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Gut microbiota composition before infection determines the *Salmonella* super- and low-shedder phenotypes in chicken

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Summary

Heterogeneity of infection and extreme shedding patterns are common features of animal infectious diseases. Individual hosts that are super-shedders are key targets for control strategies. Nevertheless, the mechanisms associated with the emergence of super-shedders remain largely unknown. During chicken salmonellosis, a high heterogeneity of infection is observed when animal-to-animal cross-contaminations and reinfections are reduced. We hypothesized that unlike super-shedders, low-shedders would be able to block the first *Salmonella* colonization thanks to a different gut microbiota. The present study demonstrates that (i) axenic and antibiotic-treated chicks are more prone to become super-shedders; (ii) super or low-shedder phenotypes can be acquired through microbiota transfer; (iii) specific gut microbiota taxonomic features determine whether the chicks develop a low- and super-shedder phenotype after *Salmonella* infection in isolator; (iv) partial protection can be conferred by inoculation of four commensal bacteria prior to *Salmonella* infection. This study demonstrates the key role plays by gut microbiota composition in the heterogeneity of infection and pave the way for developing predictive biomarkers and protective probiotics.

Introduction

Many studies have revealed the importance of heterogeneous shedding levels in the context of infectious diseases (Matthews *et al*., 2006; Slater *et al*., 2016). The infected individuals that harbour and shed a given pathogen at higher concentrations than their congeners are referred to as 'super-shedders'. This phenotype can be observed even in the case of a genetically homogeneous host population (e.g. inbred mice (Lawley *et al*., 2008) and chickens (Menanteau *et al*., 2018)).

As these super-shedders have a much higher transmission rate, they constitute a key target for epidemiological investigation and disease management. For various diseases, empirical observations and transmission modelling have revealed that 20% of the individuals contribute to 80% of all infection events (Woolhouse *et al*., 1997). Control strategies that do not take into account individual shedding heterogeneity tend to fail (Matthews *et al*., 2006; Marshall and French, 2011). Novel approaches may include early removal of the future super-shedder individuals detected using predictive biomarkers, or interventions favouring low-shedders. However, this would require a better understanding of the underlying mechanisms shaping *Salmonella* shedding patterns.

Besides, the well-documented example of *Escherichia coli* O157 (Naylor *et al*., 2003), the existence of super-shedders has been described for campylobacteriosis (Rapp *et al*., 2012) and salmonellosis (Lawley *et al*., 2008; Bearson *et al*., 2013; Menanteau *et al*., 2018). Salmonellosis is of particular concern regarding economic losses to the livestock industry and threats to human health. *Salmonella* is the second cause of human foodborne illness, causing substantial mortality and loss to the economy (Van Cauteren *et al*., 2017; EFSA, 2018). Depending on the host and serotype, *Salmonella* have the ability to cause a wide range of diseases including lethal systemic infection and asymptomatic infection. In pigs and chickens, *Salmonella* strains may induce a systemic infection, potentially leading to death, but more

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frequently evolving into a long-term asymptomatic carrier state (Velge *et al*., 2005). *Salmonella*-carrier animals are a serious issue for animal and human health. Indeed, those animals that excrete high levels of bacteria in their faeces can contaminate their congeners but also meat products during slaughter. *Salmonella* transmission to humans is thus generally due to the consumption of contaminated food of animal origin, including poultry and eggs (EFSA, 2018).

Previous studies on chicken salmonellosis have shown that host genetics and immune response can partly explain the heterogeneous resistance to *Salmonella* (Calenge *et al*., 2010; Chaussé *et al*., 2011). However, heterogeneity of infection and the presence of super-shedders have been observed when inbred chicken are experimentally infected in isolators in which animal–animal cross-contaminations and reinfections are greatly reduced. These results suggested that other parameters are involved in susceptibility and resistance to *Salmonella* colonization at the individual level. In particular, the outcome of the first exposure to *Salmonella* is of key importance: contrary to super-shedder chicks, low-shedders are able to block early colonization (Menanteau *et al*., 2018). In addition, when *Salmonella* overcomes this first barrier effect, it might persist in the host intestine for a long period even in the absence of an external source of contamination (Menanteau *et al*., 2018). This argues for a role of the gut microbiota as observed in inbred mice infected with *Salmonella* Typhimurium (Gopinath *et al*., 2012). In the present article, we addressed the role played by the chicken gut microbiota in the host susceptibility to *Salmonella* Enteritidis infection, including in the occurrence of low- and super-shedders. These terms design the animal subgroups presenting the highest and lowest levels of *Salmonella* excretion and caeca colonization over time. By metabarcoding, we revealed that the composition of gut microbiota before infection determined the degree of host susceptibility to *Salmonella* infection. In line with this result, we assessed the protective effect of several commensal bacteria strains inoculated before infection.

Results

Inoculation of adult microbiota has a protective effect in 1-day-old chicks

Hens are known to have a greater resistance to *Salmonella* infection than chicks. Indeed, *Salmonella* Enteritidis is able to induce an asymptomatic carrier state when $5x10^4$ colony-forming units (CFU) are orally inoculated in 7-day-old chicks (Sadeyen *et al*., 2004). In older chicks, the same dose has no effect. A successful experimental infection of 30-week-old hens requires a dose of $1x10^8$ CFU per hen (Sadeyen *et al*., 2006).

We aimed to test whether the gut microbiota composition of adult hens may explain this lower susceptibility. To this end, 80 one-day-old chicks were inoculated with diluted caecal samples originating from adult layers. A control group of 80 chicks was inoculated at the same age with a germ-free solution. All the chicks were then inoculated orally with *S*. Enteritidis at 7 days of age and their patterns of caecal colonization were recorded at 5, 12, 19 and 26 days after infection. In this experiment, all the chicks were reared in cages, as these conditions tend to favour high and homogeneous caecal colonization patterns through cross-contamination (Menanteau *et al*., 2018).

Figure 1A shows that caecal colonization was completely inhibited 5 days after infection, for all individuals (except one) inoculated with diluted hen microbiota. This inhibition was observed for a long period (i.e. until 26 days after infection) which may be interpreted as a long-term protective effect. By contrast, all chicks in the control group presented high and homogenous levels of *Salmonella* caecal colonization at all-time points following infection. This experiment highlights that adult hen microbiota samples may have a protective effect on 1-day-old chicks.

Axenic and antibiotic-treated chicks present particular patterns of susceptibility to Salmonella *infection*

In this second stage, we aimed to determine whether changes in the gut microbiota composition were linked to the development of a particular *Salmonella* shedding phenotype.

First, 7-day-old axenic chicks were orally infected with $5x10^4$ CFU *S*. Enteritidis in an 'axenic isolator'. This breeding device hampers the entry of environmental bacteria and favours the detection of low-shedder chicks (Menanteau *et al*., 2018). Surprisingly, none of the axenic chicks died following *Salmonella* infection. However, all of them exhibited very high *Salmonella* caecal colonization compared to the non-axenic chicks reared in cage (Fig. 1B). These levels were homogeneous contrary to what was observed in non-axenic chicks reared in isolators (Menanteau *et al*., 2018). These results suggest that the *Salmonella* super-shedder phenotype was strongly favoured in germ-free chicks and reinforce the hypothesis of a role of the microbiota in chicken resistance to *Salmonella* infection.

Second, we performed an experiment aimed at determining whether gut microbiota dysbiosis may favour the emergence of the super-shedder phenotype. For this, dysbiosis was induced using streptomycin as previously described (Lawley *et al*., 2008). This antibiotic is a large spectrum protein synthesis inhibitor able to kill both Gram-positive and Gram-negative bacteria. Moreover,

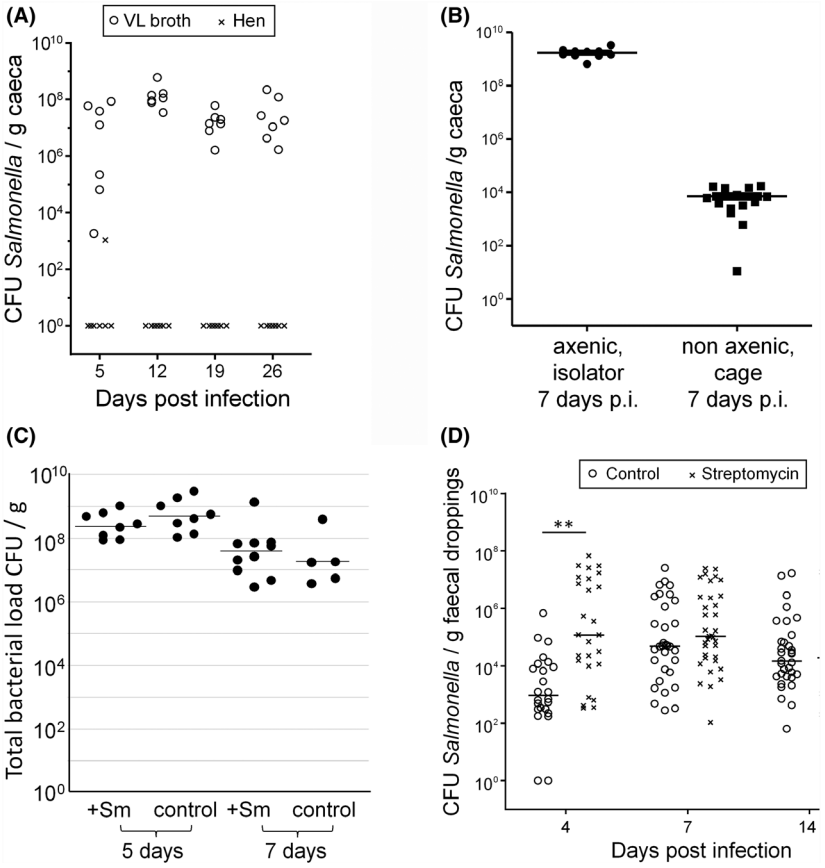

Fig. 1. Impact on *Salmonella* colonization of overall modifications of the chick gut microbiota.
A. Levels of *Salmonella* caecal colonization after an inoculation of VL broth (i.e. control chicks; empty dots) or hen microbiota (crosses) at 1 day of age, followed by an oral inoculation of *S*. Enteritidis at 7 days of age (7 individuals per time point; each cross/empty dot represents one chick). Chicks were reared in large cages.
B. Levels of *Salmonella* caecal colonization for 10 axenic and 15 SPF (non-axenic) chicks bred in an axenic- or a non-axenic-isolator, respectively. Chicks were then inoculated with *S*. Enteritidis at 7 days of age. All chicks survived.
C. Counts of cultivable bacteria in the faecal samples collected at 5 and 7 days of age from 35 chicks treated with streptomycin ('+Sm'; one chick/dot). Streptomycin was orally inoculated at 1 day of age and added into the drinking water until 4 days of age. The chicks were bred in cage until 4 days of age and next relocated in isolator. A control group of 35 chicks was bred in the same conditions except the exposure to streptomycin ('control'; one chick/dot). No significant differences were found between both groups. Bacterial cultures were carried out on rich medium (BHI) under aero-anaerobic conditions at 41°C. Bacterial counts were performed after 24 and 48 h.
D. Levels of *Salmonella* faecal excretion at 11, 14 and 21 days of age for the 'control' and streptomycin ('+Sm') groups after an oral inoculation of *S*. Enteritidis at 7 days of age. Median values and results of the t-tests comparisons were represented on the figure.

streptomycin is cleared 2 days after its removal from drinking water. Indeed, a previous study conducted in a mouse model showed that the initial number of commensal bacteria was restored 48h after treatment with streptomycin, despite qualitative modification of the microbiota (Lawley *et al*., 2008). However, to ensure that the *Salmonella* strain would not be killed by the residual streptomycin, a spontaneous streptomycin resistant mutant of our *S*. Enteritidis LA5 strain was used here to perform the experimental infection. In this experiment, chicks were first exposed to streptomycin through oral inoculation at 1 day of age, and then through drinking water containing streptomycin until 5 days of age. The bacteriological analyses performed on faecal samples on days 5 and 7 revealed similar numbers of commensal bacteria cultured on a rich culture medium (BHI), during 24 and 48h under aero-anaerobic conditions (Fig. 1C). However, different morphology patterns between the streptomycin-treated and control groups were observed. The streptomycin treatment had thus induced qualitative rather than quantitative differences in the gut microbiota composition as previously described in mice with 16S rRNA gene quantifications (Lawley *et al*., 2008).

Analyses of *Salmonella* shedding patterns, 4 days after infection, revealed dramatic differences between the streptomycin-treated and control groups (Fig. 1D). Indeed, faecal dropping of streptomycin-treated animals contained 100 fold more CFU of *Salmonella* than those of the control group. The streptomycin-mediated changes in the gut microbiota composition could have

favoured *Salmonella* colonization. However, differences between these groups were no longer detected 7 and 14 days after infection.

Low and super-shedder phenotypes can be transferred through gut microbiota transplantation

The results above support an influence of the gut microbiota composition on *Salmonella* colonization and shedding levels. To demonstrate the role of gut microbiota in the low- and super-shedder phenotypes, independently of the other factors, we investigated whether it was possible to transfer these phenotypes through microbiota transplantation.

To this end, a first group of 30 chicks (Group 1; Fig. 2A) was initially kept in a cage for 4 days during which period the chicks acquired their gut microbiota. They were then relocated into an isolator. It is important to note that although the isolators used in these studies strongly decreased the level of cross-contamination among the chickens, they did not hamper the development of gut microbiota in chicks (Menanteau *et al*., 2018). Before infection, faecal droppings were collected at 5 and 7 days of age and were immediately deep-frozen to reduce the depletion of particular taxa. At 7 days of age, chicks were infected with the *S*. Enteritidis strain LA5. Faecal samples were collected at 11 and 14 days of age, and caecal samples at 21 days of age. The chicks were clustered into low- or super-shedder categories by hierarchical clustering as previously described according to the levels of *Salmonella* found in the postinfection samples (Menanteau *et al*., 2018). Pre-infection faecal samples were then pooled to prepare two mixes; the first one contained samples taken from three chicks that became super-shedders after infection ('super-shedder' mix [SSM]); the second contained samples taken from three chicks that became low-shedders after infection ('low-shedder' mix [LSM]). In a second step, two batches of 30 one-day-old chicks, bred separately in isolator, were either orally inoculated with the SSM or LSM after hatching (Group 2, Fig. 2A). At 7 days of age, they were inoculated with *S*. Enteritidis LA5. Samples collected at 11, 14 days of age (faeces) and 21 days of age (caeca) were used for *Salmonella* counts (Fig. 2B).

The chicks of Group 2 presented contrasting shedding levels depending on which mix they had received. SSM-inoculation led, 4 days after infection, to higher levels of faecal excretion compared to the chicks inoculated with LSM mix (Fig. 2B; the median levels were CFU g^{-1} = 4.41×10^5 and 8.18×10^2, respectively, $P < 0.001$). The levels observed after LSM-inoculation was similar of those obtained for chicks not transferred with any mix (i.e. CFU $g^{-1} = 9.37 \times 10^2$, see (Menanteau *et al*., 2018)). In addition, SSM-inoculated chicks presented much higher and more homogeneous *Salmonella* caecal colonization than LSM-inoculated chicks (Fig. 2B; the median values were CFU $g^{-1} = 1.30 \times 10^7$ and 4.85×10^5 respectively, $P < 0.001$).

A multivariate analysis was performed to assess the relationships among the gut microbial compositions of donors and receivers. For this, the shedding levels at 4, 7 and 14 days after infection were summarized using a PCA framework (Fig. 2C). We highlighted a relationship between each categories and Principal Component 1 (SSM donors: $P = 0.03$, LSM donors: $P = 0.11$, SSM receivers: $P < 0.001$, LSM receivers: $P < 0.001$). Moreover, donors and receivers for SSM or LSM tended to be clustered on Principal Component 1: the mean coordinates being positives for the SSM and negatives for the LSM. Thus, individual positions on the PCA plot support the hypothesis that shedding patterns of group 2 chicks were related to the bacterial composition of the transferred mix. Consequently, chicks can be either sensitized or protected against *Salmonella* infection through a microbiota transfer. This suggests in turn that particular bacterial species may favour or inhibit *Salmonella* colonization.

Gut microbiota composition is modified by Salmonella *infection*

Differences in gut microbiota composition before and after infection in low and super-shedder chicks were assessed using an amplicon-based sequencing of V3/V4 variable region of 16S rRNA genes. For this purpose, 30 chicks were reared in an isolator and infected with *S*. Enteritidis, at 7 days of age. Faecal samples were collected on days 5, 7, 11, 14 and the caecal content and caecal mucus were sampled on day 21. The chicks were then clustered into low, super and intermediate shedders on the basis of a PCA (Fig. 3A and B). For further metabarcoding characterization, a subset of 13 individuals was defined by selecting equivalent numbers of chicks within the three shedding categories (Fig. 3A and B).

On day 5, most of the OTUs found in chick microbiota were assigned to the *Firmicutes* phylum and to the *Lactobacillaceae* and *Lachnospiraceae* families (60.02%, 24.26% of total microbiota at 5 days of age; Fig. S1). On day 21 (caecal samples), the *Lachnospiraceae* was the predominant family (66.21%). Among the 49 OTUs presenting significant changes, most of them were assigned to the genera *Anaerotruncus*, *Bacillus*, *Enterococcus*, *Lactobacillus*, *Lachnoclostridium* and *Streptococcus* (Table S1A). This was confirmed by variance analyses of genus frequencies (Table S1B). Pairwise comparisons of sample types (i.e. faecal samples collected at 5, 7, 11, 14 days and caecal and mucus samples collected at 21 days) revealed a successive replacement of

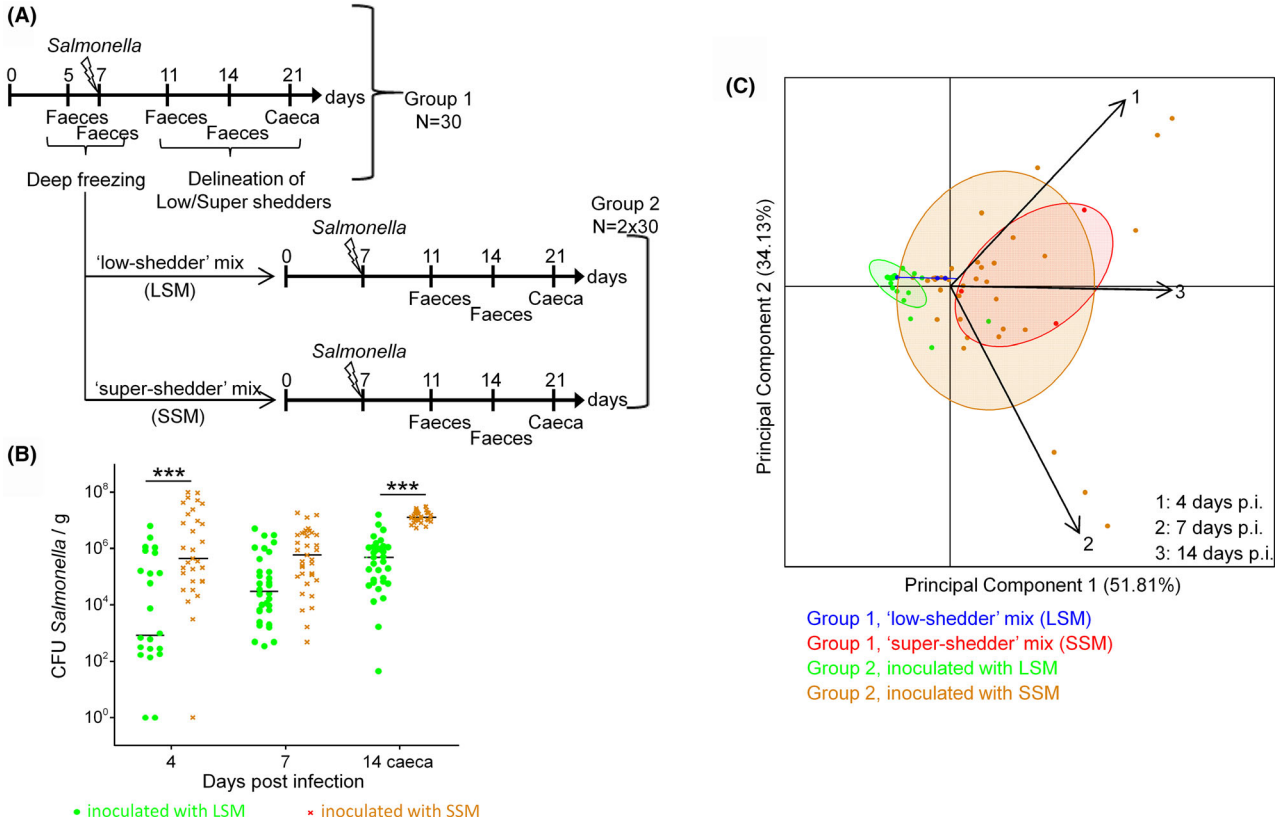

Fig. 2. *Salmonella* colonization following a gut microbiota transfer from low- or super-shedder chicks.
A. Experiment and sampling designs. Group 1: chick gut microbiota samples were collected before *Salmonella* infection at 5 and 7 days of age and were immediately frozen with glycerol. Chicks were then infected at 7 days of age and microbiota samples were collected at 11, 14 (faeces) and 21 (caeca) days of age. According to the levels of *Salmonella* counted in the faecal samples, chicks were clustered into low, intermediate and super-shedder phenotypes. Thereafter, faecal samples collected on days 5 and 7 (i.e. before infection) were assigned to the low or super-shedder categories. Two mixes of samples were prepared using either samples taken from low-shedders (i.e. 'low-shedder mix' or LSM) or super-shedders ('super-shedder mix' or SSM). The mixes were used to inoculate the chicks of Group 2 at day 1. At 7 days of age, chicks of group 2 were orally inoculated with *S.* Enteritidis. Excretion levels in Group 2 were determined at 11 and 14 days of age and caecal colonization at 21 days of age.
B. Levels of *Salmonella* excretion and caecal colonization for the chicks of Group 2 inoculated with the LSM (green dots) or SSM (orange crosses) mixes. Student t-tests were used to compare chicks inoculated with SSM/inoculated with LSM.
C. PCA summarizing the distribution of the shedding levels among LSM/SSM-inoculated individuals (in green and orange respectively) and individuals used to prepare the LSM/SSM (blue/red).

Lactobacillus genus by the *Enterococcus*, *Streptococcus* and *Eubacterium hallii* from 5 to 11 days of age (Table S2). In addition, we observed differential abundances between 14 days-faecal samples and 21 days-caecal samples depending on preferences for oxidative metabolism (i.e. increase of anaerobes genera *Anaerotruncus* and a *Lachnospiraceae*, and decrease of facultative aerobic genera *Enterococcus* and *Streptococcus*). These results are in line with previous studies focusing on the early dynamics of caecal microbial community (Mead and Adams, 1975; Mead, 1989). In addition, the main difference observed between the caecal and mucus samples was the significantly higher prevalence of the *Bacillus* genus in mucus samples, a genus including obligate aerobes and facultative anaerobes, which may reflect the expected proximo-distal oxygen gradient in the intestine (Donaldson *et al*., 2016).

Diversity patterns across 'sample types' were then summarized using four α-diversity indices. Under this framework, we observed a significant increase in α-diversity from day 5 to day 21 for all the indices under consideration (Fig. 4A; $P < 0.001$). This confirms that the isolators used in this study did not hamper the development of a complex and diverse microbiota. The caecal samples collected on day 21 yielded the highest α-diversity values of the whole series for all the four indices considered (e.g. with a mean Shannon diversity $H' = 3.67$), which were even higher than the values found for caecal mucus samples collected at the same time ($H' = 2.93$). Consequently, the α-diversity of caecal and faecal

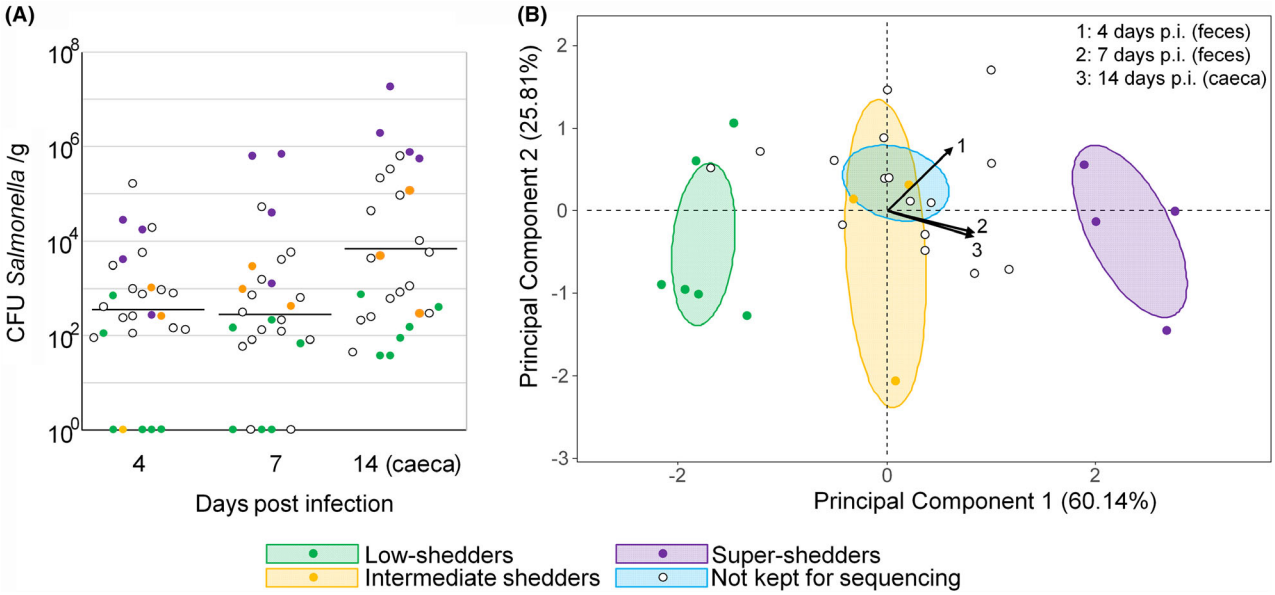

Fig. 3. *Salmonella* faecal excretion and caecal colonization after an oral inoculation. For the purpose of the gut microbiota metabarcoding, 13 chicks were taken from a group of 30 chicks. On the day of hatch, all chicks were housed in a conventional battery cage system in the same room for 4 days to acquire a gut microbiota. Then, they were relocated in isolator at 5 days of age and orally inoculated with $5x10^4$ of the *S.* Enteritidis strain at day 7. Fresh faecal samples were collected at 4, 7, 11, 14 days of age; caecal and mucus samples at 21 days of age. The faecal and caecal samples recovered were split in two parts; one part was immediately frozen in liquid nitrogen for DNA extraction and 16S rRNA gene sequencing and the second part was used to quantify *Salmonella* load.
A. Levels of *Salmonella* faecal excretion and caecal colonization of all inoculated chicks. A PCA summarizing the Log_{10} values of *Salmonella* counts at 11, 14, 21 days of age was used to assign the chicks into three shedding categories (i.e. low- [green], intermediate [orange] and super-shedders [purple]). The 13 chicks kept for metabarcoding are represented by coloured solid dots according to the shedding category; the other chicks are represented by empty dots.
B. PCA plot summarizing the Log_{10} values of *Salmonella* counts. According to their position in the plot, chicks were assigned to three shedding categories (i.e. low- [green], intermediate [orange] and super-shedders [purple]). The 13 chicks kept for metabarcoding are represented by coloured solid dots; the other chicks are represented by empty dots

samples presented significant differences (Table 1). The measures of β-diversity revealed highly similar taxonomic profiles within each sample type; significant relationships were found between the sample type and all the four β-diversity indices under consideration ($P < 0.0001$; see Fig. 4B illustrating the distribution of Bray–Curtis distances). Moreover, the β-diversity was clearly different between faecal (day 5, 7, 11, 14) and caecal (day 21, mucus) samples (Fig. 4B).

Salmonella *shedding level is related to the gut microbiota composition*

The previous α- and β-diversity analyses revealed that differences of microbiota composition were strongly shaped by 'sample type', defined by origin of the sample (faecal, caecal, caecal mucus) and the chick's age at the time of sampling. We, thus, investigated the relationship between *Salmonella* shedding level and gut microbiota composition, taking into account sample types as a potential bias.

First, variance analyses showed that α-diversity indices were correlated to the shedding level categories (i.e. super, intermediate and low-shedders), even when the sample type (i.e. including age) was taken into account. We obtained significant results ($P < 0.05$) for three of the four indices under scrutiny (the Observed diversity index; the Chao1 index; the Shannon index); these differences relied on slight differences between low-shedders compared to intermediate, and low-shedders compared to super-shedders (Table 1). The low-shedder chicks presented the lowest α-diversities for

Fig. 4. Diversities of gut microbiota before and after *Salmonella* infection for all sample types. The gut microbiota diversities of the 13 chicks orally inoculated with the *S.* Enteritidis strain at 7 days of age and reared in an isolator for 21 days were characterized. Faecal samples were collected at 4, 7, 11, 14 days of age (D5, D7, D11, D14); caecal and mucus samples were collected at 21 days of age (D21 and D21 mucus). The gut microbiota composition was determined by metabarcoding.
A. Distributions of the 4 measures of α-diversity taken into consideration (i.e. observed diversity, Chao1, Shannon and Inverse Simpson indices) for all sample types.
B. Multidimensional scaling summarizing the distribution of Bray–Curtis distances among the sample types (β-diversity)

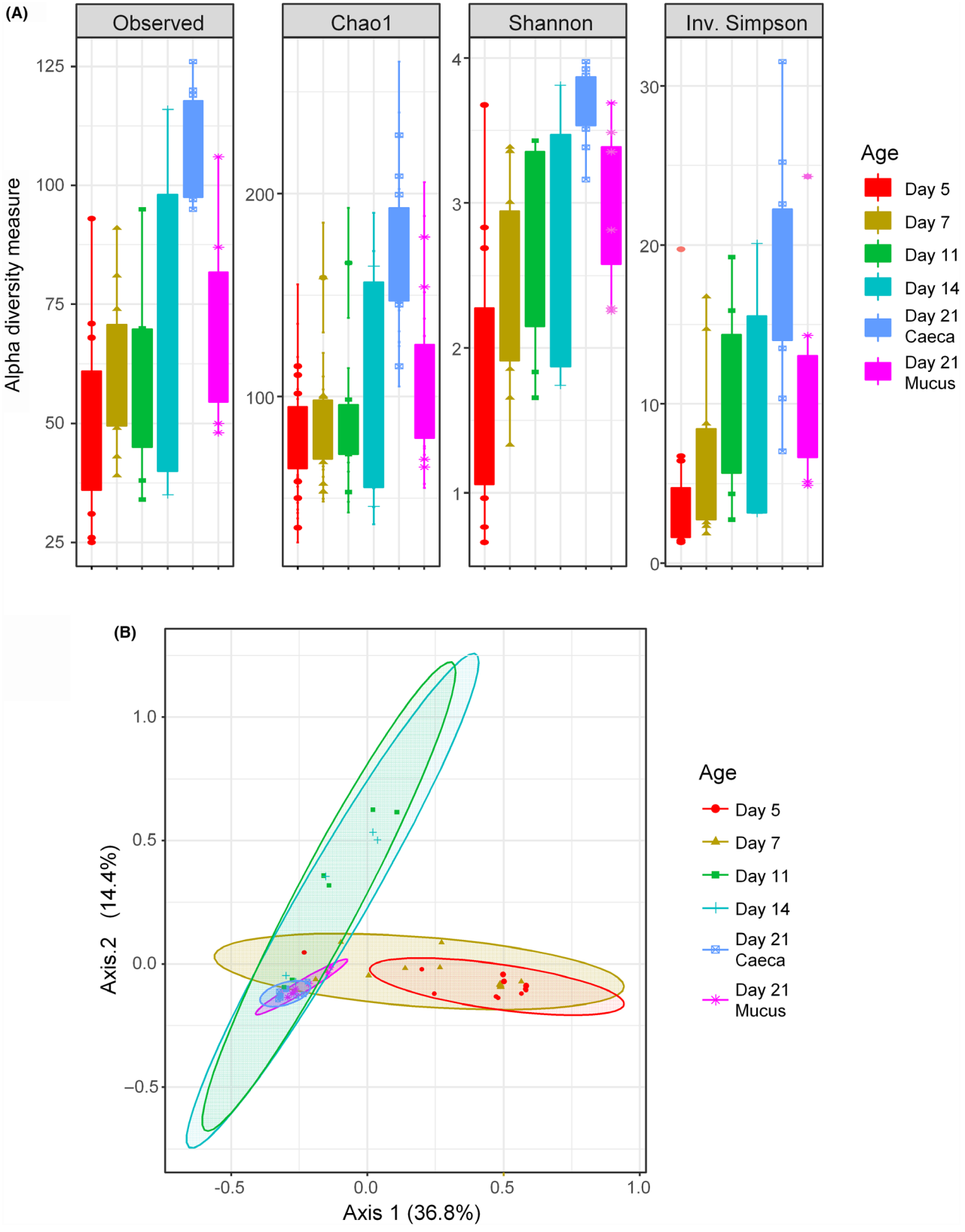

Table 1. Comparison of alpha-diversities between sample types.

		Observed diversity (R)	Chao1	Shannon	Inverse Simpson
Model 1[a]:Diversity ~ SampleDepth	Day 21 vs. Day 5	**$P < 0.001$**	**$P < 0.001$**	**$P < 0.001$**	**$P < 0.001$**
	Day 21 vs. Day 7	**$P < 0.001$**	**$P < 0.001$**	**$P = 0.009$**	**$P = 0.001$**
	Day 21 vs. Day 11	**$P < 0.001$**	**$P = 0.002$**	NS	$P = 0.089$
	Day 21 vs. Day 14	**$P = 0.006$**	**$P = 0.004$**	NS	$P = 0.066$
Model 2[b]:Diversity	Low-shedders vs. Intermediate	**$P = 0.038$**	NS	$P = 0.062$	NS
	Low-shedders vs. Super-shedders	$P = 0.058$	NS	NS	NS

For both models, post hoc Tukey tests were done to compare the modalities; significant (in bold; $P < 0.05$) and marginally significant ($P < 0.10$) are here reported.
a. The first model used for variances analyse only included the categorical variables 'sample type', 'sample depth' and 'individual'.
b. The second model included the 'infection level' as additional variable.

three indices under consideration (e.g. for the Shannon index, the mean values were $H = 2.30$, 2.92 and 2.76 for low, intermediate and super-shedders, respectively). Analysing β-diversity indices confirmed the homogeneity of microbial compositions within the shedding categories, for all the indices under consideration ($0.007 < P < 0.024$). Moreover, we observed a clear modification of β-diversity after infection and thus related to age and infection; this is illustrated by Figure 4B (for the faecal samples we can compare day 5 and 7 on one side and 11, 14, on the other side).

The relationships between α-diversity and shedding levels were then investigated without pre-defined shedding categories, but instead comparing the *Salmonella* counts. For this, generalized linear models were designed using α-diversity as a dependent variable and *Salmonella* counts at 11, 14 and 21 days of age as explanatory variables. This revealed that high levels of *Salmonella* were correlated to high α-diversities at 11 and 21 days of age (at 11 days: Chao1, $P = 0.003$; at 21 days: Observed diversity, $P = 0.003$; Shannon, $P < 0.001$; Inverse Simpson: $P < 0.001$). In contrast, negative correlations were found at 14 days of age (Shannon, $P = 0.013$; Inverse Simpson, $P = 0.002$).

We next undertook a comparison of the shedding categories at the genus level. For this, we tested for differences between low, intermediate and super-shedder categories, in each sample type, and for each genus separately (Table 2). Despite the reduced number of samples included in each sample type category, out of 54 genera, we were able to detect a total of nine presenting significant different abundances between shedding categories in a given sample type. When considering only the genera presenting a reasonably high relative abundance (> 5%), two of them were identified in the low-shedder chicks: *Enterococcus* (at 7 days of age) and *Streptococcus* (at 7 and 11 days of age) (Fig. 5A and B). In order to rule out possible assignment errors or clustering bias, a similar analysis was performed using QIIME choosing the RDP classifier-

Table 2. Genera presenting significant differences of mean relative abundance between shedding categories for each sample type.

Sample type	Genus	Relative abundance (%)	*P*-value
Day 5	*Intestinibacter*	0–0.13	0.015
	Bacillus	0.00–2.70	0.022
Day 7	*Streptococcus*	0.03–13.25	0.029
	Enterococcus	0.05–49.52	0.043
Day 11	Lachnospiraceae NK4A136 group	0–0.29	0.008
	Streptococcus	0.16–59.1	0.043
Day 14	*Not enough data*		
Day 21 (caecal samples)	*Eubacterium* oxidoreducens group	0.05–0.19	0.042
	Lachnospiraceae FE2018 group	0.57–4.05	0.044
	Marvinbryantia	0.05–0.46	0.046
Day 21 (mucus samples)	*Anaerostipes*	0.04–1.18	0.045

approach for taxonomic assignment (Wang *et al*., 2007; Caporaso *et al*., 2010). Under this scheme, we observed that *Enterococcus* was the only abundant genus that simultaneously presented high frequencies at 7 days of age ($6.43\% < f_{Enterococcus} < 38.16\%$) and significant differences between the shedding categories ($P = 0.031$).

This was confirmed by PCA summarizing the genera frequencies observed before infection (i.e. at 4 and 7 days of age) in the three shedding categories (Fig. 5 C). The main part of the inertia was here represented by Principal Component 1 (PC1; 80.83%). The *Lactobacillus* genus has the main contribution in the total variance of PC1 (i.e. 84.9% of the total variance after projection of all variables on this component). The PC1 being itself mainly correlated to the age of chicks ($P = 0.02$), the PCA suggested that the differences of the gut microbial compositions from 5 to 7 days are mainly driven by the abundance of *Lactobacillus*. Concerning the Principal Component 2 (PC2, representing 12.1% of the total variance), we observed that *Enterococcus* and *Lachnoclostridium* were the two genera that contributed to its

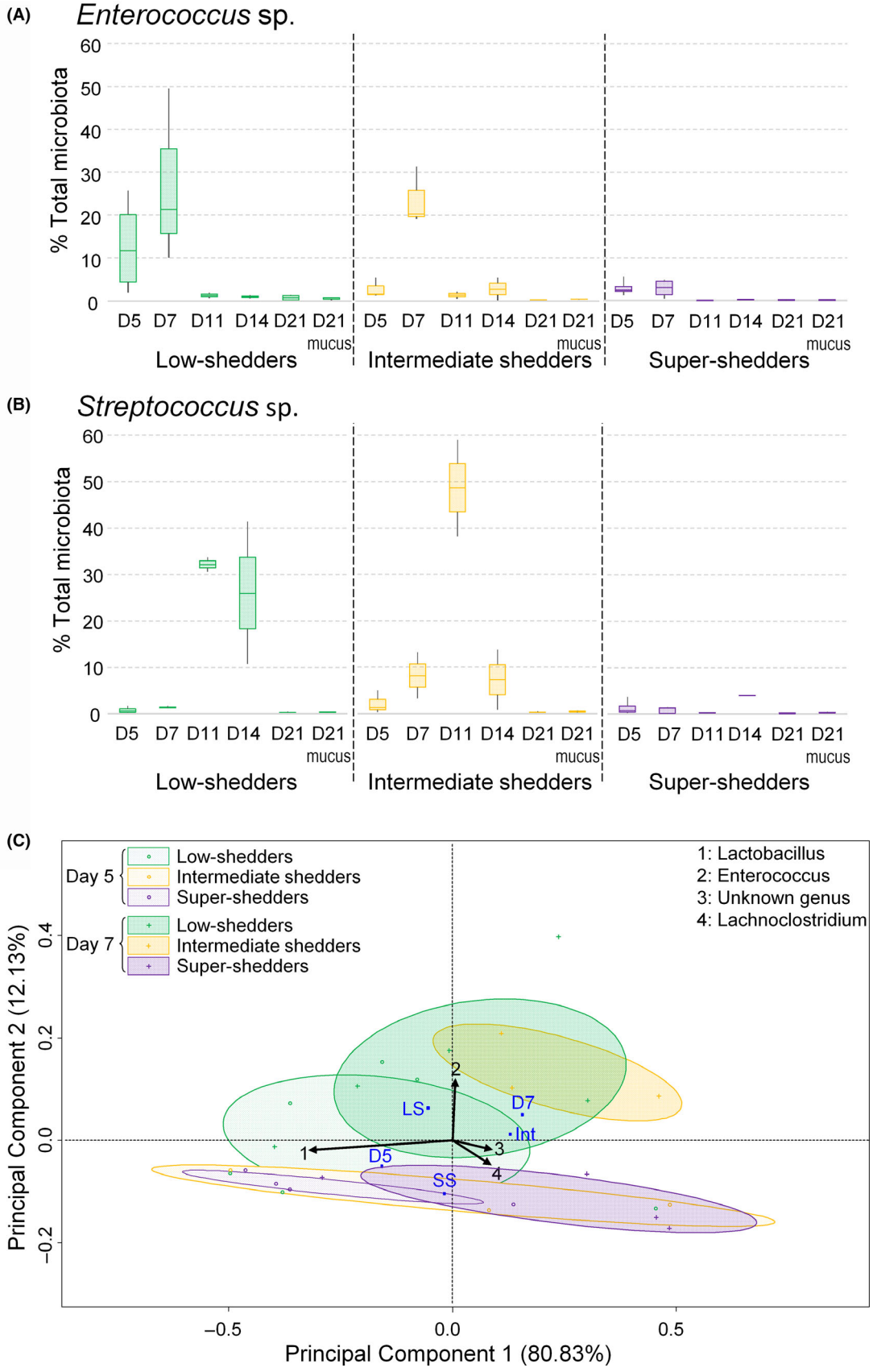

Fig. 5. Genus level-differences of the gut microbial compositions among shedding categories before and after *Salmonella* inoculation. Relative abundances of the genera *Enterococcus* (A) and *Streptococcus* (B) within each shedding phenotypes. In the same experiment described Figure 4, we characterized the gut microbiota of 13 chicks orally inoculated with the *S*. Enteritidis strain at 7 days of age and reared in an isolator for 21 days. Faecal samples were collected at 5, 7, 11, 14 days of age (D5, D7, D11, D14); caecal and mucus samples were collected at 21 days of age (D21 and D21 mucus). According to the levels of *Salmonella*, the 13 chicks were clustered into the three shedding categories: low (green), intermediate (orange) and super-shedders (purple), using the results of the PCA (Fig. 3B). The gut microbiota composition was determined by 16S metabarcoding. Mean comparisons among low, intermediate and super-shedders were conducted using Student t-tests; significant p-values were obtained at 5 (*Enterococcus*), 7 (*Enterococcus* and *Streptococcus*) and 11 (*Streptococcus*) days of age (see Table 2). (C) A Principal component analysis was carried out to summarize the distribution of the relative abundances among all samples at the genus level before infection. The 4 genera contributing at least for 5% of variance represented by the two Principal Components are reported on the plot. Blue dots represent the centres of gravity for each age (D5 = 5 days of age, D7 = 7 days of age) and the shedding categories (i.e. low-shedders [LS], intermediate [Int], super-shedders [SS]). Confidence ellipses summarize individual positions within each age and shedding category

variance (80.2% and 12.8% respectively). In addition, PC2 presented a significant relationship with the shedding categories (*F*-test; $P = 0.02$). In particular, we observed significant differences between the low and super-shedder group (*t*-tests; $P = 0.023$ and 0.008) and the rest of individuals. Thus, according to the information highlighted by PC2, *Enterococcus* genus was here the main taxonomic feature allowing to predict the low- or super-shedder phenotypes (see also Fig. 5A).

Enterococcus faecium *alone did not protect against* Salmonella *colonization*

In the previous experiment, OTU taxonomic assignation did not enable the species level identification. Nevertheless, the main *Enterococcus*-related OTU involved in the shedding phenotype (88.5% of *Enterococcus* total abundance) should be assigned to *Enterococcus faecium*. Indeed, the corresponding sequence mostly matched to *E. faecium* – associated sequences (42.2% of all best hits, compared to 15.0% for *E. durans*, 8.0% for *E. hirae*, 5.4% for *E. faecalis*). In addition, a PCR made using specific primers of *Enterococcus* species (Deasy *et al*., 2000; Jackson *et al*., 2004) confirmed the presence of *E. faecium*.

For these reasons, *E. faecium* was regarded as the main feature predicting the low- and super-shedder phenotypes. In order to highlight a direct causal effect, we tested the inhibitory power of *E. faecium* strains on *Salmonella* colonization. Two strains of *E. faecium* (MT10 and MT11), originating from a faecal sample of white leghorn (PA12) chickens, were orally inoculated in a group of 30 chicks reared in a large isolator (CFU = 1×10^9 per chick) at 1 and 6 days of age. A control group was inoculated using the bacterial medium alone. At 7 days of age, all the chicks were orally inoculated with *S*. Enteritidis LA5. Under these conditions, we did not observe any significant differences between the *Salmonella* shedding levels of the *E. faecium*-inoculated and control groups (at any time point; $0.11 < P < 0.61$) (Fig. 6A). However, a great heterogeneity of *Salmonella* excretion levels was observed in both groups, which hampered us to detect a significant effect of the *E. faecium* inoculation. A second experiment performed with a spontaneous rifampicin resistant M10 strain showed that *E. faecium* inoculated after hatching may highly colonized the gut of chicks throughout the experiment. Indeed, at 11, 14, 21, 28 days of age, we respectively recovered $CFU/g_{E.\ faecium} = 2.51 \times 10^6$, 1.58×10^6, 2.51×10^6, 6.31×10^4 in faecal samples; at 21 days of age, we found $CFU/g_{E.\ faecium} = 1.26 \times 10^5$ in caecal samples. However, we did not observe any significant decrease of *S*. Enteritidis colonization in the *E. faecium* M10 strain-inoculated group even if it tended to be lower (data not shown).

Enterococcus faecium *associated with three other commensal bacteria partly protect against* Salmonella *colonization*

This lack of direct effect of *E. faecium* could be related to the strains we isolated. Moreover, as it is rare for one taxon to be sufficient in providing colonization resistance, we tested the direct effect of *E. faecium* in association with well-defined commensal bacteria. For this mix of 4 bacteria, we used the *E. coli* Nissle 1917, the *Lactobacillus rhamnosus* strain (DSM 7133), the *Clostridium butyricum* strain (DSM 10702) and the *Enterococcus faecium* DSM 7134 strain, which together have an immunomodulatory effect in chicks. Inoculated separately, none of these bacteria were able to protect chicks from *S*. Enteritidis colonization. For example, after 7-day postinfection, the level of *S*. Enteritidis caeca colonization was 9.26 log10 CFU x g^{-1} in axenic chicks and was 9.20 log10 CFU x g^{-1} when *E. coli* Nissle was inoculated at 1 days of age, and at 8.73 log10 CFU x g^{-1} when *Enterococcus faecium* was inoculated at 1 days of age. Moreover, alone the *Clostridium butyricum* strain was unable to significantly colonize the intestine. Together, the four bacteria were able to colonize gut of axenic chicks (Fig. S2) and, orally inoculated the day of hatch, they induced a significant one to two Log_{10} decrease of *S*. Enteritidis excretion; this effect was maximal 1 and 2 weeks postinfection but was still significant 3 weeks postinfection

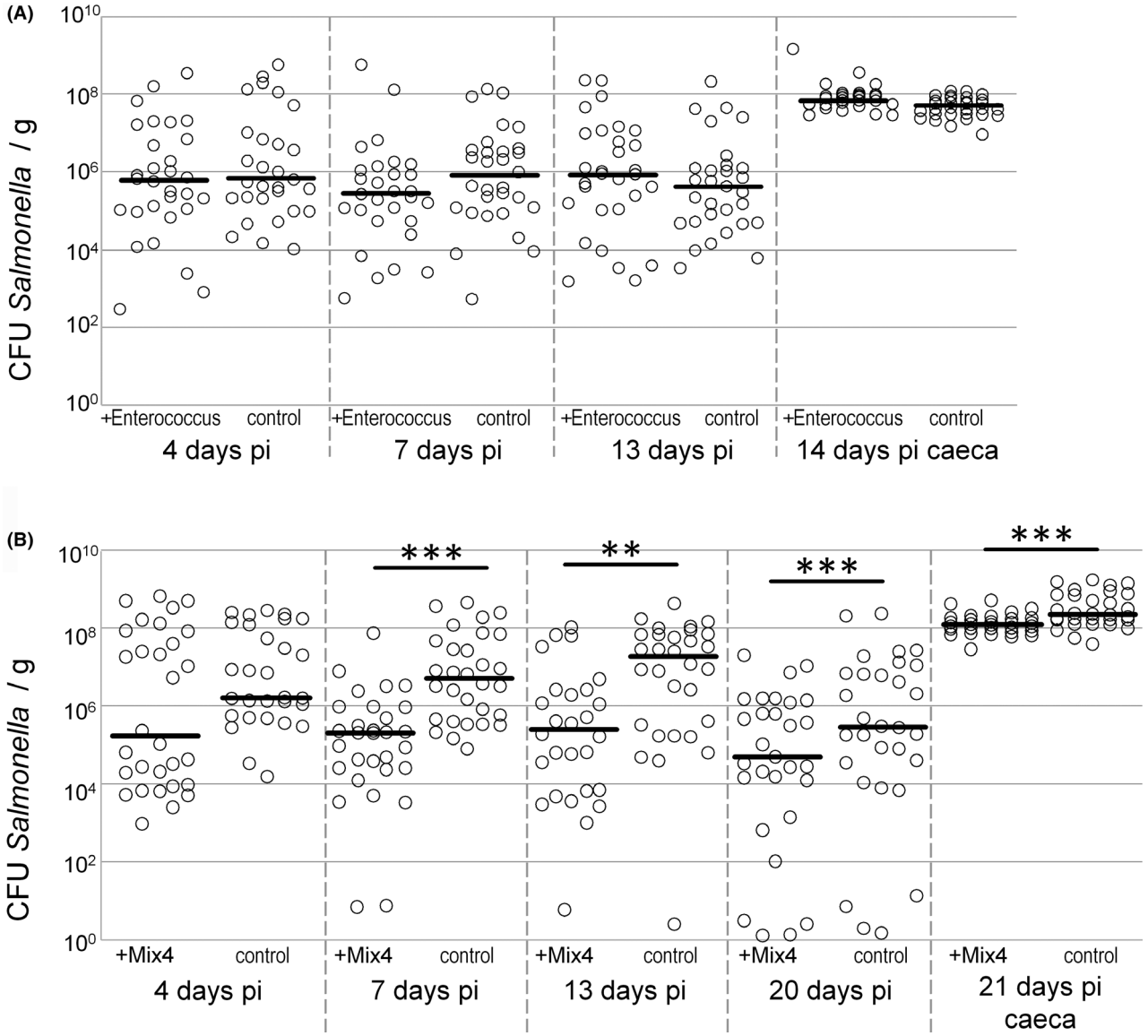

Fig. 6. *Salmonella* faecal excretion and caecal colonization following an inoculation of commensal bacterial strains
A. To measure the protective activity of *Enterococcus faecium, a* group of 35 chicks ('+Enterococcus') was inoculated with the *E. faecium* strains at 1 and 6 days of age and relocated to isolators at 4 days of age. A second group of 30 chicks ('control') was reared under similar conditions, except that they were inoculated at 1 and 6 days of age with sterile PBS. At 7 days of age, the chicks of both groups were orally inoculated with *S.* Enteritidis; faecal samples were collected at 4, 7, 13 days after infection and caecal samples 14 days after infection.
B. To maesure the protective activity of the mix of 4 commensal bacteria (*Enterococcus faecium, E. coli* Nissle 1917, *Lactobacillus rhamnosus, Clostridium butyricum*), only one oral inoculation was performed on the day of hatch (+Mix4). The second group was inoculated at the same time with PBS (control). Immediately after the first inoculation, chicks were relocated to non-sterile isolators. At 7 days of age, the chicks of both groups were orally inoculated with *S.* Enteritidis; faecal samples were collected at 4, 7, 13, 20 days after infection and caecal samples 21 days after infection. In all cases, levels of *Salmonella* faecal excretion and caecal colonization were measured after homogenization of caecal and faecal samples in TSB. Then, serial 10-fold dilutions were plated on *Salmonella–Shigella* medium containing nalidixic acid (20 μg ml^{-1}). The mean counts of *S.* Enteritidis CFU in faecal droppings or caeca were calculated per gram at each time point. Median values and results of the *t*-tests comparisons were represented on the figure.

(Fig. 6B). An interesting effect of this flora was an increase of the number of low-shedders concomitant with a decrease of the number of super-shedders. For example, at 2-week postinfection, 21 chicks out of 30 excreted more than 1×10^6 bacteria per g faeces in the control group (super-shedder) compared to 10 chicks when the 4 bacteria were inoculated. In contrast, only one chick out of 30 excreted less than 1×10^4 bacteria per g faeces in the control group (low-shedder) compared to 8 chicks when the 4 bacteria were inoculated (Fig. 6B). This result suggested that in association with other commensal bacteria, *Enterococcus faecium* could

have a protective activity against *Salmonella* colonization and thus could be directly related to the low-shedder phenotype, in addition to being a biomarker.

Discussion

Past studies have demonstrated that the outcome of *Salmonella* Enteritidis infection is an interplay of bacterial virulence traits (Bogomolnaya *et al*., 2008), host genetic factors (Calenge *et al*., 2010) and immune response (Chaussé *et al*., 2011). Other likely factors have been suggested, including dosage of pathogen exposure, diet, host age and immune status. In the present study, we highlighted that the composition of the chicken gut microbiota determined the levels of *Salmonella* colonization and the emergence of super-shedder individuals. These results were obtained under strict experimental conditions allowing alternative hypotheses to be ruled out (e.g. polymorphism among *Salmonella* strains or host genetics). Indeed, the chicks originated from the same lineage (inbred to more than 99%) and were inoculated with the same *Salmonella* inoculum. Moreover, hens of this lineage were bred under SPF conditions. Lastly, the chicks used for most of the experiments were infected in a controlled breeding system consisting of isolators. The isolators include several devices aimed at reducing cross-contaminations between individuals. However, these facilities did not hamper either the development of a gut microbiota, or its variability between animals (Menanteau *et al*., 2018), or the expected chicken gut microbiota temporal dynamics (Videnska *et al*., 2014).

In particular, we showed that changes in the chicken gut microbiota composition dramatically modified the outcome of *Salmonella* colonization. In the absence of gut microbiota (i.e. axenic hosts), all chicks became super-shedders after *Salmonella* colonization. By contrast, inoculation of gut microbiota originating from adult hens protected chicks from *Salmonella* colonization and carrier state as in part demonstrated by Varmuzova *et al*. (2016). In addition, these authors showed that the transfer of samples taken from younger chicken failed to protect against *Salmonella* infection.

Furthermore, we showed that chicks were more prone to develop a super-shedding phenotype in an isolator after an antibiotic-mediated modification of the gut microbiota composition. As previously described (Videnska *et al*., 2013), this effect was only observed over a short period. This may correspond to the transient perturbation of the gut microbiota following the complete clearance of streptomycin. In this hypothesis, the gut microbiota of both control and streptomycin-treated groups converged towards the same bacterial composition resulting in similar levels of *Salmonella* colonization. As alternative hypotheses, one may suggest that either the functional profiles of the microbiome of the two groups had converged (independently of the taxonomic composition), resulting in similar susceptibility to *Salmonella* colonization; or *Salmonella*'s virulence factors had overcome colonization resistance in the control group. Whatever the cause, the increase in *Salmonella* colonization observed after the streptomycin treatment was already observed in a mouse model (Lawley *et al*., 2008). This may be interpreted as a higher predisposition for *Salmonella* colonization, possibly due to the disappearance of one or more commensal bacteria involved in the protective effect or to the modification of inflammation or to a spike in compounds such as free sialic acids or fucose, which favour *Salmonella* growth. Altogether, these results suggest that chicks tend to become super-shedders when their gut microbiota lacks specific features able to outcompete *Salmonella* or presents certain features which favour *Salmonella* growth.

Our results also show that the gut microbiota, present during the chicks' first exposure to *Salmonella,* partly determines the shedding phenotype they will subsequently develop. This was confirmed by the transfer of the super-shedder phenotype using microbiota samples originating from super-shedder chicks. The super-shedder phenotype acquired in this way lasted for more than two weeks after infection as demonstrated by a high and homogeneous caeca colonization. This was is in line with our previous results, which demonstrated that super-shedder chicks remained super-shedders for several weeks after their relocation into an isolator reducing reinfections (Menanteau *et al*., 2018). The role of the early gut microbiota, present just before the first exposure was also confirmed by gut microbiota metabarcoding. For example, the genus *Enterococcus* was more abundant before infection in the chicks that later became low-shedders. In addition, the abundance of *Streptococcus* is higher in low-shedders just before and after infection.

Altogether, these results may have several implications. First, they open the way to developing microbiota biomarkers; in particular, *Enterococcus faecium* might be an interesting tool to detect future shedding phenotypes, and therefore flocks including 'at risk' chickens. It should be noted that these results were obtained mainly using faecal samples. This kind of biological material could enable the use of a non-lethal, cost-effective detection method to predict the susceptibility to *Salmonella* infection or the outcome of *Salmonella* infection. Such fast, robust and early diagnostic methods could be applied to control strategies of chicken salmonellosis by aiming to remove future super-shedder individuals before infection or to intervene at the level of gut microbiota in order to prevent super-shedding (Callaway *et al*., 2004). The transfer from experiment to veterinary applications should take into account that microbiota sources and

transmission among chickens in the field may differ considerably from the conditions in isolators used in this study (Kubasova *et al*., 2019). However, the development of microbiota markers is one of the most promising avenues for microbiota-targeting therapies (Haiser and Turnbaugh, 2012; Lemon *et al*., 2012).

Second, the finding that low-shedder chicks harboured a higher relative abundance of *Enterococcus* sp. before infection is of particular interest regarding the potential development of protective probiotics. Negative interactions between *Enterococcus* and *Salmonella* have been documented in several studies using *in vitro* (Theppangna *et al*., 2006) and *in vivo* approaches (Carina Audisio *et al*., 2000; Carter *et al*., 2017). In order to assess the protective activity of *E. faecium*, we performed an *in vivo* assay with several strains isolated from a chicken faecal sample. However, despite the fact that this strain actually colonized the caeca after its inoculation at 1 day of age, it did not provide any protection against *Salmonella*.

The higher abundance of *E. faecium* in low-shedder chicks before infection could be explained without any protective effect. For instance, *E. faecium* might not have inhibited *Salmonella* colonization by itself, but an undetected feature could have been involved. This would disqualify this bacterium as a potential probiotic, but would argue for its use as biomarker of the chicks' susceptibility to *Salmonella* infection. Another hypothesis is that the strains used were different to those conferring protection. A second, non-exclusive, hypothesis was that *E. faecium* alone is not sufficient to induce a strong protective effect, but should be rather used in combination with other bacteria within a probiotic cocktail. To test this last hypothesis, we performed an experiment where we measured the protective activity of a mix of four commensal bacteria including *E. faecium*. Interestingly, oral inoculation of the mix of 4 commensal strains induced a 10 to 100 fold decrease in the *S*. Enteritidis colonization. As these commensal bacteria were unable to confer protection independently, the protection seems to arise from their synergy.

The potential negative relationship between this mix of commensal bacteria and *Salmonella* raises questions on the mechanisms at play in this competition. Given the current knowledge on microbial ecology in the context of gut microbiota, different non-exclusive hypotheses can be postulated. First, there may be a competition for the replication niche, as already described in pigs infected by *S*. Typhimurium (Levine *et al*., 2012). Second, a direct negative interaction may be caused by bacteriocins produced by *Enterococcus* (Hanchi *et al*., 2018). Third, some probiotic effects are based on bacterial metabolic by-products; these include lactic acid, which is potentially involved in the antagonistic effect of *E. faecium* and *Lactobacillus* species against several *Salmonella* species (Carina Audisio *et al*., 2000). Protective activity of the short chain fatty acids produced by the *Clostridia* has been largely described (El Aidy *et al*., 2013). Similarly, recent advances regarding the mechanisms of *salmonella* infection, have pointed out that an increased intestinal oxygenation, following the depletion of butyrate-producing bacteria, may favour its colonizaztion. This may in turn favour *Salmonella* colonization (Rivera-Chávez *et al*., 2016). Recently, it has been shown that facultative aerobes, such as *Lactobacillus,* may challenge *Salmonella* and thus have a protective effect in chicks (Litvak *et al*., 2019). Fourth, we cannot exclude that flagellated *Enterococci* (Turner *et al*., 2016) trigger and activate a TLR5-dependent innate immune response, an important pathway for *Salmonella* pathogenesis in chickens (Iqbal *et al*., 2005). To verify these different hypotheses, a precise study of the role played by each of these bacteria will have to be carried out with isolated bacteria and in consortia.

Thus, the knowledge gained through this work will be used to develop new predictive biomarkers to identify the 'at risk' animals but also to develop tailored starting microbiota potentially able to outcompete *Salmonella* in human and farm animals through microbial community engineering approaches (Brugiroux *et al*., 2016).

Experimental procedures

Ethics approval

The experiments with chickens were carried out in strict accordance with French legislation. All animal care and use adhered to French animal welfare laws. The protocols for this study were approved by the French Ministry of education, higher education and research (Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche) under the protocol N° APAFIS#5833-20l60624l6362298 v3. The principles of reduction, replacement and refinement were implemented in all the experiments.

Housing conditions

The chicks were reared either in a conventional battery cage system or in an isolator. An isolator is an experimental breeding system allowing cross-contaminations to be reduced among host individuals through a constant removal of air and sterilization of faeces (see Menanteau *et al*., 2018 for more details), and control of diet and environmental contamination. Chicks for only one experiment (see below) were germ free because in such housing systems a microbiota may be acquired from the bacteria found in the hatchery. For this experiment, white leghorn chicks (PA12 lineage) were used. They

originated from the specific-pathogen-free (SPF) flock of the PFIE (INRAE, Val de Loire, France), a core facility specialized in experimental animal infections. The *Salmonella*-free status of chicks was checked by both serological and bacteriological tests. Chicks were fed *ad libitum*, had free access to drinking water, and a 12:12 L:D lighting scheme was applied. Food and water were only sterilized for the axenic chicks.

Culture and inoculation of bacteria

For all except one experiment, we used either the nalidixic acid-resistant *Salmonella* Enteritidis phage type 4, strain LA5 or a spontaneous streptomycin resistant clone of this strain. This well-characterized LA5 strain was isolated from a 4-day-old broiler chicken (Dibb-Fuller *et al*., 1999; Grépinet *et al*., 2012). To prepare the inocula, *Salmonella* was cultured aerobically in trypticase soy broth (TSB) (BioMérieux) supplemented with 20 μg ml^{-1} of nalidixic acid (Sigma-Aldrich) for 24 h at 37°C with shaking. The cultures were centrifuged at 4500 $\times$ g for 20 min at room temperature 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 $\times$ 10^5 CFU ml^{-1} and chicks were orally inoculated with a volume of 0.2 ml at 7 days of age. An aliquot of the same inoculum was used throughout the experiments.

We used the *S*. Enteritidis clone 1009 (Duchet-Suchaux *et al*., 1995) instead of strain LA5 for one experiment aiming to infect chicks after a transfer of hen microbiota. The inoculum was prepared in the same way as above. Both 1009 and LA5 strains behave similarly in all chicken models.

The *Enterococcus faecium* strains (MT10 and MT11) were isolated from the faecal droppings of white leghorn (PA12) chicks originating from the SPF flock of the PFIE (INRA, Val de Loire, France; see below). After suspension in PBS, bacterial cultures were prepared on bile esculin agar (BEA) medium. Colonies presenting morphological characteristics of the *Enterococcus* sp. were identified by multiplex PCR using specific genus-specific primers (E1 5′-TCAACCGGGGAGGGT-3′ and E2 5′-ATTACTAGCGATTCCGG-3′) and species-specific primers for *E. faecalis* (FL1 5′-ACTTATGTGACTAACTTAACC-3′ and FL2 5′-TAATGGTGAATCTTGGTTTGG-3′) and *E. faecium* (FM1 5′-GAAAAAACAATAGAAGAATTAT-3′ and FM2 5′-TGCTTTTTTGAATTCTT CTTTA-3′) (Deasy *et al*., 2000; Jackson *et al*., 2004). DNA extraction was carried out using the NucleoSpin Microbial DNA kit following the manufacturer's instructions (Macherey-Nagel France, Hoerdt, France). A spontaneaous rifampicin resistant strain was recovered from the BEA medium supplemented with 100 μg ml^{-1} rifampicin. They were orally inoculated in a group of 30 chicks reared in a large isolator (CFU = 1 $\times$ 10^9 per chick) at 1 and 6 days of age, whereas the control group only received PBS medium. *Salmonella* Enteritidis infection was then performed by oral inoculation of 5 $\times$ 10^4 bacteria/chick at 7 days of age (see below). Two independent experiments were performed with the M10 and M11 strains and another one with the rifampicin resistant M10 strain.

A mix of four commensal bacteria was developed based on our results and on the literature. For this mix of 4 bacteria, we used the *E. coli* Nissle 1917 (Boudeau, *et al*., 2003), the *Lactobacillus rhamnosus* strain (DSM 7133), the *Clostridium butyricum* strain (DSM 10702) and the *Enterococcus faecium* DSM 7134 strain. Each bacterium was cultured independently and mixed just before oral inoculation. The inoculum was prepared by mixing 2.5 ml of an overnight culture of Nissle 1917 strain grown in 10 ml BHI medium (Difco) at 37°C without agitation, 2.5 ml of an overnight culture of *E. faecium* strain grown in 10 ml BHI medium at 37°C without agitation, 2.5 ml of an overnight culture of *Lactobacillus rhamnosus* strain grown in 10 ml BHI medium at 37°C into an anaerobic jar with gas pack CO_2 gas generator (BD BBL) and 2.5 ml of a two-days culture of *Clostridium butyricum* strain grown in Wilkins Chalgren medium without shaking under N2 atmosphere at 37°C into an anaerobic jar culture with gas pack CO_2 gas generator. Number of bacteria in the inoculum was measured with TSA plates (Biorad), M. enterococcus plates (Difco), Man, Royosa et Sharpe (MRS) plates (Biorad) and in mass in TSA containing ammonium citrate (0.5 g l^{-1}) and sodium metabisulphite (1 g l^{-1}) at 37°C for 48 h, respectively. MRS and TSA plates were incubated anaerobically into a jar with gas pack CO_2 gas generator. Just after hatch, chicks were orally inoculated with 200 μl of this mix. The inoculum corresponded to 7.7 $\times$ 10^6 CFU of *E. coli* Nissle 1917, 1.1 $\times$ 10^7 CFU of *E. faecium*, 1.3 $\times$ 10^3 CFU of *Clostridium butyricum* and 2.5 $\times$ 10^6 CFU of *Lactobacillus rhamnosus*.

Infection after hen microbiota transfer

The microbiota samples of adult layers (21–25 weeks of age) were kindly provided by F. Humbert (AFSSA Ploufragan). They were diluted in VL broth with glycerol (10%), a culture medium favouring anaerobes, and stored under deep-freeze conditions (−80°C). Two groups of 80 white leghorn (PA12) chicks were reared for 33 days in cages. Each chick from the first group was orally inoculated at 1 day of age with 0.2 ml of diluted hen microbiota thawed at room temperature. The chicks of the second (control) group were orally

inoculated with the same volume of VL broth with glycerol. In addition, all chicks were orally inoculated at 7 days of age with *S*. Enteritidis strain 1009 (CFU = 5×10^4 per chick). At 5, 12, 19 and 26 days after infection 7 chicks were sacrificed and caecal samples were collected in order to quantify *Salmonella* colonization.

Infection of axenic chicks

The germ-free chicks were obtained by hatching and rearing white leghorn (PA12) chicks under sterile conditions as described by Schellenberg and Maillard with some modifications (Schellenberg and Maillard, 1973). Just after laying, the surface of clean eggs was sterilized by immersion in 1.5% Divosan (Johnson Diversey France, Fontenay-sous-Bois, France) for 5 min and then for an additional 3 min. Then, eggs were transferred into a sterile HEPA-filtered incubator. After 19 days at 37.5°C and 45% humidity, the egg surfaces were again sterilized in 1.25% Divosan for 4 min at 37°C. Eggs were then transferred for hatching to a sterile isolator with controlled humidity, ventilation and temperature. The temperature was maintained at 37.5°C for the first week and then reduced by 1°C per day to a stable temperature of 25°C. Birds were raised on an X ray-irradiated starter diet from Special Diets Services (Dietex, Argenteuil, France). Sterilized water was provided ad libitum for the entire duration of the experiment. The sterile status of chickens was confirmed by recovering fresh faecal droppings at one and four days of age and before *Salmonella* inoculation. Samples were incubated under aerobic and anaerobic conditions in tubes containing 10 ml of sterile brain-heart infusion broth. Chicks were orally inoculated with *S*. Enteritidis LA5 on day 7 and sacrificed on day14 (7 days postinfection). The level of caecal contamination was recorded as for the conventional chickens.

Infection after streptomycin treatment

For this experiment, two groups of 35 chicks were placed in two separate cages at one day of age. One of these groups was kept as controls and was not exposed to streptomycin. The chicks of the other group were orally inoculated with streptomycin at one day of age (30 mg/0.2 ml) and via drinking water (2 g l^{-1}) over the following four days. Next, all chicks were placed into two separate large isolators (2.26 m^2). At five days of age, the drinking water containing streptomycin was replaced by the same antibiotic-free water used for the rest of this experiment. All chicks were orally inoculated with the streptomycin resistant *S*. Enteritidis LA5 strain at 7 days of age; faecal samples were collected at 4, 7 and 14 days after infection.

Infection after chick microbiota transfer

A first group of 30 white leghorn (PA12) chicks was reared for 21 days. Chicks were initially reared together in a cage for 4 days to favour acquisition of gut microbiota. They were then relocated to isolators for the remaining 17 days (Group1; Fig. 4A). At 7 days of age, chicks were orally inoculated with *S*. Enteritidis LA5 (CFU = 5×10^4 per chick). Faecal droppings were collected before infection at 5 and 7 days of age, and after infection at 11, and 14 days of age; caecal samples were collected at 21 days of age after necropsy. Faecal droppings were recovered by gently pressing the abdomen of chicks. The samples recovered before infection were immediately frozen in liquid nitrogen and stored in tubes containing physiological serum (0.5 ml) combined with glycerol (50%). After infection, a hierarchical clustering, summarizing the levels of faecal excretion and caecal colonization of *Salmonella*, was performed using Euclidean distance and 'ward' linkage to cluster chicks into low-, intermediate- and super-shedder categories, as described by Menanteau *et al*. (2018)). Next, two mixes of microbiota were prepared; on the one hand, three faecal samples collected before infection (i.e. at 5 and 7 days of age) from low-shedder chicks were pooled and diluted in PBS ('low-shedder' mix); on the other hand, three faecal samples collected before infection from super-shedder individuals were pooled and diluted ('super-shedder' mix). The two resulting mixes were used to inoculate orally (0.2 ml) one-day-old chicks of a second group (Group 2, Fig. 4A). Group 2 consisted of 70 chicks divided into two subgroups of 35 individuals; the first was exposed to the 'super-shedder' mix and the second to the 'low-shedder' mix. After oral inoculation, the chicks were kept in two separate cages for four days, to harmonize within group gut microbiota. They were then relocated to two separate isolators. At 7 days of age, all chicks were inoculated with the *S*. Enteritidis strain LA5 (CFU = 5×10^4 per chick) and remained in their isolator for 21 days. Faecal samples were collected at 5, 7, 11, 14 and days of age and caecal samples at 21 days of age. Bacteriological analyses were performed as described below, on the samples collected 4, 7 and 14 days after infection to determine the levels of faecal excretion and caecal colonization of *Salmonella*.

Infection of chicks to measure the microbiota composition and the effect of commensal bacteria

On the day of hatch, all chicks were marked individually with leg tags and were housed in a conventional battery cage system in the same room for 4 days to acquire a gut microbiota. They were then randomly divided into groups of 30, relocated to large isolators at 5 days of age where

they were orally inoculated with $5x10^4$ of the *S.* Enteritidis LA5 strain on day 7 as previously described (Menanteau *et al.*, 2018). Fresh faecal samples were collected before and after infection (at 5, 7, 11, 14, 21 days of age) and caecal colonization was determined after necropsy at 21 days of age. To collect caecal mucus samples, the second caeca was washed and gently stirred using physiological serum until removal of the faecal content. Two additional washes were conducted using physiological serum containing 0.1% Tween 80 to recover mucus. The resulting mix was shaken vigorously and rapidly frozen in liquid nitrogen for further processing.

The faecal and caecal samples recovered were divided into two parts; one was immediately frozen in liquid nitrogen for DNA extraction and 16S rRNA gene sequencing, and the other was used to quantify the *Salmonella* load. For this purpose, caecal and faecal samples were homogenized in TSB and serial 10-fold dilutions were plated on *Salmonella–Shigella* medium containing nalidixic acid (20 μg ml^{-1}). 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 in 30 ml TSB to reveal contamination below the detection threshold. After 24 h at 37°C, these cultures were plated on *Salmonella–Shigella* medium containing nalidixic acid and incubated for 24 h. After enrichment, the detection threshold was one bacterium per organ.

To measure the effect of *Enterococcus faecium* or the effect of the mix of four commensal bacteria on *Salmonella* colonization, two groups of 35 chicks were used. One group was orally inoculated with two clones of *Enterococcus faecium* (MT10 and MT11) at 1 and 6 days of age (5×10^8 CFU of each clone/0.2 ml per chick). With the mix of 4 commensal bacteria, only one oral inoculation was performed on the day of hatch. The second group was inoculated at the same time with PBS. Immediately after the first inoculation, chicks were relocated to non-sterile isolators. At 7 days of age, chicks were orally inoculated with 5×10^4 of the *S.* Enteritidis LA5 strain. Fresh faecal samples were collected at 4, 6, 11, 14 and 20 days of age and caecal samples at 21 days of age. The faecal and caecal samples recovered were used to quantify *Salmonella* load and the *E. faecium* resistant to rifampicin.

16S rRNA gene sequencing

Microbial DNA was extracted using the QIAamp DNA Stool mini-kit following the manufacturer's instructions (Qiagen, Hilden, Germany). Briefly, 25 mg of faecal or caecal contents were transferred to a tube with lysis buffer and sterile zirconium beads. Samples were homogenized at maximum speed (FastPrep FP120) for four cycles of 45 s (with cooling between cycle 2 and 3), and then heated at 70°C for 15 min. Following centrifugation (5 min, 16 000 *g*, 4°C), a second extraction step was carried out. The two supernatants were pooled for the DNA purification step. Proteinase K was added and the sample was heated at 70°C for 10 min to degrade proteins. Ethanol was then added and DNA was purified using QIAamp columns as described by the manufacturer. The sample was eluted in 200 μl of Tris-EDTA buffer AE (Qiagen, Hilden, Germany). DNA quantity and quality were measured with a Nanodrop spectrophotometer and then diluted to the same concentration of 5 ng ml. PCR amplification was performed using the forward primer 5′-*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG* – MID – GT – CCTACGGGNGGCWGCAG-3′ and reverse primer 5′ - *GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG* – MID – GT – GACTACHVGGGTATCTAATCC-3′. The sequences in italics served as the index and adapter ligation, whereas the underlined sequences enabled amplification over the V3/V4 region of 16S rRNA genes. MIDs represent different sequences of 5, 6, 9 or 12 base pairs in length, which were used to differentiate samples within the sequencing pools. PCR amplification was performed using a HotStarTaq Plus MasterMix kit (Qiagen, Hilden, Germany). The resulting PCR products were purified using AMPure beads. In the next steps, the concentration of PCR products was determined using spectrophotometry, the DNA was diluted to 100 ng μl^{-1} and groups of 14 PCR products with different MID sequences were indexed with the same indices using Nextera XT Index Kit following the manufacturer's instructions (Illumina, San Diego, CA, USA). Prior to sequencing, the concentration of differently indexed samples was determined using a KAPA Library Quantification Complete kit (Kapa Biosystems, Boston, MA, USA). All indexed samples were diluted to 4 ng μl^{-1} and 20 pM phiX DNA was added to a final concentration of 5% (v/v). Sequencing was performed using MiSeq Reagent Kit v3 and MiSeq apparatus according to the manufacturer's instructions (Illumina, San Diego, CA, USA).

The16S rRNA gene sequencing included 78 samples collected from 13 chicks. The read assembly yielded a total of 3, 649 597 16S rRNA gene sequences. After removal of the chimeric and non-informative sequences, and of the samples sequenced with a low coverage ($N_{reads} < 500$), we kept 50 samples (totalling 185 889 sequences). The sequences were clustered into 442 OTUs.

OTUs relative abundances

An initial quality-trimming step of the resulting raw reads was performed using TrimmomaticPE 0.30 (Bolger *et al.*, 2014). Data were then uploaded on FROGS analysis pipeline (Escudie *et al.*, 2018) which was used for all

further steps of microbiota characterization. First, paired-end reads from each sample were clustered by allowing no mismatch in MID sequences. They were next selected using an expected read size of 292 bp, and a total amplicon size ranging from 350 bp to 550 bp with a mean of 460 bp. The resulting sequences were clustered using Swarm (Mahé *et al*., 2014). For this, we used 1 and 3 as the values of aggregation distance parameters (for the denoising and final clustering steps, respectively). OTUs including chimeric sequences were then removed using VSearch (Rognes *et al*., 2016). Other quality control steps included removal of very rare OTUs (relative abundance < 0.00005% of the total read numbers; present in less than three of the 78 samples) and those including sequences matching phiX sequences recorded in a specific databank (Escudie *et al*., 2018). Finally, the resulting OTUs were classified using an NCBI Blast + search within Silva SSU 123 database (Camacho *et al*., 2009; Quast *et al*., 2013).

Diversity indices

Diversity assessment was based on the Observed diversity, Chao1, Shannon and inverse Simpson α-diversity indices and the Jaccard, Bray-Curtis, Unifrac and weighted Unifrac β-diversity indices. To compute the last two indices, we used genetic distances estimated using the Tamura & Nei distance model with uniform mutation rates and equal nucleotide frequency (Tamura and Nei, 1993). The computations were performed using R-package Phyloseq and MEGA 7.0.20 (McMurdie and Holmes, 2013; Kumar *et al*., 2016).

Statistical analyses

Statistical information regarding each experiment is described within the figure legends and in the Results section.

Principal component analyses (PCA) were performed using the R-package FactoMineR (Lê *et al*., 2008). Differences in means between two groups were assessed using Student *t*-tests; one-way ANOVAs were performed to compare more than two groups, except for the comparison of mean β-diversity indices which were conducted using PERMANOVA (9999 permutations). Student t-tests and ANOVA were performed using the dedicated native R tools (R Development Core Team, 2015) or STAMP 2.1.3 (Parks *et al*., 2014); PERMANOVA tests were performed using the adonis() function of the R-package Vegan (Oksanen *et al*., 2013).

Differential abundances for each OTU were assessed following the hypothesis that abundances in each sample followed negative binomial distributions. Under this scheme, relative abundance of each OTU may be modelled by fitting a generalized linear model. Significant logarithmic fold-change ratios were detected using Wald tests and Benjamini-Hochberg adjustment for multiple testing ($P < 0.01$). The computations were performed using the R-package DESeq2 (Love *et al*., 2014).

Multivariate modelling of *Salmonella* relative abundances was performed using linear mixed effects models, allowing random effects to be taken into account. These computations were performed using R-package nlme (Pinheiro *et al*., 2014). Multivariate modelling of α-diversities was conducted using generalized linear models using the dedicated R function glm(). Poisson regression was used for indices equivalent to OTUs count (Observed diversity and Chao1 index).

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Conflict of interest

None declared.

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Data availability statement

The raw 16S rRNA gene sequencing data reported in this paper have been deposited in the Sequence Read

Archive (SRA) of the European Nucleotide Archive (ENA) with the accession numbers biosamples ERS3428982 to ERS3429031 of the bioproject PRJEB32628.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Family-level gut microbial compositions before and after *Salmonella* infection. For 16S metagenomic analysis, 13 chicks from a group of 30 chicks reared in an isolator for 21 days, and orally inoculated with the *S*. Enteritidis LA5 strain at day 7 were investigated. Faecal samples were collected at 5, 7, 11, 14 days of age (D5, D7, D11, D14); caecal and mucus samples at 21 days of age (D21 caeca D21 mucus). After DNA extraction, gut microbiota composition was determined by 16S rRNA gene sequencing. Some samples had to be discarded after quality control steps. Only the 20 most abundant families are reported here, using different colours, all other families being included into the category "Others".

Fig. S2. Levels of intestinal colonization of the four commensal bacteria. At day of hatch, germ-free chicks were orally inoculated with 200 µl of a bacterial inoculum containing 6.59 10^7 CFU of *Escherichia coli* Nissle 1917, 4.32 10^7 CFU of *Enterococcus faecium* DSM 7134, 8.90 10^3 CFU of *Clostridium butyricum* DSM 10702 and 1.24 10^7 CFU of *Lactobacillus rhamnosus* DSM 7133. The inoculum was prepared by mixing 2.5 mL of an overnight culture of Nissle 1917 strain grown in 10 ml BHI medium (Difco) at 37°C without agitation, 2.5 ml of an overnight culture of DSM 7134 strain grown in 10 ml BHI medium at 37°C without agitation, 2.5 ml of an overnight culture of DSM 7133 strain grown in 10 ml BHI medium at 37°C within an anaerobic jar with gas pack and 2.5 ml of a two-days culture of DSM 10702 strain grown in 100 ml DSMZ Medium 110 (Oxoid) at 37°C into an anaerobic jar culture with gas pack under agitation. Presence of each bacterial strain was verified at days 5, 8, 15, 28, 44 and 55 post inoculation by plating serial dilutions of a fecal sample in Nacl 0.9 g l^{-1} onto MRS plates (Biorad) for *Lactobacillus rhamnosus*, M. enterococcus plates (Difco) for *Enterococcus faecium*, Drigalsky plates (Biorad) for *Escherichia coli*, and TSA plates supplemented with 0.5 g l^{-1} ammonium ferric citrate and 1 g l^{-1} sodium metabisulphite for *Clostridium butyricum*. TSA and MRS plates were incubated anaerobically into a jar with gas pack.

Table S1. OTUs and genera showing significant differential abundances over the whole time series in chicks inoculated with *Salmonella* at 7 days of age. For 16S metabarcoding analyses, 13 chicks were considered from a group of 30 chicks. On the day of hatch, all chicks were housed in a conventional battery cage system in the same room for 4 days to acquire a gut microbiota. Next, at 5 days of age, they were relocated in isolator and orally inoculated with 5×10^4 of the *S*. Enteritidis 'LA5' strain at Day 7. Fresh faecal samples were collected at 4, 7, 11, 14 days of age; caecal and mucus samples at 21 days of age. Microbial DNA was extracted and 16S rRNA genes were sequenced as described in material and methods. (A) OTUs' abundances among samples were modelled using a Generalized Linear Model approach under the hypothesis of a negative binomial response variable. Threshold were adjusted using Benjamini-Hochberg's method. Each OTUs was designated by its deepest unambiguous taxonomic assignation (i.e. when available, at the genus level; otherwise using assignation at higher taxonomic ranks) and a unique arbitrary number (in parentheses). (B) Multiple testing of genus abundance according to sample categories (at 4, 7, 11, 14 using faecal samples, and 21 days using caecal and mucus samples); significance threshold was adjusted using Bonferronni's method.

Table S2. Differentially abundant OTUs between pairs of sample type categories.

