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Adult EEG Reactivity in Rat Offspring After an Uncomplicated Birth Is Not Significantly Altered by Gestational Antibiotics

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Abstract

Prolonged gestational antibiotic exposure was previously reported to impair EEG reactivity in adult offspring after perinatal asphyxia. It remained unclear to what extent the effect depended on perinatal insult. We assessed adult EEG reactivity after uncomplicated birth using the same gestational treatment paradigm. Pregnant Wistar rats received antibiotics (AB), probiotics (P), combined treatment (AB&P), or control (C). Adult offspring (AB: 5; P: 8; AB&P: 7; C: 9; 14 males) underwent intermittent photic stimulation during chloral-hydrate-induced EEG burst suppression. Reactivity to 60 s stimulation was quantified in 180 s trials using burst count reactivity (BCR) and the burst-suppression reactivity index (BSRi). After baseline burst count filtering, 200 trials were retained across groups. BCR did not differ significantly between groups. No multiplicity-corrected pairwise comparison identified a specific treatment-associated difference in BCR or BSRi. Mixed-effects models accounting for repeated trials likewise showed no significant overall group effect. Our small cohort did not provide evidence for a long-term effect of gestational antibiotic or probiotic exposure following uncomplicated birth. Together with the previous report, we suggest that gestational antibiotic administration could impair resilience to perinatal asphyxia at an exposure level without a readily detectable effect after uncomplicated birth.

Keywords: burst suppression; EEG reactivity; maternal antibiotics; gut microbiome; neurodevelopment; perinatal asphyxia; uncomplicated birth

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Received: 15 September 2026
Revised: 25 October 2026
Accepted: 30 October 2026
Published: 31 October 2026

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1. Introduction

Antibiotics are frequently used during pregnancy for maternal and fetal indications, and appropriate treatment should not be regarded as intrinsically harmful. Prolonged or broad-spectrum exposure can, however, alter the maternal gut microbiome and other maternal biological responses, raising the question of whether such exposure has consequences for offspring neurodevelopment that persist beyond birth [1,2]. Recent population-based and meta-analytic studies suggest that associations between prenatal antibiotic exposure and neurodevelopmental outcomes require cautious interpretation, particularly because familial and clinical factors may contribute to the observed associations [3,4].

The maternal gut microbiome changes during pregnancy together with host metabolism and contributes to the biochemical and immunological environment in which the fetal brain develops [5,6]. Experimental depletion of the maternal microbiota was shown to alter fetal neurodevelopment, including thalamocortical axon development [7]. Furthermore, antibiotics can modify maternal immune responses [8] and produce biological conditions distinct from germ-free models [9].

Experimental studies have reported diverse offspring consequences after maternal antibiotic exposure, with effects depending on treatment window, species, and the function examined. Periconceptional exposure has been associated with altered anxiety-related and social behaviour in rats [10], gestational exposure with changes in offspring microbiota and immunity in mice [11], and other gestational protocols with changes in learning and hippocampal structure [12]. Maternal treatment confined to the early postnatal period has also been followed by behavioural changes in adult offspring [13].

Abnormalities observed early in development do not necessarily remain equally apparent in adulthood. Maternal antibiotic exposure has been associated with reduced locomotor activity at 4 weeks that was no longer detectable at 7 to 8 weeks, despite persisting changes in exploratory behaviour [14]. Conversely, adult offspring may show selective abnormalities, such as altered tactile sensitivity, while many other sensorimotor functions remain relatively preserved [7]. Adult brain function therefore needs to be measured directly rather than inferred from developmental or behavioural outcomes.

Electroencephalographic (EEG) reactivity during burst suppression provides a controlled way to probe cortical responsiveness in the adult brain. Burst suppression consists of alternating periods of high-amplitude activity and marked suppression and is influenced by anaesthetic state and cerebral metabolic synaptic dynamics [15–17]. Sensory input can recruit additional bursts despite this suppressed background [18]. In our previous experimental work, intermittent photic stimulation reduced suppression, and suppression-ratio-based reactivity was sensitive to persistent functional impairment after hypoxic–ischaemic injury, including perinatal asphyxia (PA) [19]. This paradigm therefore provides a functional endpoint with which to ask whether a developmental exposure leaves a detectable alteration in adult cortical responsiveness.

PA occurs during continuing brain maturation and can have long-term consequences that extend beyond the initial hypoxic–ischaemic insult [20,21]. Using our previously established Wistar rat model of maternal dysbiosis induced by prolonged gestational antibiotic exposure [22], we recently reported that adult offspring exposed to both prolonged gestational antibiotics and the PA protocol (9% O_2 and 20% CO_2 in N_2 for 90 min) induced in offspring on postnatal day 6 showed impaired EEG reactivity to photic stimulation compared with controls [23]. Furthermore, the effect was partly attenuated by probiotic co-administration during gestation, as measured by both burst count reactivity (BCR) and the burst-suppression reactivity index (BSRi). It remained unclear to what extent the detrimental effect depended on PA.

The aim of this study was to assess whether prolonged gestational antibiotic exposure at a level associated with greater impairment after PA is also associated with a demonstrable impairment of EEG reactivity when birth is uncomplicated by PA. Rather than focusing solely on the effect of antibiotics, we used the same four-group gestational treatment paradigm as in the previous study, where the rats were subjected to PA [23]. Pregnant Wistar rats were randomized to receive antibiotics (AB), probiotics (P), combined treatment (AB&P), or control (C). Adult offspring, born naturally, underwent a sham procedure matching the PA procedure on postnatal day 6. The rats were weaned at day 21. We assessed EEG reactivity to photic stimulation in 2-month-old offspring during chloral-hydrate-induced EEG burst suppression using the same two measures: BCR and BSRi. In contrast to the PA study with a matching design, we did not identify a statistically significant treatment-specific difference in EEG reactivity when birth was uncomplicated.

2. Materials and Methods

2.1. Animals and Experimental Design

Pregnant Wistar rats were assigned to four gestational treatment groups: antibiotics (AB), probiotics (P), antibiotics plus probiotics (AB&P), or control (C), matching our previous study design [23]. Neurophysiological investigations were performed in offspring at 2 months postnatal under deep general anesthesia with EEG activity maintained at the burst-suppression level. The cohort comprised 29 offspring (20 dams): C, 9 (6 dams); AB, 5 (4 dams); AB&P, 7 (6 dams); and P, 8 (4 dams). Overall, 14 of 29 animals were male (48.3%). The proportion of males was 55.6% in C (5/9), 40.0% in AB (2/5), 42.9% in AB&P (3/7), and 50.0% in P (4/8).

After completion of the recordings, animals were euthanized by cervical dislocation under deep general anesthesia. All procedures were approved by the Local Committee for Animal Research, Carol Davila University of Medicine and Pharmacy, Bucharest, Romania (7845/25.03.2021), and complied with Directive 2010/63/EU on the protection of animals used for scientific purposes. The project was authorized by the Ilfov Directorate for Veterinary Health and Food Safety (project authorization no. 37/23.08.2023) and approved by the Ethics Committee of the Cantacuzino National Military Medical Institute for Research and Development (CE 319/2023).

2.2. Gestational Treatment

The maternal treatment protocol was identical to that used in the matched PA study [23]. From gestational day 11 until birth, dams in the AB and AB&P groups received a broad-spectrum antibiotic cocktail in drinking water consisting of ampicillin (1 mg/mL), vancomycin (0.5 mg/mL), neomycin (5 mg/mL), and meropenem (1 mg/mL). Dams in the P and AB&P groups received Vivomixx (Mendes, Lugano, Switzerland) containing *Lactobacillus* spp., *Bifidobacterium* spp., and *Streptococcus thermophilus* at 6.1×10^8 CFU/mL from gestational day 1. In the AB&P group, antibiotics were administered overnight (16:00–08:00) and probiotics during the remaining hours to avoid simultaneous exposure. Drinking water was replaced every 24 h, and maternal water intake and body weight were monitored throughout gestation.

2.3. EEG Recording and Reactivity Assessment

The electrode implantation and EEG recordings were previously described in detail [19]. Anesthesia was induced with 3.0% isoflurane in a sealed chamber, followed by intraperitoneal ketamine (60 mg/kg) and xylazine (5 mg/kg). Supplemental intramuscular ketamine (60 mg/kg) was administered as needed to maintain surgical depth, confirmed by absence of the tail-pinch response. Animals were secured in a stereotaxic frame. After

shaving and disinfection, a midline scalp incision was made to expose the cranium. $FeCl_3$ was applied to the bone surface to improve dental cement adhesion. Custom epidural electrode arrays were constructed from silver wires soldered to 2.54 mm pitch pin headers and insulated with acrylic resin along the shaft, leaving only the distal tips exposed. Electrode holes were drilled at the following coordinates relative to bregma: 5 mm anterior, ±2.35 mm lateral; and 6 mm posterior, ±3.35 mm lateral. Electrodes were placed in epidural contact with the dura mater and secured with dental cement. Animals received 0.5 mL intraperitoneal saline for hydration and recovered in their home cages for 2 days before EEG recordings. It should be noted that, for simplicity, the cortical EEG is hereafter referred to simply as EEG.

For EEG recordings, burst suppression was induced with intraperitoneal chloral hydrate (0.4 g/kg) after isoflurane induction. If visually assessed suppression occupied less than 50% of the epoch, up to two supplemental chloral hydrate doses of 0.1 g/kg were administered. Recordings were stopped when the EEG recovered toward a nearly continuous pattern. Signals were acquired using a BIOPAC MP150 system at 1000 Hz, 10 dB gain, and a 1–35 Hz hardware bandpass filter, using a two-channel fronto-parietal bipolar montage. The ground electrode was placed at the tail base with conductive gel. Stimulation voltage and cardiac activity were recorded in parallel. Chloral hydrate was retained to match the previously published EEG reactivity protocol under burst suppression, where sensory-evoked burst recruitment can be studied reproducibly.

Intermittent photic stimulation was delivered through a light-emitting diode placed in front of the right eye at 0.5 Hz, while the left eye was covered and the room was kept dark. Stimulation was controlled by a custom MATLAB (version 2022b, MathWorks, Inc., Natick, MA, USA) script and delivered in 60 s stimulation epochs alternating with 60 s stimulus-free intervals. The two EEG channels were designated ipsilateral and contralateral relative to the stimulated eye.

2.4. EEG Processing and Burst-Suppression Quantification

Raw recordings were converted to Hierarchical Data Format version 5 (HDF5) and anonymized before analysis; a de-identification key was stored separately to preserve blinded processing. EEG signals were preprocessed with a zero-phase fourth-order Butterworth bandpass filter (1–35 Hz).

Burst annotation was performed using BURST (Burst-sUppression Reviewer-Supervised analysis Tool) [23], a custom Python graphical user interface for semi-automated, reviewer-supervised trial-by-trial analysis of burst-suppression recordings. The tool imported preprocessed EEG, reconstructed stimulus triggers from the recorded stimulation channel by rising-edge detection, and derived a rectified and smoothed EEG envelope from the primary channel. Candidate burst intervals were generated using a relative threshold workflow: a noise reference estimate was computed from sliding window minima of the rectified signal and scaled by a fixed amplitude threshold of four times baseline. The resulting binary burst-suppression representation was refined using minimum duration constraints (burst $\geq$ 0.45 s; suppression $\geq$ 0.50 s). A single blinded reviewer inspected each automatically proposed annotation and could accept, edit, add, delete, or reposition burst intervals before final trial validation. Trials were excluded when transient artifacts, signal loss, or unstable baseline activity precluded reliable annotation. Manual review was performed by a single blinded annotator, using the same rule-based workflow across groups.

Each trial comprised three consecutive 60 s epochs: stimulation-free baseline (PRE), intermittent photic stimulation (STIM), and stimulation-free recovery (POST). Epoch boundaries were referenced to the recorded stimulation signal to compensate for possible trigger drift. Figure 1A shows the trial structure within the recording session, and Figure 1B illus-

trates the annotated raw EEG and the corresponding rectified-smoothed envelope used for candidate burst detection.

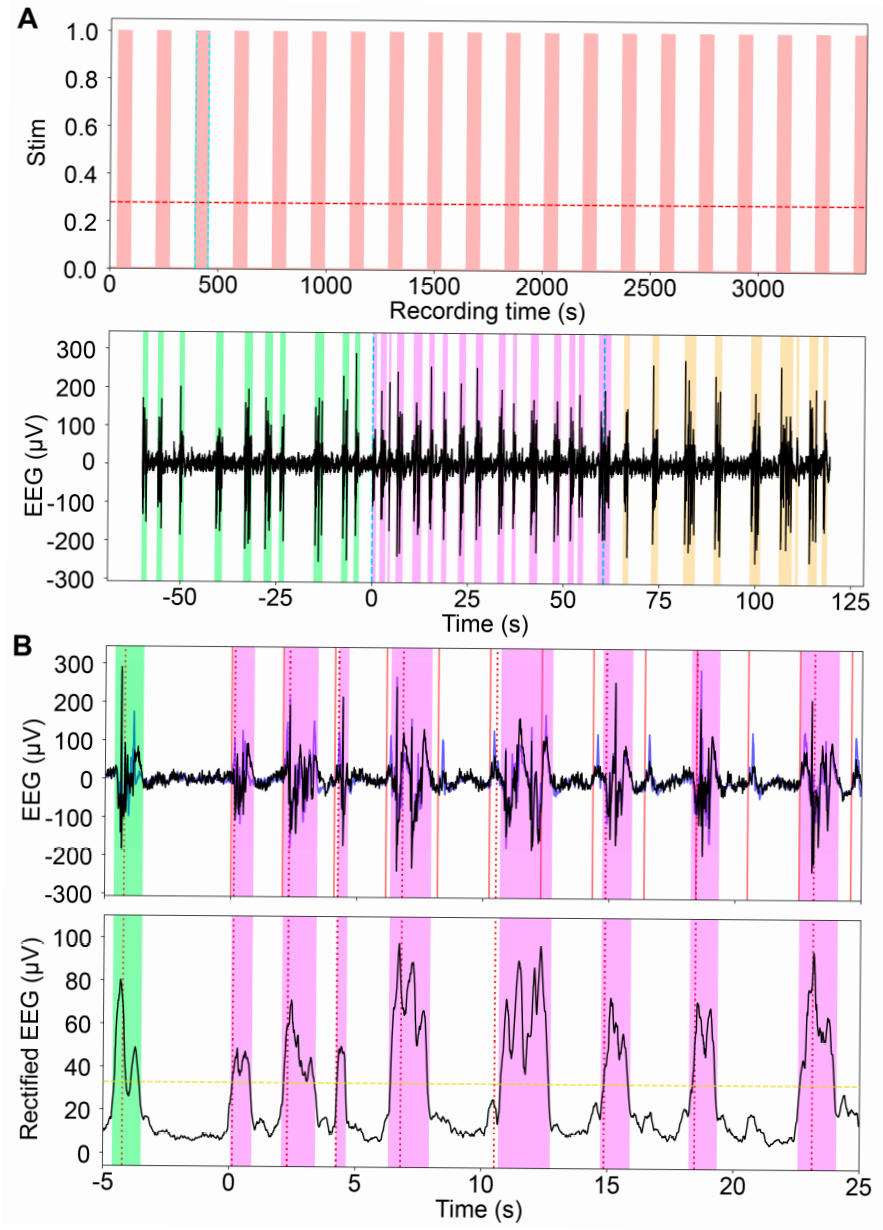


Figure 1. Burst-suppression EEG recording and semi-automated burst annotation in a representative rat after prolonged gestational antibiotic exposure. (**A**) Recording overview and trial structure. Top: complete recording session showing intermittent photic stimulation epochs (red shading) alternating with stimulus-free intervals; the cyan dashed vertical lines mark the trial expanded below. Bottom: one complete trial, with PRE baseline (green), STIM photic stimulation (magenta), and POST recovery (orange). The ipsilateral raw EEG trace is shown in black, and accepted burst intervals are shaded in pink. (**B**) Annotated signal detail and envelope-based candidate detection. Top: 30 s window centered on stimulation onset, with photic stimuli indicated by red vertical lines, manually reviewed burst boundaries by dashed vertical lines, and accepted bursts by pink shading. The contralateral EEG channel is superimposed in blue to illustrate visually evoked responses that were not always followed by full bursts. Bottom: rectified and smoothed EEG envelope used for threshold-based candidate generation, with the detection threshold shown as a yellow dashed line and accepted burst intervals shaded in pink. This illustrative antibiotic-exposed recording documents the scoring procedure; group comparisons are presented in Figures 2 and 3. Abbreviations: EEG, electroencephalography; PRE, pre-stimulation; STIM, stimulation; POST, post-stimulation.

Burst count reactivity (BCR) was defined as BCR = BCStim − BCPre, where larger values indicate greater burst recruitment during stimulation for a given pre-stimulation baseline. The suppression-ratio-based burst-suppression reactivity index was defined as BSRi = (SRPre − SRStim) / SRPre, normalizing the stimulation-evoked reduction in SR to the pre-stimulation level. This normalization accommodates differences in starting suppression but does not, by itself, establish equivalent anaesthetic depth. Larger BSRi values indicate stronger EEG reactivity.

2.5. Data Analysis and Statistics

All analyses were performed in Python 3.13 using pandas 2.3 for data management, NumPy 2.3 for numerical computation, SciPy 1.16 for inferential statistics, statsmodels 0.14 for ANOVA, mixed models, and post hoc tests, and scikit-learn 1.7 for supplementary utilities. The significance threshold was $\alpha = 0.05$. When multiple comparisons were performed, the correction method is specified below.

Individual offspring were the EEG observation units, consistent with the PA study [23].

To reduce between-group differences in pre-stimulation burst count (BCPre) before comparing reactivity, we used an exhaustive search of integer lower and upper BCPre limits. For each candidate interval, the trials falling within that interval were retained and their four-group baseline BCPre difference assessed by one-way ANOVA. Among intervals with an omnibus $p > 0.05$, the interval retaining the most trials was selected. Thus, the procedure identifies a broad common range of pre-stimulation burst frequencies while maximizing usable recordings; it does not imply that a non-significant baseline test establishes identical anaesthetic depth. The same selection rule was applied independently to the PA and uncomplicated-birth cohorts, allowing their retained BCPre ranges to differ.

Group-level summaries are reported as mean ± standard deviation (SD) unless otherwise stated. Standard error of the mean (SEM) and median values are reported where relevant. Sample sizes are reported at both trial and animal levels. Between-group differences in continuous variables (BCPre, BCR, and BSRi) were assessed with one-way ANOVA using scipy.stats.f_oneway. Significant omnibus effects were followed by Tukey's honestly significant difference (HSD) post hoc testing using statsmodels.stats.multicomp.pairwise_tukeyhsd, controlling the family-wise error rate across all pairwise comparisons. For completeness, exploratory group-level effect sizes are reported alongside the main BCR and BSRi tests. Post hoc BCR contrasts following a non-significant omnibus test are presented descriptively, not as independent evidence of a group effect.

The effect of recording epoch (PRE, STIM, POST) within each group was assessed using trial-level repeated-measures ANOVA (RM-ANOVA), treating individual trials as the repeated unit, with statsmodels AnovaRM. Post hoc pairwise comparisons between epochs were conducted using paired *t*-tests with Holm correction across the three epoch pairs (PRE-STIM, STIM-POST, and PRE-POST). BCR was calculated per trial as BCR = BCStim − BCPre. BSRi was calculated as BSRi = (SRPre − SRStim) / SRPre; trials with SRPre = 0 were excluded from BSRi analyses.

The relationship between suppression ratio (SR) and burst count (BC) was characterized for each group and epoch using ordinary least-squares (OLS) linear regression (scipy.stats.linregress), with Pearson r, R^2, slope, intercept, and *p*-value reported for each group × epoch combination. To test whether the SR–BC slope was modulated by epoch, group, or their interaction while accounting for within-animal trial clustering, an LMM was fitted with SR as the outcome and BC, Epoch, Group, all two-way interactions, and the BC × Epoch × Group interaction as fixed effects, with Animal as a random intercept. The significance of the interaction terms was evaluated using likelihood ratio tests (LRTs) comparing the full model with reduced models fitted by maximum likelihood. Following

the hierarchical model structure, the BC × Epoch and BC × Group interaction blocks were tested by jointly removing each two-way interaction and its corresponding three-way interaction (BC × Epoch × Group), resulting in 8 and 9 degrees of freedom, respectively. The three-way interaction was evaluated separately by removing only the BC × Epoch × Group term (6 degrees of freedom).

Group effects on BCR and BSRi were modeled using LMMs with Group as a fixed effect and Animal as a random intercept. The overall group effect was tested by LRT comparing the group model with an intercept-only null model, both fitted by maximum likelihood. Fixed-effect estimates and pairwise contrasts were obtained from restricted maximum likelihood fits. Pairwise contrasts were tested as linear combinations of fixed-effect coefficients using the t_test method, with Holm correction across all pairwise comparisons. The intraclass correlation coefficient (ICC) was calculated as the between-animal variance divided by the sum of between-animal and residual variances, quantifying the proportion of outcome variance attributable to stable between-animal differences. Confidence intervals refer to unadjusted individual model contrasts, whereas the corresponding Holm-adjusted p-values address the family of six pairwise comparisons.

Trial-level statistical power was estimated within the one-way ANOVA framework (statsmodels FTestAnovaPower) at $\alpha = 0.05$ with four groups. Effect sizes are reported as Cohen's f and η^2 values derived from observed between-group variance. Power was calculated at each cohort's observed effect size and cross-evaluated at the other cohort's effect size to assess comparability between the PA and uncomplicated-birth datasets. The minimum detectable effect (MDE) at 80% power is reported for each cohort. The reported post hoc power estimates describe the precision of the available datasets at specified effect sizes. As with any non-significant comparison, the possibility of a smaller undetected effect should be kept in mind, particularly in the AB group with five animals.

Sex composition was summarized descriptively by experimental group. Owing to the small expected cell counts, a four-group asymptotic chi-square test was not used to claim balanced allocation. The study was not designed for sex-specific treatment-effect inference.

3. Results

3.1. Baseline Burst Count Filtering

Prior to analysis, trials were filtered to ensure comparable baseline burst count activity (BCPre) across groups. Before filtering, the dataset comprised 280 trials from 29 animals (C: 9 animals, 78 trials; AB: 5 animals, 29 trials; AB&P: 7 animals, 69 trials; P: 8 animals, 104 trials). BCPre differed significantly across groups at this stage (one-way ANOVA: $F(3, 276) = 9.87$, $p < 0.001$). Post hoc Tukey testing revealed that this difference was driven by two pairs: C vs. P (mean difference = 3.23 bursts, $p < 0.001$) and AB vs. P (mean difference = 4.08 bursts, $p < 0.001$), while all other pairwise comparisons were non-significant (all $p > 0.06$). Group means (±SD) before filtering were C = 10.71 ± 4.82, AB = 11.55 ± 3.98, AB&P = 8.99 ± 4.88, P = 7.47 ± 4.64 bursts.

To identify an optimal BCPre inclusion window that equalized baseline activity across groups while maximizing trial retention, an exhaustive grid search was performed over all integer (minimum, maximum) BCPre pairs within the observed range (1–21 bursts). A window was considered valid if the one-way ANOVA on BCPre across the four groups yielded $p > 0.05$. Among the 120 valid windows identified, the BCPre window from 6 to 21 was selected as it retained the largest number of trials (200 trials, 29 animals, ANOVA $p = 0.153$). Accordingly, 80 of 280 eligible trials (28.6%) were excluded by the common range BCPre filter. By group, the filter retained 65/78 C, 28/29 AB, 49/69 AB&P and 58/104 P trials; all 29 animals remained represented.

3.2. Burst Count Reactivity

After BCPre filtering, BC showed a consistent transient increase during stimulation in all groups (Figure 2A–D). Trial-level RM-ANOVA demonstrated a significant epoch effect in every group (all $p < 0.001$). BC increased significantly from PRE to STIM in all groups after Holm correction (all $p < 0.001$) and decreased significantly from STIM to POST in all groups (all $p < 0.001$). Mean ± SD values were C: PRE 12.17 ± 3.81, STIM 19.09 ± 3.67, POST 11.75 ± 4.34; AB: PRE 11.82 ± 3.77, STIM 16.57 ± 5.85, POST 11.29 ± 4.35; AB&P: PRE 11.12 ± 4.15, STIM 17.16 ± 5.95, POST 10.47 ± 5.14; and P: PRE 10.64 ± 3.81, STIM 16.05 ± 7.15, POST 9.66 ± 4.89. This pattern indicates transient stimulus-evoked burst recruitment followed by recovery toward baseline.

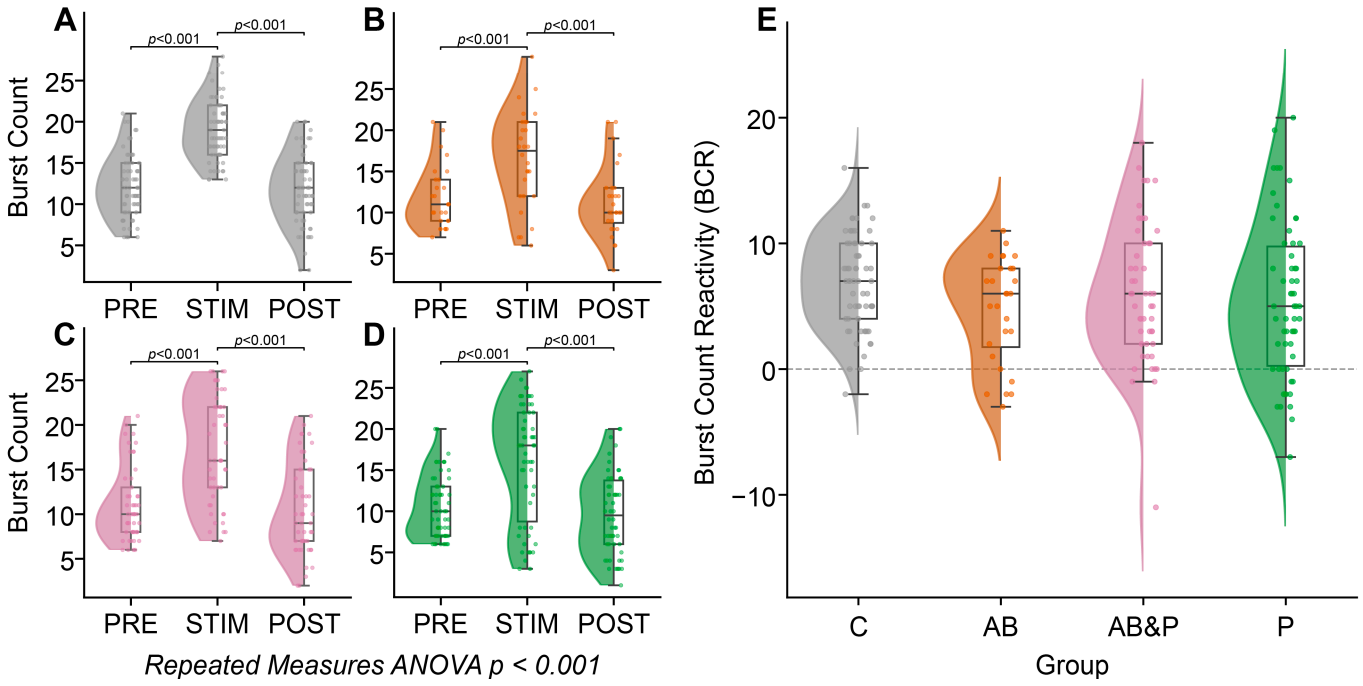


Figure 2. Burst count dynamics and burst count reactivity by treatment group. The baseline BCPre filter (6–21 bursts) retained 200 trials from 29 animals: C, 65 trials/9 animals; AB, 28/5; AB&P, 49/7; P, 58/8. Violin plots represent trial-level distributions, boxes the median and interquartile range, and dots individual trials (not independent animals). (**A**–**D**) Burst counts in PRE, STIM and POST for C, AB, AB&P and P; all groups showed increased burst count during STIM followed by recovery (within-group Holm-corrected PRE-STIM and STIM-POST $p < 0.001$). (**E**) BCR = BCStim − BCPre; the trial-level four-group test was not significant ($p = 0.204$). The corresponding model results and confidence intervals are given in the text. Abbreviations: C, control; AB, antibiotics; AB&P, antibiotics plus probiotics; P, probiotics; BC, burst count; BCPre, pre-stimulation burst count; BCStim, stimulation burst count; BCR, burst count reactivity; PRE, pre-stimulation; STIM, stimulation; POST, post-stimulation.

Baseline drift was assessed by comparing PRE and POST epochs within each group (Figure 2A–D). No PRE-POST comparison was significant after Holm correction (C: $p = 0.406$; AB: $p = 0.500$; AB&P: $p = 0.202$; P: $p = 0.072$). Thus, the stimulation epoch increase in BC was not attributable to a progressive change in background burst activity over time.

The magnitude of BCR, defined as BCStim − BCPre, did not differ significantly across groups (Figure 2E; one-way ANOVA: $F(3, 196) = 1.54$, $p = 0.204$). Exploratory pairwise comparisons did not identify a significant group difference; the ANOVA effect size was $\eta^2 = 0.023$ (Cohen f ≈ 0.154). Mean BCR values were C = 6.92 ± 3.73, AB = 4.75 ± 4.18, AB&P = 6.04 ± 5.54, and P = 5.41 ± 6.21 bursts.

The absence of a statistically significant overall group effect for BCR was also observed using an LMM with Group as a fixed effect and Animal as a random intercept (Figure 2E; 200 trials, 29 animals). The ICC was 0.496, indicating that 49.6% of BCR variance was attributable to stable between-animal differences (animal variance = 12.82; residual variance = 13.03). The LRT comparing the group model with an intercept-only model showed no significant group effect (chi-square(3) = 4.31, $p = 0.230$). All Holm-corrected pairwise contrasts were non-significant: C vs. AB, delta = 3.23, 95% CI −1.25 to 7.70, pHolm = 0.787; C vs. AB&P, delta = 0.11, 95% CI −3.79 to 4.02, pHolm = 1.000; C vs. P, delta = 2.92, 95% CI −0.85 to 6.70, pHolm = 0.776; AB vs. AB&P, delta = −3.11, 95% CI −7.77 to 1.54, pHolm = 0.787; AB vs. P, delta = −0.30, 95% CI −4.85 to 4.25, pHolm = 1.000; and AB&P vs. P, delta = 2.81, 95% CI −1.18 to 6.80, pHolm = 0.787.

3.3. Suppression Ratio and Burst-Suppression Reactivity Index

SR was derived from the finalized burst boundaries and used as a complementary time-based measure of burst-suppression dynamics. SR and BC were strongly negatively correlated across all groups and epochs (Figure 3A–D): R^2 ranged from 0.44 to 0.84, and all regressions were significant (all $p < 0.001$). The strength of the association during STIM varied by group (C: $R^2 = 0.44$; AB: $R^2 = 0.78$; AB&P: $R^2 = 0.70$; P: $R^2 = 0.84$), with similarly robust associations during PRE (C: $R^2 = 0.54$; AB: $R^2 = 0.57$; AB&P: $R^2 = 0.53$; P: $R^2 = 0.57$) and POST (C: $R^2 = 0.67$; AB: $R^2 = 0.64$; AB&P: $R^2 = 0.65$; P: $R^2 = 0.70$). An LMM including BC, Epoch, Group, and their interactions, with Animal as a random intercept, showed no significant modulation of the SR–BC slope by epoch (BC × Epoch: chi-square(8) = 11.66, $p = 0.167$), group (BC × Group: chi-square(9) = 13.05, $p = 0.161$), or their interaction (BC × Epoch × Group: chi-square(6) = 6.37, $p = 0.383$; ICC = 0.615; animal variance = 46.87; residual variance = 29.36). These results indicate a homogeneous inverse SR–BC relationship across treatment groups and recording epochs.

Consistent with the BC results, stimulation was associated with a transient decrease in SR in all groups, as reflected by the STIM epoch values included in Figure 3A–D. Within-group RM-ANOVA was significant in every group (all $p < 0.001$). SR decreased significantly from PRE to STIM in all groups (all Holm-corrected $p < 0.001$; mean ± SD − C: 68.68 ± 9.02 to 54.36 ± 12.75; AB: 78.54 ± 9.02 to 68.68 ± 12.92; AB&P: 75.38 ± 10.67 to 65.26 ± 16.04; P: 77.73 ± 10.83 to 65.04 ± 21.83) and recovered significantly from STIM to POST in all groups (all $p < 0.001$; C: 54.36 ± 12.75 to 66.18 ± 12.32; AB: 68.68 ± 12.92 to 76.36 ± 11.48; AB&P: 65.26 ± 16.04 to 74.53 ± 12.92; P: 65.04 ± 21.83 to 77.88 ± 12.87).

BSRi quantified the normalized magnitude of stimulation-evoked SR reduction (Figure 3E). Trial-level BSRi showed some heterogeneity across the four groups (one-way ANOVA: F(3, 196) = 2.79, $p = 0.042$), but no pairwise comparison was significant after Tukey correction (all $p > 0.068$). Mean BSRi values were C = 0.212 ± 0.140, AB = 0.130 ± 0.094, AB&P = 0.136 ± 0.175, and P = 0.179 ± 0.196. Because the omnibus test does not identify which treatment contrast contributes to the overall variation, it does not by itself demonstrate an antibiotic-associated difference. The four-group ANOVA effect size was $\eta^2 = 0.041$ (Cohen f = 0.207).

BSRi was further assessed using an LMM with Group as a fixed effect and Animal as a random intercept (Figure 3E; 200 trials, 29 animals). The ICC was 0.647 (animal variance = 0.015; residual variance = 0.008), indicating substantial between-animal clustering. The LRT comparing the group model with an intercept-only model did not identify a significant group effect (chi-square(3) = 7.42, $p = 0.060$). All Holm-corrected contrasts were non-significant: C vs AB, delta = 0.178, 95% CI 0.033 to 0.322, pHolm = 0.095; C vs AB&P, delta = 0.081, 95% CI −0.046 to 0.208, pHolm = 0.834; C vs P, delta = 0.130, 95% CI 0.006 to 0.253, pHolm = 0.197; AB vs AB&P, delta = −0.097, 95% CI −0.247 to 0.054,

pHolm = 0.834; AB vs P, delta = −0.048, 95% CI −0.195 to 0.099, pHolm = 0.934; and AB&P vs P, delta = 0.048, 95% CI −0.082 to 0.179, pHolm = 0.934. Thus, no specific treatment-associated difference in SR-based reactivity was established after multiplicity correction and accounting for repeated trials within animals. For interpretation, the control–antibiotic estimate is in the direction of lower AB reactivity, but its confidence interval is unadjusted, and the corresponding Holm-adjusted pairwise test is not significant.

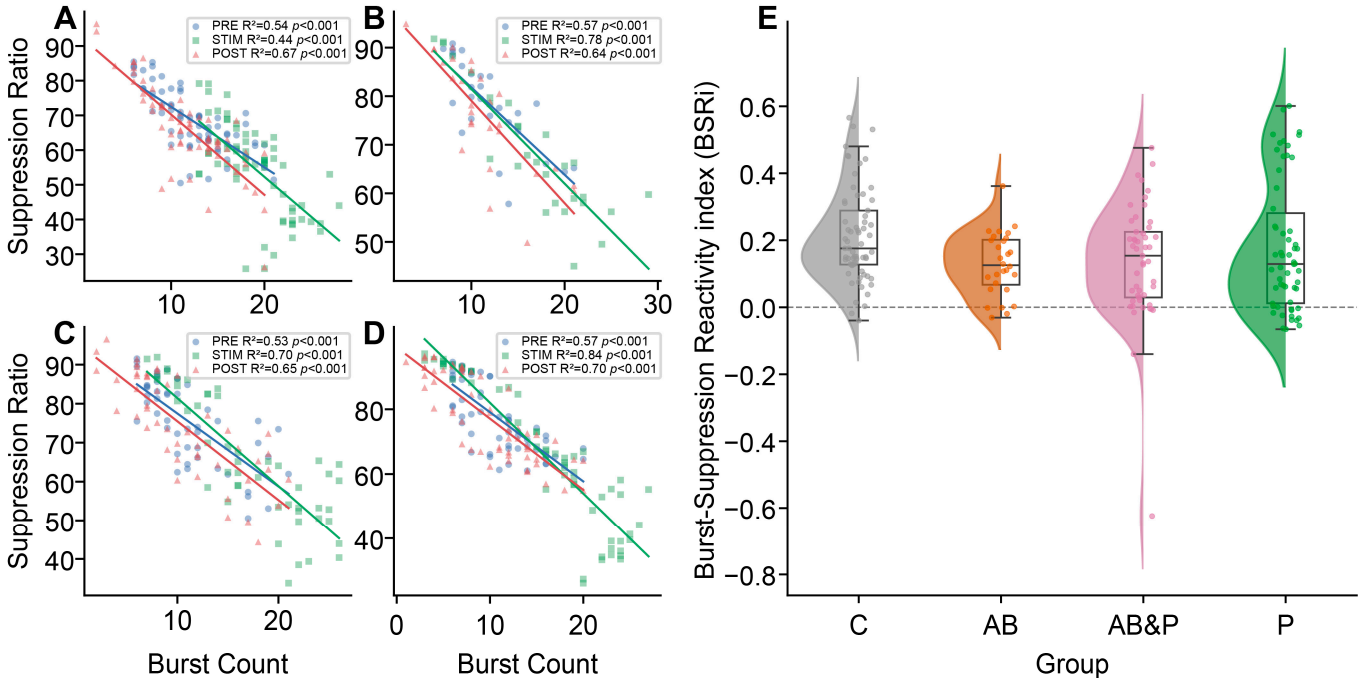


Figure 3. Suppression ratio–burst count relationship and burst-suppression reactivity index by treatment group. The same 200 trials from 29 animals were retained (C, 65/9; AB, 28/5; AB&P, 49/7; P, 58/8; trials/animals). (**A**–**D**) SR plotted against BC by PRE, STIM and POST epoch; points are individual trials and lines are group–epoch OLS fits. All associations were negative (all $p < 0.001$); detailed regression and mixed-model statistics are presented in the text. (**E**) BSRi = (SRPre − SRStim)/SRPre; positive values indicate reduced suppression during photic stimulation. The trial-level omnibus test was $p = 0.042$; the animal-intercept mixed-model omnibus test was $p = 0.060$. The distribution includes one negative AB&P trial, retained under the uniform annotation criteria. Abbreviations: C, control; AB, antibiotics; AB&P, antibiotics plus probiotics; P, probiotics; SR, suppression ratio; BC, burst count; OLS, ordinary least squares; BSRi, burst-suppression reactivity index; SRPre, suppression ratio during pre-stimulation baseline; SRStim, stimulation suppression ratio; PRE, pre-stimulation; STIM, stimulation; POST, post-stimulation.

3.4. Power and Cohort Comparability Analyses

The present uncomplicated-birth cohort analysis matched the published PA paradigm, using the same burst-count-centered design and the same BCPre filtering rule [23]. Trials were retained only when pre-stimulation burst count fell within a range that yielded no significant between-group baseline difference. The filter limits differed between cohorts because the baseline distributions differed: PA, BCPre between 8 and 15 bursts; uncomplicated birth, BCPre between 6 and 21 bursts. The selection rule was identical in both cohorts.

Following filtering, trial-level statistical power was compared between cohorts using the BCPre one-way ANOVA as a reference scenario ($\alpha = 0.05$, four groups). The harmonic mean trial count per group was 53.3 for PA and 45.1 for uncomplicated birth, a difference of 15.4%. The observed Cohen's f for the BCPre effect after filtering was f = 0.129 ($\eta^2 = 0.016$) in PA and f = 0.165 ($\eta^2 = 0.026$) in uncomplicated birth. Achieved trial-level power was 10.3% for PA and 12.4% for uncomplicated birth at their respective observed effect sizes.

Critically, when uncomplicated birth power was evaluated at the PA effect size (f = 0.129), achieved power was 9.4%, essentially identical to the PA cohort, indicating similar trial-level statistical power. The minimum detectable effect at 80% power was f = 0.471 for PA and f = 0.516 for uncomplicated birth, indicating that both cohorts are similarly positioned to detect medium-to-large effects at the trial level. Animal-level power was lower in both cohorts (PA: harmonic n = 11.0 animals/group, f = 0.279, power = 8.3%; uncomplicated birth: harmonic n = 6.9 animals/group, f = 0.134, power = 5.3%), reflecting the inherent constraint of litter-based in vivo designs, and animal-level analyses in both studies should be considered exploratory.

In the uncomplicated-birth cohort, group sex counts were C: 5M/4F; AB: 2M/3F; AB&P: 3M/4F; P: 4M/4F (14 males/29 offspring). The published PA cohort included 23 males among 45 offspring. Because group-level cell counts are small, we report sex composition descriptively rather than asserting balance from an asymptotic chi-square test. No sex-stratified treatment inference is made.

A parallel power analysis was conducted for BSRi, the primary SR-based reactivity metric. In the PA cohort, BSRi showed a large between-group effect (Cohen f = 0.482, η^2 = 0.189; $F(3, 227) = 17.60$, $p < 0.001$; mean ± SD - C-PA: 0.296 ± 0.202, AB-PA: 0.104 ± 0.106, AB&P-PA: 0.173 ± 0.148, P-PA: 0.240 ± 0.132), yielding 77.0% achieved power at 47.9 harmonic trials per group. In the uncomplicated-birth cohort, the BSRi effect was smaller (Cohen f = 0.207, η^2 = 0.041; $F(3, 196) = 2.79$, $p = 0.042$; mean ± SD - C: 0.212 ± 0.140, AB: 0.130 ± 0.094, AB&P: 0.136 ± 0.175, P: 0.179 ± 0.196), yielding 17.3% achieved power at 45.1 harmonic trials per group. The lower achieved power in the uncomplicated-birth cohort therefore reflects the smaller effect magnitude rather than substantially lower trial numbers: when uncomplicated-birth power was evaluated at the PA effect size, it reached 73.9%, whereas PA power evaluated at the uncomplicated-birth effect size fell to 18.2%. The narrower range of uncomplicated-birth group means (0.130–0.212) compared with PA group means (0.104–0.296) is consistent with smaller between-group differences in SR-based reactivity among animals not subjected to PA. These power comparisons refer to the trial-level four-group framework and do not represent a formal Treatment × PA interaction test.

4. Discussion

The principal finding was that prolonged gestational antibiotic exposure was not associated with a demonstrable treatment-specific impairment of adult EEG reactivity after an uncomplicated birth. Burst-suppression EEG remained responsive to intermittent photic stimulation in every gestational treatment group. Stimulation increased burst count and reduced suppression, followed by recovery toward baseline. Although the estimated effects tended toward lower reactivity in antibiotic-exposed offspring, neither the animal-intercept models nor the multiplicity-adjusted pairwise comparisons established a treatment-specific impairment. These findings provide the previously missing uncomplicated-birth context for our earlier observation that gestational antibiotic exposure aggravated the long-term EEG consequences of perinatal asphyxia (PA) [23].

4.1. Adult EEG Reactivity After Gestational Antibiotic Exposure

EEG reactivity was quantified using two measures, BCR and BSRi, capturing complementary aspects of photic responsiveness during burst suppression. BCR quantifies the additional bursts recruited during stimulation, while BSRi measures the relative reduction in time spent in suppression. Suppression could decrease because bursts become more frequent, longer, or both [15,24]. The inverse relationship between burst count and suppression ratio is therefore physiologically consistent. However, both measures are derived from the same burst annotations and should not be considered truly independent biological

confirmations. Although they do characterize different aspects of cortical responsiveness under deep anesthesia, their relationship to neurological function in awake animals remains uncertain. The statistical evidence also requires distinguishing overall group heterogeneity from a specific antibiotic-associated effect. BCR showed no significant overall group effect in either the trial-level or mixed-effects analysis. For BSRi, the trial-level four-group ANOVA suggested heterogeneity ($p = 0.042$), while the animal-intercept model showed a non-significant overall group effect (LRT $p = 0.060$). The mixed-model C–AB contrast indicated lower reactivity after antibiotic exposure (difference 0.178; unadjusted 95% CI 0.033–0.322), but did not remain significant after Holm correction ($p = 0.095$). A similar pattern was observed for C–P (difference 0.130; unadjusted 95% CI 0.006–0.253; Holm-adjusted $p = 0.197$). Accordingly, the data are compatible with treatment-associated differences but do not establish a specific contrast after correction; equally, they cannot be interpreted as demonstrating equivalence among groups.

The interval between prenatal treatment and adult EEG recording is relevant to this interpretation. During development of the maternal treatment model, antibiotic exposure was associated with an early righting-reflex delay [22]. This observation motivates the assessment of later function but cannot establish persistence or recovery of the same phenotype because the endpoints and cohorts differ. A preserved response in the present EEG paradigm could reflect limited effects on this particular function, compensation, or effects in domains not captured by burst-suppression reactivity. This functional specificity is consistent with reports that some offspring consequences of maternal antibiotics diminish with age [14], whereas others remain selective for particular sensory or behavioural functions [7]. Gestational exposure may also alter offspring microbiota without corresponding differences in the behavioural tasks examined [25].

Comparisons with other experimental reports additionally depend on the developmental exposure window and regimen. Long-term behavioural findings after antibiotic treatment during the early postnatal period [13] or direct exposure in adult animals [26] cannot be extrapolated directly to prolonged gestational treatment. Even within gestational models, antibiotic composition, host immune responses, and the outcomes assessed may differ [9,11]. Furthermore, more recently, prenatal ciprofloxacin exposure was associated with selective behavioural alterations in adult offspring, further emphasizing the importance of the exposure regimen and functional endpoint when assessing long-term neurodevelopmental consequences [27]. The present observation should therefore be interpreted specifically as an adult EEG reactivity result under the exposure conditions studied, rather than as evidence that gestational antibiotic treatment has no lasting neurodevelopmental consequences more broadly.

4.2. Interpretation Alongside the Previous Perinatal Asphyxia Study

Our previous study showed that prolonged gestational antibiotic exposure was associated with greater impairment of adult EEG reactivity in offspring subjected to experimental PA [23]. In that model, the prenatal treatment was administered to dams, whereas PA was induced in pups on postnatal day 6. As all offspring in the earlier cohort underwent PA, that study could not determine whether antibiotics also had an independent long-term effect on EEG reactivity. The present study addresses this unresolved question in offspring not subjected to PA. It found reactivity in every treatment group, but no statistically established antibiotic-specific impairment after accounting for repeated trials and multiple comparisons. This distinction complements, rather than negates, the earlier finding and does not rule out an independent antibiotic effect.

The directional estimates warrant attention when the two cohorts are considered together. The raw trial-mean C–AB difference in BSRi was 0.082 in the present cohort,

compared with 0.192 in the PA cohort. However, the present mixed-model estimate (0.178) is close to the latter value, and the estimates reflect different weighting and statistical approaches. The current confidence interval therefore remains compatible with a meaningful independent reduction in reactivity; these data do not establish that the antibiotic effect is intrinsically smaller without PA. Rather, the statistical evidence for treatment-specific impairment was more consistent in the PA cohort. Because the cohorts were analyzed separately, using different retained baseline burst count ranges, this contextual comparison does not provide a formal treatment-by-PA interaction test.

The probiotic results should be interpreted in the same context. In the PA cohort, probiotic co-administration partially attenuated the pattern associated with antibiotic exposure [23]. In the present cohort, no multiplicity-corrected comparison identified a difference between antibiotics alone and combined treatment. Thus, these findings do not demonstrate probiotic rescue after uncomplicated birth and do not imply that any probiotic action is restricted to offspring exposed to PA.

A biologically plausible interpretation, consistent with the proposed "double-hit" framework [22], is that gestational antibiotic exposure alters susceptibility to, or the longer-term consequences of, hypoxic–ischaemic injury. Maternal microbial and immune signaling may influence developmental protective processes [28], and experimental neonatal studies implicate intestinal microbial composition in inflammatory responses and brain injury after hypoxia–ischemia [29]. Alternatively, gestational exposure may alter repair or maturation after the insult, consistent with evidence linking gut-related processes to later neuroinflammatory and cognitive outcomes [30]. As neither acute injury severity nor the subsequent recovery trajectory was measured in the present cohort, the adult EEG findings cannot distinguish these mechanisms. The two studies therefore support investigating impaired resilience to PA as a biologically plausible hypothesis, potentially alongside an independent effect of gestational antibiotic exposure.

4.3. Limits of the Inference

Our four-drug antibiotic regimen was designed as a strong experimental maternal perturbation rather than a representation of typical antibiotic use during human pregnancy. Although maternal and offspring microbiota were not assessed in the present cohort, targeted bacterial-group quantitative PCR was used during earlier development of the maternal treatment model, supporting the rationale for the regimen. Nevertheless, because antibiotics may also affect maternal immune responses [8], attributing the adult EEG findings specifically to microbiome disruption remains indirect.

Gestational treatment was administered to dams, with four to six dams per group, as in our previous PA study [23]. We considered individual offspring as the units of EEG analysis, since the neurophysiological outcomes were measured in these animals. Mixed-effects models accounted for repeated trials within offspring but did not explicitly account for litter effects. Thus, shared maternal exposure-related factors cannot be excluded, and repeated trials do not increase the number of independently treated pregnancies. Moreover, the relatively small and unequal animal groups, particularly the five antibiotic-exposed offspring, limit the precision of treatment-effect estimates.

The data-driven trial selection based on pre-stimulation burst count (BCPre) improved comparability of spontaneous burst frequencies but resulted in unequal trial retention across groups and did not establish identical anaesthetic states. Indeed, baseline suppression ratios remained different (C: 68.68%; AB: 78.54%; P: 77.73%), as the suppression ratio reflects both burst frequency and duration. Although BSRi normalizes the stimulation-induced reduction in suppression to each trial's baseline SR, residual dependence on anaesthetic state cannot be excluded. In addition, burst annotations were validated by a single blinded

reviewer. Individual outlying observations, including a negative BSRi value in AB&P, were retained according to consistent trial quality criteria. Finally, the present cohort did not include an independent positive control demonstrating impaired EEG reactivity, although the sensitivity of this experimental paradigm was established in our previous PA studies.

The study was not designed to resolve sex-specific treatment effects. Furthermore, because the PA and uncomplicated-birth cohorts were analyzed separately and used different retained BCPre ranges, comparisons between the two studies remain descriptive rather than constituting a formal test of treatment-by-PA interaction. These considerations do not diminish the complementary value of the present findings but limit conclusions regarding the magnitude of an independent antibiotic effect and its potential interaction with perinatal asphyxia.

5. Conclusions

We assessed adult EEG reactivity following uncomplicated birth using the same gestational treatment paradigm as in our previous study, in which prolonged gestational antibiotic exposure was associated with impaired EEG reactivity in adult offspring after perinatal asphyxia. In contrast, our relatively small EEG dataset did not provide evidence of a long-term impairment associated with gestational antibiotic or probiotic exposure, although an independent effect cannot be excluded. Together with the previous report, these findings suggest that gestational antibiotic administration may compromise resilience to perinatal asphyxia at an exposure level that is not associated with a readily detectable impairment following uncomplicated birth. Further studies are needed to investigate the potential interaction between gestational antibiotic exposure and perinatal asphyxia.

Author Contributions: Conceptualization, M.S., M.-I.I., A.-M.Z. and M.M.; methodology, M.S., M.-I.I., A.-M.Z. and M.M.; software, V.-P.M., A.M., A.-V.I. and M.M.; validation, M.S., M.-I.I., A.-M.C., A.-M.Z. and M.M.; formal analysis, M.S., A.M. and M.M.; investigation, V.-P.M., M.S., A.-M.C., V.S., A.-V.I., A.-T.C. and A.B.; resources, A.-M.C. and V.S.; data curation, M.S., V.-P.M., A.M. and M.M.; writing—original draft preparation, M.S., V.-P.M., M.-I.I., A.-M.Z. and M.M.; writing—review and editing, M.S., V.-P.M., M.-I.I., A.-M.C., A.M., V.S., A.-V.I., A.-T.C., A.B., L.Z., A.-M.Z. and M.M.; visualization, M.S., V.-P.M., A.M. and M.M.; supervision, L.Z., A.-M.Z. and M.M.; project administration, A.-M.C. and A.-M.Z.; funding acquisition, A.-M.C. and A.-M.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the Sectoral Research and Development Program of the Romanian Ministry of National Defense (PSCD-1-2022-28 NEURO_Depress).

Institutional Review Board Statement: All experimental procedures were approved by the Local Committee for Animal Research, Carol Davila University of Medicine and Pharmacy, Bucharest, Romania (7845/25.03.2021, approved on 25 March 2021), and were conducted in accordance with Directive 2010/63/EU on the protection of animals used for scientific purposes. The project was authorized by the Ilfov Veterinary and Food Safety Directorate (project authorization no. 37/23.08.2023, approved on 23 August 2023) and was approved by the Ethics Committee of the Cantacuzino National Military Medical Institute for Research and Development (CE 319/2023, approved on 24 July 2023).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available from the corresponding author upon reasonable request.

Acknowledgments: The authors thank Cristian Ciotei and Mara Belcin for their assistance in conducting the experiments. During the preparation of this manuscript, the authors used Claude Opus 5 (Anthropic) and ChatGPT 5.6 (OpenAI) for the purposes of spelling, grammar, and rephrasing ideas. All study design, statistical analysis, data interpretation, and scientific conclusions were per-

formed exclusively by the authors. The authors have reviewed and edited the output and take full responsibility for the content of this publication.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AB	Antibiotic
AB&P	Antibiotics plus probiotics
ANOVA	Analysis of variance
BC	Burst count
BC_{Post}	Burst count during post-stimulation recovery
BC_{Pre}	Burst count during pre-stimulation baseline
BCR	Burst count reactivity
BC_{Stim}	Burst count during stimulation
BS	Burst suppression
BSRi	Burst-suppression reactivity index
C	Control
CFU	Colony-forming units
EEG	Electroencephalogram
HDF5	Hierarchical Data Format version 5
ICC	Intraclass correlation coefficient
LMM	Linear mixed model
LRT	Likelihood ratio test
OLS	Ordinary least squares
P	Probiotic
PA	Perinatal asphyxia
POST	Post-stimulation epoch
PRE	Pre-stimulation epoch
RM-ANOVA	Repeated-measures analysis of variance
SD	Standard deviation
SEM	Standard error of the mean
SR	Suppression ratio
SR_{Pre}	Suppression ratio during pre-stimulation baseline
SR_{Stim}	Suppression ratio during stimulation
STIM	Stimulation epoch

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