Early adversity is a potent risk factor for mental-health disorders in exposed individuals, and effects of adversity are exhibited across generations. Such adversities are also associated with poor gastrointestinal outcomes. In addition, emerging evidence suggests that microbiota-gut-brain interactions may mediate the effects of early-life stress on psychological dysfunction. In the present study, we administered an early-life stressor (i.e., maternal separation) to infant male rats, and we investigated the effects of this stressor on conditioned aversive reactions in the rats' subsequent infant male offspring. We demonstrated, for the first time, longer-lasting aversive associations and greater relapse after extinction in the offspring (F1 generation) of rats exposed to maternal separation (F0 generation), compared with the offspring of rats not exposed to maternal separation. 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 of potential therapies to ameliorate such generational effects.

Keywords
maternal separation, extinction, Pavlovian conditioning, infantile amnesia, inheritance, generational effects, probiotic

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Early adversity is a potent risk factor for mental-health problems across the life span (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 third-generation offspring, of stress-exposed parents or grandparents (for a review, see Cowan, Callaghan, Kan, & Richardson, 2016). These findings emphasize the importance of family history for an individual’s mental health. For instance, posttraumatic stress disorder in holocaust survivors was associated with higher rates of psychopathology in their adult offspring, even when those offspring were conceived and raised during peacetime (Yehuda, Bell, Bierer, & Schmeidler, 2008). Similar effects have been observed in rodents, whereby paternal stress (during either infancy or adulthood) results in altered emotion-related responding in adult offspring (Dias & Ressler, 2014; Dietz et al., 2011; Gapp et al., 2014). Although 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. To develop effective treatments, researchers will have to understand when these risks can first be detected.
during development and how mental-health risk is increased in the offspring of stress-exposed parents.

How stress effects are transmitted across multiple generations is 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, Callaghan, Kan, & Richardson, 2016; Curley, Mashoodh, & Champagne, 2011). Although distinguishing between these pathways is undoubtedly important to basic science, identifying avenues for intervention and treatment of stress-related disorders is essential for clinical treatment, regardless of the mode of transmission.

Gastrointestinal manipulations constitute one potentially useful (and thus far unexplored) group of interventions that may have a generational effect on stress-related disorders. Numerous recent examples demonstrate the links between gastrointestinal and psychological function, particularly in the context of stress. For instance, gastrointestinal disorders (e.g., irritable bowel syndrome) are highly comorbid with various forms of psychopathology, and their prevalence is increased 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, and these alterations are dependent on stress-induced changes to gut microbiota (De Palma et al., 2015). 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, 2016; Gareau, Jury, MacQueen, Sherman, & Perdue, 2007; Tillisch et al., 2015). For example, we have shown that probiotics reverse the effects of stress on learned aversive reactions in infant rats (Cowan, Callaghan, & Richardson, 2016). 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 researchers have 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 rat pups exposed to maternal separation (compared with those raised under standard conditions) exhibit faster maturation of memory for aversive events and extinction that is prone to relapse, phenomena that may be relevant to the development and treatment of mental illness (Callaghan & Richardson, 2011, 2012a, 2012b, 2013, 2014; Cowan, Callaghan, & Richardson, 2013). Specifically, under nonstressed conditions, infant rats exhibit rapid forgetting of learned associations (infantile amnesia; for reviews, see Callaghan, Li, & Richardson, 2014; Campbell & Spear, 1972) and erasure-like extinction; that is, they are less likely than adult rats to exhibit relapse effects such as reinstatement of the association (hereafter, simply reinstatement) or renewal of the association after extinction (hereafter, simply renewal; for a review, see J. H. Kim & Richardson, 2010). However, after stress caused by separation from their dams or by corticosterone treatment, infant pups exhibit excellent retention (Callaghan & Richardson, 2012a) and greater relapse after extinction compared with standard-reared rats (Callaghan & Richardson, 2011, 2014). In other words, stress appears to accelerate the developmental emergence of these behaviors, which may be an index of mental-health risk.

In the current study, we first examined whether these early putative indicators of risk after directly experienced adversity are handed down to subsequent generations via the paternal line. Second, we determined whether treatment with a probiotic ameliorated generational patterns of risk in the infant offspring of rats exposed to maternal separation. 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 paternal stress on the offspring's mental health, we used two affective-learning paradigms in infant rats: retention of aversive associations and extinction of aversive associations. To examine the effect of probiotics as a preventive measure or an active treatment for generational stress, we treated stressed fathers-to-be in their infancy or their later nonstressed infant offspring, respectively, with a probiotic before examining affective learning in the offspring.

Method

Subjects

Three generations of experimentally naive male rats derived from Sprague-Dawley rats were bred and housed at the School of Psychology, The University of New South Wales. The day of birth was designated postnatal day 0 (P0). No more than one rat from a given litter was used in a group. Rats were housed with their mother and littermates,
and food and water were 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-subjects designs, and no rodents were used in more than one experimental paradigm (with the exception of the polymerase-chain-reaction experiments, in which a single animal sometimes supplied a fecal sample as well as a gastrointestinal milk sample). On the basis of our past work examining conditioning and extinction in developing animals, we aimed for group sizes of 8 to 12 across all behavioral experiments; we have found this number to be sufficient to detect differences in conditioned responses. In some cases, the number of data points per group is lower because of animal availability at the time of the studies or data exclusion (or both). A total of 398 animals were used across all 13 experiments (for a breakdown of the number of animals in each group in each experiment, see Table S1 in the Supplemental Material).

Maternal separation

In the first generation (F0), some rats experienced maternal separation (MS rats), and some rats experienced standard rearing (SR rats). During maternal separation (P2–P14), all pups were removed from the home cage, weighed, and placed together in an incubator for 3 hr (as described previously; Callaghan & Richardson, 2011). 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 did not see any differences in weight between MS and SR pups (Callaghan & Richardson, 2011). Rats were weaned on P21 to P23 and kept in social groups (2–8 rats) that had been exposed to the same rearing conditions. No further manipulations occurred after weaning.

Breeding

To produce second-generation (F1) offspring, each MS and SR adult male was housed with a multiparous SR female. Males remained with the females for 20 days before being removed from the breeding cage. Hence, males had no contact with their offspring. One male from each MS-F0 litter was used once for breeding to produce the animals used for experiments comparing MS-F1 and SR-F1 offspring. Thus, all pups within a given group were derived from distinct ancestral lineages. Because of 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 (for further details, see the Supplemental Material).

Probiotic treatment

A commercially available probiotic was given to some F0 rats and some F1 rats (Fig. 1). The probiotic was administered via the dam’s drinking water from P2 to P14. This probiotic was composed of Lacidofil powder (95% Lactobacillus rhamnosus R0011 and 5% Lactobacillus helveticus R0052; provided by Lallemand Health Solutions, Montreal, QC, Canada; for a review of the applications and properties of this formulation, see Foster, Tompkins, & Dahl, 2011), which was rehydrated in distilled water at a concentration of 10⁸ colony-forming units per milliliter. The solution was changed every second day to ensure bacteria viability.

SYBR Green–based quantitative polymerase chain reaction (qPCR) was used to confirm the presence of L. rhamnosus R0011 in MS-F0 animals. Extraction of milk from rats’ stomachs was performed according to the procedure described by Fellows and Rasmussen (1984). DNA was extracted using a kit for isolating bacterial DNA in milk (Norgen Biotek Corporation, Thorold, ON, Canada). Using primers described by Gareau et al. (2007) and obtained from Thermo Fisher Scientific, we conducted qPCR. We used a melt-curve analysis (see Fig. S1 in the Supplemental Material) to verify the reaction specificity. Replicating our previous finding (Cowan, Callaghan, & Richardson, 2016), we detected L. rhamnosus R0011 in samples (both milk and feces) from probiotic-exposed MS-F0 pups but not in samples from vehicle-exposed MS-F0 pups (see Fig. S10 in the Supplemental Material). However, by the time the rats reached adulthood, L. rhamnosus R0011 was no longer detectable in the feces of either treatment group (see Fig. S11 in the Supplemental Material; for further details about the qPCR analysis, see the Supplemental Material).

Apparatus

Two types of chambers that differed in size, illumination, and visual characteristics were used to provide distinct contexts (Contexts A and B) for the conditioning and extinction experiments (for further details about all of the apparatus, see the Supplemental Material). The chambers for conditioning, extinction, and testing were cleaned with tap water after each rat.

Behavioral procedures

Rats in the retention experiments were conditioned in Context A on P17 and then tested in the same context 1, 10, or 12 days later. Rats in the extinction experiments were conditioned on P17 in Context A; they were then given extinction training on P18, a reinstatement treatment on P19, and testing on P20, all in Context B. Conditioning consisted of a 2-min adaptation period followed
by six presentations of a white-noise conditioned stimulus (CS; 8 dB above background noise, 10 s) that coterminated with a shock (0.6 mA, 1 s), the unconditioned stimulus (US). Extinction consisted of a 2-min adaptation period and 30 nonreinforced presentations of the 10-s CS (with a 10-s intertrial interval). Reinstatement treatment involved a single reminder shock (0.4 mA, 1 s) after a 2-min adaptation period, whereas no-reminder groups were exposed to the context for the same duration without receiving any shock. Finally, testing involved a 1-min baseline period followed by a single, continuous 2-min presentation of the CS.

Longer retention of aversive associations and greater relapse after the reinstatement treatment were considered putative indicators of vulnerability in infant rats because 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, Thrivikraman, Meaney, & Plotsky, 2001; Kalinichev, Easterling, Plotsky, & Holtzman, 2002).

For the probiotic aversive-learning experiments, rats that exhibited a freezing response (i.e., the absence of all movement except that required for respiration) more than 50% of the time at baseline were returned to the home cage for 10 min before being placed in the testing context again to extinguish the freezing response (maximum three trials of context extinction).

To assess the hypothesis that alterations in maternal behavior acted as a mechanism for the transmission of the stress phenotype across generations, we assessed maternal anxiety and maternal care; we observed no behavioral differences during these assessments (for details about these assessments, see Apparatus, Light/Dark Test, and Pup Retrieval in the Supplemental Material).

**Scoring, exclusions, and statistics**

Freezing responses in rats were scored by a time-sampling procedure; each rat was scored every 3 s as freezing or not freezing (for additional details, see the Supplemental Material). These observations were then
converted into a score to indicate the proportion of total observations scored as freezing (i.e., the freezing level). A second scorer, unaware of the experimental condition of each rat, scored a random sample (30%-45%) of all rats tested. The interrater reliability was very high across all experiments, rs = .910 to 1.000.

All data were analyzed in IBM SPSS Statistics (Version 23). Effect sizes were calculated in SPSS ($\eta^2$) or by hand (Cohen’s d and r). Cohen’s d was calculated using the equation $d = (M_2 - M_1)/\sqrt{(SD_1^2 + SD_2^2)/2}$. The r statistic was calculated using the equation $Z/\sqrt{N}$. When significant differences in pre-CS freezing levels were detected at testing (for ns and pre-CS freezing levels in all experiments, see Table S1 in the Supplemental Material), CS-elicited freezing during testing was analyzed with analysis of covariance (ANCOVA) using the pre-CS freezing scores as a covariate. In general, however, the same results were obtained whether the data were analyzed with analysis of variance (ANOVA) or ANCOVA. The exception to this was the examination of probiotic effects on the reinstatement effect, in which ANOVA without the pre-CS freezing level as a covariate resulted in a nonsignificant interaction, $F(2, 50) = 2.69, p = .078, \eta^2 = .10$. Consequently, the analysis for this experiment was performed using difference scores (i.e., percentage of CS-elicited freezing minus percentage of pre-CS freezing), although the same results were obtained when the data were analyzed by ANCOVA using the pre-CS freezing level as a covariate.

When data were normally distributed, post hoc t tests were used to determine significant interaction effects. When data were not normally distributed, nonparametric Mann-Whitney U tests were used to determine interactions. If Levene’s test for equality of variances was significant for t tests, then the adjusted t statistic and nominal degrees of freedom are reported. If the assumption of sphericity was violated when we used a mixed-design ANOVA, the Greenhouse-Geisser correction was made, but nominal degrees of freedom are reported. Any rats that were statistical outliers at testing (≥ 3.75 SD away from the mean) or that exhibited high baseline freezing levels (> 65%) at testing were excluded from subsequent analyses. This resulted in 15 exclusions across all experiments (3.76% of all rats tested; for full details, see Table S2 in the Supplemental Material).

Results

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

Effect of fathers’ stress on retention of aversive memories in offspring. We first examined whether stress caused by maternal separation affected the retention of aversive memories in F1 infant offspring. There was a main effect of paternal rearing condition, $F(1, 32) = 4.26, p = .047, \eta^2 = .12$, and an interactive effect of paternal rearing condition and retention interval, $F(1, 32) = 4.43, p = .043, \eta^2 = .12$, on freezing behavior during testing (Fig. 2a). SR-F1 rats exhibited much lower freezing levels at the 10-day interval than at the 1-day interval, which indicates forgetting ($U = 10.50, p = .008, r = .60$); however, MS-F1 rats exhibited high and similar freezing levels at both the 1-day interval and the 10-day interval, which indicates good retention ($U = 33.00, p > .250, r = −.23$). We saw the same enhanced retention in third-generation male rats (i.e., the MS-F2 grand-offspring of rats that had been exposed to maternal separation; see Fig. S2 in the Supplemental Material).

![Fig. 2](image-url) Results for the second-generation (F1) offspring of rats exposed to maternal separation (MS) or standard rearing (SR). Mean freezing levels elicited by the conditioned stimulus (CS) are graphed for each paternal rearing condition. In (a), freezing levels for MS-F1 and SR-F1 rats ($N = 37$) are graphed separately for CSs presented 1 and 10 days after conditioning. In (b), freezing levels for MS-F1 and SR-F1 rats ($N = 46$) are graphed separately for no reinstatement, reinstatement, and untrained reinstatement groups. Error bars represent ±1 SEM. Circles represent results for individual rats.
Effect of fathers’ stress on extinction in offspring.
We next examined whether infant offspring of MS and SR fathers exhibited the reinstatement effect (i.e., a return of the conditioned response after a postextinction reminder foot shock). Within-session extinction behavior was not different between groups (see Fig. S6 in the Supplemental Material). The postextinction testing data were initially analyzed as a 2 (paternal rearing condition: MS-F1 vs. SR-F1) × 2 (postextinction treatment: reinstatement vs. no reinstatement) factorial design. There was a main effect of paternal rearing condition, \( F(1, 35) = 4.66, p = .038, \eta^2_p = .12 \), and an interaction between paternal rearing condition and postextinction treatment, \( F(1, 35) = 5.08, p = .031, \eta^2_p = .13 \) (Fig. 2b). SR-F1 rats exhibited low freezing levels at testing, regardless of whether they received the reinstatement foot shock or not, \( t(19) = .57, p > .250 \). 95% CI = [−13.55, 26.90], \( d = 0.26 \). In contrast, freezing levels in MS-F1 animals were much higher in those that were given the foot shock than in those that were not given the foot shock, \( t(17) = 3.79, p = .003, 95\% \) CI = [14.09, 52.91], \( d = 1.91 \).

To ensure that the reinstatement effect was not driven by enhanced context learning (i.e., that the reinstatement foot shock in itself did not cause learning), we created another group, the untrained reinstatement group, in which rats were not conditioned but did receive the foot shock the day before testing. Considering that rats this age show poor context learning (especially in response to weak shocks), we used a small sample size of rats \( (n = 3) \) from each paternal rearing condition, and data were then collapsed across rearing conditions to create a single untrained-reinstatement group \( (n = 6) \); freezing levels in MS-F1 and SR-F1 rats in the untrained-reinstatement group were low and similar (both \( M_s = 16.67 \), \( t(4) = 0.00, p > .250, 95\% \) CI = [−39.40, 39.40], \( d = 0.00 \). Follow-up \( t \) tests showed that the MS-F1-reinstatement group was significantly different from 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, \( t(14) = 1.85, p = .088, 95\% \) CI = [−3.71, 47.75], \( d = 1.01 \) (for reinstatement and renewal effects in the F2 generation, see Figs. S3 and S4 in the Supplemental Material).

Can probiotics function as an active treatment or an effective preventive 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 2 weeks of life (P2–P14; active-probiotic-treatment group) or left them untreated (vehicle group). 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–P14) and then examined behavior in their subsequent F1-generation offspring (preventive-probiotic-treatment group; see Fig. 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 were main effects of treatment, \( F(2, 44) = 7.91, p = .001, \eta^2_p = .27 \), and of test interval, \( F(1, 44) = 15.25, p < .001, \eta^2_p = .26 \), and a significant interaction between treatment and test interval \( F(2, 44) = 4.25, p = .021, \eta^2_p = .16 \) (Fig. 3a). In a replication of our previous results, vehicle-treated MS-F1 rats exhibited excellent retention of an aversive memory across a 12-day period; for rats in the vehicle group, there was no difference between the 1-day and 12-day intervals, \( U = 43.50, p > .250, r = .03 \). However, probiotics administered in either the F0 or the F1 generation restored the age-appropriate profile of infantile amnesia in MS-F1 rats (active-probiotic-treatment group: \( U = 0.00, p = .001, r = .85 \); preventive-probiotic-treatment group: \( U = 2.00, p = .002, r = .78 \)). This finding demonstrates that probiotics were effective both as an active treatment and as a preventive treatment.

Effects of probiotic on infant extinction. MS-F1 pups were tested for the reinstatement effect after extinction. Within-session extinction behavior did not differ across groups (see Fig. S7 in the Supplemental Material). At testing, there was a significant interactive effect of treatment and reinstatement condition, \( F(2, 50) = 4.99, p = .011, \eta^2_p = .17 \), and a significant main effect of reinstatement condition, \( F(1, 50) = 4.99, p = .030, \eta^2_p = .09 \); the main effect of treatment was not significant, \( F(2, 50) = 1.68, p = .196, \eta^2_p = .06 \) (Fig. 3b). The vehicle group exhibited the reinstatement effect; freezing levels were higher in the reinstatement group relative to the no-reinstatement group, \( U = 11.50, p = .002, r = .67 \). However, the reinstatement effect was not observed for MS-F1 rats that received probiotic treatment in either the F0 or the F1 generation (active-probiotic-treatment group: \( U = 36.00, p > .250, r = .09 \); preventive-probiotic-treatment group: \( U = 32.00, p > .250, r = .09 \)). 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 after extinction in MS-F1 pups (see Fig. S5 in
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Probiotics affected neither maternal anxiety levels nor the dam’s caregiving behavior toward pups (see Figs. S8 and S9 in the Supplemental Material).

Discussion

We have reported 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 could be “inherited” by infant offspring. This is important clinically because 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) prevented the transmission of MS effects on aversive learning to the F1 generation. Likewise, treatment of
F1 pups reversed the behavioral phenotypes, demonstrating the effectiveness of probiotics as both a preventive and an active remedy. These findings suggest that behavioral phenotypes that are putatively involved in vulnerability to later-life anxiety, and that are transmitted across generations through fathers, can be effectively prevented or treated with noninvasive probiotic manipulations.

As mentioned in the introduction, how stress effects are transmitted across generations is currently the subject of intense debate. Although data from the current studies cannot distinguish whether stress effects were transferred 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; this temporary transfer of the probiotic strains to the pups’ colons was eliminated by adulthood. This strongly indicates that the specific probiotic strains used in the initial treatment were 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 leads to dramatic changes in the composition of gastrointestinal bacteria; such changes have been suggested to regulate stress-induced changes in social behavior (e.g., Zijlmans, Korpela, Riksen-Walraven, de Vos, & de Weerth, 2015; for a review, see Parashar & Udayabanu, 2016). In addition, 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, Venkatesh, Milbrandt, Gordon, & Raichle, 2015). It is possible that, in the current study, the probiotic intervention may have arrested or reversed changes in the development of aversive learning via effects on social functioning or metabolism, helping to preserve or repair infant performance. Indeed, it may be the case that either stress-induced or probiotic-induced changes in the intestinal microbiota can be passed down the generations, given that previous studies have suggested that the microbiota (or at least certain taxa) is heritable and that host genetics exert an influence on microbiota composition (Goodrich et al., 2014). In fact, this interspecies (host-microbe) interaction is likely to be bidirectional: It has also been shown that the microbiota can alter host gene expression, particularly with regard to genes involved in immune regulation (one likely candidate for the microbial effects on metabolic function; Broderick, Buchon, & Lemaitre, 2014).

Many neurotransmitters that are important for mood and that have programming effects on brain development (e.g., serotonin, γ-aminobutyric acid, or GABA) 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. Supporting this hypothesis, a recent study showed that microbial composition of the rodent gut regulates amygdala development (Stilling et al., 2015), a hub of emotional functioning. The specific strains of bacteria used in the current 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 increased by separation stress (Gareau et al., 2007; Hennessy et al., 2015). Corticosterone has also been shown to lead to accelerated development of emotion-related learning systems (i.e., long-lasting retention and high rates of relapse after extinction; for a review see Callaghan & Richardson, 2013). These data suggest the intriguing possibility that probiotics’ mechanism of action on threat responses in the current study may have involved dampening of stress-activated hormones and proinflammatory 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 been investigated only in males. This argument notwithstanding, previous research has demonstrated sex-specific generational inheritance of emotion-related responses (Franklin et al., 2010; H. K. 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, because of 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 make 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. Note that the probiotic used in the current studies already has established safety and efficacy in pediatric populations because it is frequently used in the treatment of gastrointestinal diseases (e.g., Freedman et al., 2014). Together with these past studies, the current data make a
strong case for further investigations into the clinical efficacy of these particular probiotic strains for the treatment of stress-related emotional health problems in children.

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Author Contributions
All the authors came up with the study design. B. L. Callaghan and C. S. M. Cowan carried out the experiments, analyzed the data, and drafted the manuscript. All the authors made final comments on the manuscript. B. L. Callaghan and C. S. M. Cowan contributed equally to this work.

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Declaration of Conflicting Interests
The authors declared that they had no conflicts of interest with respect to their authorship or the publication of this article.

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Supplemental Material
Additional supporting information can be found at http://pss.sagepub.com/content/by/supplemental-data

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