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**Treating generational stress: Effect of paternal stress on offspring memory and extinction development is rescued by probiotic treatment**

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## **Abstract**

Early-life adversity is a potent risk factor for mental health disorders in exposed individuals, with effects of adversity exhibited across generations. Such adversities are also associated with poor gastrointestinal outcomes, with emerging evidence suggesting that microbiota-gut-brain interactions may mediate the effects of early-life stress on psychological dysfunction. In the present study we investigated novel generational effects of an early-life stressor administered to infant males (maternal separation) on conditioned aversive reactions in their subsequent male infant offspring. We demonstrated, for the first time, longer-lasting aversive associations and greater relapse after extinction in the offspring (F1 generation) of maternally-separated rats (F0 generation). Importantly, these generational effects were reversed by probiotic supplementation, which was effective as both an active treatment when administered to infant F1 rats, and as a prophylactic when administered to F0 fathers before conception (i.e., in fathers' infancy). These findings have high clinical relevance in the identification of early-emerging putative risk phenotypes across generations and potential therapies to ameliorate such generational effects.

## Introduction

Early adversity is a potent risk factor for mental health problems across the lifespan (Lupien, McEwen, Gunnar, & Heim, 2009; Repetti, Taylor, & Seeman, 2002). Numerous epidemiological and empirical studies have now shown that similar neurobehavioral alterations are often experienced by the offspring, and even grand-offspring, of stress-exposed parents (see Cowan, Callaghan, Kan, & Richardson, 2016, for a review), emphasizing the importance of family history for an individual's mental health. For instance, parental PTSD in holocaust survivors was associated with higher rates of offspring psychopathology (reported in adulthood), even when those offspring were conceived during peacetime (Yehuda, Bell, Bierer, & Schmeidler, 2008). Similar effects have been observed in rodents via a paternal line of transmission, whereby parental stress (either during infancy or adulthood) results in altered emotion-related responding in the non-stress-exposed adult offspring (Dietz et al., 2011; Dias & Ressler, 2014; Gapp et al., 2014). While these data highlight the potency of stress across numerous generations, to date, such outcomes have been demonstrated almost exclusively in the adult offspring of stress-exposed individuals. An understanding of *when* in development these risks can first be detected and *how* mental health risk is increased in the offspring of stress-exposed parents will be required to develop effective treatments.

Explanations of how stress effects are transmitted across multiple generations are currently the subject of intense debate. Studies have suggested that such inheritance may be intergenerational (e.g., mating behavior, parenting, in utero effects) or transgenerational (e.g., germ line epigenetic alteration; Cowan et al., 2016; Curley, Mashoodh, & Champagne, 2011). While distinguishing between these pathways is undoubtedly important from a basic science perspective, from a clinical

standpoint, identifying avenues for intervention and treatment of stress-related disorders is essential, regardless of the mode of transmission.

One potentially useful (and thus far, unexplored) group of interventions that may have a generational effect on stress-related disorders involves gastrointestinal manipulations. Numerous recent examples demonstrate the links between gastrointestinal and psychological function, particularly in the context of stress. For instance, there are high levels of comorbidity between gastrointestinal disease (e.g., irritable bowel syndrome) and various forms of psychopathology, with increased prevalence in populations exposed to early life stress (e.g., Chitkara, van Tilburg, Blois-Martin, & Whitehead, 2008). In rodents, exposure to maternal separation stress increases anxiety and depression-like behaviors in adulthood, with these alterations being dependent on stress-induced changes to the gut microbiota (De Palma et al., 2015). Importantly, manipulations that affect the microbiota, such as probiotics, also have significant effects on affective functioning, emotion-related neural activity, and stress-related physiology in rodents and humans (Cowan, Callaghan, & Richardson, in press; Gareau, Jury, MacQueen, Sherman, & Perdue, 2007; Tillisch et al., 2013). For example, we have shown that probiotics administered to rodent mothers during breastfeeding reverse the effects of stress on learned aversive reactions in her infants (Cowan et al., in press). In addition, increasing evidence suggests that the microbiota is heritable (in both rodents and humans), and that microbiota manipulations can alter both gastrointestinal and neural outcomes for infant offspring (Goodrich et al., 2014; Jašarević, Howerton, Howard, & Bale, 2015). Together these data suggest that stress-induced changes to the microbiota may play a mechanistic role in the generational effects of stress, and therefore that altering the microbiota may help to ameliorate such generational patterns. To date, no one has examined whether a probiotic

treatment is effective in preventing or reversing stress effects on affective function across generations. Considering the ease of implementing probiotic interventions, understanding the generational effects of probiotics on stress-related disorders would be of high clinical value.

In previous research, we have shown that maternally-separated rat pups exhibit faster maturation of memory for aversive events and relapse-prone extinction (behaviors that may be relevant to the development and treatment of mental health; Callaghan & Richardson, 2011, 2012a, 2012b, 2013, 2014; Cowan, Callaghan, & Richardson, 2013). Specifically, under non-stressed conditions infant rats exhibit rapid forgetting of learned associations (infantile amnesia; see Callaghan, Li, & Richardson, 2014; Campbell & Spear, 1972, for reviews) and erasure-like extinction (i.e., they are less likely to exhibit relapse effects such as reinstatement or renewal following extinction; see Kim & Richardson, 2010, for a review), but after maternal separation stress or corticosterone treatment infant pups exhibit excellent retention (Callaghan & Richardson, 2012a) and greater relapse after extinction (Callaghan & Richardson, 2011, 2014). In other words, stress appears to accelerate the developmental emergence of these behaviors, which may index mental health risk. Here we examined whether these early putative indicators of risk following directly experienced adversity are handed down to subsequent generations via the paternal line. Second, we determined whether treating young stress-exposed rats or their infant non-exposed offspring with a probiotic ameliorated generational patterns of risk. We hypothesized that the offspring of stress-exposed fathers would exhibit behavioral markers of putative risk for mental illness – longer retention of aversive associations and greater relapse after extinction. We also hypothesized that treatment of fathers, or their offspring, with a probiotic would prevent, or reverse, these alterations in

affective behavior. To index the effects of parental stress on offspring 'mental health' we utilized two affective learning paradigms in infant rats: 1) retention of aversive associations, 2) extinction of aversive associations. To examine the effect of probiotics as a preventative measure or an active treatment for generational stress we treated stressed to-be-fathers in their infancy, or their later non-stressed infant offspring, respectively, with a probiotic before examining affective learning in the offspring.

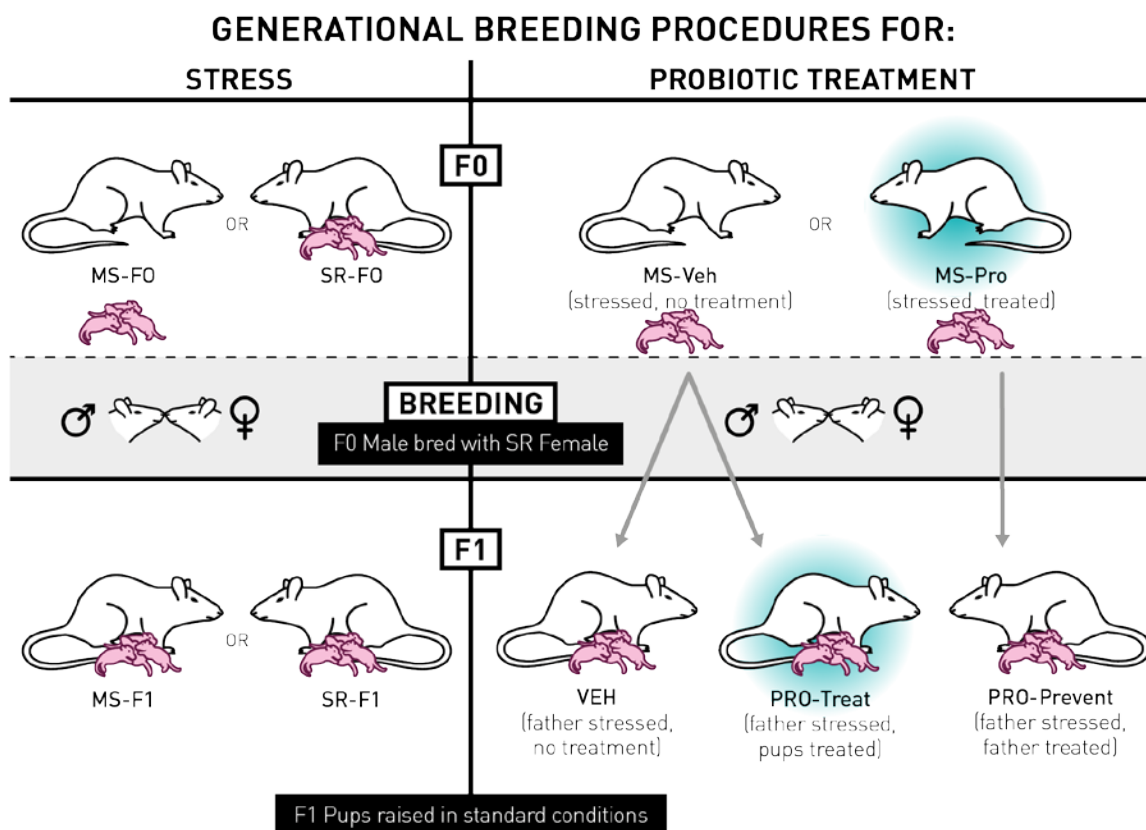
## **Methods and Materials**

**Subjects.** Experimentally naive male Sprague-Dawley-derived rats, bred and housed at the School of Psychology, The University of New South Wales, were used. The day of birth was designated postnatal day (P)0. No more than one rat per litter was used per group. Rats were housed with their mother and littermates with food and water available ad libitum. Animals were treated according to the principals of animal care and use outlined in the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*; the Animal Care and Ethics Committee at The University of New South Wales approved all procedures. All experiments were between-subject designs and no rodents were used in more than one experimental paradigm. Based on our past work examining conditioning and extinction in developing animals we aimed for group sizes of 8-12 across all behavioral experiments as we have found this sufficient to detect differences in conditioned responses. In some cases data points per group are lower due to animal availability at the time and/or data exclusion. There was a total of 398 animals used across all 13 experiments (please see Table 1 in the SOMR for a breakdown of n per group in each experiment).

**Maternal Separation.** Maternally-separated or standard-reared rats sired experimental subjects. During maternal separation (MS; P2-14) all pups were removed from the home cage, weighed, and placed in an incubator as a litter for three hours (as described previously; Callaghan & Richardson, 2011). Standard-reared (SR) animals were exposed to the same handling cues (i.e., daily weighing), but were not removed from the dam for any extended period of time. Using this procedure, we do not see any differences in weight between MS and SR pups (Callaghan & Richardson, 2011). Rats were weaned on P21-P23 and kept in social groups (2-8 rats) that had been exposed to the same rearing condition. No further manipulations occurred post-weaning.

**Breeding.** To produce second-generation (F1) offspring, maternally-separated and standard-reared adult males were each pair-housed with a multiparous standard-reared female (Figure 1). Males remained with the female for 20 days before being removed from the breeding cage. Hence, males had no contact with their offspring. For experiments comparing MS-F1 and SR-F1 offspring, one male per MS-F0 litter was used for breeding, with each male bred once. Thus, all pups within a given group were derived from distinct ancestral lineages. Due to logistical restrictions, breeding for the probiotic and F2 generation experiments was streamlined by breeding males a maximum of two times, each time with a different female. (see Supplemental Online Material for further detail).





**Figure 1.** Breeding protocols for the MS-F1 and probiotic experiments. To test the generational effects of maternal separation stress, first generation (F0) males directly exposed to either maternal separation (MS-F0) or standard rearing (SR-F0) were bred with SR females to produce a second (F1) generation (MS-F1 or SR-F1). To test the efficacy of probiotic treatments in cases of generational stress, untreated (MS-Veh) and probiotic-treated (MS-Pro) F0 males were bred with SR females. In the second (F1) generation one group was treated with a probiotic (PRO-treat) and the others were given a vehicle (VEH; PRO-Prevent); green shading indicates when the probiotic was administered. In all cases, F1 animals were raised in standard-rearing conditions and had no contact with the F0 sire.

**Probiotic Treatment.** A commercially available probiotic was administered to either the F0 or the F1 generation via the dam's drinking water from P2-14. This probiotic was comprised of Lacidofil<sup>®</sup> powder (*Lactobacillus rhamnosus* R0011, 95%, and *Lactobacillus helveticus* R0052, 5%, provided by Lallemand Health Solutions, Montreal, QC, Canada; see Foster, Tompkins, & Dahl, 2011, for a review of the applications and properties of this formulation), rehydrated in distilled water at

a concentration of  $10^9$  CFU/mL. The solution was changed every second day to ensure bacteria viability.

SYBR Green-based qPCR was used to confirm the presence of *L. rhamnosus* R0011 in MS-F0 animals. Stomach-milk extraction was performed based on the procedure described by Fellows and Rasmussen (1984). DNA was extracted using a milk bacterial DNA isolation kit (Norgen Biotek Corporation, Thorold, ON, Canada). qPCR was conducted using primers described by Gareau et al. (2007) and obtained from Thermo Fisher Scientific. A melt curve analysis (Supplemental Figure S1) verified the reaction specificity. Replicating our previous finding (Cowan et al., in press), *L. rhamnosus* R0011 was detected in samples (both milk and feces) from probiotic-exposed MS-F0 pups, but not vehicle-exposed MS-F0 pups (Supplemental Figure S10). However, by adulthood *L. rhamnosus* R0011 was no longer detectable in the feces of either treatment group (Supplemental Figure S11). See Supplemental Online Material for further details.

**Behavioral Procedures.** Rats in the retention experiments were conditioned on P17 and tested in the same context 1, 10, or 12 days later. For the extinction experiments, rats were conditioned on P17 in one context, given extinction training on P18, a reinstatement treatment on P19, and test on P20, all in a different context to training. Longer retention of aversive associations and greater relapse after the reinstatement treatment were considered as putative indicators of vulnerability in infant rats, as these behaviors are typically not observed early in development unless rodents have been exposed to maternal separation (e.g., Callaghan & Richardson, 2013), a procedure associated with increased anxiety later in adulthood (e.g., Huot et al., 2001; Kalinichev et al., 2002).

Conditioning consisted of a 2-minute adaptation period followed by six pairings of a white noise CS (8dB above background, 10s) co-terminating with a shock US (0.6mA, 1s) in context A. Extinction consisted of a 2-minute adaptation period and 30 non-reinforced presentations of the 10s CS (10s ITI) in context B. Reinstatement involved a single reminder shock (0.4mA, 1s) after a 2-minute adaptation period in context B, while no-reminder groups were exposed to context B for the same duration without receiving any shock. Finally, test involved a 1-minute baseline period followed by a single, continuous 2-minute presentation of the CS in context B. For the probiotic experiments, rats freezing >50% at baseline were returned to the home cage for 10 minutes before being placed in the test context again in order to extinguish the context freezing response (maximum three trials of context extinction). The conditioning/extinction/test apparatus were cleaned with tap water after each rat.

To assess the hypothesis that alterations in maternal behavior acted as a mechanism for the transmission of the stress phenotype across generations, maternal anxiety and maternal care were assessed on the light/dark apparatus and the pup retrieval test, respectively. No behavioral differences were observed on either of these tests (see Supplemental Online Material).

**Apparatus.** Two types of chambers that differed in terms of size, illumination, and visual characteristics were used to provide distinct contexts (A and B) for the conditioning and extinction experiments. See Supplemental Online Material for further details.

**Scoring, Exclusions, and Statistics.** Freezing responses in rats were scored by a time sampling procedure whereby each rat was scored every three seconds as freezing or not freezing (see Supplemental methods for additional details). These

observations were then converted into a percentage score to indicate the proportion of total observations scored as freezing. A second scorer, unaware of the experimental condition of each rat, scored a random sample (30-45%) of all rats tested. The inter-rater reliability was very high across all experiments,  $r_s=.910-1.000$ .

All data were analyzed in SPSS (version 23). Effect sizes were calculated in SPSS ( $\eta_p^2$ ) or by hand (Cohen's  $d$  and  $r$ ). Cohen's  $d$  was calculated using the following equation  $d = (M_2 - M_1) / SD_{\text{pooled}}$  where  $SD_{\text{pooled}} = \sqrt{(SD_1^2 + SD_2^2) / 2}$ . When significant differences in pre-CS freezing at test were detected (see Supplemental Table 1 for  $n_s$  and pre-CS freezing levels in all experiments) CS-elicited freezing during test was analyzed with ANCOVA using the pre-CS freezing scores as a covariate. However, in general the same results were obtained whether the data were analyzed with ANOVA or ANCOVA. The exception to this was in the examination of probiotic effects on reinstatement, where ANOVA without the pre-CS freezing as a covariate resulted in a non-significant interaction,  $F(2,50)=2.69$ ,  $p=.078$ ,  $\eta_p^2=.10$ . As such, the results and analysis for this experiment are presented based on difference scores (i.e., percent CS-elicited freezing less percent pre-CS freezing), although the same results were obtained if the data were analyzed by ANCOVA using pre-CS freezing as a covariate. When data were normally distributed, post-hoc t-tests were used to interrogate significant interaction effects. When data were not normally distributed, non-parametric Mann-Whitney U tests were used to interrogate interactions. When t-tests were used, if Levene's test for equality of variances was significant then the adjusted t-statistic and nominal  $df$  are reported. Whenever a mixed-design ANOVA was used, if the assumption of sphericity was violated, the Greenhouse-Geisser correction was made but nominal  $df$  are reported. Any rats that were statistical outliers at test ( $\geq 3.75SD$  away from the mean) or that exhibited high

baseline freezing (>65%) at test were excluded from subsequent analyses. This resulted in 15 exclusions across all experiments (3.9% of all rats tested; see Supplemental Table S2 for full details).

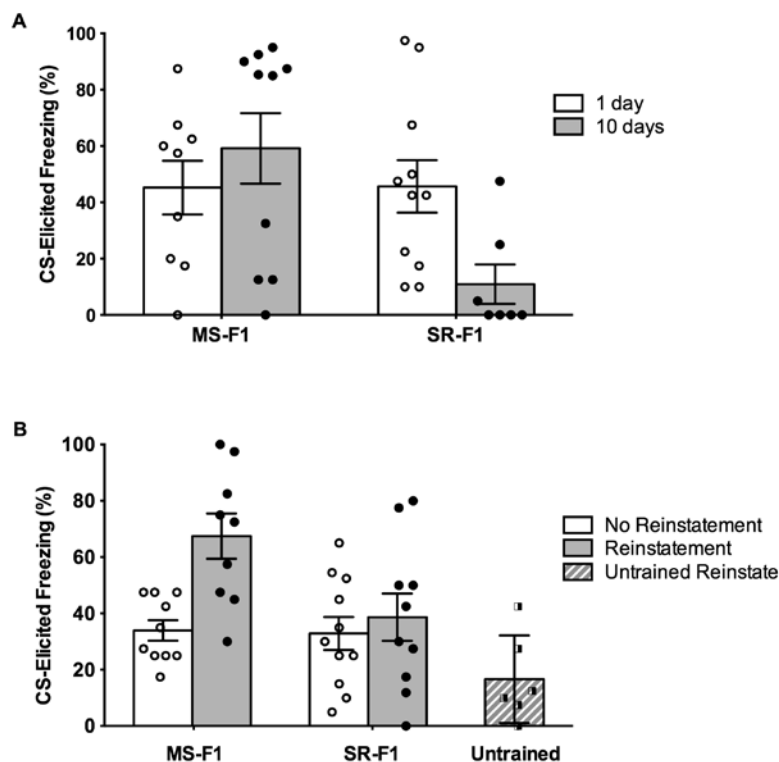
## Results

### Are generational effects of stress on learning evident in infant offspring?

**Effect of father stress on offspring retention phenotype.** We first examined whether maternal-separation stress in fathers affected the retention of aversive memories in second-generation (F1) infant offspring (see Figure 1 for a depiction of the breeding protocol). There was a main effect of paternal rearing condition,  $F(1,32)=4.26$ ,  $p=.047$ ,  $\eta_p^2=.12$ , and a paternal rearing condition by retention interval interaction,  $F(1,32)=4.43$ ,  $p=.043$ ,  $\eta_p^2=.12$ , on freezing behavior during test (Figure 2A). The median freezing response in the maternally-separated F1 (MS-F1) and standard-reared F1 (SR-F1) rats when tested one day after conditioning was 57.50 and 42.50 respectively and did not differ significantly from one another,  $U=48.00$ ,  $p>.250$ ,  $r=XX$ , indicating that rats of both lineages were equally able to learn about a CS-US association and express their conditioned response (CR) 24 hours later. However, there were large differences in median freezing responses between MS-F1 and SR-F1 rats tested 10 days after conditioning ( $Mdns=85.18$ , 0.00, respectively;  $U=10.00$ ,  $p=.014$ ,  $r=XX$ ). Indeed, while SR-F1 rats exhibited much lower median levels of freezing at the 10-day interval ( $Mdn=0.00$ ) than at the one day interval ( $Mdn=42.5$ ), indicating forgetting ( $U=10.50$ ,  $p=.008$ ,  $r=XX$ ), MS-F1 rats exhibited high and similar median levels of freezing at both 1 and 10 day intervals, indicating good retention ( $Mdns=57.50$ , 85.18;  $U=33.00$ ,  $p>.250$ ,  $r=XX$ ). Interestingly, we saw the same enhanced retention in third-generation males (i.e., grand-offspring of maternally-separated fathers; MS-F2; Supplemental Figure S2).

**Effect of father stress on offspring extinction phenotype.** We next examined whether infant offspring of MS and SR fathers exhibited the reinstatement effect (i.e., a return in CR following a post-extinction reminder foot shock). Within session extinction behavior was not different between groups (see Supplemental Figure S6). The post-extinction test data was initially analyzed as a 2 (paternal rearing condition – MSF1 vs. SRF1) x 2 (post-extinction treatment – Reinstatement vs. No Reinstatement) factorial design. There was a main effect of paternal rearing condition,  $F(1,35)=4.66$ ,  $p=.038$ ,  $\eta_p^2=.12$ , and an interaction between paternal rearing condition and post-extinction treatment,  $F(1,35)=5.08$ ,  $p=.031$ ,  $\eta_p^2=.13$  (Figure 2B). SR-F1 rats exhibited low levels of freezing at test, regardless of whether they received reinstatement or not,  $t(19)=.57$ ,  $p>.250$ , 95% CI [-15.35–26.90],  $d=.26$ . In contrast, levels of freezing in MS-F1 animals given reinstatement were much higher than for those that were not reinstated,  $t(17)=3.79$ ,  $p=.003$ , 95% CI [14.09–52.91],  $d=1.91$ . To ensure that the reinstatement effect was driven by enhanced context learning in reinstated rats (i.e., that the reinstatement foot shock in itself didn't cause learning) another group was created in which rats were not conditioned but did receive the 'reinstatement' foot shock the day prior to test. Considering rats this age show poor context learning (especially to weak shocks) a small sample size ( $n=3$ ) was used in each paternal rearing condition and data were then collapsed across rearing condition to create a single 'Untrained Reinstatement' group ( $n=6$ ; freezing levels in MS-F1 and SR-F1 'Untrained Reinstatement' rats were low and similar; both  $M_s=16.67$ ,  $t(4)=.00$ ,  $p>.250$ , 95% CI [-39.40–39.40],  $d=.00$ ). Follow-up t-tests showed that the MS-F1 'Reinstatement' group was different to the 'Untrained Reinstatement' group,  $t(13)=4.53$ ,  $p=.001$ , 95% CI [29.61–75.16],  $d=2.57$ , but the SR-F1 'Reinstatement' group and the 'Untrained Reinstatement' group did not differ,  $t(14)=1.83$ ,  $p=.088$ , 95% CI [-3.71–47.75],

$d=1.01$ . Results for the F2 generation – reinstatement and renewal effects – are presented in Supplemental Figures S3 and S4.



**Figure 2.** Mean ( $\pm$  SEM) freezing levels during test for the offspring of maternally-separated (MS-F1) and standard-reared (SR-F1) sires. A) Retention phenotype: paternal stress results in MS-F1 infants exhibiting longer-lasting retention of an aversive memory compared to SR-F1.  $N = 37$ . B) Extinction phenotype: MS-F1 males exhibit reinstatement of CS-elicited freezing following a reminder treatment, while SR-F1 males exhibit relapse-resistant extinction.  $N = 46$ .

### Can probiotics function as an effective prophylactic or an active treatment to reverse the effects of paternal stress on F1 generation offspring?

To examine whether a probiotic treatment could rescue rodents from the generational effects of stress we treated MS-F1 pups with a probiotic for the first two weeks of life (P2-14; PRO-Treat), or left them untreated (VEH). To examine whether probiotics would work prophylactically to prevent the transmission of the MS

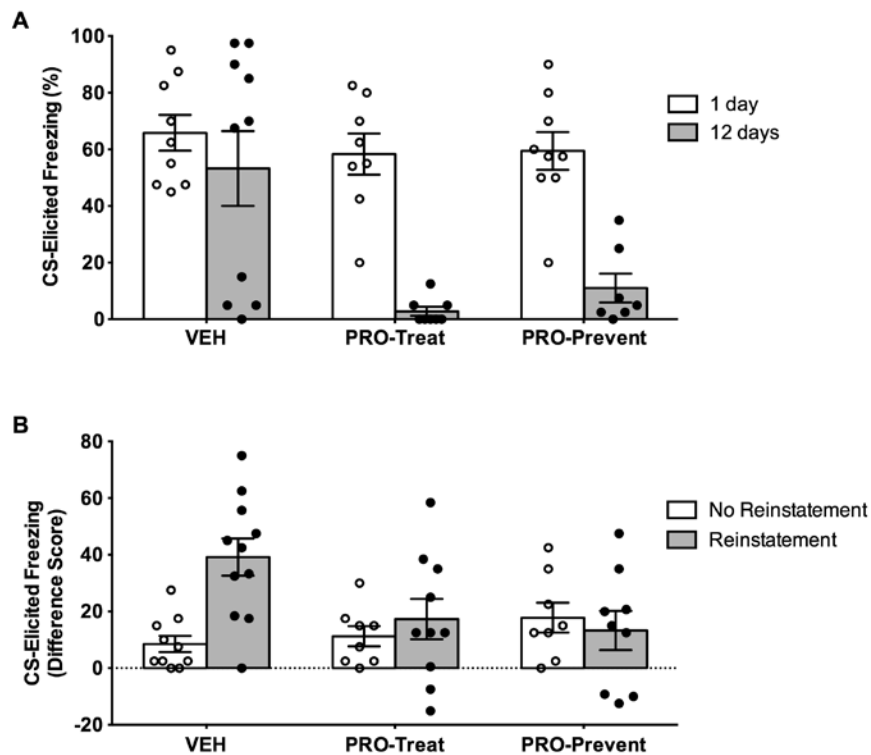
phenotype from the F0 to the F1 generation we treated MS-F0 pups with a probiotic during the period of maternal separation (P2-14) and then examined behavior in their subsequent F1 generation offspring (PRO-Prevent; see Figure 1 for a schematic of the procedure).

**Probiotic effects on infant retention.** Probiotic treatment had a strong effect regardless of when it was administered; there was a main effect of treatment  $F(2,44)=7.91, p=.001, \eta_p^2=.27$ , test interval,  $F(1,44)=15.25, p<.001, \eta_p^2=.26$ , and an interaction between treatment and test interval  $F(2,44)=4.25, p=.021, \eta_p^2=.16$  (Figure 3A). Replicating our previous results, vehicle-treated MS-F1 rats exhibited excellent retention of an aversive memory across a 12-day period (VEH group did not differ at the one and 12 day intervals,  $Mdn_{1d}=62.50, Mdn_{12d}=67.50, U=43.50, p>.250, r=.03$ ). However, probiotics administered either in the F0 or F1 generation restored the age-appropriate profile of infantile amnesia in MS-F1 rats, PRO-Treat:  $Mdn_{1d}=58.75, Mdn_{12d}=0.00, U=0.00, p=.001, r=.85$ , and PRO-Prevent:  $Mdn_{1d}=57.50, Mdn_{12d}=3.75, U=2.00, p=.002, r=.78$ , demonstrating that probiotics were effective both as an active treatment and as a prophylactic.

**Probiotic effects on infant extinction.** MS-F1 pups were tested for the reinstatement effect following extinction. Within-session extinction behavior did not differ across groups (see Supplemental Figure S7). At test, there was a significant treatment by reinstatement condition interaction,  $F(2,50)=4.99, p=.011, \eta_p^2=.17$ , and a significant main effect of reinstatement condition,  $F(1,50)=4.99, p=.030, \eta_p^2=.09$ ; the main effect of treatment was not significant,  $F(2,50)=1.68, p=.196, \eta_p^2=.06$ . The VEH group exhibited the reinstatement effect (higher freezing in the reinstatement group,  $Mdn=42.50$ , relative to no-reinstatement group,  $Mdn=5.00$ ),  $U=11.50, p=.002, r=.67$ .



However, the reinstatement effect was not observed in the PRO-Treat groups,  $Mdn_{reinst}=12.50$ ,  $Mdn_{no}=11.25$ ,  $U=36.00$ ,  $p>.250$ ,  $r=.09$ , and PRO-Prevent groups,  $Mdn_{reinst}=15.00$ ,  $Mdn_{no}=13.75$ ,  $U=32.00$ ,  $p>.250$ ,  $r=.09$  (Figure 3B). That is, probiotic treatment restored an age-appropriate, relapse-resistant profile of extinction in the next generation of infants, regardless of whether treatment was delivered post-hoc or prophylactically. Probiotics were also effective in reversing and preventing the transmission of the renewal phenotype following extinction in MS-F1 pups (see Supplemental Figure S5). Importantly, probiotics did not affect maternal anxiety levels nor the dam's caregiving behavior towards pups (Supplemental Figures S8 and S9).



**Figure 3.** Mean ( $\pm$  SEM) CS-elicited freezing during test for the offspring (F1 generation) of maternally-separated fathers following no treatment (VEH), probiotic treatment in the F0 generation (PRO-Prevent), or probiotic treatment in the F1 generation (PRO-Treat). Only VEH animals exhibit A) long-lasting memory retention and B) the reinstatement effect, whereas probiotics restore age-appropriate A) infantile amnesia and B) relapse-resistant extinction.  $N_s = 51$  &  $56$ .

## Discussion

Here we report two novel and important findings related to the emergence and treatment of generational effects of stress. First, we have shown that putative risk factors for mental disorders – persistent retention of aversive associations and relapse after extinction – emerge earlier than normal in offspring of stress-exposed fathers. These data are the first to demonstrate that stress-induced behavioral alterations in affective learning can be ‘inherited’ by *infant* offspring. This is important clinically, as transmitted behavioral alterations that are detectable early in development are a useful target for intervention. Indeed, our second finding demonstrated that such intervention (in the form of probiotics administered to F0 pups) prevents the transmission of MS effects on aversive learning to the F1 generation. Similarly, treatment of F1 pups reversed the behavioral phenotypes, demonstrating the effectiveness of probiotics as both a prophylactic and an active remedy. These findings are clinically important, suggesting that behavioral phenotypes putatively involved in vulnerability to later life anxiety, and transmitted across generations through fathers, can be effectively prevented and/or treated with non-invasive probiotic manipulations.

As mentioned in the Introduction, explanations of how stress effects are transmitted across generations are currently the subject of intense debate. While data from the current studies cannot distinguish whether stress effects were transmitted through a primarily behavioral or biological route (e.g., maternal behavior, in utero stress programming, epigenetic effects), they do suggest that microbiota alterations produced by stress might be active contributors. In the current study, probiotic

administered to nursing dams was transmitted to pups via the breast milk, resulting in temporary transfer of the probiotic strains to the pup's colon that was eliminated by adulthood. This strongly indicates that the specific probiotic strains used in the initial treatment are not directly transferred to MS-F1 offspring of probiotic-exposed fathers. However, it does not exclude the possibility that some other alteration in the overall composition of the gastrointestinal microbiota might be transmitted across generations.

Stress has a dramatic impact on the composition of gastrointestinal bacteria, which has been suggested to regulate stress-induced changes in social behavior (e.g., Zijlmans et al., 2015; also see Parashar & Udayabanu, 2016, for a review). Also, recent reports suggest that the massive metabolic demands of the developing brain are heavily dependent on the delicate balance of microbes in the gut (Goyal et al., 2015). In the current study, it is possible that the probiotic intervention may have arrested/reversed changes in the development of threat-related behaviors via effects on social functioning or metabolism, helping to preserve or repair infant performance. Indeed, it may be the case that either stress- or probiotic-induced changes in the intestinal microbiota can be passed down the generations, as previous studies have suggested that the microbiota (or at least certain taxa) is heritable, with host genetics exerting an influence on microbiota composition (Goodrich et al., 2014). In fact, this inter-species (host-microbe) interaction is likely to be bidirectional, as it has also been shown that the microbiota can alter host gene expression, particularly with regards to genes involved in immune regulation (one likely candidate for the microbial effects on metabolic function; Broderick, Buchon, & Lemaitre, 2014).

Many neurotransmitters important for mood and that have programming effects on brain development (e.g., GABA, serotonin) are produced in large quantities

as metabolites of the gut microbiota and can later enter the central nervous system (Barrett, Ross, O'Toole, Fitzgerald, & Stanton, 2012; Yano et al., 2015), potentially influencing current emotional function and neural activity, as well as the development of emotion-related circuits. Indeed, microbial composition of the rodent gut was recently shown to regulate amygdala development (Stilling et al., 2015), a hub of emotional functioning. Interestingly, the specific strains of bacteria used in this study (*L. rhamnosus* R0011; *L. helveticus* R0052) have known dampening effects on circulating stress hormones (i.e., corticosterone) and cytokines (Foster et al., 2011), both of which are upregulated following separation stress (Gareau et al., 2007; Hennessy et al., 2015) and have been shown to lead to accelerated development of emotion-related learning systems, i.e., long lasting retention and greater relapse after extinction (for a review see Callaghan & Richardson, 2013). These data suggest the intriguing possibility that the mechanism of action for probiotics on threat responses in the current study may involve dampening of stress-activated hormones and pro-inflammatory immune signaling pathways. Such possibilities provide exciting avenues for future research to develop novel and effective treatments for mental health disorders.

One limitation of this study is that all behavioral tests were restricted to male pups and a paternal line of inheritance. We opted not to examine female generational effects in the current series of experiments primarily because the effects of stress in F0 generation pups have only been investigated in males. This argument notwithstanding, previous research has demonstrated sex-specific generational inheritance of emotion-related responses (Franklin et al., 2010; Kim, Capaldi, Pears, Kerr, & Owen, 2009). Hence, it will be important to determine sex-specific effects on affective maturation inheritance and their treatment with probiotics in future studies.

In addition, due to small sample sizes, some of the analyses may be underpowered.

Follow-up studies should aim to collect data from larger samples of rodents.

Regardless of the ultimate mechanism, the ease of administration, minimal risk, low cost, and general public acceptance of probiotics makes them an ideal candidate to investigate as a first line of defense against stress-induced vulnerabilities. The fact that early life adversity is often highly comorbid with poor nutrition and gastrointestinal problems (Chitkara et al., 2008; Widom, Czaja, Bentley, & Johnson, 2012) further strengthens the case for probiotic interventions in stress and mental illness. Importantly, the probiotic used in the current studies already has established safety and efficacy in pediatric populations as it is frequently used in the treatment of gastrointestinal diseases (e.g., Freedman et al., 2014). Together with these past studies, the data presented here make a strong case for further investigations into the clinical efficacy of these particular probiotic strains for the treatment of stress-related emotional health in children.

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