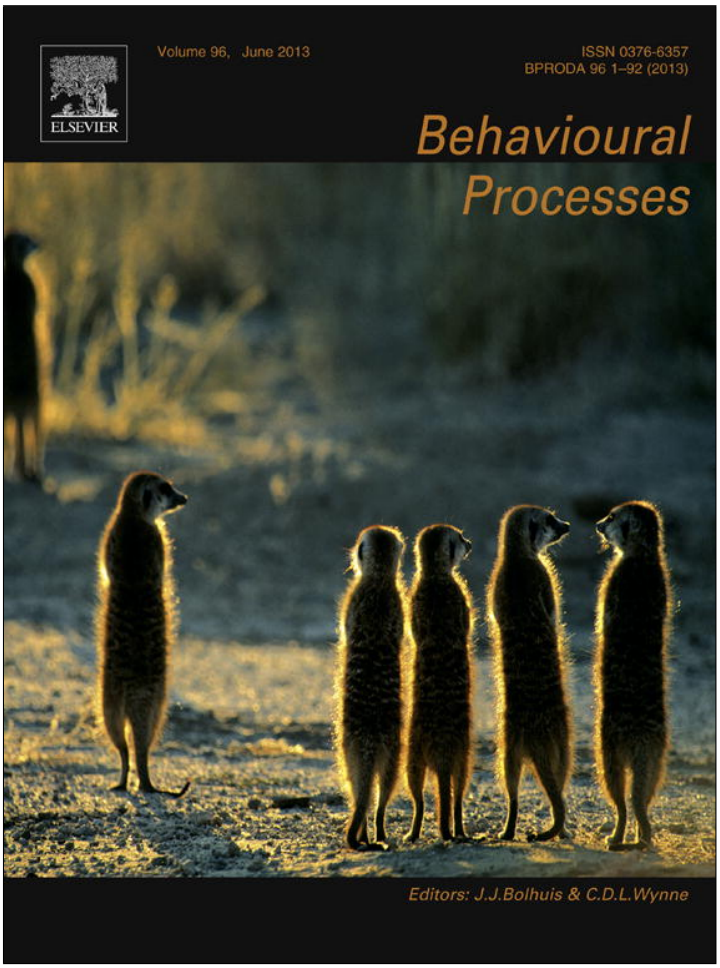

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Ingestion of *Mycobacterium vaccae* decreases anxiety-related behavior and improves learning in mice

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ABSTRACT

Coevolution of microbes and their hosts has resulted in the formation of symbiotic relationships that enable animals to adapt to their environments and protect themselves against pathogens. Recent studies show that contact with tolerogenic microbes is important for the proper functioning of immunoregulatory circuits affecting behavior, emotionality and health. Few studies have examined the potential influence of ambient bacteria, such as *Mycobacterium vaccae* on the gut–brain–microbiota axis. In this preliminary research, we show that mice fed live *M. vaccae* prior to and during a maze learning task demonstrated a reduction in anxiety-related behaviors and maze completion time, when tested at three maze difficulty levels over 12 trials for four weeks. Treated mice given *M. vaccae* in their reward completed the maze twice as fast as controls, and with reduced anxiety-related behaviors. In a consecutive set of 12 maze trials without *M. vaccae* exposure, treated mice continued to run the maze faster for the first three trials, and with fewer errors overall, suggesting a treatment persistence of about one week. Following a three-week hiatus, a final maze run revealed no differences between the experimentals and controls. Additionally, *M. vaccae*-treated mice showed more exploratory head-dip behavior in a zero maze, and *M. vaccae* treatment did not appear to affect overall activity levels as measured by activity wheel usage. Collectively, our results suggest a beneficial effect of naturally delivered, live *M. vaccae* on anxiety-related behaviors and maze performance, supporting a positive role for ambient microbes in the immunomodulation of animal behavior.

Coevolution of microbes, macrobiotic organisms and their animal hosts over the past 500 million years has resulted in the development of some symbiotic relationships that enable animals to adapt to the ambient environment and protect themselves against pathogens (Strachan, 1989; Chakrabarty, 2003; Tlaskalova-Hogenova et al., 2011; Rook, 2012). Such relationships involve bidirectional signaling between the gastrointestinal tract and the brain via neural, hormonal and immune interactions (Grenham et al., 2011). Recent work on communication between the brain–gut–microbiota axis using rodents (Berick et al., 2011; Grenham et al., 2011; Bravo et al., 2012), monkeys (Bailey et al., 2004), pigs (Barnes et al., 2012) and humans (Knowles et al., 2008; Khani et al., 2012) has deepened our understanding of how such symbiotic relationships can influence animal behavior. Studies with germ-free animals allow evaluation of the effects of the microbiota on the CNS; antibiotic studies provide insight on how use of broad-spectrum antibiotics can modulate the microbiome and affect behavior; infection studies show that enteric pathogens can induce anxiety-like behaviors in animals; probiotic studies show beneficial effects on the intestinal tract and improved behaviors associated with anxiety-related conditions (see Bravo et al., 2012). For example, Li and colleagues (2009) reported that alterations in the diversity of enteric bacteria influence memory and learning in mice, Clarke et al. (2012) found sex-specific regulation of hippocampal serotonin associated with anxiety using germ-free mice and Bravo et al. (2011) further demonstrated that *Lactobacillus rhamnosus* influences emotional behavior in mice through the GI tract with involvement of the vagus nerve and gamma-amminobutyric acid (GABA) system. This research provides evidence about how changes in the gut microbiota can lead to modification in CNS function with ramifications for behavior.

Homeostatic function and behavior, however, can be influenced not only by normal and disrupted enteric microbiota associations, but by organisms present in the ambient environment as well. Rook and Brunet (2002) have proposed and championed the "old friends" hypothesis as a way to explain the explosion of allergic, chronic inflammatory and autoimmune disorders present among people living in developed nations. They suggest that contemporary urban

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lifestyles have disrupted long established relationships during prenatal, neonatal and adulthood with coevolved organisms such as helminths, soil and water microbes, farm animals and pets that are typically recognized as harmless by the innate immune system and induce an anti-inflammatory response. Since allergies are mediated by T helper (T_H2) lymphocytes, and autoimmunity is mediated by T helper (T_H1/T_H17) lymphocytes, the immune dysregulation caused by lack of exposure to "old friends" likely involves disruptions not only in innate immunity but to the adaptive immune system as well. Such dysregulation of immunoregulatory circuits of the immune system may also potentially affect mood, cognitive function and behavior (Rook et al., 2003, 2012; Raison et al., 2010; Rook, 2012). These same homeostatic processes are likely important to the behavioral ecology of all mammals.

A microbe that has been the subject of several studies investigating the hypothesis that extant nonpathogenic organisms can improve behavioral health outcomes is *Mycobacterium vaccae*. *M. vaccae* is an aerobic bacterium found in temperate environments and animals are likely exposed to it through contact with water, soil and vegetation (Sneath et al., 1986; Gomez et al., 2001; Kazda et al., 2009). As an aerobe, it cannot colonize the anaerobic GI tract of animals and is thought of as a transient commensal (Rook and Brunet, 2005). *M. vaccae* was used in clinical trials in which terminal lung cancer patients were inoculated with heat-killed *M. vaccae.* Treated patients showed improved emotional health and general cognitive function (O'Brien et al., 2004). These findings led to speculation that an immune response to *M. vaccae* antigens might involve a ubiquitous neurotransmitter such as serotonin that plays a role in mood, arousal and learning (Leussis and Bolivar, 2006; Cools et al., 2007; Hohmann et al., 2007; Cifariello et al., 2008). Thus, an immune response to this microbe might positively impact behavior influenced by emotionality.

Examining this idea in a mouse model, Lowry et al. (2007) tested the hypothesis that peripheral exposure to *M. vaccae* antigen causes a T helper cell response that activates brain serotonergic systems in mice. Their research demonstrated that mice injected with heat-killed *M. vaccae* antigen experienced (1) a T_H1 and T regulatory cell biased immune activation of a subset of serotonergic neurons located in the dorsal raphae nucleus (DRI) of the brainstem and that project to the hippocampus and other forebrain regions, (2) elevated serotonin metabolism in the ventromedial prefrontal cortex, and (3) a reduction in stress-related emotional behavior in the forced swim test. Prior to this, Hunt et al. (2005) showed that heat-killed *M. vaccae* could influence immunocompetence through GI tract interaction in mice after administration by gavage.

Several researchers document the effect of immunomodulation on cognition and psychiatric disorders (Brynskikh et al., 2008; Miller, 2010; Yirmiya and Goshen, 2011). Integration of Lowry et al.'s (2007) findings and recent research on the nature of brain–gut–enteric microbiota interactions encouraged us to ask: Could ingestion of *M. vaccae* alter anxiety behavior and influence learning in mice? We hypothesized that if *M. vaccae* decreases stress response through an immune system activation of serotonin pathways, then mice that ingest *M. vaccae* may show superior complex maze performance and fewer anxiety-related behaviors than control mice.

1. General methods: all experiments

1.1. Animals

For all experiments, male, BALB/c specific pathogen free mice were obtained from Charles River Laboratories when they were about 38 days old, housed individually in an isolated animal room under a 12 h light/dark cycle and at a constant 25 °C temperature, and fed Carolina Biological Supply Company Mazuri rodent pellets (5663) (ad libitum). This mouse strain was used to maintain consistency with the mice used by Lowry et al. (2007). Each mouse was placed in an individual polycarbonate cage with a wire bar lid used to hold the water bottle and feed. Carefresh Natural Premium pet bedding, obtained from Carolina Biological Supply Company, was placed directly into the cage allowing the absorption of urine and the animal to burrow and/or den. To allow the mice to become acclimated to their new setting, the experiments were started when mice were 52 days old, and weighted approximately 21–25 g.

1.2. Ethical note

All animal experiments were conducted in accordance with the 2010 US Animal Welfare Act under animal use protocols (#01-2010 and #01-2011) and animal husbandry standard operating procedures approved by the Sage Colleges Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering. At the completion of each study animals were humanely euthanized using CO_2.

1.3. M. vaccae

M. vaccae (15,483) was purchased from the American Tissue Cell Culture (ATCC) and stored at 5 °C until reconstituted. *M. vaccae* was grown in nutrient broth for four days at 37 °C and stored in a refrigerator until needed. Aliquots of 0.1 mL, containing approximately 4.5×10^6 CFU/mL (determined by a standard plate count) was applied to the food vehicle of treatment mice, as appropriate.

1.4. Food vehicles

All mice were denied food for 24 h before administration of food vehicles. The food vehicle given the experimental mice consisted of a piece of white *Wonder* bread (produced by Hostess Brands), approximately 1 cm × 1 cm, onto which 0.1 mL of *M. vaccae* was aseptically pipetted. The bread was coated on the same side with a thin layer of store brand creamy peanut butter to increase palatability. Control mice received a food vehicle like that given the experimental animals (1 cm × 1 cm square of white bread coated with peanut butter), but which lacked *M. vaccae*. Treatment mice in experiments 2 and 3 received a food vehicle identical to that given the control mice, i.e. it lacked *M. vaccae*.

2. Experiments 1–3: Complex maze experiments

2.1. Methods

2.1.1. Sample

In experiments 1–3, ten mice constituted the treatment group and eight mice constituted the control group. The same mice were used through the progression from experiments 1 to 3.

2.1.2. Complex maze

A Hebb–Williams style complex maze was used in this study (Fig. 1). This type of maze is widely used in measuring spatial learning tasks and working memory with rodents (Shore et al., 2001; Parle et al., 2006). This maze operates on appetitive rather than aversive principles.

The mice were tested in a maze free of bedding or other materials. The maze was a square Plexiglas box (14 cm high, 45 cm × 45 cm) consisting of five rows, 9 cm wide, with five door openings, 8 cm wide. The start box was 9 cm × 13 cm in size. Eight turns are required to reach the end point of the unobstructed maze.

Three levels of maze difficulty were used in experiment 1. Each successive level involved additional turns and openings and longer

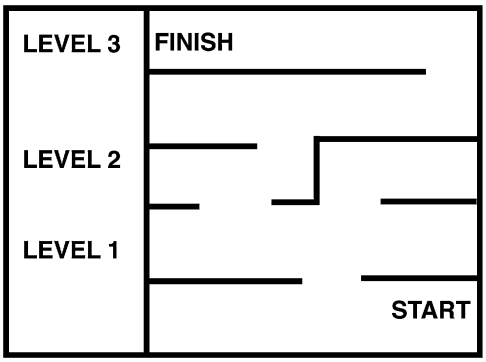

Fig. 1. Illustration of the maze layout. Mice were placed in the maze at the start box, and rewards were placed at the finish of level 1, level 2 or level 3 as appropriate. (Level 3 finish is shown here.)

maze run length. This was accomplished by closing off pertinent door openings with Plexiglas barriers that were attached to the top of the maze walls with small clips. Level 1 total maze run distance was 54 cm, level 2 was 64 cm and level 3 was 136 cm.

For level 1, barriers blocked off the two door openings in the second row of the maze. This required that a minimum of two turns to be made to reach the food reward. For level 2, barriers were removed from the second row of the maze that established level 1, and a barrier was instead placed in the door opening of the third row of the maze. This required that a minimum of four turns be made to reach the food reward. For level 3, all barriers were removed and the maze was completely unobstructed, requiring a minimum of eight turns to reach the food reward. Level 3 of the maze was used in all testing in experiments 2 and 3. After each mouse was tested, the maze was sanitized with 70% alcohol, rinsed with water and allowed to dry completely.

2.1.3. Anxiety-related behaviors

Mice demonstrate a variety of anxiety-related behaviors (Blanchard et al., 2001, 2003; Leussis and Bolivar, 2006; Bailey and Crawley, 2009; Gould, 2010; Smolinsky et al., 2010). Seven separate anxiety-related behaviors (Table 1) were observed and scored.

2.1.4. Analysis

Maze trials were videotaped (SONY Handicam DCR-HC85). An experienced observer blind to the treatment for each mouse assignment scored the anxiety-related behaviors from the tapes. All statistical analyses used IBM Statistical package for the Social Sciences (SPSS), version 20, and all reported values are means and standard errors of the means (S.E.M.). Comparisons of two independent means were made using a two-tailed t-test ($P < 0.05$). Comparisons among means in experimental designs with multiple between subjects factors were analyzed using analysis of variance (ANOVA, $P < 0.05$) followed, when appropriate, by post hoc analysis using pairwise comparisons with Bonferroni corrections. Comparisons of within-subjects factors were performed using repeated measures analysis of variance ($P < 0.05$) followed, where appropriate, by post hoc pairwise comparisons using Bonferroni corrections. For experiments 1–3, a different observer rescored 8% of the tests for an interobserver reliability estimate of 92% for the scored anxiety-related behaviors. Maze running errors were scored as the total number of three types of errors: wrong turns moving forward; backtracking; and direct return to start box (Winocur and Moscovitch, 1990; Devan et al., 2006).

Table 1
Ethogram of anxiety-related behaviors.

Behavior pattern	Description
1. Defecation	Number of fecal boli released per trial
2. Elongation	Mouse moves with a low-back, stretched posture or movement while keeping hind feet stationary; number of events per trial counted
3. Grooming	Mouse uses front paws to rub face and whiskers; number of events per trial counted
4. Immobilization	Mouse remains motionless for three or more seconds; number of events per trial counted
5. Latency to start	Number of seconds mouse spends in start box after initial placement until hindquarters cross the start box boundary to the maze
6. Return to start	Mouse moves from the start box to other area of the maze and then returns to start box and remains for at least a second; number of events per trial counted
7. Wall climbing	Mouse puts both front paws on maze wall while on hind legs; all events within 15 s period were scored as one event

3. Details of individual experiments

3.1. Experiment 1: Effect of M. vaccae

3.1.1. Methods

To determine whether mice that ingested live *M. vaccae* perform differently in a maze than control mice, experimental mice ($N = 10$) were immunologically primed by placing a food vehicle on the wire bar lid of their cages on two occasions: 21 days and 7 days prior to the start of maze testing in experiment 1. Since *M. vaccae* was incorporated into the food reward of the experimental mice at the finish of each maze run, those mice received additional *M. vaccae* during the 12 maze trials of experiment 1. Control mice were given a food vehicle on days −21 and −7 as well, but it lacked *M. vaccae*. Likewise, the food rewards at the finish of the maze runs of control mice lacked *M. vaccae*.

Maze testing was a repeated measures design at three levels of maze difficulty. All mice were tested during each trial and all testing occurred on Sunday, Tuesday and Thursday. Four trials were conducted at level 1, one each on Sunday, Tuesday, Thursday, and Sunday. Likewise, four trials were conducted at level 2, one each on Tuesday, Thursday, Sunday and Tuesday. Finally, four trials were conducted at level 3 of the unobstructed maze, one each on Thursday, Sunday, Tuesday and Thursday. This resulted in 12 trials over a 4-week period.

Start time was recorded when all four paws of the mouse touched the floor of the maze. Completion time for the maze was scored when the mouse ate the food reward for a full three seconds to ensure commitment to ingestion. The order in which individuals from the experimental and control groups were tested was alternated each time a mouse was tested. Time to finish the maze was recorded and demonstrated anxiety-related behaviors were scored.

3.1.2. Results

3.1.2.1. Maze run time. Twelve trials of testing at three maze difficulty levels revealed that mice that ingested *M. vaccae* completed the maze twice as fast ($X = 55.2 \pm 10.6$ s, $N = 10$) as control mice ($X = 116.8 \pm 11.8$ s, $N = 8$). A repeated measures ANOVA showed that ingestion of *M. vaccae* had a significant effect on the time it took for experimental and control mice to complete the maze (ANOVA: $F_{1,16} = 15.08$, $P = 0.001$). A main effect by maze level was observed as well. Mauchly's test indicated that the assumption of sphericity had been violated, $\chi^2 = 16.3$, $P < 0.05$, therefore degrees of freedom were corrected using Greenhouse–Geisser estimates of sphericity ($\varepsilon = 0.6$), (ANOVA: $F_{1.2,\ 19.2} = 30.26$, $P = 0.0001$). Bonferroni post hoc tests showed that performance differed at levels 1 and 2 of the maze ($M_{diff} = 91.65$, 95% CI [49.79, 133.52]), and at levels 1 and 3 of the maze ($M_{diff} = 95.93$, 95% CI [49.79, 142.1.]), but not at levels 2 and 3 of the maze (Fig. 2). The group treatment by maze level

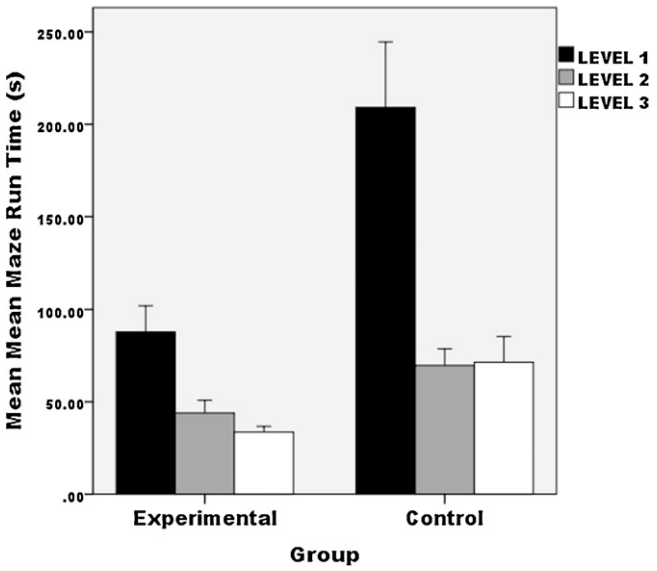

Fig. 2. Comparison of the effect of ingestion of *M. vaccae* on maze run time at each level of the complex maze in experiment 1. Experimental mice completed the maze faster than control mice at each level of the maze with the largest difference in performance seen at level 1.

interaction was also significant (ANOVA: $F_{1.2,19.2} = 6.96$, $P = 0.013$). Post hoc comparison of means and confidence interval between the two groups at each level revealed that while experimental mice performed better than the control mice at all levels of the maze, their performance at level 3 was not different than their performance at level 2 (Table 2).

3.1.2.2. Anxiety-related behaviors. A mixed measures ANOVA with Bonferroni corrections was performed for each of the anxiety behaviors, with the group (experimental [$N = 10$] versus control [$N = 8$]) as the between-subjects independent variable, the maze level (level 1 versus level 2 versus level 3) as the within-subjects variable, and the duration or count or the anxiety behaviors as the dependent variables. For all of the behaviors except return to start, Mauchly's tests of sphericity were violated and the degrees of freedom were corrected using Greenhouse–Geisser estimates of sphericity (ε). Table 3 presents the main effects. The main effect of maze level was observed for all seven behaviors. Four behaviors exhibited treatment (group) effects: elongation, immobilization, grooming and latency to start. A maze level by treatment interaction was shown for three behaviors: immobilization, grooming and latency to start.

Table 2
Summary of repeated measures ANOVA multiple comparisons of *M. vaccae* treatment by maze level for maze run time in experiment 1.

Maze	Level 1 *X* (SEM) 95% CI	Level 2 *X* (SEM) 95% CI	Level 3 *X* (SEM) 95% CI
Exp	87.9_a(23.5) [38.05, 137.7]	44.0_b(7.4) [28.25, 59.65]	33.6_b(8.6) [15.47, 51.78]
Control	209.1_c (26.3) [153.39, 264.8]	69.7_a (8.3) [52.16, 87.27]	71.5_a (9.6) [51.17, 91.76]

Note. CI = confidence interval. Different subscript letters indicate statistically significant differences $P < 0.05$.

Anxiety-related behaviors for both experimentals and controls decreased from maze level 1 to maze level 3. Post hoc comparisons are reported only for the behaviors which had both a significant group effect and maze level effect. The most common pattern was a decrease in anxiety-related behaviors between levels 1 and 2, with no significant differences between levels 2 and 3. Bonferroni post hoc tests showed that both the experimentals and the controls exhibited significantly fewer immobilizations between maze level 1 and maze level 2 ($X_{diff} = 0.70$, 95% CI [0.214,1.17], $P = 0.004$), and maze level 3 ($X_{diff} = 0.63$, 95% CI [0.110,1.153], $P = 0.015$), which were not significantly different from each other. Likewise, for latency to start, both the experimentals and the controls exhibited significantly fewer immobilizations between maze level 1 and maze level 2 ($X_{diff} = 63.22$, 95% CI [29.90, 96.55], $P = 0.0001$), which were not significantly different from each other. This pattern was the same for the grooming behavior (levels 1–2: $X_{diff} = 0.38$, 95% CI [0.056, 0.71], $P = 0.019$; levels 1–3: $X_{diff} = 0.49$, 95% CI [0.07, 0.90], $P = 0.019$). For elongation a different pattern emerged: elongations at level 1 and 3 ($X_{diff} = 2.92$, 95% CI [2.04, 3.81], $P = 0.0001$) and at level 2 and 3 ($X_{diff} = 2.10$ 95% CI [0.95, 3.25], $P = 0.0001$) were significantly different, but not between levels 1 and 2. For return to start, wall climbing and defecation, the pattern of decline was mixed for both experimentals and controls in terms of whether a significant difference from level 1 was observed at level 2 first or not until level 3, and the group effect was not significant for these behaviors.

Table 3
Summary of main effects and interaction effects for anxiety behaviors in experiment 1.

Behavior	Main effects		Interaction
	Level	Group	
Immobilization	$F_{1.17,18.7} = 11.88$ $P = 0.002$	$F_{1,16} = 12.99$ $P = 0.002$	$F_{1.17,18.7} = 12.96$ $P = 0.03$
Grooming	$F_{1.26,20.11} = 8.99$ $P = 0.005$	$F_{1,16} = 11.02$ $P = 0.004$	$F_{1.26,20.11} = 6.02$ $P = 0.018$
Latency to start	$F_{1.01,16.19} = 23.7$ $P = 0.001$	$F_{1,16} = 12.99$ $P = 0.002$	$F_{1.01,16.19} = 7.49$ $P = 0.014$
Elongation	$F_{1.51,24.11} = 22.61$ $P = 0.001$	$F_{1,16} = 5.84$ $P = 0.03$	
Return to start	$F_{2,32} = 9.38$ $P = 0.001$		
Wall climbing	$F_{1.48,23.70} = 5.50$ $P = 0.02$		
Defecation	$F_{1.39,22.31} = 8.6$ $P = 0.004$		

Table 4
Summary of repeated measures ANOVA multiple comparisons of *M. vaccae* treatment by maze level for anxiety behaviors in experiment 1.

Maze Behavior	Level 1 *X* (SEM) 95% CI	Level 2 *X* (SEM) 95% CI	Level 3 *X* (SEM) 95% CI
Immobilization			
Exp	0.2_a(0.3) [−0.30, 0.75]	0.0_b (0.0) [−0.8, 0.8]	0.03_b (0.1) [−0.15, 0.20]
Control	1.3_c (0.3) [0.73, 1.9]	0.2_a (0.0) [0.6, 0.25]	0.3_a (0.1) [0.6, 0.44]
Grooming			
Exp	$0.2_{a,d}$ (0.2) [−0.27, 0.57]	$0.1_{a,d}$ (0.1) [−0.11, 0.31]	0.1_a (0.1) [−0.12, 0.22]
Control	1.1_b(0.2) [0.70, 1.60]	0.4_c(0.1) [0.18, 0.64]	$0.3_{a,c,d}$ (0.1) [0.07, 0.44]
Latency to start			
Exp	31.5_a (17.5) [−5.52, 68.57]	$3.8_{b,e}$ (2.0) [−0.34, 8.0]	3.5_b (1.8) [−0.33, 7.33]
Control	108.2_c (19.5) [66.80, 149.64]	9.5_d (2.2) [4.82, 14.15]	$8.1_{d,e}$(2.0) [3.78, 12.35]

Note. CI = confidence interval. Different subscript letters indicate statistically significant differences $P < 0.05$.

The treatment by maze level interaction was significant for three of the behaviors: immobilization, grooming and latency to start (Table 4). These three behaviors show the pattern of performance in which controls at maze levels 2 and 3 approximate that of experimentals at maze level 1. The significant interaction indicates that the difference between the groups in these anxiety-related behaviors due to treatment was present at maze level 1 but not at levels 2 or 3 of the maze. Although controls exhibited more returns to start, wall climbing and defecation than experimentals at each maze level, these differences were not significant in interaction across the maze levels. By the third level of the maze, the controls were showing fewer anxiety-related behaviors, approximating the lower levels of anxiety behaviors expressed by the experimentals at the first two levels. The measure of latency to start appeared to be the best indicator of initial anxiety for the experimentals and the controls, with a large mean differences at maze level 1 compared to maze levels 2 and 3. While experimentals showed significantly less hesitation to start than controls at each level, this was the only behavior in which the controls at both levels 2 and 3 exhibited less of the particular anxiety behavior the experimentals at level 1.

The patterns of reduced run time and reduced demonstration of anxiety-related behaviors were similar for both experimentals and controls across the three levels of the maze. This suggests that as anxiety-related behaviors decline, running speed improves.

3.1.2.3. Errors. Mice treated with *M. vaccae* demonstrated fewer errors ($X = 3.7 \pm 0.6$, $N = 10$) across all three levels during maze runs than the control mice ($X = 4.6 \pm 0.7$, $N = 8$), but these differences were not statistically significant.

4. Experiment 2: *M. vaccae* removal

4.1. Methods

To determine what would happen to complex maze performance and anxiety-related behaviors when *M. vaccae* was no longer administered, both experimental and control mice were tested only at level 3 of the maze without *M. vaccae* in the food reward. To maintain the same maze testing schedule that was used in experiment 1, experiment 2 began three days following the last test day of experiment 1. All mice were subsequently tested three times a week for four weeks, yielding a total of 12 trials. Time to finish the maze and demonstrated anxiety-related behaviors were recorded as for experiment 1.

4.2. Results

4.2.1. Maze run time

In the consecutive set of 12 trials at level 3 of the complex maze without *M. vaccae* in the food reward, experimental mice continued to complete the maze twice as fast ($X = 21.6 \pm 10.1$ s, $N = 10$) as the control mice ($X = 47.0 \pm 11.3$ s, $N = 8$). A repeated measures ANOVA over the course of the 12 trials, however, revealed that these differences were not statistically significant. Further analysis of differences in the maze performance of experimental ($X = 18.8 \pm 6.3$ s, $N = 10$) and control mice ($X = 50.1 \pm 7.1$ s, $N = 8$) at trials 1 and 2 (ANOVA: $F_1 = 15.33$, $P = 0.001$) and experimental ($X = 26.0 \pm 6.3$ s, $N = 10$) and control mice ($X = 49.5 \pm 7.0$ s, $N = 8$) at trials 1, 2 and 3 (ANOVA: $F_{1,2} = 6.92$, $P = 0.018$) revealed that experimental mice completed the maze faster than control mice and that these differences were statistically significant. At trials 4–12 of maze testing, however, statistically significant differences in the maze run time of the two groups were not observed.

4.2.2. Anxiety-related behaviors

Repeated measures ANOVA for each of the seven anxiety-related behaviors indicated that experimental ($X = 0.1 \pm 0.04$, $N = 10$) and control mice ($X = 0.3 \pm 0.05$, $N = 8$) differed significantly from one another in only one behavior, grooming ($F_{1,16} = 10.73$, $P = 0.005$).

4.2.3. Errors

Experimental mice demonstrated fewer errors ($X = 2.2 \pm 0.6$, $N = 10$) than control mice ($X = 4.1 \pm 0.7$, $N = 8$) during the 12 trials of maze testing. Analysis of these results show that mice who previously ingested *M. vaccae* displayed significantly fewer errors than control mice (ANOVA: $F_{1,16} = 4.53$, $P = 0.049$). There was no main effect of trial number, or group by trial interaction. This indicates that even though experimental mice were not running faster than control mice over the course of the 12 trials of testing at level 3, they were making less errors in the maze than the control mice.

5. Experiment 3: Strength of memory

5.1. Methods

To determine how well mice remembered the maze pattern, all mice were rested for three weeks and one final maze test was conducted at level 3, seven weeks after the experimental mice had last been exposed to *M. vaccae*. No *M. vaccae* was administered in the food reward at this time. Time to finish the maze and demonstrated

anxiety-related behaviors were recorded as for experiments 1 and 2.

5.2. Results

The experimental mice completed the maze faster ($X = 12.9 \pm 3.0$ s, $N = 10$) than control mice ($X = 20.0 \pm 4.6$ s, $N = 8$), and with fewer anxiety-related behaviors ($X = 0.8 \pm 0.2$, $N = 10$) than control mice ($X = 1.1 \pm 0.3$, $N = 8$). However, these differences were not statistically significant. Similarly, the experimental mice demonstrated fewer errors ($X = 1.9 \pm 0.6$, $N = 10$) during maze runs than the control mice ($X = 2.4 \pm 0.6$, $N = 8$), but the differences in errors were not statistically significant.

6. Experiment 4: Elevated Zero Maze

6.1. Methods

To evaluate the effects of *M. vaccae* treatment on anxiety-related behaviors in addition to those measured during a complex maze learning task, an elevated zero maze was employed. The elevated zero maze (EZM) examines anxiety behaviors based on the premise that mice have an aversion for open, more illuminated spaces (Jonas et al., 2010) and allows for exploration uninterrupted by a central space, such as in the elevated plus maze (Shepherd et al., 1994; Walf and Frye, 2007; Braun et al., 2011).

6.2. Subjects

Forty-one mice were divided into a control group ($N = 11$) who did not receive *M. vaccae* in their food vehicle and three treatment groups ($N = 30$) of 10 mice each. Mice in the treatment groups all received *M. vaccae* in their food vehicle but differed from one another in the time of testing in the EZM following their last *M. vaccae* treatment: 12 h ($N = 10$), 18 h ($N = 10$) or 24 h ($N = 10$).

6.3. Elevated zero maze

The maze (Med Associates, St. Albans, VT) consisted of a circular platform (7.0 cm wide with a 45.5 cm inner diameter) that was elevated 64.5 cm above the floor. It was equally divided into two closed quadrants and two open quadrants. The two closed areas had walls on both sides that were 20.5 cm in height. The open areas lacked walls, but were bordered by a narrow lip of clear plastic (0.5 cm high) to diminish the likelihood of mice falling onto the floor. The activity of the mice was monitored by an overhead camera and scored by visual observation of behaviors.

6.4. M. vaccae exposure

Experimental mice were exposed to *M. vaccae* using the immunological priming schedule utilized in experiment 1. Experimental mice were given a food vehicle on the wire lid of their cages which contained *M. vaccae* on three occasions: 3 weeks before maze testing, 1 week before maze testing and on the day before EZM testing. Control mice were given a food vehicle on the same schedule as experimental mice, but which lacked *M. vaccae*.

6.5. EZM testing protocol

EZM testing occurred in a room separate from the home colony and under dim light (approximately 70 lux) using a protocol described by Walf and Frye (2007). Each mouse was transported to the testing room and placed within the closed area of the EZM at the boundary to the open area, facing inward. EZM testing lasted for 5 min. Placement in each of the two closed areas of the maze was alternated between subjects. After each mouse was tested, the maze was sanitized with 70% alcohol, and allowed to dry completely.

We scored three behaviors from the EZM trials: number of entries into the open maze area, time spent in the open area, and the number of head dips from the closed area and from the open area. Head dips are a standard ethological measurement used in EZM testing indicative of motivation to explore and risk assessment (Shepherd et al., 1994; Bourin et al., 2007; Walf and Frye, 2007). Entry into the open area of the maze was recorded when all four paws were in the open area. Time spent in the open area was scored as the percentage of time that mice spent with all four paws in the open area. Head dips are defined as the mouse looking over the edge of the maze by arching the neck and pointing the nose down toward the floor. Head dip from the closed area was scored when one or more paws remained in the closed area while the mouse looks over the edge. Head dip from open area was scored when the mouse had four paws in the open area while looking over the edge of the maze.

6.6. Analysis

Experiment 4 zero maze trials were videotaped using the same equipment as in experiments 1–3. An experienced observer who was blind to the treatment assignment scored the entries into the open area, time spent in the open area and head dipping behaviors from the tapes.

6.7. Results

There were no statistically significant differences between experimentals and controls in the time spent in the open area and in the number of entries into the open area from the closed area. Mice did differ in the number of combined head dips (combined open and closed area head dips) (ANOVA: $F_{3,41} = 3.01$, $P = 0.042$). Post hoc pairwise comparisons revealed that there was a significant difference in the head dipping behavior of the 12 h group ($X = 13.0 \pm 1.55$, CI [9.86,16.14]) and the control group ($X = 8.50 \pm 1.62$, CI [5.21, 11.79]), but no significant differences between the 18 h and 24 h groups.

7. Experiment 5: activity testing

7.1. Methods

7.1.1. Subjects

Fifteen mice (8 = treatment, 7 = control) were individually housed in home cages with standard running wheels. Rotation of the wheels was recorded by magnetically activated counters (Mini Mitter, Respironics Company).

7.1.2. M. vaccae exposure

Experimental mice were exposed to *M. vaccae* at three different times: on the day before the wheels were released (day zero), on day 14 and on day 21.

7.1.3. Activity testing protocol

Mice were acclimated for two weeks within the activity cages with the wheels immobilized. On the day following the first exposure of experimental mice to *M. vaccae*, the wheels were released. Running activity was collected from the counters daily for the next 23 days, and the mean km distance traveled/day was calculated.

7.1.4. Results

The mean daily distance traveled by the experimental mice ($X = 6.3 \pm 1.4$ km, $N = 8$) did not significantly differ from the control mice ($X = 6.8 \pm 1.7$ km, $N = 7$) over the 23 days of activity testing.

8. Discussion

This research shows that ingestion of live *M. vaccae* prior to and during a complex maze learning task (experiments 1–3) reduced maze run time and anxiety-related behaviors in BALB/c mice. Four of the seven measured anxiety-related behaviors, immobilization, grooming, latency to start and elongation, were significantly different in the *M. vaccae* treated group as compared to the control group (Table 3). These effects do not appear to be due to differences in generalized activity levels related to treatment with *M. vaccae*. Experiment 5 did not show differences in wheel running activity due to treatment with *M. vaccae*. Additional evaluation of anxiety using a standard anxiety-testing maze, EZM, revealed only differences in one measure: head dipping.

In complex maze experiment 1, mice given *M. vaccae* showed superior performance compared to controls with both faster maze run time and reduced expression of anxiety-like behaviors. Although maze level effects were significant overall, the primary difference was seen at level 1 for both running time and anxiety behaviors. The difference in performance of the two groups of mice was greatest during early exposure to the maze when the novelty of the task might have been most anxiety-provoking to these animals. The behavior latency to start reveals hesitation to exit the start box and enter the maze. The experimentals entered the maze more readily than the controls at every level. Even though at level 1 both experimentals and controls exhibited much more hesitation than they did at levels 2 and 3, experimentals were approximately three times less hesitant than controls at each maze level (Table 4). These results indicate that *M. vaccae* treatment may have abated anxiety in the experimentals which affected both motivation to run through the maze and expression of anxiety-related behaviors. The other three behaviors for which there was a significant group effect (immobilization, grooming and elongation) showed a similar pattern. The experimentals and controls did not differ in errors at each level, and the run time of the two groups was similar by level 3 despite the fact that the maze difficulty increased at each maze level. Therefore the performance differences across the levels within each group may be primarily due to increasing familiarity with the maze. The main outcome of this experiment is that *M. vaccae* treated mice showed superior maze run time and diminished anxiety compared to the controls, and that difference was most pronounced in early maze trials.

The reduction of anxiety-related behaviors resulting from *M. vaccae* ingestion may have allowed more rapid complex maze investigation, resulting in reduced run time once the maze is learned at each maze level. If we consider these results in the context of the findings of Lowry et al. (2007) who demonstrated that injected *M. vaccae* antigen stimulates brain serotonin production and decreases stress-related behaviors in mice for a short period of time in a forced swim test, then it seems feasible that ingestion of live *M. vaccae* may stimulate serotonin production through immunological mechanisms. This suggests that ingested *M. vaccae* may upregulate DRI serotonergic neurons that modulate stress responsive behaviors. Plasticity in the stress response influencing cognitive behavior can be modulated through a T cell response. While many studies (Grenham et al., 2011; Bravo et al., 2012; Clarke et al., 2012) show that microbiota can activate innate and adaptive immune mechanisms that influence anxiety and behavior, our research indicates that ingestion of an ambient bacterium which is not part of the enteric microbiome may have this same beneficial effect.

In a consecutive set of 12 trials without *M. vaccae* treatment at level 3 of the maze (experiment 2), experimental mice continued to run the maze faster and with less anxiety-related behaviors than control mice. This pattern was only statistically significant, however, during the first three trials of maze testing in this experiment. It appears that while *M. vaccae* ingestion had an effect on maze performance of experimental mice for about a week after *M. vaccae* removal, this effect was not long lasting. Among the test behaviors, a significant group difference was only observed for grooming. This indicates that anxiety-behaviors were no longer influencing maze performance. However, there were significant differences in the number of maze navigational errors with control mice demonstrating two times as many errors as experimental mice. The experimental mice may have remembered the complex maze pattern better than the controls for the 4-week duration of experiment 2. Lastly, following a three-week rest period (experiment 3), a final trial revealed no statistically significant differences in run time, anxiety-related behaviors or errors between the two groups. The similarities in maze performance and demonstrated anxiety-related behaviors may have occurred because, after 25 maze trials, both groups of mice knew the maze pattern equally well.

Although our study did not aim to elucidate the underlying neuroimmunological mechanisms potentially responsible for the results we observed, those mechanisms must interact with several higher order behavioral systems. *M. vaccae* treatment may have increased motivation to run the maze due to a facilitatory interaction with rewarding properties inherent in maze exploration itself, the action of running, and/or to an acutely rewarding feature of the *M. vaccae* in the food vehicle given at the end of each maze trial in experiment 1. Acquisition of conditioned cues may have been heightened by the treatment resulting in increased performance times and anxiety reduction which would then have facilitated familiarization with the maze. In experiment 1, after every four trials, the maze complexity and distance to complete the maze were increased. Both the experimental mice and control mice exhibited fewer anxiety-related behaviors with each subsequent level of the maze despite increasing complexity. However, the experimentals continued to exhibit fewer anxiety-related behaviors than the control mice at each new level of the maze. A further consideration is the level of stress reactivity exhibited by various mouse strains. BALB/c is considered to be a stress reactive mouse strain (Palumbo et al., 2009). *M. vaccae* treatment might produce a different outcome relative to the stress profile of the strain.

In order to examine the effect of ingested *M. vaccae* on anxiety behavior in a non-cognitive task, we observed performance in an elevated zero maze (experiment 4). The experimental and control groups did not differ in their willingness to enter the open areas of the maze whether tested 12, 18 or 24 h after the last exposure to ingested *M. vaccae* or the placebo. However, the experimental and control groups did differ in head dip behavior in the 12 h test. As reviewed in Shepherd et al. (1994), including ethological measures of behavior indicative of risk assessment and exploration add clarity and reliability to the standard measures of open arm entries and time spent, elucidating anxiolytic and anxiogenic drug effects. In experiment 4, we observed significant differences in the exploratory risk assessment behavior of head dipping. Head dipping in elevated mazes is commonly considered to be an exploratory movement (Bourin et al., 2007) with increased head dips being indicative of decreased anxiety (Braun et al., 2011). Head dipping in the EZM may be related to exploration and risk assessment involved in "looking for an escape route" behavior. In this way it may be related to behaviors required for exploring, spatially navigating, and learning a complex maze such

as that used in experiments 1–3. Thus, *M. vaccae* treatment may affect the motivational system involved in exploration of novel and potentially threatening environments, and enhance spatial memory. Moreover, the results of experiment 5 indicate that the behavioral differences observed in mice treated with *M. vaccae* versus controls in experiments 1–4 were likely not due to effects on basic activity level. It should be noted, however, that the small sample size used in this experiment may not have provided enough power to detect a treatment effect of *M. vaccae* exposure on activity. Future research should be conducted to answer this question more fully.

Raison et al. (2010) speculate that the mammalian microbiome plays a critical role in the development of the immune system and the maintenance of human health. Saprophytic mycobacteria are common in the environment (Kazda et al., 2009) and while they do not replicate in the gut, were likely to have always been present in the gastrointestinal tract of our ancestors due to contact with mud and water (Rook, 2010). Repeated, long-term exposure to *M. vaccae* could serve as an adaptive mechanism for the development of tolerance responses to stressful situations. Further, such a scenario dovetails with the old friends hypothesis (Raison et al., 2010). Thus, by upsetting the long established relationship of our immune system to ambient bacteria such as *M. vaccae*, complex behaviors such as learning could be negatively affected through the dysregulation of immunoregulatory responses coupled to the neuromodulation of emotionality.

While recent research has shed light on the ability of the microbiota to influence behavior via neural, hormonal and immune interactions (Li et al., 2009; Bravo et al., 2011; Clarke et al., 2012), it is surprising to think that a common ambient microbe may modulate anxiety behaviors. Our research provides initial data suggesting that ingestion of live *M. vaccae* can reduce anxiety behaviors related to exploration of novel environments, and exert a previously unreported influence on learning in mice. This effect on behavior was fast acting, observed by the first maze run trial, but the effect diminished with *M. vaccae* removal. This suggests that *M. vaccae* may act as a kind of pharmabiotic, inducing short-term physiological changes affecting behavior.

The impact of exposure to microbes on animal behavior in natural environments is unknown. We could hypothesize, for example, that differential exposure to microbes such as *M. vaccae* may influence the expression of behavioral phenotypes related to being a "wanderer" or a "resident" (as in prairie voles, *Microtus ochrogaster*, Getz et al., 1993; Solomon and Jacquot, 2002; Ophir et al., 2008) with implications for spatial exploration related to differential reproduction. Research that incorporates a behavioral ecological perspective on brain–gut–microbe interactions is necessary to understand the underlying mechanisms that shape the evolution of those interactions. Our results contribute preliminary evidence of an adaptively significant behavioral response of mice to *M. vaccae* ingestion that could have arisen from the coevolution of mammalian neuroimmunological systems and ambient microbes.

Conflict of interest statement

All authors declare that there are no conflicts of interest.

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