

# Heterogeneity of Persistence of *Salmonella enterica* Serotype Senftenberg Strains Could Explain the Emergence of this Serotype in Poultry Flocks

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## Abstract

*Salmonella enterica* serotype Senftenberg (*S. Senftenberg*) has recently become more frequent in poultry flocks. Moreover some strains have been implicated in severe clinical cases. To explain the causes of this emergence in farm animals, 134 *S. Senftenberg* isolates from hatcheries, poultry farms and human clinical cases were analyzed. Persistent and non-persistent strains were identified in chicks. The non-persistent strains disappeared from ceca a few weeks post inoculation. This lack of persistence could be related to the disappearance of this serotype from poultry farms in the past. In contrast, persistent *S. Senftenberg* strains induced an intestinal asymptomatic carrier state in chicks similar to *S. Enteritidis*, but a weaker systemic infection than *S. Enteritidis* in chicks and mice. An in vitro analysis showed that the low infectivity of *S. Senftenberg* is in part related to its low capacity to invade enterocytes and thus to translocate the intestinal barrier. The higher capacity of persistent than non-persistent strains to colonize and persist in the ceca of chickens could explain the increased persistence of *S. Senftenberg* in poultry flocks. This trait might thus present a human health risk as these bacteria could be present in animals before slaughter and during food processing.

**Citation:** Boumart Z, Roche SM, Lalande F, Virlogeux-Payant I, Hennequet-Antier C, et al. (2012) Heterogeneity of Persistence of *Salmonella enterica* Serotype Senftenberg Strains Could Explain the Emergence of this Serotype in Poultry Flocks. PLoS ONE 7(4): e35782. doi:10.1371/journal.pone.0035782

**Editor:** Roy Martin Roop II, East Carolina University School of Medicine, United States of America

**Received:** August 17, 2011; **Accepted:** March 21, 2012; **Published:** April 24, 2012

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**Funding:** This work was supported by grants from the Institut National de Recherche Agronomique (INRA) and the French agency for food, environmental and occupational health safety (Anses). Zineb Boumart holds a Doctoral fellowship funded by Anses and INRA. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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## Introduction

*Salmonella* spp. are among the main causes of food-borne bacterial gastroenteritis in industrial countries. The serotypes most frequently associated with human illness are Enteritidis and Typhimurium, mainly due to the consumption of contaminated chicken eggs and meat [1–3]. Although the prevalence of these serotypes has fallen over the last decade in Europe, the emergence of some other *Salmonella* serotypes has been observed at the same time. For example, in recent years the prevalence of *Salmonella enterica* serotype Infantis among poultry and humans has increased [4,5]. This is not yet the case with *Salmonella enterica* serotype Senftenberg (*S. Senftenberg*), but the recent emergence of this serotype in poultry flocks is a cause of concern.

In poultry production in the past, *S. Senftenberg* was frequently isolated from hatcheries but gradually disappeared during animal rearing [6]. On the poultry farm, development of complex digestive microbiota [7] and maturation of the immune system, allowed *S. Senftenberg* strains to be eliminated from infected chicks. However, the traits of this serotype have changed and it has now become more frequent in animals and on poultry farms. Some strains are able to persist throughout the rearing period, thus

representing a potential risk for consumers [8–10]. This health hazard is strengthened by the existence of several intestinal and hospital-borne infections in humans linked to *S. Senftenberg* [11,12].

The aim of our study was to investigate the existence of strains with different levels of persistence in chickens which could explain the increased presence of this serotype in animals and on poultry farms, and to determine whether some strains represent a potential risk to human health. Avian isolates of *S. Senftenberg* from hatcheries and poultry farms, together with human strains were collected, and the genetic relationship between these isolates was assessed using Pulsed-Field Gel Electrophoresis (PFGE). Based on this analysis, some isolates were selected to investigate their ability to persist and colonize chickens, invade avian and human cells and induce systemic infection in mice.

## Materials and Methods

### Bacterial strains

Avian *S. Senftenberg* strains (n = 105) were obtained from monitoring programs, prevalence surveys and private laboratories. Human *S. Senftenberg* strains (n = 29) were provided by the

French National Reference Centre for *Salmonella*, Institut Pasteur, Paris.. All isolates were collected from independent sources at different dates. Possible redundant strains were discarded. The *S. Enteritidis* LA5 wild-type strain is a field isolate from infected chickens [13].

### Genetic analysis

Pulsed-Field Gel Electrophoresis of 134 isolates of *S. Senftenberg* was performed using the *Xba*I restriction enzyme following the procedure developed by the Centre for Disease Control (CDC) [14] and as previously described [15]. The Simpson index of diversity was used to calculate the ability of the method to discriminate between patterns [16].

Multi-Locus Sequence Typing (MLST) was performed on a subset of 23 selected from the 134 isolates, as previously described [17]. Data were submitted to the MLST database website (<http://mlst.ucc.ie/mlst/dbs/Senterica>).

### Animal experiments

Based on our previous genetic study, 12 strains of *S. Senftenberg* were selected to check their ability to colonize broiler chickens. Spontaneous rifampicin-resistant strains of *S. Senftenberg* (100 µg/ml) were used to facilitate the detection of the inoculated strains. To validate the use of these strains, we demonstrated that the acquisition of rifampicin resistance did not modify the virulence level of the strains. One-day-old chicks (JA957 line) were orally infected with  $10^5$  colony-forming units (CFU) of *S. Senftenberg* and 15 chicks were sacrificed every week for five weeks post-infection (p.i). Cecae were removed, homogenized in buffered peptone water, serially diluted, plated on Brilliant Green agar (BG) with rifampicin and incubated at 37°C. In the absence of growth, samples were enriched in Modified Semi-solid Rappaport Vassiliadis medium (MSRV) at 41.5°C for 24–48 h and plated on BG agar with rifampicin.

In a second set of experiments, two persistent (SS308, SS209) and two non-persistent (SS304, SS278) strains were selected from the 12 strains and used to inoculate chicks orally. Spleens and ceca were removed aseptically every week p.i. One cecum was used to determine the bacterial level and the second for histological analysis. Sera were taken from 10 infected and 10 uninfected animals every week.

The virulence potential of *Salmonella* strains was analyzed in the mouse model. Six- to eight- week-old female BALB/c mice (Janvier, France) were orally inoculated with  $5 \times 10^8$  CFU of persistent (SS209, SS308), non-persistent (SS278, SS304) and human (SS291) *S. Senftenberg* strains, or the *S. Enteritidis* LA5 strain, as previously described [18]. At three days p.i, the ileum, colon, mesenteric lymph nodes (MLN) and spleen were aseptically removed, homogenized in TSB, serially diluted and plated on *Salmonella-Shigella* agar (37°C, 24 h). Homogenates were kept overnight at 37°C for enrichment. For lethality experiments, survival was recorded twice a day and at 28 days p.i, the ileum, colon, MLN and spleen of surviving animals were removed, and bacterial colonization was assessed. Animal experiments were carried out in strict accordance with French recommendations. The protocol for the study using chicks and mice was approved by the ComEth Afssa/ENVA/UPEC (10-0032) and the Val de Loire (N 2011/10) Ethics Committee for Animal Experiments respectively.

### Histological analyses

Samples of cecal tissue in the proximal region (5 mm) were taken from six animals per group and analyzed according to microdissecting procedures, as previously described [19]. The

heights of 10 villi and the depths of 20 crypts were measured in six animals per group using an optical microscope and viewer analysis software (visilog 6.3, Noesis), as previously described [20]

### Quantification of the humoral immune response of chicks

Serum samples from infected and healthy animals were taken every week to detect immune response markers (IgM and IgY) using an ELISA assay, performed as previously described with some modifications [21]. The 96-well ELISA plates were coated with 100 µl of bacterial suspension ( $10^7$  bacteria/well) containing the four heat-inactivated *S. Senftenberg* strains (SS278, SS304, SS209, SS308). Samples were diluted and incubated in secondary antibodies, either peroxidase-conjugated Goat anti-chicken IgM or Rabbit anti-chicken IgY, and revealed using tetramethylbenzide (TMB) according to the supplier's instructions (Uptima, France). All the experiments were performed in duplicate.

### Invasion and multiplication assays

Human adenocarcinoma HT29 (ECACC n°85061109), chicken hepatocellular carcinoma LMH [22] and avian HD11 and murine J774 macrophages (ECACC n°85011428) were maintained under the conditions described previously [23,24]. Prior to infection, macrophages were activated with LPS (0.05 µg/ml) in the medium without antibiotics. Bacterial invasion and multiplication were quantified using a gentamicin protection assay, as previously described [25].

### Statistical analyses

Data from the mice experiments were analyzed using the Systat 9.0 software (SPSS Inc., USA). Differences between means were determined using a one-way analysis of variance (ANOVA) followed by a student *t*-test. Values of  $P \leq 0.05$  were considered significant.

Data from the chicken experiments were analyzed using proc glm in SAS®. For cecal colonization, we performed an analysis of variance to study the response log CFU/g in terms of the effects of day, strain and day\*strain interaction. To identify persistent from non-persistent strains, level of the day factor was determined from 19 days to 26 or 33 days p.i. The effects (day, strain and day\*strain) are significantly different from zero at the 0.05 level. Pairwise comparisons of the day\*strain effect were carried out and the p-values of hypothesis tests were adjusted using the Tukey method. The strains are clustered from the distance matrix calculated by 1 - p-values and a complete linkage as agglomerative algorithm. The heatmap and dendrogram summarize the results of the comparison tests of the means of the strains.

An ANOVA was performed on the results of spleen colonization to study the response log CFU/g in terms of the effects of day, strain and day\*strain interaction. When the interaction day\*strain was significant, the pairwise comparison was performed. In addition, we were interested by the contrast between persistent (SS308, SS209) and non-persistent (SS304, SS278) strains. Histological analysis data were analysed using Stat-View 5.0 software (Abacus concepts, Berkeley, CA, USA). Differences between the means of villus heights or crypt depths were determined using the Kruskal-Wallis test followed by the Mann-Whitney test at a 5% level of significance ( $P \leq 0.05$ ).

## Results

### Genetic analysis of *S. Senftenberg* isolates

PFGE typing of the 134 isolates resulted in 87 different patterns, with the majority grouped into 20 clusters ( $\geq 90\%$  similarity). For

the entire panel, the Simpson index of diversity was 0.99 showing the high diversity of the strains discriminated using XbaI. The 20 clusters corresponded to four groups according to the origin of the strains (Figure 1). Eleven clusters (group A) consisted mainly of avian strains isolated from hatcheries or at the beginning of the rearing period. One cluster (group D) consisted exclusively of avian strains isolated at the end of the rearing period. The PFGE analysis also enabled human isolates to be separated. Half of these were in the same cluster and were genetically close to avian strains (group C), but the other human isolates were grouped in genetically distant clusters (<67% of similarity) (group C). The other clusters consisted of strains of different origins (hatchery and poultry farm) (group B).

The MLST analysis of the 23 isolates revealed that five isolates (SS308, SS267, SS270, SS5816 and SS3845) exhibiting genetically close PFGE patterns, belong to the same sequence type (ST196). These five isolates show more than 84% of similarity, and are genetically distant from the other strains (67% of similarity). All the other strains tested, which belong to genetically distant PFGE clusters, share the same ST14 sequence type (figure 1).

### Cecal contamination level of *S. Senftenberg* strains

On the basis of the genetic relationship between the isolates and their origin (hatchery or poultry farm), 12 rifampicin-resistant strains were selected to study their ability to colonize and persist in broiler chickens: six strains isolated at the end of the rearing period from groups A (SS278), C (SS41, SS270) and D (SS308, SS47, SS267); five strains from hatchery or the beginning of the rearing period from groups B (SS304, SS209) and A (SS17, SS13, SS48), and one human strain (SS291) (figure 2). The heatmap and dendrogram based on the distance matrix calculated by 1 - *p*-values and a complete linkage as agglomerative algorithm allowed the identification of highly persistent, intermediate and non-persistent strains (figure 3). The three strains identified as being persistent in this study (SS308, SS47, SS209) were able to colonize ceca of chicks and persist at high levels until the end of the experiment (6 to 7 log CFU/g) (Figure 2A). The number of bacteria increased significantly from day 1 to day 5 p.i. ( $P \leq 0.05$ ), remaining high and almost stable from day 5 to day 26 or 33 p.i. For the two strains identified as non-persistent (SS304, SS278), a peak value of about 5 log<sub>10</sub> CFU/g was observed on day 5 p.i, but the number of bacteria in the ceca significantly decreased to values under 0.5 log CFU/g 26 days p.i. The intermediate strains are significantly different than persistent and non-persistent strains (figure 3). Among the intermediate strains, two clusters can be observed. Strains SS270, SS48 and SS41 persisted and colonized the ceca for four to five weeks post infection (>5 log CFU/g) but at significantly lower levels than the persistent strains. For strains SS13, SS17, SS291 and SS267 the number of bacteria decreased after 12 days post-infection but at colonization levels which were significantly higher than those of the non-persistent strains (~3 to 4 log CFU/g).

Persistent (SS308, SS209) and non-persistent (SS304 and SS278) strains were selected for a second trial to confirm their cecal contamination level and to analyze further their virulence potential. The results (Figure 2B) confirmed the ability of strains to persist or not in chicks and confirmed that different *S. Senftenberg* strains exhibited different levels of colonization and persistence in poultry.

### Spleen colonization level of *S. Senftenberg* strains

To determine whether *S. Senftenberg* strains are able to induce systemic infection, we studied their ability to colonize the spleen of chickens. For the two persistent strains we noted a spike in spleen

colonization frequency at day 5 p.i (90%) and day 19 p.i (60%) respectively, but with low colonization levels (<2.5 log<sub>10</sub> CFU/g). The spleens of chickens infected with the non-persistent strains were only slightly colonized, and less than 20% of samples were positive after enrichment (Figure 4). An analysis of variance shows that all the effects (strain, day, and the interaction day\*strain) are significant ( $p < 0.05$ ). In this condition, a pairwise comparison showed that only few significant differences were however observed between the strains to a particular day, probably due to high variability between animals. The test of the difference between the group of persistent (SS308, SS209) and non-persistent strains (SS304, SS278) was significant at level 0.05. These differences between persistent and non-persistent strains suggest that translocation of the intestinal barrier was greater for persistent strains. However, all the *S. Senftenberg* strains tested showed a poor capacity to invade the spleen compared to invasive serotypes such as *S. Enteritidis* for which the spleen colonization level is usually superior to 4 log CFU/g [26,27].

### Antibody response induced by *S. Senftenberg* strains

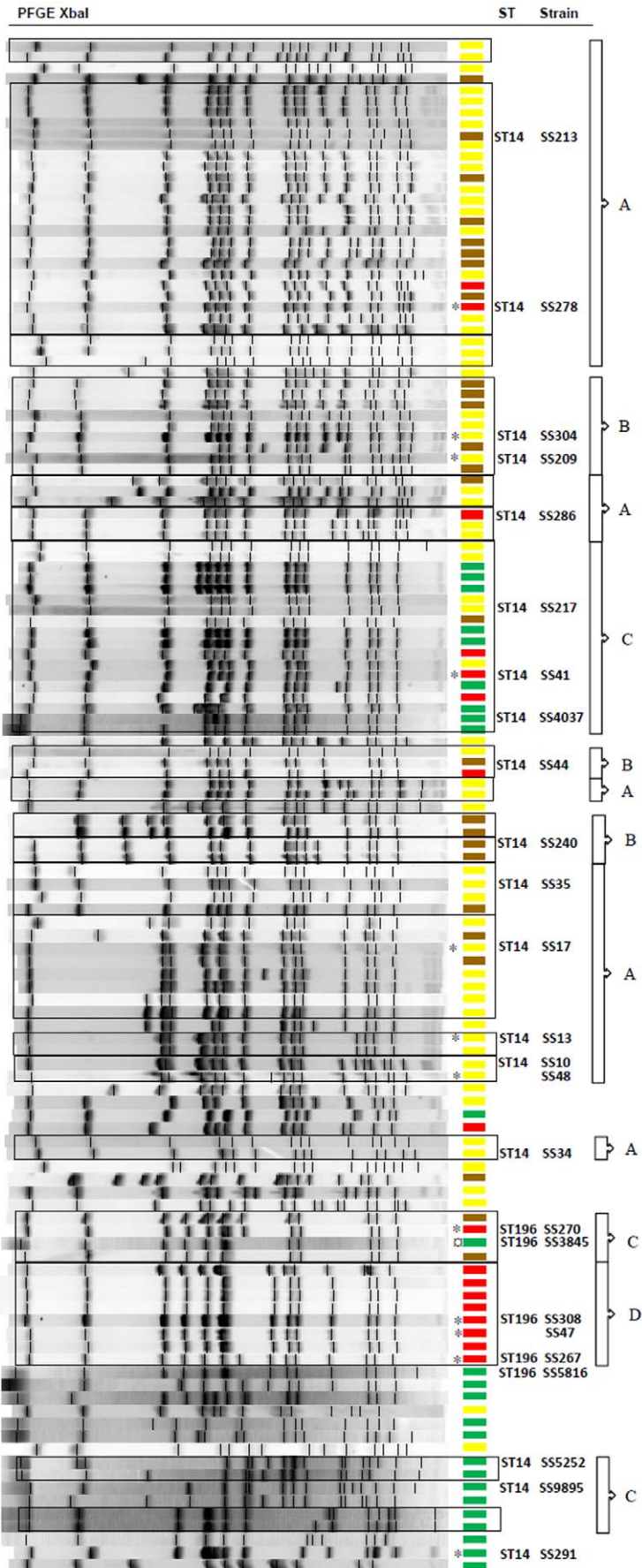
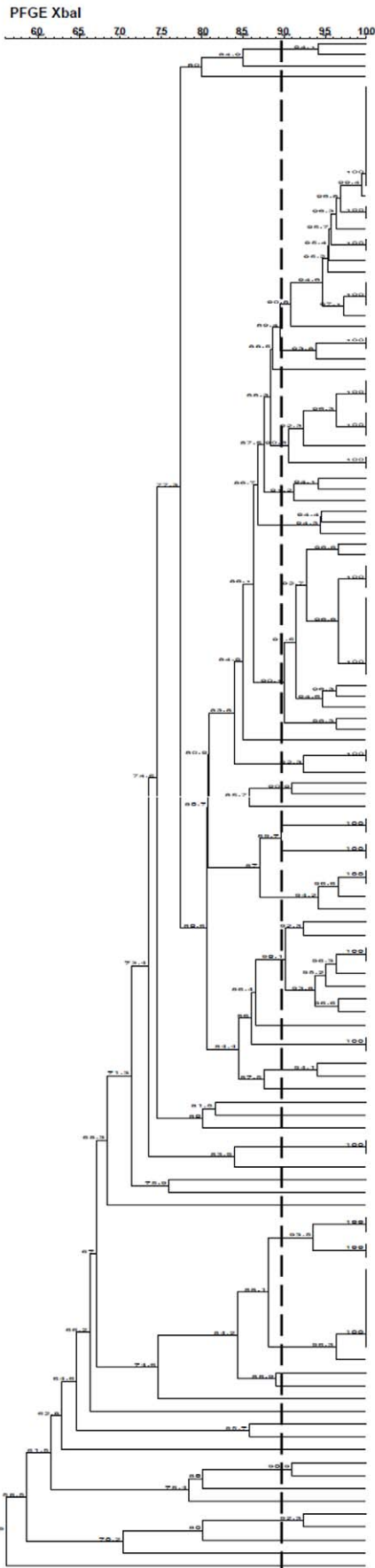
To investigate whether *S. Senftenberg* is detected as a pathogen by chicks, serum samples from infected and healthy animals were collected every week to test for immune response markers (IgM and IgY) using an ELISA assay. The healthy *Salmonella*-free animals were slightly positive for IgM and IgY antibodies. Consequently, the mean antibody titers of these control animals were subtracted from those of infected animals. Our results show that the IgM antibody response induced by *S. Senftenberg* strains was very low (OD = 0.01 at 5 days pi to less than 0.2 at 26 days pi) compared to that of chicks infected by *S. Gallinarum* (OD = 1.7), an invasive serotype responsible for systemic infection [28]. The lack of specific IgY response against *S. Senftenberg* strains (DO < 0.05) suggests that the very low IgM response observed in infected chickens is not specific to *S. Senftenberg* strains.

### Effect of persistent and non-persistent *S. Senftenberg* strains on intestinal morphology

To assess the effect of *S. Senftenberg* strains on the intestinal barrier, the height of cecal villi and depth of crypts of chickens infected with persistent SS308 and non-persistent SS304 strains were measured (table 1). At five days p.i, the villus height and crypt depth of infected birds were not significantly different from each other but were significantly lower than those of healthy animals ( $P \leq 0.05$ ), suggesting an alteration of intestinal mucosa in all infected birds. No significant difference in villus height and crypt depth was observed between infected chickens at 26 days p.i. However, the villus height of SS304 infected birds was significantly lower than that of the control group ( $P \leq 0.05$ ).

### The in vitro virulence of *S. Senftenberg* strains

To investigate whether the low level of spleen colonization of infected chickens was related to poor ability to infect enterocytes or to destruction by spleen macrophages, the invasiveness of *S. Senftenberg* strains was tested in vitro. We first demonstrated that the selection of rifampicin-resistant mutants did not impact on the invasiveness capability of *S. Senftenberg* strains because no difference in adhesion, entry and intracellular levels were observed with LMH cells, except for the wild-type SS304 strain which showed a slightly higher multiplication level (~1 log) than the SS304 rifampicin-mutant strain (data not shown). Our results show that all *S. Senftenberg* rifampicin-resistant strains were 10- to 100-fold less invasive than *S. Enteritidis* LA5 strain in both avian and human epithelial cells (LMH and HT29). This was not related



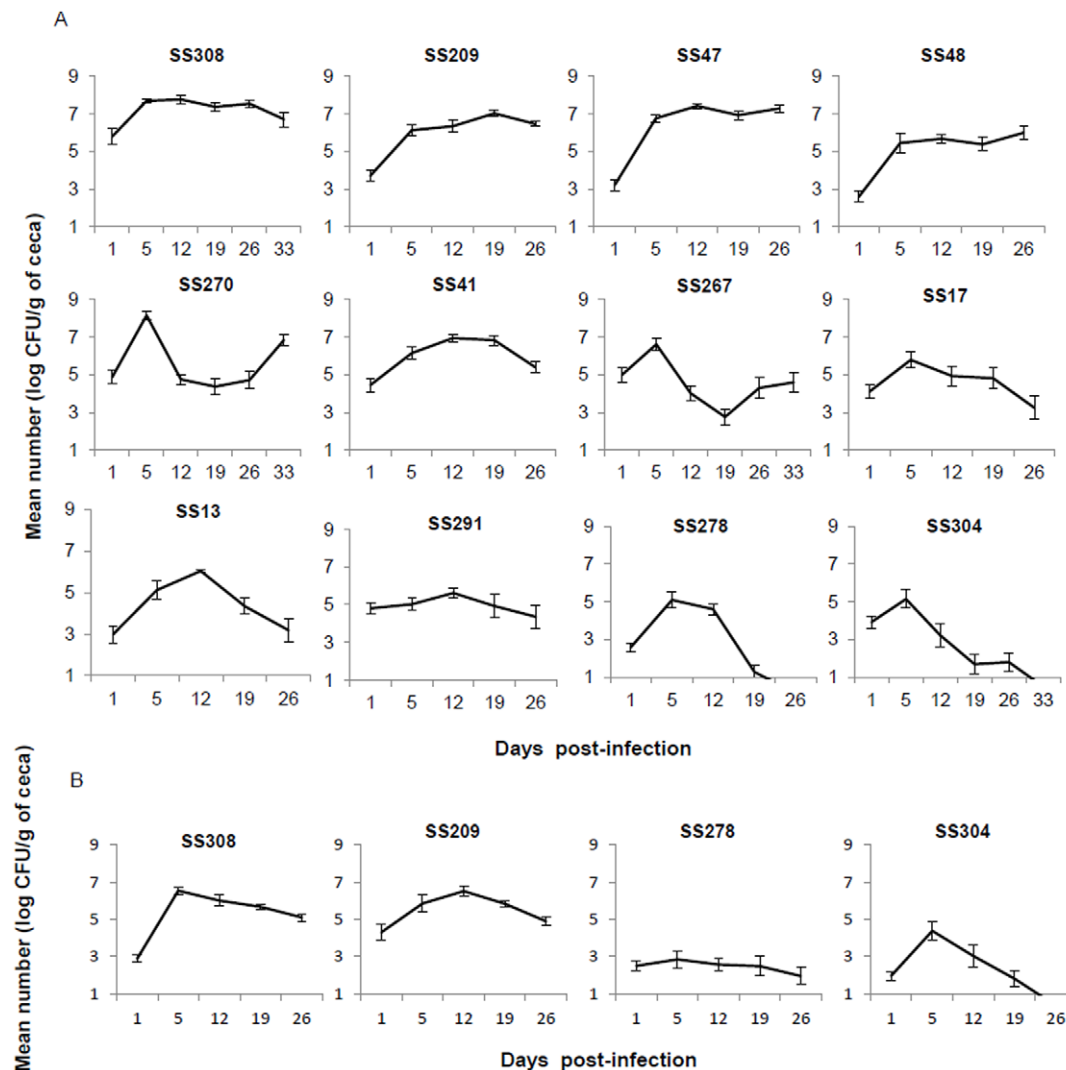
**Figure 1. PFGE and MLST analysis of *S. Senftenberg* strains.** Dendrogram generated by Bionumerics software showing PFGE fingerprint of 134 isolates of *Salmonella* Senftenberg from avian and human sources. ST: MLST sequence type of 23 isolates. Broken line indicates the division into clusters corresponding to 90% similarity with the Dice coefficient. Clusters are framed in black. Fragments ranging between 28.8 kpb and 1135 kpb were included. **Yellow rectangle:** Strains isolated from hatcheries or poultry farms (<3 weeks), **brown rectangle:** strains isolated from poultry farms (3–15 weeks) or animal feed, **red rectangle:** strains isolated at the end of the rearing period (>15 weeks) and **green rectangle:** human strains. A, B, C, D: groups of clusters. \*Strains selected for the chicken experiments. □ This Profile is representative of five genetically identical human strains.  
doi:10.1371/journal.pone.0035782.g001

to poor cell adhesion capacity, because no significant differences were observed between strains (data not shown). With LMH cells (Figure 5A), all *S. Senftenberg* strains had almost the same invasion and multiplication level, except for one non-persistent strain SS304 which appeared less invasive. With HT29 cells, the non-persistent SS304 and persistent SS209 strains had a lower invasion level than that of the other strains, but this was unrelated to their persistence phenotype. In contrast to *S. Enteritidis*, the number of intracellular bacteria for all *S. Senftenberg* strains at 20 h p.i decreased or remained stable in HT29 cells (Figure 5B). In

both avian HD11 and murine J774 macrophages, the behavior of *S. Senftenberg* strains was very similar to that of *S. Enteritidis*. All these strains were able to invade macrophages at high levels and to survive in LPS-activated macrophages (Figure 5C–D).

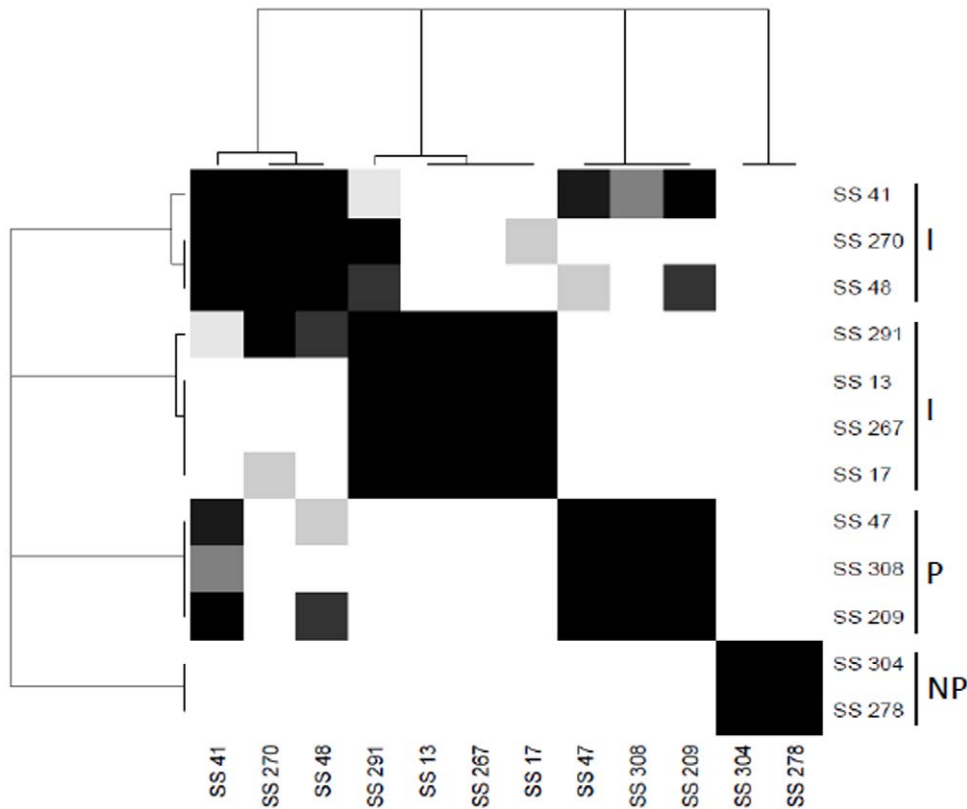
#### Virulence of *S. Senftenberg* strains in a murine model

The experiments performed on chicks showed that *S. Senftenberg*, like *S. Enteritidis*, induced an asymptomatic carrier state. However, in the mouse, *S. Enteritidis* induces a typhoid-like disease. To assess the pathogenicity of *S. Senftenberg* in a murine



**Figure 2. Cecal persistence and colonization levels of *S. Senftenberg* isolates in infected chicks.** Number of CFU of *Salmonella* Senftenberg rifampicin-resistant strains per gram of cecum of chicks orally infected with  $10^5$  CFU. (A) Enumeration was performed up to 33 days post-infection for strains SS304, SS308, SS270, SS267, and 26 days p.i for strains SS17, SS291, SS209, SS13, SS48, SS47, SS278, SS41. (B) Cecal load of chicks inoculated with four selected strains (SS308, SS209, SS304, SS278) in a second experiment up to 26 days p.i. The data presented correspond to the mean of positive and negative cecum samples  $\pm$  SEM.  
doi:10.1371/journal.pone.0035782.g002

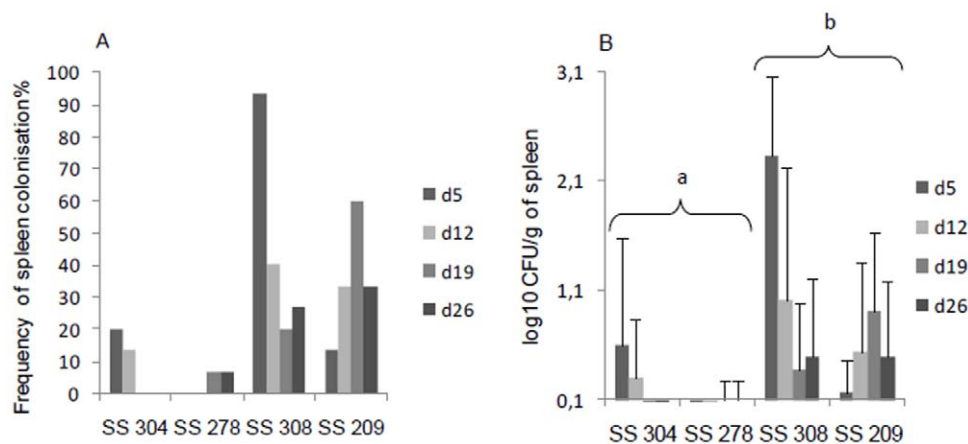




**Figure 3. Statistical analysis comparing the cecal colonization levels of *S. Senftenberg* rifampicin-resistant strains in chicks.** Dendrogram and heatmap show the clustering of strains from a complete linkage as agglomerative algorithm and the distance matrix calculated by 1-p-values where p-values were obtained from comparisons of means of strain colonization levels during the period 19 to 26 or 33 days p.i and adjusted using Tukey's method for multiple comparisons. Strains belonging to the same black square are similar and significantly different from the other strains. I: intermediate strains, P: persistent strains, NP: non-persistent strains.  
doi:10.1371/journal.pone.0035782.g003

model, BALB/c mice were orally inoculated with persistent (SS308, SS209), non-persistent (SS278, SS304) or a human isolate (SS291). At three days p.i, intestinal colonization was 2 to 3  $\log_{10}$  less than with *S. Enteritidis* ( $P < 0.05$ ). By contrast, the level of

infection of the ileum by the persistent strain SS308 was similar to *S. Enteritidis*. In the MLN, there was a significant difference in the colonization level between *S. Enteritidis* and *S. Senftenberg* strains. Non-persistent strains were found at lower frequencies and



**Figure 4. Spleen colonization of chicks infected with *S. Senftenberg*.** Broiler chickens were orally infected with  $10^5$  CFU. A) Frequency of spleen colonization by persistent (SS308, SS209) and non-persistent (SS278, SS304) strains of *S. Senftenberg* at different times post-infection. B) Number of CFU of *S. Senftenberg* per gram of spleen. Spleen samples which were positive after enrichment were given a value of 33 CFU. These results correspond to the mean of 15 animals (positives and negatives)  $\pm$  SEM.\* Letters (a-b) indicate a significant difference in means of spleen colonization levels between the groups of persistent (SS308, SS209) and non-persistent strains (SS304, SS278) at the 0.05 level.  
doi:10.1371/journal.pone.0035782.g004

**Table 1.** Measurement of proximal cecal villus height and crypt depth of control chicks and animals infected with persistent SS308 and non-persistent SS304 strains of *S. Senftenberg* at 5 and 26 days post-infection (infection at 1 day of age).

	Parameters	Healthy animals	SS308	SS304
5 days p.i	Villus height ( $\mu\text{m}$ )	498 $\pm$ 46 <sup>a</sup>	404 $\pm$ 63 <sup>b</sup>	390 $\pm$ 22 <sup>b</sup>
	Crypt depth ( $\mu\text{m}$ )	98 $\pm$ 6 <sup>a</sup>	84 $\pm$ 7 <sup>b</sup>	90 $\pm$ 9 <sup>b</sup>
26 days p.i	Villus height ( $\mu\text{m}$ )	678 $\pm$ 48 <sup>a</sup>	634 $\pm$ 112 <sup>ab</sup>	518 $\pm$ 42 <sup>b</sup>
	Crypt depth ( $\mu\text{m}$ )	128 $\pm$ 12 <sup>b</sup>	118 $\pm$ 6 <sup>b</sup>	110 $\pm$ 9 <sup>b</sup>

<sup>a,b</sup>The mean of proximal cecal villus heights and crypt depths  $\pm$ SD. Different letters in the same line indicate a significant difference with the Mann-Whitney test ( $P\leq 0.05$ ).

doi:10.1371/journal.pone.0035782.t001

significantly lower levels than persistent strains ( $P<0.05$ ). The human isolate SS291 showed an intermediate level of colonization between that of the persistent and non-persistent strains (Figure 6A). Unlike the LA5 strain, which is able to infect the spleen at a high level ( $>4 \log_{10}$ ), no viable bacteria were recovered in the spleen of mice, except those of two animals infected with the persistent strain SS308 and detected only after enrichment (data not shown). This result is consistent with the inability of *S. Senftenberg* to establish a lethal systemic infection. Mice infected with *S. Senftenberg* showed no signs at any time during infection, whereas *S. Enteritidis* inoculated mice died between 7 and 11 days p.i. At 28 days p.i., all *S. Senftenberg* strains persisted in the ileum and colon. However, the persistent strain SS308 showed a higher ileum and colon colonization level, and only persistent strains were able to persist in the MLN (Figure 6B). No viable *S. Senftenberg* strains were detected in the spleen.

To confirm that the selection of rifampicin-resistant mutants was not responsible for the lack of systemic infection of *S. Senftenberg* strains, the spleen colonization levels of mice orally infected with the mutant or wild-type strains were compared. Our results showed no significant differences between the wild-type and rifampicin-resistant strains, none of the strains were able to colonize the spleen of mice (data not shown).

Overall, these results show a poor ability of *S. Senftenberg* to induce systemic infection compared to other serotypes such as *S. Enteritidis* and suggest that persistent strains have a greater ability to colonize and persist in animal tissues than non-persistent strains.

## Discussion

In poultry production, *S. Senftenberg* is generally associated with the hatchery environment, but it has recently become more frequent in animals and on poultry farms. Furthermore, some strains have been implicated in severe human infections throughout the world [29,30].

In this study, experimental infection demonstrated that the cecal contamination capacity of chickens varied according to the *S. Senftenberg* strain involved. We identified persistent strains able to persist and colonize ceca at high levels for five weeks, and non-persistent strains which colonized ceca at lower levels and were practically eliminated five weeks p.i. The existence of distinct persistent phenotypes within *S. Senftenberg* could explain the recent emergence of this serotype in poultry flocks.

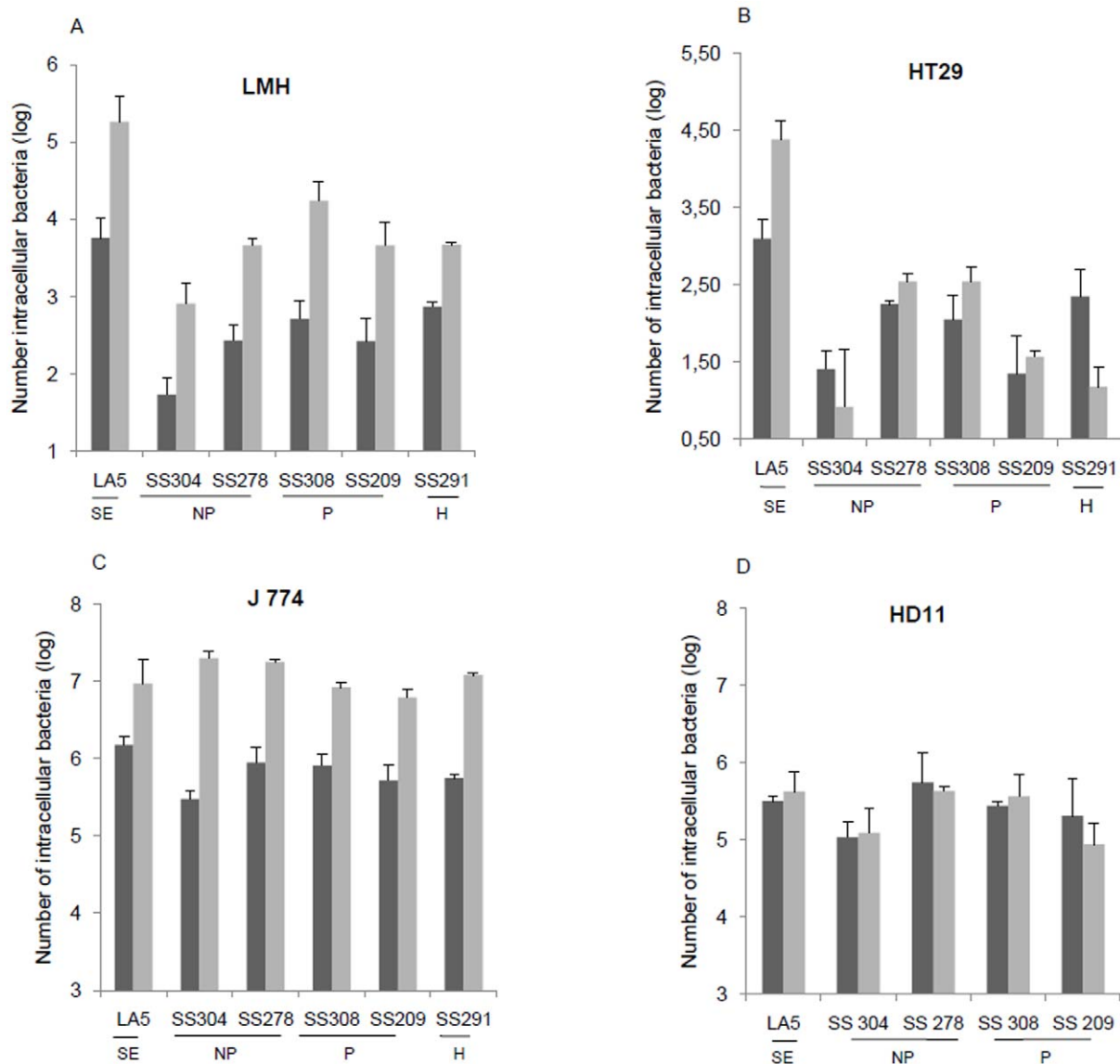
The genetic analysis of the 134 *S. Senftenberg* isolates shows that strains isolated at the end of the rearing period (superior to 15

weeks) are distributed on nearly all branches of the dendrogram and thus belong to different PFGE clusters. Moreover, the two persistent strains identified by the *in vivo* infections were distant from each others by PFGE analysis (66.3% of similarity) and have two different MLST sequence type. This result suggests that the persistence trait has been acquired independently in non-genetically related strains. However, it is interesting to note that the strains belonging to the MLST sequence type ST196 are grouped within two closely related PFGE clusters and correspond to avian strains isolated at the end of the rearing period (group D) or to human isolates (group C). Moreover two out of three of the persistent strains in chickens (SS308, SS47) belong to these PFGE clusters suggesting that ST196 could be used to identify some persistent strains. Similarly, PFGE analysis also allowed human isolates to be separated, half of which were in the same cluster while the others were in genetically distant clusters.

Among the 12 isolates tested *in vivo*, only two were identified as non-persistent. One was the SS304 strain from a cluster of group B and originated from hatchery, which is in line with its non-persistence in our chicken experimental infection. The other strain SS278 was isolated after 24 weeks of rearing. This apparently contradictory finding could be explained by external contamination or persistence in the environment. This hypothesis is supported by the fact that the SS278 strain belongs to PFGE cluster common to group A strains. Moreover, no checks were carried out during the rearing period to test for isolates originating from hatcheries or one-day-old chicks, thus explaining the persistence of some of these isolates in animals. That was the case of the two strains SS209 and SS48 isolated from one-day-old animal feces and hatchery respectively which were able to colonize and persist in chickens experimentally infected.

Although we have shown that persistent *S. Senftenberg* strains are able to induce an asymptomatic carrier state in chicks, in a similar way to *S. Enteritidis*, the hallmark of *S. Senftenberg* is its poor capacity to induce systemic infection in either chicks or mice. *S. Senftenberg* strains induced fewer morphological changes in the intestine of chicks than described for *S. Enteritidis* during the first days of infection [31], colonized the spleen at a low level, and consequently did not induce an antibody response. Similarly, in the mouse model, which is the gold standard for assessing *Salmonella* virulence [32], there was a high level of intestinal colonization but no spleen colonization, which can in part be explained by the poor ability to infect MLN. One hypothesis is that *S. Senftenberg* is unable to survive in macrophages and is thus eliminated before reaching the internal organs [33]. However, our *in vitro* results, showing that *S. Senftenberg* strains were able to invade and survive in activated macrophages at the same levels as *S. Enteritidis*, suggest that this hypothesis is not valid. Another possible explanation is that the low virulence of *S. Senftenberg* is related to its low capacity to translocate the intestinal barrier. This hypothesis is supported by the observation that all *S. Senftenberg* strains were less invasive for avian and human epithelial cells than *S. Enteritidis* and were unable to multiply within human enterocytes, unlike *S. Enteritidis*. This is probably not related to an absence of the *Salmonella* pathogenicity island-1 (SPI-1), as previously suggested [34], because the *invA* and *hilA* virulence genes were detected in all *S. Senftenberg* strains used in our study (data not shown).

When comparing persistent and non-persistent strains, it can be seen that they vary in their capacity to colonize and persist in animal tissues. However, persistence of *S. Senftenberg* strains is not correlated with the invasion and multiplication levels in epithelial cells and activated macrophages. These findings suggest that the difference of colonization levels in chicken spleen and



**Figure 5. Cell invasion capacity of *S. Senftenberg* strains.** In vitro invasion and intracellular multiplication were performed in epithelial cell lines (HT29 and LMH) and macrophages (HD11 and J774). Persistent (P) (SS308, SS209) and non-Persistent (NP) (SS304, SS278) strains were analyzed in addition to a human isolate (H) (SS291). Cells were infected at a MOI of 10 CFU/cell and the number of intracellular bacteria was determined 2 h (black) and 20 h (grey) post-infection. Data representing the mean  $\pm$  SD of at least two independent experiments carried out in duplicate. The invasive strain of *Salmonella* Enteritidis LA5 was used as a positive control. doi:10.1371/journal.pone.0035782.g005

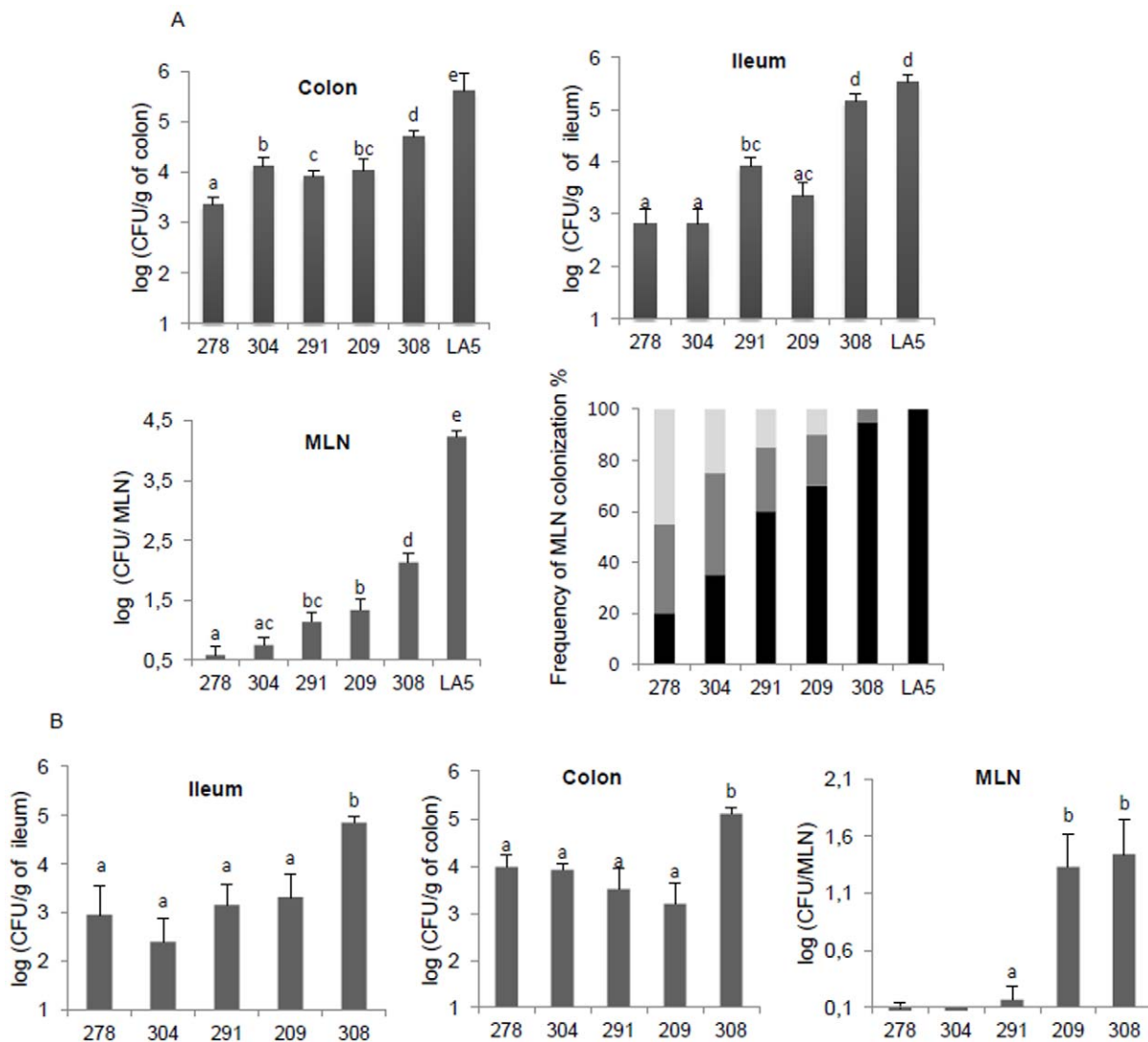
mice MLN between strains could be attributed to the difference in the bacterial load in intestinal contents. Nevertheless, the greater persistence in the MLN of persistent strains does not support this hypothesis and suggests that non-persistent strains are also impaired in their ability to resist the innate immune response.

In our study, we also showed that the human strain SS291 colonized the ceca of chicks and the intestinal tract and MLN of mice at levels between those of persistent and non-persistent strains. Moreover, like all the *S. Senftenberg* strains tested, the human strain did not induce systemic disease in mice. One hypothesis is that the infected humans had some other factors that facilitated the infection process. Another hypothesis is that the systemic infection model of the mouse is not suitable for testing the virulence of *S. Senftenberg* in humans. This later hypothesis is

supported by the data obtained from a food-borne disease outbreak by Hu *et al.* who identified several *S. Senftenberg* clinical isolates from immunocompetent patients infected with no other pathogens [34]. In line with our results, they showed that *S. Senftenberg* isolates from humans could not induce an intestinal pathology or systemic infection in the mouse model of enterocolitis. Overall, we consider that all *S. Senftenberg* strains are potentially pathogenic for humans; persistent strains would thus present a greater risk for human health than non-persistent strains, due to their ability to persist and colonize the intestine, while non-persistent strains would be eliminated before slaughter and food processing.

Human clinical cases caused by the Senftenberg serotype are not necessarily linked to avian sources. In fact, the results of our





**Figure 6. Mouse infection by *S. Senftenberg* strains.** Groups of BALB/c mice were orally infected with  $5 \times 10^8$  bacteria of persistent (SS308, SS209), non-persistent (SS304, SS278) and human (SS291) *S. Senftenberg* strains ( $n=10$  for each group). (A) At day 3 p.i., the number of bacteria detected in the ileum, colon and mesenteric lymph nodes (MLN) was determined. The *S. Enteritidis* LA5 strain was used as a positive control ( $n=6$ ). The results of two independent experiments were pooled for analysis. The frequency of MLN colonization by *Salmonella* strains was calculated after direct detection (**black**) or enrichment (**grey**). **White** histogram corresponds to negative MLN samples. (B) Number of bacteria in the same organs determined 28 days p.i. Data correspond to the mean of positive and negative animals  $\pm$  SEM of two independent experiments. Letters (a–e) indicate a significant difference between the different groups of infected mice ( $P < 0.05$ ). doi:10.1371/journal.pone.0035782.g006

PFGE analysis show that although numerous human isolates are genetically close to avian isolates, others are grouped in distant clusters suggesting a divergent evolution. We could thus suppose that these human isolates are mainly related to sources other than poultry or the area of contamination (e.g., travel-acquired infection). This hypothesis is supported by recent outbreaks of infection of non-avian origins, such as the one linked to pre-packed basil [35]. This serotype can adhere to plant leaves [36] and is also able to survive in a hypersaline environment, and it is thus one of the main serotypes associated with seawater and seafood products [37,38]. Further research to study the virulence and persistence capacity of these human strains of different origins would be of interest.

To conclude, the increased presence of *S. Senftenberg* on poultry farms could be explained by differing capacities of the

persistent and non-persistent strains to colonize and survive in chick and mouse tissues. Persistent strains could also be more able to survive in the intestinal content of chicken. The role played by these factors requires further investigation.

## Acknowledgments

We thank the staff of the Plateforme d'Infectiologie Expérimentale (INRA, Nouzilly) for caring for the mice and participating in the experiments, the Service d'Élevage et d'Expérimentation en Pathologie Animale of Anses for animal experiments and participating in the experiments, and the Pôle Expérimental Avicole de Tours (INRA, Nouzilly). We also thank the Agence Nationale de Sécurité Sanitaire de l'alimentation of Maison Alford, Paris, which performed the PFGE analysis of 19 human isolates included in this study.

## Author Contributions

Conceived and designed the experiments: ZB SMR FL IVP IG FXW PV MC CHA PM. Performed the experiments: ZB SMR FL IVP PM.

Analyzed the data: ZB SMR IVP IG FXW PV MC CHA PM. Contributed reagents/materials/analysis tools: ZB SMR IVP IG FXW PV MC. Wrote the paper: ZB PV.

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