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RESEARCH ARTICLE

Effects of gut microbiota transfer on emotional reactivity in Japanese quails (*Coturnix japonica*)

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ABSTRACT

The interaction between the gut microbiota (GM) and the brain has led to the concept of the microbiota–gut–brain axis but data for birds remain scarce. We tested the hypothesis that colonization of germ-free chicks from a quail line selected for a high emotional reactivity (E+) with GM from a line with low emotional reactivity (E−) would reduce their emotional behaviour in comparison with germ-free chicks from an E+ line colonized with GM from the same E+ line. The GM composition analysis of both groups revealed a shift in terms of microbial diversity and richness between day 21 and day 35 and the GM of the two groups of quails were closer to each other at day 35 than at day 21 at a phylum level. Quails that received GM from the E− line expressed a lower emotional reactivity than quails colonized by GM from the E+ line in tonic immobility and novel environment tests carried out during the second week of age. This result was reversed in a second tonic immobility test and an open-field run 2 weeks later. These behavioural and GM modifications over time could be the consequence of the resilience of the GM to recover the equilibrium present in the E+ host, which is in part driven by the host genotype. This study shows for the first time that a GM transfer can influence emotional reactivity in Japanese quails, supporting the existence of a microbiota–gut–brain axis in this species of bird.

KEY WORDS: Microbiota–gut–brain axis, Emotion, Genetics, Bird

INTRODUCTION

Studies on the role of the gut microbiota (GM) in many aspects of physiology, including immunity, digestion and metabolism (Greer and O'Keefe, 2011; Kamada et al., 2013; O'Hara and Shanahan, 2006), and particularly in brain development (Grenham et al., 2011; Sampson and Mazmanian, 2015) are increasing. Indeed, it has recently been shown that microorganisms hosted by the gut could influence many brain functions and consequently the behaviour responses. For example, anxiety-like behaviour and hypothalamic–pituitary–adrenal (HPA) axis activity are impaired in germ-free rodents (Sudo et al., 2004; Campos et al., 2016; Crumeyrolle-Arias et al., 2014). This has led to the concept of a microbiota–gut–brain axis (Cryan and Dinan, 2012; Mayer et al., 2015). Treatment with probiotics (microorganisms that have beneficial effects for the host when administered in adequate amounts) have anxiolytic effects on rodents and humans (Foster and McVey Neufeld, 2013; Messaoudi et al., 2011). Bercik et al. (2011) showed that a GM transfer between BALB/c mice (strain genetically prone to exacerbate anxiety) and NIH Swiss mice (strain genetically prone to moderate anxiety) allowed modification of the behavioural phenotypes of these mouse strains: the GM transfer induced reduced anxiety in BALB/c mice in a step-down test while the NIH Swiss mice expressed increased anxiety. In addition, the contribution of the GM in several human diseases such as autism, Alzheimer's disease or Parkinson's disease is increasingly recognized (Collins et al., 2012; Sherwin et al., 2017). However, evidence supporting the concept of the microbiota–gut–brain axis in birds is lacking and only a few authors have investigated the effects of GM on behaviour in this animal model. Azeem (2013) showed that the administration of the probiotic *Bacillus amyloliquefaciens* helped to reduce distress calls and agonistic behaviour in turkeys. More recently, a decrease in emotional reactivity in a tonic immobility test and memory improvement have been described by Parois et al. (2017) following continuous supplementation with the probiotic *Pediococcus acidilactici* in Japanese quails.

Because of its precocial character, the Japanese quail (*Coturnix japonica*, Temminck & Schlegel 1849) is a model particularly adapted to this type of study because it has the capacity to live without its mother in early life, thus limiting the influence of maternal microbiota. Furthermore, germ-free quails showed a reduction in emotional reactivity in various situations of fear and social perturbation (Kraimi et al., 2018). All these results suggest that the GM are able to act on emotional behaviour in some birds, as previously observed in rodents. In order to strengthen this idea, we performed a microbiota transfer experiment to determine the influence of GM in this species. A quail line (E+) genetically selected for its high emotional reactivity level, characterized by a long tonic immobility duration, was colonized with the GM from a quail line selected for its low emotional reactivity level (E−), characterized by a short tonic immobility duration. Emotional reactivity is characterized by behavioural and physiological responses to a challenging situation (Boissy, 1995). Tonic immobility is an innate behaviour of widespread passenger motor inhibition in animals and can be induced in birds by a brief restraint (Gallup, 1979). It has been shown that the duration of maintenance of this tonic immobility behaviour is positively correlated with the fear of the bird in other tests such as open-field or emergence tests. Furthermore, frightening stimuli presented before the induction of tonic immobility (noise, placement in an unfamiliar environment, etc.) extend the tonic immobility duration of the individual (Jones, 1986; Mills and Faure, 1991). We thus hypothesized that colonization with the GM of the E− line would reduce the emotional reactivity of the E+ line.

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MATERIALS AND METHODS

All animal care procedures were carried out in accordance with the guidelines set by the European Communities Council Directive (Directive 2010/63/UE) and with French legislation on animal research. The protocol was 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 protocol no. 201707131037724.V2-10607. The principles of reduction, replacement and refinement were implemented in all experiments.

Egg disinfection

Fertilized eggs were obtained from a line genetically selected for its high level of emotional reactivity, characterized by a long tonic immobility duration (E+ line) (Mills and Faure, 1991). Birds were housed at the avian experimental unit of INRA, Tours (UE PEAT, INRA, Nouzilly, France). Eggs from females of the 62nd generation were collected every 90 min and disinfected with Divosan following the procedure described previously (Kraimi et al., 2018) in the facilities of the Plate-Forme d'Infectiologie Expérimentale (PFIE, UE-1277, INRA Centre Val de Loire, Nouzilly, France).

Animals and housing

After disinfection, the eggs were placed in sterile isolators until hatching. Control for germ-free status was performed through aerobic and anaerobic culture of freshly voided faecal samples (in resazurin thioglycolate, serum-enriched BHI and blood agar and incubated at 35 and 25°C). The day following hatching (day 1), we transferred 36 chicks into two large sterile isolators of identical dimensions and organization (Fig. 1A) in the same room (18 chicks per isolator and treatment, both sexes included because sexual dimorphism is not distinguishable at this age). Quails were wing-banded for identification. Autoclaved water and γ-irradiated (45 kGy, Scientific Animal Food and Engineering, Augy, France) feed pellets for chicks (metabolizable energy: 12.2 MJ; crude protein: 204 g kg^{-1}) were provided *ad libitum*. The temperature of the isolators was maintained at 40–38°C for the first 3 days and was progressively reduced to 20°C. The light cycle was 24 h per day until day 4, when it was gradually reduced by 1 h of light per day until reaching a minimum of 10 h of light per day. We provided enrichment of the chick's living environment in the isolators by including wood shaving dust baths and by placing previously sterilized new objects (glass or plastic balls) in the isolators on successive days.

Bacterial inoculation

The donor quails were female adults (13 weeks old and from the 62nd generation) from E+ and E− lines (see below) and were killed by occipital sinus pentobarbital injection (0.5 ml). The caecal collection was carried out immediately after death in a disinfected room with autoclaved tools and close to a Bunsen burner. The pair of caeca were opened and their contents gently removed to avoid including the mucosa. The caecal contents were then mixed aerobically in a solution of 500 µl of sterile glycerol+cysteine and frozen at −80°C. On the day of inoculation (day 2), the inocula were thawed and diluted in 10 ml of sterile physiological saline. The chicks were colonized with GM from a conventional adult of their E+ line in one isolator (18 chicks, group M+) or from a conventional adult of the opposite line with a low emotionality trait (E−) in the other isolator (18 chicks, group M−). GM transfers into quails were performed in the isolators by a trained experimenter and consisted of an oral gavage with 100 µl of the diluted bacterial solution. Quails were then closely monitored for 24 h after the inoculation step.

Microbiota composition analysis

Sample collection and microbial DNA extraction

Caecal contents and faeces used for comparison of the two lines were collected before the experiment from n=11, 13 week old E+ female quails and n=11, 13 week old E− female quails at the avian experimental unit (UE PEAT) in a disinfected room with

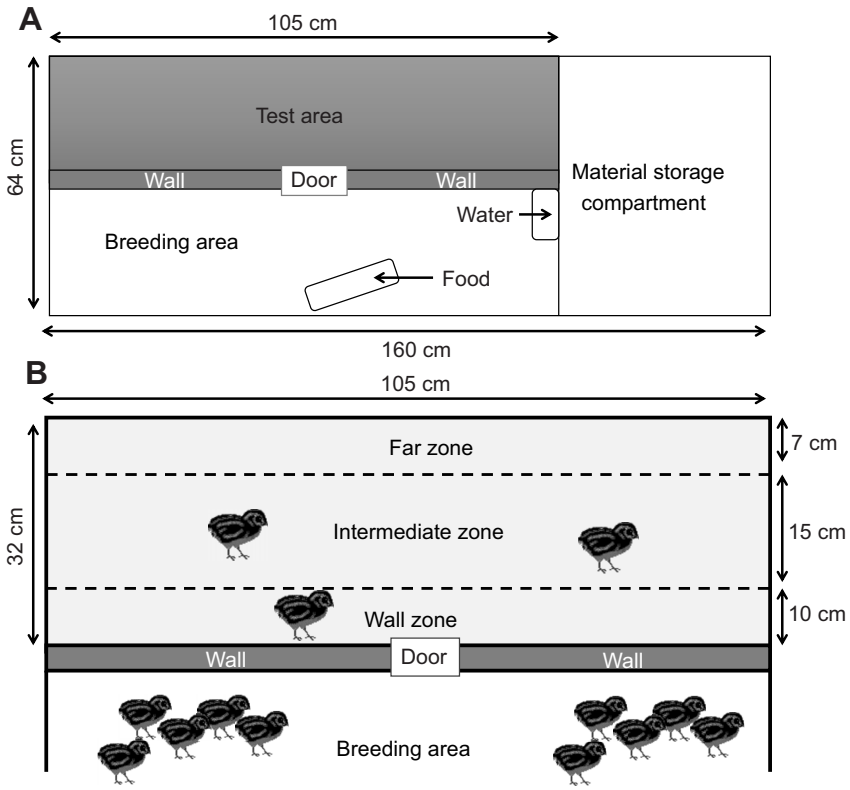

Fig. 1. Experimental set up. (A) Different areas of the isolators (160×64 cm). The test area was separated from the breeding area, which contained food and water. (B) Different zones used for the novel environment test (the wall zone, the intermediate zone and the far zone). The wall zone represents proximity with the other quails.

autoclaved tools and close to a Bunsen burner (resulting in 22 faecal samples and 22 caecal samples in total). The E+ and E− donors used for the inoculation were part of these two groups of 11 females. Birds were killed by occipital sinus pentobarbital injection (0.5 ml) and the pair of caeca were opened and their contents gently removed to avoid including the mucosa.

Faeces from quails of the two experimental groups M+ and M− were collected individually inside the isolators on day 21 by placing the quail in a box containing a γ-irradiated (45 kGy, Scientific Animal Food and Engineering) plastic sheet at the bottom. The plastic sheet was changed between each quail, which allowed individual samples to be obtained. At day 35, faeces were collected before necropsy in sterile conditions under a microbiological safety workbench with autoclaved tools. Quails were killed by occipital sinus pentobarbital injection (0.5 ml).

Caecal contents and faeces collected were frozen at −80°C before DNA extraction. Microbial DNA extraction was carried out using the QIAamp DNA mini-kit (ref. 51306, Qiagen Inc., Courtaboeuf, France), according to the procedure previously described (Mignon-Grasteau et al., 2015). In brief, 25 mg of thawed digestive contents were mixed in 1 ml of lysis buffer and homogenized at maximum speed (frequency 30 s^{-1}) with 0.4 g of sterile zirconium beads in a tissuelyser mixer (Retsch MM400, Haan, Germany) for 3 min, followed by heating at 70°C for 15 min. After centrifugation at 16,000 ***g*** and 4°C for 5 min, the supernatant was conserved at ambient temperature and 300 µl lysis buffer was added. The homogenization steps were repeated on the pellet and followed by a second centrifugation (5 min, 16,000 ***g***, 4°C). The two supernatants were pooled and homogenized for the DNA purification and filtration step. Proteinase K and AL buffer (Qiagen Inc.) were added to the supernatants and the mix was heated at 70°C for 10 min to remove proteins. The sample was transferred to a tube containing pure ethanol for the purification step using a QIAamp column as described by the manufacturer. The sample was then eluted in buffer AE (10 mmol l^{-1} Tris-Cl, 0.5 mmol l^{-1} EDTA, pH 9.0; Qiagen Inc.). Measurement of microbial DNA purity was performed with a NanoDrop spectrophotometer based on the 260/280 nm and 260/230 nm OD ratios.

Amplification of 16S rRNA gene by PCR

PCR amplification of the bacterial 16S rRNA gene on DNA extracts was performed using primers designed to amplify from the highly conserved sequences of the V4–V5 regions (forward: CTTTCCC-TACACGACGCTCTTCCGATCTGTGYCAGCMGCCGCGGTA; reverse: GGAGTTCAGACGTGTGCTCTTCCGATCTCCCCGY-CAATTCMTTTRAGT). PCR reactions were run in 96-well plates in a final volume of 50 µl (made up with H_2O) containing 5 µl 10× PCR buffer, 4 µl dNTP mix (2.5 mmol l^{-1}), 0.5 µl Taq DNA polymerase (5 U $µl^{-1}$), 1 µl of each primer (20 µmol l^{-1}) and 10 ng DNA. The PCR program used consisted of an initial denaturation step at 94°C for 2 min followed by 30 cycles at 94°C for 60 s, 65°C for 40 s, 72°C for 30 s and a final extension step of 72°C for 10 min. PCR product size was checked by 2% agarose gel electrophoresis before the sequencing step.

16S rRNA sequencing

V4–V5 region full-length reads were obtained using Illumina Miseq 250 bp paired end reads. The resulting PCR products were purified and loaded onto the Illumina MiSeq cartridge according to the manufacturer's recommendations. The quality of the run was checked internally using the PhiX control (following the manufacturer's instructions) and, with the help of the previously integrated index, each pair-end sequence was assigned to its sample. Each pair-end sequence was assembled using Flash software (Magoč and Salzberg, 2011) with at least a 10 bp overlap between the forward and reverse sequences, which allowed 10% mismatch (Lluch et al., 2015). The absence of contamination was verified with a negative (no-template) control during the PCR. Four bacterial samples that are run routinely in the sequencing facility were used in parallel with the current samples to control for the quality of the stitching procedure. The resulting DNA sequences for the 16S rRNA were clustered in operational taxonomic units (OTU) with Usearch v8.1.1861 (Edgar, 2010) using the Uparse pipeline (Edgar, 2013) to create a table of abundance. The sequences can be accessed from the NCBI Sequence Read Archive (SRA) under project number PRJNA523994. Taxonomic affiliation was performed on the RDP Train Set 15 with a confidence score threshold of 80%.

Growth

The quails were weighed individually in the isolators on day 7, 14, 21 and 33, using a system connected to an external balance.

Behavioural tests

The isolators were separated into two equal parts using an opaque separating wall. One half, containing the feed and water, was dedicated to breeding while the other was specifically used for the behavioural tests (Fig. 1A).

Behavioural tests were always carried out by the same experimenter with sterile gloves and equipment and they were recorded with a camera fixed above each isolator.

Novel environment test

Inspired by the open-field test, we measured the behavioural reactions of the quails when they were placed in the test area of the isolator for the first time. On day 12, the quails were introduced for the first time into the test area in groups of three (to limit the social isolation component) for a period of 5 min and we measured the time spent and the number of entries and displacements in each of the following zones (Fig. 1B) for each individual with Observer XT (version 12.5) software: (1) the 'wall' zone, close to the wall separating congeners; (2) the 'far' zone, far from the wall separating congeners; and (3) the 'intermediate' zone, between the wall and far zones. Each quail was initially placed in the wall zone and visits to the intermediate and far zones were considered to be exploratory behaviour related to low emotional reactivity.

Tonic immobility test

The standard tonic immobility test (Gallup, 1979; Jones, 1986; Mills and Faure, 1991) was performed on day 15 and day 29 to assess the emotional reactivity of quails. The quail was placed on its back in a U-shaped plastic cradle in the test area of the isolator and restrained for 10 s (with one hand on the sternum and the other lightly cupping the head of the bird). The experimenter remained virtually motionless and silent. If the bird took more than 10 s before it righted itself, the duration of tonic immobility was recorded. After five inductions without tonic immobility, a duration value of 0 s was noted. In contrast, if the quail did not rise after 10 min, the test was stopped and a maximum value of 600 s was recorded for tonic immobility duration. The parameters measured during the test were: the number of induction attempts and the duration of tonic immobility. Individuals with a low number of inductions and a long duration of tonic immobility were considered to be very emotional. A tonic immobility index was also calculated after the test with the following formula: index=(6−number of inductions)×tonic immobility duration. This index gives higher

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weighting to tonic immobility induced easily and lower weighting to tonic immobility requiring many inductions.

Open-field test

On day 33, the quails were subjected to an open-field test outside the isolators to assess emotional reactivity to a novel environment. As the test device was too large to be placed in an isolator, the test was performed in a clean and sterilized room. Each quail was removed from the isolator, alternating between treatments, and carried individually in a transport box where it remained for 5 min in order to let it calm down and limit fear reactions associated with removal from the isolator. The quail was then placed in the centre of a square arena (80 cm×80 cm×29 cm) made of wood with a floor made of yellow waterproof plastic under 50 lx light conditions and allowed to freely explore the test arena for 3 min. A camera was fixed directly above the arena and, using Ethovision XT tracking software, we recorded the locomotor activity (total distance travelled), and the time spent in the centre zone, intermediate zone and peripheral zone of the open field. All these components made it possible to reveal freezing behaviour and inactivity associated with increased emotional reactivity in an unfamiliar place, i.e. the open field, in quails and chicks (Calandreau et al., 2011; Jones et al., 1991, 1992, 1995). At the end of each test session, the quail was returned to its isolator using a transport box and the test arena was cleaned with disinfectant.

Statistical analysis

For the microbiota data, the Shannon index, Simpson diversity index and Chao1 richness estimator were calculated on the rarefied OTU table (McMurdie and Holmes, 2013) using the vegan package (https://cran.r-project.org/package=vegan). For microbiota samples collected in females before the experiment, statistical differences in diversity were analysed using an ANOVA with line (E+ or E−) and gut source (caecal contents or faeces) as fixed effects. For faecal microbiota samples collected in colonized quails during the experiment, group (M+ and M−) and sex were used as fixed effects. In addition, the relative abundance of bacterial phyla, order and families was compared with Wilcoxon–Mann–Whitney tests for differences between groups and Wilcoxon signed-rank tests for differences between ages. To identify the OTUs that were different between the two lines, a Wilcoxon–Mann–Whitney test with a Benjamini–Hochberg procedure correction was performed on the core OTUs (that were present in all the samples in each group).

For behavioural data, generalized linear mixed models (GLMM, package 'lme4') were used, with group (M+ or M−), sex and the interaction between sex and group as fixed effects and with the order in which the quails were tested as the random effect. We used GLMM with different rules than normal when needed: a GLMM with Gamma errors was used for the total distance and the time spent in the various zones during the novel environment test and the open-field test, and a GLMM with Poisson errors was used to compare the number of inductions in the tonic immobility test, and the number of displacements and entries in the different zones in the novel environment test. In the novel environment test, where three quails were tested together, the trio number was used as the random effect.

Growth data were first log transformed and then tested using a generalized linear model with group as the main factor, age as the repetition factor and identification of each quail as the random factor.

All results are presented as means±s.e.m. The significance level was set at $P \leq 0.05$ and $0.05 < P < 0.10$ was considered as a trend. All statistical analyses were performed with RStudio software (version 1.1.453).

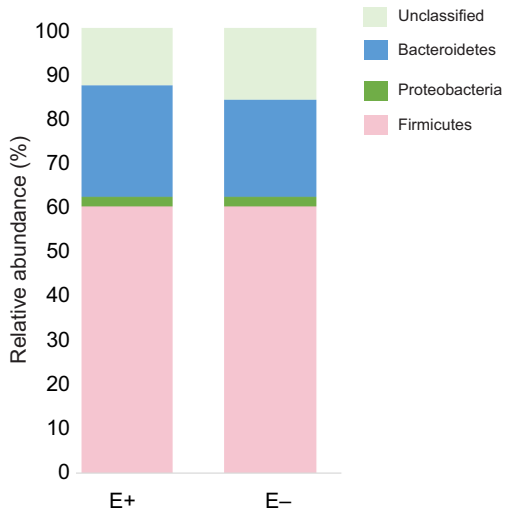

Fig. 2. Relative abundance of major bacterial phyla in the caecal contents of E+ and E− quails. Phyla with an abundance <1% are not shown.

RESULTS

Comparison of GM between E+ and E− lines

The richness estimator Chao1, and the Shannon and the Simpson indexes all revealed a higher diversity in the caecal contents than in the faecal samples (P=0.04, P<0.001, P<0.001, respectively). No line effect was found for any of these indexes (P=0.78, P=0.75, P=0.64, respectively). No differences in the relative abundance of major bacterial phyla in the caecal contents were identified between the two quail lines (Fig. 2). Regarding the OTUs present in all caecal contents, three were found to be significantly different and six tended to be different between the E+ and E− line (Table 1).

Effects of GM transfer on growth

The growth data revealed an increase in mass with age of the quails (P<0.0001) but no differences between M+ and M− groups ($F_{1,118}$=0.005, P=0.94).

Effects of GM transfer on emotional behaviour

The sex effect and the interaction with the group were not significant for any of the behavioural test measures (P>0.1).

Compared with M+ group, quails that received GM of the E− line made fewer entries (2.06±0.6 versus 4.58±1.5, χ^2=5.06, P=0.024) and

Table 1. Relative abundance and taxonomy of operational taxonomic units (OTUs) that differ between the caecal contents of E+ and E− quails

		Abundance (%)		
OTU ID	Classification	E+	E−	*P*-value
OTU22	*Gemmiger*	1.54±0.36	0.33±0.09	<0.01
OTU70	*Clostridium* cluster XIVa	0.46±0.09	0.18±0.04	<0.01
OTU304	*Anaerofilum*	0.22±0.05	0.04±0.01	0.01
OTU20	*Clostridium* cluster IV	1.19±0.30	0.50±0.13	0.06
OTU33	*Coprobacillus*	0.74±0.19	0.38±0.05	0.06
OTU5804	*Coprococcus*	0.43±0.09	0.18±0.04	0.06
OTU42	Erysipelotrichaceae *incertae sedi*	0.34±0.09	0.16±0.03	0.06
OTU62	*Clostridium* cluster XIVa	0.28±0.05	0.14±0.03	0.06
OTU113	*Clostridium* cluster XIVa	0.14±0.02	0.07±0.02	0.06

The results are expressed as means±s.e.m. (n=11 E+, n=11 E−). OTUs with P-values <0.10 are shown.

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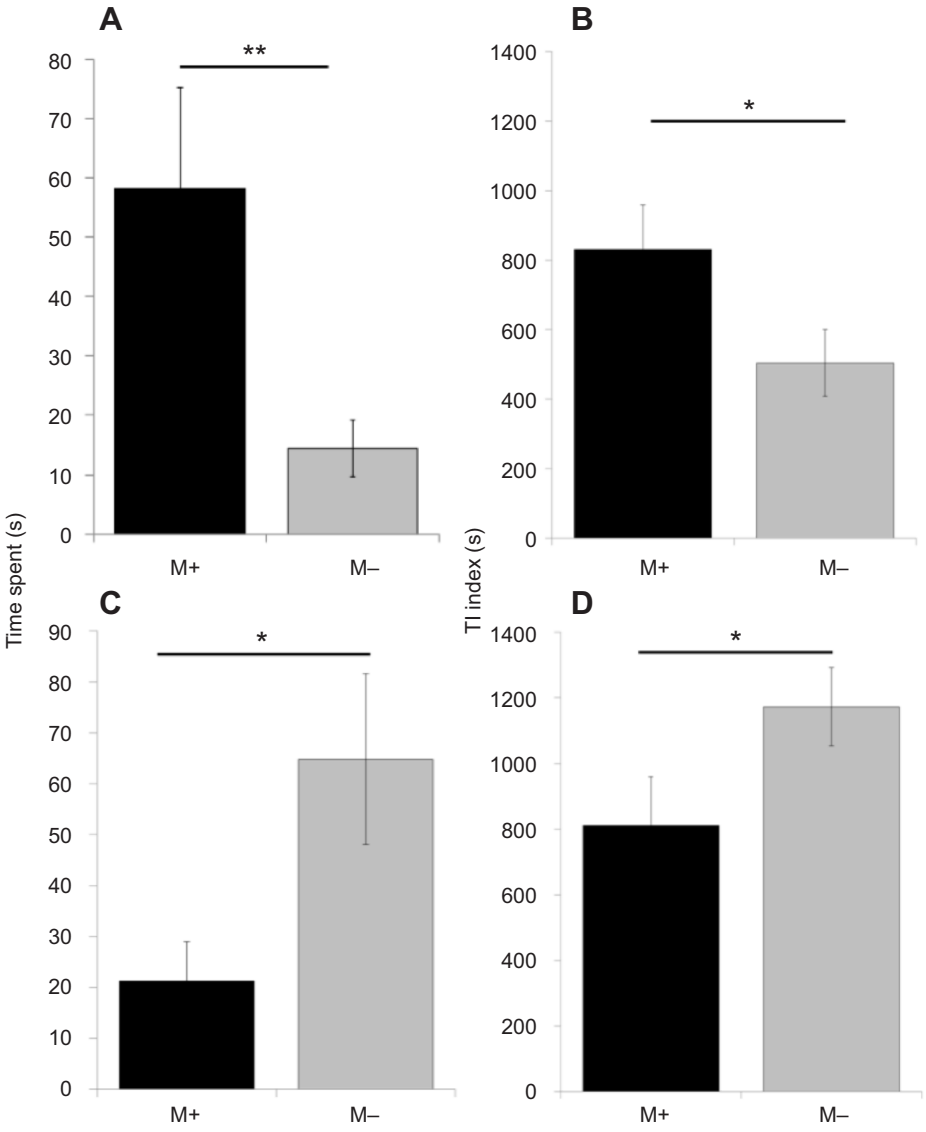

Fig. 3. Responses of M+ and M– groups to behavioural tests. (A) Time spent in the wall zone during the novel environment test (day 12). (B,D) Tonic immobility (TI) index of the M+ and M– quails during the tonic immobility test performed on day 15 (B) and day 29 (D). (C) Time spent in the centre zone during the open-field test (day 33). The results are expressed as means±s.e.m. *$P \leq 0.05$, **$P \leq 0.01$.

spent less time in the wall zone (Fig. 3A), but more time in the far zone in the novel environment test (200.3±13.1 versus 94.6±20.7 s, $\chi^2=6.52$, $P=0.01$). No significant difference was found for the time spent in the intermediate zone between the M+ quails (96.8±9.4 s) and M– quails (68.5±10.5 s, $\chi^2=2.36$, $P=0.12$). Regarding the number of entries in the intermediate zone and far zone, no significant differences were revealed between the groups.

During the tonic immobility test carried out on day 15, quails of the M– group tended to require a higher number of inductions (1.8±0.3 versus 1±0.0, $\chi^2=3.02$, $P=0.08$) and tended to show a shorter duration of tonic immobility (111.8±21.1 versus 166.1±25.6 s, $\chi^2=2.78$, $P=0.09$) than the M+ group. In the same way, the tonic immobility index was significantly lower in the group M– (Fig. 3B).

In the second tonic immobility test, performed on day 29, the number of inductions did not differ between the M+ and M– groups (1.2±0.2 versus 1.3±0.1, $\chi^2=0.09$, $P=0.77$, respectively) but quails of the M– group showed a longer tonic immobility duration (235.4±23.2 versus 166.8±28.7 s, $\chi^2=4.16$, $P=0.04$) and greater tonic immobility index than the M+ group (Fig. 3D).

In the open-field test, compared with the M+ quails, the M– quails spent significantly less time at the periphery (5.4±3.9 versus 23.51±12.8 s, $\chi^2=5.69$, $P=0.02$) and more time in the centre of the open field (Fig. 3C). There was no difference between the M+ and M– groups for the distance travelled (respectively, 384.6±97.1 versus 218.6±46.4 cm, $\chi^2=1.04$, $P=0.31$) in the test area.

Comparison of GM between M+ and M– groups

The sex effect and the interaction with the group were not significant for any of the microbiota measures ($P>0.1$).

At day 21, the M– group was more diverse than the M+ group (Chao1=301.27±12.1 versus 251.75±22.2, $P=0.04$; Shannon=3.50±0.1 versus 2.93±0.2, $P=0.01$; Simpson=0.89±0.02 versus 0.82±0.03, $P=0.02$, respectively). In contrast, at day 35, the Shannon and Simpson diversity indexes for faeces of the M– group were significantly lower than those for the M+ group (3.67±0.1 versus 4.09±0.1, $P=0.01$; 0.92±0.01 versus 0.95±0.01, $P=0.03$, respectively), while no difference was found between the two groups for the Chao1 richness estimator (300.30±14.01 versus 330.95±14.36, $P=0.16$). Regarding the relative abundance of major bacterial phyla, significant differences were revealed between M+ and M– quails at day 21 (Fig. 4A) but these differences were not found at day 35 (Fig. 4B).

At a family level, significant differences were detected at day 21 (Fig. 5A) and day 35 (Fig. 5B) between M+ and M– groups. In both

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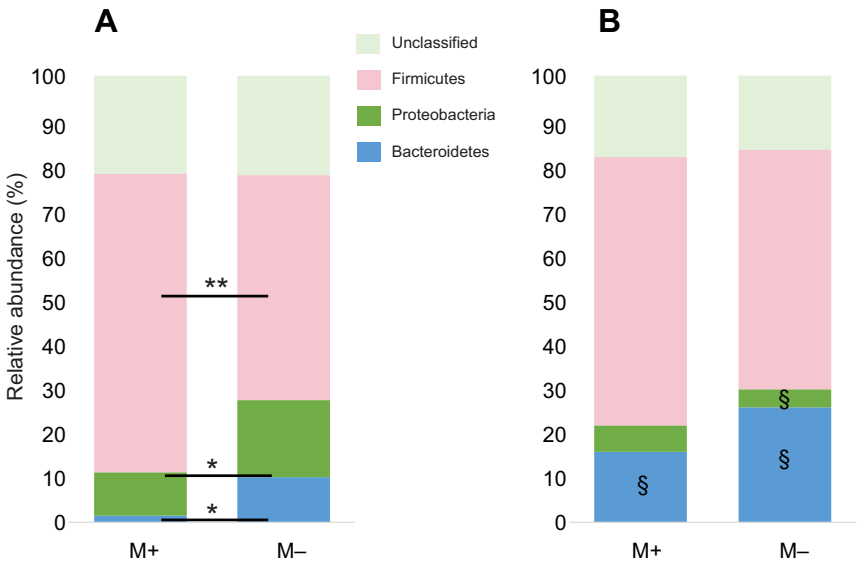

Fig. 4. Relative abundance of major bacterial phyla in the faeces of M+ and M– groups. (A) Day 21. (B) Day 35. Phyla with an abundance <1% are not shown. Differences between groups: *$P \leq 0.05$, **$P \leq 0.01$. Differences between day 21 and day 35: §$P \leq 0.05$.

groups, a significant modification of the GM composition with age was revealed at the phylum and family level (Figs 4 and 5).

As shown in Fig. 5, the proportion of unclassified bacteria was high at a family level. At day 21, these unclassified bacteria belonged to the phyla Bacteroidetes (0.52±0.13% in M+ group versus 3.49 ±1.38% in M– group, P=0.13), Proteobacteria (7.65±2.34% in M+ group versus 18.10±4.61% in M– group, P=0.19) and Firmicutes (21.86±3.41% in M+ group versus 33.01±4.46% in M– group, P=0.10). At day 35, the unclassified bacteria belonged to the phyla Bacteroidetes (4.38±1.06% in M+ group versus 5.82±1.57% in M– group, P=1.00), Proteobacteria (3.74±0.94% in M+ group versus 2.56±1.44% in M– group, P=0.01) and Firmicutes (55.13±3.44% in M+ group versus 61.09±3.62% in M– group, P=0.33). In the M+ group, a significant increase in Bacteroidetes (P=0.003) and Firmicutes (P=0.001) unclassified bacteria was revealed between day 21 and day 35. Bacteria responsible for this increase belonged to the order Bacteroidales (0.16±0.08% at day 21 versus 2.87±0.87% at day 35, P=0.001), Clostridiales (7.28 ±2.04% at day 21 versus 28.79±3.62% at day 35, P=0.001) and Erysipelotrichales (0.02±0.01% at day 21 versus 0.37±0.28% at day 35, P=0.005). In the M– group, an increase was also observed between day 21 and day 35 for Firmicutes unclassified bacteria (P=0.003) but a decrease was revealed for Proteobacteria unclassified bacteria (P=0.005). Bacteria responsible for these effects belonged to the order Clostridiales (16.84±3.15% at day 21 versus 36.47±5.15% at day 35, P=0.012), Enterobacteriales (17.79±4.57% at day 21 versus 2.51±1.44% at day 35, P=0.007) and Rhizobiales (0.02±0.01% at day 21 versus 0.003±0.001% at day 35, P=0.005).

DISCUSSION

Differences in GM between the E+ and E– lines

It has been shown in Japanese quails that a greater richness and diversity in GM is found in the caecum in comparison with the other gastro-intestinal tract compartments (Wilkinson et al., 2016). This observation led us to use caecal microbiota for inoculation and is in accordance with our results showing higher values of Chao1, Shannon and Simpson indexes in caecal contents than in faeces of our quails.

The caecal contents of the E+ and E– lines did not differ in terms of microbial richness, diversity and phyla abundance but several

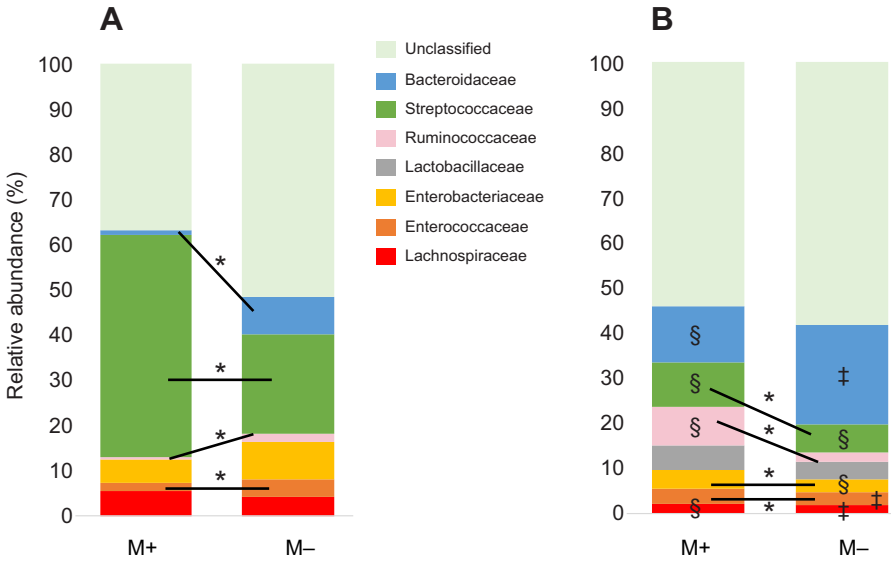

Fig. 5. Relative abundance of major bacterial families in the faeces of M+ and M– groups. (A) Day 21. (B) Day 35. Families with an abundance <1% are not shown. Differences between groups: *$P \leq 0.05$. Differences between day 21 and day 35: §$P \leq 0.05$, ‡$0.05 < P \leq 0.1$.

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differences in abundance were revealed at the OTU level: the OTUs that differed the most between the lines belonged to the Firmicutes phylum, the abundance of which is often altered by stress (Bailey et al., 2011; Pusceddu et al., 2015), anxiety (Bangsgaard Bendtsen et al., 2012) or depressive disorder (Jiang et al., 2015).

Colonization with E− GM does not influence the growth of quails

GM transfer did not affect quail growth in our study. The absence of an effect on the growth of quails is reassuring because it reinforces the idea that the behavioural data obtained are linked to the GM and not to disruption of body mass. It is also in line with results obtained with germ-free Japanese quails, which showed that body mass was not affected by this condition (Kraimi et al., 2018).

Colonization with E− GM reduces emotional reactivity in quails in early life

Quails colonized with the GM of the E− line made fewer entries and spent less time in the wall zone but more time in the far zone in the novel environment test, suggesting a reduced emotional reactivity in this group. Indeed, in this test, the quails are initially placed in the wall zone in front of the door and the most emotional individuals are too afraid to explore and spend time in this introduction zone, as is the case in the centre of an open-field test outside isolator (Calandreau et al., 2011; Jones et al., 1991). Furthermore, it can be assumed that the wall zone of the isolator represents a reassuring place, because of its proximity to the congeners. Conversely, less-emotional quails move and explore the entire test area, even the farthest zones.

During the first tonic immobility test carried out at 15 days of life, quails of the M− group tended to remain for a shorter time in tonic immobility and to require a greater number of inductions than the M+ group. We showed in a previous experiment that the absence of microbiota induced an even greater decrease in tonic immobility duration (Kraimi et al., 2018). Furthermore, the tonic immobility index, which combines these two parameters, giving more weight to easily induced tonic immobility and lower weight to tonic immobility requiring several inductions, was significantly lower in the M− group. This also reflects a decrease in emotional reactivity in these quails compared with those of the M+ group. Nevertheless, the values of tonic immobility remained high, which indicates that colonization with the GM of the E− line allowed modification of the behavioural phenotype of the E+ quails by decreasing their emotional reactivity but did not reverse it completely. A similar result was also found by Bercik et al. (2011) in a GM transfer between two strains of mice genetically prone to high or moderate anxiety-like behaviour. As these quails have been selected for several generations using this tonic immobility test, such a result enhanced by the behavioural responses in the novel environment test represents significant proof that colonization with a different GM can reduce the emotional reactivity of these birds, in accordance with our hypothesis.

Colonization with M− microbiota increases emotional reactivity in quails from day 29

Surprisingly, in the second tonic immobility test performed on day 29, quails of the M− group showed a greater tonic immobility duration and tonic immobility index than those of the M+ group. In contrast to the first tonic immobility test, colonization with the E− GM seems to have made the E+ quails more emotional than those colonized with the E+ microbiota. One could question whether this is a consequence of the repetition of this test but this is unlikely because of the long period between the two tests (14 days). In addition, the tonic immobility values for the M+ group were similar between the two tests. Only the emotional reactivity in the M− group was modified.

In the open-field test, we also observed a reversal of results in comparison with the novel environmental test conducted at the beginning of the experiment. Indeed, compared with the M+ group, the M− quails spent significantly less time in the periphery and more time in the centre of the open field, where they were initially introduced, which is characteristic of a high emotional reactivity in these quails (Calandreau et al., 2011; Jones et al., 1991).

Intriguingly, while colonization with E− GM reduced the emotional reactivity of E+ quails until day 15, according to our hypothesis, it then led to an increase in the emotional responses of these quails later in the experiment. This behavioural modification through time could be explained by GM modifications as described below.

GM differences between the M+ and M− groups

Microbial diversity and richness were greater in faeces of quails colonized with GM from the E− line at day 21 but not at day 35. At day 35, the M− group had lower microbial diversity than the M+ quails but a similar microbial richness. At a phylum level also, the GM composition evolved with time. Indeed, at 21 days of age the M− group had a greater abundance of Proteobacteria and Bacteroidetes but a lower abundance of Firmicutes; these differences were not found at day 35. It can be assumed that the host genetics plays a role in this GM modification over time. However, the effect of age could be also important in the resilience effect. We revealed an increase of the Bacteroidetes phylum between day 21 and day 35 in both groups but a decrease of the Proteobacteria phylum in the M− group. Regarding lower classification levels, we showed that the relative abundance of Bacteroidales (strictly anaerobic), Clostridiales (strictly anaerobic) and Erysipelotrichales (facultatively anaerobic) orders and Bacteroidaceae (strictly anaerobic), Ruminococcaceae (strictly anaerobic) and Lachnospiraceae (facultatively anaerobic) families increased between day 21 and day 35 in the M+ group while the Streptococcaceae (facultatively anaerobic) family decreased between these two periods. In the M− group, we found a decrease in the relative abundance of the orders Enterobacteriales (facultatively anaerobic) and Rhizobiales (facultatively anaerobic) and the families Streptococcaceae (facultatively anaerobic), Enterobacteriaceae (facultatively anaerobic) and Enterococcaceae (facultatively anaerobic) between day 21 and day 35 but an increase of the Clostridiales order (strictly anaerobic) and Bacteroidaceae (strictly anaerobic) and Lachnospiraceae (facultatively anaerobic) families between these two ages. These results supported the demonstration that anaerobic conditions increase with the age of the animal (Mancabelli et al., 2016; Qu et al., 2008; Roto et al., 2015). They also showed that GM evolves with time in both groups but this evolution is not the same in the two groups, probably as a result of host genetics (Ding et al., 2017).

The E+ and E− lines differed for several OTUs, indicating an impact of the genetic selection. Several studies have demonstrated an influence of the host genome on the composition of the GM. It appears that selective pressure could be imposed by the host to sort the microbiota it hosts and allow the development of some bacteria at the expense of others (Ley et al., 2006; Kurilshikov et al., 2017). This explains why different genetic lines fed the same diets and reared in the same conditions present differences in GM composition. For example, two lines of chickens genetically selected for digestive efficiency have different GM compositions (Mignon-Grasteau et al., 2017). Other studies have also highlighted

several heritable bacterial taxa (Goodrich et al., 2014) and associations between host single nucleotide polymorphisms and bacterial taxa (Bonder et al., 2016). It is therefore possible that E+ quails gradually modified the inoculated E− GM towards a microbial composition closer to that of the E+ line, as already demonstrated in another study of reciprocal microbiota transfer between mice and zebrafish (Rawls et al., 2006). Indeed, after GM transfer, mice and zebrafish reconstituted their original GM in terms of the relative abundance of microbial communities (Rawls et al., 2006). Thereby, the behavioural modification through time in the M− quails could be a consequence of the GM modification observed between day 21 and day 35. After a while, the inoculated E− GM would be modified to correspond to the appropriate E+ GM and this shift in GM composition would cause an increased in emotional reactivity in these quails.

Conclusion

In conclusion, as we have shown in a previous study with germ-free quails (Kraimi et al., 2018), the GM influences the emotional reactivity of Japanese quails. Indeed, in the present study, as we hypothesized, colonization from birth with GM from a line with low emotional reactivity reduced the emotional behaviour of quails from a line with high emotional reactivity. However, this effect did not seem to persist after 15 days of age and even seemed to be reversed. A change in the GM composition over time seems to be the cause of this behavioural modification but further research is needed to understand the physiological mechanisms involved in the microbiota–gut–brain axis in our model and identify the molecules liberated by microbiota that can modify the behaviour of Japanese quails. This GM transfer experiment confirms that the GM can influence the behaviour of Japanese quails, as demonstrated in rodents and humans, and that host genetics influences GM composition.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: N.K., L.C., O.Z., P.V., E.G., C.L.; Methodology: N.K., L.C., O.Z., K.G., C.D., P.V., E.G., S.L., C.P., C.L.; Software: N.K., L.C., O.Z., C.L.; Validation: N.K., P.V., C.L.; Formal analysis: N.K., O.Z., K.G., C.D., P.V., C.P., C.L.; Investigation: N.K., C.L.; Resources: N.K., C.L.; Data curation: N.K., O.Z., C.L.; Writing - original draft: N.K.; Writing - review & editing: N.K., L.C., O.Z., K.G., C.D., P.V., E.G., S.L., C.P., C.L.; Visualization: N.K., L.C., C.L.; Supervision: C.L.; Funding acquisition: C.L.

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Data availability

The 16S rRNA sequences can be accessed from the NCBI Sequence Read Archive (SRA) under project number PRJNA523994.

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