

Jornal de Pediatria



- Breastfeeding increases microbial community
- ³ resilience[☆],☆☆

4 Q1 Isabel I. Carvalho-Ramos^a, Rubens T.D. Duarte^b, Katia Brandt^c,
 Marina B. Martinez^a, Carla R. Taddei^{a,d,*}

- ^a Universidade de São Paulo (USP), Faculdade de Ciências Farmacêuticas, Departamento de Análises Clínicas e Toxicológicas,
- São Paulo, SP, Brazil
- ^b Universidade Federal de Santa Catarina (UFSC), Departamento de Microbiologia, Imunologia e Parasitologia, Florianópolis,
- SC, Brazil
- ¹⁰ ^c Universidade Federal de Pernambuco (UFPE), Programa de Pós Graduação em Saúde da Criança e do Adolescente, Recife, ¹¹ PE, Brazil
- ¹² ^d Universidade de São Paulo (USP), Escola de Artes, Ciências e Humanidades, São Paulo, SP, Brazil
- Received 14 November 2016; accepted 30 May 2017

14 **KEYWORDS** 15 Breastfeeding: 16 Intestinal microbiota; 17 Dendrogram analysis; 18 Microbial resilience; 19 Food: 20 Antibiotic 21 **Q**2 23 24 25 26 27 28 29

Abstract

Objective: Since the present group had already described the composition of the intestinal microbiota of Brazilian infants under low social economic level, the aim of the present study was to analyze the microbial community structure changes in this group of infants during their early life due to external factors.

Methods: Fecal samples were collected from 11 infants monthly during the first year of life. The infants were followed regarding clinical and diet information and characterized according to breastfeeding practices. DNA was extracted from fecal samples of each child and subjected to PCR-DGGE analysis.

Results: The results revealed a pattern of similarity between the time points for those who were on exclusive breastfeeding or predominant breastfeeding. Although there were changes in intensity and fluctuation of some bands, the DGGE patterns in the one-year microbial analysis were stable for breastfeeding children. There was uninterrupted ecological succession despite the influence of external factors, such as complementary feeding and antibiotic administration, suggesting microbiota resilience. This was not observed for those children who had mixed feeding and introduction of solid food before the 5th month of life.

* Corresponding author.

E-mail: crtaddei@usp.br (C.R. Taddei).

http://dx.doi.org/10.1016/j.jped.2017.05.013

0021-7557/© 2017 Sociedade Brasileira de Pediatria. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Pediatria 🕅

^{*} Please cite this article as: Carvalho-Ramos II, Duarte RT, Brandt K, Martinez MB, Taddei CR. Breastfeeding increases microbial community resilience. J Pediatr (Rio J). 2017. http://dx.doi.org/10.1016/j.jped.2017.05.013

[🎌] Study conducted at Universidade de São Paulo (USP), Faculdade de Ciências Farmacêuticas, São Paulo, SP, Brazil.

+Model

PALAVRAS-CHAVE

Aleitamento materno;

Microbiota intestinal;

Análise de

Resistência

microbiana:

Antibiótico

Alimentação;

dendrograma;

2

30

ARTICLE IN PRESS

Carvalho-Ramos II et al.

Conclusion: These results suggested an intestinal microbiota pattern resilient to external forces, due to the probiotic and prebiotic effects of exclusive breastfeeding, reinforcing the importance of exclusive breastfeeding until the 6th month of life.

© 2017 Sociedade Brasileira de Pediatria. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/ 4.0/).

Aleitamento materno aumenta a resiliência da comunidade microbiana

Resumo

Objetivo: Como nosso grupo já havia descrito a composição da microbiota intestinal de neonatos brasileiros em baixo nível socioeconômico, o objetivo deste estudo foi analisar alterações estruturais da comunidade microbiana deste grupo de neonatos no início de sua vida devido a fatores externos.

Métodos: Amostras fecais foram coletadas mensalmente de 11 neonatos durante o primeiro ano de vida. Os neonatos foram acompanhados com relação a informações clínicas e nutricionais e caracterizados de acordo com práticas de amamentação. O DNA foi extraído das amostras fecais de cada criança e submetido a análise através da técnica de Reação em Cadeia da Polimerase – Eletroforese em Gel de Gradiente Desnaturante (PCR-DGGE).

Resultados: Os resultados revelaram um padrão de similaridade entre seus próprios pontos temporais em indivíduos em aleitamento materno exclusivo ou predominante. Apesar de variações na intensidade e flutuação de algumas bandas, o padrão DGGE na análise microbiana de um ano foi estável em crianças em aleitamento materno. Houve sucessão ecológica ininterrupta apesar da influência de fatores externos, como alimentação complementar e administração de antibióticos, sugerindo resiliência da microbiota. Isso não foi observado nas crianças com alimentação heterogênea e introdução de alimentos sólidos antes do quinto mês de vida.

Conclusão: Nossos resultados sugerem um padrão de microbiota intestinal resiliente a forças externas, devido a efeitos probióticos e prebióticos do aleitamento materno exclusivo, reforçando a importância do aleitamento materno exclusivo até o sexto mês de vida.

© 2017 Sociedade Brasileira de Pediatria. Publicado por Elsevier Editora Ltda. Este é um artigo Open Access sob uma licença CC BY-NC-ND (http://creativecommons.org/licenses/by-nc-nd/4. 0/).

60 Introduction

The intestinal microbiota is an important key in health and disease.¹ It is well known that the intestinal microbiota compositions of newborns and children are influenced by birth,² diet,³ geographic region, and environmental influences.^{4,5}

New methods in 16S rRNA sequencing^{6,7} are rewriting the 65 understanding about the relationship between bacteria and 66 human host, and the use of these methods for characteriz-67 ing the intestinal microbiota of infants and children living 68 in developed countries has received increasing attention.^{3,8} 69 However, few studies conducted in developing countries 70 have corroborated the global observation of inter-individual 71 variability,^{4,9} despite the differences in intestinal micro-72 biota composition. All these findings may contribute to the 73 worldwide understanding of how a geographic region and its 74 environmental contamination influence or do not influence 75 the intestinal microbiota establishment. 76

The authors previously described the microbiota composition of a group of children living at a low socioeconomic level in Brazil, characterized by low rates of *Staphylococcus* in early ages¹⁰ and a high abundance of *Escherichia* at the 12th month of age.⁵ However, information about how the microbial community structure changes in the short term due to external factors was missing. Denaturing gradient gel electrophoresis (DGGE)¹¹ has demonstrated the dynamics of the diversity in the intestinal microbial community can be assessed to determine microbial structural differences between environments¹² and their changes over time.¹³ The present study analyzed the microbial community structure of this group of children monthly during their first year of life, using DGGE.

Material and methods

Subjects and samples

A group of 11 children living at a low socioeconomic level was analyzed, as previously described.^{5,10} The infants were vaginally delivered at the University Hospital of the University of São Paulo (HU-USP). Information concerning the socioeconomic condition of the family and sanitary conditions was collected monthly, during medical appointments. The child's diet was monitored regularly, concerning the period of lactation and timing of the introduction of new foods. The occurrence of infections, health complications, and medication uses were recorded. Fecal samples were collected from babies on the first 2 days, the 7th day, and every month up to the 12th month of life. The mothers were instructed to collect the fecal sample immediately after elimination

59

JPED 548 1-10

84

85

87

88

89

90

91

92

93

94

95

96

97

98

99

100

101

102

103

104

Breastfeeding and intestinal microbial resilience

Child #	Gender	Antibiotic use (time)	Breastfeeding practice ^a
1	F	Amoxicillin (9th month – 7 days)	Complementary feeding
2	F	NO	Complementary feeding
3	F	Cephalexin (1st month – 7 days)	Predominant breastfeeding
6	Μ	Amoxicillin(7th m month – 10 days)	Complementary feeding
7	F	NO	Complementary feeding
8	F	Amoxicillin (5th month – 10 days)	Complementary feeding
12	Μ	Amoxicillin (2nd and 9th months – 7 days)	Exclusive breastfeeding
13	Μ	NO	Exclusive breastfeeding
14	Μ	Amoxicillin (9th month – 10 days)	Exclusive breastfeeding
15	Μ	Cephalexin (10th month - 10 days)	Complementary feeding
17	Μ	Amoxicillin (6th month – 10 days)	Exclusive breastfeeding

Modified from Taddei et al.⁵

^a According to World Health Organization indicators for assessing breastfeeding practices.¹³

with a standardized sterile spoon, place it in a sterile plastic container, and keep it in a freezer (-20 °C) until the appointment some hours later. Samples were transported all the way to the laboratory in an ice-filled polystyrene container, numbered (Table 1S – supplementary material) and immediately stored at -80 °C until DNA extraction, performed in two to three weeks after the collection.

Breastfeeding practices

The children enrolled in this study were characterized 114 according to feeding practices, based on WHO Indicators 115 for Assessing Breastfeeding practices,¹⁴ as follows: exclusive 116 breastfeeding for those children who received only breast 117 milk; predominant breastfeeding for those who received 118 breast milk, water and water based drinks, and fruit juice; 119 and complementary feeding for those who received breast 120 milk and any food including non-human milk (Table 1). Also, 121 according to the WHO indicators,¹⁴ the time recommended 122 for exclusive breastfeeding was up to the 6th month of age. 123

124 DNA extraction

DNA was extracted from stools using Stool QIAmp DNA
 Mini kit (Qiagen[®], CA, USA) according to the manufacturer's instructions. The extracted DNA from stool was quantified
 with a NanoDrop spectrophotometer (Thermo Scientific[®], MA, USA) and kept at -20 °C until use.

Polymerase chain reaction (PCR) amplification for DGGE

The PCR reaction for DGGE was performed using primers for 132 the V3 hypervariable region of the 16S rRNA gene (F338GC: 133 5'-CGC CCG GC CCG GGC CGC GGC GGC GGC CGG GGG 134 GCA GGG GCC TAC GGG AGG CAG CAG-3' and R518: 5'-ATT 135 GCT GCT ACC GCG GG-3').¹⁵ The reaction was optimized to 136 a final volume of 25 $\mu\text{L:}$ 1X buffer, 1.5 mM MgSO4, 0.2 mM 137 dNTPs, 0.2 mM primers, 0.1 U Hi-Fidelity Taq polymerase 138 (Invitrogen[®], CA, USA), and 20 ng DNA. The reactions were 139 performed using the program described by Muyzer et al.¹⁵ 140

DGGE of PCR amplicons

PCR amplicons were separated by DGGE by using the specifications of Muyzer et al.¹⁵ and the Decode system (BioRad Laboratories[®], CA, USA). Bionumerics v.5.1 (Applied Maths[®], Belgium) was used for the evaluation of diversity profiles of each sample, calculation of the Dice coefficient, and cluster analysis. The analysis considered band presence/absence, the GC%-derived position of each band and the relative intensity of the bands to reflect the shared microbial community and the abundance of each population, respectively. The Bray-Curtis coefficient was used to create a similarity matrix among each sample.¹⁶ A cluster analysis was performed from the similarity matrix with Primer 6+.¹⁷ The correlation between samples and band profiles generated by DGGE was determined by correspondence analysis (CA) performed with the program Canoco v4.5 (Biometris[®], Wageningen, Netherlands). The CA biplot was constructed with inter-species distance scaling, where each child was considered a distinct sample and each DGGE band was considered a unique species. No sample or species-weights were specified. In order to make data visualization clearer, three groups of DGGE profiles were created for each sample: (a) 0-5 months; (b) 6-11 months; (c) >12 months. Grouping circles were automatically drawn by the Canoco software for the three profile groups. In order to test whenever the type of diet influenced the microbial community, children were grouped into exclusive (child #12, 13, 14, and 17) and complementary (child #1, 2, 3, 6, 7, 8, and 15) breastfeeding. Child #3 was included in the complementary group for this analysis. A PERMANOVA analysis¹⁸ was done using the similarity matrix described above. A total of 999 permutations were done among DGGE profiles of children between the two diet groups. Since the design is unbalanced (different number of children in each group), a Type III sum of squares was used. The critical p-value was adjusted by Bonferri correction for multiple comparisons (alpha = 0.05; adjusted *p*-critical = 0.005).

Ethical considerations

This research was approved by the Ethics Committee of the HU-USP (under registration number 574/05). All of the mothers enrolled in the research signed an informed consent.

141

142

143

144

145

146

147

148

149

150

151

152

153

154

155

156

157

158

159

160

161

162

163

164

165

166

167

168

169

170

171

172

173

174

175

176

177

178

179

180

4

182 **Results**

183 Clinical information

From the 11 children enrolled in this study, four had a 184 diet characterized as exclusive breastfeeding up to the 6th 185 month of age. One child had a diet characterized as pre-186 dominant breastfeeding up to the 6th month of age, with 187 the introduction of tea with honey at the 3rd month, and 188 the other six children had a diet characterized as comple-189 mentary feeding up to the 5th month of age (Table 1), with 190 the introduction of non-human milk, fruits, and yogurt at the 191 3rd and 4th months of age. At the 6th month of age, most 192 of the children were eating soft food such as soup, fruits, 193 fruit juice, and yogurt. At the end of the 12th month of life, 194 all children were eating a diverse solid diet, which included 195 meat, grains (rice, beans, peas, and lentils), wheat flour, 196 fiber (greens), fruits, milk (formula and/or breast milk), and 107 vogurt. Seven received an antibiotic for respiratory infection 198

RTICLE IN P

¹⁹⁹ during the study (Table 1).

200 Molecular analysis of infants' microbiota by DGGE

Fecal DNA of each child was extracted and the variable V3 201 region of the 16S rRNA gene was amplified. Clustering analy-202 sis was performed for all time points of all children to search 203 for some correlation (Fig. 15 - supplementary material). It 204 205 was not possible to correlate any pattern of similarity with breastfeeding practice by analyzing the dendrogram. There-206 fore, each DGGE profile was subjected to clustering analysis 207 and a dendrogram was obtained for each child (data not 208 shown). The individual results revealed an increase in bands 209 over the months, indicating an increase in microbiota diver-210 sity and complexity, and in inter-individual differences in 211 DGGE profiles, without a unique pattern seen for all chil-212 dren. 213

However, the PCR-DGGE profile of each child revealed 214 an interesting pattern of similarity between time points 215 for those who were on exclusive breastfeeding or predomi-216 nant breastfeeding (Fig. 1). The microbiota profile of those 217 children showed a persistence of prominent bands even 218 after weaning and/or introduction of solid food, and also 219 a gradual increase in the number of bands over time, sug-220 gesting an ecological succession. This was not observed 221 for those children who had mixed feeding and introduc-222 tion of solid food before the 5th month of life (Fig. 2), for 223 whom a varied profile was observed, without similarities at 224 the same time point. The PERMANOVA analysis comparing 225 exclusive and complementary breastfeeding revealed signif-226 icant differences between these groups (p = 0.004; adjusted 227 p-critical = 0.005). According to this result, the microbial 228 community of each breastfeeding group is different at a 95% 229 confidence level. 230

231 Exclusive breastfeeding

The DGGE profile of children under exclusive breastfeeding showed a microbial profile characterized by a stable pattern of bands, with common bands during the study time, with a slight increase or decrease in intensity (Fig. 1A–D), even during the influence of external factors, such the introduction of solid food or antibiotic administration. In the first weeks of life, there were a few bands that disappeared in subsequent months, but then a pattern was established, with an increase in bands, indicating an increase in diversity. After solid food introduction into the diet, the band profile changed, with the disappearance of some bands, and the appearance of others, although the prominent bands remained present with no differences in intensity, suggesting an ecological succession, and an increase in diversity.

Children #12, 13, 14, and 17 were on exclusive breastfeeding up to the 6th month of age, and soft food and fruit juice were then introduced during this month. Only child #13 did not receive an antibiotic, while the others were given an antibiotic once or twice to treat upper respiratory infections (Table 1). For children who received an antibiotic, the diversity was reduced, and there was a fluctuation in the intensity of the bands in the month following drug administration. However, the microbial profile remained stable, and diversity increased in the subsequent months.

Although there were changes in intensity and fluctuation of some bands, the DGGE pattern in the one-year microbial analysis was stable for breastfeeding infants. There was ecological succession with food introduction or antibiotic administration, without losing the microbial community, suggesting microbiota resilience. This evolution was also reflected in the CA. An ecological succession was observed after the introduction of food for all of these infants; at the 6th month of age, with a shift in microbial profile, and after the 12th month of age, the microbial community seemed to be preserved (Fig. 3A).

Predominant breastfeeding

The DGGE profile in this diet was similar to one observed in exclusive breastfeed. Only one child (#3) had a diet characterized as predominant breastfeeding, with an introduction of tea with honey at the third month of age (Fig. 1E). This child had a skin infection at the 7th day of life¹⁰ and was treated with cephalexin for ten days. The DGGE profile showed a reduction in bands in the months following antibiotic administration, with a recovery of diversity at the 5th month of age, with an evident pattern in the microbial community. The introduction of soft food at the 6th month did not seem to disturb this pattern, and the community structure was observed up to the end of the study time. Even the introduction of tea did not affect this pattern. This profile was also noticed in the CA, which indicated the change in microbial community structure after the 5th month of age (Fig. 3A).

Complementary feeding

The DGGE profiles observed for the children not on exclusive breastfeeding (Fig. 2) showed a fluctuation in bands,286without a restoration of the initial microbial pattern. They288had a mixed feeding at the second month of life, and solid289food was introduced at the 3rd or 4th month of age. The290DGGE profile did not show a pattern of ecological succession as seen with the children described above, but rather291

270

271

272

273

274

275

276

277

278

279

280

281

282

283

236

237

238

239

240

241

242

243

244

- 284
- 285

Breastfeeding and intestinal microbial resilience



Fig. 1 Polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE) profiles of exclusive (A–D) and predominant (E) breastfeeding children. (A) child #12; (B) child #13, (C) child #14, (D) child #17; (E) child #3. The administration of antibiotic is indicated by the arrow.

a fluctuation in bands over the study time. There was an 293 increase in bands over time in a bigger proportion of the 294 profile compared to breastfeeding children; however, the 295 microbial community structure was not preserved, and no 296 pattern could be identified. The children under complemen-297 tary food were #1, 2, 6, 7, 8, and 15. Only children #2 and 298 7 did not receive any antibiotics during the study time, and 299 even so, the DGGE profiles were dispersed without a defined 300

pattern. For those who received antibiotics, the number of the bands in the DGGE profile decreased during the antibiotic administration time (Fig. 2), and an increase in bands was observed in subsequent months, but with a different microbial community. CA showed a shift in microbial profile after the introduction of non-human milk or soft food before the 6th month of age, disturbing the microbial community (Fig. 3B).

6

ARTICLE IN PRESS



Fig. 2 Polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE) profiles of complementary breastfeeding children: children #1, #2, #6, #7, #8, and #15. The administration of antibiotic is indicated by the arrows.

309 Discussion

This group had already described; using qPCR and *16S rRNA* library construction, the composition of intestinal microbiota of this group of children was analyzed in some points during their first year of life.^{5,10} In the present study, however, the microbial community structure was analyzed in a monthly basis, revealing a more detailed view of these changes over time.

The dendrogram analysis did not show any correlation between clustering profile and breastfeeding practice, since there were inter-individual differences between the

318

Breastfeeding and intestinal microbial resilience

7



Fig. 3 Correspondence analysis of intestinal microbial community over time for each child with: (A) exclusive (children #12, 13, 14, and 17) and predominant (child #3) breastfeeding. (B) complementary feeding.

children, as discussed previously.⁵ On the other hand,
the analysis of the individual's profile allowed a clearer
interpretation of intestinal microbial community structure,
showing an increase in bands over the months, indicating
an increase in microbiota diversity and complexity, as previously discussed.^{3,5,19}

Bacterial resilience was described as the rate at which microbial composition returns to its original composition after being disturbed,²⁰ due to physiological features and adaptation mechanisms in the new environment. If abundance was reduced by a disturbance, some bacteria group may benefit from the new conditions and then increase in abundance, restoring the original composition.²⁰

The analysis of DGGE results of this group of children revealed an interesting profile of microbiota community resilience in children fed exclusively with breast milk, supported by the PERMANOVA analysis, in which the microbiota profile was different from those under complementary feeding (p < 0.005). Children #12, 13, 14, and 17 were on exclusive breastfeeding up to the 6th month of life, and after the introduction of solid food and/or antibiotic administration, an increase in band number was noted, suggesting an

340

341

8

ARTICLE IN PRESS



increase in microbial diversity. Moreover, the DGGE patterns
 were not disturbed and a structure of the community was
 shared in each child, as seen by CA. Despite the impact on
 the diversity of bacterial genera, the introduction of solid
 food and/or antibiotic administration had little effect on
 bacterial community structure, showing evidence of micro biota resilience.

Breastfeeding is universally recognized as the ideal way to feed infants with a complete source of nourishment,²¹ and also it effectively reduces morbidity and mortality from diarrhea and other infectious diseases.²²

Human milk is a source of symbiotic and probiotic bacteria²³ and prebiotics²⁴ for the baby's gut, such as human milk oligosaccharides (HMOs). HMOs are not digested by the

354

Breastfeeding and intestinal microbial resilience

human gut, and therefore, the role of intestinal microbiota
 is crucial for their hydrolysis.²⁴ In this way, HMOs increase
 the population of beneficial bacteria by both probiotic
 and prebiotic effects, and, consequently, the intestinal
 microbiota of infants is dominated by *Bifidobacterium* and
 Lactobacillus.^{25,26}

The introduction of solid food in children's diet provides a 362 great change in intestinal microbiota diversity. 13,27,28 At the 363 end of the 6th month of age, all children had a diverse solid 364 food diet, which included meat, grains (rice, beans, peas, 365 and lentils), wheat flour, fiber (greens), fruits, milk (for-366 mula and/or breast milk), and yogurt.⁵ The DGGE patterns 367 of the children studied showed an increase in bands, suggest-368 ing also an increase in microbiota diversity. In children with 369 complementary feeding, the DGGE profile showed a fluctu-370 ation in bands during the time studied, and also no sharing 371 of common bands according to CA, corroborating the DGGE 372 results. Furthermore, the early introduction of solid food in 373 the baby's diet seemed to be associated with instability in 374 the microbiota community. 375

Child #3 had a diet characterized as predominant breast-376 feeding, with the introduction of tea with honey at the 377 3rd month of age and breast milk up to the 6th month 378 of age. The DGGE pattern was similar to that of children 379 on exclusive breastfeeding, and the tea and honey did not 380 seem to disturb the microbial ecology over time. Despite the 381 change in microbial richness,¹⁰ the administration of antibi-382 otics did not affect microbial resilience, as evident also for 383 children on exclusive breastfeeding. 384

Antibiotic administration is also another external fac-385 tor that has a negative impact on intestinal microbiota 386 diversity,^{5,29} with a perturbation of microbiota resilience.³⁰ 387 However, the three children (#12, 14, and 17) who were 388 given oral antibiotics showed a reduction in bacterial 389 diversity without disturbance of the intestinal microbial 390 community. The microbial community appeared to be 391 restored in the subsequent month after antibiotic admin-392 istration. 393

The DGGE profile of child #14 demonstrated a marked 30/ decrease in microbial diversity after the introduction of solid 395 food, with the restoration of diversity after the administra-396 tion of antibiotics. However, the community structure was 397 not altered, and the CA showed a pattern of shared bands 398 without disturbances. Interestingly, the medical chart for 399 this child showed a decline in growth curve after the 6th 400 month, with signs of malnutrition over time. This child had 401 less to eat due to socioeconomic factors, and the mother 402 replaced food with breast milk, which could explain the 403 decrease in microbial diversity and the maintenance of 404 microbiota resilience. 405

The microbial profile of child #17 demonstrated a fluctu-406 ation in bands and appeared to give rise to a microbiota with 407 a new profile, although it grouped with the other samples 408 sharing the same bands, suggesting microbiota resilience. 409 It is possible that this change was due to antibiotic admin-410 istration concomitant with the introduction of solid foods. 411 The effects of antibiotics on the microbiota did not allow 412 413 new bacteria from foods or the prebiotic effects of foods to 414 produce microbiota changes from the 6th to 10th month of life. 415

⁴¹⁶ In the previous studies enrolling this group of Brazil-⁴¹⁷ ian children,^{5,10,25} the authors showed a notable absence of *Staphylococcus* as intestinal colonizer¹⁰ and an abundance of *E. coli*,⁵ corroborating the worldwide knowledge of the importance of the influence of external factors in intestinal microbiota colonization. These children had a milk diet, and the intestinal microbiota was dominated by *Bifidobacterium* and *Lactobacillus*.²⁵ However, none of these studies were able to associate specific profiles or changes in microbiota composition with exclusive breast feeding.

Using DGGE, the intestinal microbial community structure during the first year of life of the children studied was determined, and the results suggested the resilience of the intestinal microbiota pattern to external forces, here represented by antibiotic administration and the introduction of solid food. The maintenance of a stable bacterial community in the infants' gut may provide eubiose effects in health over the following years. After birth and up to the weaning period, human milk is the only nutrition recommended by WHO.¹⁴ To the best of the authors' knowledge, this is the first report showing the association of exclusive breastfeeding with microbial community resilience, reinforcing the importance of exclusive breastfeeding up to the 6th month of life, as recommended by the WHO.¹⁴

Funding

FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) No. 06/55141-4 to MBM and FAPESP No. 11/51196-7 to CRT.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

Financial support was from FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo): No. 06/55141-4 to MBM and No. 11/51196-7 to CRT. Dr. A. Leyva provided English editing of the manuscript.

Appendix A. Supplementary data

Supplementary with this data associated arti-452 cle can be found, in the online version, at 453 doi:10.1016/j.jped.2017.05.013. 454

References

- 1. Sekirov I, Russell SL, Antunes LC, Finlay BB. Gut microbiota in health and disease. Physiol Rev. 2010;90:859–904.
- 2. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habits in newborn. PNAS. 2010;107:11971–5.
- 3. Fallani M, Amarri S, Uusijarvi A, Adam R, Khanna S, Aguilera M, et al. Determinants of the human infant intestinal microbiota after the introduction of first complementary foods in infant samples from five European centres. Microbiology. 2011;157:1385–92.

418

419

420

421

422

423

424

425

426

427

428

429

430

431

432

433

434

435

436

437

438

439

440

441

442

443

444

444

446

447

448

449

450

451

455

456

457

458

459

460

461

462

463

464

465

+Model

Carvalho-Ramos II et al.

10

467

475

476

485

486

487

488

- 4. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, et al. Impact of diet in shaping gut microbiota 468 revealed by a comparative study in children from Europe and 469 rural Africa. PNAS. 2010;107:14691-6. 470
- 471 Taddei CR, Oliveira FF, Duarte RT, Talarico ST, Takagi EH, Ramos Carvalho II, et al. High abundance of Escherichia during the 472 establishment of fecal microbiota in Brazilian children. Microb 473 Ecol. 2014;67:624-34. 474
 - 6. Metzker ML. Emerging technologies in DNA sequencing. Genome Res. 2005;15:1767-76.
- 7. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone 477 CA, Turnbaugh PJ, et al. Global patterns of 16S rRNA diver-478 sity at a depth of millions of sequences per sample. PNAS. 479 480 2011:108:4516-22.
- 8. Azad MB, Konva T, Maughan H, Guttman DS, Field CJ, Chari 481 RS, et al. Gut microbiota of healthy Canadian infants: pro-482 files by mode of delivery and infant diet at 4 months. CMAJ. 483 2013;185:385-94. 484
 - 9. Lin A, Bik EM, Costello EK, Dethlefsen L, Hague R, Relman DA, et al. Distinct distal gut microbiome diversity and composition in healthy children from Bangladesh and the United States. PLoS ONE. 2013;8:e53838.
- 10. Brandt K, Taddei CR, Takagi EH, Oliveira FF, Duarte RT, Irino 489 I, et al. Establishment of the bacterial fecal community 490 during the first month of life in Brazilian newborns. Clinics. 491 2012;67:113-23. 492
- 11. Ercolini D. PCR-DGGE fingerprinting: novel strategies for detec-493 tion of microbes in food. J Microbiol Methods. 2004;56: 494 297-314. 495
- 12. Dehingia M, Devi KT, Talukdar NC, Talukdar R, Reddy N, Mande 496 SS, et al. Gut bacterial diversity of the tribes of India and com-497 498 parison with the worldwide data. Sci Rep. 2015;22:18563.
- 13. Favier CF, Vaughan EE, De Vos WM, Akkemans AD. Molecular 499 monitoring of succession of bacterial communities in humans 500 neonates. Appl Environ Microbiol. 2002;68:219-26. 501
- 14. World Health Organization (WHO). Indicators for assessing 502 infant and young child feeding practices. Part I: Definition. 503 Geneva: WHO; 2008. 504
- 15. Muyzer G, Waal EC, Uitterlinden AG. Profiling of complex micro-505 bial populations by denaturing gradient gel electrophoresis 506 analysis of polymerase chain reaction-amplified genes coding 507 for 16S rRNA. Appl Environ Microbiol. 1993;59:695-700. 508
- 16. Suchodolski JS, Ruaux CG, Steiner JM, Fetz K, Williams DA. 509 Application of molecular fingerprinting for qualitative assess-510 ment of small-intestinal bacterial diversity in dogs. J Clin 511 Microbiol. 2004;42:4702-8.

- 17. Clarke KR, Gorley RN. PRIMER v6: user manual/tutorial. Plymouth: PRIMER-E: 2006.
- 18. Anderson M, Ter Braak CJ. Permutation tests for multi-factorial analysis of variance. J Stat Comput Simul. 2003;73:85-113.
- 19. Scholtens PA, Oozeer R, Martin R, Ben Amor K, Knol J. The early settlers: intestinal microbiology in early life. Annu Rev Food Sci Technol. 2012;3:425-47.
- 20. Allison SD, Martiny JB. Resistance, resilience, and redundancy in microbial communities. PNAS. 2008;105:11512-9.
- 21. González-Chica DA, Gonçalves H, Nazmi A, Santos IS, Barros AJ, Matijasevich A, et al. Seasonality of infant feeding practices in three Brazilian birth cohorts. Int J Epidem. 2012;41: 743-52.
- 22. German JB, Dillard CJ, Ward RE. Bioactive components in milk. Curr Opin Clin Nutr Metab Care. 2002:5:653-8.
- 23. Martín R, Jimenez E, Heilig H, Fernandez L, Marín ML, Zoetendal EG, et al. Isolation of bifidobacteria from breast milk and assessment of the bifidobacterial population by PCR-denaturing gradient gel electrophoresis and quantitative real-time PCR. Appl Environ Microbiol. 2009;75:965-9.
- 24. Barile D, Rastall RA. Human milk and related oligosaccharides as prebiotics. Curr Opin Biotechnol. 2013;24:214-9.
- 25. Talarico ST, Santos FE, Brandt K, Martinez MB, Taddei CR. Anaerobic bacteria in the intestinal microbiota of Brazilian children. Clinics. 2017;72:154-60.
- 26. Fallani M, Young D, Scott J, Norin E, Amarri S, Adam R, et al. Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breastfeeding, and antibiotics. J Pediatr Gastroenterol Nutr. 2010;51: 77-84.
- 27. Roger LC, Mccartney AL. Longitudinal investigation of the faecal microbiota of healthy full-term infants using fluorescence in situ hybridization and denaturing gradient gel electrophoresis. Microbiology. 2010;156:3317-28.
- 28. Nielsen S, Nielsen DS, Lauritzen L, Jakobsen M, Michaelsen KF. Impact of diet on the intestinal microbiota in 10-month-old infants. J Pediatr Gastroenterol Nutr. 2007;44:613-8.
- 29. Tanaka S, Kobayashi T, Songjinda P, Tateyama A, Tsubouchi M, Kiyohara C, et al. Influence of antibiotic exposure in the early postnatal period on the development of intestinal microbiota. FEMS Immunol Med Microbiol. 2009:56:80-7.
- 30. Gibson MK, Pesesky MW, Dantas G. The yin and yang of bacterial resilience in the human gut microbiota. J Mol Biol. 2014;426:3866-76.

550

551

552

553

554

555