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(Article begins on next page)

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## FECAL MICROBIOTA IN BREAST-FED INFANTS AFTER ANTIBIOTIC THERAPY

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Heading: Microbiota after antibiotic therapy

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## ABSTRACT

### Objectives

To evaluate modifications of gut microbiota after antibiotic therapy in breastfed infants.

### Study design

~~We recruited~~ 26 exclusively breastfed infants younger than 5 months hospitalized for pneumonia ~~and treated with ceftriaxone (at dosage of 50 mg per kilo per day -administred intramuscularly)~~ ~~were recruited. and we analyzed~~ intestinal microbiota at day 0 - before starting antibiotic administration - at the end of the therapy (5 days after) and after 15 days after the stop ~~was analyzed.~~

Stool samples were collected and immediatly diluted and cultured on ~~several~~ selective media to detect total bacteria, *Enterobacteriaceae*, ~~e~~ *Enterococci* and ~~l~~ *Lactobacilli*. Statistical analysis was performed by using Wilcoxon test.

### Results

After 5 days of antibiotic therapy we observed a significant reduction in total fecal bacterial count ( $p = 0.003$ ) and in ~~faecal concentration of~~ *Enterobacteriaceae* ( $p = 0.001$ ), *Enterococci* ( $p = 0.000$ ) and *Lactobacilli* load ( $p = 0.000$ ), in comparison with control group at day 0. Conversely, ~~The values of~~ bacterial count values for all ~~the species~~ bacteria detected after 15 days from the end of therapy are significantly increased and similar to control group counts.

### Conclusion

Our findings showed that gut microbiota was significantly ~~changes~~ modified after antibiotic therapy; exclusively breastfeeding may be relevant in promoting the re-establishment of gut microbiota composition in early infancy.

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## BACKGROUND

At birth, the gut is sterile and within few hours, bacteria start to appear in the feces (I-II). The first bacteria colonizing the neonatal gut are aerobic or facultatively anaerobic, ~~like such as~~ *Enterobacteriaceae*, ~~e~~ *Enterococci* and ~~s~~ *Staphylococci* (III). ~~During their growth they~~ They consume oxygen making the intestinal milieu suitable for the proliferation of anaerobic species such as *Bifidobacterium* spp., *Clostridium* spp. and *Bacteroides* spp. (IV). ~~Type of delivery influences gut microbiota~~ In the first period of life: ~~maternal~~ faecal and vaginal maternal bacteria colonized infants born by spontaneous delivery, whereas infants born through caesarean section are exposed to ~~bacteria-microorganisms originating~~ from hospital environment and health care workers. Intestinal ~~bacterial-microbial~~ composition is influenced by gestational age, hygiene measures ~~and~~ environmental factors ~~and in the first days of life and type of feeding practices~~; in particular, breast fed infants are less colonized by *Clostridium difficile* and *Escherichia coli* than formula fed infants (V, VI). ~~Intestinal bacterial composition is also influenced by Antibiotics-antibiotic therapy~~ ~~can that could also influence the composition of faecal microbiota with different effects depending on antibacterial class, specific spectrum of the agent, dose, route of administration, pharmacokinetic and pharmacodynamic properties.~~ Antibiotics are designed to attack specific bacterial pathogens ~~but, in the process, they indiscreetly~~ **reduce the number of beneficial human microbiota microorganisms that, particularly in infants, is and part of the gut-associated lymphatic tissue, particularly in infants (A).** Studies investigating microbiota modifications induced by antibiotic therapy are still limited and mainly performed in adults (VII). In particular, no literature data are available ~~in literature~~ on bacterial changes after administration of parenteral ceftriaxone, one of the main 3<sup>rd</sup> generation chepalosporin used in infancy.

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## AIM

This study was performed to evaluate both modifications occurring in intestinal microbiota in infancy after ~~ceftriaxone antibiotic therapy with ceftriaxone and to evaluate the~~ re-establishment of

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~~the gut microbiota within 2 weeks after treatment in exclusively breastfed infants. For this reason we analyze gut microflora before starting antibiotic therapy (T0), after 5 days of drug administration (T5) and 15 days later (T20).~~

## MATERIALS AND METHODS

### Study group and patients selection

~~26 infants, affected by pneumonia and treated with parenteral ceftriaxone, were recruited in by the Department of Paediatrics (Regina Margherita Children Hospital, Turin, Italy). They were all born at term, adequate for gestational age, with a birth weight between 2500 and 4000 g, exclusively breastfed, aged < 6 months and affected by pneumonia. Infants were excluded if they had clinical evidence of gastrointestinal disorders or if they had received other antibiotics or probiotics in the week preceding recruitment.~~

Parents gave written consent to the inclusion of their infants in the study. The ~~study was approved by the local ethicals committee approved the study.~~

### Study Design and Sample Collection

All enrolled infants received parenteral ceftriaxone 50 mg/Kg per day for 5 days ~~for pneumonia treatment.~~

~~Stool samples were collected from each infant before starting antibiotic therapy (T0 = day of hospital admission), after 5 days of drug administration (T5 = the last day of antibiotic therapy) and day 20 (T20 = 15 days after the end of antibiotic therapy).~~

~~on day 0 (day of hospital admission, before starting antimicrobial treatment), day 5 (the last day of antibiotic therapy) and day 20 (15 days after the end of antibiotic therapy).~~

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The faecal samples were transported to the Department of Public Health and Microbiology Laboratories and processed within 1 h.

### Bacteriological Analysis

All faecal samples were weighted and diluted 1:10 with normal saline solution. Further serially dilutions ( $10^{-2}$ - $10^{-9}$ ) were made in normal saline solution and small volumes samples (100  $\mu$ l) were plated on different media: Brain Heart Infusion Agar (BHA; Biolife, Milan, Italy) for total aerobic counts; Azide Maltose Agar (KF; Biolife) to detect enterococci; Chromogenic Coliform Agar (CCA; Biolife) to detect *Enterobacteriaceae*; Rogosa Bios agar to detect lactobacilli (RG; Biolife, Milan, Italy), Brain Heart Infusion Agar (BHA; Biolife), Azide Maltose Agar (KF; Biolife), Gram Negative Anaerobe Selective Medium (GN; Oxoid, Garbagnate Milanese, Italy) and *Clostridium difficile* Agar (CLO; bioMérieux, Rome, Italy), Chromogenic Coliform Agar (CCA; Biolife).

All plates were incubated at 37°C; for 3 days under aerobic (BHA, KF and CCA plates) were incubated for 24 h under aerobic conditions; microaerophilic (RG for 3 days under microaerophilic conditions; and anaerobic (GN, and CLO, RG under) anaerobic conditions. The colony counts of the different dilutions were recorded and the microbial counts were reported as cfu/g (colony forming unit/ (CFU) per gram of faeces).

The qualitative analysis was performed by Gram staining and by morphological, colorimetric and biochemical methods (api-Api Strep System; bioMérieux bioMérieux, Rome, Italy).

### Statistical methods

All statistical calculations were performed with commercially available software (SPSS for Windows release 15.0 SPSS Inc., Chicago, IL, USA).

Data are shown as median and (interquartile range) for continuous variables as appropriate. The Wilcoxon test was used to evaluate differences between paired samples. All reported *p* values are two-sided and differences were considered to be significant when  $p < 0.05$ .

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## RESULTS

The first microbial analysis was performed before starting antibiotic therapy with ceftriaxone at Time0. In these faecal samples median-Median of total bacterial count was  $7.80E+09$  ( $2.89E+10$ ), *Enterobacteriaceae* were  $2.06E+09$  ( $1.51E+10$ ), *e*Enterococci were  $8.30E+08$  ( $3.05E+09$ ) and whereas *L*actobacilli were  $3.87E+07$  ( $3.04E+07$ ). No clostridia was found. The second microbial analysis, performed after 5 days of ceftriaxone administration (T5) showed a significant decreased reduction, in comparison with control group at T0, in count of total bacterial count  $5.55E+07$  ( $3.90E+09$ ) ( $p = 0.003$ ) and in each bacterial group: *Enterobacteriaceae* was  $9.75E+06$  ( $6.90E+08$ ), ( $p = 0.001$ ), *e*Enterococci were  $3.03E+03$  ( $1.59E+06$ ) ( $p = 0.000$ ) and *L*actobacilli were  $0.00E+01$  ( $1.47E+04$ ) ( $p = 0.000$ ). We detected *Clostridium difficile* in 1 infant. At day-T20 median of total bacterial count increased with bacterial load of was  $9.20E+09$  ( $1.36E+10$ ), *Enterobacteriaceae* were  $2.17E+09$  ( $6.06E+09$ ), *e*Enterococci were  $3.53E+07$  ( $1.66E+09$ ) and *L*actobacilli were  $1.46E+07$  ( $2.23E+08$ ). No *Clostridia* was found at T0. However significant differences in gut microflora between T0 and T20 (total bacterial count  $p = 0.228$ , *Enterobacteriaceae*  $p = 0.182$ , enterococci  $p = 0.534$  and *l*actobacilli  $p 0.316$ ) were observed.

The microbial analysis of stool samples after 5 days of ceftriaxone showed a significant reduction in total bacterial count ( $p = 0.003$ ) and in each bacterial group (*Enterobacteriaceae*  $p = 0.001$ , *Enterococci*  $p = 0.000$  and *Lactobacilli*  $p = 0.000$ ). We detected *Clostridium difficile* in 1 infant.

We didn't find significant differences in gut microflora between T0 and T20 (total bacterial count  $p = 0.228$ , *Enterobacteriaceae*  $p = 0.182$ , *Enterococci*  $p = 0.534$  and *Lactobacilli*  $p 0.316$ ).

## DISCUSSION

Cephalosporins are broad-spectrum antimicrobial agents and they could cause great quantitative and qualitative ecological disturbance in normal gut microbiota (VII); this risk is high in particular for agents with biliary excretion, like ceftriaxone. Antibiotic administration can cause quantavite and

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qualitative ecological disturbances in the normal gut microbiota. They attack specific bacterial pathogens but, in the process, they can reduce the number of beneficial bacteria that, particularly in infants, is part of the gut associated lymphatic tissue (VII). These effects vary not only between antibiotic class, but also between the specific agent in each class. Ceftriaxone is one of the most used antibiotics in paediatric age. In this study we evaluated the modifications that occur in faecal microbiota after an antibiotic therapy performed with ceftriaxone in exclusively breast fed infants. Cephalosporins are broad spectrum antimicrobial agents and they could cause great disturbance in intestinal microflora; this risk is high in particular for agents with biliary excretion, like ceftriaxone. We decided to analyze modifications induced by ceftriaxone because it is one of the most used antibiotics in paediatric age. Studies conducted in adult healthy volunteers showed a significant suppression of anaerobic intestinal microflora (IX-X). Another study, conducted in adult patients undergoing colon-rectal surgery, demonstrated a suppression—significant reduction of enterobacterial count, with only minor changes in Gram positives aerobic flora and anaerobes (XI). As In literature there are only scanty studies concerning the changes induced in gut microbiota by ceftriaxone in pediatric age, in this study we evaluated the modifications in faecal microbiota after ceftriaxone therapy in exclusively breast-fed infants. Microbial analysis, performed after 5 of ceftriaxone administration showed a decreased count of total bacterial count; in particular there was a reduction in *Enterobacteriaceae*, enterococci and lactobacilli. These findings are in according with those of Borderon et al. who demonstrated a disappearance of *Enterobacteriaceae* and a reduction in concentration of *Pseudomonas spp.*, *Streptococci* and *Staphilocoecus-Staphylococcus aureus* (XII). Another study, conducted by Fujita et al. with only ~~on~~ 3 patients, showed a disappearance of b *Bifidobacteria* and *Enterobacteriaceae*, with a preservation of *Streptococcus spp.* and *Staphilocoecus-Staphylococcus spp.* (XIII). In our study the second microbial analysis, performed after 5 of ceftriaxone administration showed a decreased count of total bacterial count; in particular there was a reduction in concentration of *Enterobacteriaceae*, *Enterococci* and *Lactobacilli*.

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In literature is reported that antibiotics directed against anaerobes (aminopenicillins, cephalosporins and clindamycin) cause more often diarrhoea (XIV-XV). It is known that *Clostridium difficile* can be responsible for antibiotic (i.e aminopenicillins, cephalosporins and clindamycin) associated diarrhoea (XIV-XV-XVI); we found this ~~bacteria~~ bacterium only in 1 patient after ceftriaxone therapy with Ceftriaxone, but he it didn't present modifications in stool frequency (non capisco cosa significhi questa frase). Anyway a limited number of studies has been conducted in infancy to evaluate the modifications that occur in gut microflora after ~~an~~ antibiotic therapy and the recovery of its original composition. In our patients we didn't observe any change in stool frequency and nobody presented antibiotic associated diarrhoea.

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We decided to analyze only exclusively breast-fed infants because a relationship between human milk and infant health had been recorded periodically for thousands of years (XVII). Epidemiologic studies found that artificially fed infants are three to ten fold higher risk of disease, in particular enteric infections leading to diarrhoea (XVIII-XIX). It is known that there are different mechanism by which human milk acts on intestinal mucosa: it accelerates maturation of the gut barrier function, it contains bioactive components including IgAs and it attenuate early inappropriate inflammatory reactions (XX). Some studies underlined the role of breast-feeding in influencing gut microflora: in particular human milk is associated with ~~an~~ higher colonization by ~~Bifidobacteria~~ Bifidobacterium spp. and a lower ~~concentration in~~ bacterial load due to Enterobacteriaceae, Clostridia ~~Clostridium spp.~~ and *Bacteroides spp.* (XXI).

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According to our results, we can hypothesize a protective role of breast-feeding in ~~re-establish~~ establish normal gut microbiota in a short period of time after antibiotic therapy.

Some meta-analyses have been completed on the use of probiotics for the prevention of antibiotic associated diarrhoea in the general population. The authors concluded that probiotics administration significantly reduce the relative risk of AAD in adults (XXII, XXIII). A recent Cochrane Review underlines the role of probiotics like *Lactobacilli* in the prevention of antibiotic associated diarrhoea

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(AAD) (XXIV), non c'entra tanto con il nostro studio perchè i bambini dello studio non hanno avuto

diarrea.

Larger studies, using molecular techniques are warranted not only to study but also to prevent the changes that occur in gut microbiota after antibiotic therapy and its consequences. It would be interesting to understand if probiotic administration during or after antibiotic therapy can speed up the re-establishment-establishment of normal count of Lactobacilli, in order to prevent antibiotic associated diarrhoea, in particular in formula-fed infants.

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