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1 **Early-life gut microbiota and neurodevelopment in preterm infants: any role for *Bifidobacterium*?**

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24

25 **Abstract**

26 Despite the well-recognized importance of proper gut microbiota assembly for the child’s future health, the
27 connections between the early-life gut microbiota and neurocognitive development in humans have not been
28 thoroughly explored so far. In this pilot observational study, we aimed to unveil the relation between dynamic
29 succession of the gut microbiota in very low birth weight infants during the first month of life and their
30 neurodevelopment, assessed at 24-month corrected age. According to our data, the early-life gut microbiota of
31 preterm infants with normal vs impaired neurodevelopment followed distinct temporal trajectories with peculiar
32 compositional rearrangements. In this context, early *Bifidobacterium* deficiency seem to constitute a negative
33 biomarker of adverse neurological outcomes.

34 *Conclusion:* our data might pave the way for future in-depth studies focusing on potential impact of
35 bifidobacteria or specific microbiota patterns on neonatal neurodevelopment and lay the foundation for
36 microbiome-based clinical practices to modulate altered profiles and improve long-term health.

37

38 **Keywords:** very low birth weight; preterm infants; gut microbiome; neurodevelopment; *Bifidobacterium*.

39

40 **What is Known:**

- 41 - Preterm infants are at increased risk for adverse neurological outcomes and gut microbiota dysbiosis.
- 42 - The gut microbiota and the nervous system share critical developmental windows in early life.

43 **What is New:**

- 44 - The absence of *Bifidobacterium* at 30 days of life in preterm infants is associated with neurodevelopment
45 impairment in early childhood.
- 46 - The administration of *Bifidobacterium* strains could promote optimal neurocognitive development in fragile
47 infants.

48

49 **Abbreviations**

- 50 CA: correct age
- 51 GQ: general development quotient
- 52 IQR: interquartile range
- 53 NI: neurodevelopmental impairment
- 54 VLBW: very low birth weight

55 **Introduction**

56 Preterm infants are at increased risk for adverse neurological outcomes and gut microbiota dysbiosis [1]. While
57 the association of gut microbiota dysbiosis with short-term clinical outcomes is widely studied, its relationship
58 with long-term outcomes remains largely unknown. Interestingly, the gut microbiota and the nervous system
59 share critical developmental windows in early life. Recently, the French EPIFLORE prospective observational
60 cohort study on very preterm newborns found out that the gut microbiota at week 4 after birth exhibited
61 bacterial patterns that varied according to gestational age, perinatal characteristics, individual treatments, and
62 neonatal intensive care unit strategies; furthermore, early gut microbiota features were associated with 2-year
63 outcomes, even after adjustment for confounders [2]. While animal model studies have shown a direct
64 connection between early-life microbiota and neurocognitive development, data in humans are scarce.
65 Therefore, our aim was to investigate associations between gut microbiota dynamic features during the first
66 month of life in very low birth weight (VLBW) preterm infants and neurodevelopment in early childhood.

67

68 **Materials and methods**

69 Preterm infants with gestational age <32 weeks and/or VLBW were enrolled after birth and followed
70 longitudinally up to 24-month corrected age (CA) within a prospective pilot observational study. Stool samples
71 were collected at 1, 4, 7, and 30 days of life. Microbial DNA was subjected to 16S rRNA Illumina sequencing
72 as previously described [3]. Bioinformatics and statistics are detailed in Supplementary Methods.
73 Neurodevelopment was assessed at 24-month CA by revised Griffiths Mental Development Scale (GMDS-R),
74 as a part of neurodevelopmental follow-up of preterm infants. The psychologist performing the Griffiths Mental
75 Development examination were blinded to microbiota analysis. GMDS-R General Development Quotient (GQ)
76 was calculated using standardized score tables for the English infant population (mean \pm SD, 100.5 \pm 11.8), as
77 no standardized data are available for the Italian population. Normal development was defined as a GQ score
78 \geq 88.7, and cut-offs for mild or moderate/severe neurodevelopmental impairment (NI) were 88.6 and 76.8,
79 respectively [4]. The Ethical Board of S. Orsola Hospital (Bologna, Italy) approved the study (study ID
80 25/2014/U/Oss) and written informed consent was obtained from infants' parents.

81

82 **Results**

83 Twenty-seven preterm infants were recruited (14 female [51.9%], 21 born to Caucasian mothers [77.8%], 2 to
84 Asian mothers [7.4%], 4 to African mothers [14.8%]). Median (interquartile range - IQR) gestational age was

85 30.6 (28.6-33.6) weeks and median (IQR) birth weight 1,196 (917-1,374) g. At 24-month CA, 21 infants had
86 normal neurodevelopment and 6 showed NI (3 mild and 3 moderate/severe NI cases). Infants with NI had higher
87 need for surfactant administration. No other difference in clinical characteristics was described between infants
88 with vs. without NI. Detailed clinical characteristics of the recruited infants stratified by neurodevelopmental
89 outcome at 24 months are shown in **Table 1**.

90 As for microbiota assessment, no significant differences were found in GM alpha diversity between study
91 groups over time, except for a trend towards greater diversity in infants with NI at day 30 ($p=0.17$, Wilcoxon
92 test) (**Figure 1a**). On the other hand, beta diversity analysis revealed distinct temporal trajectories between
93 infants with NI and those with normal neurodevelopment ($p\leq 0.05$, PERMANOVA) (**Figure 1b**). Furthermore,
94 based on the unweighted UniFrac metrics, at day 1 and 30, there was significant segregation between the two
95 types of NI (mild vs moderate/severe, $p\leq 0.046$). At the taxonomic level (**Figure 1c**), compared to infants with
96 normal neurodevelopment, those with NI tended to be enriched in *Enterococcaceae* at day 7 and 30 ($p=0.2$,
97 Wilcoxon test). Interestingly, despite an early overrepresentation of *Bifidobacteriaceae* in the gut microbiota of
98 infants with NI ($p=0.05$), their levels cleared by day 7 and tended to be lower than those of infants with normal
99 neurodevelopment at day 30 ($p=0.1$). Notably, at day 30, *Bifidobacterium* abundance was positively correlated
100 with the 24-month GQ score ($p=0.01$, $\tau=0.449$; Kendall rank correlation test) (**Figure 1d**). The major
101 represented species were *B. longum* and *B. breve*, neither of which were found in the gut microbiota of infants
102 with NI (**Figure 1e**).

103

104 **Discussion**

105 Through this prospective pilot observational study, we shed some light on the connections between
106 the early-life gut microbiota dynamic assembly and neurocognitive development of preterm infants
107 in early childhood. In particular, we found a relationship between both dynamic patterns (i.e., beta
108 diversity trajectories) and static features (i.e., relative taxon abundance at certain timepoints) of the
109 gut microbiota during the first month of life with neurodevelopmental outcomes at 24-month CA.

110 Our findings appear to be in line with those of the recent EPIFLORE study, showing that early
111 microbiota is associated to later neurodevelopment [2]. Very recently, a systems-level analysis of the
112 gut microbiota, immune system, and neurophysiological development during hospitalization up to term
113 equivalent age of 60 extremely preterm infants (with gestational age <28 weeks and birth weight < 1000g)

114 revealed that *Klebsiella*-dominated gut microbiota communities are highly predictive for brain damage and are
115 associated with a pro-inflammatory immunological profile [5]. This study suggested that aberrant development
116 of the gut-microbiota-immune-brain axis could contribute to the onset and/or aggravation of brain injury in
117 extremely preterm infants. To the best of our knowledge, our study is the first study reporting on the
118 association between early colonization with *Bifidobacterium* in preterm infants and neurodevelopment
119 in early childhood: specifically, the absence of *Bifidobacterium* at 30 days of life appeared to be
120 associated with NI. *Bifidobacterium* spp. are known to play a pioneering role in the healthy
121 development of the infant gut microbiota, contributing to the fine-tuning of the immune system and
122 potentially exerting neuroprotective effects, mainly through the modulation of the production and
123 release of neuroactive substances [6, 7]. The absence and/or low abundance of *Bifidobacterium*
124 might thus constitute a biomarker of vulnerability and immaturity, and this observation could
125 potentially lead to early intervention strategies aimed at promoting optimal neurodevelopment in
126 preterm infants during neonatal intensive care unit hospitalization and after discharge. Some
127 limitations of our study need to be acknowledged, especially the small number of subjects included in
128 our monocentric cohort. Furthermore, another limitation is constituted by the time window of
129 microbiota analysis, as stool samples were collected only at days 1,4,7, and 30, making us blind to
130 microbiota changes after the first month of life. However, although preliminary, we believe that the results
131 of the present study are promising. Further studies in larger cohorts, possibly with other omics techniques (e.g.,
132 metagenomics and metabolomics) and animal models, are needed to provide additional evidence and
133 mechanistic insights. Once the role of *Bifidobacterium* in promoting optimal neurocognitive development in
134 preterm infants is confirmed, it would be reasonable to design further trials evaluating microbiome-based
135 clinical practices, including both microbiome-modifying strategies and the use of *Bifidobacterium* strains as
136 probiotics, aimed at modulating unbalanced profiles and favoring the long-term health of these fragile infants.

137

138

139 **Declarations**

140 **Funding:** No specific funding was received to assist with the preparation of this manuscript.

141 **Conflicts of interest/Competing interests:** The authors have no conflicts of interest to declare that are relevant
142 to the content of this article.

143 **Availability of data and material:** Raw sequencing reads are available in the National Center for Biotechnology
144 Information Sequence Read Archive (Bioproject ID PRJNA783925) .

145 **Code availability:** Not applicable.

146 **Authors' contributions:** AA, PB and LC designed the study protocol. IB, MB, ST, EB, and AS performed data
147 acquisition and analysis. IB, MB, ST, and AA wrote the first draft of the manuscript, which was revised
148 critically by AS, EB, PB, and LC. All the authors gave final approval of the version to be submitted and agree to
149 be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any
150 part of the work are appropriately investigated and resolved.

151 **Ethics approval:** The Ethical Board of S. Orsola Hospital (Bologna, Italy) approved the study (study ID
152 25/2014/U/Oss). The study was performed in accordance with the ethical standards of the Declaration of
153 Helsinki.

154 **Consent to participate:** Written informed consent was obtained from the parents.

155 **Consent for publication:** Parents signed informed consent regarding publishing their data.

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 186

187 **Figure legends**

188 **Figure 1 Early-life gut microbiota assembly in very low birth weight infants with normal or impaired**
 189 **neurodevelopment. a**, Boxplots showing alpha diversity, measured according to the inverse Simpson index, in
 190 stool samples from preterm infants with neurodevelopmental impairment (NI_yes) or with normal
 191 neurodevelopment (NI_no), collected on days 1, 4, 7 and 30 of life. #, $p=0.17$; Wilcoxon test. **b**, Principal
 192 Coordinates Analysis (PCoA) based on weighted (left) and unweighted (right) UniFrac distances, showing all
 193 samples colored by time point. Symbols indicate the presence or absence of NI, and the arrows represent the
 194 direction of temporal variations of the gut microbiota in each study group. **c**, Boxplots showing the relative
 195 abundance distribution of bacterial families differentially represented between study groups over time. *,
 196 $p=0.05$; #, $p\leq 0.2$; Wilcoxon test. **d**, Scatter plot of correlation between *Bifidobacterium* relative abundance at
 197 day 30 and General Development Quotient score at 2 years of corrected age ($p=0.01$, $\tau=0.449$; Kendall rank
 198 correlation test). **e**, Hierarchical Ward-linkage clustering based on Kendall correlation coefficients of the relative
 199 abundance of *Bifidobacterium* spp. in stool samples from preterm infants at 30 days of life. Samples are color-
 200 coded by study group in the vertical bar (same colors as panel A). *, unclassified species

201

202 **Table 1. Characteristics of the study population stratified by neurodevelopmental outcome at 24-month**203 **corrected age.**

Variable	Normal neurodevelopment (n=21)	Neurodevelopmental impairment (n=6)	p-value
Mother's origin Italy, <i>No. (%)</i>	15 (71)	3 (50)	0.37
Female, <i>No. (%)</i>	12 (57)	2 (33)	0.38
Birth weight, <i>median (IQR), g</i>	1200 (1041-1385)	909 (800-1389)	0.21
Gestational age, <i>median (IQR), weeks</i>	30.6 (28.6-33.7)	29 (26.2-32.3)	0.43
Culture proven sepsis, <i>No. (%)</i>	0	1 (17)	0.22
Necrotizing enterocolitis, <i>No. (%)</i>	0	0	
Respiratory distress syndrome, <i>No. (%)</i>	15 (71)	6 (100)	0.28
Surfactant administration, <i>No. (%)</i>	4 (19)	4 (67)	0.04
Intraventricular haemorrhage, <i>No. (%)</i>	1 (5)	1 (17)	0.40
Patent ductus arteriosus, <i>No. (%)</i>	4 (19)	2 (33)	0.59
Exclusive human milk during first week, <i>No. (%)</i>	19 (90)	5 (83)	0.55
Exclusive human milk during first month, <i>No. (%)</i>	18 (86)	3 (50)	0.10

204

