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## OPEN Probiotic *L. reuteri* treatment in stressed rats improves maternal care and corrects corticosterone-BDNF-oxytocin balance offering insights for maternal *postpartum* distress treatment

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Mothers experiencing depressive symptoms or gestational stress are at risk of developing maternal distress, which, in its most severe form, can progress to *postpartum* depression, profoundly affecting maternal well-being and infant care. Using a late-gestation restraint stress model, we investigated whether *postpartum* treatment of lactating dams with probiotic *Limosilactobacillus reuteri* (*L. reuteri*) could reverse stress-induced disruptions in maternal behavior and neurobiology. *L. reuteri* effectively improved maternal care, and restored reactivity to pup separation in stressed dams. *Postpartum* *L. reuteri* also corrected corticosterone-oxytocin imbalances in plasma, normalized hypothalamic oxytocin levels, the unglycosylated and glycosylated oxytocin receptor isoforms and it regulated the ratio of precursor to mature brain-derived neurotrophic factor (BDNF). Additionally, *L. reuteri* restored gut microbiota balance, increasing *Lactobacillus* spp. while reducing pathogenic strains in fecal pellets of stressed dams during lactation and post-weaning, correlating with both maternal oxytocin and corticosterone plasma levels. This study is the first to demonstrate the therapeutic efficacy of a probiotic and its molecular support in counteracting the adverse effects of gestational stress on maternal care through brain-gut axis mechanisms. These findings support probiotic-based interventions as safe, complementary preventive strategy for improving maternal health in conditions involving impaired maternal care and microbiota dysbiosis.

**Keywords** Gestational stress, *Limosilactobacillus reuteri*, Oxytocin and oxytocin receptor, BDNF, Corticosterone, Gut microbiota

Maternal pathologies such as *postpartum* depression (PPD) that include a reduced maternal care are underdiagnosed and difficult to treat, and has a strong impact on both maternal health and child development<sup>1</sup>. Indeed, medication for PPD typically involves conventional antidepressants or allopregnanolone analogs (brexanolone or zuranolone), but with a risk of transfer to the baby through breastfeeding<sup>2</sup>. PPD could represent one of the factors of early-life stress, contributing to the development of disorders through changes in epigenetic programming transmitted by alterations in maternal care<sup>3,4</sup>. A reduced maternal care has been associated with

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increased methylation of the gene encoding the glucocorticoid receptor and a prolonged corticosterone (CORT) response to stress in the adult offspring<sup>5</sup>. In addition, enhancing maternal care through early adoption has been shown to reverse the effects of perinatal stress (PRS) on regulating the hypothalamic–pituitary–adrenal axis in adult offspring<sup>6</sup>. In the PRS rat model, the reduced maternal care caused by gestational stress was associated with increased glucocorticoids and decreased oxytocin (OT) levels in the offspring<sup>7</sup>. Both animal and human studies link perturbed OT transmission to low maternal care or neglect in the early *postpartum* period<sup>8</sup>. For example, in humans, increased methylation of the OT encoding gene during pregnancy predicts mother intrusiveness in the *postpartum* period<sup>9</sup>. The action of OT is mediated by G-protein coupled receptors (oxytocin receptors OTRs), which are found in brain regions involved in maternal behavior, including the hypothalamus<sup>10</sup>. Maternal behavior in rats has been linked to changes in OTR binding in response to postweaning isolation<sup>11</sup> and, in humans, polymorphic variants of the OTR-encoding gene interact with early parental experience to influence the development of psychiatric disorders later in life<sup>12</sup>. Therapeutic interventions aimed at enhancing OT signaling may enhance maternal behavior and hold promise in the treatment of PPD<sup>13</sup> and other disorders induced by maternal distress, characterized by low maternal care. In line, *postpartum* treatment with the OTR agonist, carbetocin, restored maternal care in stressed rat dams and corrected the pathological phenotype of their offspring<sup>7,14</sup>.

Recent studies have disclosed an intricate interplay between OT and brain-derived neurotrophic factor (BDNF) in the hippocampus, a key brain region in stress-related disorders<sup>15,16</sup>. A reciprocal interaction between OT/OTR and BDNF/TrkB (the receptor activated by the mature form of BDNF) also occurs in hypothalamic neurons<sup>17</sup>, and BDNF enhances maternal care by activating TrkB receptors in OT neurons<sup>18</sup>. Thus, the hypothalamic BDNF/OT axis is also a candidate drug target for therapeutic intervention in stress-related disorders.

Of note, the OT/OTR system is also involved in mechanisms of gut–brain communication<sup>19,20</sup>, and affects the composition of the gut microbiota<sup>21</sup>. Additionally, OT levels are influenced by the gut microbiota, with the intestinal epithelium reducing OT under its regulation<sup>22</sup>. Gut microbiota plays a key regulatory role in depression progression<sup>23</sup>, and it is likely an additional key causative factor in PPD<sup>24</sup>. Changes in gut microbiota composition are linked to PPD and reduced maternal care<sup>24,25</sup>. In mice models, gut microbiota dysbiosis has been associated with changes in maternal care<sup>26</sup> and causally related to altered exploratory behavior in a mouse model of PPD<sup>27</sup>.

Probiotics, or psychobiotics<sup>28</sup>, show promise in treating stress-related disorders of the offspring<sup>20,29–31</sup> and emerging evidence highlights their beneficial effects on mental health, sharing transcriptomic signatures with antidepressants<sup>32</sup>. The impact of probiotics on maternal mental health remains underexplored, despite its critical importance given that maternal distress can profoundly influence both parental outcomes and consequently offspring development<sup>33</sup>. In this context, *Limosilactobacillus reuteri* (*L. reuteri*) ATCC PTA 6475, originally isolated from human breast milk, has demonstrated OT-activating properties<sup>19,34,35</sup>, suggesting its potential to influence maternal neuroendocrine function and behavior. While another strain, *L. reuteri* PBS072, has been shown to improve maternal mood and breastfeeding quality in healthy women, it has not been associated with effects on maternal care<sup>36</sup>. Thus, the specific impact of *L. reuteri* on maternal care in models of gestational stress remains unexplored. Using the PRS rat model, this study uniquely investigates the effects of *postpartum* treatment with *L. reuteri* in lactating dams exposed to gestational stress. It examines its influence on maternal care towards pups, the balance between plasmatic CORT and OT levels, as well as the hypothalamic OT/BDNF axis in association with mother's gut microbiota composition. We also provided a complementary assessment of mother's behavior in two paradigms not-directed towards pups such as locomotor reactivity to a novel environment and exploration in the elevated-plus maze. By focusing on the mother rather than the offspring, this study is groundbreaking, providing valuable insights into how probiotic treatment can support maternal health and stress resilience during the *postpartum* period. Our data pave the way to the development of a new strategy aimed at enhancing maternal care in mothers with low mood, as occurs in PPD. This strategy, based on the use of a probiotic, is safe, and may limit the consequences of a low maternal care on the developmental trajectories of the offspring.

## Methods and materials

### Ethics

All experiments were carried out in accordance with the ARRIVE Guidelines (<https://arriveguidelines.org>) and followed the rules of Directive 2019/10/10 of the Council of the European Communities. This project was approved by Ethic Committee CEEA-75 (Comité d'Ethique en Expérimentation Animale Nord-Pas de Calais) and by the MESRI (Ministère de l'Enseignement supérieur, de la Recherche et de l'Innovation; APAFiS #33,654). No human endpoints were set in the present study.

### Animals

25 nulliparous Sprague Dawley female rats (Charles River, France) weighing approximately 250–275 g, aged 8 weeks were purchased from Charles River (France) and group-housed (five females/cage) in individually ventilated cages (IVCs; Tecniplast, Italy) and kept under standard conditions at a temperature of 21–22 °C, humidity of 50% to 60%, with a 12 h light/dark cycle (lights on 7 am: lights off 7 pm) with ad libitum access to food and water. After 10 days of group housing (to encompass two estrous cycles and optimize estrous cycle synchronization), each female was individually housed with a sexually experienced male rat for one week. Following that, a gain of at least 10 g was considered as an index of pregnant status. As bedding, a mixture of sawdust and cellulose was provided and a chewable food stick was added as minimal environmental enrichment condition. During gestation enrichment was improved by adding 20 gr of cellulose during the last week of gestation as nesting material. After delivery, no culling was performed, and to ensure comparable litter conditions

across groups, the total number of pups per litter and the male-to-female ratio were recorded throughout the postpartum period, with no significant differences observed (supplementary S1A).

### Gestational stress procedure

14 pregnant females were randomly assigned to a stress procedure, following our standard protocol<sup>6</sup>, while the remaining females were left undisturbed (control unstressed group) during gestation in their home cages. Starting on the approximately 11th day of pregnancy and continuing until delivery, the dams were restrained (3 times/day/45 min) in a transparent plastic cylinder with conical end caps and exposed to bright light (979 Lux). During stress procedure, females were daily inspected for their well-being and any expected or unexpected adverse events (i.e. abrupt drop of body weight sign of spontaneous abortion or degradation of fur) were recorded by the animal care givers and the experimenters. Dam's body weight in both stressed and unstressed groups was monitored weekly during gestation and during the *postpartum* period (PP) at PP7 and PP21, when weaning of pups occurred. After birth, the pups remained undisturbed with their mother until weaning.

### Culture and treatment with *L. reuteri* during lactation

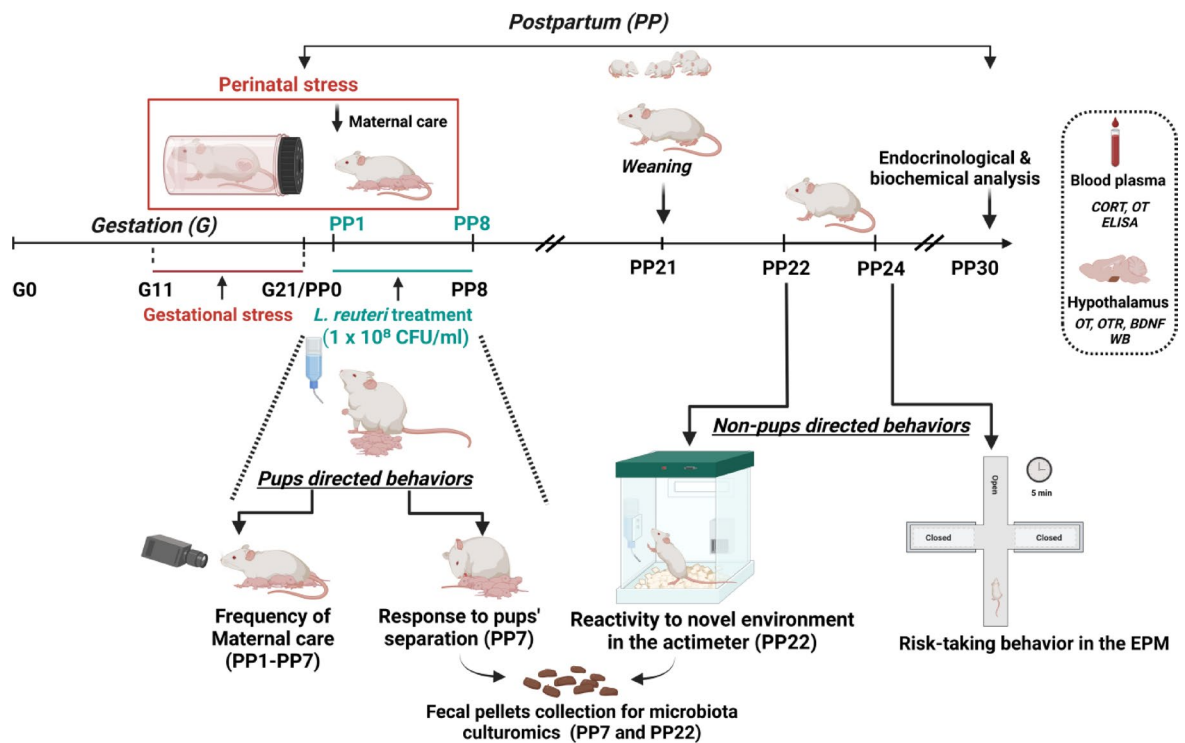
To modulate the OT system, lactating dams were given a probiotic treatment with *L. reuteri* ATCC PTA 6475 (BioGaia AB, Eslöv, Sweden) during the first *postpartum* week (PP1-PP8).

*L. reuteri* cultures were grown anaerobically in MRS broth. The bacterial growth was assessed by serial dilution plating on MRS agar, followed by colony counting and calculation of CFU/ml. After centrifugation, bacterial cells were resuspended in PBS and frozen at -80 °C. Live cultures were added to the dams' drinking water, which was thoroughly mixed and vortexed according to BioGaia's recommendations and established protocols from the literature<sup>19</sup>. The solution was replaced daily to ensure consistent dosing and minimize dosage variability.

The probiotic group consumed  $1 \times 10^8$  CFU/ml, with each rat ingesting between  $0.78 \times 10^{10}$  to  $0.79 \times 10^{10}$  CFU per day, while the control group received PBS-treated water. Both groups consumed the treated water ad libitum.

### Experimental design

The experimental design and timeline (Fig. 1) included four groups: stressed mothers treated with *L. reuteri* from PP1 to PP8 (STRESS+*L. reuteri*; n=8) or water (STRESS+water; n=6), and their unstressed controls (CONT+*L. reuteri*; n=5, CONT+water; n=6). Females began pregnancy at 2.5 months of age and were



**Fig. 1.** Experimental design and timeline. Gestational stress was induced during the last 10 days of pregnancy, followed by *L. reuteri* treatment from *postpartum* day 1 (PP1) today 8 (PP8). Maternal care and response to pup separation were evaluated during the first week of lactation. After weaning, dams were assessed for locomotor reactivity to novel environment using an actimeter (PP22) and exploratory and risk-taking behavior in the elevated-plus maze (PP24). Microbiota analysis in fecal pellets was conducted at PP7 (after maternal response to pup separation test) and PP22 (after the locomotor activity test in the actimeter). On PP30, oxytocin (OT), its receptor (OTR), and brain-derived neurotrophic factor (BDNF) were analyzed in the hypothalamus, and the corticosterone/oxytocin (CORT/OT) balance was measured in plasma.

approximately 3.5 months old when the study concluded. Pup-directed behaviors, including pup-directed care and maternal response to pup separation, were assessed during the first week of lactation (PP1–PP7) and at PP7, respectively. Non-pup directed behavior as locomotor reactivity to a novel environment and exploration in the elevated-plus maze (EPM) were evaluated on PP22 and PP24. To assess gut microbiota composition, fecal samples were collected at PP7 and PP22. Tissue collection (hypothalamus and trunk plasma) was performed at postnatal day 30 (PP30) via decapitation. Anesthesia was not used in order to avoid potential interference with protein content. Sample size estimates were based on prior behavioral and biochemical studies conducted in our laboratory and calculated using STATISTICA software, with the power of the experiment set at 80%. For behavioral and biochemical data, each dam served as the experimental unit.

## Pup-directed behaviors during lactation

### *Maternal care*

The active behavior of the mother in the nest (nursing behavior, licking, carrying pups, and arched back over pups) was manually scored from 7 to 8 am every minute (60 observations/h with 1 h of observation per day) with small infrared cameras (AMC, France) positioned on the dams' cages rack during the first four *postpartum* days. The 7 am time point was selected based on previous observations<sup>7</sup>. Additionally, we collected observations at 3 pm on 3rd day. The data obtained were expressed as a percentage of licking/nursing with respect to the total number of observations per day and across all days, as also reported in previous investigations<sup>14</sup>.

### *Maternal response to pup separation test*

Maternal reactivity following a 15-min separation from pups on *postpartum* day 7 (PP7) was assessed alongside natural home-cage behavior. Maternal responsiveness was assessed by measuring the latency to initiate pup contact (first licking) after separation. The dam was weighed in a separate cage while pups were scattered in the home cage. Upon return, the latency to lick the first pup and maternal behaviors, including time spent nursing or self-directed activities, were scored over 10 min.

## Non-pup-directed behaviors after weaning

### *Locomotor reactivity to novel environment*

At PP22, dams were assessed for their locomotor reactivity to a novel environment in an actimeter equipped with infrared sensors (beams) for locomotor activity detection, (Imetronic, Bordeaux, France). Each female was placed in one of the actimeter cages for 90 min. The amount of locomotor activity was represented by the frequency of infrared sensor interruptions.

### *Exploratory activity and risk-taking behavior in the elevated-plus maze (EPM)*

At PP 24, dams were assessed for their number of visits and time spent in each arm in the EPM according to our previous protocol<sup>37</sup> with a video camera tracking (EthoVision, Noldus, The Netherlands). The exploratory behavior was measured through the number of visits in both the open and closed arms, and risk-taking behavior as the presence of the rat in the open arms.

## Gut microbiota culturomics analysis in fecal samples

For quantification of culturable bacteria, fecal samples were collected from lactating dams at PP7 during the 15-min pup separation and at PP22 following exposure to the actimeter. Feces were cultured on bacterial media focusing on the most representative anaerobic and aerobic culturable bacteria that may undergo changes during intestinal dysbiosis, with the main bacteria species analyzed as *Lactobacillus spp* (*L. spp*), that englobes different species of *lactobacilli* among them *L. reuteri*, and pathogenic strains including *Escherichia coli* (*E. coli*) and *Enterococcus faecalis* (*E. faecalis*). Fecal material was weighed and homogenized in PBS. Serial dilutions ranged from  $10^{-2}$  to  $10^{-3}$  were performed, each dilution was spread on petri dishes. Aerobic bacteria were isolated on MacConkey (MCK) and bile-esculin-azide (BEAz) culture media, respectively. For anaerobic bacteria, petri dishes containing Man, Rogosa, Sharpe (MRS) culture medium at 37 °C. All bacterial plates were incubated at 37 °C and read after 24 h. To identify bacterial colonies in the Petri dishes, 1.5 µL of matrix solution ( $\alpha$ -cyano-4-hydroxycinnamic acid; Bruker Daltonics) dissolved in 50% acetonitrile, 47.5% water, and 2.5% trifluoroacetic acid was added to the bacterial colonies, which were then analyzed using mass spectrometry (MALDI-TOF; Microflex-Bruker Daltonics, Leipzig, Germany).

## Biochemical analysis

Hypothalamus was dissected, immersed in liquid nitrogen, and rapidly frozen at  $-80$  °C. Measurement of OTR and BDNF protein expression in the hypothalamus by western blotting (WB) was performed in crude synaptosomal fractions according to a previous established protocol using subsequent centrifugations in HEPES-buffered sucrose of the homogenized tissue<sup>38</sup>. Each target protein was run on two separate gels, with samples from six animals per group. To ensure consistency and allow quantitative comparison, a sample from the CONT water-treated group was used as an internal control on both gels. Antibodies against BDNF (1:500, #282,051-AP, Proteintech) and oxytocin receptor (OTR; 1:250, #sc-33209, Santa Cruz) were used, with  $\beta$ -actin (1:1000, #A5316, Sigma) as the loading control. Anti-BDNF detected proBDNF (32 kDa), truncated BDNF (tBDNF, 28 kDa), and mature BDNF (mBDNF, 14 kDa); anti-OTR detected unglycosylated (45 kDa) and glycosylated (55 kDa) isoforms. After immunoblotting, membranes were sectioned by molecular weight when feasible to analyze both target proteins and  $\beta$ -actin on the same blot. Bands were visualized using ECL (ThermoFisher) and digitized with the FUSION® system. Optical density (O.D.) was quantified using ImageJ, normalized to  $\beta$ -actin, and expressed as a ratio relative to the CONT group.

### Endocrinological analysis

Plasma assessment of OT and CORT levels was performed in trunk blood samples collected around PP30 with rat/ELISA kit (OT, sensitivity 9.4 pg/ml-CUSABIO # CSB-E14197r; CORT, sensitivity 6.1 ng/mL-Demeditec # DEV9922). OT levels in the hypothalamus were measured by ELISA test (Enzo ADI-901-153A; sensitivity 15 pg/ml) using 100  $\mu$ l of the supernatant obtained after the first centrifugation (15,000  $\times$  g, 20 min, 4 °C) of homogenized hypothalamic tissue in HEPES-buffered sucrose. Tissues used for WB and Elisa were from the same animals. Results were normalized to protein quantity after a BCA assay and expressed as pg/ $\mu$ g protein.

### Statistical analyses

STATISTICA 8.0 (Stat Soft. Inc) was used for power analysis (power goal set at 0.8), sample size calculation, and data analysis. The normality of data distribution was assessed using the Shapiro–Wilk test, and then a Two-ways ANOVA with Newman-Keuls post-hoc (NK) or Planned Comparisons (p.c) tests were applied. Independent variables included group with stress\* (stress vs. cont) and probiotic\* (*L. reuteri* vs. water). Pearson correlations analysis was used to investigate association between behavioral, endocrinological and neurochemical parameters. A p-value < 0.05 was considered significant. All graphs were created with GraphPad PRISM Version 10.2.3 (MA, USA).

## Results

### The weight-reducing effect of postpartum *L. reuteri* during the postpartum period is independent of gestational stress

Administration of *L. reuteri* (Fig. 2A) led to a significant decrease in body weight, as reflected in the average measurements taken on *postpartum* day (PP7) and *postpartum* day (PP21), regardless of whether the rats had been exposed to stress during gestation (n = 5–8 rats/group;  $F_{(1,21)} = 4.55$ ,  $p = 0.04\#$ ). Maternal stress during pregnancy had previously been associated with reduced body weight, as observed at gestational days 14 (G14) and 20 (G20) (supplementary S1B).

### Appetitive value of *L. reuteri* postpartum administration

Probiotic oral intake increased during the first *postpartum* week, regardless of gestational stress (n = 5–8 rats/group; *probiotic effect*  $F_{(1,21)} = 8.27$ ,  $p = 0.009\#\#$ ; average/week, supplementary S1C). However, within the stressed group, *L. reuteri* intake was higher compared to the water-treated group during the 7-day treatment period (*stress x treatment x day interaction*,  $F_{(6,126)} = 2.13$ ,  $p = 0.05 * \#\$$ ; p.c.  $\#\#p < 0.01$  or  $\#p < 0.05$ ) (Fig. 2B).

### Postpartum *L. reuteri* corrected pup-directed behaviors in stressed mothers

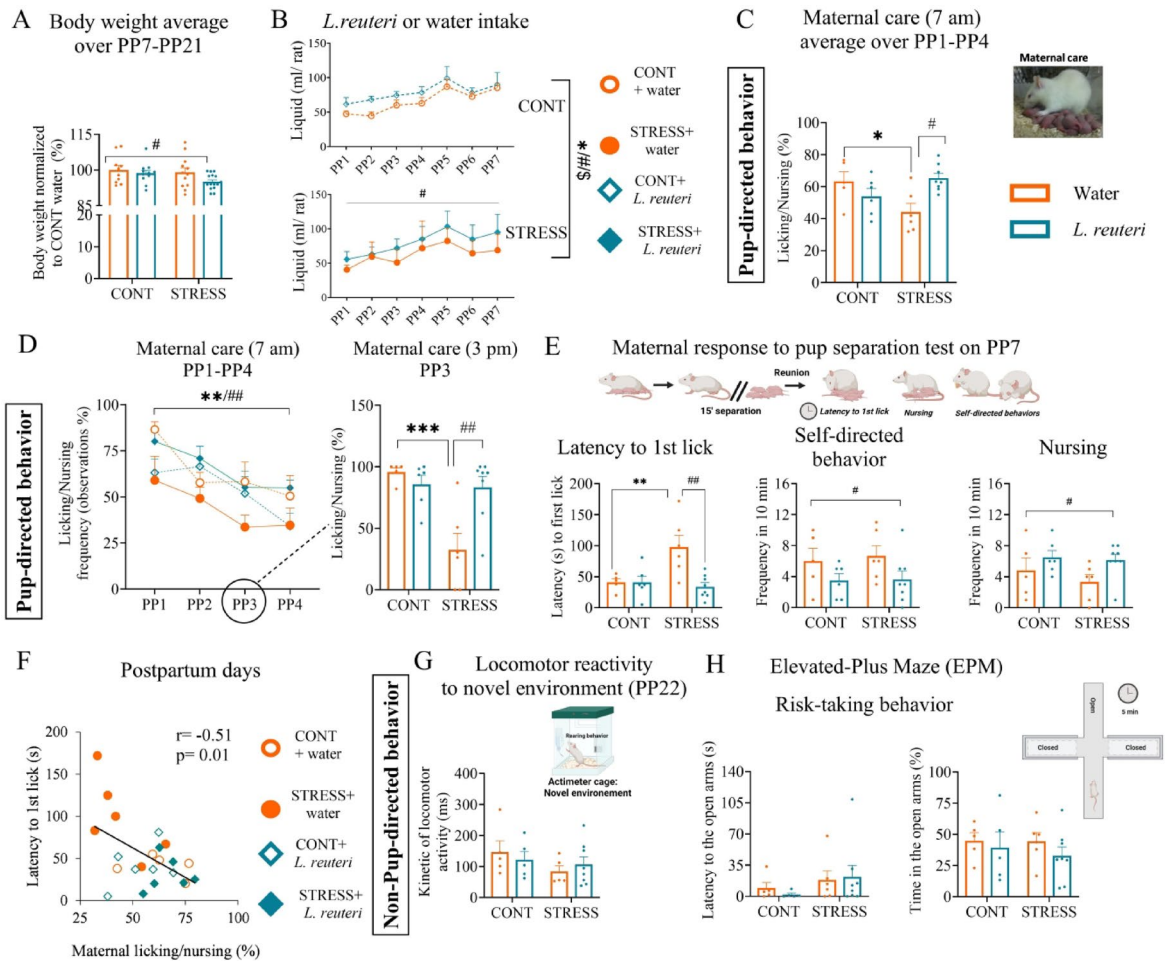
*Maternal care* (PP1–PP4): Gestational stress reduced maternal care (licking/nursing amount) (Fig. 2C, D) during the first *postpartum* week at 7 am, and *L. reuteri* rescued that deficit (n = 5–8 rats/group, *stress x probiotic interaction*  $F_{(1,21)} = 10.49$ ,  $p = 0.003**/\#\#$ ; NK, *stress vs. cont* in water groups,  $p = 0.023*$ ; *water vs. L. reuteri* in the stress group,  $p = 0.021\#$ ). The same profile was observed at 3 pm on 3<sup>rd</sup> day (Fig. 2D) (n = 5–8 rats/group, *stress x probiotic interaction*  $F_{(1,21)} = 9.89$ ,  $p = 0.004**/\#\#$ ; NK *stress vs. cont* in water groups,  $p = 0.0008***$ ; *water vs. L. reuteri* in the stress group,  $p = 0.01\#\#$ ). *Maternal reactivity to pup separation* (PP7): Stressed dams + water displayed a longer latency to contact pups after separation compared to controls, which was corrected by *L. reuteri* (Fig. 2E) (n = 5–8 rats/group, *stress x probiotic interaction*,  $F_{(1,21)} = 7.63$ ,  $p = 0.01**/\#\#$ ; NK, *stress vs. cont* in water groups,  $p = 0.002**$ ; *water vs. L. reuteri* in the stress group  $p = 0.004\#\#$ ). During the 10 min following the pups' reunion, we measured mother nursing and self-directed behaviors (grooming and eating) (Fig. 2E). While gestational stress had no impact on these parameters, we observed a main effect of *L. reuteri*, which increased nursing behavior and reduced self-directed behavior in both stressed and control groups (n = 5–8 rats / group; *probiotic effect, nursing*,  $F_{(1,21)} = 4.58$ ,  $p = 0.044\#$ ; *self-directed behavior*,  $F_{(1,21)} = 4.98$ ,  $p = 0.03\#$ ). Altogether, maternal care and licking latency were strongly associated ( $r = -0.51$ ,  $p = 0.01**$ , Fig. 2F), with *L. reuteri* enhancing maternal licking/nursing and reducing licking latency in stressed dams.

### Gestational stress and postpartum *L. Reuteri* do not influence non-pup directed behaviors

Following weaning, rat dams were assessed for non-pup-directed behaviors, including reactivity to a novel environment in the actimeter (PP22) and exploratory and risk-taking behavior in the elevated-plus maze (EPM, PP24). *Locomotor Reactivity* (Fig. 2G): Neither stress nor *L. reuteri* treatment affected total locomotor activity (n = 5–8 rats/group; *stress x probiotic interaction*,  $F_{(1,19)} = 0.75$ ,  $p = 0.39$ ). *Exploratory and Risk-Taking Behavior in the EPM* (Fig. 2H and supplementary S1D): Activity levels (visits to open/closed arms), risk-taking measures (time spent in open arms, latency to enter open arms, and visits to open arms), and total visits were similar across groups (n = 5–8 rats/group; supplementary S1D: total visits,  $F_{(1,20)} = 0.32$ ,  $p = 0.57$ ; visits to open arms,  $F_{(1,20)} = 0.41$ ,  $p = 0.71$ ; Fig. 2H: time spent in the open arms,  $F_{(1,20)} = 0.13$ ,  $p = 0.71$ ; latency to open arms,  $F_{(1,20)} = 0.24$ ,  $p = 0.62$ ).

### Gestational stress induced gut dysbiosis, which was restored by postpartum *L. reuteri* treatment

Culturomics analysis of fecal samples (Fig. 3) revealed that both gestational stress and *L. reuteri* had an impact on the gut microbiota composition. At PP7 (Fig. 3A), stressed dams exhibited lower *Lactobacillus spp* and higher *E. coli* and *E. faecalis* compared to controls, which displayed similar levels of species composition. *L. reuteri* treatment selectively restored microbial balance in the stressed group, by increasing *Lactobacillus spp* and reducing *E. coli* and *E. faecalis*, underscoring its potential to counteract stress-induced dysbiosis (n = 5–6 rats/group; *L. spp: stress x probiotic interaction*,  $F_{(1,19)} = 17.17$ ,  $p = 0.0005***/\#\#\#$ ; *probiotic effect*  $F_{(1,19)} = 9.76$ ,  $p = 0.005**$ ; NK *water vs. L. reuteri* in the stress group,  $p = 0.0004\#\#\#$ . *E. coli: stress effect*,  $F_{(1,19)} = 8.35$ ,  $p = 0.009**$ ;

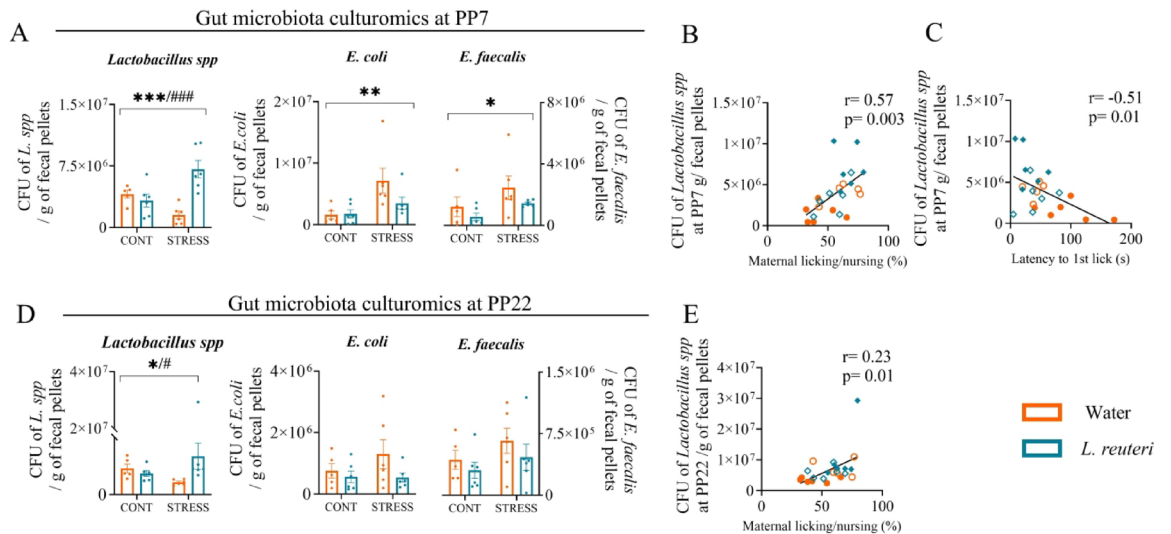


**Fig. 2.** Postpartum *L. reuteri* corrected pup-directed behaviors. *L. reuteri* decreased body weight average over PP7 and PP21 during the postpartum period independently of the stress groups (A). Stressed female rat dams exhibited more liquid consumption with *L. reuteri* over the 7 days treatment (ml/ rat) (B) Gestational stress reduced maternal care via the reduction of licking/nursing during PP1-PP4 (measured at 7 am), while *L. reuteri* given to stressed dams corrected it (C). The same profile was observed at the 3 pm time point on day 3 (D). For maternal response to pup separation on PP7, gestational stress increased latency to lick which was reduced by *L. reuteri* (E). The probiotic increased nursing behavior and reduced self-directed behaviors in both groups following reunion with pups. Maternal licking/nursing (averaged across the four days) and latency to first lick in the maternal response to pup separation test were correlated. Pearson’s correlation coefficient (*r*) values and related *p* values are reported (F). At PP22, both gestational stress and postpartum *L. reuteri* did not impact the reactivity to the novel environment in the actimeter (G). At PP24, gestational stress and postpartum *L. reuteri* did not influence neither exploratory nor risk-taking behavior in the EPM (H). Pearson’s correlation coefficient (*r*) values and related *p* values are reported. All values are means  $\pm$  S.E.M. (*n* = 5–8 rats/group), \* *p* < 0.05, \*\* *p* < 0.01, \*\*\* *p* < 0.0001 for CONT vs. STRESS; and # *p* < 0.05 and ## *p* < 0.01 for water vs. *L. reuteri* corresponding group. For interaction effects, \*\*/## < 0.01 for stress and probiotic interaction and \*/#/\$ *p* < 0.05 for stress, probiotic, and day interaction.

NK stress vs. cont in water groups, *p* = 0.02\*; water vs. *L. reuteri* in the stress group, *p* = 0.05#. *E. faecalis*: stress effect,  $F_{(1,19)} = 4.44$ , *p* = 0.04\*). Furthermore, *L. spp* at PP7 showed a strong positive correlation with maternal care (*r* = 0.57, *p* = 0.003) (Fig. 3B) and negatively with latency to first lick (*r* = -0.51, *p* = 0.01) (Fig. 3C). Interestingly, by PP22, the same microbiota deficit persisted in *L. spp* while no significant differences were observed for *E. coli* or *E. faecalis* (*n* = 5–6 rats/group; *L. spp*: stress  $\times$  probiotic interaction,  $F_{(1,19)} = 4.4$ , *p* = 0.04\*/#) (Fig. 3D). Additionally, the amount of *Lactobacillus* (*L. spp*) at PP22 remained positively correlated with maternal care (*r* = 0.23, *p* = 0.01) (Fig. 3E).

**Impact of maternal stress and correction of postpartum *L. reuteri* on the plasmatic CORT /OT balance and the OT /BDNF balance in plasma and the hypothalamus**

**Plasma CORT and OT balance** (Fig. 4A): Stressed-water dams showed higher plasma CORT and lower OT levels than controls. *L. reuteri* normalized these levels in stressed dams (*n* = 5–6 rats/group; CORT: stress  $\times$  probiotic interaction,  $F_{(1,19)} = 15.88$ , *p* = 0.0007\*\*/###; NK, stress vs. cont in water groups, *p* = 0.0001\*\*\*; water vs. *L. reuteri*



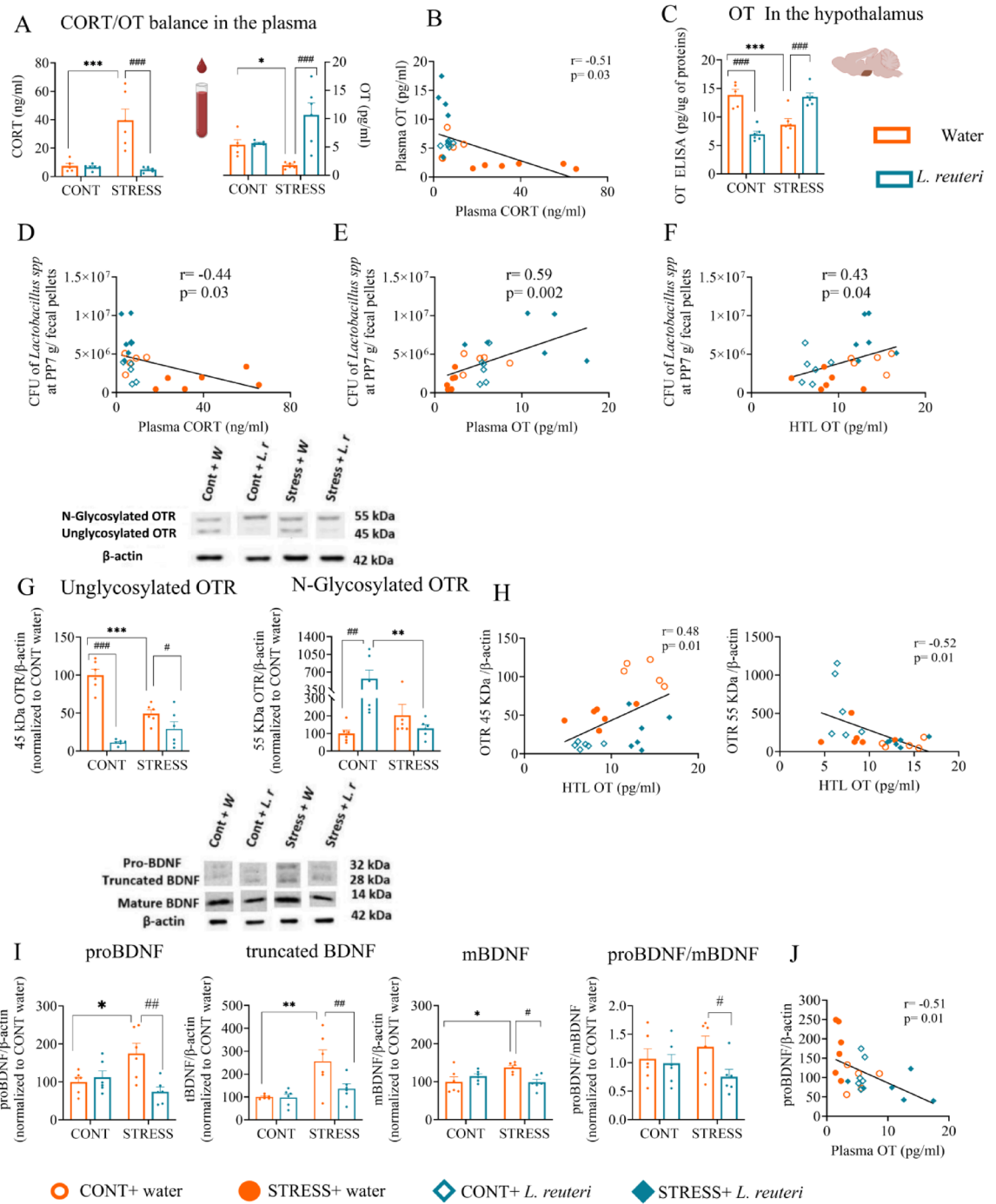
**Fig. 3.** Postpartum *L. reuteri* Treatment Restores Gut Microbiota and Correlates with Improved Maternal Behavior in Stressed Dams at PP7 and PP22. At PP7, culturomics in fecal samples showed gut dysbiosis in stressed dams which was rescued by postpartum treatment *L. reuteri* (A). Similarly, gestational stress related gut dysbiosis persisted showing the same profile PP22 which was rescued by postpartum treatment *L. reuteri* (B). At PP7 *L. spp* levels in fecal pellets correlated with maternal behavior (C) and latency to first lick in the pup separation test (D). Similarly, at PP22, *L. spp.* correlated with maternal behavior (E). Pearson's correlation coefficient ( $r$ ) values and related  $p$  values are reported. All values are means  $\pm$  S.E.M. ( $n = 5\text{--}8$  rats/group), \*  $p < 0.05$  and \*\*  $p < 0.01$  for CONT vs. STRESS; corresponding group. For interaction effects, \*/#  $< 0.01$  and \*\*\*/###  $< 0.001$  for stress and probiotic interaction.

in the stress group,  $p = 0.0002###$ ; OT: stress  $\times$  probiotic interaction,  $F_{(1,19)} = 12.90$ ,  $p = 0.002**/#$ ; NK, stress vs. cont in water groups,  $p = 0.05^*$ ; water vs. *L. reuteri* in the stress group,  $p = 0.0004###$ ). A significant negative correlation was observed between plasma OT and CORT ( $r = -0.51$ ,  $p = 0.01^{**}$ ) (Fig. 4B). Hypothalamic OT levels (Fig. 4C): stressed-water dams had reduced OT levels, which were restored by *L. reuteri*. Interestingly, *L. reuteri* increased OT in stressed dams but it reduced it in controls ( $n = 5\text{--}6$  rats/group, stress  $\times$  probiotic interaction,  $F_{(1,19)} = 50.48$ ,  $p = 0.000001^{***/###}$ ; NK, stress vs. cont in water groups,  $p = 0.0008^{***}$ ; water vs. *L. reuteri* in the stress group,  $p = 0.0006###$ , and  $p = 0.0002###$  in the cont group). Gut microbiota at PP7, *L. spp.* levels displayed opposing correlation profiles with plasma hormone concentrations—negatively correlating with CORT ( $r = -0.44$ ,  $p = 0.03$ ) while positively correlating with OT ( $r = 0.59$ ,  $p = 0.002$ ) (Fig. 4D, E). Notably, *L. spp.* levels in fecal pellets also exhibited a positive correlation with hypothalamic OT ( $r = 0.43$ ,  $p = 0.04$ ) (Fig. 4F).

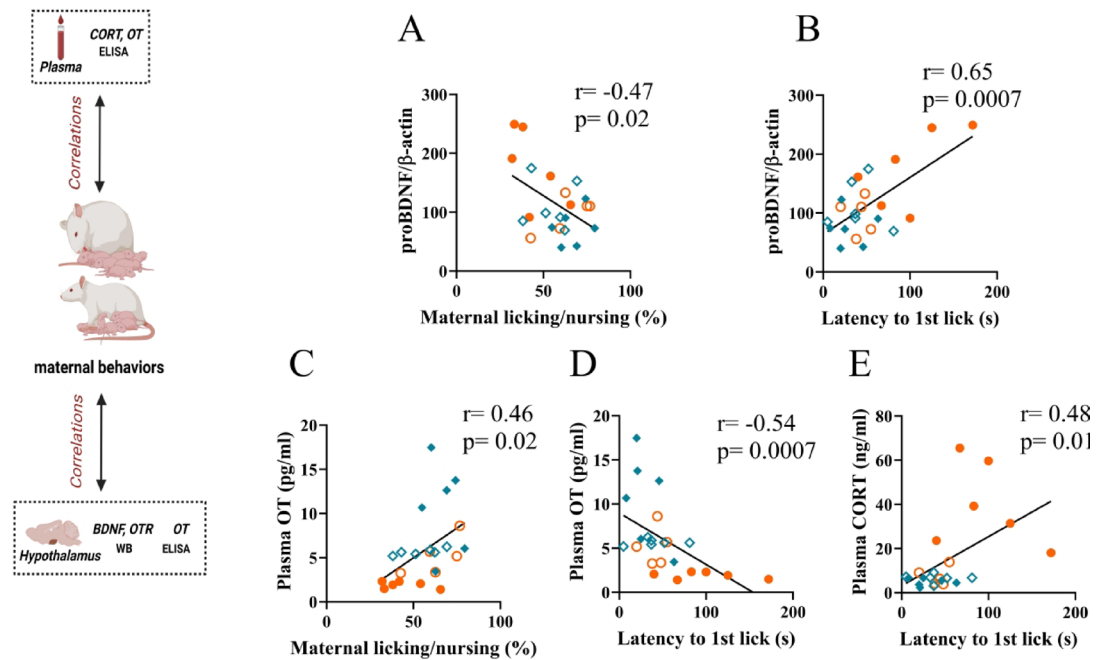
OT Receptor (OTR) Isoforms (Fig. 4G): Gestational stress reduced the 45 kDa unglycosylated OTR isoform and increased the 55 kDa N-glycosylated isoform, effects modulated by *L. reuteri*. In stressed dams, *L. reuteri* reduced both OTR isoforms while in controls it had opposite effects (45 kDa,  $n = 6$  rats/group, stress  $\times$  probiotic interaction,  $F_{(1,20)} = 25.44$ ,  $p = 0.00006^{***/###}$ ; NK, stress vs. cont in water groups,  $p = 0.0002^{***}$ ; water vs. *L. reuteri*,  $p = 0.04\#$  in the stress group and  $p = 0.0001###$  in the cont group; glycosylated 55 kDa,  $n = 6$  rats/group stress  $\times$  probiotic interaction  $F_{(1,20)} = 8.73$ ,  $p = 0.007^{**/#}$ ; NK, water vs. *L. reuteri* in cont group  $p = 0.008\#$ ; stress vs. cont in *L. reuteri* groups,  $p = 0.008^{**}$ ). Total OTR levels (unglycosylated + N-glycosylated) were reduced by stress and *L. reuteri* (supplementary S1E). Positive and negative correlations were observed between hypothalamic OT and 45 kDa ( $r = 0.48$ ,  $p = 0.01$ ) and 55 kDa OTR isoforms ( $r = -0.52$ ,  $p = 0.01$ ) (Fig. 4H). BDNF Isoforms (Fig. 4I): stressed mothers showed higher proBDNF and mBDNF levels, both reduced by *L. reuteri* ( $n = 6$  rats/group; proBDNF, stress  $\times$  probiotic interaction,  $F_{(1,20)} = 9.72$ ,  $p = 0.005^{**/#}$ ; stress vs. cont in water groups,  $p = 0.02^*$ ; water vs. *L. reuteri* in the stress group,  $p = 0.004\#$ ); mBDNF, stress  $\times$  probiotic interaction,  $F_{(1,20)} = 10.04$ ,  $p = 0.004^{**/#}$ ; NK, stress vs. cont in water groups,  $p = 0.05^*$ ; water vs. *L. reuteri* in the stress group,  $p = 0.05\#$ ). tBDNF showed a similar profile (tBDNF, stress  $\times$  probiotic interaction,  $F_{(1,20)} = 4.31$ ,  $p = 0.05^*/\#$ ; NK, stress vs. cont in water groups,  $p = 0.01^{**}$ ; water vs. *L. reuteri* in the stress group,  $p = 0.007\#$ ). *L. reuteri* did not affect BDNF isoforms in control unstressed dams. The proBDNF/mBDNF ratio was reduced by *L. reuteri* in stressed dams (probiotic effect,  $F_{(1,20)} = 3.39$ ,  $p = 0.08$ ; *p.c.* water vs. *L. reuteri* in the stress group,  $p = 0.03\#$ ). No correlation was found between hypothalamic OT and plasma CORT levels or between OTR and plasma CORT or plasma OT. However, in the BDNF and OT Correlation proBDNF negatively correlated with plasma OT ( $r = -0.51$ ,  $p = 0.001$ ) (Fig. 4J). Stress + water and stress + *L. reuteri* groups were distinctly clustered in significant correlations, highlighting the restorative effects of *L. reuteri* in stressed dams.

### Maternal behavior correlates with BDNF levels in the hypothalamus and the plasmatic CORT/OT balance

Associations were analyzed between maternal behaviors (nursing/licking averaged over four days and latency to lick pups after reunion) hypothalamic OT/BDNF and the plasmatic CORT/OT balance (Fig. 5). BDNF and



**Fig. 4.** Impact of maternal stress and correction of *postpartum* *L. reuteri* on the CORT/ OT balance and the oxytocinergic /BDNF balance in plasma and the hypothalamus. Gestational stress disrupted the plasmatic CORT/ OT balance and *postpartum* *L. reuteri* restored it (A) and CORT were correlated in plasma (B). Gestational stress reduced OT levels in the hypothalamus (C). At PP7, *L. spp.* levels in fecal pellets were correlated with plasma CORT (D) and OT levels (E), as well as with OT levels in the hypothalamus (F). Protein expression of unglycosylated (45 kDa) and N-glycosylated (55 kDa) OTR in the hypothalamus (G). Hypothalamic OT correlated with both OTR isoforms (H). proBDNF, tBDNF, mBDNF and proBDNF/mBDNF ratio in the synaptosomal fraction of hypothalamus (I). proBDNF correlated with the OT plasma (J). Pearson's correlation coefficient (r) values and related p values are reported. All values are means ± S.E.M. (n = 6 rats/group), \* p < 0.05, \*\* p < 0.01 and \*\*\* p < 0.001 for CONT vs. STRESS, and # p < 0.05, ## p < 0.01 and ### p < 0.001 for water vs. *L. reuteri* corresponding group.



**Fig. 5.** Correlation analysis. Maternal nursing/licking behavior (averaged across PP1-PP4) and maternal response to pup separation (latency to first lick) correlated with proBDNF in the hypothalamus (A, B) and with the CORT/OT balance in the plasma (C-E). Pearson's correlation coefficient ( $r$ ) values and related  $p$  values are reported ( $n = 5-6$  rats / licking latency in the pup separation test group).

**Maternal Behavior:** Notably, BDNF levels were correlated with maternal behavior: proBDNF was negatively linked to maternal nursing/licking ( $r = -0.47$ ,  $p = 0.02$ ) and positively with maternal response to pup separation as indicated by higher latency to lick ( $r = 0.65$ ,  $p = 0.007$ ) (Fig. 5A, B). Plasma CORT/OT balance and maternal care: Plasma OT positively correlated with maternal care ( $r = 0.46$ ,  $p = 0.02$ ) (Fig. 5C). Licking latency correlated negatively with OT and positively with CORT balance (OT:  $r = -0.54$ ,  $p = 0.007$ ; CORT:  $r = 0.48$ ,  $p = 0.01$ ) (Fig. 5D, E). Stress+water and stress+*L. reuteri* groups were distinctly clustered in significant correlations, highlighting the restorative effects of *L. reuteri* in stressed dams.

## Discussion

We demonstrated for the first time that probiotic *L. reuteri* treatment in lactating rats restores maternal caregiving behaviors disrupted by gestational stress, which are known not only to negatively impact offspring development but also to exert long-term programming effects<sup>11,14</sup>.

Notably, stressed dams exhibited no significant deficits in non-caregiving behaviors, consistently with *postpartum* depression (PPD), which primarily affects bonding, responsiveness, and maternal care<sup>39</sup>. *L. reuteri* regulated the plasmatic imbalance between CORT and OT responses, by enhancing the OT/OTR system and restoring the disrupted OT/BDNF balance caused by gestational stress in the hypothalamus, a brain region that is particularly involved in the CORT/OT hormonal regulation of maternal care (see Introduction and<sup>6,7,10</sup>). Also, the hypothalamus plays a key role in the regulation of body weight, sleep/wake cycle, response to stress<sup>29</sup>, and sociability<sup>40</sup>, which are all compromised in depressive disorders, including PPD. Here, treatment with *L. reuteri* significantly reduced dam body weight during lactation, regardless of stress exposure. This effect might be related to changes in hypothalamic OTR expression because treatment with the OTR agonist, carbetocin, was shown to affect body weight in lactating dams<sup>7</sup>. Interestingly, PPD is associated with weight gain<sup>41</sup>, and the weight-lowering effect of *L. reuteri* observed here and in other studies<sup>42,43</sup> may also help to mitigate metabolic complications of PPD. Moreover, we observed that stressed dams showed a preference for *L. reuteri* over water, indicating its appetitive value. This behavior may reflect altered stress responses and a compensatory increase in *L. reuteri* consumption to counter stress-induced dysbiosis, promoting resilience and adaptation. Notably, this preference highlights the potential for improved adherence to probiotic treatment in lactating mothers experiencing stress-related disorders.

Stressed dams exhibited intestinal dysbiosis, with reduced *Lactobacillus spp.* and increased pathogenic bacteria, including *E. coli* and *E. faecalis*. Probiotic treatment with *Lactobacillus reuteri* during lactation selectively restored fecal microbiota balance and intestinal homeostasis in stressed dams but had no effect on controls. Notably, gut dysbiosis and the counteractive effects of *L. reuteri* emerged by the end of the first *postpartum* week and persisted after pup weaning. A clinical study found late-pregnancy anxiety negatively associated with beneficial microbes like *Eubacterium* and *Oscillospira*, while heightened anxiety correlated with increased *Peptostreptococcaceae* and *Peptococcaceae*, bacterial groups linked to stress and poor health outcomes, supporting our findings<sup>44</sup>, thus reinforcing the relevance of maternal microbial signatures on maternal mental

health. However, evidence on microbiota changes in pregnant or lactating women with depressive symptoms remains scarce. Also, while prenatal stress-induced changes in microbiota composition—specifically the reduction of *Lactobacillus* strains—have been reported in adult rat offspring<sup>31,45</sup>, and in young non-human primate offspring<sup>46</sup> in association with maternal factors, this study provides the first direct evidence for maternal stress impacting the maternal gut microbiome and underscore the potential of *L. reuteri* to counteract maternal dysbiosis. Moreover, maternal dysbiosis was strongly associated with deficient maternal care both at PP7 and PP22, reinforcing the link between *Lactobacillus* abundance and maternal care across *postpartum* stages.

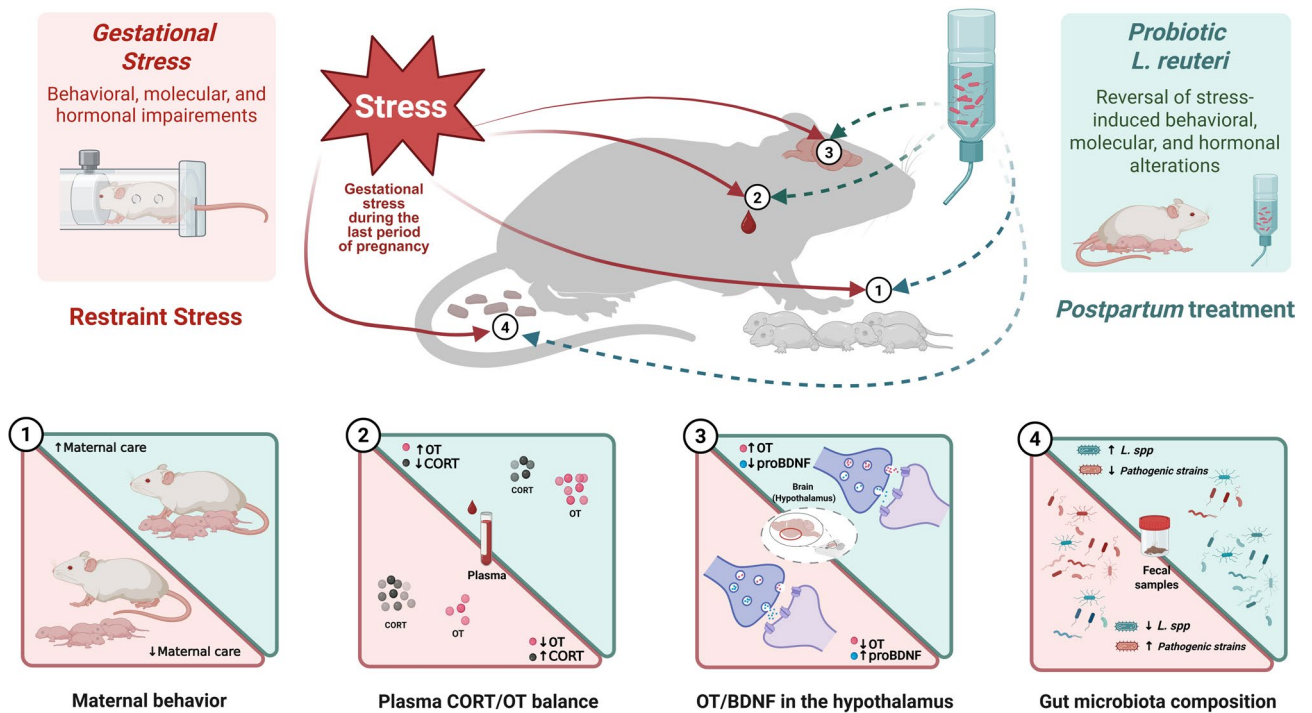
Remarkably, our study revealed that in stressed dams, reduced maternal care correlated with elevated CORT and decreased OT plasma levels, both restored by *L. reuteri* treatment. This restoration highlights the intricate interplay between CORT and maternal care, as CORT not only regulates metabolic and stress response but also plays a critical role in maternal care<sup>45</sup>. However, while elevated CORT levels during *peripartum* can impair maternal care<sup>47,48</sup>, the OT system is known to positively regulate these behaviors<sup>7,8</sup>. Here, changes in OT levels were specifically correlated with changes in the reduced licking/nursing behavior, whereas both OT and CORT levels were correlated with the latency of dams to the first-lick of their pups, which is an index of maternal responsiveness to pup separation. Of note, there is evidence for a strong negative correlation between plasma OT levels and PPD symptoms<sup>13,49,50</sup>, raising the attractive possibility that treatment with *L. reuteri* may improve PPD by enhancing the OT system. Microbiome composition in *Lactobacillus* at *postpartum* day 7 displayed opposing correlation profiles with the CORT/OT balance in plasma, correlating negatively with elevated CORT and positively with decreased OT. Again, *L. reuteri* treatment reversed this profile. Interestingly, at *postpartum* day 7 the amount of *Lactobacillus* in stressed dams not only positively correlated with maternal care (as also observed for *L. spp* at PP22), but also negatively with the higher latency to the first-lick, which is under the influence of CORT response<sup>47–49</sup>. These findings underscore the role of CORT/OT balance in shaping gut microbiota and suggest gut microbiota influences *postpartum* depression (PPD)-related maternal behavior via the HPA axis<sup>24</sup>. Of note, *L. spp* positively correlated also with hypothalamic OT, further supporting a gut-brain axis interaction in the regulation of maternal behavior and stress response.

The effect of gestational stress and *L. reuteri* administration on hypothalamic OTR levels was more intriguing. We detected two OTR isoforms through immunoblot analysis, with the 55 kDa isoform corresponding to the N-glycosylated form. However, it is currently unclear whether N-glycosylation influences OTR expression and/or function<sup>51</sup>. The reduction in OTR levels found in the hypothalamus of stressed dams is in line with previous studies showing a down-regulated OTR in the medial preoptic area (MPOA) of low-licking rat mothers<sup>52</sup>. Thus, it appears that a down-regulation of hypothalamic OTR is linked to stressful conditions affecting maternal behavior, and, interestingly, increased DNA methylation of the OTR gene promoter has been linked to risk of PPD<sup>53</sup>.

Several studies have focused on how maternal stress causes changes in BDNF levels in the brain of the offspring<sup>54–56</sup>. We examined for the first time how BDNF behaves in a brain region of lactating mothers with less care of the pups as a result of gestational stress. It is established that mBDNF is produced by the enzymatic cleavage of proBDNF, and measurements of the proBDNF/mBDNF ratio may provide insights on the effect of a particular treatment on BDNF turnover rate. Similarly, to other pro-hormones, proBDNF may also be active on its own, and while mBDNF activates TrkB receptors causing receptor homodimerization and signaling, proBDNF binds to p75 neurotrophin receptor. As opposed to mBDNF, proBDNF negatively regulates synaptic plasticity and may induce apoptotic neuronal death<sup>57</sup>. The proBDNF/mBDNF ratio can be targeted by therapeutic intervention in stress-related disorders, as shown by the evidence that antidepressants promote proBDNF cleavage into mBDNF<sup>58</sup>. Here, gestational stress enhanced proBDNF levels to a greater extent than mBDNF levels in the hypothalamus, and treatment with *L. reuteri* lowered the proBDNF/mBDNF ratio in stressed dams, but not in control dams. Thus, it is possible that under pathological conditions, probiotics affect the conversion of proBDNF into mBDNF similarly to antidepressants. Our correlation findings indicate that enhanced hypothalamic proBDNF levels are detrimental to pup licking/nursing behavior and maternal responsiveness to pup separation. Considering the interplay between OTR and mBDNF-TrkB receptors in the hypothalamus<sup>17,18</sup>, therapeutic strategies to reduce proBDNF synthesis or enhance its conversion into mBDNF may be valuable in the treatment of low maternal caregiving behavior associated with PPD. Here, neuroendocrinological analyses conducted several weeks after the assessment of maternal care reveal correlations with post-weaning markers, underscoring *L. reuteri*'s lasting impact on molecular brain pathways associated with maternal care. Maternal microorganisms play a crucial role in the development of the infant gut microbiota, with *Lactobacillus* being among the first genera to colonize the infant gut<sup>59</sup>. Evidence indicates that *Lactobacillus* from the mother's breast milk and mucosa is transferred to the infant, supporting the concept of mother-to-infant microbial transmission<sup>60,61</sup>. Thus, it is both logical and plausible that *L. reuteri*'s ability to reduce maternal gut dysbiosis may enhance the transfer of beneficial bacteria to the infant, thereby promoting infant well-being.

In conclusion, as illustrated in Fig. 6, we showed that maternal care during lactation and the associated microbial profiles (at PP7) reflect stable, trait-like characteristics that can predict later gut dysbiosis (at PP22) and alterations in CORT/OT levels, in line with developmental programming models linking early-life indicators to long-term health trajectories. Furthermore, our findings highlight the effectiveness of *L. reuteri* in enhancing maternal caregiving behaviors, thereby identifying a novel CORT/OT/BDNF pathway through which *L. reuteri* promotes maternal well-being. Our results position *L. reuteri* as a safe and effective “psychobiotic” capable of complementing existing therapies to alleviate *peripartum* psychological burden, improve maternal mental health, and strengthen the mother-infant bond, often weakened by maternal distress, including *postpartum* depression.

## Modeling the Effects of Postpartum *L. reuteri* in Stressed Dams



**Fig. 6.** Modeling the Effects of Postpartum *L. reuteri* in Stressed Dams. Postpartum treatment with *L. reuteri* mitigated the impact of peripartum stress on maternal care behaviors, plasma CORT/OT balance, and levels of proBDNF and OT/OTR in the hypothalamus. Additionally, the probiotic restored gut microbiota composition during lactation and after pup weaning. Stressed dams treated with *L. reuteri* during the first week of lactation exhibited improved pup-directed maternal behaviors, lower plasma CORT levels, and higher OT levels. These changes were accompanied by the normalization of proBDNF and OT/OTR levels in the hypothalamus.

### Data availability

All data generated or analyzed during this study are included in this manuscript.

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## Author contributions

R.B., S.M., and S.M-F designed the experiments. R.B. and V.C. set up the probiotic treatment. R.B. carried out all behavioral experiments, biochemical analysis, schematic and graphical presentations. A.G. provided additional support for biochemical and statistical analysis as well as schematic and graphical assistance. R.C. and B.S. performed microbiota culturomics analysis in fecal samples. R.B., A.G., H.B. and S.M-F. performed data analysis. R.B., F.N., S.M. and S.M-F. wrote the manuscript. A.H–L. provided editorial inputs.

## Declarations

### Competing interests

The authors declare no competing interests.

### Additional information

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