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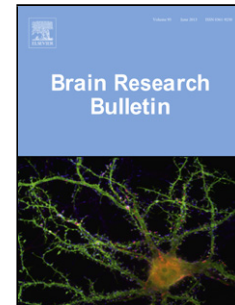


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# The influence of a probiotic supplementation on memory in quail suggests a role of gut microbiota on cognitive abilities in birds

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## Highlights

- Quail supplemented with *Pediococcus acidilactici* had a better memory.
- *Pediococcus acidilactici* had no consistent effect on the fear of quail.
- Traits of emotional reactivity did not explain the improvement of memory.

## Abstract

The gut microbiota is involved in host behaviour and memory in mammals. Consequently, it may also influence emotional behaviour and memory in birds.

Quail from two genetic lines with different fearfulness (LTI: long tonic immobility,  $n=37$ ; STI: short tonic immobility,  $n=32$ ) were either or not supplemented with a probiotic (*Pediococcus acidilactici*) from hatching. Emotional reactivity was measured in a tonic immobility test (d6 and 7 of age) and two open-field tests (d13 to 15; d22 to 24). Memory was measured in a test rewarded with mealworms, where quail had to remember the cups previously visited (d34 to 36). Quail endured a 5-days stress period from days 17 to 21 to help revealing the potentially beneficial effect of the probiotic.

As expected, STI quail were less fearful compared to the LTI quail ( $p < 0.05$ ). Probiotic supplementation had no effect on most measures of emotional reactivity ( $p > 0.05$ ), except in the tonic immobility test where supplemented STI quail had lower immobility duration ( $p = 0.0001$ ). Regarding the memory test, the two lines had similar performances. Quail fed with probiotics made fewer errors ( $p = 0.040$ ). There was no significant correlation between traits of emotional reactivity and of memory.

In conclusion, the supplementation with *Pediococcus acidilactici* as a probiotic, affected a specific trait of emotional reactivity in STI quail, and improved memory in both lines, which strengthens the idea that the influence of gut microbiota on the host behaviour and memory seen in mammals is shared by birds.

**Keywords:** Microbiota; memory; emotional reactivity; probiotic; bird.

## 1. Introduction

The gut microbiota plays a major role in the immunity of its host [1]. It is also involved in host metabolism, central nervous system development and even host behaviour, leading to the concept of the brain-gut-microbiota axis [2].

Emotional behaviour is modulated by the gut microbiota [3]. This has been strengthened through experiments highlighting an altered anxiety-like or depressive-like behaviour in germ-free compared to specific pathogen-free rodents [4–6]. In addition, the administration of a probiotic (*Lactobacillus rhamnosus*) to mice showed anxiolytic effects [7], whereas a bacterial infection with *Campylobacter jejuni* or *Citrobacter rodentium* resulted in increased anxiety-like behaviour [8,9].

Emerging evidence also supports a role of the gut microbiota on cognitive functions, especially memory. Germ-free mice have been shown to have impaired working memory compared to specific pathogen-free mice [10]. Moreover, chronic treatment with antibiotics or infection with *Citrobacter rodentium* in mice have been shown to decrease both working and non-spatial memories, especially under acute stress conditions [10,11]. The supplementation of rodents with probiotics (e.g. strains of *Lactobacillus* and *Bifidobacterium* genera) improved or recovered memory abilities [10,12,13].

Moreover, many experiments demonstrated effects only in stressed or anxious animals [10,13,14]. Furthermore, in germ-free experiments, differences have been found between more or less emotive rodent strains. The absence of gut microbiota leads to reduced anxiety in strains genetically prone to moderate emotivity (strains NMRI and Swiss) [5,15,16]. In contrast, studies using strains exhibiting an exacerbated emotivity (strains F344 and BALB/c) have demonstrated an anxiogenic effect of the absence of gut microbiota [4,17]. Thus, the gut microbiota influence on behaviour appears to depend on the host's genetic background. Rodent models that are characterized by different levels of anxiety have largely contributed to demonstrate the relationship between emotional behaviour or anxiety and memory and learning [18–21].

While the important role of gut microbiota on behaviour and memory has been well demonstrated in rodents, such influences have not been investigated in birds. Only influences on immunity, metabolism and growth performance have been investigated in this phylum [22–24]. Consequently, the hypothesis of the present study was that a supplementation with a

probiotic as a mean to change the gut microbiota composition of birds could influence their emotivity and memory abilities. The model of the Japanese quail (*Coturnix japonica*) was selected for its capacities to live without its mother in early life, thus limiting the influence of maternal microbiota. Two genetically-selected lines characterised by different emotional reactivity levels were studied since gut microbiota influence on behaviour could depend on the host's genetic background, especially their anxiety trait.

## 2. Materials and methods

Animal care procedures were conducted in accordance with the guidelines set by the European Communities Council Directive (86/609/EEC) and with the French legislation on animal research (<http://ethique.ipbs.fr/sdv/charteexpeanimale.pdf>; accessed 15 January 2011). Furthermore, the regional ethics committee approved the protocol (authorization number 2012-01-3).

### 2.1. Animals and housing

Sixty-nine female Japanese quail (*Coturnix japonica*) from two lines genetically selected on a short or a long duration of tonic immobility (STI: Short Tonic Immobility; LTI: Long Tonic Immobility) [25], were bred under the same conditions from hatching until the end of the experiment on the farm of the avian experimental unit of Tours (UE PEAT, INRA). Quail were from the 54<sup>th</sup> generation of selection of lines. The control group was composed of 19 LTI quail and 16 STI quail, whereas the supplemented group was composed of 18 LTI quail and 16 STI quail.

On hatching day, chicks were wing-banded. Then, as sex determination is difficult at this age, they were separated in 8 different cages with their male siblings (16 quail per cage both sex included). Quail from divergent lines, as well as from different treatments were raised in separated cages (2 cages per line and per treatment). The siblings were spread between the control group and the supplemented group to randomize genetic influences. The average weight was balanced between treatment groups. On Day 30, to prevent mounting assaults,

females were separated from the males and grouped per line and per treatment in 4 distinct cages. All the experimental procedures were only done on females.

The cages (l × L × h: 0.85 × 0.95 × 0.25 m) were all located in the same room. The temperature of the room was 40°C on Day 0 and it was decreased progressively to reach 25°C at 20 days of age. Light intensity in each cage varied between 1 and 40 Lux according to the location in the cage. On the day of hatching, photoperiod lasted 24 hours and decreased progressively to 16 hours per day on Day 10. Chicks received an acoustic enrichment over 8 hours per day, via a radio, from Monday to Friday.

## 2.2. Feeding management and probiotic distribution

Feed was presented as pellets for chicks (Metabolisable Energy = 2 900 kcal/kg; Crude Protein = 260 g/kg). The probiotic species used was *Pediococcus acidilactici* (Bactocell®, Lallemand), since it is used in birds [26,27] and it belongs to the *Lactobacillaceae* family, among which several genera have been shown to have psychotropic effects. For the supplemented group, probiotic was included in feed in micro-capsule before pelleting in order to protect it (Bactocell ME®), and the final concentration in pellets was  $2.54 \times 10^6$  CFU per gram of feed. This concentration was determined with a microbiological culture. The supplementation was given from the day of hatching to the end of the tests at 36 days of age. For the first two weeks, a supplement of Bactocell Drink® was added to water (2 g/l of water,  $2.5 \times 10^6$  CFU per gram). The mean daily intake of *P. acidilactici* per quail chick could then be estimated between 15 and  $22 \times 10^6$  CFU during the first week of life. To minimize any crossed contamination, materials used for care and for testing of supplemented animals were different from those used for control animals. Water and pellets were available *ad libitum*.

## 2.3. Period of stress

A procedure modified from Laurence et al. [28] was applied to the quail from the 17<sup>th</sup> to the 21<sup>st</sup> day of age to induce stress in order to try to directly induce disruption in the gut microbiota composition and/or modifications in the gut wall physiology. Indeed, it has been shown that a state of stress may induce impairment of intestinal microbiota [29]. Thus, the application of a

procedure of stressing actions could reveal easily the beneficial effect of probiotics. The procedure to induce a stress consisted of a succession of negative repeated stimulations. During this period, animals were submitted to five daily stimulations in an unpredictable way and did not receive the acoustic enrichment. The negative stimulations employed were: putting the group of individuals in small unusual cages without moving the cages, putting in small unusual cages and moving cages on a trolley, disruption in cages (noises during days and nights, introduction of novel objects, air-spraying, handling with gloves) and isolation in buckets. Each stressor was used only once per day and stressors were administered at random times to increase unpredictability and decrease animal habituation to stressors. It has been shown that this procedure of negative stimulation induces a state of chronic stress in Japanese quail [28,30].

#### 2.4. Behavioural tests

Before each test, animals belonging to a same cage were put together in a pre-test cage (l=65 × w=40 × h=32cm), for a minimum of 15 min. In this pre-test cage, there was an enrichment with wood-shavings, water and feed *ad libitum*, as well as sound enrichment. This precaution minimized fear reactions while taking chicks from the large cages.

##### 2.4.1. Tonic immobility test

The tonic immobility test was performed to evaluate emotional reactivity in Japanese quail [31]. This test was performed at 6 to 7 days of age.

The induction of the behaviour was made for 10 seconds. Numbers of induction attempts necessary to induce tonic immobility and the duration of the immobility were measured. After five inductions without tonic immobility, the quail was deemed not to be susceptible and a duration of 0 seconds was recorded. If the bird failed to right itself after 5 minutes, a maximum of 300 seconds was recorded. Quail with the higher numbers of induction or duration were considered to be the most fearful.

##### 2.4.2. Open-field test



An open-field test was also performed to test emotional reactivity in Japanese quail [31]. This test was used to evaluate behaviours of fear regarding an unknown place and social isolation. This test was performed twice: before the period of stress (13 to 15 days of age) and right after (22 to 24 days of age).

The open-field arena was 80 × 80 cm large with linoleum for floor covering. It was considered that the chick is especially frightened if it stays in the middle of this arena, where it was placed, and rarely explores the peripheral area [32,33]. Each chick was separated from its group and put in the arena for 5 minutes. The chick's trajectory was tracked by a camera with the software Ethovision XT (Noldus Information Technology B.V., Wageningen, The Netherlands). The measures made were the time spent in peripheral area (width of 10 cm) and the total distance traveled.

#### 2.4.3. *Memory test*

This test evaluated the memory of individuals by monitoring the ability of the quail to remember that a cup was already visited. To complete the test, animals had to collect 8 mealworms in 8 cups.

The 8 cups were placed in the arena used for the open-field test (Figure 1). The wall of the box was identified by four shapes: a cross, a line, a triangle and a square to provide spatial landmarks for the quail. The 8 cups were placed in staggered rows and in a way to be placed at an equal distance from each other. The test was carried out over 3 successive days for each 4 groups from 34 to 36 days of age.

During the week prior to the test, quail learnt to eat mealworms for 4 days. The first day mealworms were put in their home cage. Then, the quail were placed in individual cages with mealworms in a cup. The quail that never managed to eat mealworms were eliminated for that test. The remaining subjects were 13 LTI and 14 STI quail for the control group, and 15 LTI and 15 STI quail for the supplemented group. For the last three days of the week, animals

were placed four by four in the box test with one worm in each of the 8 cups in order to limit bias due to a new location.

The feed pellets in the home cages were removed at least 3 hours before the test for the quail to be hungry. At the beginning of each test, the animal was placed at the same place in the arena (Figure 1) and it had 10 minutes maximum to find the 8 mealworms. If it managed to do the task in a shorter time, the test was stopped and the duration was registered.

The measures made during the test were: the duration to complete the test, the succession of cups visited and the number of errors done. Moreover, when a quail has visited a cup, then other cups and then finally came back to the original cup, the interval between the two visits to the same cup was monitored. If the interval was very short (0 to 1 cup), it was considered as an immediate error. However, if the interval was long (3 cups or more), it was considered as a delayed error. The rate of immediate or delayed errors corresponded to the number of times when the quail made this kind of errors balanced by the number of total visits made by the quail. The variables analysed were the total duration to achieve the test, the number of visits, the global rate of error (number of all errors confounded divided by the total number of visits) and the rates of immediate and delayed errors.

## 2.5. Statistical analysis

Statistical analyses were performed with the software R [34]. The two variables of the tonic immobility test (number of inductions and total duration) were normalized by logarithmic transformation. Other variables were normal without transformation. In all the statistical analysis,  $p < 0.05$  was considered statistically significant, and  $0.05 \leq p < 0.1$  as a trend.

Mixed effects model for repeated measures with the treatment, the genetic line, the day, all the double interactions as fixed effects and the animal being included as a random effect were used to test the effect of treatment on memory traits (total duration, number of visits, global rate of error). These analyses were done with the function lme from the R package nlme [35]. The effects of the line and the day (from 1 to 3), as well as the interactions were removed from the model when  $p > 0.05$ . Because no difference between days was obtained regarding the

number of visits and the global rate of errors, the rates of immediate and delayed errors were averaged on the three days of test. Tonic immobility traits, open-field traits and the rates of immediate and delayed errors were tested in two-factor ANOVA with the treatment, the genetic line and their interaction in the model. The line effect and the interaction between the two effects were removed from the model when  $p > 0.05$ . These ANOVA were estimated with the function `lm` of the R software. The `lsmeans` function from the R package `lsmeans` [36] was used to perform pairwise comparisons with the FDR correction when interactions were significant ( $p < 0.05$ ).

Pearson's correlations were calculated between traits of emotional reactivity and memory traits.

### 3. Results

#### 3.1. Emotional reactivity tests

##### 3.1.1. Tonic immobility test

There was no interaction between the line and the treatment effects on the number of inductions needed to induce tonic immobility ( $p > 0.05$ ). This number was lower in LTI quail compared to STI ( $p < 0.001$ ) and it tended to be higher in quail fed with probiotics compared to controls ( $p = 0.095$ ).

There was an interaction between the line and the treatment effects on tonic immobility duration ( $p = 0.01$ ). Tonic immobility was shorter in STI compared to LTI (Figure 2). It was also shorter in STI quail fed with probiotics compared to controls ( $p = 0.0001$ ) while it was not different between control and probiotic quail from the LTI line (Figure 2).

##### 3.1.2. Open-Field tests

During the first open-field test, there was no interaction between the line and the treatment effects on the total distance travelled and on the time spent in the peripheral area of the arena ( $p > 0.05$ ). The distance walked in the open-field arena was higher in STI quail in comparison to the LTI ones ( $p < 0.0001$ ) without any effect of the probiotic. The time spent in the peripheral

area tended to be higher in STI quail compared to LTI quail ( $p = 0.061$ ) and there was no significant effect of the probiotic.

During the second open-field test, there was no interaction between the line and the treatment effects on the total distance travelled or on the time spent in the peripheral area of the arena ( $p > 0.05$ ). The total distance was higher for the STI quail compared to the LTI quail ( $p = 0.0001$ ). The time spent in the peripheral area was also higher in STI compared to LTI ( $p = 0.018$ ) and it tended to be lower in the probiotic quail compared to the control ones ( $p = 0.090$ ).

### 3.2. *Memory test*

The time used to eat all the worms and hence complete the test was affected by the interaction between line and day effects ( $p = 0.033$ ; Figure 3). This duration decreased from Day 1 to Day 2 and from Day 2 to Day 3 in LTI and from Day 1 to Day 2 only in STI quail. It was lower in quail fed with probiotics compared to controls ( $p = 0.020$ ).

The numbers of visits to the cups and the global rate of errors were neither affected by the day, nor the line, the treatment effects nor any interactions between these variables ( $p > 0.05$ , number of visits =  $21.2 \pm 0.7$ ).

The mean rate of immediate errors for the three days of test was not affected either by the line, by the treatment or by the interaction between these two variables ( $p > 0.05$ ) (Figure 4). The mean rate of delayed errors was lower in probiotic-fed quail compared to controls ( $p = 0.040$ ), but it was not affected by the line and the day effects or the interaction between these variables ( $p > 0.05$ ) (Figure 4). The mean rate of immediate errors was lower than the mean rate of delayed errors ( $p = 0.002$ ) (Figure 4).

### 3.3. *Correlations between emotional reactivity and memory*

There were no significant Pearson's correlations between the emotional reactivity traits (number of inductions and duration of the tonic immobility, time spent in peripheral area and

the total distance traveled in the open-field) and the memory traits (total duration to achieve the test, number of visits, global, immediate and delayed errors rates) ( $p > 0.05$ ).

#### 4. Discussion

Two studies using fish [37] and one study on weaning piglets [38] have previously demonstrated that the administration of the strain of *P. acidilactici* resulted in a change in the gut microbiota composition. Therefore, in the present study, it was postulated that the administration of the bacteria strain from hatching has also changed the gut microbiota composition of the quail.

##### 4.1. Influence of the probiotic on emotional reactivity

Prior to the chronic stress period, emotional reactivity was measured through tonic immobility duration and activity in an open-field. The probiotic supplementation reduced tonic immobility duration in STI quail but not in LTI quail, although STI quail are usually less reactive than LTI quail in many fear tests [39]. It had no significant effect on behaviour in the open-field in both lines. This suggests that the supplementation of probiotic would have differential effects on emotional reactivity according to the behavioural trait and also according to the intrinsic reactivity of each quail. The reason why we found an effect of the probiotic only in STI quail may be related to their vagus activity. Indeed, their parasympathetic activity is significantly higher than LTI quail and they therefore have greater vagus nerve activity [40]. In rodents, there is much evidence of the anxiolytic effect of probiotics, especially those composed with strains belonging to *Lactobacillus* and/or *Bifidobacterium* genera [14,41–46] and two studies demonstrated a major involvement of the vagus nerve. Bercik et al. [47] have shown a reduced anxiety in a step-down test after treatment with *Bifidobacterium longum* in a mice model of colitis and this supplementation was not effective in vagotomized mice. Similarly, vagotomy abolished the anxiolytic effect of a chronic consumption of a strain of *Lactobacillus rhamnosus* in the elevated plus maze test and open-field test in BALB/c mice [7].

These results raise the question of the importance of host genotype and its sensitivity to changes in the gut microbiota, postulated to occur through *P. acidilactici* supplementation [37,

38]. It has also been shown that there are links between host genotype and the composition of the gut microbiota both in mice [48] and birds [49]. The absence of intestinal microbiota leads to reduced anxiety in some strains [5,15,16] and an increase in other strains [4,17]. Crumeyrolle-Arias et al. [4] suggested that the microbiota would buffer the host's emotional reactivity, enhancing it in poorly reactive strains (such as Swiss) and lowering this reactivity in fearful strains (BALB/c).

The probiotic strain used could also explain the results differences between our two reactivity emotional tests (tonic immobility and open-field test). Indeed, a study that assessed the effect of two *Bifidobacteria* strains of probiotics on the anxiety of mice revealed a probiotic strain and behavioural test-dependent efficiency [50]. They showed that a strain of *Bifidobacterium breve* led to lower anxiety in the elevated plus maze test but had no effect in the open field test, whereas the results were the other way round when they used a strain of *B. longum*. Therefore, it is important to note that the animal model, the genetic line, the bacterial strain and the behavioural test can conduct to different effects of probiotic on emotional measures.

#### 4.2. Influence of the probiotic on memory

In the memory test, the global rates of errors were not different between the three days. This observation suggested that the test would enable to test short-term memory which is independent between days. Quail managed to complete the test in shorter time as the days progressed probably because they felt more confident and moved quickly in the device. Two different errors were distinguished: the immediate and the delayed errors. The rate of immediate errors was lower than the rate of delayed mistakes. Indeed, quail better remembered the cup visited just before, whereas it was more difficult to remember a succession of cups. There was no effect of the line on the two rates of errors, showing that the memory abilities of both lines would be equal. Quail supplemented with probiotics showed a lower rate of delayed errors in comparison to non-supplemented quail. This result supports the idea of an improvement of memory abilities of quail fed continuously from hatching with *P. acidilactici*.

These beneficial effects of a probiotic have already been observed in rodents. Indeed, supplementation of diabetic rats with a probiotic formulation containing *Lactobacillus acidophilus*, *Lactobacillus fermentum* and *Bifidobacterium lactis* improved alterations of spatial learning and consolidated memory in the Morris water maze [12]. Moreover, probiotic supplementation in mice with *Lactobacillus helveticus* in high-fat diet has also improved spatial memory in a Barnes maze test [44]. The provision of *B. longum* and *B. breve* to BALB/c mice made them discriminate two objects faster than control. Mice fed the *B. longum* made also fewer errors in the Barnes maze and got higher performances in a fear conditioning test [13].

## 5. Conclusion

The continuous supplementation with the probiotic *Pediococcus acidilactici* from the day of hatching provoked an improvement of the memory in both lines of quail genetically selected for tonic immobility. The improvement of memory did not appear to be related to a reduction in fearfulness in both lines. The present study suggests that the gut microbiota is able to modify emotional behaviour and memory in birds. According to the trait studied, these changes would depend on the host genotype, as it was previously observed in rodents.

## Conflict of interest statement

None

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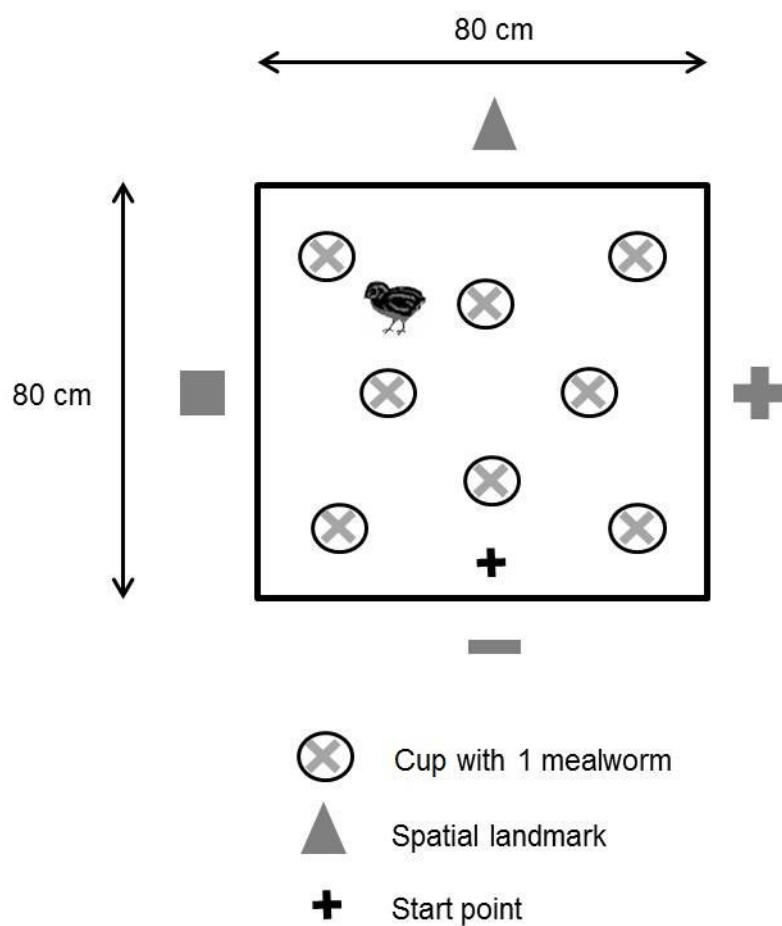
## Figures

Figure 1: Drawing of the device used for the memory test.

Figure 2: Effect of supplementation with a probiotic on tonic immobility duration of female quail of the two lines genetically selected on this criterion (LTI: Long Tonic Immobility, STI: Short Tonic Immobility) (Control group:  $n = 19$  LTI and 16 STI quail; Supplemented group:  $n = 18$  LTI and 16 STI quail). Results are presented as mean  $\pm$  SE. Letters were attributed for significantly different values  $a < b < c$  ( $p < 0.05$ ).

Figure 3: Effects of supplementation with a probiotic and days on time needed to complete the test of evaluation of the memory for LTI (Long Tonic Immobility) and STI (Short Tonic Immobility) female quail for the three days of test (Control group:  $n = 13$  LTI and 14 STI quail; Supplemented group:  $n = 15$  LTI and 15 STI quail). Results are presented as mean  $\pm$  SE. Letters were attributed for significantly different values  $a < b < c < d$  ( $p < 0.05$ ), regarding the day effect.

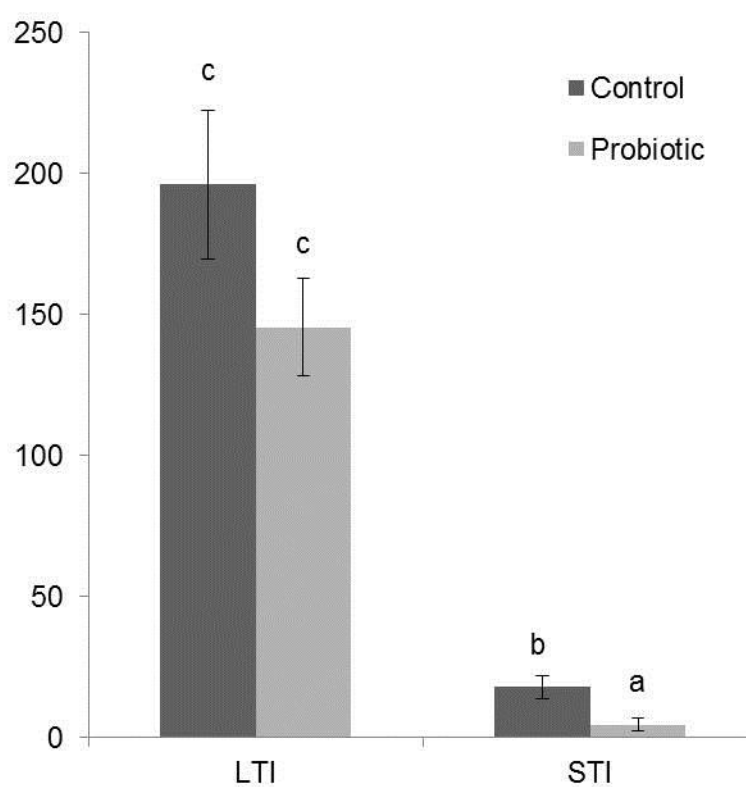
Figure 4: Effect of supplementation with a probiotic on the rates of immediate and delayed errors for LTI (Long Tonic Immobility) and STI (Short Tonic Immobility) female quail (mean for the three days of test) (Control group:  $n = 13$  LTI and 14 STI quail; Supplemented group:  $n = 15$  LTI and 15 STI quail). Results are presented as mean  $\pm$  SE. Letters were attributed for significantly different values  $a < b$  ( $p < 0.05$ ).



Figr-1



## Tonic immobility duration (s)

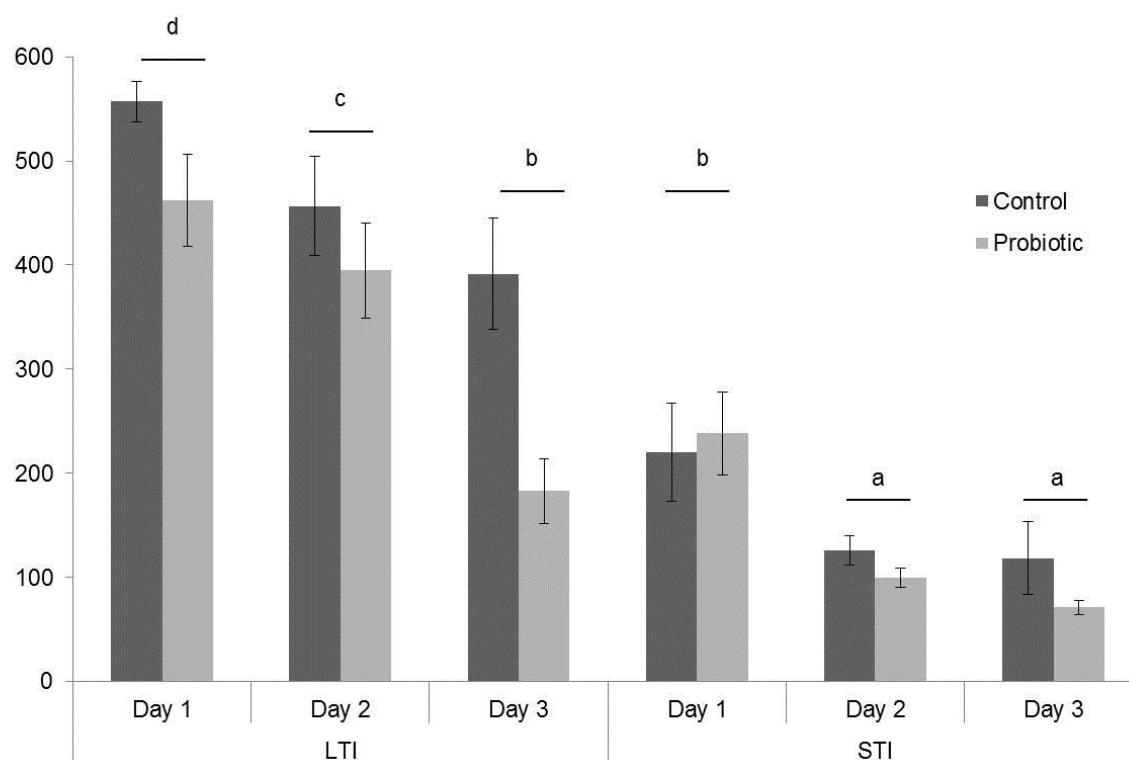


Figr-2

Fig-3

Total duration in the memory test (s)

Treatment effect: Probiotic < Control ( $p = 0.02$ )



Figr-4

Rate of errors

Error effect: Immediate < Delayed ( $p = 0.002$ )