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Role of systemic infection, cross contaminations and super-shedders in *Salmonella* carrier state in chicken

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Summary

Carriage of *Salmonella* is often associated with a high level of bacterial excretion and generally occurs after a short systemic infection. However, we do not know whether this systemic infection is required or whether the carrier-state corresponds to continuous reinfection or real persistence in caecal tissue. The use of a *Salmonella* Enteritidis *bamB* mutant demonstrated that a carrier-state could be obtained in chicken in the absence of systemic infection. The development of a new infection model in isolator showed that a marked decrease in animal reinfection and host-to-host transmission between chicks led to a heterogeneity of *S.* Enteritidis excretion and colonization contrary to what was observed in cages. This heterogeneity of infection was characterized by the presence of super-shedders, which constantly disseminated *Salmonella* to the low-shedder chicks, mainly through airborne movements of contaminated dust particles. The presence of super-shedders, in the absence of host-to-host transmission, demonstrated that constant reinfection was not required to induce a carrier-state. Finally, our results suggest that low-shedder chicks do not have a higher capability to destroy *Salmonella* but instead can block initial *Salmonella* colonization. This new paradigm opens new avenues to improve understanding of the carrier-state mechanisms and to define new strategies to control *Salmonella* infections.© 2018 Society for Applied Microbiology and John Wiley & Sons Ltd

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Introduction

Salmonella are enteric bacteria recognized as an important economic and public health problem throughout the world. *Salmonella* infections are the second most common cause of food poisoning and the first cause of foodborne illnesses resulting in hospitalization and death in the developed world [20 000 hospitalizations and 400 deaths in United States; first cause of foodborne related death in France (27%)] (Scallan *et al*., 2011; Van Cauteren *et al*., 2017). They also result in a significant loss to the agri-food industry and represent a substantial burden on healthcare system. Non-typhoidal *Salmonella* are passed to humans mainly through poultry products or pork meat. Consequently, to reduce human contaminations, focus has been placed on developing intervention strategies to produce safe live animals and wholesome food products (Callaway *et al*., 2004).

One hallmark of *Salmonella* pathogenicity is its ability to induce a wide disease spectrum (Velge *et al*., 2005). Depending on the hosts and serotypes, *Salmonella* can induce diseases ranging from gastro-enteritis to typhoid fever. In chicken and pigs, *S.* Enteritidis and *S.* Typhimurium, the two main serotypes isolated in human infections, do not usually induce disease symptoms (Mughini-Gras *et al*., 2014). *Salmonella* carriage is the ability of bacteria to persist in the host organism for many weeks up to many years without causing any symptoms, making the identification of carrier animals difficult. *Salmonella*-carrier animals are a serious food safety issue, because the infected animals, which often excreted high level of bacteria in their feces, can contaminate meat products.

In chicken, this persistence generally occurs after a short systemic infection that may either lead to death of very young birds or develop into caecal asymptomatic persistence, which is often accompanied by a high level of bacterial excretion, facilitating *Salmonella* transmission to counterparts (Poppe, 2000). *Salmonella* is transmitted between birds through contact with infected individuals, fomites, ingestion of materials contaminated with faeces

and/or airborne routes (Velge *et al*., 2005). In cattle and mice, transmission efficiency has been linked to increased levels of faecal *Salmonella*, with high-shedding hosts often named super-shedders being responsible for most of the transmission (Matthews *et al*., 2006; Gopinath *et al*., 2012).

However, despite the importance of *Salmonella* persistence as a reservoir of disease, little is known about the mechanisms at play when *Salmonella* induces a carrier state. Moreover, the factors facilitating persistence and enabling host-to-host transmission have been largely neglected (Gopinath *et al*., 2012). For example, we do not actually know whether systemic infection is required for the carrier state to become established and to develop and/or whether it is necessary for the immune response stimulation. The establishment of the carrier state actually corresponds to a balance between the capacity of the bacteria to overcome the host response and the ability of the host immune system to control the infection (Ruby *et al*., 2012). Moreover, the fact that these *Salmonella*-carrier animals represent a significant reservoir with the potential to contaminate neighbouring naïve animals as well as the environment, and that they shed the pathogen in high enough numbers to transmit infection, raises the question of the real persistence capability of *Salmonella.* In other words, does *Salmonella*-carrier state correspond to persistence in caeca or is it due to a constant recontamination of chickens?

In order to understand better the mechanisms of *Salmonella*-carrier state in chicken, we analyzed the respective role of systemic infection and animal recontamination. For this purpose, we used a *Salmonella bamB* mutant altered in its ability to induce systemic infection and developed a new infection model in an isolator where the host-to-host transmissions are greatly decreased.

Results and discussions

Salmonella-carrier animals are a serious food safety issue, especially those shedding high levels of *Salmonella* in their faeces, thereby contaminating: (1) neighbouring naïve animals; (2) slaughter plants and meat products during processing; (3) edible crops when manure is used as a soil amendment; and (4) water supplies when slurry (manure) runs off into waterways. However, mechanisms leading to persistence of *Salmonella* are poorly understood. Studies to elucidate the mechanisms leading to the carrier state are sometimes performed on farm animals, but the large majority of data have been obtained from mouse models. In Nramp1+/+ (SLC11A1) mice (CBA mice and 129sv), which are more resistant to *Salmonella* systemic infection than the Nramp1-/- mice, oral infection by *Salmonella* results in a chronic systemic infection (Ruby *et al*., 2012). However, the mouse models do not seem to be representative of the carrier state observed in chicken and pigs where no persistent systemic infection occurs. In these farm animals, systemic infection precedes persistence in caeca and disappears from spleen and liver, a few weeks after infection (Virlogeux-Payant *et al*., 2003). A previous study has shown that an intravenous inoculation of *Salmonella* can lead to caecal persistence (Girard Santosuosso *et al*., 1998), it is, therefore, important to determine whether systemic infection is required to induce *Salmonella* carrier state in caeca and/or it determines the development of the carrier state.

Systemic infection is not required for long term intestinal persistence in chicks

The kinetics of spleen infection presented in Fig. 1A confirms that *S.* Enteritidis induced a transient systemic infection. The first weeks postinfection spleens of all chicks were infected after oral infection (Fig. 1C); then *Salmonella* was not detected in spleen 3 to 4 weeks postinfection (pi) even though a few bacteria could still be detected in this organ from weeks 4 to 6 pi in a few animals as previously described (Berthelot-Hérault *et al*., 2003). This has been also described for liver (Virlogeux-Payant *et al*., 2003). In contrast to the short-term infection of the spleens, *S.* Enteritidis persisted in the caeca of the same birds for longer than 9 weeks (Fig. 1B) and at very high levels. This level was above 1×10^6 *Salmonella*/g of caeca up until 7 weeks postinfection.

When a *bamB* mutant (formely called YfgL), which is altered in outer-membrane protein biogenesis and in expression of the major virulence factors (Amy *et al*., 2004), was orally inoculated in chicks, systemic infection was dramatically altered (Fig. 1A). This is demonstrated by the very low level of spleen infection by the mutant compared to the wild-type strain, which was close to the detection threshold throughout the time course of the experiment ($p < 0.001$). Moreover, 30% to 50% of the animals had no *Salmonella* in their spleen even after enrichment (Fig. 1D). Analysis of the caeca of the same birds showed a very high level of infection in all birds and no significant difference was detected between chicks inoculated with the wild-type or the mutant strain, except at 1 week postinoculation (Fig. 1B). This difference at 1 week pi is in line with previous results in one-day-old chicks and in mice (Amy *et al*., 2004; Fardini *et al*., 2007). Taken together, the high level of caeca infection of all chicks by the *bamB* mutant concomitant to the absence of spleen infection of half of the chicks strongly suggests that systemic infection is not required for long-term persistence of *Salmonella* in caeca.

BamB is an outer membrane lipoprotein included in a complex that has been shown to be involved in the

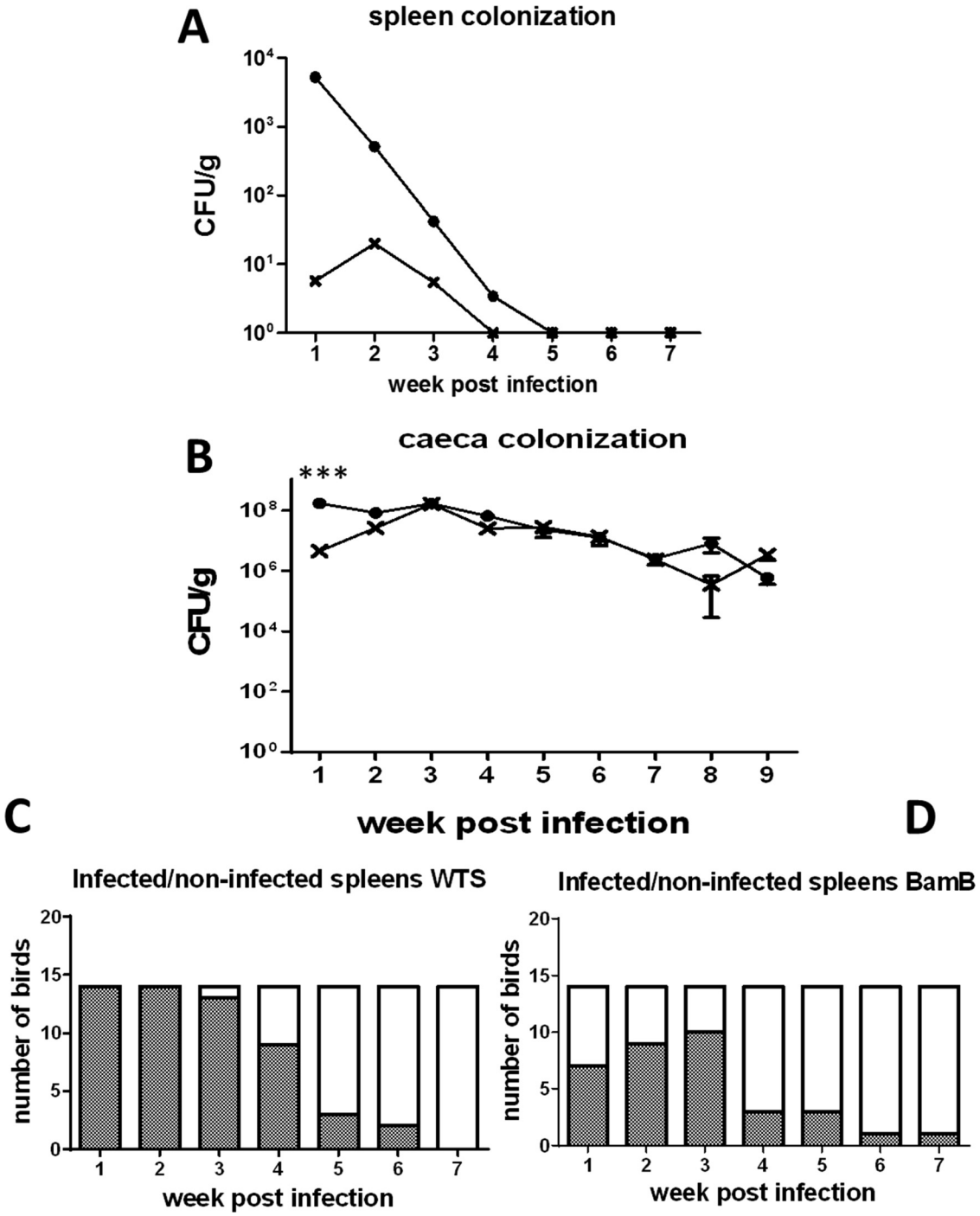

Fig. 1. Role of systemic infection in development of an asymptomatic carrier state in chick orally infected by *Salmonella* Enteritidis.Specific-pathogen-free chicks (126) of the PA12 line were orally inoculated with 5×10^4 CFU of the wild type LA5 strain (dot) or its isogenic *bamB* mutant (x) in large cages. Each weeks, 14 chicks from each group were killed by carbon dioxide gas inhalation. The mean numbers of *S.* Enteritidis CFU per gram of spleen (A) and caeca (B) were compared by one way analysis of variance (ANOVA) and analysed by the Bonferroni–Dunn test (***, $p < 0.001$). When necessary, the sample homogenates were enriched to reveal contamination below the detection threshold. The frequency of spleen colonization was described for the wild type strain (C) and for the *bamB* mutant (D).

assembly of outer membrane β-barrel proteins in *E. coli* and *Salmonella* (Malinverni and Silhavy, 2011; Namdari *et al*., 2012). The deletion of *bamB* leads to a drastic virulence defect in mice, characterized by a low spleen infection level (Fardini *et al*., 2007). Various explanations for the caeca infection by the Δ*bamB* mutant strain contrary to the spleen can be put forward. First, we previously demonstrated that deletion of *bamB* led to a

downregulation of the genes involved in the biosynthesis of the 3 T3SS of *Salmonella*, that is, T3SS-1, T3SS-2 and the flagella, which are required for systemic infection (Hume *et al*., 2017). Bohez *et al*. demonstrated that colonization of caeca and shedding of a Δ*hilA* mutant strain, which did not express the T3SS-1 related genes, was greatly decreased compared to the wild-type strain and that this strain was more or less eliminated from the chicken gut four weeks postinfection (Bohez *et al*., 2006). Thus, our results suggest that a low level of expression of the main virulence factors is sufficient to induce caecal colonization and persistence. A second explanation, which is not exclusive, is that additional invasion and colonization mechanisms exist in *Salmonella*, like the T3SS-1 independent entry mechanisms (Velge *et al*., 2012). A third explanation is that caecal persistence of *Salmonella* corresponds mainly to a constant reinfection of birds and that host-to-host transmission determines the carrier state at the flock level.

Low levels of host-to-host transmission induces a heterogeneity of *Salmonella Enteritidis* infection

To address this hypothesis, we developed a new model of infection in isolator where animal reinfections and host-to-host transmission between chicks were decreased. Afterwards we compared the infection of chicks reared in large cage and in isolator. When chicks were infected in large cages, the hallmark of *Salmonella* carrier state was the high level of caeca colonization observed in all chicks for several weeks, at levels remaining above 1×10^6 CFU/g (Fig. 1B). Interanimal variability of caeca colonization is presented in Fig. 2A for the same experiment. These high homogeneous levels of chick infection were also observed for *Salmonella* excretion when we collected fresh faecal samples (mean excretion level above 1×10^6 CFU/g the first four weeks postinfection; Fig. 2B). It is not related to the number of chicks per cage as we observed similar levels of caeca colonization when 100 (Fig. 2A) or 10 (Fig. 2B) chicks were infected in a large cage but at a similar density. Similar high homogeneous levels of excretion and caeca colonization have been observed with other chicken lines infected in cages (Chausse *et al*., 2011) and with another *S.* Enteritidis strain (Sadeyen *et al*., 2004).

Frequent host-to-host transmissions occur in the classic experimental designs (i.e., using large cages) (De Vylder *et al*., 2011). To determine whether *Salmonella* carrier state was related to a continuous reinfection of chicks or whether *Salmonella* could actually persist in caeca in the absence of re-infection, it was necessary to block any host-to-host transmission between chicks. To this end, we infected one chick in a small isolator. For this experiment, ten independent replicates were performed. In isolators,

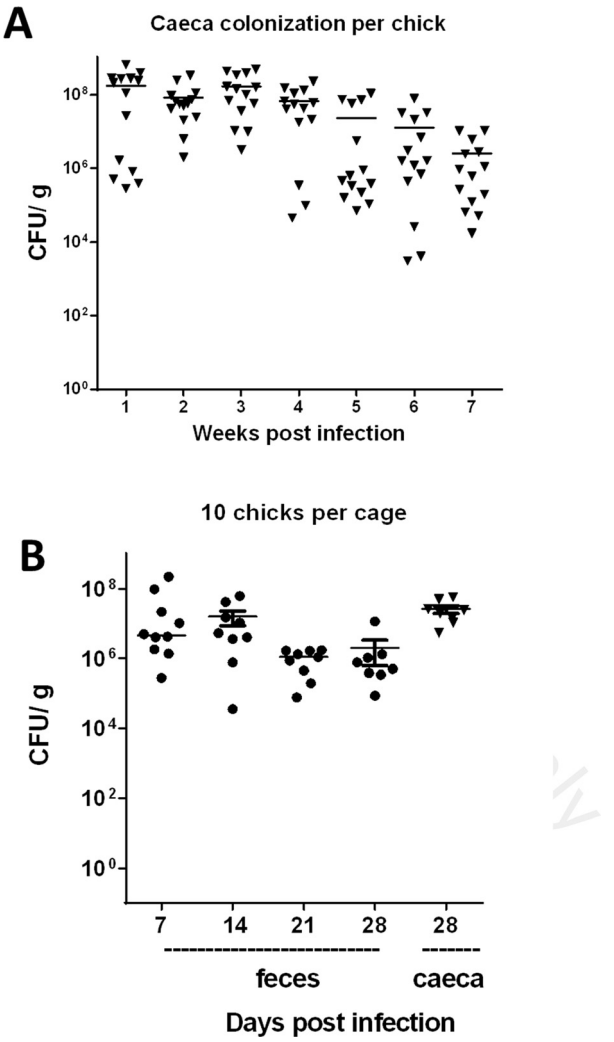

Fig. 2. Interanimal variability of chicks infected in cage by *Salmonella* Enteritidis.Comparison of the level of *S.* Enteritidis colonization in caeca of 100 chicks orally inoculated with 5×10^4 CFU of the wild type LA5 strain (A) and of 10 chicks orally inoculated with the same dose (B). In the first case, 14 chicks/week were necropsied to measure caeca colonization. In the last case, fresh faecal excretion was followed for 4 weeks (dot) and caeca colonization (triangle) was monitored at necropsy, 28 days postinfection. Each point represents one chick. *Salmonella* colonization and excretion were expressed as the mean of *Salmonella* per gram.

host-to-host transmission and subsequent reinfections were greatly hampered but chicks are not axenic. Oral infection with *S.* Enteritidis led to heterogeneous patterns of infection (Fig. 3). The median level of faecal excretion was very low compared to that found in cages [6×10^1 *Salmonella*/g faeces 7 days after infection, vs. 4×10^6 *Salmonella*/g faeces for chicks reared in large cages (Figs 3 and 2B)]. Similarly, the median level of caeca colonization four weeks p.i. was more than 1 000 fold greater in cages than in isolators (2.5×10^7 vs. 1.5×10^4 *Salmonella*/g). The median values included very different results; in isolators, around 20%–50% of chicks excreted no

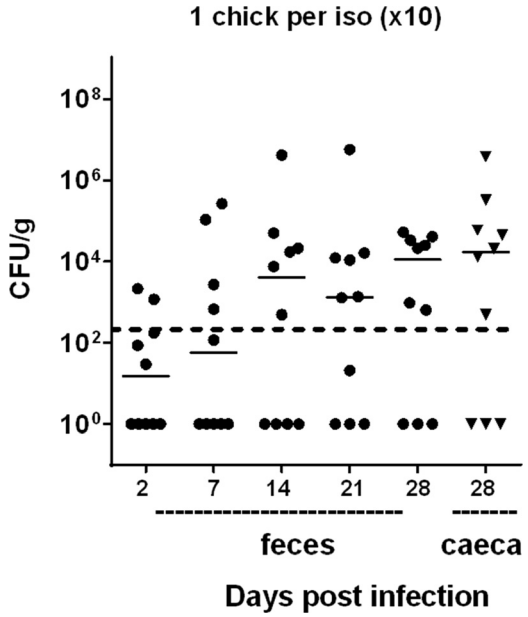

Fig. 3. Interanimal variability when chicks are individually reared in isolator.To analyse the role of animal–animal transmission, one chick, reared in an isolator, was orally inoculated with 5×10^4 CFU of the wild type LA5 strain. Fresh faecal excretion was followed for 4 weeks (dot) and caeca colonization (triangle) was monitored at necropsy, 28 days postinfection. Ten isolators were used and consequently each point represents a repetition of this experiment (one chick/isolator). In isolator, due to the heterogeneity of infection, *Salmonella* colonization and excretion were expressed as the median of *Salmonella* per gram.

bacteria, and only a few chicks excreted a level of *Salmonella* comparable to that was observed in cages (i.e., *Salmonella*/g > 1×10^5). Moreover, some chicks only excreted *Salmonella* 2 or 3 weeks postinoculation (5 positive chicks after 7 days vs. 7 positive chicks after 21 days).

Overall, the development of this new model of infection, where individual chicks were maintained under strict isolation suggests that when host-to-host transmission are greatly reduced, *S.* Enteritidis induces heterogeneous infection. Heterogeneity of infection is characterized by the presence of some chicks excreting no or few bacteria in their faeces and others excreting high levels, which are very close to levels observed in the classic cage model.

However, housing a single chick per isolator could induce stress and may in turn alter chick susceptibility to *Salmonella* colonization. We, therefore, performed an infection with 10 and 30 chicks in larger isolators. For these experiments, the density of chicks per isolator was close to that observed in large cages. For the sake of comparison, the results found for three separate isolators containing 10 chicks were pooled (Fig. 4A) and compared with those obtained with 30 chicks in one isolator (Fig. 4B).

This experiment confirmed that when host-to-host transmission was greatly decreased, chicks presented highly heterogeneous levels of infection, whatever the number of chicks living together. This first underlines the efficiency of isolators in controlling host-to-host transmission and secondly, it indicates that isolation induced-stress probably did not explain infection heterogeneity when a single chick was reared in an isolator. These results also show that homogeneity of *Salmonella* infection observed in cages is most likely related to a high recontamination rate of chicks.

This model in different sized isolators is, thus, suitable for studying the interaction of *Salmonella* with chicks in the absence of or with a small degree of host-to-host transmission between animals. The model in a cage corresponds to the addition of several variables: (1) infection of a single animal, (2) survival of the bacteria in the environment and (3) bacterial transmission to a congeners and host recontamination. This new model will help to understand how *Salmonella* establishes an initial interaction with the chick intestine, to identify the bacterial and host factors involved in intestinal colonization independently of the other variables, and to test the impact of preventive and therapeutic treatments on these crucial steps in the development of the carrier state.

Heterogeneity of *Salmonella Enteritidis* infection is related to the presence of super-shedder and low-shedder chicks

This new and original model of chick infection by *Salmonella* has allowed us to demonstrate that when reinfections and host-to-host transmissions are greatly reduced, *S.* Enteritidis induces heterogeneous excretion and caecal infection. A principal component analysis (PCA) was performed to better analyze heterogeneous *Salmonella* counts (Fig. 4A and B). A strong collinearity was observed among the measures performed at 28 days using either faecal or caecal samples. This suggests that levels of *Salmonella* in faecal samples were representative of those in caeca. This good correlation was only observed when we used fresh faecal samples and not when we used cloacal swabs (data not shown). In Fig. 4B, we can observe that numerous chicks had a low level of excretion at 4 and 7 days postinfection (inferior to log 3,0) and a low level of caeca contamination (inferior to log 4.0). These chicks are in green in the corresponding PCA (Fig. 4D). Several chicks had a high level of excretion at 4-and 7 days postinfection (superior to log 4.2) and a very high level of caeca contamination (superior to log 6.0). These chicks correspond to numbers 8, 38, 23, 41 (in purple in the Fig. 4D). Some chicks had a low level of excretion 4 days pi but later they had a very high level of excretion and caeca contamination. These chicks correspond to numbers 12 and 39 of Fig. 4D. A few chicks had a high level of excretion 4 days pi

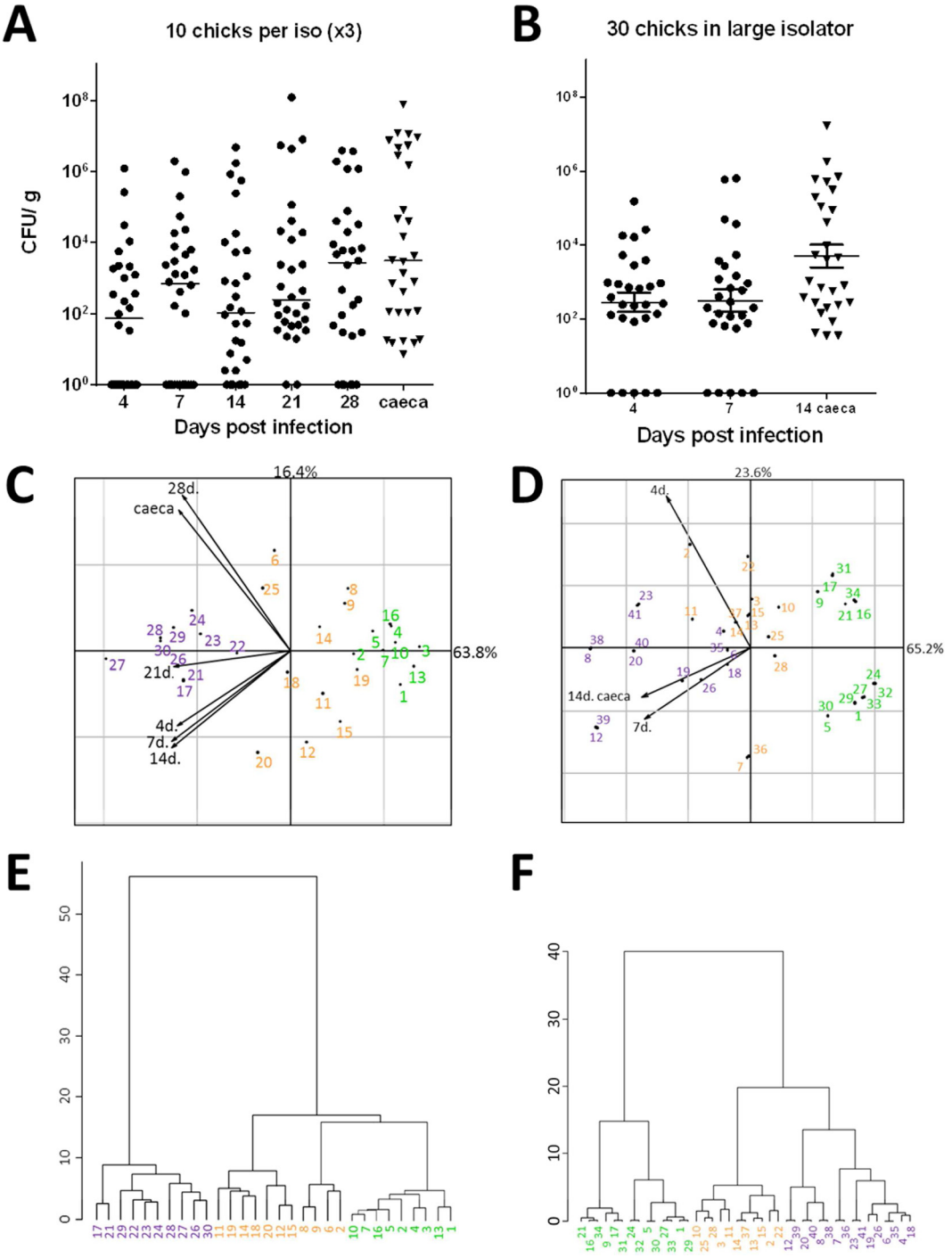

Fig. 4. In absence of host-to-host transmission, *S.* Enteritidis induced heterogeneous excretion and caecal colonization, with the presence of Low and super-shedder chicks.Ten (A, C, E) or 30 (B, D, F) chicks reared in one isolator, were orally inoculated, at 7 days of age, with 5×10^4 CFU of the wild type LA5 strain. Fresh fecal excretion (dot) was followed for 4 weeks (A) or 2 weeks (B) and caeca colonization (triangle) was monitored at necropsy 4 weeks (A) or 2 weeks (B) postinfection. The results of 3 different medium isolators containing 10 chicks were merged (A) and compared to the results obtained with one isolator containing 30 chicks (B). In isolator, due to the heterogeneity of infection, *Salmonella* colonization and excretion were expressed as the median of *Salmonella* per gram. Each point represents one chick. Principal component analysis (C, D) and hierarchical clustering (E, F) were performed on the log number of *Salmonella* per gram. For these analyses, kinetics of fresh fecal excretion per chick were taken into account as well as the level of caeca colonization. Each number corresponds to one chick. Chicks considered as the super-shedders are noted in purple, whereas chicks considered as the low-shedders are noted in green and those considered as the Intermediate in orange.

(superior to log 4.2) but a low level of excretion and caeca contamination after this date (No. 2 and 22 Fig. 4D). Similar observations can be observed on Fig. 4A and its corresponding PCA (Fig. 4C).

A hierarchical clustering indicates that chicks may be clustered in a simple fashion, as illustrated in Fig. 4E and F. A first group included chicks having no or few bacteria in faecal droppings, and a low quantity in caeca (green), and designated here as 'Low-shedder' chicks. A second cluster (purple) corresponded to chicks having very high levels of caecal colonization ($>5 \times 10^4$ *Salmonella*/g) comparable to the values observed in cage; these chicks were designated here as 'super-shedder'. The combined analysis of the PCA and the hierarchical clustering suggests that an intermediate group can also exist (caecal colonization $< 5 \times 10^4$/g). This cluster tends either to be associated with super-shedders in a 30-chick isolator, or to low-shedder in 10-chick-isolator. This suggests that when more chicks were kept together, more tended to cluster in the super-shedders category. This may be explained by the existence of host-to-host transmission in large isolators but which occur at a low frequency compared to cages. In line with this, when a naïve chick was placed in an isolator already containing 10 infected chicks, it often became positive within 7 to 14 days (data not shown).

Higher microbiota exchanges occur in large cages than in isolators

To determine the levels of host-to-host transmissions in large cages and in isolators, we used quantitative PCR to compare roughly the evolution of faecal microbiota composition of chicks reared in the two housing conditions. For this, a first PCA analysis was performed in order to define arbitrary microbiota clusters, on the basis of 10 bacterial group abundances at 7 days of age (2 bacterial groups were not detected in these chicks). A second PCA analysis was performed to analyze the microbiota composition within the clusters at 5 weeks of age. A multinomial regression analysis was used to assess differences among clusters. At the end of the experiment, differences were no longer detected for the chicks reared in cages (Fig. 5A and B). This result suggests that gut microbiota tended to become homogeneous when chicks were reared in cages, probably as the result of frequent host-to-host transmissions. By contrast, microbiota clusters were still significantly different at 35 days of age when chicks were reared in isolators ($p < 0.03$) (Fig. 5C and D). This result suggests that gut microbiota evolved with time but they did not become homogeneous among chicks in an isolator. Taken together, these results strongly suggested that the level of host-to-host transmissions was low in isolators compared to cages.

When a chick is a super-shedder, it remains a super-shedder even without host-to-host transmission

To determine why some chicks became super-shedders and other low-shedders, we hypothesized that heterogeneity of infection observed in isolators could be because low-shedder chicks were more able to eliminate *Salmonella.* According to this hypothesis, the lack of low-shedder chicks in large cages could be explained by more frequent recontamination events. To address this hypothesis, 7-day-old-chicks reared in large cages were first infected and then kept in their cage for 7 days to favour host-to-host transmission and favour a high and homogeneous levels of *Salmonella* excretion [median level = 7×10^6 *Salmonella*/g (Fig. 6)]. Twenty chicks were then transferred into isolators and levels of faecal and caecal excretion were measured over time (Fig. 6). Our hypothesis was that if some chicks were able to destroy *Salmonella*, the level of bacteria in faecal droppings should decrease when host-to-host transmission disappeared, at least in a few chicks. Figure 6 shows that after transfer into isolator, despite a strong decrease in host-to-host transmission, the level of bacterial faecal excretion remained very high for all chicks and similar to levels observed when chicks were in cages. This result demonstrates that heterogeneity of *Salmonella* infection is not related to the ability of low-shedder chicks to eliminate *Salmonella* and strongly suggests that it is linked to the chick's ability to block the initial *Salmonella* infection.

Taken together, these results allow us to respond to our hypothesis saying that caecal persistence of *Salmonella* could corresponds mainly to a constant reinfection of birds and that host-to-host transmission determines the carrier state at the flock level. The presence of super-shedders, with a high level of caeca colonization, similar to that observed in the large cage model and excreting a high level of *Salmonella* as early as 4 days postinfection and throughout the experiment, demonstrates that *Salmonella* is able to persist in a bird even in the absence of reinfection and host-to-host transmission. The hypothesis suggesting that persistence of *Salmonella* corresponds mainly to a constant animal–animal reinfection is thus not valid at least for some animals. However, the existence of low-shedder chicks under the same conditions demonstrates that host-to-host transmissions between chicks are required to induce a homogeneous and high level of *Salmonella* infection at the flock level. The data showing that a chick which is a super-shedder remains a super-shedder even without host-to-host transmission strongly suggests that these low-shedder chicks do not have a higher ability to eliminate *Salmonella* than the super-shedders, but that they are more able to block the primary colonization by *Salmonella.* Despite being able to block this initial colonization, in the presence of Supper

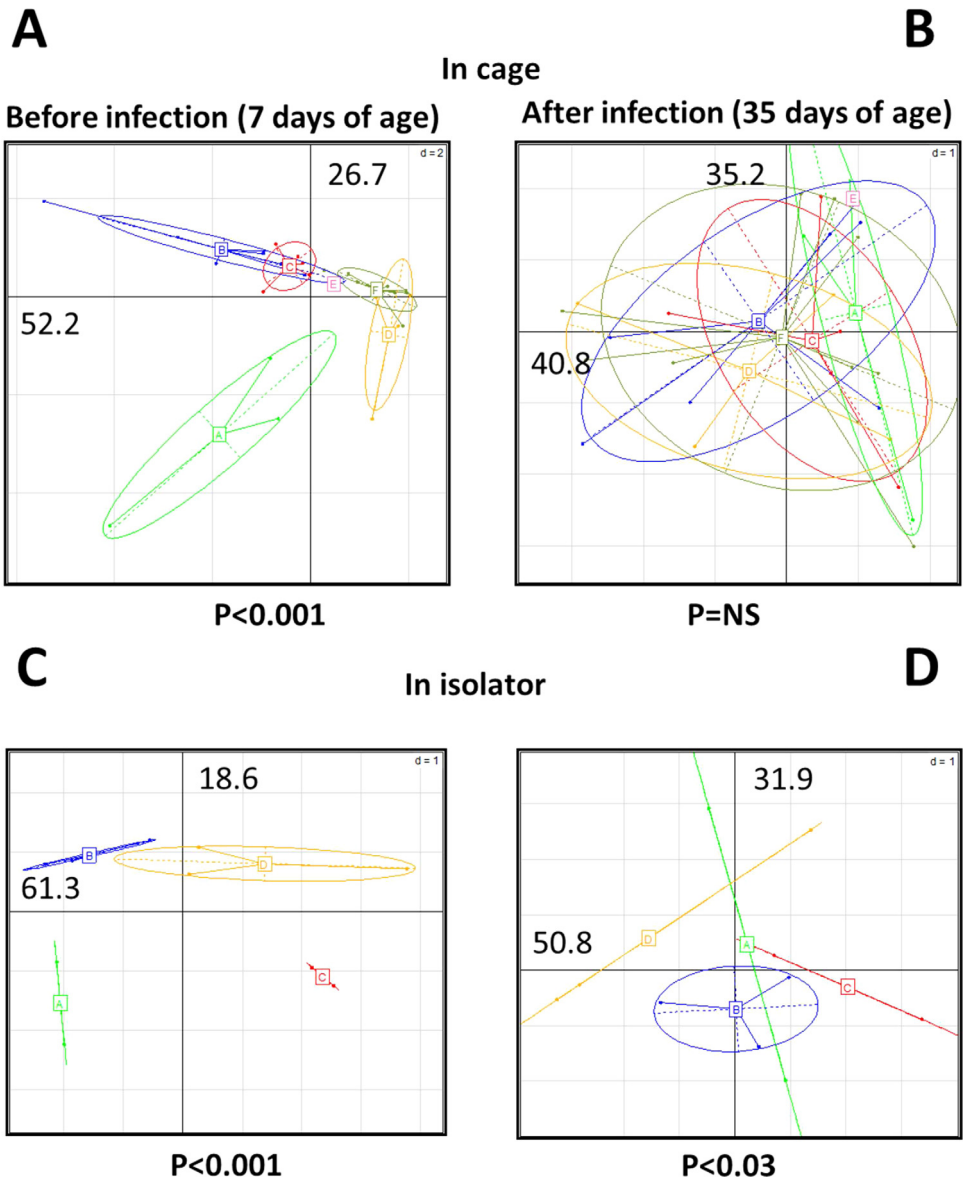

Fig. 5. Microbiota composition evolved differently in chicks reared in cage and in isolator.Chick microbiota composition was analysed by quantitative PCR enabling to quantify eight different bacterial groups. Fresh faecal samples were recovered from chicks reared in cage (A, B) or in isolator (C, D) before infection (A, C) and after wild type *S.* Enteritidis infection (B, D). Relative abundance of each bacterial group or genus per chick was described by a principal component analysis. Several clusters were defined before infection (7 days of age), on the basis of PC coordinates. For this, we only considered PC representing ~80% of total variance (respectively, 6 clusters and PC1 to 3 for chick bred in cage; 4 clusters and PC1 and 2 for chicks bred in isolators). Microbiota composition at 35 days of the same clusters was next summarized by PCA analysis (B, D). The relation among cluster category and PC coordinates was assessed using multinomial regression analyses.

shedders these low-shedders are unable to maintain their colonization resistance being overwhelmed by this continual excretion of *Salmonella* and in-turn become super shedders themselves. This hypothesis is strengthened by the fact that when we orally inoculated 1×10^8 bacteria instead of 5×10^4 *S.* Enteritidis to chicks raised in isolator, all chicks became super-shedders (data not shown).

Host-to-host transmission occurs mainly through contaminated aerosols and dust particles in the air

Host-to-host transmission of a pathogen ensures its successful propagation and maintenance within a host population. We demonstrated that host-to-host transmissions between chicks were essential for *Salmonella* carrier state to develop at the flock level. For this reason, identifying the main routes of host-to-host transmission may be

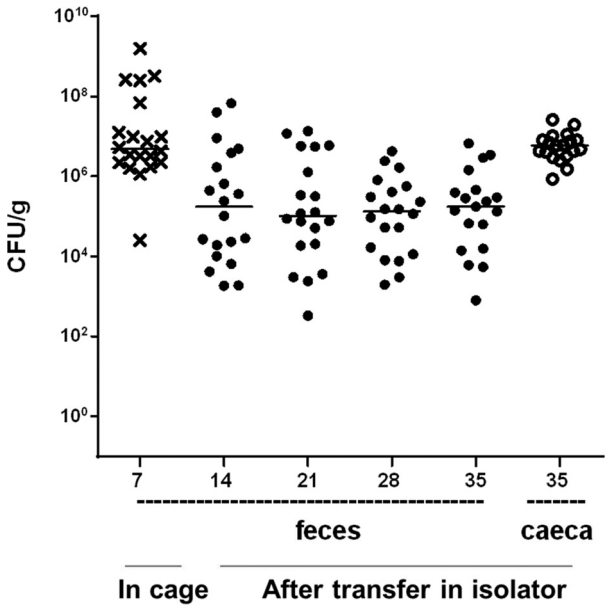

Fig. 6. *Salmonella* Enteritidis colonization of chicks infected in cage and then transferred in isolator.Twenty chicks reared in one cage, were orally inoculated with 5×10^4 CFU of the wild type LA5 strain at 7 days of age. After 7 days, chick fecal samples were recovered and chicks were transferred into one isolator. *Salmonella* excretions in fresh fecal samples were followed for 5 weeks postinfection in cage (x) or in isolator (dot). Caeca colonizations (open circle) were monitored at necropsy.

of key importance. In chicken, the faecal oral route has been described as the main source of reinfection (Holt *et al*., 1998). We tested this hypothesis by placing fine mesh wire on the floor of the isolators in order to favour presence of faeces on the floor. However, under these conditions we did not observed a higher frequency of super-shedders (data not shown). The second source of reinfection that has been described is airborne movement of *S.* Enteritidis (Davies and Wray, 1994; Gast *et al*., 1998). As it is impossible to decrease the flow rate of the ventilation in the isolator or the filtration of air, we tested this hypothesis in the cage model. For that purpose, we put naïve chicks into a small cage and kept them close (i.e., 5 meters away) to a cage containing infected chicks. Figure 7 shows that the 'naïve' animals were rapidly contaminated by *Salmonella* and at high levels, similar to those obtained in the cage containing infected chicks. Figure 7A and B show that in the cage containing noninoculated chicks, all chicks quickly excreted very high levels of *Salmonella* and no statistical difference could be detected between the two groups (Fig. 7C). The only difference was related to the level of *Salmonella* in caeca, which was significantly lower in the naïve chick group than in the inoculated chick group ($p < 0.001$) but remained a very high level of infection (6.7×10^7/g vs. 4.1×10^8 *Salmonella* respectively). This experiment showed that chicks infected under 'natural conditions' that is to say not by oral injection of bacteria, exhibited similar levels of excretion and caeca contaminations as those observed in our model which involved oral inoculation with 5×10^4 *Salmonella.*

Bacterial source tracking revealed that only a few *Salmonella* were recovered from lung samples, but high levels of bacteria were found in drinking water and feed suggesting that infection was very likely transmitted mainly by oral ingestion. In light of these results, we suggest that host-to-host transmission mainly occurs through contaminated dusts or aerosols which can contaminated food/water from the cage housing the noninfected animals as previously suggested (Nakamura *et al*., 1994). This experiment was repeated in another room and thus under different environmental conditions and the same results were obtained. Interestingly, in a third experiment, we failed to reproduce airborne transmission and *Salmonella* contamination when the cage playing the role of the contamination transmision did not include super-shedder chicks. In this experiment, where the *Salmonella* excretion levels for all chicks were inferior to 3×10^3/g in the infected cage, no chick excreted *Salmonella* in the naïve chick group even after 3 weeks. This strongly supports a major influence of the super-shedder phenotype on *Salmonella* infection patterns at the flock level.

Although the initial route of *S.* Enteritidis transmission appears to be through contaminated dust particles or aerosols, further studies are required to understand the mechanisms of this transmission. However, our results suggest that the presence of super-shedders is required for airborne horizontal transmission of *Salmonella.* These observations are of great importance to improve guidelines on control measures in poultry farms and highlight the great importance of ventilation in buildings.

The presence of super-shedder and low-shedder animals has already been described in pigs following inoculation with equivalent doses of *S.* Typhimurium (Huang *et al*., 2011). This has also been found in inbred mice, where only one subset of infected mice shed high levels of *Salmonella* and transmitted infection (Lawley *et al*., 2008). However, in these models, the ratio of excretion levels between super-shedder and low-shedder ranged from 10 to 1000, while the order of magnitude in our experiments was clearly higher (i.e. 1×10^6). This is probably related to the very low level of host-to-host transmission in our isolators in contrast to that observed in the other models.

More generally, heterogeneity in infectious disease dynamics, where a small subset of the population is responsible for the majority of new cases, is now commonplace (Woolhouse *et al*., 1997; Lloyd-Smith *et al*., 2005; Gopinath *et al*., 2014). The 20–80 rule refers to the general pattern where 20% of hosts contribute to around

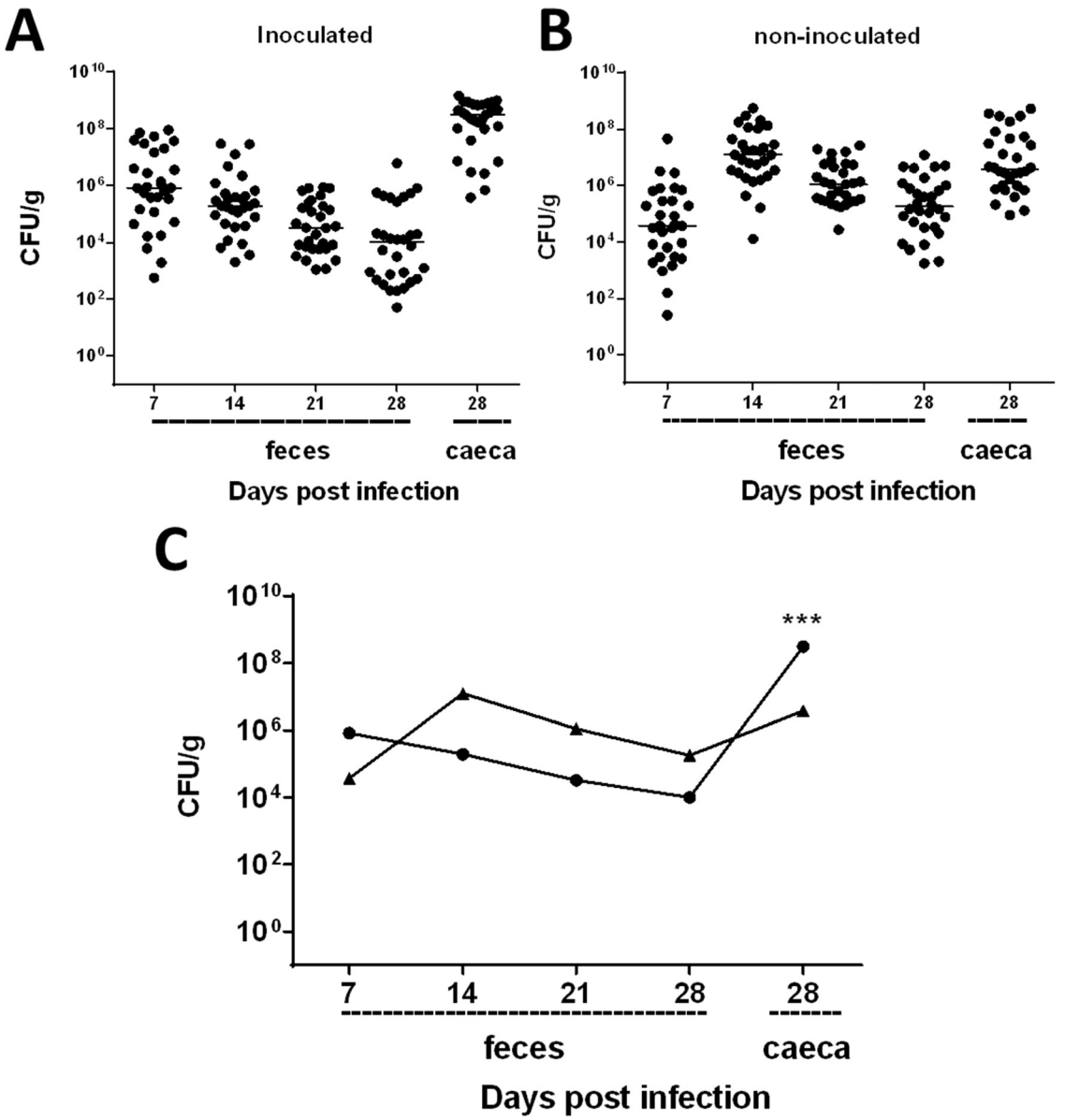

Fig. 7. Airborne transmission of *S.* Enteritidis between chicks reared in cage.*Salmonella* shedding of infected and uninfected chicks reared in cages located in the same room. Fifty chicks of the first large cage were orally inoculated at 7 days of age, with 5×10^4 CFU of the wild-type LA5 strain. The chicks of the small cage (30 chicks), distant of 5 m, were not inoculated but under the air flow of the first cage. *Salmonella* fecal shedding and caeca colonization from the same 30 chicks were monitored by culturing fresh fecal samples and caeca from the inoculated chicks (A) and the uninfected chicks (B). The numbers of *S.* Enteritidis CFU per gram of caeca and fecal samples were compared by the Wilcoxon–Mann–Whitney test (C) and the median values of the CFU are presented for inoculated (dot) and noninoculated (triangle) chicks (***, $p < 0.001$).

80% of the transmission potential of a pathogen (Woolhouse *et al*., 1997). There is evidence that super-spreaders or super-shedder hosts may emerge in many other human and animal diseases. The most frequent zoonoses (salmonellosis, colibacillosis and campylobacteriosis) are no exception to this general rule (Chase-Topping *et al*., 2008; Bearson *et al*., 2013), and the link between the presence of livestock super-shedders and the occurrence of human infections has been established for *E. coli* (Chase-Topping *et al*., 2008). This phenomenon has key implications for infectious disease dynamics and control. To improve the microbial safety of food and to develop new preventive measures for controlling zoonosis, we have to take into account this heterogeneity of infection and target the super-shedders. Identifying those hosts would enable controlling over approximately 80% of the pathogen transmission events in the population (Galvani and May, 2005). Therefore, prediction, identification and elimination of the super-shedders represent important facets of infectious disease management and preparedness plans.

In conclusion, we have demonstrated that *Salmonella* can persist for more than four weeks in some chicks in the absence of host-to-host transmission and thus that constant reinfection is not required to induce a long-term carrier state. We have also demonstrated that systemic infection is not required to cause persistence in caeca and thus it does not determine the development of carrier state. This work has highlighted the crucial role of host-to-host transmission in the spread of *S.* Enteritidis and in the establishment of a homogeneous carrier state at the flock level. When host-to-host transmissions are greatly reduced, *S.* Enteritidis induces a heterogeneous infection where 10%–30% of inoculated chicks are super-shedders functioning as a reservoir for the pathogen and continually disseminate *Salmonella* to the low-shedder chicks. Heterogeneity of infection is a multifactorial process involving numerous parameters such as the genetics of the host and thus the host immune status. Several resistant and susceptible chicken lines have been described according to these differences (Sadeyen *et al*., 2004; Fife *et al*., 2009). However, in the present study heterogeneity of infection was also observed even though we used the same *Salmonella* strain, dose and chicken line (inbred to more than 99% and reared under SPF conditions). Our results suggest that the low-shedder phenotype is related to chicks, which do not have a higher ability to eliminate *Salmonella* as observed in resistant chicken lines (Sadeyen *et al*., 2004; Calenge *et al*., 2010) but instead are more efficient at blocking the initial *Salmonella* colonization. This resistance to *Salmonella* colonization seems only observed for low doses of *Salmonella* often observed in field conditions as when we orally inoculated high doses of *Salmonella* only obtained with super-shedders, all chicks directly became super-shedders. Several mechanisms related to colonization resistance and/or a barrier effect have been described (Van Immerseel *et al*., 2005; Endt *et al*., 2010; Brugiroux *et al*., 2016). Our model in an isolator is thus a suitable model to understand why some animals are Low-shedder while others are super-shedders. This work opens new avenues: (i) to analyze genes that are involved in persistence independently of the transmission step, which involves coping mechanisms in the environment; (ii) to identify the host immune response and the gut microbiota composition involved in low-shedder chicks; (iii) to define new strategies to control *Salmonella* infection by identification of the super-shedders and targeting their elimination or treatment.

Experimental procedures

Ethical statement

Animal experiments were carried out in strict accordance with French legislation. The experiments were approved by the French Ministry of education, higher education and research (Ministère de l'éducation nationale, de l'enseignement superieur et de la recherche) under the protocol No. APAFIS#5833-20l6062416362298 v3. The principles of reduction, replacement and refinement were implemented in all the experiments.

Salmonella strains

The nalidixic acid-resistant *Salmonella* Enteritidis Phage Type 4 LA5 strain was used. This well-characterized strain was isolated from a 4-day-old broiler chicken (Dibb-Fuller *et al*., 1999; Grepinet *et al*., 2012). For a small number of experiments, a spontaneous streptomycin resistant clone of LA5 or a LA5*yfgL::aphT* was used. This latter mutant, also named *bamB::aphT* mutant, is impaired in its outer membrane biogenesis and T3SS expression, and consequently is greatly attenuated for virulence in mice (Fardini *et al*., 2007). To prepare the inocula, the strains were cultured aerobically in trypticase soya broth (BioMérieux) supplemented with 20 μg/ml of nalidixic acid (Sigma-Aldrich) for 24 h at 37°C with shaking. When necessary, streptomycin or kanamycin were added (at 500 μg/ml or 25 μg/ml respectively). The cultures were centrifuged at 4500 × g for 20 min at 20°C and the pellets were suspended in phosphate buffered saline (PBS) containing 50% glycerol. The bacterial suspensions were aliquoted, frozen and stored at –80°C. On the inoculation day, the bacterial suspensions were diluted in PBS to obtain 2.5×10^5 colonies forming units/ml. The frozen aliquots from the same initial inoculum were used throughout the experiments.

Housing systems

All white leghorn chicks (PA12) used in this study originated from the same Specific-Pathogen-Free (SPF) flock reared at the platform of animal infectious diseases PFIE (INRA Val de Loire, France). The *Salmonella*-free status of all chicks was tested by analyses of blood and faecal samples. All chicks had free access to drinking water and were fed ad libitum. A 12:12 L:D lighting scheme was applied.

Two housing systems were used, a modulated large cage system and an isolator system. Each large cage was installed in a separate room with the same environmental conditions (feed, water, temperature, air humidity, lighting scheme). All rooms were equipped with a high efficiency particulate air filter (HEPA). The cages could be modulated and provided a maximum area of 4 m^2 that was designed for 150 chickens. Chicks were reared on a slatted flooring, which was covered with wire mesh on the floor: 1 cm × 1cm for chicks up to 10 days of age, and 2 cm × 2 cm for chicks over 10 days of age. Under

these conditions, only a small quantity of faeces was present on the floor. On the day of hatching, all chicks were marked individually by means of a leg tag.

Three types of isolator were used. Small isolators, measuring 0.24 m^2 were used for 1 chick per isolator; medium size isolators, measuring 0.90 m^2 were used to house 10 chicks; large isolators, measuring 2.26m^2, were used for 30 chicks. In the isolators host-to-host transmissions between chicks and subsequent reinfections were severely hampered through efficient filtration of air with HEPA filters and with an air change rate above 54 m^3/h. Wire mesh similar to that used in large cages was used on the floor allowing faeces to fall directly into decontaminant solution of quaternary ammonium solution resulting in immediate sterilization. Finally, feed and water containers were suspended above floor level in order to limit direct contamination through animal droppings. White leghorn chicks (PA12) housed in isolators were not germ free because they acquired their microbiota in the hatchery and then in the room where they were reared in battery cages for 7 days. After one week, chicks were infected via the oral route using the same dose as that used in our classic large cage model (5×10^4 bacteria) and then transfered to isolators.

Infection of chicks

To assess infection by the *bamB* mutant, newly hatched white leghorn chicks (PA12) were randomly divided into two large cages of 126 and placed in two rooms each with controlled airflow and temperature. The absence of *Salmonella* in faecal samples was checked systematically in birds before experimental infection. At 7 days of age, chicks were inoculated orally with 0.2 ml of bacterial suspension containing either wild type *S.* Enteritidis LA5 or the isogenic *bamB* mutant (5×10^4 CFU/chick). Each week, 14 animals were sacrificed by carbon dioxide inhalation. Spleen and caeca were removed aseptically from each animal for quantification of *Salmonella* load. Organs were homogenized in trypticase soy broth (TSB) and serial 10-fold dilutions were plated on *Salmonella–Shigella* (SS) medium containing nalidixic acid (20 μg/ml). The mean counts of *S.* Enteritidis CFU in organs were calculated per gram at each time point. When necessary, the sample homogenates were enriched to reveal contamination below the detection threshold. Enrichment was performed by diluting crushed organ in 30 ml tryptic soy broth. After 24 h at 37°C these cultures were plated on SS medium containing nalidixic acid and incubated for 24 h. Under these conditions, the detection threshold after enrichment is one bacteria per organ.

The other infections in the large cages were performed with the wild type strain, under the same conditions but with different numbers of chicks.

For the air transmission experiments, a large cage (4 m^2) and a small cage (2 m^2) were placed in a large room (35 m^2). In the large cage, 50 chicks were orally inoculated with *S.* Enteritidis at 7 days of age. In the small cage, positioned 5 m away, 30 chicks were not inoculated but were under the airflow, which is directed from the inoculated cage to the noninoculated cage at 8.5 m/s. Great care was taken to avoid any transmission between the cages due to humans. It is important to note here that chicks were reared on a slatted flooring with only a small quantity of faeces on the floor.

To follow *Salmonella* excretion level when chicks were infected in large cages and then transferred in isolator, 85 chicks, reared in large cage (4 m^2), were orally infected at 7 days of age and maintained in this cage for 7 days. Seven days postinfection, 20 infected chicks were transferred in two medium-size isolators (10 chicks per isolators). At 7, 14, 21, 28 days postinfection (for the transmission via air), and at 7, 14, 21, 28, 35 days postinfection (for the transfer in isolator), fresh faeces were sampled. At the end of the experiment, animals were sacrificed by carbon dioxide inhalation and caeca were removed aseptically from each animal for quantification of *Salmonella* load. Caeca and fecal samples were homogenized in trypticase soy broth (TSB) as previously described and serial 10-fold dilutions were plated on SS medium containing nalidixic acid (20 μg/ml). The mean counts of *S.* Enteritidis CFU in faecal droppings or caeca were calculated per gram at each time point. When necessary, the sample contents were enriched as previously described.

For infection in isolators, on the hatch day, all chicks were marked individually with leg tags and were housed in a conventional battery cage system in the same room for 1 week to acquire a gut microbiota. One week later, chicks were randomly divided into groups of 10, individually infected by the oral route with 5×10^4 *S.* Enteritidis wild type as previously described and were transferred into 3 different medium size-isolators (0.9 m^2). Similar experiment was performed but with large isolators (2.26 m^2) where 30 infected chicks where introduced. Fresh faecal samples were collected before and after infection (2 or 4, 7, 14, 21, 28 days of age) and caeca colonization was determined after necropsy. *Salmonella* load was assessed as for the cages. When necessary, the sample contents were enriched.

Microbiota characterisation

Microbiota composition of faeces was analysed on faecal droppings, which were collected by gently pressing the chick abdomen. Fresh faeces were directly frozen in dry ice and stored at -80°C until further analysis. Microbial DNA was extracted using the QIAamp DNA mini-kit

following the manufacturer's instructions (QIAGEN, ref 51306). Briefly, 25 mg of faeces were transferred to a tube with lysis buffer and sterile zirconium beads. Samples were homogenized in the FastPrep FP120® instrument for 4 × 45 s at a speed of 6.0 m/s. The tubes were placed in ice for 5 min after two cycles. This step was followed by heating for 15 min at 70°C and centrifugation (5 min, 16 000 g, 4°C) before a second extraction step was carried out. The two supernatants were pooled for DNA purification. Proteinase K was added and the samples were heated for 10 min at 70°C to remove proteins. Ethanol was then added and the samples were purified using a QIAamp column as described by the manufacturer. DNA was eluted in Tris-EDTA buffer AE (Qiagen).

The amount and quality of DNA were measured with a Nanodrop spectrophotometer. 16S rDNA was quantified using qPCR to determine the number of 16S rDNA copies of total bacteria and the main bacterial groups in the chicken gut in the Firmicutes phylum (lactobacillales and Clostridiales orders, and *Clostridium coccoides* and *C. leptum* groups) and the Proteobacteria phylum (Enterobacteriales order, and *Escherichia coli* species), the Bacteroides-Prevotella group, the Bifidobacteriales order and the *verrucomicrobiales* order and *fusobacteria* phylum. Primers used to achieve this are described in Supporting Information Table S1. Briefly, quantitative PCR reactions were run in triplicate in 384 well plates in a final volume of 10 µl. The EpMotion 5070 liquid handling robot (Eppendorf, Le Pecq, France) was used to distribute the master mix and DNA. Reactions for bacterial groups consisted of 5 µl of Light Cycler 480 SYBR Green I Master Mix (Roche, Meylan, France), 0.5 µl of 10 µM for each primer, 1.5 µl of nuclease-free water and 2.5 µl of template DNA at the appropriate dilution. Amplification was carried out with a Light Cycler 480 (Roche, Meylan, France) as follows: 10 min at 95°C, followed by 45 cycles of denaturation (10 s at 95°C), annealing (30 s) and extension (30 s at 72°C). Following amplification, melting curves were analyzed to assess the specificity of the amplified product. Standard curves were generated by amplification of serial 10-fold dilutions of DNA extracted from a pure culture of each targeted bacteria. The copy number of 16S rDNA for each reaction was calculated from the standard curves and determined by the second derivative maximum method.

Statistical analyses

Results were compared by analysis of variance and means were analysed using Student's *t*-tests for homogeneous infections as previously described (Sadeyen *et al*., 2004). In contrast, median and a nonparametric test were used for heterogeneous infections (Wilcoxon–Mann–Witney test). To determine the super-shedder and low-shedder phenotypes, the levels of *Salmonella* excretion were analyzed using a principal component analysis. Chicks clusters were inferred using a hierarchical clustering analysis based on Ward's distance (Ward, 1963). Correlation among chick clusters (defining a categorical variable) and PCA coordinates were tested using multinomial regression. All computations were performed using the dedicated R-packages Rcmdr, nnt ADE4TkGUI, VGAM and the R native function h-clust (Venables and Ripley, 2002; Thioulouse and Dray, 2007; R Development Core Team, 2008; Fox and Bouchet-Valat, 2017).

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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:

Table S1. Primers used for gut microbiota analysis by quantitative PCR.