Impact of Host Genetics on Caecal Microbiota Composition and on *Salmonella* Carriage in Chicken

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Research Article

Keywords: Caecal Microbiota, Salmonella Carriage, Gallus gallus, Host Genetics

Posted Date: September 17th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-76645/v1

Abstract

Background *Salmonella* Enteritidis (SE) is one of the major causes of human foodborne intoxication through the consumption of contaminated poultry products. Genetic selection of animals more resistant to *Salmonella* carriage and the modulation of gut microbiota are two promising ways of decreasing individual *Salmonella* carriage. This study aims to identify the main genetic and microbial factors controlling the individual levels of *Salmonella* carriage in chickens (*Gallus gallus*) in controlled experimental conditions. Two-hundred and forty animals from the White Leghorn inbred lines, N and 6_1, were infected by SE at 7 days of age. After infection, animals were kept in isolators to reduce the recontamination of birds by *Salmonella*. Caecal contents were sampled at 12 days post-infection and used for DNA extraction. Microbiota DNA was used to measure individual counts of SE by digital PCR and to determine the bacterial taxonomic composition through a 16S rRNA gene high-throughput sequencing approach.

Results Results confirmed that the N line is more resistant to *Salmonella* carriage than the 6_1 line, and that intra-line variability is higher for the 6_1 line. Furthermore, the 16S analysis showed strong significant differences in microbiota taxonomic composition between the two lines. Out of 617 Operational Taxonomic Units (OTUs), over 390 were differentially abundant between the two lines. Furthermore, within the 6_1 line, we found a difference in the microbiota taxonomic composition between high and low *Salmonella* carriers, with 39 differentially abundant OTUs. Finally, via metagenome functional prediction based on 16S data, we identified several metabolic pathways potentially associated to microbiota taxonomic differences (*e.g.* butyrate metabolism) between high and low carriers.

Conclusions Overall, this study demonstrates that the caecal microbiota composition of the N and 6_1 lines is influenced by the host genetics, which could be one of the reasons why these lines differ for their *Salmonella* carriage in experimental infection conditions.

Background

Reducing *Salmonella* carriage in chicken flocks is a challenge to ensure human food safety and enhance breeding viability. The consumption of contaminated poultry products can indeed cause human food intoxications. The reported number of human salmonellosis outbreak cases due to the consumption of contaminated chicken meat and eggs in Europe was over 4,000 from 2014 to 2016, with more than 93% due to the Enteritidis serovar (EFSA Journal, 2019). Control of hygiene, detection of several serovars of *Salmonella* and culling of contaminated flocks or vaccination in farms aim to decrease this number. Nevertheless, the economic impact of human salmonellosis remains estimated at approximately 3 billion euros a year (EFSA Journal, 2019). Developing new strategies to improve the control of *Salmonella* propagation in poultry livestock is therefore essential to respond to this challenge.

In adult chickens, *Salmonella enterica* Enteritidis (SE) does not induce symptoms and can stay in the organism for a long time [1]. Carrier animals excrete the bacteria in the environment, thus increasing the

bacteria propagation and facilitating the contamination of all animals. Previous studies identified several quantitative trait loci (QTL) associated with *Salmonella* carriage in the White Leghorn inbred lines N and 6_1 [2–4], showing the contribution of host genetic variations in the resistance to *Salmonella* carriage. However, the effects associated with the identified loci are weak, which would imply a large polygenic control of this trait. It does not allow a direct application in commercial lines by marker-assisted selection of more resistant animals. The weak effects of the identified loci might also be due to a biased estimation of individual *Salmonella* carriage, due to the recurrent recontamination of birds by *Salmonella* excreted by other birds within the timeframe of experimental infections. This recontamination probably leads to a homogenization of *Salmonella* carriage in chicken flocks. The use of isolators, strongly reducing this recontamination, actually led to a much increased variation among birds in the experimental White Leghorn line PA12 [1], allowing the description of three categories of birds according to their shedding: super-, intermediate- and low-shedders. These isolators allow a control of the air purity and limit faecal-oral recontaminations between birds by the use of grids on the floor and decontamination of faecal drops.

Additionally, many studies demonstrate the importance of the gut microbiota for host health, in poultry as well as in every livestock species and in human. In human, disruptions of the intestinal microbiota can lead to many kinds of non-infectious diseases by altering the host physiology and metabolism and triggering inflammation [5]. It is now very clear that the host and its intestinal microbiota both contribute to the expression of many phenotypic traits of interest in livestock species [6]. In chicken, it is also well established that intestinal health is the result of complex functional interactions between intestinal microbes and host immunity [7]. In particular, the adult gut microbiota in chicken has been consistently identified as a protective factor to prevent the colonisation of intestines of young chicks by *Salmonella* sp. through a mechanism called competitive exclusion [8–10]. Indeed, the spraying of an adult microbiota on young chicks or the use of probiotics are already efficiently used in commercial flocks to reduce the *Salmonella* Enteritidis load [11–14]. Many studies have also assessed the efficiency of nutritional strategies aiming at reducing the *Salmonella* Enteritidis load by a modulation of the intestinal microbiota leading to an improvement of the host immunity [15–18]. Such strategies include prebiotics or probiotics boosting the production of beneficial metabolites, the modulation of host immunity, and the improvement of intestinal barrier function [19]. To understand the underlying biological mechanisms of such strategies, and to identify the bacterial taxa actually competing with *Salmonella*, it is important to analyse the impact of *Salmonella* infection on the host microbiota composition. Comparing infected and non-infected animals, such studies sought to identify OTU signatures of *Salmonella* infection. For example in pigs, Argüello et al. identified bacteria from the class *Clostridia* that could prevent *Salmonella* Typhimurium colonisation [20]. In chicken, the family *Ruminococcaceae*, which are more abundant in non-infected animals at 4 days post infection (dpi), could be a signature of *Salmonella* infection [21].

Overall, it seems both host genetic variations and intestinal microbiota composition could explain variations in individual *Salmonella* Enteritidis carriage in chicken. Furthermore, genetic studies on other phenotypic traits, such as digestive efficiency [22], body weight [23] or feather-pecking in laying hens [24]

showed that host genetic variations influence the intestinal microbiota composition in chicken. In these studies, authors identified several QTLs and single nucleotide polymorphisms (SNPs) associated with specific bacterial species that may explain the phenotype and calculated heritabilities of these bacteria abundances.

In this study, we investigated the combined influence of host genetic variations and gut microbiota composition on caecal *Salmonella* Enteritidis (SE) load. Using the chicken N and 6_1 inbred lines, we studied the impact of genetics on both the *Salmonella* carriage and microbiota composition at the individual level, using the isolator model, which highly decreases the exchanges of gut microbiota between animals [1]. We infected 240 animals with *Salmonella* Enteritidis at 7 days post hatching. We collected caecal contents at 12 dpi to assess both individual microbiota taxonomic composition and SE counts. Our aims were:

(i) To assess the intra-line individual variability of *Salmonella* load and of the caecal microbiota composition;

(ii) To compare *Salmonella* load and caecal microbiota composition of the two lines, in order to assess the existence of a genetic control of these two parameters;

(iii) To identify putative microbial signatures of low/high *Salmonella*

Methods

Experimental design

Two experiments were conducted with a total of 240 animals of the White Leghorn inbred lines N and 6_1 (both males and females, with an equal distribution). For each experiment, chicks from both lines (n=120; 60 animals from each line) hatched together with free access to food and water at the experimental unit PEAT (Pole d'Expérimentation Avicole de Tours, Nouzilly, France). They were immediately transferred to the PFIE unit (Plateforme d'Infectiologie Expérimentale, INRA, Nouzilly, France) where they were reared together in battery cages. On day 7, each chick was orally infected with *Salmonellaenterica* Enteritidis (Strain 775 [LA5 Nal20Sm500], 5.10^4 cfu/0.2 mL/chick) and animals were transferred into four isolators to decrease oro-faecal recontaminations as described previously [1]. Two isolators contained chicks from line N and two other isolators contained chicks from line 6_1, with 30 birds per isolator. Sibs and half-sibs were separated into the two isolators available for each line, in order to prevent confounding between an intra-line genetic effect on *Salmonella* carriage and an effect of the isolator. Isolators with birds from line 6_1 in the first experiment contained birds from line N in the second experiment, in order to prevent confounding between isolator and line effects. Then, 12 days post-infection, animals were euthanized with CO2 according to the French regulation for experimental chickens and caecal contents were gently collected so as not to remove the intestinal mucosa, weighted, transferred in cryotubes and immediately

frozen in liquid nitrogen and stored at -80°C until use. All animal procedures has been approved by the Ethic committee (APAFIS#5833-2016062416362298v3) and authorised by the French Government.

DNA extraction

Individual caecal DNA was extracted from an average of 200 mg of frozen caecal contents as previously described [25]. In brief, samples were incubated at 70°C for 1 hour with 250 µl of guanidine thiocyanate buffer (4 M guanidine thiocyanate–0.1 M Tris (pH 7.5) and 40 µl of 10% N-lauroyl sarcosine–0.1 M phosphate buffer (pH 8.0)) and 500 µl of 5% N-lauroyl sarcosine. One volume (750 µl) of 0.1-mm-diameter silica beads (Sigma) was added, and tubes were shaken for 10 min at the maximum speed of a MM200 Mixer Mill (Retsch, Germany). Tubes were vortexed and centrifuged at 14000 rpm for 5 min at 4°C. After recovery of the supernatant, 30 µl of Proteinase K (Chemagic STAR DNA BTS kit, Perkin Elmer, USA) were added and samples were incubated for 10 min at 70°C at 250 rpm in Multi-Therm (Benchmark Scientific, USA), then for 5 min at 95°C for enzyme inactivation. Tubes were centrifuged at 14000 rpm for 5 min at 4°C and supernatants were transferred into a deepwell plate. The plate was loaded on the nucleic acid workstation Chemagic STAR (Hamilton, Perkin Elmer, USA) and the extraction protocol was performed with Chemagic STAR DNA BTS kit (Perkin Elmer, USA) by the @BRIDGe platform (INRAE, Jouy-en-Josas, France) according to the manufacturer's instructions. DNA concentration was measured by fluorometric quantification (Qubit) and DNA samples were stored at -20°C.

Quantification of *S.* Enteritidis by ddPCR

Individual abundances of *Salmonella* Enteritidis in caecal contents at day 19 were obtained by Droplet Digital PCR using the QX200 Droplet Digital PCR system (Bio-Rad) at the @BRIDGe platform (INRAE, Jouy-en-Josas, France). Each DNA sample was diluted at 1:2 and 1:5 in a final volume of 5 µl to be under PCR saturated conditions. Droplets were generated from the 20 µl of the total volume of amplification mix and genomic DNA (15000 to 20000 droplets per sample). The amplification targeted a region of the *InvA* gene specific to *Salmonella* Enteritidis using specific primers, as described by Daum et al. [26] (Additional file 1). PCR cycling condition started with an enzyme activation step at 95°C for 10 min, followed by 40 cycles at 94°C for 30 min and 60°C for 1 min, and ended by an enzyme deactivation step at 98°C for 10 min. For all cycling steps, we used a 2.5°C/sec ramp rate.

For each sample, the number of copies of *Salmonella* per gram of caecal content was calculated from the average number of copies of *Salmonella* per µl of the two dilutions, assuming that each amplified copy of the *InvA* gene corresponds to one *Salmonella* bacterium. Data were analysed with a log transformation of the copies of *Salmonella*. Analyses of variance (Anova) were performed to test the significance of differences of the copies of *Salmonella* between conditions (line, sex, experiment or isolator) using the anova function in R software version 3.5.1 (Type I sum of squares).

PCR and Sequencing of 16S rRNA Genes

Amplification of the V3-V4 hyper-variable region of the 16S rRNA coding gene was performed at the INRAE @BRIDGe platform. Universal V3-V4 primers (Additional file 1) were used for the first PCR reaction. PCR cycling conditions were as follows: an initial denaturation step (94°C for 10 min), 35 cycles of amplification (94°C for 1 min, 68° C for 1 min and 72°c for 1 min) and a final elongation step at 72°C for 10 min. Amplicons were then purified using magnetic beads (Clean NA, GC biotech B.V., The Nederlands) and the concentration was controlled using a Nanodrop spectrophotometer (Thermo Scientific, USA). In the second PCR, samples were multiplexed and the second pair of primers was used (Additional file 1). An initial denaturation step (94°C for 10 min), 12 cycles of amplification (94°C for 1 min, 65°C for 1 min and 72°C for 1 min) and a final elongation step at 72°C for 10 min were performed. Amplicons were purified and concentration was controlled as described for the first PCR reaction. One run on an Illumina MiSeq was used to sequence amplicons (2 x 250 paired-end reads) according to the standard protocol.

Bioinformatic and statistical analyses

Identification of Operational Taxonomic Units (OTUs) was performed by using the FROGS pipeline [27]. FastQC program to control quality and the Cutadapt program to find and remove adapter sequences from sequencing reads, and R1 and R2 reads were merged and filtered (at Phred >= Q20) by using the Flash program [28]. OTUs were identified using the Swarm program [29]. Chimera OTUs were removed using the VSEARCH program [30], taxa filtering was performed with a minimum abundance threshold of 0.005% as proposed by [31]. Finally, phylogenetic affiliations were identified using the Silva database by using the blastn+ program [32]. OTUs representing less than 0.5% of global reads and samples with less than 10,000 reads were removed.

The phyloseq (1.24.2) and vegan (2.5-3) packages were used with R to perform the diversity analysis on normalized data. Alpha diversity was measured using the Shannon index and beta diversity using the Whittaker index. Analyses of variance (Anova) were performed to test the significance of differences of alpha and beta diversity between conditions (line, sex, experiment or isolator). We evaluated the Bray-Curtis distances using the env_fit method that we plotted in an NMDS (Non-Metric Multidimensional Scaling) representation. Permutational Multivariate Analyses of Variance (Permanova) were used to test for the significance of differences of diversity according to variations in lines, experiments, isolators and sex. The R package metagenomeSeq (1.24.1) was used for the identification of differentially abundant (DA) OTUs between lines N and 6_1 and between high and low carriers within line 6_1. The OTU table was first normalised, and the model was fitted using the fitZIG method by including experiment, isolator and sex as cofactors. Heatmaps were built using the function plotMRheatmap on significant DA OTUs. Then, OTUs were aggregated by family and genus with the command aggregateByTaxonomy to identify DA families and genera. Functional gene families and MetaCyc pathway were predicted using the PICRUST2 package. DA KEGG ortholog (KO) and DA MetaCyc pathway were identified with the R package DESeq2 (1.26.0). DA MetaCyc pathways were aggregated at the super-pathway level using MetaCyc database [33].

Metadata table, unrarefied OTU table, and corresponding taxonomic classifications have been included as Additional files 2, 3 and 4, respectively.

Results

Abundance of *S.* Enteritidis in caecal contents

We obtained an average of 3.38 log10 copies of DNA target sequences per gram of caecal content for line 6_1, and 2.55 log10 copies for line N (Figure 1). An Anova including genetic line, experiment, isolator and sex as factors showed a significant difference (P<0.001) of ISC (Individual *Salmonella* Carriage) between the two lines (Table 1 and Additional file 5, Table 1). This difference between lines was also significant when considering each experiment separately (Table 1). We also observed that ISC is more variable in line 6_1 than in line N (P<0.001), with standard deviations of 1.3 and 0.7, respectively. This higher ISC variation in line 6_1 was more marked in experiment 1 than in experiment 2 (P<0.001), with standard deviations of 1.61 vs. 0.84 (Figure 1). ISC was not significantly different between males and females (Additional file 5, Table 1). Furthermore, the mean ISC between the two experiments was significantly different (P<0.001) for line N (2.2±0.7 in experiment 1 vs. 2.9±0.4 in experiment 2) but not for line 6_1. Finally, within each experiment, there were no significant differences between isolators containing line 6_1, but there were significant differences between isolators containing line N (P<0.01).

Table 1: *S.* Enteritidis abundance in caecal contents of chickens 12 days after oral infection.

Line	Log 10 mean values of copies of DNA target sequences per g of caecal contents		
	Exp1	Exp2	All Exp
N	2.21±0.74	2.86±0.43	2.55±0.68
6	3.41±1.61	3.35±0.84	3.38±1.29
p-value*	**1.86e-06**	**3.84e-05**	**9.59e-10**

*Anova F-test test using all factors

In line 6_1 for the experiment 1, we were able to identify by hierarchical clustering, two extreme groups of fifteen samples, for which we have usable information on their microbiota, that were considered to be low and high carriers. The low-carriers group displayed a mean of 1.8±0.6 log10 copies of DNA per gram of caecal content, while the high-carriers group had a mean of 5.2±0.6 log10.

Structure and diversity of bacterial community

Due to strict controls of DNA quality after extraction and amplification, only 228 samples with at least 10 ng of DNA from the 240 initial samples were sequenced. After sequencing, we obtained 8,300,144 reads with an average of 32,464 reads per sample. From these reads, 617 OTUs were identified with a

percentage of affiliation of 99% at the family level and 65% at the genus level. Samples with a total number of reads lower than 10,000 were removed, so that 182 samples from the 228 sequenced samples were finally analysed: 86 samples from line 6_1 and 95 from line N.

At the phylum level, *Firmicutes*, followed by *Proteobacteria* and *Bacteroidetes* (Figure 2), dominated the bacterial composition. At the family level, we observed a predominance of *Lachnospiraceae* and *Ruminoncoccaceae*, followed by the *Enterobacteriaceae*, *Clostridiales* and *Bacillaceae* (Figure 2 and Additional file 5, Table 2).

The analysis of alpha diversity with the Shannon index did not show a significant difference between lines, experiments, or sex (Additional file 5, Table 3). Using the Whittaker index, we observed a significant difference of beta diversity between experiments ($P<0.0001$), corresponding to 0.24 for experiment 1 vs. 0.20 for experiment 2. However, we did not observe any difference in beta diversity between lines (0.20 for both). Comparing high- and low-carriers in line 6_1, we identified no significant difference in alpha diversity and richness, but we identified a significant difference in beta diversity (Additional file 5, Table 3).

Non-metric multidimensional scaling and differentially abundant OTUs between lines

The analysis of the beta diversity using the Bray-Curtis distance and an NMDS representation (Figure 3) showed that the microbiota composition is clearly clustered according to the genetic line. This clustering is observed for each experiment individually but is more contrasted in experiment 1 (Figure 3). The Permanova analysis confirmed this line effect ($P<0.001$) after correcting for the experiment, isolator and sex effects, whether merging data from the two experiments or considering each experiment separately (Additional file 5, Table 4). The global microbiota composition was also significantly different between experiments and isolators, but not significantly different between males and females (Additional file 5, Table 4).

Of the 617 OTUs identified, we observed a total of 390 significant DA OTUs between lines over the two experiments, after controlling for the isolator effect (Additional file 5, Table 5). When analysing experiments separately, a heatmap showed a more strongly defined clustering for experiment 1, with 388 DA OTUs between lines (Figure 3), whereas for experiment 2, we observed 284 DA OTUs. A total of 187 DA OTUs were found to be in common between the two experiments.

In the following analyses, we aggregated the 617 OTUs in order to compare compositions at higher taxonomic ranks, which led to 14 families and 51 genera. We identified 9 DA families and 31 DA genera between lines over the two experiments, after adjusting for the isolator effect (Figure 4 and Additional file 5, Table 6). For experiment 1 and experiment 2, we respectively identified 9 and 5 DA families and 24 and 29 DA genera (Figure 4). At these levels of aggregation, significant DA families and significant DA genera between lines are not the same when comparing the two experiments, and some are inversely abundant. Nevertheless, *Christensenellaceae* is in both cases a family that is more abundant in the resistant line N. At the genus level, *Tyzzerella 3*, *Lachnoclostridium*, *Marvinbryantia*, *Ruminococcaceae UCG-013* are more abundant in line 6_1 (susceptible) and *Ruminococaceae UGC-004*, *Ruminococcus 1*, *Pseudomonas*,

Pseudoflavonifractor, *Christensenellaceae R-7 group* and *Ruminococcaceae UGC-014* are more abundant in line N (resistant).

High and low carriers in line 6_1 and the family *Christensenellaceae*

In order to assess a potential difference of microbiota composition between high and low carriers in line 6_1, we used an NMDS representation for the first experiment (Figure 4, A). We chose experiment 1 because it had the largest variability in individual *Salmonella* carriage, which allowed for the identification of high and low carriers. The Permanova analysis showed a significant (P<0.01) difference of microbiota composition between both groups. The metagenomeSeq analysis led to the identification of 39 DA OTUs between high and low carriers (Figure 4, B and Additional file 5, Table 7). After aggregation, we obtained one DA family: *Christensenellaceae*, and three DA genera: *Ruminococcaceae NK4A214* group, *Ruminiclostridium 5* and *Christensenellaceae R-7* group (Figure 5, C and Additional file 5, Table 8).

A comparison of the results in DA analyses between lines and between high and low carriers led to the identification of a common, DA family: the *Christensenellaceae*. *Christensenellaceae* is more highly abundant in low carriers than in high carriers in line 6_1 and is also more highly abundant in line N, which is more resistant (i.e. low carrier) to *Salmonella.* The same observation can be made for the DA genus *Christensenellaceae R7-group*, which is more abundant in low carriers as well as in the resistant line N for both experiments. A regression analysis led to a significant correlation (P-value = $4.11e^{-06}$) between ISC and *Christensenellaceae* abundance.

Functional gene prediction

Using PICRUST2, we predicted 4,954 KEGG Orthologies (KO) and 323 pathways over all lines and experiments. Using DESeq2, we identified 1,590 DA KO between lines N and 6_1, adjusting for the effect of the experiment, and 69 DA pathways (false discovery rate (FDR) < 0.01). In line 6_1, the analysis between high and low carriers in experiment 1, adjusting for the effect of the isolator, led to the identification of 507 DA KO and 75 DA pathways (FDR > 0.01). The 64 DA pathways in common for the comparisons between line N and 6_1 and between high and low carriers were aggregated at the super-pathway level and are represented in Figure 6.

Discussion

Using a model of *Salmonella* infection in isolators limiting animal-animal recontaminations and animal reinfection and thus homogenisation of individual *Salmonella* carriage [1], we confirm the existence of variability linked to genetics and microbiota composition. We evidenced gut microbiota differences associated to both the genetic line (N vs 6_1) and individual *Salmonella* carriage status (high *vs.* low ISC carriers). On average, line N had less *Salmonella* abundance than line 6_1, suggesting a genetic control of the carriage. In line 6_1, the larger observed variability allowed us to identify contrasted groups of low and

high carriers. Finally, through our caecal microbiota taxonomic analysis, we identified taxa and metabolism pathways that may be associated with *Salmonella* carriage.

In both experiments conducted, line N was more resistant and microbiota taxonomic composition was clearly different between both lines. Nevertheless, ISC was higher for line N in the second experiment and the DA OTUs between lines were not strictly the same between experiments. These differences in microbiota composition might be due to the impact of the immediate environment on individual microbiota composition. Although conditions were controlled to be similar between both experiments, even slight changes might lead to differences in the primo-colonisation of the intestinal tract [34, 35]. The hatching environment might have differed between both batches and/or the two batches of eggs might have carried different microbes. Those potential differences might have affected the bacterial gut primo-colonisation of the newborn chicks. As a result, a difference in microbiota composition before infection could affect the microbiota composition after infection. In the following section, since ISC and microbiota composition differed slightly between experiments, we will focus only on results validated in both experiments.

Impact of the chicken genetic line on the Individual *Salmonella* Enteritidis carriage

Previous studies showed the impact of host genetics on *Salmonella* carriage, using different infection models. Significant differences in *Salmonella* Enteritidis carriage in commercial or local chicken breed have already been identified [36–38]. Our results with lines N and 6_1 confirm previous results obtained without using isolators with the same lines, with on-floor grouped rearing [2, 39], confirming that line N is a lower *Salmonella* Enteritidis carrier than line 6_1. Previous studies validated candidate genes associated with resistance to *Salmonella* Enteritidis carriage, such as SLC11A1 and TLR4 [40–42] and identified QTLs on several chromosomes i [2–4]. Nevertheless, due to the highly polygenic control of carrier-state in these lines, with many loci with weak effects involved [43], identification of causal genes at the QTLs has not been yet possible. The infection of birds in isolators, which would allow for much larger individual variability [1], might be a way to improve future QTL detection studies and identification of causal genes. Interestingly, we observed a larger variability in ISC in line 6_1, confirmed in two independent experiments. This larger variability of ISC generates new hypotheses: could this ISC variability be caused by intra-line genetic variations, or by a variability of the microbiota composition?

Impact of the genetic line on the microbiota composition

In both experiments conducted, we showed a clear difference of the caecal microbiota composition between lines N and 6_1 at 12 days post-infection, which suggests the existence of a host genetic control of this composition. For each experiment, birds from lines N and 6_1 were hatched in the same environment at the same time, and raised together until the experimental infection, so that the initial microbial environment was similar for all birds tested before infection. Differences in caecal microbiota composition between lines can therefore not be attributed to differential exposures to environmental microbes before infection. Furthermore, for experiment 1, we observed no significant difference of

microbiota composition between the two isolators used for the same line (P>0.1, Additional file 5, Table 4). Thus, we cannot associate differences in microbiota composition between lines with isolators in experiment 1. In experiment 2, we observed a significant difference of microbiota composition between the two isolators used for the same line (P<0.01, Additional file 5, Table 4). It can be argued that isolators might be populated by different microbial populations, which could in turn influence the caecal microbiota composition after infection. Howerver, isolators were sterilized between each experimentation. In addition, we inverted the isolators containing each line in the second experiment, and still observed a similar difference between lines in terms of caecal microbiota composition. Moreover, since the utilisation of isolators decreases the oro-faecal recontamination of commensal microbiota as well as pathogenic bacteria, the caecal microbiota composition of each chicken can mature isolated from the impact of the other birds.

Since we analysed caecal microbiota composition after infection, we cannot exclude that the infection by *Salmonella* affected this microbiota composition. Does the higher susceptibility of line 6_1, with a higher number of *Salmonella* in the caeca, causes a shift in microbiota composition? Comparing seven different time points after infection with SE, Liu et al. showed 18 genera significantly DA between treated and control group [44]. Nevertheless, it was shown in other studies that infection with SE does not have a huge impact on gut microbiota composition. Videnska et al. showed that the infection significantly impacts only the *Lactobacillaceae*, while Zeng et al. identified only a lower abundance of *Ruminococcaceae* at 4 dpi in an infected group [21, 45]. Although we lack samples collected from a control group before infection to verify this hypothesis, we hypothesize that SE infection presumably had a weak impact on microbiota composition.

A study on *Campylobacter* resistance between lines N and 6_1 was conducted by Chintoan-Uta and colleagues [46]. Their objectives were to transfer microbiota from the resistant line to the susceptible line and to study the *Campylobacter* carriage. First, they showed no significant difference of microbiota composition between donor birds from the N and 6_1 lines at 21 days, despite a clustering on an NMDS representation. The authors hypothesized that the low number of samples, only five per line, could likely explain this non-significant result. Secondly, they showed that at 21 days in the recipient line after transplantation, the genetic line had a significant impact on microbiota composition, while the transplanted microbiota did not. This supports our hypothesis that genetics have a strong impact on the composition of the microbiota, at least 3 weeks after hatching in lines N and 6_1. Nevertheless, Chintoan-Uta et al. did not show that transplantation of microbiota composition from the resistant line to the susceptible induces a decrease of *Campylobacter* infection, and they concluded that the microbiota does not have an impact on the mechanism of *Campylobacter* resistance, even if heritable genetic differences exist. Nevertheless, their conclusion could be the result of a lack of power since few samples were tested.

In chicken as well as in other livestock species and in human, the genetic control of the intestinal microbiota composition is well documented. In human studies, the high sensitivity of the gut microbiota to a myriad of parameters, especially differences in diet, makes statistical analyses complex due to many confounding factors. In spite of these difficulties, some bacteria known to be associated with immunity

seem to be heritable and candidate genes have been identified [47]. For example, the family *Christensenellaceae*, which are the most heritable bacteria in the study of Goodrich et al. in human, show a heritability of 0.39, while other studies reported an heritability of 0.62 for this family [48, 49]. Candidate genes in human that might be associated with *Christensenellaceae* abundance, such as ILR23 or *FUT2,were also identified* [50, 51]. In chicken, studies identified differences in microbiota composition between genetic lines, for instance in two chicken lines that differ in their susceptibility to bacterial infections [52] or in two divergent genetic lines for body weight [53]. Another study compared four commercial lines and an indigenous Indian breed, which revealed a significant impact of the genetic background on microbiota composition and led to the identification of 42 specific biomarkers [54]. At least two studies identified moderate heritabilities of bacteria families and several QTLs involved in the control of these bacterial abundances [22, 55].

Finally, we conclude that this clear difference of microbiota composition between lines after *Salmonella* infection is probably caused by host genetic variations between lines N and 6_1. We also showed a difference in ISC between lines. Therefore, in contrast to Chintoan-Uta's conclusion for *Campylobacter*, we formulate the hypothesis that genetic variations between lines N and 6_1 might indirectly influence *Salmonella* carriage through an influence on the microbiota composition. This does not exclude other potential pathways, in particular those involving host immunity. For example, the candidate genes SIVA1, implicated in a cell death mechanism in extracellular trap production, or SLC11A1, associated with heterophil extracellular trap production and phagocytosis of SE, may be implicated in leukocyte function and in the inhibition of intracellular bacterial growth by depleting metal ions [56].

Relation of caecal microbiota diversity and composition with individual *Salmonella* Enteritidis carriage

The colonisation and the adhesion of commensal bacteria covering the mucosal epithelium constitute a protective biofilm by their competitive exclusion (CE) function. Studies affirm that CE is currently the best approach to decrease *Salmonella* colonization in chicken in commercial production [9].

In our study, all animals were experimentally infected and the identification of signature bacteria was performed by comparing chickens with differences of individual *Salmonella* carriage between lines or intra-lines. Our hypothesis is that the abundance of commensal, potentially competitive bacteria is higher in the microbiota of resistant chickens, thus preventing the colonisation of *Salmonella* in the intestinal tract. The genetic background could in part control this abundance of competitive bacteria. We first compared the microbiota of line 6_1 (susceptible) to line N (resistant), and we subsequently compared the high and low carriers in line 6_1. Thus, differences of caecal microbiota diversity or composition between lines and between high and low carriers could be indicative of potential signature bacteria taxa of high and low ISC.

The weak correlation of microbiota diversity with ISC

The average value of microbiota beta diversity within a group measures the similarity of microbiota composition of each chicken of this group compared to all the chickens of the group. The higher the

value of beta diversity, the more individual microbiota in the group differ from each other. We compared here two groups: line N vs. line 6_1.

We showed that the microbiota beta diversity is not significantly different between lines (Whittaker index = 0.2 for the two lines). Thus, the difference of ISC between lines and the larger ISC variability in line 6_1 cannot be related to a difference in beta diversity of the caecal microbiota. Similarly, the average alpha diversity was not significantly different between lines and cannot be related to the observed differences of ISC. Thus, at 12 dpi, it seems that differences in genetic backgrounds do not have an impact on the microbiota diversity. Furthermore, the comparison of high and low carriers in line 6_1 showed that alpha diversity cannot be associated with differences in ISC. Comparing chickens infected and non-infected with SE, Liu and colleagues (2018) showed a slight decrease of richness at 14 dpi in the infected group, but no significant difference for the Shannon index [44]. At 10 dpi, another study similarly showed no significant difference between infected and non-infected groups [21]. These observations corroborate ours and lead us to conclude that the infection with SE or the level of SE carriage do not affect the OTU diversity in the caecal microbiota.

However, we identified a significant difference in beta diversity between high and low carriers within line 6_1: individual microbiota of high carriers are more similar compared to the individual microbiota of low carriers, which harbour more differences. Does the higher level of *Salmonella* drive the microbiota to a more similar composition in high-carriers? Or are these animals more susceptible to *Salmonella* because some shared characteristics of their microbiota lead to a less efficient competitive exclusion? These questions remain open; as we also cannot exclude that genetic variations within line 6_1 could explain these differences in caecal microbiota beta diversity.

Correlation between individual microbiota composition and Individual *Salmonella* Enteritidis carriage and functional analysis

DA OTUs and pathways identified between lines could potentially be associated with differences in ISC. Likewise, DA OTUs and pathways identified between high and low carriers in line 6_1 could be associated with ISC.

Short-Chain Fatty Acids (SCFAs) metabolic pathway

We showed that the SCFAs metabolic pathway would be more abundant both in the resistant line N and in low carriers in the susceptible line 6_1. Thus, the production of SCFAs could be associated with low *Salmonella* carriage. This result is coherent with many studies showing a relationship between *Salmonella* colonisation and SCFA. Many studies showed that the production of SCFAs, such as butyrate, by the intestinal microbiota has beneficial effects for the host. Studies have conferred to SCFAs several functions: the regulation of immune cell function [57], the activation of macrophages [58] or the maintenance of the oxygen balance preventing dysbiosis [59]. In mice, Zhou and collaborators showed in 2017 that butyric acid could increase the host capacity to resist to pathogen infection by restoring the

gastrointestinal barrier [60], and in 2019, they showed that butyric acid can decrease the inflammation in chicken [61]. Besides, we know that an increase of inflammation makes electron acceptors available to *Salmonella* for oxygen respiration, which in turn increases the ability of *Salmonella* for competition and colonisation [62]. More specifically, it was shown that the rise of SCFAs on *in vitro* culture of avian intestinal cells decreases the pathogenicity of *Salmonella*, blocking its entry into the organism [63]. Thus, line N and low carriers in line 6_1 could carry a beneficial microbiota for *Salmonella* resistance.

Catechol degradation pathway

The catechol degradation pathway was also more abundant in both the resistant line N and in the low *Salmonella* carriers of the susceptible line 6_1. Several studies report the association of catechol with *Salmonella* virulence. *Salmonella* have the ability to produce auto-inducers 3 (AI-3), which have a similar chemical structure as the catecholamine from the catechol family [64, 65]. In chicken and in pigs, studies have already showed that a treatment with catecholamine, e.g. Norepinephrine, increases *Salmonella* colonization in the host and *Salmonella* spread in the environment [66, 67]. Two mechanisms have been described to explain this phenomenon: the iron availability and the quorum sensing signal. A study in mice on *Salmonella* Typhimurium showed that molecules from the catechol family increase the iron availability for *Salmonella* by chelating iron III in their aromatic function and increasing iron accumulation in macrophages, which in turn facilitate *Salmonella* colonisation [68]. Besides, catechol plays a role of quorum sensing signal for the production of biofilm, thus increasing the virulence of *Salmonella* during host infection [64, 69]. Outside the host, this biofilm increases the *Salmonella* capacity to resist on eggs or meat, rising salmonellosis risks in human [70]. Thus, the microbiota capacity of catechol degradation in line N and low carriers in line 6_1 could be beneficial for *Salmonella* resistance.

Family Christensenellaceae

We showed that the *Christensenellaceae* family is more abundant in low *Salmonella* carriers (Figure 4). This bacteria family was already associated with beneficial impact on health in human and in mice [49]. For example, it has been associated with longevity [71], with beneficial impacts on obesity [48, 72] and with metabolic health [49]. The ability of *Christensenellaceae* to produce butyric acid confers to these bacteria a real health interest (refer to the section "*Short-Chain Fatty Acids metabolic pathway*"). Interestingly, in parallel, it was shown that *Christensenellaceae* is one of the most heritable bacterial families of the human intestinal microbiota [48]. This leads us to formulate the hypothesis that host genetic variations between lines N and 6_1 might cause variations in *Christensenellaceae* abundance, which in turn could affect *Salmonella* resistance. However, to our knowledge, the heritability of *Christensenellaceae* has not been assessed in chicken. Finally, Azcarate-Peril et al. showed that the use of Galacto-Oligosaccharides (GOS) as a prebiotic in chicken increases the clearance of *Salmonella* Typhimurium after infection and interestingly also increases the *Christensenellaceae* abundance [73]. Indeed, *Christensenellaceae* have the capacity to metabolize GOS unlike the host or *Salmonella*, which could be in disfavour of *Salmonella* [73, 74].

Family Enterobacteriaceae, Butyrate producers and anaerobisation

The family *Enterobacteriaceae* was more abundant in the resistant line (Figure 4). These bacteria are in competition with *Salmonella* for the respiration of oxygen [75] and for the use of nutrients as iron [76]. These bacteria are also able to product bacteriocine to inhibit the proliferation of *Salmonella* [75]. The family *Ruminococcaceae* and the genera *Flavonifractor*, *Pseudoflavonifractor*, *Anaerostipes* and *Intestinimonas*, which are butyrate producers [77, 78], were also more abundant in the resistant line. We have already described the beneficial effects of butyrate (see the section "*Short-Chain Fatty Acids metabolic pathway ")*. We can add that, according to Litvak and colleagues in 2019, the combined effects of butyrate production, maintaining a low oxygen concentration, and oxygen respiration by competitive bacteria such as *Enterobacteriaceae*, lead to an anaerobisation of the lumen and thus, a decrease of the capacity of *Salmonella* colonisation [75]. Our results are compatible with this hypothesis.

Family Ruminococcaceae and inflammation

We showed that the *Ruminococcaceae* family is less abundant in the susceptible line 6_1 (Figure 4). It has been shown that a decrease of *Ruminococcaceae* can be associated with an increase of inflammation [78] and thus an increase of *Salmonella* competition [62].

Other bacteria

Other interesting bacteria associated with low carriage were identified in experiment 2 but not confirmed in experiment 1. This is the case of *Lactobacillus*, which were more abundant in the resistant line. Some species of these bacteria, used as a probiotic, were shown to significantly decrease the *Salmonella* Enteritidis carriage [79, 80] and are also associated with the acceleration of *Salmonella* Typhimurium clearance in chicken [73]. Nevertheless, bacteria beneficial for health have also been found with a higher abundance in the susceptible line. For example, the *Blautia* genus, which is more abundant in line 6_1 in experiment 2 (not confirmed in experiment 1), is a butyrate producer [77] and has beneficial anti-inflammatory effects [81]. Thus, looking at individual taxa might not be sufficient. More likely, we suggest that the total balance of beneficial bacteria has an impact on the *Salmonella* resistance, which supports the idea of studying the aggregated contributions of several taxa to the same metabolic pathway.

Conclusions

We showed an impact of the genetic line on individual *Salmonella* Enteritidis count in caeca and on caecal microbiota taxonomic composition. We also showed associations between the abundances of bacterial taxa and metabolic pathways with the ISC status (high *vs.* low carriers), which were previously associated with resistance to *Salmonella* Enteritidis. Most notably, we identified an overrepresentation of the Short-Chain Fatty Acids metabolic pathway and the Catechol degradation pathway, as well as the *Christensenellaceae, Enterobacteriaceae* and *Ruminococcaceae* families in low-carriers. Based on these observations, we hypothesize that genetic differences between lines N and 6_1 may influence the level of *Salmonella* carriage by influencing the abundances of beneficial bacteria. Combining information on host

genetics and gut microbiota composition is useful to sharpen the prediction of complex traits such as resistance to pathogens. Our study showed that both caecal microbiota and the host genetic background play a role in the mechanisms leading to *Salmonella* colonization resistance in chickens. Future studies should decipher the genes that potentially control differences in bacterial abundances in these lines.

List Of Abbreviations

SE: *Salmonella* Enteritidis

OTU: Operational taxonomic units

QTL: Quantitative trait loci

DPI: Days post infection

SNP: Single nucleotide polymorphisms

NMDS: Non-metric multidimensional scaling

DA: Differentially abundant

KO: KEGG ortholog

ISC: Individual *Salmonella* carriage

FDR: False discovery rate

SCFAs: Short-chain fatty acids

GOS: Galacto-Oligosaccharides

Declarations

Ethics approval

All animal procedures has been approved by the Ethic committee (APAFIS#5833-2016062416362298v3) and authorised by the French Government.

Consent for publication

Not applicable

Availability of data and material

Sequencing data analysed during the current study are available in the NCBI Sequence Read Archive (SRA) database under the Bioproject accession number PRJNA649900. All data generated and analysed

during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

Funding

Joint program of Instituts Carnot Santé Animale (ICSA) and Pasteur Maladies Infectieuses (PMI). The PhD of Anais Cazals is funded by Région Ile de France (50%) and the Animal Genetics Division of INRAE (50%).

Authors' contributions

AC analyzed the data and wrote the manuscript. FC, BB and PV conceived the study. NB, JLC, MNR, DJ and CB performed laboratory assays. PM, NB, FC, JLC performed animal experiments and collected samples. JE and AR supervised bioinformatics and biostatistics analysis. FC, JE, AR, BB and PV contributed and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank our colleagues from the experimental unit PEAT who respectively raised the parents of the animals studied and furnished the chicks used for our study. We also thank colleagues from the experimental unit PFIE, who efficiently monitored the experiments and collected the samples and colleagues from the SPVB research team, who helped collect the samples.

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Figures

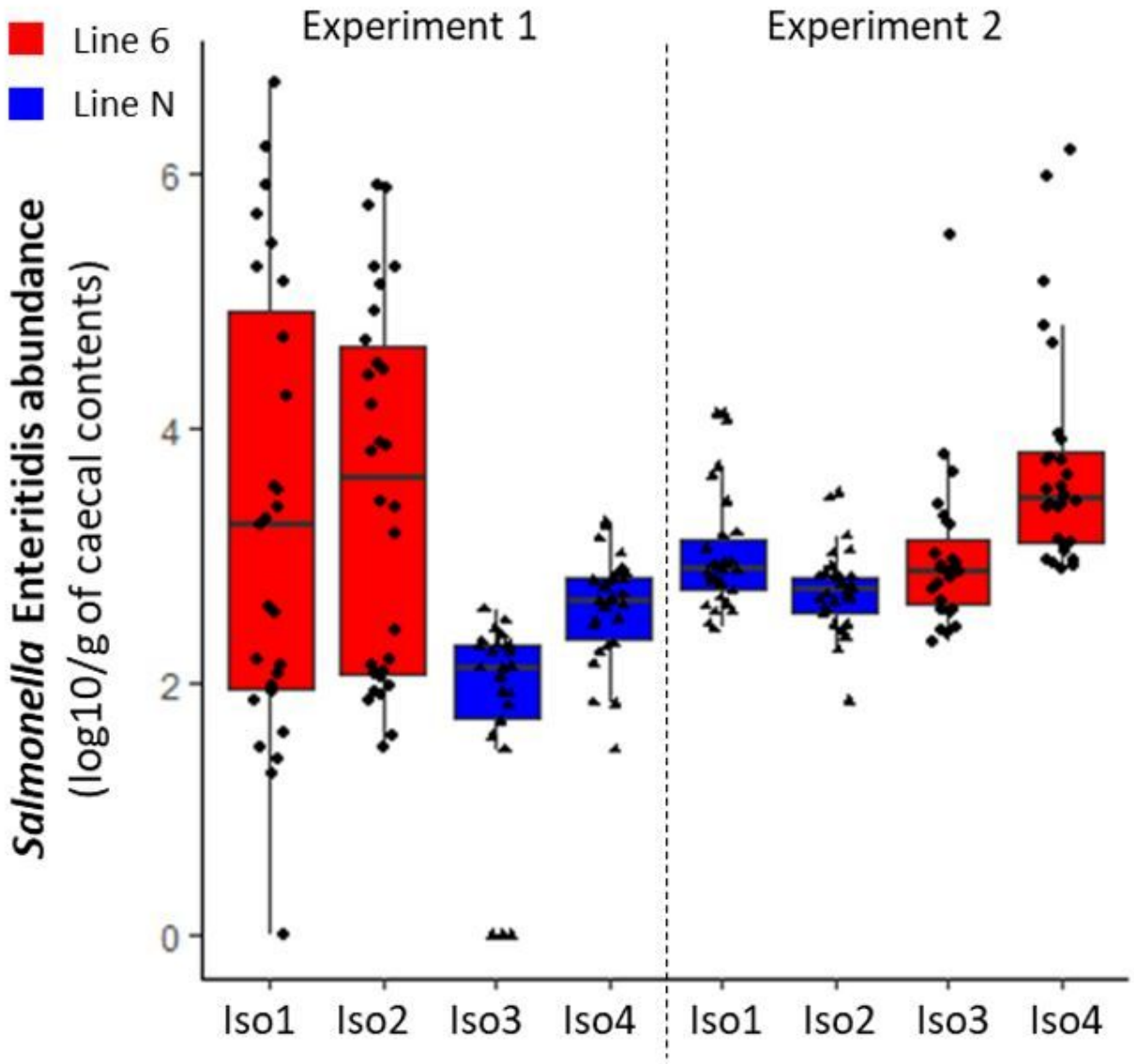

Figure 1

Salmonella Enteritidis abundance at 12 dpi in caecal contents. Salmonella Enteritidis abundance at 12 dpi in caecal contents of chickens from lines N and 61 infected with S. Enteritidis (log10/g of caecal contents) according to the experiment and the isolator. We observe a significant difference of Salmonella carriage between lines and between experiments. In line 61, the carriage is more variable between chicks, particularly for the experiment 1.

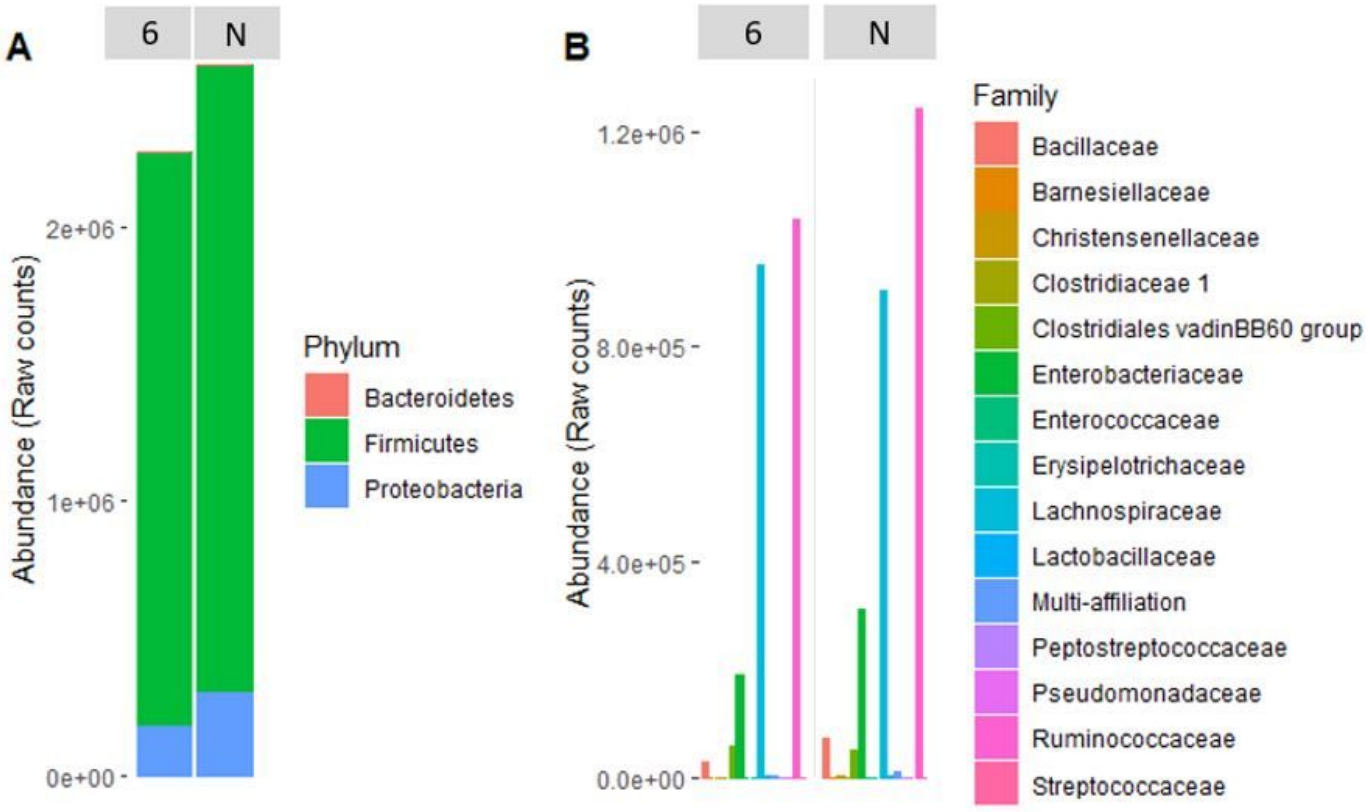

Figure 2

Microbiota composition at phylum and family levels for lines N and 61. Microbiota composition using raw counts at phylum level (A) and family level (B) for lines N and line 61. The most abundant phylum is Firmicutes followed by Proteobacteria and Bacteroidetes. We observed no significant difference at this phylum level between lines. At the family level, we observed first, Ruminococcaceae and Lactobacillaceae, followed by Enterobacteriaceae, Clostridiales and Bacillaceae.

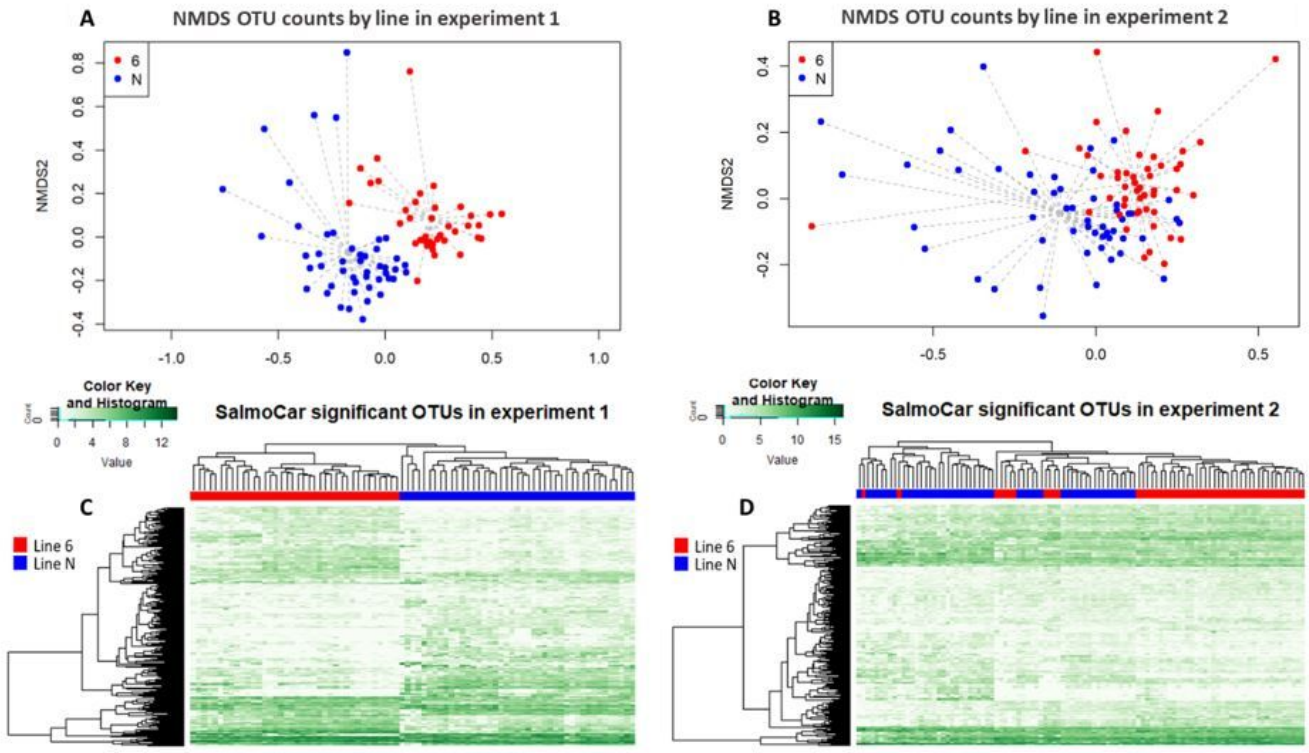

Figure 3

Non-metric multidimensional scaling representations and heatmaps comparing lines N and 61 for caecal microbiota composition. Non-metric multidimensional scaling (NMDS) representations of the OTU composition of caecal contents using the Bray-Curtis distance. These figures compare the composition between samples from lines N and 61 in experiment 1 (A) and experiment 2 (B). We can observe a significant difference of microbiota composition between lines after correcting for the isolator and sex (Permanova, P<0.001). Heatmaps of the caecal DA OTUs between lines N (in blue) and 61 (in red) in experiment 1 (C) and in experiment 2 (D).

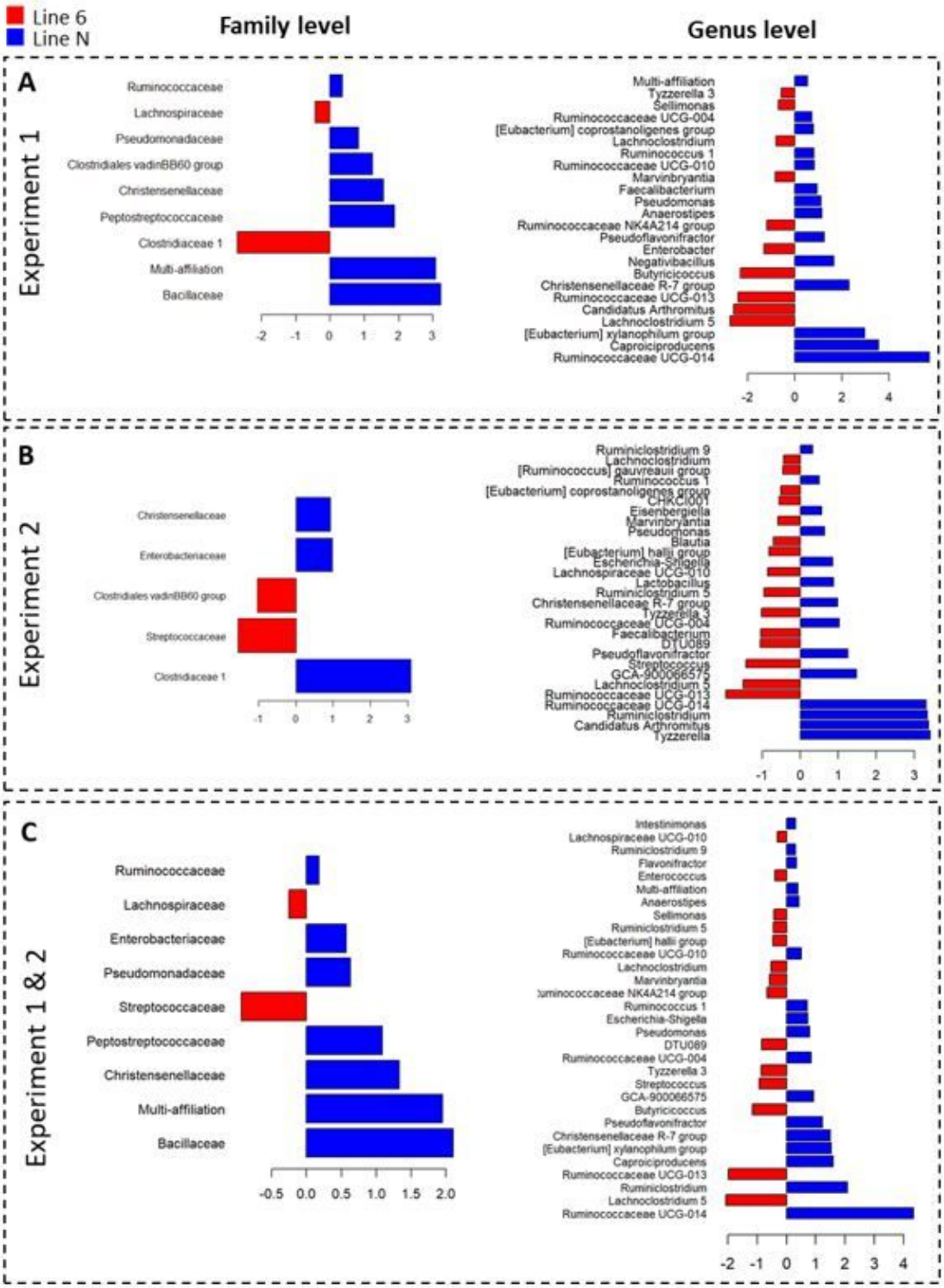

Figure 4

Differentially abundant families and genera between lines N and 61 according to the experiment. DA families and genera between lines N and 61 for experiment 1 (A), experiment 2 (B) and experiments 1 and 2 together (C). Representation of the log2 fold-changes for the comparison between lines (blue indicates greater abundance in line N, red greater abundance in line 61).

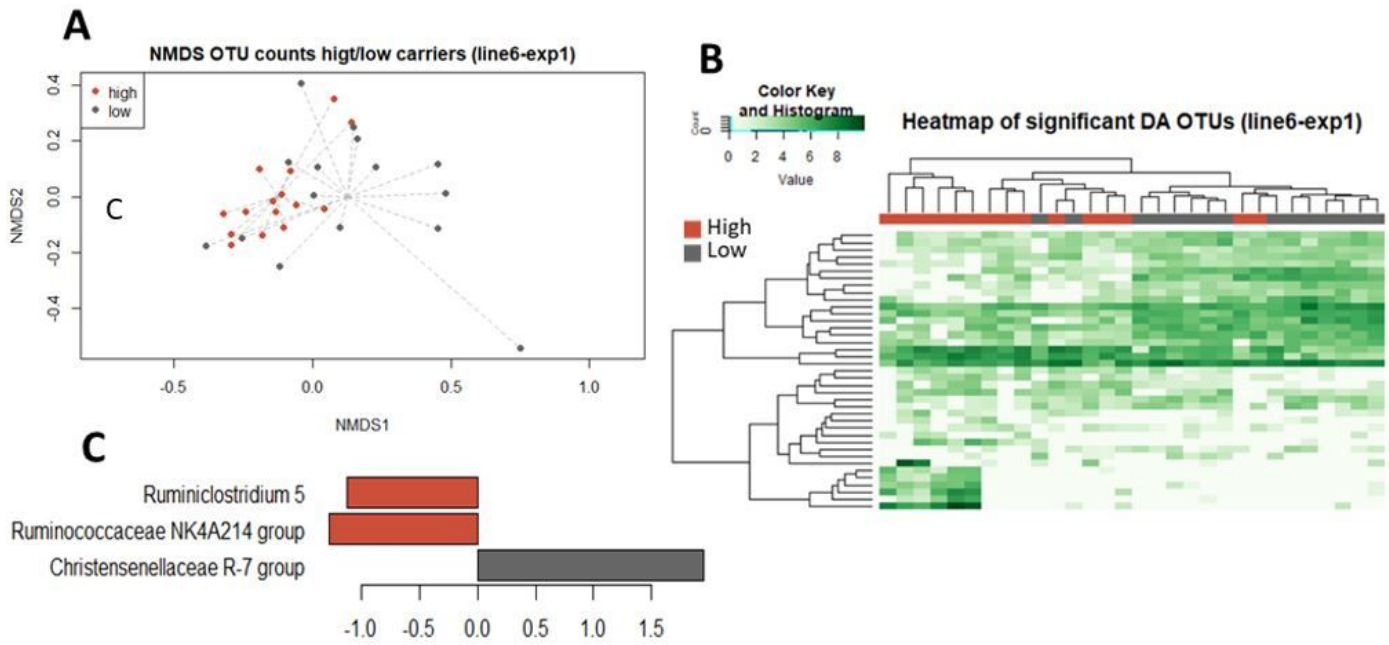

Figure 5

NMDS representation, heatmap and significant DA genera for high and low carriers (line 61- exp1) (A) NMDS representation of the caecal microbiota of high and low carriers in line 61 (Bray-Curtis distance). High and low carriers are significantly different (Permanova, P-value < 0.01); caecal microbiota from different isolators are not significantly different (Permanova, P-value > 0.5). (B) Heatmap representation of the 39 DA OTUs between high- and low-carriers in line 61 for experiment 1. (C) The significant DA genera between high and low carriers for line 61 in experiment 1 (Log2 Fold-Changes).

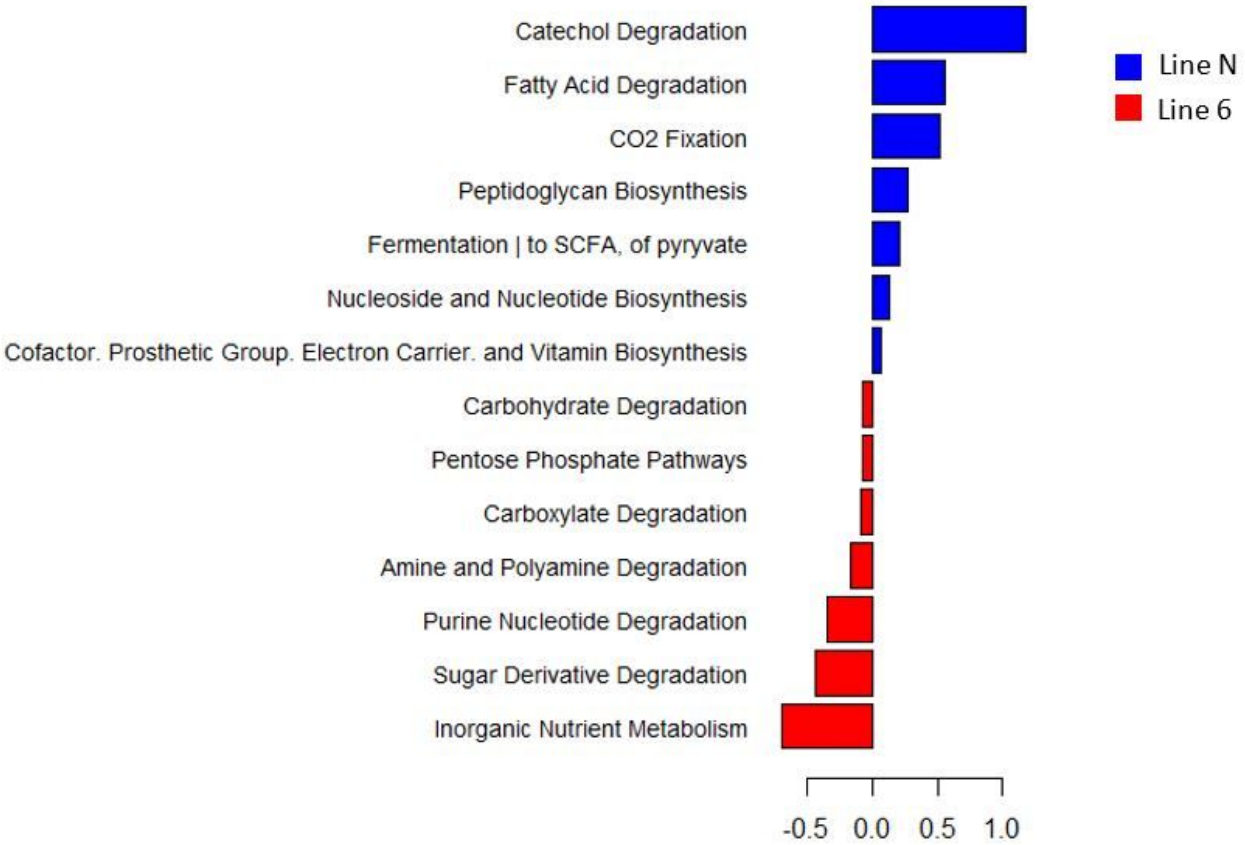

Figure 6

DA super-pathways in common between lines N and 61 and high/low carriers in line 61. DA super-pathway (MetaCyc database – DESeq2) in common for the comparisons of caecal microbiota between lines N and 61 and between high and low carriers in line 61. Representation of the log2 fold-changes for the comparison between lines (blue indicates greater abundance in line N, red greater abundance in line 61).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfiles.docx
- additionalfile5.xlsx
- additionalfile4.txt
- additionalfile3.txt
- additionalfile2.txt
- additionalfile1.txt