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ABSTRACT

Chronic stress and the gut microbiota appear to comprise a feed-forward loop, which contributes to the development of depressive disorders. Evidence suggests that memory can also be impaired by either chronic stress or microbiota imbalance. However, it remains to be established whether these could be a part of an integrated loop model and be responsible for memory impairments. To shed light on this, we used a two-pronged approach in Japanese quail: first stress-induced alterations in gut microbiota were characterized, then we tested whether this altered microbiota could affect brain and memory function when transferred to a germ-free host. The cecal microbiota of chronically stressed quails was found to be significantly different from that of unstressed individuals with lower α and β diversities and increased *Bacteroidetes* abundance largely represented by the *Alistipes* genus, a well-known stress target in rodents and humans. The transfer of this altered microbiota into germ-free quails decreased their spatial and cue-based memory abilities as previously demonstrated in the stressed donors. The recipients also displayed increased anxiety-like behavior, reduced basal plasma corticosterone levels and differential gene expression in the brain. Furthermore, cecal microbiota transfer from a chronically stressed individual was sufficient to mimic the adverse impact of chronic stress on memory in recipient hosts and this action may be related to the *Alistipes* genus. Our results provide evidence of a feed-forward loop system linking the microbiota-gut-brain axis to stress and memory function and suggest that maintaining a healthy microbiota could help alleviate memory impairments linked to chronic stress.

1. Introduction

An emerging body of literature recognizes the microbiota-gut-brain axis (MGBA) as a complex and bidirectional network of interactions between the gut microbiota and the brain impacting brain health and cognitive function (Cryan et al., 2019; Fröhlich et al., 2016). In the last decade, several human and animal studies have reported links between the gut microbiome and brain-related diseases like anxiety, depression or memory deficits by demonstrating major effects of gut microbiota manipulation on these disorders (Liu et al., 2020; Sherwin et al., 2017).

Whether modifications in microbiota composition impact brain function remains elusive although the involvement of immune (proinflammatory cytokines), neural (spinal and vagus nerves), metabolic (short-chain fatty acids), endocrine and neurotransmitter pathways have been suggested (Cryan et al., 2019). A recent study (Chevalier et al., 2020) provided mechanistic evidence suggesting that stress, diet

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and gut microbiota generate a pathological feedforward loop that contributes to depressive disorders via the central endocannabinoid system. However, such a loop has not been demonstrated for other disorders that can be induced by stress, for instance cognitive alterations. Indeed, chronic stress is known to induce dysbiosis in the gut characterized by changes in gastrointestinal motility and increased intestinal permeability leading to a "leaky gut" allowing bacteria and pathogens to cross the epithelial barrier.

Chronic stress has also well-known negative effects on memory (Gaelle et al., 2019; Sandi, 2013). Gut microbiota may contribute to these effects of chronic stress on memory. For example, Li et al. (2009) showed an improvement in spatial memory abilities, measured using the hole-board apparatus, in mice with dietary-induced shifts in bacteria diversity (Li et al., 2009). A high-fat diet also led to alterations in gut microbiota composition and memory impairments in mice subjected to the Morris water maze test or the fear conditioning test (Bruce-Keller et al., 2015; Jørgensen et al., 2014). In addition, comparisons between specific pathogen-free mice and germ-free mice significantly helped to highlight the link between the MGBA and memory. In 2018, Lu and his colleagues (Lu et al., 2018) showed significant deficits of memory in germ-free mice, which supports the important role of the microbiota in memory development. More recently an inoculation of germ-free mice with *Lactobacillus* species has also been suggested to improve short-term memory in the passive avoidance memory test (Mao et al., 2020). Indeed, many studies have provided evidence of the positive effects of *Lactobacillus* and *Bifidobacterium* probiotic supplementation on memory capacities in mice using the Y-maze and Barnes maze tests, object recognition test, or fear conditioning test (Bravo et al., 2011; Savignac et al., 2015; Yang et al., 2020) but also in human volunteers with several memory questionnaires (Bagga et al., 2018). Conversely, administration of antibiotics induces deleterious effects on memory as shown in mice subjected to the social transmission of food preference test and novel object recognition test (Desbonnet et al., 2015; Fröhlich et al., 2016).

Although the links between chronic stress and memory and gut microbiota and memory have been demonstrated, the question still remains as to whether or not chronic stress could induce memory impairments via gut microbiota changes alone. The aim of the present study was to provide evidence of a feed-forward loop system linking the MGBA to stress and memory function showing that a chronic stress state induces gut dysbiosis which in turn may affect the brain and memory function. Japanese quails were used because we have already shown that their anxiety-like behavior and memory properties are impacted by a chronic stress procedure (unpredictable repeated negative stimuli for 21 days) (Lormant et al., 2021, 2020) and gut microbiota manipulations (germ-free model, microbiota transfer and probiotic supplementation) (Kraimi et al., 2019, 2018; Parois et al., 2017). Moreover, Japanese quail have recently been suggested as a relevant model to study the involvement of gut microbiota in stress processes (Lyte et al., 2021) and are widely used in many areas of physiology, mainly for development investigation (Morris et al., 2020). Here, the approach of cecal microbiota transfer (CMT) was used involving the transfer of microbiota from a chronically-stressed individual to germ-free naive quails to investigate whether the CMT induced any negative consequences on quails' spatial and cue-based memory abilities as previously demonstrated in the stressed donors (Lormant et al., 2020). Additional analysis of plasma corticosterone levels, short-chain fatty acid activity, KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway predictions of microbiome and gene expression in the brain were carried out to reveal a stress loop linking the gut microbiota to memory function.

2. Material and methods

All the animal care procedures were carried out in accordance with the guidelines set by the European Community's Council Directive. The protocol was approved by the French Ministry of education, higher education and research under the protocol N° APAFIS# 201707131037724. V3 - 10607. We used a line genetically selected for its long tonic immobility duration and therefore a high level of emotional reactivity (Lormant et al., 2020).

The timing plan of the experiment and methodological details are reported in the Supplementary Material and Methods.

2.1. Chronic mild stress procedure in donors and microbiota sample collection

At 15th day of age, male quails were divided in two groups, i.e. unstressed and stressed groups. From the age of 17–40 days, quails from the stressed group were exposed to unpredictable repeated negative stimuli four times per day and once per night while quail from the control group were just visited by a human four times per day according to the procedure previously described (Lormant et al., 2021, 2020). Each negative stimulus lasted 30 min, continuously or not (confinement in a corner of the home cage, disturbances in the home cage, cage shaking, unexpected sounds, crowding, novel environment, transport stress). Negative stimuli and visits occurred at random times and a given stimulus was never used twice per day in order to increase unpredictability and decrease animal habituation to the stress procedure.

Cecal contents were collected from 4 adult males which had previously been subjected to the chronic stress procedure and from 4 adult males which had not been stressed (unstressed quails). They were mixed in 500 μL of sterile glycerol (used as a cryoprotectant) + cysteine (used as an antioxidant) and stored at – 80 °C (Chu et al., 2017).

2.2. Animals and housing

Disinfected eggs that were incubated for 17 days were placed in sterile isolators and germ-free chicks hatched in the following days. Two days after hatching (Day 2), the chicks were transferred in six large sterile isolators (see Supplementary Material and Methods**;** see Kraimi et al. (Kraimi et al., 2018)). Six females and six males were kept in each isolator. In order to avoid mating in the isolator and excessive stocking density, female quails were eliminated at Day 14 and only males were conserved for plasma corticosterone assays and memory tests.

2.3. Bacterial inoculation

On the day of transfer to the isolators (Day 2), we colonized the chicks of three of the isolators (group STRESS-T) with the cecal microbiota from a unique donor male randomly chosen among the stressed quails. We chose to use only one donor instead of a pool to avoid uncontrolled effects by mixing different microbial communities, called "coalescence" (Rillig et al., 2016). The chicks from the three other isolators (group CONTROL-T) were colonized with the cecal microbiota from another donor male from the unstressed quails. The cecal content was thawed and diluted aerobically in 12 mL of sterile physiological saline. Each chick was colonized by oral gavage with 100 μL of this microbiota suspension.

The cecal contents of recipient females at Day 14 and recipient males at Day 36 were also collected for analysis of microbiota composition.

2.4. Microbiota composition analysis

Microbial DNA extraction was performed using the QIAamp DNA mini-kit (ref #51306, Qiagen Inc., Courtaboeuf, France) following the procedure previously described (Kraimi et al., 2019).

PCR amplification of the bacterial 16 S rRNA gene on DNA extracts was carried out using the primers designed to amplify from highly V4-V5 conserved regions (Fadeev et al., 2021).

2.5. Anxiety-like behavior tests

Each isolator was divided into two equal areas using an opaque

separating wall. One area was dedicated to rearing, while the other half was used for the behavioral tests (Kraimi et al., 2018).

On Day 7, in order to assess the anxiety-like behavior in a novel environment we introduced the quails in groups of three in the test area for the first time for 5 min to measure for each individual the average time spent (time/number of entries) in the wall zone of the test area (see Supplementary Material and Methods).

On Day 12 we measured the anxiety-like behavior of the quails during a period of separation from their congeners. The quails were individually placed in the test area for 5 min and the number of times the quail entered in the wall zone of the test area was recorded.

On Day 13, we measured the behavioral reactions of quails in the presence of a novel object. Each quail was placed for 5 min in the test area in a sterilized white plastic corridor which contained a sterilized red plastic ball. The number of quails attempting to escape from the object was counted as an indication of fear.

After the memory tests, male quails were subjected to an open-field test outside the isolators to assess anxiety-like behavior to a novel environment. Each quail was placed in the center of the open-field (square arena, 80 cm × 80 cm × 29 cm) and allowed to freely explore the test arena for 5 min and we recorded the total distance traveled.

2.6. Plasma corticosterone levels

Plasma corticosterone levels of male quails were measured on Day 14 for the basal value and after a stress (restraint in a crush cage for 10 min) on Day 15.

2.7. Memory tests

Memory tests were performed on males with 18 quails in the STRESS-T group and 16 quails in the CONTROL-T group.

2.7.1. Training

The training phase took place after familiarization to mealworms and habituation to the test device. In the training phase, seven cups were covered with white paper and only the rewarded cup was covered with black paper. The rewarded cup contained two to three live mealworms and the location of this cup remained the same throughout the whole training period. Quails underwent two training trials per day with an interval of 30 min between each trial. Finding the reward is a task which can be solved by quails by either learning that the black cup contains the reward (cue-based memory) or by learning the spatial location of the rewarded cup (spatial memory). Quails were placed in the arena at one of three different randomly distributed entry points. The trial was stopped when quails found the mealworms in the rewarded cup or after a maximum test duration of 300 s. The latency and the number of cups visited before finding the rewarded cup were recorded for each trial. After 4 consecutive days we stopped the training phase when all the quails took on average less than 35 s and made fewer than 3 mistakes before reaching the black rewarded cup without a significant difference of performance between the two treatments.

2.7.2. Probe tests

The day following the last training trial, quails completed two different tests (spatial and cued test). In both of these, no mealworms were placed in the cups to avoid any olfactory cue. In the first, the spatial test, all the cups were white to assess whether quails used their spatial memory to locate the position of the rewarded cup (spatial cup). The tested quail was introduced at a different entry than the three used for the training phase and was allowed to explore the arena freely for 2 min. The latency and the number of cup visits before finding the spatial cup (used as indicator of memory errors) were recorded. The second test was performed 3 days after the spatial test. This cued test was a displacement test in which the black cup was placed in a different position from that of the training period. This test was used to determine the memory system engaged to solve the task: if a quail went first to the spatial cup from the previous test, it indicates that it used a dominant spatial strategy and if it visited the black cup (the cued cup), it indicates that a dominant cue-based strategy based on the cup color was used (Kim and Baxter, 2001). The latency and the number of cup visits before finding the spatial or the cued cup were also recorded in this test.

2.8. Gene expression in the brain

At Day 36, quails were decapitated post-euthanasia and brains removed for dissection of the hippocampus, arcopallium and hypothalamus regions. All the samples were then stored at − 80 °C until analysis.

Total RNA was extracted from frozen brain tissue and quantitative PCR was performed to measure gene expression of glucocorticoid receptor (GR), mineralocorticoid receptor (MR), brain derived neurotrophic factor (BDNF), proliferating cell nuclear antigen (PCNA) and corticotropin releasing hormone receptor 1 (CRHR1). Different housekeeping genes were used. Hippocampus mRNA levels were normalized to SUZ12 gene expression, arcopallium mRNA levels to GAPDH and PGK1 genes expression, hypothalamus mRNA levels to GAPDH, PGK1 and β-actin genes expression (see Supplementary Material and Methods).

2.9. 2. 9. Short-chain fatty acid (SCFA) analysis in fecal samples

Fecal contents from male quails were collected individually inside the isolators at Day 6 and 20. All the samples were stored at – 80 °C before SCFA analysis. See Supplementary Material and Methods for more details.

2.10. Statistical analysis

The results are presented as means ± SEM. All statistical analyses were performed with R (version 3.5.1) and RStudio software (version 1.1.463).

For the gut microbiota composition analysis, differential abundances at the genus level among stressed quails, unstressed quails, STRESS-T and CONTROL-T groups were assessed using Welch *t*-tests corrected using Bonferroni's approach. The computations were performed using STAMP v 2.1.3.

Finally, the samples were compared using PICRUST2 v. 2.2.0_b in order to infer microbial gene content from 16 S rRNA gene data and associated enrichment of metabolic pathways. The distribution of pathway abundances was visualized by PCA, using STAMP v 2.1.3; differential pathway abundances were assessed using Welch's *t*-tests corrected using Bonferroni's method.

Behavioral data were analyzed using generalized linear mixed models (GLMM; package 'lme4' v 1.1–19) with group (STRESS-T or CONTROL-T), sex and interaction between group and sex as the fixed effects and the order in which the quails were tested as the random effect. In the novel environment test, the trio number was used as the random effect. In the case of repeated measures as in the habituation and learning phase of the memory tests, group and day were used as the fixed effects with the order of passage as the random effect. A GLMM with Gamma errors was used for the total distance traveled, the latencies and the time spent in the various zones during the different behavioral tests. A GLMM with Poisson errors was used to compare the number of entries in the different zones of the tests and the number of cups visited in the memory tests. During the novel object test, we compared the number of quails that escaped using a Chi2 test.

Corticosterone data were first log-transformed and then tested using a generalized linear model (GLMM; package 'lme4' v 1.1–19) with group as the main factor and the order of collection as the random factor.

Gene expression data and SCFA concentration were also analyzed with the same generalized linear mixed models with Gamma law and the

group as the fixed effect.

3. Results

3.1. Stress-induced alterations in gut microbiota composition are transferred via cecal microbiota transfer

We first analysed the effects of the chronic stress procedure on the composition of quails' cecal microbiota. A total of 415 OTUs were found among the samples. Higher alpha diversities were observed in the unstressed quails (Fig. 1a). Furthermore, differential abundance assessed at the phylum level revealed higher relative abundances of the *Firmicutes* in the unstressed quails ($p < 0.01$) and of the *Bacteroidetes* in the stressed quails ($p < 0.05$) (Fig. 2a). At the genus level, differential abundance was observed for only one genus, *Alistipes* sp. ($p < 0.05$; Fig. 2b), out of the 69 observed in our dataset. This genus was mainly represented by the OTU1 (85.5% of the sequences assigned to Alistipes sp. with > 99% identity). The OTU1 was found to be more abundant in the stressed quails (relative abundances: 35.2% ± 2.0% and 0.7% ± 0.5% respectively in the stressed and unstressed quails).

After CMT into germ-free naive quails, we compared cecal microbiota composition of germ-free recipients colonized with the cecal microbiota of a quail randomly picked either from a group of unstressed quails (CONTROL-T group) or from a group of stressed quails (STRESS-T group).

Cecal contents collected at Day 14 showed differences in the relative abundance of the major phyla between STRESS-T and CONTROL-T groups. Therefore, firstly we assessed overall differences using diversity indexes which revealed a higher cecal microbial diversity for the CONTROL-T quails (Fig. 1a). The CONTROL-T and STRESS-T groups compared at OTU level using Bray-Curtis distances revealed high between-group differences and weak within-group differences ($p < 0.001$, Fig. 1b). Secondly, we observed differential abundances reflecting the cecal microbial composition already observed in the donor quails. At the phylum level, the *Firmicutes* were thus more abundant in the CONTROL-T quails ($p < 0.001$), whereas the *Bacteroidetes* and *Actinobacteria* were more abundant in the STRESS-T quails ($p < 0.001$ and $p < 0.001$ respectively) (Fig. 2a). At the genus level, differential abundances were found for 40 genera, but only *Alistipes* sp. presented reasonably high relative abundances (> 5% in mean; $p < 0.001$; Fig. 2b) with higher abundance in the STRESS-T group. This genus was mainly represented by OTU1 and OTU2 (respectively 84.9% and 14.0% of the sequences assigned to *Alistipes* sp.). Thirdly, the functional diversity was inferred from the taxonomic profiling, using the PICRUST2 approach for function (i.e. E.C. numbers) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway prediction. The results indicated an average NSTI score of 0.23 ± 0.06 for the 415 OTUs, showing that the predictions were poorly supported for a fraction of the OTUs. However, the NSTI scores were respectively 0.12 and 0.11 for the two OTUs involved in the main differences between the STRESS-T and CONTROL-T groups, OTU1 and OTU2. We found 300 enriched KEGG pathways; among them, 78 pathways presented significant differences between STRESS-T and CONTROL-T groups (Fig. 1c; Supplementary Table 1). In particular, we observed that the microbiota from STRESS-T had a reduction in tryptophan synthesis (EC:4.2.1.20) while its catalysis was enriched compared to CONTROL-T (EC:4.1.99.1; Fig. 1d), mainly through the *Alistipes* genus which represent 15/26 of the species having tryptophanase.

The cecal contents collected at Day 36 showed few differences between STRESS-T and CONTROL-T groups. The levels of alpha diversities were lower than those observed at Day 14 and were similar between the CONTROL-T and STRESS-T groups (Fig. 1a). The Bray-Curtis distances revealed qualitative differences between the groups ($p < 0.001$; Fig. 1b). However, we did not observe significant alterations in differential abundances at the phylum level (Fig. 2a) and, at the genus level, only one category presented differential abundances and a reasonably high abundance (5.0%). This category, referred as unknown, included all uncertain genera assigned to the Lachnospiraceae family (> 5% in mean

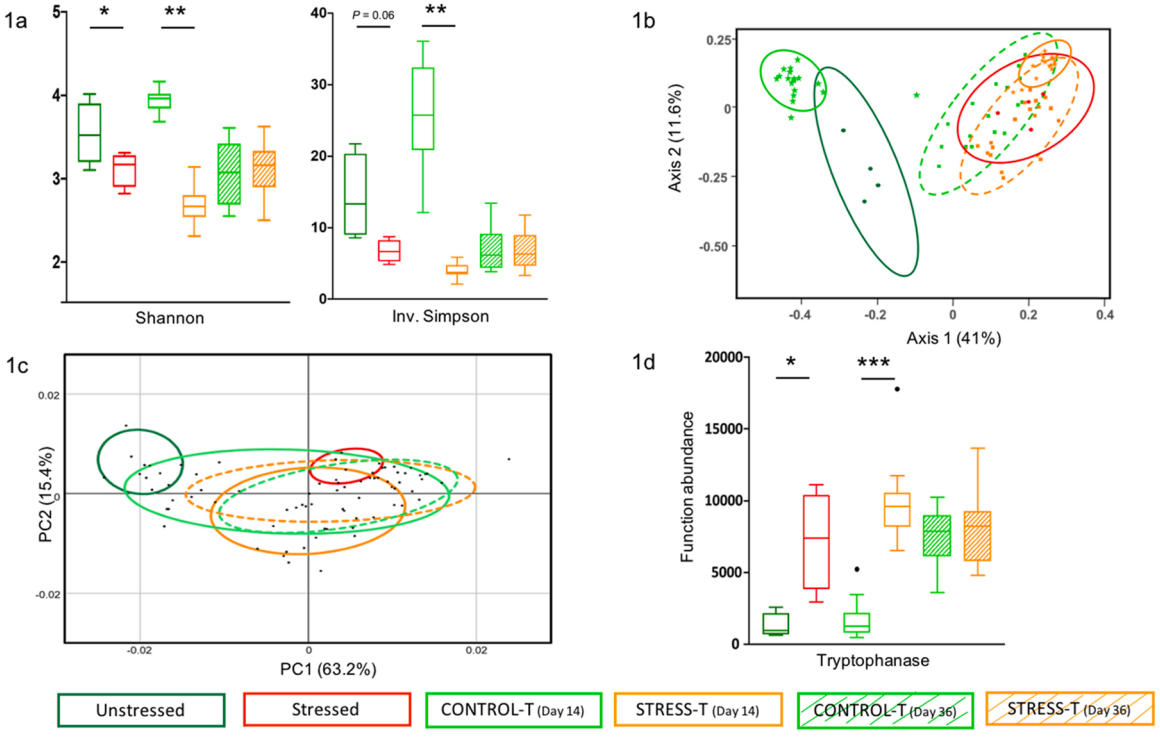

Fig. 1. Comparison of the operational taxonomic units in cecal contents of Unstressed (n = 4) and Stressed (n = 4), CONTROL-T (n = 18) and STRESS-T (n = 17) groups at Day 14 and CONTROL-T (n = 16) and STRESS-T (n = 18) groups at Day 36 for (a) Shannon and Inverse Simpson α-diversity indexes, (b) non Metric Multidimensional Scaling (NMDS) representation of Bray–Curtis distances, (c) PCA visualization of the pathway enrichment analysis derived from the taxonomic profiles, (d) Function prediction levels of the tryptophanase activity (EC:4.1.99.1).

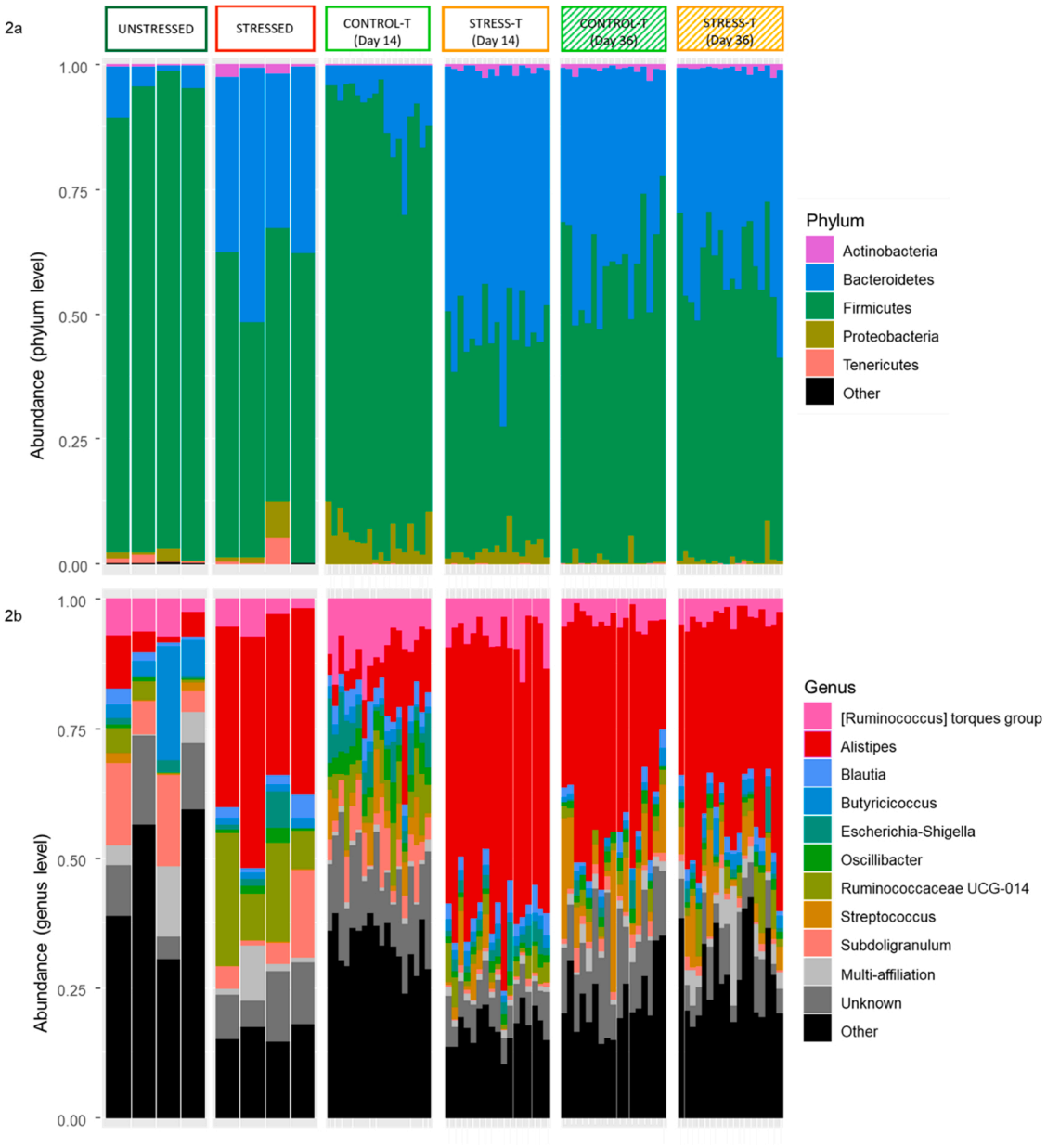

Fig. 2. Relative abundance of major bacterial phyla (a) and genera (b) in the cecal contents of unstressed (n = 4) and stressed (n = 4) quails, CONTROL-T (n = 18) and STRESS-T (n = 17) groups at Day 14 and CONTROL-T (n = 16) and STRESS-T (n = 18) groups at Day 36.

abundance; $p < 0.001$; Fig. 2b). In line with this, functional predictions revealed only a few differences between the CONTROL-T and STRESS-T group at Day 36, including two pathways associated with tetrapyrrole biosynthesis (PWY-5189 and PWY-5188; Fig. 1c) and seven functions mainly associated with these pathways.

3.2. Cecal microbiota transfer from stressed quails to germ-free naive quails results in increased anxiety-like behavior and impaired spatial and cue-based memory

In a novel environment test, quails from the STRESS-T group spent significantly more time on average in the wall zone, corresponding to where they were introduced and which indicated a fear-induced reduction of exploration (Fig. 3a).

Fear of novelty was also investigated using a test that involved introducing a novel object (red plastic ball). The quails that fled far from the object were twice as numerous in the STRESS-T group as in the CONTROL-T group (Fig. 3b).

In the open-field test, quails of the STRESS-T group traveled significantly shorter distances than those of the CONTROL-T group (Fig. 3c), which indicates a state of enhanced stress.

When separated from their congeners by a wall in the social separation test, the STRESS group individuals entered significantly more into this wall zone (Fig. 3d) which reveals increased locomotor activity in this zone, indicating higher anxiety-like behavior in this situation of social isolation.

In the memory testing, quails were individually habituated to the test arena and trained in the spatial learning task before the tests. During the 4 days of training, the quails of both groups learned to find the rewarded cup without treatment effect on the latency to visit the rewarded cup and the number of cups visited before reaching the rewarded cup. Latency to visit the rewarded cup decreased over time independently of the treatment (day effect: $\chi^2 = 24.29$, $p < 0.0001$; treatment effect: $\chi^2 = 0.78$, $p = 0.38$; interaction day*treatment: $\chi^2 = 0.58$, $p = 0.44$, Fig. 4a) and the number of cups visited before reaching the rewarded cup also decreased without treatment effect (day effect: $\chi^2 = 7.11$, $p < 0.01$;

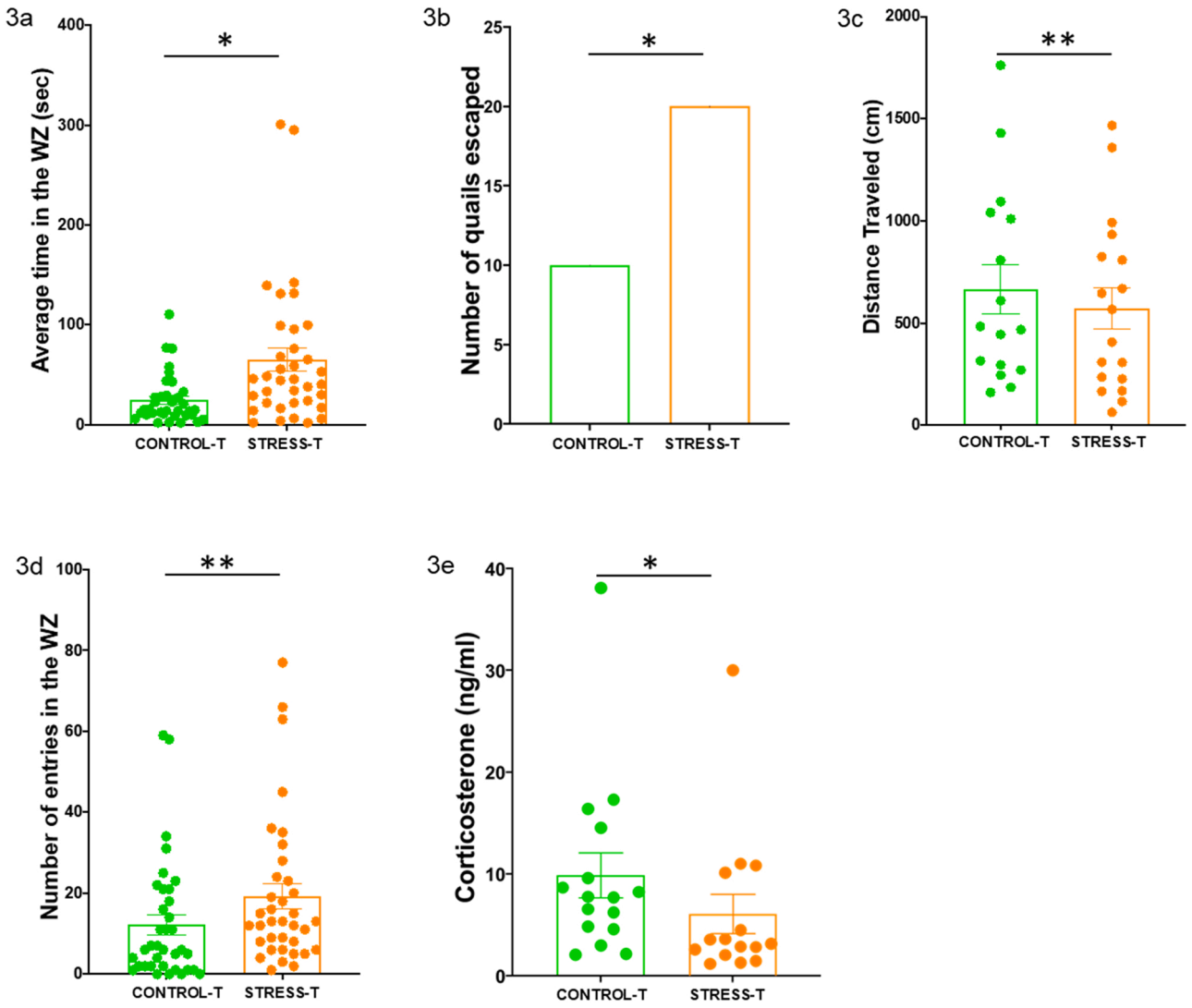

Fig. 3. (a) Average time spent in the wall zone (WZ) during the novel environment test in the CONTROL-T (n = 36) and STRESS-T (n = 36) groups. (b) Number of quails that escaped from the device during the novel object test in the CONTROL-T (n = 36) and STRESS-T (n = 36) groups. (c) Distance traveled during the open-field test in the CONTROL-T group (n = 16) and the STRESS-T group (n = 18). (d) Number of entries in the wall zone (WZ) during the social separation test in the CONTROL-T (n = 36) and STRESS-T (n = 36) groups. (e) Basal plasma corticosterone levels on Day 14 in the CONTROL-T group (n = 16) and the STRESS-T group (n = 15). The results are expressed as mean values ± SEM. * $p < 0.05$, * * $p \leq 0.01$.

treatment effect: $\chi^2 = 0.18$, $p = 0.67$; interaction day*treatment: $\chi^2 = 0.36$, $p = 0.54$, Fig. 4b). After training, a test to evaluate spatial memory was performed. During this test, all the cups were unrewarded and quails had to find the previously rewarded cup - at an unchanged location - using only spatial information since all cups had a white cover. This test revealed spatial memory was impaired in the STRESS-T group compared to the CONTROL-T group. Quails of the STRESS-T group tended to take more time (Fig. 4c) and visited significantly more cups before reaching the location of the previously rewarded cup (Fig. 4d).

During the cued test, all the cups were unrewarded, the location of the cued cup was modified and we measured whether the individuals looked for the location of the cup usually rewarded (spatial memory) or for the cue that was associated with the reward during training (cue-based memory; black cover). Quails of the STRESS-T group took significantly more time (Fig. 4e) and tended to make more visits than CONTROL-T quails before reaching the cued cup (Fig. 4f).

3.3. Microbiota transplantation influences corticosterone levels in the plasma, SCFA concentration in the feces and gene mRNA expression in the brain

We assessed the impact of CMT on plasma corticosterone levels of recipient quails both before and after acute stress. Plasma corticosterone levels at baseline were significantly lower in the STRESS-T group than in the CONTROL-T group (Fig. 3e). The magnitude of the increase in corticosterone after contention (level induced by stress of contention minus basal level) tended to be higher in STRESS-T quails than in CONTROL-T quails (6.02 ± 1.9 vs -1.7 ± 2.8, $\chi^2 = 3.54$, $p = 0.07$) and the plasma corticosterone levels obtained after 10 min of restraint stress were not significantly different between the two groups (8.7 ± 0.9 for CONTROL-T group vs 11.3 ± 2.5 for STRESS-T group, $\chi^2 = 0.05$, $p > 0.10$).

The fermentation activity of the gut microbiota was measured through the quantitative analysis of SCFA contained in fecal contents at Day 6 and Day 20 after CMT. Fecal samples at Day 6 revealed significantly higher concentrations in the CONTROL-T group for caproate, isovalerate and isocaproate (Supplementary Table 2). No significant

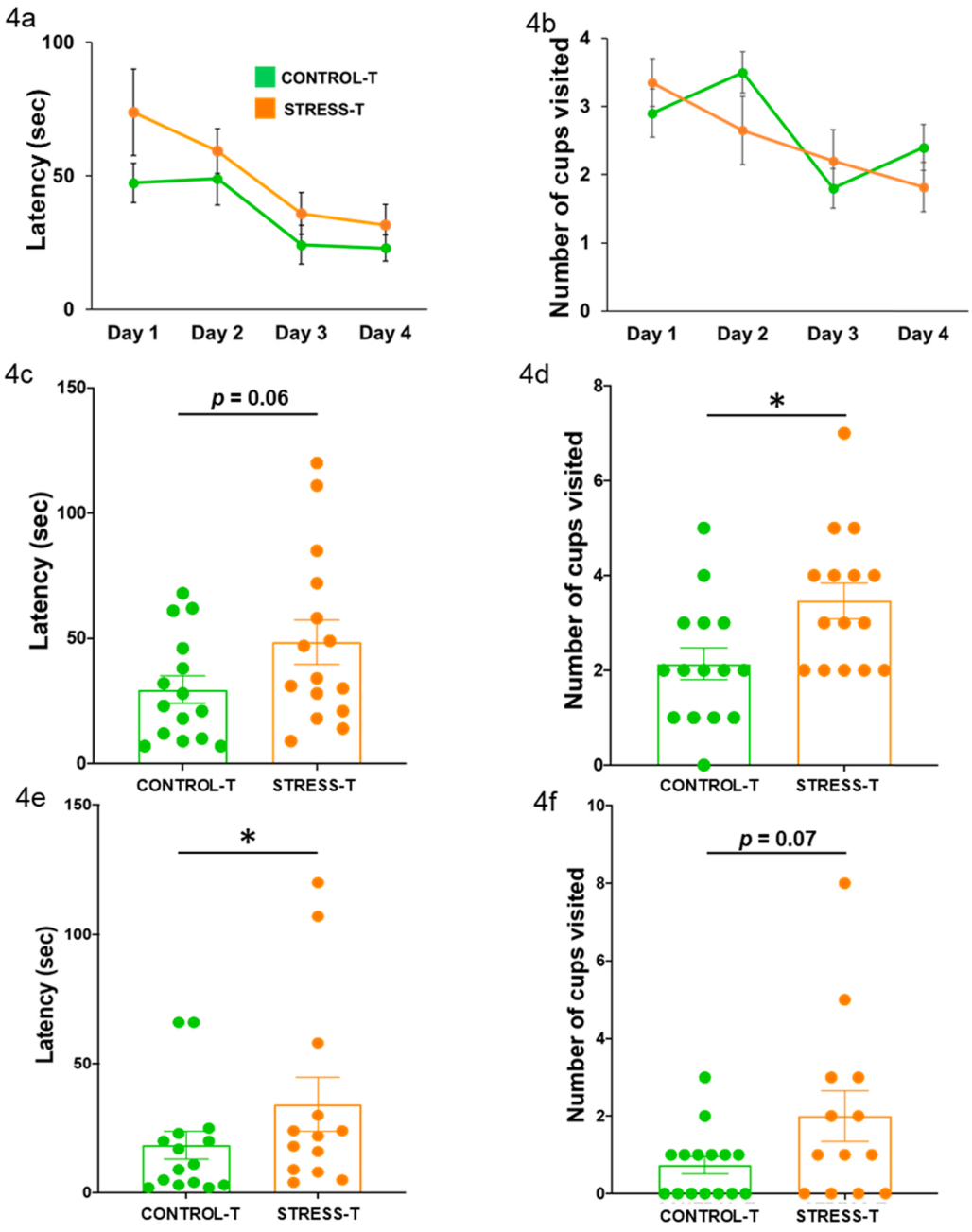

Fig. 4. Latency (a) and number of cups visited (b) before reaching the rewarded cup during the 4 days of training in the CONTROL-T group (n = 15) and the STRESS-T group (n = 15). Latency (**c**) and number of cups visited (**d**) before finding the location of the previous rewarded cup during the spatial test in the CONTROL-T group (n = 15) and the STRESS-T group (n = 15). Latency to reach the cued cup (**e**) and number of cups visited before reaching the cued cup (**f**) in the CONTROL-T group (n = 15) and the STRESS-T group (n = 14) during the cued test. The results are expressed as mean values ± SEM. * $p < 0.05$.

differences in SCFA composition were found at Day 20.

Finally, we assessed CMT effects on gene expression in the hippocampus, the arcopallium and the hypothalamus, which are brain structures involved in cognitive processing, control of fear behavior and regulation of the HPA axis, respectively. In the hippocampus, *CRHR1* expression was significantly lower in quails of the STRESS-T group (Fig. 5a). No significant differences were found in the arcopallium (Fig. 5b). The expression of *CRHR1* and *PCNA* were significantly reduced in the hypothalamus of the STRESS-T group compared to the CONTROL-T group (Fig. 5c). BDNF expression tended to be higher in the arcopallium (Fig. 5**b**) and lower in the hypothalamus of the STRESS-T group compared to the CONTROL-T group (Fig. 5c).

4. Discussion

Chronic stress is not only recognized to have a major impact on gut physiology and microbiota composition (Madison and Kiecolt-Glaser, 2019) but it is also known to be an important risk factor for brain-related dysfunctions such as memory impairments (Sandi, 2013). Moreover, Chevalier and collaborators (Chevalier et al., 2020) recently showed that chronic stress and the gut microbiota generate a feedforward loop that contributes to depressive disorders. In the present research, we investigated whether a similar loop system could exist between gut microbiota and memory deficits associated with chronic stress conditions. Using Japanese quails raised in a unique microbial controlled-environment inside isolators, we demonstrated that germ-free host quails receiving cecal microbiota from a donor quail subjected to chronic stress showed clear impairments in memory and also increased anxiety-like behavior when compared to germ-free quails implanted with cecal microbiota from unstressed quail. To induce chronic stress in quails we used a procedure of unpredictable negative stimulations, which has been thoroughly validated in this line of Japanese quail (Lormant et al., 2020). Although this procedure has profound impacts on behavior and physiology, its potential impact on gut

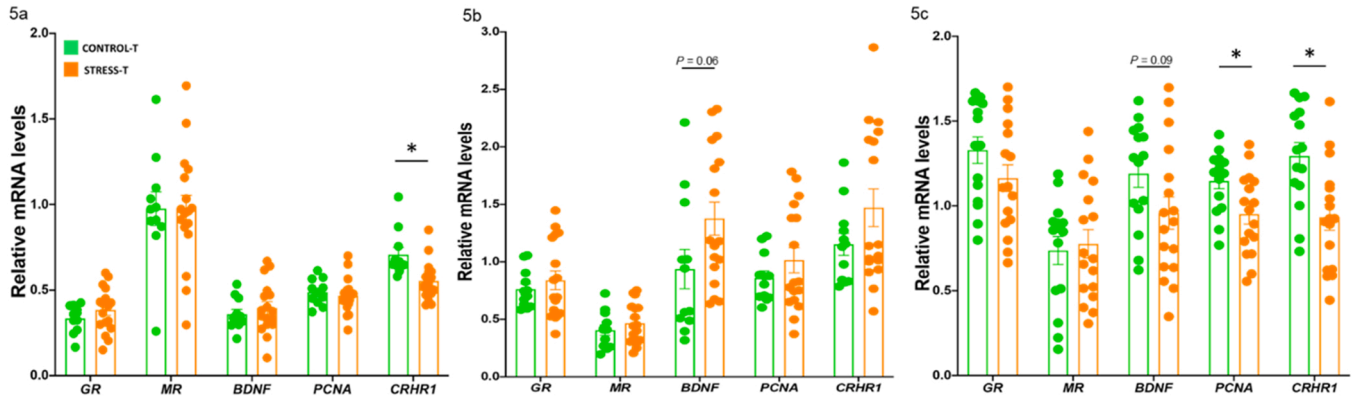

Fig. 5. Gene expression in the hippocampus (a, CONTROL-T: n = 11, STRESS-T: n = 17), the arcopallium (b, CONTROL-T: n = 12, STRESS-T: n = 17) and the hypothalamus (c, CONTROL-T: n = 15, STRESS-T: n = 17). The results are expressed as mean values ± SEM. * $p < 0.05$.

microbiota has not been investigated to date.

The composition of cecal microbiota of quails subjected to the chronic stress procedure differed from that of unstressed quails. The main differences related to one OTU assigned to the *Alistipes* genus (OTU 1; more abundant in stressed quails), which belongs to the *Bacteroidetes* phylum, known to be altered by various forms of stress, including exposure to early-life maternal separation stress (Pusceddu et al., 2015) and social stress (Bharwani et al., 2016). *Alistipes* sp. is a genus already known to be favored by various kinds of induced stress in different models. This includes models in which mice were subjected to a water immersion restraint stress (Li et al., 2017) or mice housed on a grid floor and stressed by this rearing condition (Bangsgaard Bendtsen et al., 2012). Interestingly, increased abundance of *Alistipes* sp. has also been found in the gut microbiota of depressive human patients (Jiang et al., 2015).

The oral inoculation of the modified microbiota in recipient quails resulted in a higher anxiety level than in quails inoculated with the microbiota from unstressed quail. The rigorous use of germ-free chicks and controlled conditions in isolators demonstrates that individuals of the STRESS-T group were more anxious as they displayed an overall decrease in exploration in the novel environment test and the open-field test and increased activity during social isolation. This increase in anxiety-like behavior mimics the one reported in quails subjected to the chronic stress procedure (Calandreau et al., 2011a). The higher anxiety-like behavior of STRESS-T quails in response to novelty was confirmed by the novel object test during which the quails escaped twice as much in the STRESS-T group compared to the CONTROL-T group. Again, this result is in line with the increased neophobia described in chronic stress quails (Favreau-Peigné et al., 2016) and further strengthens the implication of gut microbiota in neophobia responses that we recently characterized in this quail line (Kraimi et al., 2018).

Our study showed that colonization with the cecal microbiota from a stressed individual affects spatial memory and cue-based memory. During the training session, the quails of both groups learned the task similarly, which allows us to interpret the test responses in terms of specific memory capacity with no bias of motivation, vision ability or learning.

Spatial memory, which consists in relating positions of visual cues in the environment, is indeed a privileged target of chronic stress in mammals and also in birds (Lindqvist and Jensen, 2009). Unlike spatial memory, which is a form of explicit memory, cue-based memory is an implicit memory system based on a simple cue-response association (Squire and Dede, 2015). Previous studies have shown correlations between modifications of the gut microbiota and impaired memory performances (Gareau, 2016). However, no study has established a causal link between the alterations in gut microbiota induced by chronic stress and memory deficits. The CMT protocol we used enables us to demonstrate for the first time the causal role of the gut microbiota in stress-induced memory impairments by showing that an altered gut microbiota alone is able to induce the negative effects of chronic stress on spatial and cue-based memory (Fig. 6). This pivotal result is in line with a recent study that showed that a transfer of gut microbiota from old mice to young mice was sufficient to reproduce the cognitive decline associated with aging (Lee et al., 2020) and suggests that memory impairments are mediated by gut microbiota in many cases. The results of the CMT protocol provide evidence of a feed-forward loop system linking the microbiota-gut-brain axis to stress and memory function (Fig. 6). This evidence suggests that future research should target gut microbiota composition and not only neurobiological pathways to prevent stress-induced memory alterations.

Furthermore, our data revealed differential regulation of several genes in the brain according to the cecal microbiota used for colonization. In the hippocampus and the hypothalamus, colonization with the cecal microbiota from a stressed individual reduced *CRHR1* expression and the level of plasma corticosterone. *CRHR1* is an essential regulator of the HPA axis; its hippocampal expression has also been shown to be reduced by maternal separation in mice (Reshetnikov et al., 2018) and *CRHR1*-deficient mice are unable to mount a corticosterone response to stress (Timpl et al., 1998). Moreover, this reduction in plasma corticosterone levels under basal conditions has previously been described in European starling (Cyr and Michael Romero, 2007; Rich, 2005) and in this line of quail subjected to a chronic stress procedure (Calandreau et al., 2011b), whereas more acute stress increases corticosterone levels in this species (Lyte et al., 2021). We found reduced *PCNA* expression in the hypothalamus of the STRESS-T group, which suggests decreased cell proliferation. We also noted that in the STRESS-T group *BDNF* expression tended to reduce or increase in the hypothalamus and arcopallium respectively, suggesting changes in brain plasticity mechanisms. However, we did not detect any significant differences in mRNA expression levels in any of the three brain structures for the nuclear receptors *GR* and *MR* involved in the negative feedback of the HPA axis.

We looked at the cecal microbiota composition to understand more clearly whether microbiota transfer can modulate anxiety-like behavior and memory performance. As expected, the cecal microbiota transfer also led to different cecal microbiota composition in the recipient quails which can explain the behavioral, cognitive and physiological differences observed. At Day 14, cecal contents of STRESS-T quails showed lower microbial alpha diversity, a lower abundance of *Firmicutes* and a higher abundance of *Actinobacteria* and *Bacteroidetes* than cecal contents of CONTROL-T quails. Interestingly, these results are very similar to those observed in stressed or unstressed donors, which suggests successful microbiota transfer. In addition, the greatest differences in OTUs between the two groups were assigned to the *Alistipes* genus with higher abundance in the STRESS-T than CONTROL group. As previously mentioned, the *Alistipes* genus has already been linked to stress and depression in mice and humans (Bangsgaard Bendtsen et al., 2012; Li

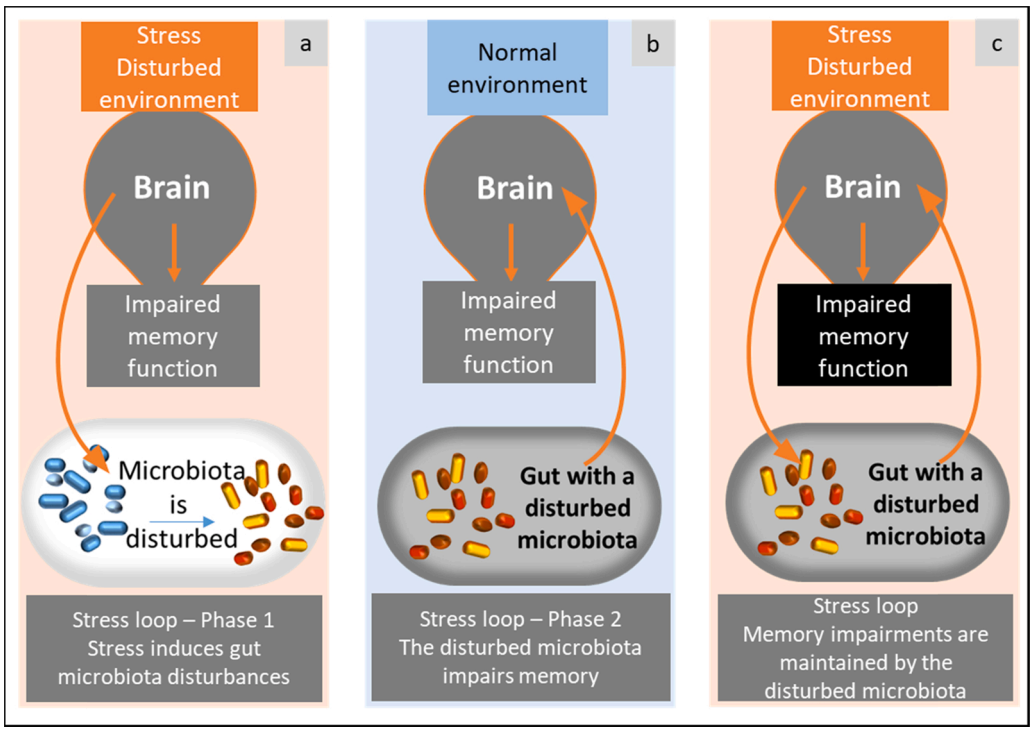

Fig. 6. A vicious loop via gut microbiota. Stress and disturbed environment conditions (a) induce alterations in the gut microbiota. This disturbed gut microbiota is able to induce impaired memory by itself, even if the environment is not stressful (demonstration in the present study, b). As a consequence, the gut microbiota and brain would result in a loop that maintains memory impairments (c).

et al., 2017) and could perhaps serve as a biomarker of stress. An increasing body of literature supports different explanations for the mechanisms by which *Alistipes* could play a role in the MGBA. Detrimental effects of *Alistipes* would be related to the permeability of the gut induced by microbial dysbiosis which allows molecules such as lipopolysaccharides (LPS) to enter into the bloodstream leading to neuroinflammation and behavioral alterations. Indeed, LPS in *Alistipes* is known to be pro-inflammatory, leading to a higher expression of CD161 and CCR6/integrin Beta7 by Th17 cells and a decrease in the anti-inflammatory butyrate-producing bacteria (Parker et al., 2020). *Alistipes* can also produce sulfonolipids (SLs), a type of sphingolipid that has anti-inflammatory effects by suppressing the activation of the cytokines like TNF-α in mice (Walker et al., 2017). In a bird model, it has been shown that *Alistipes* bacteria are also able to express glutamate decarboxylase, which is the producer of ɣ -aminobutyric acid (GABA) from glutamate, in the cecal microbiota of chickens (Polansky et al., 2016). The explanation that best fits our results and our microbiome functional analysis is that of the tryptophan amino acid (Trp) pathway and the serotonergic system since *Alistipes* species are indole-positive and possess the tryptophanase enzyme which directly produces indole from Trp and may lead to a disruption of the serotonergic balance in the host (Agus et al., 2018). Since the link between the serotonergic system and HPA axis is well-recognized now (O'Mahony et al., 2015), the alterations of behavior and HPA axis activity found in our study could be explained by the high abundance of *Alistipes* species and their action on the Trp pathway. In addition, our results on the tryptophanase function in the gut microbiota are in line with several studies showing a link between Trp metabolism in the gut and behavioral changes during chronic stress (Deng et al., 2021; Mir et al., 2020). These findings in *Alistipes* make this bacterial genus an important candidate in the interaction between the gut microbiota and the stress system and corroborate the implication of microbial Trp metabolism in behavioral and neurological stress-induced changes.

The fermentation activity of the gut microbiota assessed through the quantitative analysis of the SCFA contained in fecal contents showed higher concentrations of caproate, isocaproate and isovalerate, but lower proportions of acetate in CONTROL-T quails. Together, these data showed that CONTROL-T and STRESS-T quails had different gut microbiota fermentation activities, which strongly supports the involvement of SCFA in the microbiota-gut-brain communication in vertebrates (Cryan and Dinan, 2012; Erny et al., 2015) and is in line with recent results showing that microbiota changes induced by chronic stress affect lipid metabolism and the generation of endocannabinoids (Chevalier et al., 2020).

At the end of the experiment after 36 days, there were fewer differences in cecal microbiota between STRESS-T and CONTROL-T groups. This could reflect an age effect and an evolution of the cecal microbiota with time or a change of environment during the memory test procedure. However, both groups still showed differences in terms of anxiety-like behavior, cognition and gene expression in brain structures. This may imply that there is a critical period in early life during which the initially implanted microbiota would have irreversible consequences on cerebral, behavioral and cognitive development even after re-colonization with a different microbiota. This hypothesis is supported by several rodent studies and would imply that a critical period may exist in all vertebrates (Heijtz et al., 2011; Neufeld et al., 2011). These long-term effects of the microbiota suggest that the origin of certain cognitive disorders should not only be investigated in the gut microbiota present at the time of the onset of the disorders, but also in previous intestinal changes. This suggests that the prevention of memory alterations due to stress must target an immediate return to a state of equilibrium in the composition of the microbiota in order to avoid possible long-term effects. We suggest that the prevention of the cognitive disorders with diet intervention, or probiotic supplementation for example in individuals subjected to stress conditions at a young age inducing dysbiosis could be an interesting avenue to explore.

5. Conclusion

In conclusion, we showed that gut microbiota alone is sufficient to

mimic stress effects on cognition and impair memory abilities. These data substantiate the existence of a stress loop connecting the gut to memory development that implicates the gut microbiota as a component that has to be considered in greater depth in future studies on stress processes. Interestingly, our findings add more evidence to the role of *Alistipes* genus as a potential biomarker of stress in vertebrates because of its link with the tryptophan metabolism pathway. These data suggest that maintaining a healthy microbiota could help alleviate memory impairments linked to chronic stress.

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Declarations

Illumina sequence data have been deposited at National Center for Biotechnology Information (NCBI), under the BioProject PRJNA 527873.

CRediT authorship contribution statement

CL and NK designed the study with the help of LC and SR. CL and NK performed the experiments with the technical help of JL, CP, PC and TC. FL performed the chronic stress procedure. The DNA microbial extraction and PCR steps were carried out by KG and NK. OZ was in charge of 16 S rRNA gene sequencing and FK performed the statistical analysis of microbiota data. AF and MPM were in charge of plasma corticosterone measures. JL, NK, AVC, VC and HD performed RNA extraction and qPCR on brains. CP performed SCFA analysis. NK and CL wrote the manuscript. All the authors reviewed and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data Availability

The datasets used during the current study are available from the corresponding author on reasonable request and they are available at https://doi.org/10.15454/JYITK4.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.psyneuen.2021.105594.

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