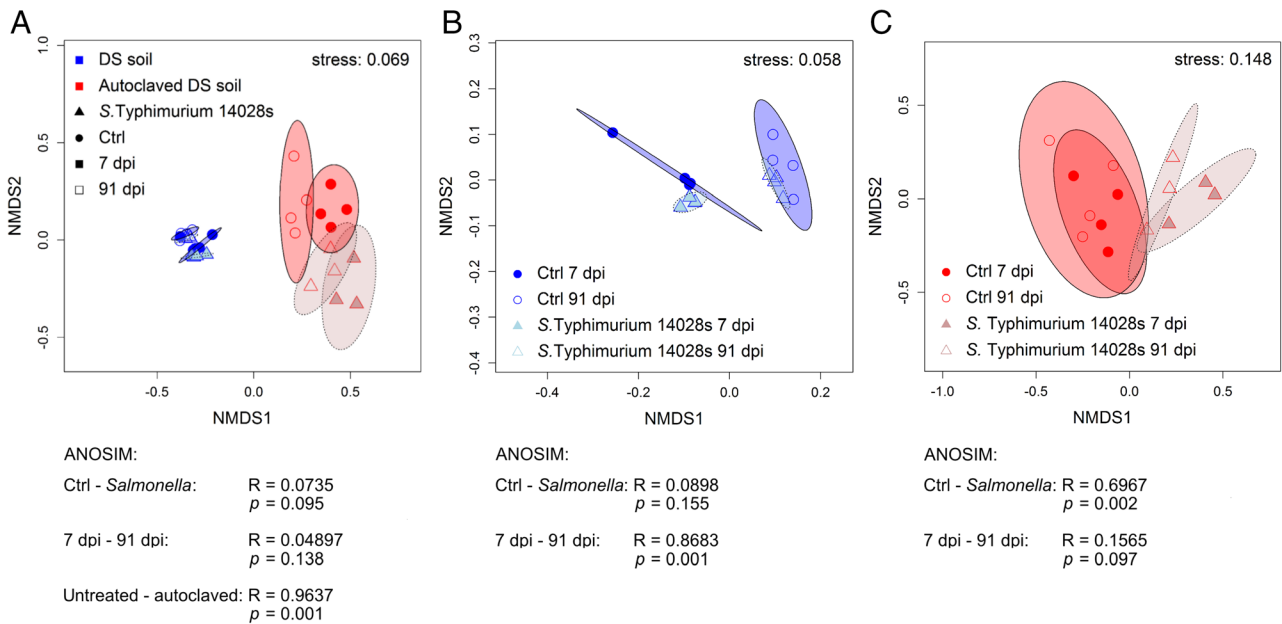


Fig. 5. Non-metric multidimensional scaling (NMDS) of the microbial communities found in untreated and autoclaved DS soil. Community composition, based on the 16S rRNA gene amplicon sequencing (Bray–Curtis community dissimilarities based on OTUs), was assessed in untreated DS soil (A and B blue symbols) and autoclaved DS soil (A and C red symbols), 7 days (closed symbols) and 91 days (open symbols) days post-inoculation (dpi) with *S. Typhimurium* 14028s (triangles). MgCl₂ (10 mM) was used as mock control (circles). The ellipses show 95% confidence intervals. The differences between controls and samples containing *Salmonella*, untreated soil and autoclaved soil and samples were taken after 7 and 91 days were tested using ANOSIM in R 3.4.0 (R Foundation, Austria) with the ‘vegan’ package.

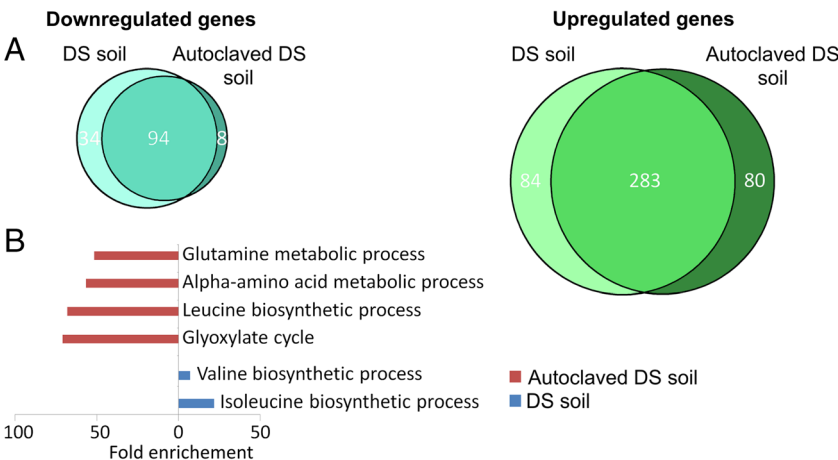


Fig. 6. Differential expressed genes and enriched gene ontology (GO) terms. Transcriptional analysis of *Salmonella* genes differentially expressed during incubation (24 h) in different conditions: Untreated DS soil suspension (DS soil); autoclaved DS soil suspension (Autoclaved DS soil); or in mineral medium, used as a control. RNA-Seq approach was performed using the HiSeq Illumina platform. The Euler diagram shows significantly down-regulated genes (teal) and upregulated genes (green) compared with mineral medium ($q < 0.05$) (A). The bar plot shows GO-terms that were significantly enriched in one treatment compared with the other (false-discovery rate (FDR) < 0.05) (B). Enriched genes connected to specific GO terms were identified using the PANTHER platform using Fisher’s exact test with FDR.

effect was strengthened by the observed enhanced persistence in soil inoculated with only 10% of the microbial community but the same total bacterial abundance. In this case, the observed effect was intermediate between a reduced and natural diversity. Our results indicate that different, possibly low abundant microbial populations, may suppress the persistence of *S. enterica* in an additive manner. Additional factors influencing the persistence of *Salmonella* might be the release of nutrients from dead cells and the reduced number of bacteria-grazing Protozoa after autoclaving. In addition, the

presence of antibiotic-producing bacteria, such as *Arthrobacter* or *Streptomyces*, in autoclaved soil is very intriguing. The produced antibiotics could affect the persistence of other bacteria (and *Salmonella*) and therefore reshaped the community structure. Different *Streptomyces* were already reported to be reduced in the presence of *Salmonella* in soil (Turpin *et al.*, 1992). While *Arthrobacter* was found to be enriched in all samples, *Streptomyces* were only enriched in autoclaved DS samples without *Salmonella*, raising the possibility of a reciprocal effect between *Streptomyces* and *Salmonella*.

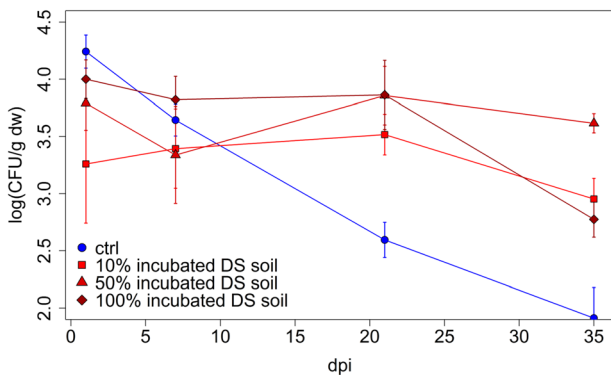


Fig. 7. Persistence of preadapted *Salmonella* in soil. Number of colony-forming units (CFU) (g dw)⁻¹ was calculated in DS soil, which contained *Salmonella* for 180 days before the experiment starts. Fresh DS soil was inoculated with either *Salmonella* obtained from LB plates adjusted to the calculated CFU (blue lines, circles) or with DS soil, which contained *Salmonella* for 180 days in different ratios. DS soil was incubated for 180 days (red lines) with *Salmonella enterica* serovar Typhimurium strain 14028s prior the experiment and used to inoculate fresh DS soil (at day 0) with 10% (rectangles), 50% (triangles) or 100% (diamonds) of the *Salmonella*-containing soil. *Salmonella* CFU was enumerated using XLD medium supplemented with rifampicin. The *Salmonella* CFU counts are further plotted for dpi. Error bars represent the standard deviation ($n = 4$). Slopes of linear regressions were analysed by a generalized linear model using the mixed procedure (Supporting Information Table S1).

Firmicutes were present in high abundance 7 days after autoclaving the DS soil. The ability to produce endospores is advantageous, as one of the multiple survival mechanisms upon physical and chemical stresses (Reineke *et al.*, 2013).

In the animal host, *Salmonella* encounters and adapts to different environmental conditions like temporary acidity, digestive processes of the host and nutritional limitations due to the nutrient uptake by the host (Dandekar *et al.*, 2014). Similar in a soil environment, *Salmonella* needs to adapt to low nutrient availability. Analysis of short-term transcriptional changes between *S. Typhimurium* 14028s exposed for 24 h to suspensions of autoclaved or untreated DS soil compared with mineral medium revealed multiple changes in its gene expression pattern. Different water-soluble compounds in the soil suspensions are probably key factors. The expression of genes involved in translation, more precisely protein metabolism and peptide biosynthesis were considerably increased in *S. Typhimurium* 14028s exposed to either DS soil suspensions, untreated or autoclaved. While more general cellular functions were upregulated in both treatments, the majority of differentially expressed genes between the autoclaved and the untreated soil treatment were mainly assigned to amino acid biosynthesis processes. Genes involved in the biosynthesis of glutamine and alpha-amino acids were strongly upregulated when cells were exposed to untreated DS soil suspensions, genes responsible for

leucine valine and isoleucine biosynthesis were strongly regulated in cells exposed to suspension of autoclaved DS soil. Among the alpha-amino acid-related genes, three genes were assigned to the synthesis of tryptophan (*trpCBD*). The tryptophan metabolism is required for effective biofilm formation on hard surfaces (Hamilton *et al.*, 2009). When exposed to DS soil suspension, the enhanced tryptophan synthesis might provide an advantage for *Salmonella* to cope with this distinct environment. Besides tryptophan, also expression of genes controlling glutamine biosynthetic processes was upregulated in *S. Typhimurium* 14028s exposed to untreated soil. Furthermore, genes matching glyoxylate metabolism-related GO terms were enriched after exposure to autoclaved DS soil suspension compared with suspension of untreated DS soil. Wang *et al.* (2010) demonstrated the importance of amino acid acetylation for the regulation of carbon metabolic processes in *Salmonella* and particularly the branching between citrate cycle and the glyoxylate bypass. The glyoxylate cycle allows utilizing simple carbon compounds as a carbon source, in a case where more complex sources, such as starch, cellulose, cellobiose, chitin and so on are not available. We assume that the regulations of amino acid biosynthesis and glyoxylate metabolic processes in soil-related environments are interconnected and involved in the adaptation to this ecosystem.

In this study, we show that such an adaptation can take place when untreated DS soil was inoculated with soil containing previously adapted *Salmonella*. However, such phenomena could be analysed from different perspectives. For example in the case of *Pseudomonas fluorescens*, the community shifts were stronger when bacteria were soil pre-adapted (Gómez *et al.*, 2016). It appears that also the soil community changes during inoculation with foreign bacteria. Mallon *et al.* (2018) proposed a legacy effect of the soil community, even after an unsuccessful establishment scenario. Several reports hypothesized that particularly *Proteobacteria* may occupy niches otherwise taken by *Salmonella*. Cooley *et al.* (2003) investigated the influence of soil autoclaving on the colonization of *Arabidopsis thaliana* with *Salmonella* Newport and *E. coli* O157:H7. The authors found that the *Gammaproteobacterium Enterobacter asburiae* suppressed epiphytic growth of *S. enterica* under sterile conditions. In contrast, in our study, *Alphaproteobacteria* were found to be more abundant in all samples with untreated soil compared with samples with autoclaved soil, *Rhizobiales* being the most abundant order. The results showed, that parts of the community were shifted, which is in line with previously published reports (Horňák and Corno, 2012; Mallon *et al.*, 2018). Taken together, a legacy effect which is in line with the enhanced persistence in soil with low microbial diversity together with the

pre-adaptation of *Salmonella* could represent the essential pre-conditions for the successful invasion. However, the used methods have some limitations. Although, we could not amplify sufficient amounts of 16S rRNA gene amplicon sequences directly after autoclaving, suggesting that the 16S rRNA of dead cells is degraded to a high extent, we cannot exclude that 16S rRNA gene amplicon sequences might originate from dead cells. The prevention of strong perturbation seems therefore to be crucial in order to avoid contamination of crops in field.

Our results demonstrated that *S. enterica* adapts to agricultural soil conditions and may survive for several months, depending on the resident soil microorganisms. In untreated soil, particular microorganisms prevented the establishment of *Salmonella* competing for nutrients or *via* negative interactions (e.g. antimicrobials). As a very promising strategy to prevent the colonization of crop plants in the field, we therefore suggest to sustain the diversity and activity of the soil microbiota. Thus, preserving the diversity and activity of the soil microbiota is an important aspect contributing to soil, plant and human health, as highlighted in the one health concept.

Experimental procedures

Salmonella strains used in this study

Salmonella enterica serovar Senftenberg (*S. Senftenberg*; Elviss *et al.*, 2009) was obtained from Nicola Holden, The James Hutton Institute, Dundee, and John Coia SSSCDRL, Glasgow, Scotland, UK. The *Salmonella enterica* serovar Typhimurium strain 14028s (*S. Typhimurium* 14028s) was kindly provided by Isabelle Virlogeux-Payant, INRA, Centre Val de Loire, France. *Salmonella enterica* serovar Typhimurium LT2 DSM 18522 (*S. Typhimurium* LT2) was obtained from the DSMZ, Braunschweig, Germany. Spontaneous rifampicin-resistant mutants were isolated from LB agar supplied with rifampicin (50 mg l^{-1}). *Salmonella* was cultured over-night in Luria Bertani (LB) broth (Carl Roth, Germany) supplemented with rifampicin (50 mg l^{-1}) at 37°C until the stationary phase, centrifuged at $1500g$ for 10 min and washed twice with 10 mM MgCl_2 prior inoculation.

Soil inoculation experiments

Soil microcosm experiments were performed with 25 or 50 g of the soil, filled into 50 ml conical tubes or 100 ml flasks, respectively. Sandy soil, an arenic-luvisol (Rühlmann and Ruppel, 2005) with minor amounts of silty sand and 5.5% clay (diluvial sand, DS), sieved through 2–4 mm was used. The soil parameters can be found in Schreiter *et al.* (2014). To reduce the microbial diversity and abundance of resident microbes, autoclave bags were filled with DS soil and autoclaved (121°C , 20 min)

three times with 1 day in between each autoclaving event, resulting in a soil with a severe reduced microbial community. Subsequently, the soil was adjusted to 50% of its maximum water holding capacity (WHC_{max}), resulting in final water content of 112 ml kg^{-1} soil (Schreiter *et al.*, 2014). In the first experiment, soil was inoculated with *Salmonella* cells resuspended in 10 mM MgCl_2 to obtain final cell-counts of $10^6 \text{ CFU (g dw)}^{-1}$ and incubated in closed bottles at 20°C in four different treatments: (i) untreated DS soil without *Salmonella*, (ii) untreated DS soil with *Salmonella*, (iii) autoclaved DS soil without *Salmonella* and (iv) autoclaved DS soil with *Salmonella*. Each treatment was represented by quadruplicates in three independent repetitions. The first repetition was conducted over a time course of 161 days, the second and third over 147 days.

In the second experiment, 20 g soil was autoclaved and reinoculated with a suspension of microbiota isolated from the same amount of DS soil (20 g) or 10% of the DS soil (see below). For all treatments, DS soil was inoculated with *Salmonella* cells resuspended in 10 mM MgCl_2 to obtain final bacterial cell-counts of $10^6 \text{ CFU (g dw)}^{-1}$ and incubated in closed bottles at 20°C in eight different treatments: (i) untreated DS soil without *Salmonella*, (ii) untreated DS soil with *Salmonella*, (iii) autoclaved DS soil without *Salmonella*, (iv) autoclaved DS soil with *Salmonella*, (v) autoclaved DS soil reinoculated with 10% community without *Salmonella*, (vi) autoclaved DS soil reinoculated with 10% community with *Salmonella*, (vii) autoclaved DS soil reinoculated with 100% community without *Salmonella* and (viii) autoclaved DS soil reinoculated with 100% community with *Salmonella*.

Next, 180 days after the start of the first experiment, the *Salmonella* containing soil was used to inoculate untreated soil in the third experiment. As a control DS soil was inoculated with *Salmonella* cells resuspended in 10 mM MgCl_2 to obtain final cell-counts of $10^4 \text{ CFU (g dw)}^{-1}$ adjusted to the calculated CFU in the samples from the first experiment and all samples were incubated in closed bottles at 20°C in four different ratios (*Salmonella* containing soil/untreated soil): (i) 0/10 (inoculated control), (ii) 1/9, (iii) 5/5 and (iv) 10/0. The bottles were filled to about one-third v/v and opened under sterile conditions every 14 days to ensure aeration.

Extraction of microbiota from soil

For the preparation of the inoculum used in the second soil microcosm experiment, the water-extractable fraction was used. DS soil was mixed with 4 mm glass beads (Karl Hecht, Germany) and sterile water in the ratio 2:1:2 and shaken for 24 h at 200 rpm. The supernatant was transferred to 50 ml conical tubes and centrifuged at $1000g$ for 15 min twice and subsequently at $3500g$ for

30 min. The supernatant was then discarded and the precipitate was used as inoculum. For the 100% inoculum, the microbiota isolated from 100 g soil was reinoculated in 100 g autoclaved soil. For the 10% inoculum, the microbiota of 10 g was reinoculated in 100 g soil, incubated for 7 days at 20°C, extracted again and reinoculated in 100 g soil to ensure a similar total abundance. In order to verify the cell density, the CFU number of total culturable bacteria was assessed on R2A plates incubated for 1 day.

Quantification of Salmonella with cultivation-independent and -dependent methods

To enumerate *Salmonella*, every second week 1 g of soil was transferred to 50 ml conical tubes and suspended in 9 ml of 10 mM MgCl₂ by vortexing. Quadruplicates of serial dilutions were dropped on R2A agar (Merck, Germany), supplemented with cycloheximide (100 mg l⁻¹), to estimate the total culturable aerobic CFU counts and XLD (Carl Roth), supplemented with rifampicin (50 mg l⁻¹), to determine the CFU counts of *Salmonella*. The CFU counts were determined after incubation at 20°C (R2A for total culturable bacteria) and 37°C (XLD for *Salmonella*) for 24 h. For verification of the results and evaluation of a possible VBNC state of *Salmonella*, a cultivation-independent enumeration was performed. Total DNA was extracted from 0.5 g soil on days 7 and 91 in the second repetition using the FastDNA SPIN Kit for Soil and further purified using the GENECLEAN SPIN Kit (MP Biomedicals, Germany) according to the manufacturer's instructions. The *Salmonella*-specific gene *invA* was quantified by qPCR as described by Zimmermann (2014). The *invA* fragment from the strain LT2, cloned into the pGEM-T vector (Promega, Germany), was used as quantification standard. The amplification efficiencies were between 82.1% and 98.0% and the *R*² values were between 0.991 and 1. Possible inhibitions were not tested. Both quantification methods were adjusted to the dry mass of the samples. We calculated a specific correction factor to estimate the density of *Salmonella* in our samples. The gene copy number and the CFU counts were compared immediately after soil inoculation with *Salmonella*. Since the obtained gene copy number was significantly lower than the corresponding CFU counts, we adjusted the qPCR results to the CFU counts for each *Salmonella* strain and used the resulting factor in the following calculations (Supporting Information Fig. S1).

Analysis of the prokaryotic diversity using 16S rRNA gene amplicon sequencing

For 16S rRNA gene amplicon sequencing, we used samples from the second soil inoculation experiment, collected

on days 0, 7 and 91 and extracted DNA as described above. The 16S rRNA gene sequences were amplified using primers flanking the V3 and V4 region (Sundberg *et al.*, 2013) and spacer and adapter barcode tags were added (Nunes *et al.*, 2016). Products with sizes smaller than 200 bp were removed using Agencourt AMPure XP beads (Beckman Coulter) according to manufacturer's instructions. The samples were pooled and adjusted to equimolar concentrations, using the DNA Clean and Concentrator-5 kit (Zymo Research). High-throughput amplicon sequencing, 2 × 250 bp, paired-end using the MiSeq platform (Illumina) was carried out following manufacturer's instructions. Raw sequence reads were first trimmed of primer sequences using cutadapt (Martin, 2011) and read pairs for which both primers were found are retained for subsequent analysis. The sequences were then merged, clustered in OTU using UPARSE (Edgar, 2013) with 97% pairwise sequence similarity threshold and chimeras were removed using UCHIME (Edgar *et al.*, 2011). The taxonomic annotation was performed using mothur 'classify.seqs' and 'method = wang' with a minimum bootstrap probability of 0.8 (Schloss *et al.*, 2009) against RDP database trainset 16 (Cole *et al.*, 2014). In total 2390 OTUs were identified. Between 5716 and 114,843 sequences were retrieved per sample. The alpha-diversity of the samples was calculated using species richness, Pielou's evenness and Shannon index, where the total reads were rarified to 5716, repeated 100 times and averaged. NMDS was conducted using the 'vegan' package in R 3.4.0 (R Foundation, Austria). The raw sequences were deposited in the Sequence Read Archive database (NCBI) with the Bioproject ID PRJNA510723. The raw 16S sequences were deposited in the Sequence Read Archive database (NCBI) with the Bioproject ID PRJNA510723.

Determination of Salmonella transcriptomes in different environments using RNA-Seq

To determine the transcriptional activity of *S. Typhimurium* 14028s in soil-related environments, we designed a new experimental approach using suspension of untreated or autoclaved DS soils (see below) and compared the transcriptional activity in each soil suspension to the activity in a mineral medium in which *Salmonella* was able to persist without changes in the CFU counts over 24 h. *Salmonella* was adjusted to OD_{600nm} = 1. Two millilitres of this suspension were pipetted into cellulose ester dialysis tubes with a pore size of 100 kDa (Spectrum Europe, The Netherlands). Closed dialysis tubes were placed in 50 ml conical tubes containing: (i) 30 ml of a mineral medium (1× concentrated M9 salts (Sigma-Aldrich, Germany), 2 mM MgSO₄ and 1.23 mM glucose in sterile deionized water); (ii) 10 g untreated DS soil and 20 ml MgCl₂; or (iii) 10 g autoclaved DS soil and 20 ml MgCl₂. All treatments were

performed in triplicates. The conical tubes were incubated at 28°C for 24 h while shaking at 180 rpm. Thereafter, the total RNA in 2 × 0.5 ml from each dialysis tube was stabilized using RNeasy Protect and extracted using the RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. The rRNA was removed using the Ribominus Kit (Thermo Fisher, Germany) with some modifications concerning the specificity of *Salmonella* 23S rRNA (Mühlig *et al.*, 2014). For the fragmentation of RNA, a Covaris S220 Focused-ultrasonicator (180 s, 175 W peak power, 10% putty, 200 cycles) was used. The fragments were dephosphorylated and phosphorylated using antarctic phosphatase (NEB, Germany), SUPERase In and T4-PNK (Thermo Fisher) to gain a common phosphorylation state for all fragments. The library preparation was conducted with the TruSeq Small RNA Library Prep Kit (Illumina) according to the manufacturer's instructions using the SuperScript IV cDNA synthesis Kit (Thermo Fisher). The RNA sequences were deposited in the Gene Expression Omnibus database (NCBI) with the accession number GSE132633 (GSM 3884781-GSM3884783 for MM, GSM3497443-GSM3497445 for untreated DS soil, GSM3497458-GSM3497460 for autoclaved DS soil).

Bioinformatics analysis of the transcriptome sequencing

The sequence (50 bp single-end Illumina HiSeq) analyses were performed using Bowtie2 (Version 0.6), cufflinks (Version 2.2.1.0), cuffmerge (Version 2.2.1.0), cuffquant (Version 2.2.1.1) and cuffdiff (Version 2.2.1.5; Langmead *et al.*, 2009; Trapnell *et al.*, 2010), where the FPKM (Fragments Per Kilobase Million) and the significant differences between transcriptional profiles of *S. Typhimurium* 14028s exposed to soil suspensions and mineral medium were calculated based on q -value < 0.05 and change of twofold (Supporting Information Table S6). Significantly enriched genes related to specific GOs were identified with the web-based tool PANTHER (<http://geneontology.org>) using the PANTHER Overrepresentation Test (Released 2019-07-01), *Salmonella* Typhimurium (all genes in database), GO biological process complete and Fisher's exact test with false discovery rate (FDR). The Euler diagrams were created with R 3.4.0 (R Foundation, Austria) using the 'eulerr' package.

Statistical analysis

For the statistical analysis of inoculation experiments, we used ANOVA with post-hoc Tukey conducted with R 3.4.0 (R Foundation, Austria) using the 'multcomp' package (Hothorn *et al.*, 2008). Slopes of linear regressions of *Salmonella* CFU were analysed by a generalized linear model using the mixed procedure (SAS 9.4; SAS institute Inc., Cary, NC). Tukey tests were performed with the

procedure glimmix of SAS. The microbial community composition was statistically analysed with generalized linear model fit and a likelihood ratio test post-hoc false discovery rate multiple correction test, and the statistical analysis of the NMDS plots was performed with ANOSIM using the 'vegan' package in R 3.4.0 (R Foundation, Austria). The comparison between CFU counts and the qPCR approach was analysed with Student's t -test.

Acknowledgements

We thank Nicola Holden and John Coia for providing us the *S. enterica* serovar Senftenberg strain. We thank Lena Rauch for the development of the mineral medium. S.J. and J.S. were funded by the Federal Office for Agriculture and Food (Bundesanstalt für Landwirtschaft und Ernährung, BLE), Grants 13HS026 and 13HS029.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors contributions

J.S., S.J., R.G., K.S., A.S. designed the concept. J.S., S.J., A.S. performed the experiments and laboratory work. J.S., S.J., S.J.S, J.N. processed the sequences. K.N. helped in transcriptome sequencing. J.S. and J.N. analysed the data. J.S. drafted the manuscript. All authors read and approved the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1: Comparison between cultivation dependent and independent methods. Quadruplicates of serial dilutions were dropped on XLD agar supplemented with rifampicin (50 mg l⁻¹) to determine the CFU counts of *Salmonella*. The colonies were counted after 24 h incubation at 37°C. For the cultivation-independent enumeration of *Salmonella*, total DNA was extracted and purified. The *Salmonella* specific gene *invA* was quantified by real-time PCR. The red dotted line symbolizes the limit of quantification in the cultivation dependent method. qPCR values were adjusted to the CFU

counts measured before and the resulting correction factor was used for further calculations.

Figure S2: Enumeration of total culturable bacteria

The number of colony-forming units (CFU) per g dw (log CFU per g soil) was calculated for untreated DS soil (blue) and autoclaved DS soil (red). R2A medium with cycloheximide was used. Plates were incubated for 24 h at 28°C. The CFU counts are plotted against time days post-inoculation (dpi). Error bars represent the standard deviation ($n = 4$). Slopes of linear regressions were analysed by a generalized linear model using the mixed procedure (Supporting Information Table S1).

Figure S3: Non-metric multidimensional scaling (NMDS) of the microbial community composition excluding OTUs assigned to *Salmonella*. Community composition, based on the 16S rRNA gene amplicon sequencing, was assessed in autoclaved DS soil (red symbols), untreated DS soil (blue symbols), 7 days (closed symbols) and 91 days (open symbols) post-inoculation with *S. Typhimurium* 14028s (triangles). MgCl₂ (10 mM) was used as mock control (circles). The OTUs assigned to *Salmonella* were manually removed before the analysis. The ellipses show 95% confidence intervals. The Bray–Curtis community dissimilarities are based on OTUs from 16S rRNA gene amplicons. ANOSIM was performed using the 'vegan' package in R 3.4.0 (R Foundation, Austria).

Table S1: Statistical analysis of the CFU number in soils during the experiments performed using Tukey test. Number of colony-forming units g⁻¹ dry soil (log CFU g⁻¹ dw⁻¹) was calculated in different experiments and plotted against days post-inoculation (dpi). The resulting slopes were compared with each other using Tukey test. Different small letter, indicated in bold, represents differences in estimate values at $p < 0.05$. The analysis of *Salmonella* persistence in untreated DS soil and autoclaved DS soil (Fig. 1) is presented in A, the slopes of all repetitions were used in this analysis; Impact of microbial diversity (Fig. 2 and Supporting Information Fig. S2) was analysed in B; Effect of adaptation to soil environment (Fig. 7) was analysed in C.

Table S2: Analysis of Variance (ANOVA) with post-hoc Tukey HSD on the persistence of *Salmonella* in soil under different conditions. Logarithm of colony-forming units/g dry soil (log CFU (g dw)⁻¹) was calculated in untreated DS soil and autoclaved DS soil in three independent experiments. To compare the persistence of *Salmonella* in the different experiments, the slope was calculated for every sample. The relations between autoclaved and untreated soil, the three *Salmonella* strains Senftenberg, Typhimurium 14028s and LT2 for each of the three independent experiments were calculated using an ANOVA with post-hoc Tukey HSD.

Table S3: Relative abundance of prokaryotic phyla

Diluvial sand (DS) soil was used as untreated or autoclaved soil and inoculated with *S. Typhimurium* 14028s on day 0. MgCl₂ (10 mM) was added to mock control samples without *Salmonella*. Total DNA was sampled immediately before inoculation or 7 and 91 dpi. The relative abundance of phyla was calculated as percentage of 16S rRNA gene sequences belonging to a particular phylum in each sample. Each experimental variant was conducted in at least three replicas.

Table S4: Alpha-diversity in all samples and influence of the different effects. Community diversity, based on the 16S rRNA amplicon sequencing, was assessed in autoclaved DS soil, untreated DS soil, 0, 7 and 91 dpi with *S. Typhimurium* 14028s. $MgCl_2$ (10 mM) was used as control. The alpha-diversity was calculated using the OTUs and species richness (SR), Pielou's evenness (Pielou) and Shannon index (Shannon). To show, that the change of diversity was independent of *Salmonella*'s abundance, the Shannon index was calculated without *Salmonella*-assigned OTUs and compared using a *t*-test. The relations between the diversity indices in autoclaved and untreated soil, 7 and 91 dpi, and the inoculation of *Salmonella* or the mock control were calculated using an ANOVA with post-hoc Tukey HSD.

Table S5: Significantly enriched gene ontology (GO) terms in the different conditions. Transcriptional analysis (RNA-Seq) of *Salmonella* genes differentially expressed during incubation (24 h) in different conditions: untreated DS soil suspension; autoclaved DS soil suspension; or in mineral medium (MM). Enriched genes related to specific GO terms were identified using the PANTHER Overrepresentation Test using Fisher's exact test with false discovery rate (FDR) correction. The table shows all GO-terms that were significantly enriched in one treatment compared with another (FDR < 0.05).

Table S6: Differentially expressed genes. Transcriptional analysis (RNA-Seq) of *Salmonella* genes differentially expressed during incubation (24 h) in different conditions: untreated DS soil suspension; autoclaved DS soil suspension; or in mineral medium (MM).