Original Article

Antibiotic Therapy in Neonates and Impact on Gut Microbiota and Antibiotic Resistance Development: A Systematic Review

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Study protocol registration: PROSPERO CRD42015026743

Word count synopsis: 249

Word count in main text: 4160 words

Running title: Systematic review on neonatal antibiotic therapy, gut microbiota and antibiotic resistance

ABSTRACT

OBJECTIVES: To systematically review the impact of antibiotic therapy in the neonatal period on changes in the gut microbiota and/or antibiotic resistance development.

METHODS: Data sources were PubMed, Embase, Medline and the Cochrane Database, supplemented by manual searches of reference lists. Randomised controlled trials (RCTs) and observational studies were included if they provided data on different categories of antibiotic treatment (yes versus no, long versus short duration and/or broad versus narrow spectrum regimens) and subsequent changes in the gut microbiota and/or antibiotic resistance development. We evaluated risk of bias using the Cochrane Handbook, adapted to include observational studies. When appropriate, we used the vote-counting method to perform semi-quantitative meta-analyses. We applied the Grades of Recommendation, Assessment, Development and Evaluation approach to rate the quality of evidence (QoE).

RESULTS: We included 48 studies; three RCTs and 45 observational studies. Prolonged antibiotic treatment was associated with reduced gut microbial diversity in all three studies investigating this outcome (very low QoE). Antibiotic treatment was associated with reduced colonization rates of protective commensal anaerobic bacteria in four of five studies (very low QoE). However, all three categories of antibiotic treatment were associated with an increased risk of antibiotic resistance development, in particular multi-drug resistance in Gram-negative bacteria, and we graded QoE for these outcomes as moderate.

CONCLUSIONS: We are moderately confident that antibiotic treatment leads to antibiotic resistance development in neonates, and it may also induce potentially disease-promoting gut microbiota alterations. Our findings emphasize the need to reduce unnecessary antibiotic treatment in neonates.

INTRODUCTION

Upon birth, infants are suddenly exposed to a wide range of bacteria colonizing mucoepithelial surfaces, including the gut.[1] The subsequent development of the infant gut microbiota is dynamic, non-resilient and shaped by factors like mode of delivery, feeding, diet and environment.[2-4] A healthy gut microbiota has a crucial role in the development of the immune systems, digestive functions and protection against infections.[4-6] The commensal aerobic and anaerobic bacteria are also essential for colonization resistance; the ability to prevent invasion and persistent carriage of pathogenic and antibiotic resistant bacteria.[7]

Antibiotics are the most commonly prescribed medications in the neonatal unit.[8] However, antibiotic overuse in early life disrupts the actively developing gut microbiota causing "bacterial dysbiosis", which is associated with an increased risk of early adverse outcome such as necrotizing enterocolitis and fungal infections.[9] Early antibiotic exposure has also been associated with allergic diseases, obesity, diabetes and inflammatory bowel disease later in life.[10-14] Overuse of antibiotics, particularly broad-spectrum antibiotics, applies a selection pressure which favours antibiotic resistant bacteria and decreases colonization resistance.[7, 15] The currently observed increase in resistance to aminoglycosides and ampicillin among Gram-negative bacteria have begun to threaten this traditional combination as empiric treatment for neonatal sepsis.[16, 17] Moreover, worldwide the emergence of ESBL-producing Enterobacteriaceae presents major challenges in managing neonatal sepsis.[18] Globally, an estimated 200 000 neonatal deaths are attributed to resistant organisms each year.[19] However, the relative impact of different types of antibiotic exposure on the actively developing gut microbiota composition and antibiotic resistance development is not fully understood.

The purpose of the current systematic review is to identify, critically appraise, and synthesize evidence from studies reporting different categories of antibiotic therapy in neonates and their impact on the gut microbiota and/or antibiotic resistance development. We

included both observational studies and randomised clinical trials (RCTs) in line with suggestions from the Cochrane group stating that systematic reviews of adverse effects will usually need to include non-randomised studies in addition to RCTs.

METHODS

This review was reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses following a registered protocol and according to the recommendations given by the Cochrane Handbook for Systematic Reviews and Interventions.[20-22] We recently published a systematic review on early clinical adverse effects of neonatal antibiotic treatment from the same research protocol.[9] For this review, our primary research question was "Are different categories of antibiotic treatment in neonates associated with different changes in gut microbiota composition and/or differences in antibiotic resistance development?"

Search Strategy

We developed our search strategy in consultation with an epidemiologist, a librarian, a paediatric pharmacologist and a neonatologist. We searched PubMed, Embase, Medline and the Cochrane Database using MeSH-Terms and free text searches with no time restrictions (last search 22nd of December 2016). The first search was conducted with MeSH terms in PubMed, Medline and the Cochrane Database by combining "Infant, Newborn" and "Anti-Bacterial Agents" with one of two outcome terms: "Drug Resistance, Bacterial" or "Microbiota". The Embase database uses its own key words, and we combined "Newborn" and "Antibiotic Agent" with either "Antibiotic Resistance" or "Microbiome". The second search was conducted using free text in PubMed, Medline and Embase combining the keywords: "Infant, low Birth Weight" or "Infant, Postmature" or "Infant, Premature" or "Infant, Newborn" with "Anti-Bacterial Agents" or "Antibiotics" and one of the following: "Antibiotic Resistance" or "Antibacterial Drug Resistance" or "Microbiota" or "Microbiome"

or "Microbiomes" or "Gut flora". We examined reference lists of included studies and relevant reviews to identify additional eligible studies. We then combined all citations and excluded duplicates or triplicates. We did not contact authors for supplementary information and we did not perform searches in the grey literature.

Study Selection and Eligibility Criteria

A study was eligible for review if it reported different categories of intravenous antibiotic treatment in the neonatal period and evaluated their impact on changes in the gut microbiota and/or antibiotic resistance development. If infants were born prematurely we defined the neonatal period up to 44 weeks postmenstrual age. We compared three different categories of antibiotic therapy: (1) Antibiotic treatment yes versus no, (2) antibiotic treatment long versus short and (3) antibiotic treatment broad versus narrow spectrum. For category (2), we suggested in advance that "prolonged" antibiotic exposure was always ≥ 3 days or the longest regimen among two antibiotic regimens compared. For category (3), we always defined regimens including third-generation cephalosporins or carbapenems as broad-spectrum regimens when compared to regimens containing aminoglycosides for coverage against Gram-negative bacteria. This definition was based on previous reports indicating that empiric therapy containing a third-generation cephalosporin for Gram-negative coverage induces significantly more resistance than a regimen containing an aminoglycoside.[15] If two similar regimens were compared, the regimen with the broadest spectrum was labelled broad-spectrum. Both RCTs and observational studies such as cohorts, case-control studies, and cross-sectional studies were eligible for inclusion. We included case-control studies reporting on the prespecified outcomes if data on antibiotic therapy prior to the outcomes were presented as extractable data in cases and controls. We excluded case reports and case series, studies with a non-neonatal or non-human population, studies that were written in other

languages than English and studies that investigated antenatal antibiotics, oral antibiotics and/or low-dose intravenous vancomycin prophylaxis.

Screening, Data Extraction, and Management

Two reviewers (JWF and EE) independently screened search results and assessed each potentially eligible study per our predetermined inclusion and exclusion criteria. We only excluded studies that we agreed were irrelevant according to our predefined criteria. A third researcher (CK) had the decisive vote in case of disagreement. We extracted the following information from included studies; author, year, country, study design, study population, including gestational age (GA) and birth weight (BW), comparison of outcomes between groups with different categories of antibiotic treatment and, if available, risk estimates with 95% CI for the specific outcome.

Gut microbiota analyses were based on faecal samples using both standard culture-based methods and culture-independent methods relying on DNA amplification and sequencing.[23] After reviewing the articles presenting data on gut microbiota we decided to present data from these studies in three main categories; microbial load, microbial diversity and microbial composition, clearly acknowledging some overlap between these categories. We defined microbial load as the total number of bacteria in a sample, microbial diversity as the number of different bacterial genus or species in a sample and microbial composition as the taxonomical composition in a sample. Antibiotic resistance development was based on detection of antibiotic susceptibility patterns in bacteria isolated from blood, urine, cerebrospinal fluid, faeces, tracheal aspirates and/or the skin surface. We defined MDR bacteria as bacteria resistant to ≥ 2 unrelated classes of antibiotics or broad-spectrum antibiotics.[24-28] Included in this category were ESBL-producing Gram-negative bacteria, carbapenem-resistant *Acinetobacter baumannii* (CRAB) and Gram-negative bacteria resistant to third-generation cephalosporins. Antibiotic resistant bacteria that did not meet any of these criteria were defined as “other antibiotic resistant bacteria”.

We applied a simple vote-counting method to investigate whether the different categories of antibiotic therapy had any effect on the outcomes of interest.[22] Studies were classified based on whether they showed a reduction in the outcome measure, no effect or an increase in the outcome measure following antibiotic treatment. When appropriate, outcomes were presented in vote-count figures.

Assessment of Methodological Quality

Methodological quality was assessed by using the Cochrane Handbook of Systematic Reviews of Interventions and recently published suggestions on how to assess risk of bias and confounding in observational studies.[22, 29] Five domains related to risk of bias were assessed for each study included: Selection, Performance, Detection, Reporting and Confounding. Risks of bias were judged as low, high or unclear for each domain. The risk of reporting bias was considered unclear in studies that did not have a previously published protocol. The risk of detection bias was considered high in studies that examined gut microbiota with culture-based methods, unclear in studies that applied 16S rRNA sequencing techniques and low in studies that applied shotgun metagenome sequencing techniques. Two reviewers (JWF and EE) assessed the risks of bias for of each study. Disagreements in the categorization process were resolved after discussion between JWF, EE and CK.

We applied the Grades of Recommendation, Assessment, Development and Evaluation Working Group (GRADE) approach to rate the quality of evidence (QoE) for each relevant outcome category.[30] This approach specifies four levels of quality from high to very low, which define the degree to which its' estimates of effects or associations can be trusted.[22, 30] RCTs started as high QoE and observational studies started as low QoE.[30] Several factors could either downgrade or upgrade the quality rating.

RESULTS

Overview of Included Studies

From 3380 identified studies, we reviewed 137 potentially eligible full-text articles. Forty-eight studies met our inclusion criteria: three RCTs published between 2000 and 2013[15, 31, 32] and 45 observational studies published between 1974 and 2016 (Figure 1).[24-28, 33-73] Two articles presented data from the same study population and were defined as one study.[34, 35] Antibiotic treatment was the randomized intervention in two out of three included RCTs.[15, 31, 32] Among the 45 observational studies, there were 22 prospective cohort studies, 12 case-control studies, seven before-after studies and four retrospective cohort studies. There were large variations regarding the categories of antibiotic therapy studied. Tables S1-S2 (available as Supplementary data at JAC online) display the main characteristics and primary outcomes of interest from the 48 included studies.

Risk of Bias and Quality of Evidence (QoE)

Figure S1a-b (available as Supplementary data at JAC online) display risk of bias assessments for each included study. Outcomes adjusted for differences in populations were reported in 16/45 (36%) observational studies.[25, 26, 28, 39, 44, 46, 50, 52, 55, 57, 63, 64, 67, 69-71] Five of these studies used stratification or multivariate analysis to adjust for antenatal antibiotic treatment as a potentially confounding variable. None of the RCTs were included in public registries.

We graded the QoE as very low for the outcomes microbial load and microbial diversity in relation to the three different categories of antibiotic treatment due to inclusion of observational studies with serious risk of bias and inconsistent results. We graded the QoE as very low for the outcomes relating to microbial composition after antibiotic treatment (Figure 2a-d). We graded the QoE as moderate for the outcomes relating to antibiotic resistance development due to inclusion of observational studies that either had large effect sizes (yes

versus no and broad versus narrow) or a dose-response effect (long versus narrow) after antibiotic treatment (Figure 3a-c).

Gut Microbiota Composition

Nineteen studies reported on antibiotic exposure and impact on the gut microbiota composition (Table SI). There were two RCTs[31, 32] and 17 observational studies.[33-49, 73] Three studies reported outcome data from both antibiotic treatment yes versus no and broad versus narrow spectrum,[34, 37, 47] and one study reported outcome after antibiotic treatment yes versus no and long versus short.[42] The remaining 15 studies reported outcome data from one category of antibiotic treatment. To examine gut microbiota composition, nine studies used 16S rRNA gene-sequence analysis,[33, 38-40, 42, 44-46, 49] one used fluorescent in situ hybridisation techniques,[32] one used deep shotgun metagenome sequence analysis[48] and eight used standard culture-based methods.[31, 34-37, 41, 43, 47, 73] The included studies reported primarily taxonomic data with different hierarchical details on i) Enterobaceriaceae, ii) obligate commensals anaerobic bacteria (e.g. bacteroides, lactobacilli and bifidobacteria etc.), iii) clostridia and/or iv) Gram-positive cocci.

Microbial Load

Three studies (296 neonates) compared the impact of antibiotic treatment (yes versus no) on microbial loads.[32, 34, 40] One study (165 neonates) found increased microbial loads,[34] one RCT (113 preterm neonates) found decreased microbial loads,[32] while one study (18 term neonates) found no significant differences in microbial loads following antibiotic treatment.[40] A small study of extremely low birth-weight neonates found an inverse correlation between the duration of antibiotic therapy and the microbial load on day 30 of life.[41]

Microbial Diversity

Four studies (159 neonates) compared microbial diversity after antibiotic treatment (yes versus no).[40, 42, 44, 49] Two studies (112 preterm neonates) reported decreased diversity among antibiotic treated neonates[42, 49] and two studies (47 neonates) reported no significant differences.[40, 44] Three studies (224 preterm neonates) examined the impact of antibiotic therapy duration (long versus short) on microbial diversity, and all three found decreased diversity following prolonged therapy.[41, 44, 48]

Microbial Composition

Figure 2 displays the results of studies reporting the impact of antibiotic treatment (yes versus no) on microbial composition. Nine studies focused on Enterobacteriaceae; four reported an increase and five studies reported unchanged composition after antibiotic treatment, mainly ampicillin plus an aminoglycoside (Figure 2a).[33, 34, 36, 37, 40, 42, 43, 46, 47] Five studies focused on different commensal obligate anaerobes showing a clear trend towards reduced colonization rates following treatment (Figure 2b).[35, 36, 38, 40, 43] In the five studies focusing on clostridia, there were equivocal results (Figure 2c).[36, 39, 40, 45, 46] Finally, four studies focused on Gram-positive cocci, and these studies showed either unchanged or higher colonization rates after antibiotic treatment (Figure 2d).[33, 36, 37, 40]

Two studies (n=983) reported Enterobacteriaceae colonization rates after treatment with broad versus narrow spectrum antibiotics.[37, 47] Both studies found lower colonization rates following third-generation cephalosporin treatment. One study of preterm infants (n=76) reported lower colonization rates of clostridia in those who received ≥ 10 days of antibiotic therapy compared with shorter duration.[39] Another study with preterm infants (n=74) reported higher colonization rates of staphylococci in those who received ≥ 5 days of antibiotic treatment compared with shorter duration therapy.[42] Finally, two studies (n=104) compared

the impact of antibiotic therapy (broad versus narrow) on abundance and/or colonization rates with staphylococci, but neither found any significant differences.[37, 42]

Antibiotic Resistance Development

Thirty-one studies, two RCTs[15, 31] and 29 observational studies,[24-28, 37, 50-72] evaluated the risk of antibiotic resistance development after antibiotic exposure (Table S2). Five studies reported outcome after antibiotic treatment yes versus no and broad versus narrow spectrum.[27, 37, 53, 55, 67] Two studies reported outcome after antibiotic treatment long versus short duration and broad versus narrow spectrum.[26, 64] Two studies reported outcome after antibiotic treatment yes versus no and long versus short duration.[25, 57] The remaining 23 studies assessed only one category of antibiotic therapy.

Nine studies reported on both infections and colonization with antibiotic-resistant bacteria,[24, 51, 57, 58, 60, 62, 65, 67, 68] while 15 studies only reported on colonization,[15, 25-27, 31, 37, 53-56, 59, 61, 66, 69, 72] and seven studies only reported on infections.[28, 50, 52, 63, 64, 70, 71] MDR bacteria were varyingly defined as bacteria resistant to both third-generation cephalosporins and aminoglycosides[55, 58] or bacteria resistant to ≥ 2 or ≥ 3 unrelated classes of antibiotics.[24-28] Thirty of 31 studies focused solely on antibiotic resistance development in Gram-negative bacteria. Among these, 20 studies focused on MDR Gram-negative bacteria.

MDR Gram-negative bacteria

Figure 3 displays the results of the 20 studies reporting the impact of antibiotic exposure on rates of infection and/or colonization with MDR Gram-negative bacteria. Nine studies reported data after antibiotic treatment yes versus no, and the majority reported increased rates of MDR Gram-negative bacteria following treatment (Figure 3a).[25, 27, 55, 57, 59, 63, 67, 69, 70] Five studies reported data after long versus shorter duration of treatment, and the majority found significantly more MDR Gram-negative bacteria after prolonged treatment (Figure

3b).[25, 26, 56, 57, 64] Thirteen studies reported data after treatment with broad spectrum versus narrow spectrum antibiotics, and the overwhelming majority reported higher rates of MDR Gram-negative bacteria following treatment with broad spectrum antibiotics (Figure 3c).[15, 24, 26-28, 50, 51, 55, 58, 64, 65, 67, 71]

Other antibiotic resistant bacteria

Four studies (n=1825) compared the impact of antibiotic treatment (yes versus no) on antibiotic resistant bacteria that were not MDR according to our definition.[37, 52, 53, 66] One study (n=584) found a higher rate of prior antibiotic treatment in neonates colonized with antibiotic resistant *Escherichia coli* and/or *Klebsiella pneumonia*.[66] One study (n=953) found an increased incidence of TEM-1 genes in *E. coli* strains in neonates following antibiotic therapy.[53] Two studies (n=288) found no statistically significant associations between antibiotic treatment (yes versus no) and subsequent antibiotic resistance development.[37, 52] Two studies compared the impact of antibiotic therapy duration;[61, 72] one of them (n=1180) found significantly longer prior antibiotic treatment among neonates colonized with antibiotic resistant Gram-negative bacteria,[72] while the other (unknown number of neonates) found no correlation between the duration of treatment and gentamicin-resistant Gram-negative bacteria.[61]

Eight studies (n=3029) compared the impact of broad- versus narrow-spectrum antibiotic treatment.[31, 37, 53, 54, 60, 62, 68, 72] One RCT (n=276) found higher colonization rates with ampicillin-resistant *Acinetobacter baumannii* following treatment with penicillin and gentamicin compared with ampicillin and gentamicin.[31] One study (n=440) found a higher rate of both ampicillin and cefuroxime resistance in Gram-negative bacteria following treatment with ampicillin compared with cefuroxime.[62] One study (n=118) found a higher rate of gentamicin resistance among Gram-negative bacteria following treatment with gentamicin compared with amikacin.[68] The remaining five studies (n=2195) did not formally test for

statistically significant differences when comparing broad versus narrow spectrum regimens,[37, 53, 54, 60, 72] but 3/5 studies (n=1258) reported increased rates of antibiotic resistance following broad-spectrum therapy.[54, 60, 72]

DISCUSSION

Key Findings

To our knowledge, this is the first systematic review to examine antibiotic therapy in neonates and its impact on gut microbiota and/or antibiotic resistance development. The primary findings were the lack of RCTs and large high-quality observational studies and the heterogeneity regarding methodology and outcomes among the included studies. Despite this, there were several salient features in this review.

First, prolonged antibiotic therapy was associated with reduced gut microbial diversity.[41, 44, 48] Decreased gut microbial diversity has been associated with early adverse outcomes such as NEC, and may have potential long lasting consequences through increased likelihood of obesity and inflammatory diseases.[10, 49, 74-77] Combined, these findings imply that prolonged exposure to antibiotic treatment in the neonatal period may increase the likelihood of disease, either in the neonatal period or later in life. However, QoE for this outcome was graded as very low. It is possible that neonatal antibiotic therapy, regardless of treatment length, leads to decreased microbial diversity, but the included studies in this category were small and two out of four studies did not detect a significant difference.[40, 42, 44, 49] Second, four out of nine studies reported increased abundance and/or colonization rates of Enterobacteriaceae following neonatal antibiotic treatment, while none of the studies reported reduced abundance.[33, 34, 36, 37, 40, 42, 43, 46, 47] In the majority of these studies, the empiric regimens consisted of ampicillin and gentamicin. We speculate that intravenous ampicillin also has an impact on Gram-positive gut bacteria despite being mainly secreted through the kidneys,[78] while intravenous gentamicin mainly covering Gram-negative bacteria

in the blood stream,[79] has a very low penetration into the gut. Combined, this may give undue benefits to the Gram-negative Enterobacteriaceae. In contrast, third-generation cephalosporin therapy may lead to a relatively lower abundance of Enterobacteriaceae as both Gram-negative and Gram-positive bacteria are within their spectrum of activity.[79] However, QoE for this outcome was again graded as very low, and even though overgrowth of Enterobacteriaceae in the human gut has previously been associated with NEC, inflammatory bowel disease and chronic fatigue syndrome there is no strong evidence of any causal relationship.[74, 76, 80-82]

Third, antibiotic treatment in the neonatal period was strongly associated with reduced abundance of protective commensal anaerobic bacteria such as bifidobacteria, lactobacilli and/or bacteriodes.[35, 40, 43] These bacteria provide colonization resistance against antibiotic resistant bacteria and potentially pathogenic bacteria such as Enterobacteriaceae and *Clostridium difficile.*[7] Moreover, it is well known that bifidobacteria may reduce expression of inflammatory response genes and stimulates genes promoting the integrity of the mucosal barrier. The QoE for this outcome was graded as very low, but our results are in line with findings in adult populations showing decreased diversity, reduced colonization rates of obligate anaerobes and increased colonization rates of *Proteobacteria* following antibiotic exposure.[83-85] Furthermore, our findings are biologically plausible as reduced numbers of bifidobacteria and lactobacilli seem to increase the risk of necrotising enterocolitis in preterm infants with an exaggerated inflammatory response.[82, 86-90] In adults some studies have found larger changes in the gut microbiota than oral microbiota following antibiotic treatment, with larger resilience in the oral communities. [84,85] However, we believe that the gut microbiota is of highest clinical relevance, both as the largest reservoir for antibiotic resistant bacteria and because the gut is characterised as the motor of multiple organ dysfunction syndrome.

Fourth, all three categories of neonatal antibiotic treatment investigated in this review were clearly associated with an increased risk of antibiotic resistance development, in

particular ESBL-producing Gram-negative bacteria and other MDR bacteria. These findings were based on moderate QoE. Antibiotic resistance genes exist even in the absence of antimicrobial drugs.[91, 92] Moreover, overuse of antibiotics may lead to increased antibiotic resistance through several mechanisms.[91, 93] Antibiotics apply a direct selection pressure that gives significant advantages to bacteria expressing resistance genes.[94] Antibiotic treatment also contributes to changes in the human gut-associated resistome, which comprise numerous functional antibiotic resistance genes in the gut microbiota.[95] Gibson and colleagues recently found that only a fraction of antibiotic resistance genes that are enriched after a specific antibiotic therapy are unique to the particular antibiotic given.[96] Finally, antibiotic treatment appears to reduce colonization resistance against antibiotic resistant bacteria through the collateral destruction of obligate anaerobic bacteria.[7, 97] An increase in the gut resistome and a decrease in colonization resistance could theoretically increase horizontal transfer of antibiotic resistance genes from commensals to potential pathogens.[98] Although *in vivo* horizontal transfer between commensals and pathogens in the gut microbiota remains to be shown, there is evidence of exchange of antibiotic resistant genes between environmental bacteria and human pathogens.[99]

Strengths and Limitations

The primary strength of this study is our rigorous and sensitive search strategy based on a previously registered search protocol. Additionally, the adverse impact of the developing infant gut microbiota is of great clinical and scientific interest. The main limitations were the lack of RCTs and the diverse studied outcomes which made meta-analysis impossible to perform. Instead, we applied a semi-quantitative vote-counting method to assess the effect of neonatal antibiotic treatment on relevant outcomes. This method has limitation as it usually fails to take account of the population sizes and methodological quality of pooled studies. Still, vote counting may be an effective method to assess ranking of outcomes.[100] Moreover,

we attempted to improve the method by presenting the differential weight of each study with squares corresponding to sample size.

The majority of studies included were small and there was a large heterogeneity in study designs, outcomes, categories of antibiotic treatment and methodological quality. Observational studies are prone to biases and confounding, and only a third of the included studies attempted to adjust for confounding through multivariable regression analysis. Evidence from observational studies is usually considered to be of low quality. However, well designed observational studies have been shown to provide similar results to RCTs and they can therefore be useful for detecting rare adverse outcomes by allowing larger sample sizes and longer lengths of follow up than RCTs for lower costs.[101] We used the GRADE approach to assess QoE. Overall, we graded QoE as very low for all outcomes presented in the gut microbiota category. In contrast, we considered the QoE as moderate in the antibiotic resistance category due to large effect sizes and a dose-response effect. Based on current evidence we are therefore moderately confident that all types of antibiotic treatment lead to increased rates of antibiotic resistance.

All included studies published prior to 2007 used culture-based techniques to examine the gut microbiota composition. It has been estimated that < 20% of environmental bacteria can be grown in defined growth media. This increases the risk of detection bias in older studies.[102] Sequencing-based techniques also have limitations. Studies relying on 16S rRNA analysis allow only a coarse sorting of bacteria mainly at phylae level. Deep shotgun metagenom sequencing allows for finer distinction at genus or species level, but it is of crucial importance to standardize sampling and temperature control during the pipeline up to DNA extraction in order to obtain valid results.[103] Moreover bioinformatic presentations are often challenging to understand and interpret.

We also acknowledge that our definition of broad-spectrum and narrow-spectrum antibiotics is somewhat arbitrary as most of the narrow-spectrum regimens covered both

Gram-negative and Gram-positive bacteria. However, our study confirms previous findings clearly suggesting that antibiotic regimens containing third-generation cephalosporins or carbapenems are more frequently associated with antibiotic resistance development than regimens with aminoglycosides for Gram-negative coverage.[15, 24, 26, 28, 50, 55, 64, 65, 67, 71] Finally, we decided to exclude studies that only examined antenatal antibiotic treatment, despite the frequent use of intrapartum antibiotic prophylaxis for prevention of neonatal infections and its reported effects on the infant gut microbiota and carriage of antibiotic resistance genes.[104] The focus of this review was on neonatal antibiotic treatment given for suspected neonatal infection, and the isolated effects of antenatal antibiotics, given to infants that did not receive antibiotics after birth, were beyond the scope of this study.

Implications and Conclusion

This systematic review highlights the profound impact on the gut microbiota and antibiotic resistance development exerted by antibiotic treatment in neonates. Antibiotic exposure in the neonatal period appears to induce varying potentially disease-promoting alterations in the gut microbiota, but quality of evidence was very low for outcomes investigated in this review. However, we are moderately confident, based on data from this review, that antibiotic treatment leads to antibiotic resistance development, in particular in Gram-negative bacteria. This clearly threatens current empiric antibiotic regimens and is a finding of great concern.

In conclusion, the findings from this systematic review, along with the findings from our recent systematic review on early adverse outcome of neonatal antibiotic therapy[9], strongly emphasize the need to reduce unnecessary antibiotic treatment in neonates. Important steps to reduce the burden of neonatal antibiotic therapy include improving preventive measures such as hand hygiene, stopping antibiotic therapy after 36-48 hours if only vaguely suspected infection and no growth in the blood culture and restricting the empiric use of broad-spectrum antibiotic treatment.[105, 106]

Funding

Drs Fjalstad and Esaiassen are recipients of funding for their PhD studies from the Regional Health Authorities in North Norway. This study was supported by internal funding. The funding source had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

Transparency declarations

None to declare.

Author contributions

JWF reviewed all relevant titles, abstracts and full-text articles, assessed quality, extracted data, and drafted the initial manuscript. EE reviewed all relevant titles, abstracts and full-text articles, extracted data, assessed quality, and revised the manuscript. LKJ contributed to study design, methodological assessment and revised the manuscript. JvdA contributed to study design and revised the manuscript. CK conceptualized and designed the study, reviewed relevant abstracts and articles, assessed quality, and revised the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work. JWF, EE and CK have full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure 1. Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) Flow diagram

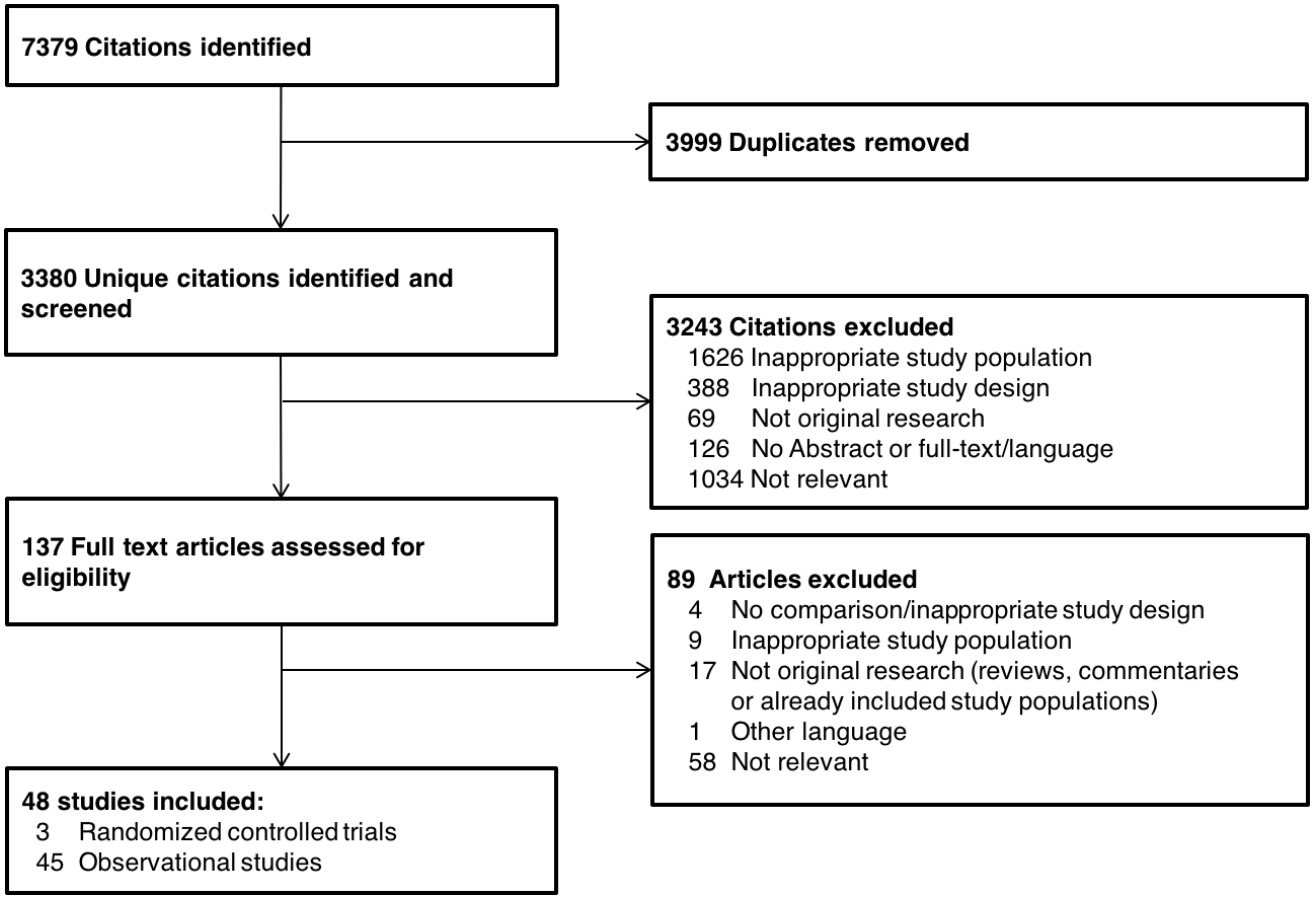


Figure 2. Vote count on gut microbial composition after antibiotic exposure – compared to no antibiotic exposure.
The sizes of squares are proportional to study populations. * symbolizes a lack of testing for statistical significance.

a) Enterobacteriaceae (9 studies; 1407 neonates)

Study	Abundance and/or colonization rates Lower	Unchanged	Higher	Specific outcome	Abundance or colonization rates
Arboleya, 2015		■		Enterobacteriaceae	Abundance
Bennet, 1986 & 1987			■	*Klebsiella*/*Enterobacter* spp.	Colonization rates
Blakey, 1982*		■		Enterobacteriaceae	Colonization rates
Bonnemaison, 2003*		■		Enterobacteriaceae	Colonization rates
Fouhy, 2012			■	Enterobacteriaceae	Colonization rates
Greenwood, 2014			■	*Enterobacter* spp.	Colonization rates
Hall, 1990		■		Coliforms	Colonization rates
La Rosa, 2014			■	Gammaproteobacteria	Abundance
Tullus, 1988		■		Enterobacteriaceae	Colonization rates

We graded quality of evidence as very low due to inclusion of observational studies with very serious risks of bias.

b) Commensal obligate anaerobes (5 studies; 304 neonates)

Study	Abundance and/or colonization rates Lower	Unchanged	Higher	Specific outcome	Abundance or colonization rates
Bennet, 1986 &87				*Bifidobacterium* spp.	Colonization rates
				*Lactobacillus* spp.	
				*Bacteriodes* spp.	
Blakey, 1982 *				*Lactobacillus* spp.	Colonization rates
				*Bacteriodes* spp.	
Butel, 2007				*Bifidobacterium* spp.	Colonization rates
Fouhy, 2012				*Bifidobacterium* spp.	Colonization rates
				*Lactobacillus* spp.	
Hall, 1990				*Bifidobacterium* spp	Colonization rates
				*Lactobacillus* spp.	

We graded quality of evidence as very low due to inclusion of observational studies with very serious risks of bias.

c) *Clostridium* species (5 studies; 248 neonates)

Study	Abundance and/or colonization rates			Specific outcome	Abundance or colonization rates
	Lower	Unchanged	Higher		
Blakey, 1982*	■			*Clostridium* spp.	Colonization rates
Ferraris, 2012		■		*Clostridium* spp.	Colonization rates
Fouhy, 2012			■	*Clostridium* spp.	Abundance
Jenke, 2013		■		*C. difficile*	Colonization rates
La Rosa, 2014	■			*Clostridium* spp.	Colonization rates

We graded quality of evidence as very low due to inclusion of observational studies with very serious risks of bias and inconsistent results.

d) Gram-positive cocci (4 studies; 116 neonates)

Study	Abundance and/or colonization rates			Specific outcome	Abundance or colonization rates
	Lower	Unchanged	Higher		
Arboleya, 2015			■	*Staphylococcus* spp.	Abundance
Blakey, 1982*		■		*S. aureus*	Colonization rates
Bonnemaison, 2003*		■		*Staphylococcus* spp.	Colonization rates
		■		*Enterococcus* spp.	
Fouhy, 2012			■	*Enterococcus* spp.	Abundance

We graded quality of evidence as very low due to inclusion of observational studies with very serious risks of bias.

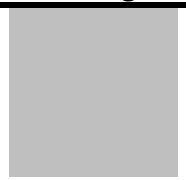
Figure 3. Vote count on infection and/or colonization with MDR Gram-negative bacteria following antibiotic exposure. The sizes of squares are proportional to study populations. † symbolizes multivariate regression analysis. NDA; no data available.

a) Antibiotic exposure - compared to no antibiotic exposure (9 studies; 2509 neonates)

Study	Infection and/or colonization rates Lower Unchanged Higher	Risk estimates	Specific outcomes	Colonization or infection
Calil, 2001		OR 2.5, 95% CI 1.08-5.77†	MDR *E. cloacae*	Colonization
Crivaro, 2007		Not available	ESBL producing *S. marcescens* & *K. pneumoniae*	Colonization
Duman, 2005		RR 14.05; 95% CI 1.19-164.62	ESBL producing *Enterobacteriaceae*	Colonization
Giuffre, 2016		Not available	MDR Gram-negative bacteria	Colonization
			ESBL producing Gram-negative bacteria	Colonization
Kumar, 2014		OR 26.04, 95% CI 3.51-35.45†	Carbapenem-resistant *A. baumannii*	Infection
Millar, 2008		Not available	MDR *Enterobacteriaceae*	Colonization
Pessoa-Silva, 2003		OR 3.23, 95% CI 0.99-10.49	ESBL-producing *K. pneumoniae*	Infection
Rettedal, 2013		OR 5.5; 95% CI 5.6-15.3†	ESBL-producing *K. pneumoniae*	Colonization
Sehgal, 2007		OR 17.80, 95% CI 1.91-165.54†	ESBL-producing Gram-negative bacteria	Infection

We graded quality of evidence as moderate due to inclusion of observational studies with large effect estimates.

b) Antibiotic exposure - long duration compared to shorter duration (5 studies; 4281 neonates)

Study	Infection and/or colonization rates Lower Unchanged Higher	Risk estimates	Specific outcomes	Colonization or infection
Cantey, 2016		Not available	MDR Gram-negative bacteria	Colonization
Crivaro, 2007		OR 1.32, 95% CI 1.02-1.70†	ESBL producing *S. marcescens* & *K. pneumoniae*	Colonization
Giuffre, 2016		Not available	MDR Gram-negative bacteria	Colonization
Le, 2008		OR 1.04, 95% CI 1.01-1.07†	ESBL-producing Gram-negative bacteria	Colonization
		OR 3.09, 95% CI 1.28-7.49†	ESBL-producing *Enterobacteriaceae*	Infection
Mammina, 2007		Not available	MDR Gram-negative bacteria	Colonization

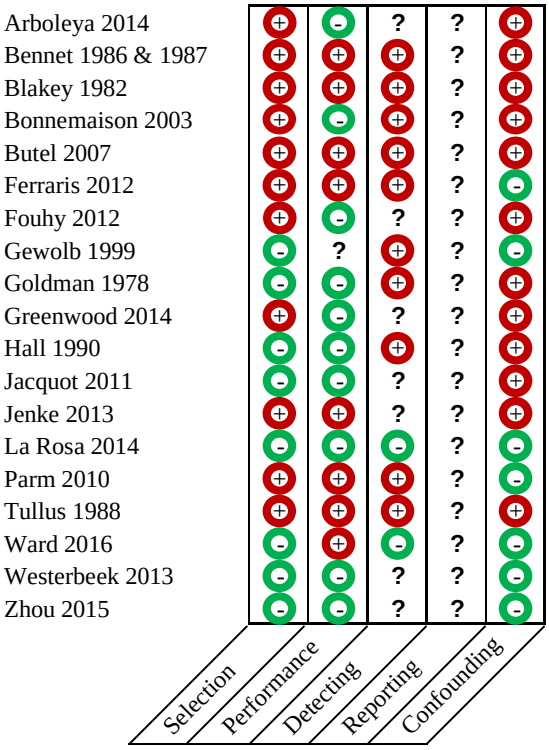
We graded quality of evidence as moderate due to inclusion of observational studies that demonstrated a dose-response effect.

c) Antibiotic exposure - Broad spectrum– compared to narrow spectrum (13 studies; 4016 neonates)

Study	Infection and/or colonization rates Lower Unchanged Higher	Risk estimates	Specific outcomes	Colonization or infection
Abdel-Hady, 2008		OR 4.9, 95% CI 1.1-21.5†	ESBL-producing *K. pneumoniae*	Infection
Acolet, 1994		Not available	Cefotaxime-resistant *E. cloacae*	Colonization
Calil, 2001		Not available	MDR *E. cloacae*	Colonization
De Araujo, 2007		Not available	MDR Gram-negatives	Colonization
De Champs, 1994		Not available	MDR *E. cloacae*	Colonization
De Man, 2000		RR 3.14, 95% CI 1.76-5.56	Cefotaxime-resistant Gram-negatives	Colonization
Le, 2008		OR 33.73, 95% CI 1.02-1136.20†	ESBL producing *Enterobacteriaceae*	Infection
Linkin, 2004		Not available	ESBL producing *Enterobacteriaceae*	Infection
Mammina, 2007		Not available	MDR Gram-negatives	Colonization
Millar, 2008		Not available	MDR *Enterobacteriaceae*	Colonization
Pessoa-Silva, 2003		OR 4.60, 95% 1.48-14.31	ESBL-producing *K. pneumoniae*	Colonization
Thatrimontrichai, 2013		Not available	Carbapenem-resistant *A. baumannii*	Infection
Thatrimontrichai, 2016		OR 4.4; 95% CI 1.2-15.6†	Carbapenem-resistant *A. baumannii*	Infection

We graded quality of evidence as moderate due to inclusion of observational studies with large effect estimates.

Figure S1. Risk of bias graph: review of authors' judgements about each risk of bias item for each included study and the two outcomes. (a) studies reporting on changes in gut microbiota (n=20). (b) studies reporting on changes in antibacterial resistance development (n=31).



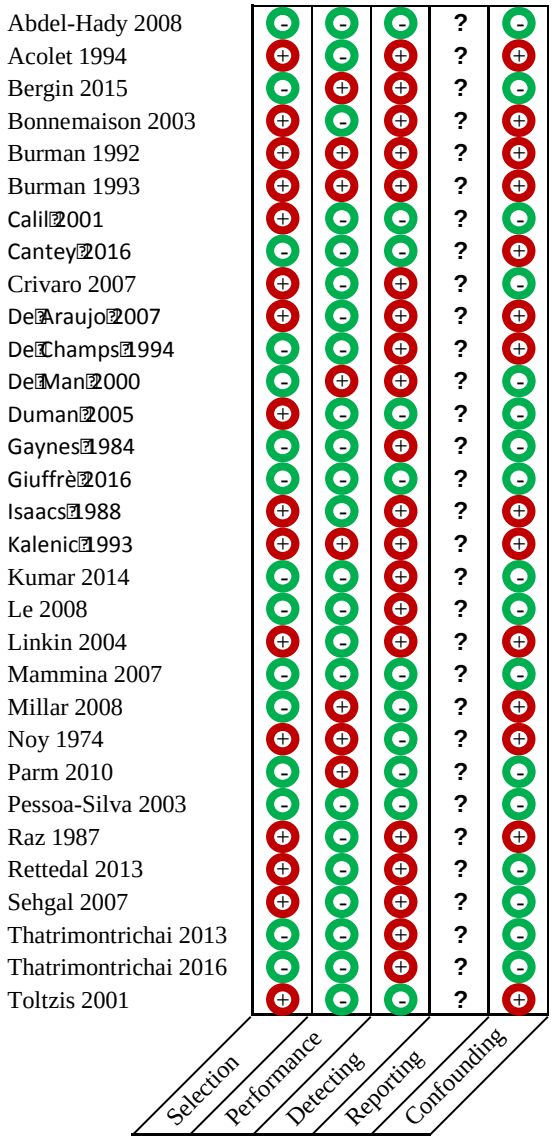


Table S1. Studies reporting previous antibiotic exposures and the effect on gut microbiota: Summary of main characteristics and results

Study	Design	N	GA and BW	Empiric regimen	Categories of antibiotic exposure and changes in gut microbiota
Arboleya et al., 2015 (Spain)	Prospective cohort	40	All GAs	EOS: AMP + GEN, LOS: VAN + AMK	**Yes vs. no:** Composition: ↓ *Staphylococcus* spp. & *Comamonadaceae*
Bennet et al., 1986 & 1987 (Sweden)	Prospective cohort	164	All GAs	NDA	**Yes vs. no:** Load: ↑; Composition: ↑ *Klebsiella*/*Enterobacter* spp., ↓ Anaerobes, ↓*Bifidobacterium* spp., ↓*Lactobacillus* spp., ↓*Bacteriodes* spp. **Broad vs. narrow:** Composition: ↑ *Enterococcus* spp., ↑ *S. faecalis*
Blakey et al., 1982 (Australia)	Prospective cohort	28	GA ≤ 36 weeks	EOS: PEN + GEN	**Yes vs. no:** Composition: No difference*
Bonnemaison et al., 2003 (France)*	Prospective cohort	30	All GAs	EOS: AMX + NET ± CTX	**Yes vs. no:** Composition: No difference **Broad vs. narrow:** Composition: No difference*
Butel et al., 2007 (France)	Prospective case-control	52	GA 30 - 35 weeks	NDA	**Yes vs. no:** Composition: No significant difference
Ferraris et al., 2012 (France)	Retrospective cohort	76	GA < 36 weeks	NDA	**Yes vs. no:** Composition: ↑ *C. butyricum* **Long vs. short:** Composition: ↓ *Clostridium* spp.
Fouhy et al., 2012 (Ireland)	Prospective cohort	18	GA ≥ 37 weeks	AMP + GEN	**Yes vs. no:** Composition: ↑ Enterobacteriaceae, ↑Gammaproteobacteriae, ↑ Peptostreptococcaceae, ↑ *Enterococcus* spp., ↑ *Clostridium* spp., ↓ *Lactobacillus* spp., ↓ *Bifidobacterium* spp., ↓ *Bacteriodetes*
Gewolb et al., 1999 (USA)	Prospective cohort	29	BW < 1000 g	EOS: AMP + GEN, LOS: VAN + CTX	**Long vs. short:** Load: ↓; Diversity: ↓
Goldmann et al., 1978 (USA)	Prospective cohort	63	All GAs	NDA	**Long vs. short:** Composition: ↑ *Klebsiella* spp., ↑ *Enterobacter* spp., and/or ↑ *Citrobacter* spp.
Greenwood et al., 2014 (USA)	Prospective cohort	74	GA ≤ 32 weeks	EOS: AMP + GEN	**Yes vs. no:** Diversity: ↓; Composition: ↑ *Enterobacter* spp. **Long vs. short:** Composition: ↑ *Enterobacter* spp., ↓ *Staphylococcus* spp.
Hall et al., 1990 (UK)	Prospective cohort	42	GA ≤ 33 weeks	NDA	**Broad vs. narrow:** Composition: ↓ *Lactobacillus* spp.
Jacquot et al., 2011 (France)	Prospective cohort	29	GA ≤ 30 weeks	EOS: AMK + (1) PEN or (2) AMP or (3) CTX, LOS: VAN + AMK	**Yes vs. no:** Diversity: No significant effect **Long vs. short:** Diversity: ↓
Jenke et al., 2013 (Germany)	Prospective cohort	68	GA < 27 weeks	NDA	**Yes vs. no:** Composition: ↑ *C. difficile*
La Rosa et al., 2014 (USA)	Prospective cohort	58	BW ≤ 1500 g	NDA	**Yes vs. no:** Composition: ↑ Gammaproteobacteria (GA ≥ 26 weeks), ↓ *Clostridium* spp. (GA ≤ 28 weeks)
Parm et al., 2010 (Estonia)	RCT	276	All GAs	EOS: (1) PEN + GEN or (2) AMP + GEN	**Broad vs. narrow:** Composition: ↑ *S. haemolyticus*, ↑ *S. hominis*, ↑ *K. pneumonia*, ↓ *Enterococcus* spp. ↑ *S. aureus*

Tullus et al., 1988 (Sweden)	Retrospective cohort	953	All GAs	AMP + GEN	**Yes vs. no:** Composition: ↓ *E. coli* **Broad vs. narrow:** Composition: No significant difference
Ward et al., 2016 (USA)	Case-control	166	All GAs	EOS: AMP + GEN	**Long vs. short:** Diversity: ↓
Westerbeek et al., 2013 (Netherlands)	RCT	113	GA < 32 weeks ± BW < 1500 g	NDA	**Yes vs. no:** Load: ↓
Zhou et al., 2015 (USA)	Case-control	38	GA < 32 weeks	NDA	**Yes vs. no:** Diversity: ↓

Outcomes: Load; the total number of bacteria in a sample, Diversity; the number of bacterial genus or species in a sample, and Composition; the taxonomical composition in a sample. Categories: **Yes vs. no** compares neonates exposed to antibiotics with non-exposed neonates, **Long vs. short** compares long and short treatment durations, **Broad vs. narrow** compares broad spectrum antibiotic treatment to narrow spectrum treatment. *; did not test for statistical significance, RCT; randomized controlled trial, GA; gestational age, PNA; post-natal age, BW; birth weight, g; gram, EOS; early onset sepsis, AMP; ampicillin, GEN; gentamicin, LOS; late onset sepsis, VAN; vancomycin, AMK; amikacin, NDA; no data available, PEN; penicillin, AMX; amoxicillin, NET; netilmicin, CTX; cefotaxime

Table S2. Studies reporting on previous antibiotic exposures and the risk of antibacterial resistance: Summary of main characteristics and results

Study	Design	N	Empiric regimen	Categories of antibiotic exposure and changes in antibacterial resistance
Abdel-Hady et al., 2008 (Egypt)	Prospective cohort	380	NDA	**Broad vs. narrow:** ↑ ESBL producing *K. pneumoniae* infection
Acolet et al., 1994 (UK)	Case-control	60	EOS: AMX + CTX, LOS: CTX	**Broad vs. narrow:** ↑ CREC colonization
Bergin et al., 2015 (USA)	Case-control	258	NDA	**Broad vs. narrow:** No significant difference
Bonnemaison et al, 2003 (France)	Prospective cohorts	30	EOS: AMX + NET ± CTX	**Yes vs. no:** Did not assess significance **Broad vs. narrow:** Did not assess significance
Burman et al., 1992 (Sweden)	Retrospective cohort	953	EOS: (1) AMP + GEN or (2) CTX	**Yes vs. no:** ↑ TEM-1 in *E. coli* **Broad vs. narrow:** No significant difference
Burman et al., 1993 (Sweden)	Retrospective cohort	46	EOS: (1) AMP + GEN or (2) CTX	**Yes vs. no:** *E. cloacae*: ↑ MIC to ampicillin, cephalotin, cephalexin
Calil et al., 2001 (Brazil)	Prospective cohort	342	EOS: AMX + (1) GEN or (2) CRO, LOS: OXA + (1) GEN or (2) CRO	**Yes vs. no:** ↑ MDR *E. cloacae* colonization **Broad vs. narrow:** ↑ MDR *E. cloacae* colonization
Cantey et al., 2016 (USA)	Before-after study	2502	EOS: AMX + GEN, LOS: OXA + GEN	**Long vs. short:** No significant difference
Crivaro et al., 2007 (Italy)	Case-control	167	AMP + GEN	**Yes vs. no:** ↑ ESBL-producing *S. marcescens and K. pneumoniae* **Long vs. short:** ↑ ESBL-producing *S. marcescens and K. pneumoniae*
De Araujo et al., 2007 (Brazil)	Before-after study	995	PEN & GEN	**Broad vs. narrow:** ↑ MDR GNB
De Champs et al., 1994 (France)	Before-after study	636	(1) AMP + GEN or (2) AMP + AMK	**Broad vs. narrow:** ↑ Gentamicin-resistant, cephalosporin-resistant, and MDR *E. cloacae*, ↑ Amikacin-resistant *P. aerunginosa*; ↓ Gentamicin & amikacin-resistant GNB, MRSE
De Man et al., 2000 (Netherlands)	RCT	436	EOS: (1) PEN + TOB or (2) AMX + CTX, LOS: FLU + (1) TOB or (2) CTX	**Broad vs. narrow:** ↑ Colonization with cefotaxime-resistant *Enterobacter* spp. & GNB
Duman et al., 2005 (Turkey)	Prospective cohort	118	NDA	**Yes vs. no:** ↑ ESBL-producing Enterobacteriaceae colonization
Gaynes et al., 1984 (USA)	Case-control	32	(1) PEN or (2) AMP + (1) GEN or (2) KAN	**Yes vs. no:** ↑ Aminoglycoside-resistant *E. coli*
Giuffrè et al., 2016 (Italy)	Prospective cohort	1152	SAM + GEN	**Yes vs. no:** ↑ MDR GNB colonization **Long vs. short:** ↑ MDR & ESBL-producing GNB colonization

Isaacs et al., 1988 (UK)	Before-after study	NDA	EOS: PEN + (1) NET or (2) GEN, LOS: FLU + (1) NET or (2) GEN	**Long vs. short:** No significant difference
Kalenic et al., 1993 (Croatia)	Before-after study	440	(1) AMP + GEN or (2) CXM + GEN	**Broad vs. narrow:** ↓ Ampicillin-resistant GNB, cefuroxime-resistant GNB & cefuroxime-resistant *K. pneumoniae*
Kumar et al., 2014 (India)	Case-control	65	NDA	**Yes vs. no:** ↑ CRAB blood stream infections
Le et al., 2008 (USA)	Before-after study	250	EOS: AMP + GEN, LOS: VAN + (1) CTX or (2) TOB	**Long vs. short:** ↑ ESBL-producing Enterobacteriaceae infection **Broad vs. narrow:** ↑ ESBL-producing Enterobacteriaceae infection
Linkin et al., 2004 (USA)	Case-control	10	NDA	**Yes vs. no:** ↑ ESBL-producing Enterobacteriaceae
Mammina et al., 2007 (Italy)	Prospective cohort	210	EOS: SAM + GEN	**Long vs. short:** ↑ MDR GNB colonization **Broad vs. narrow:** ↑ MDR GNB colonization
Millar et al., 2008 (UK)	Prospective cohort	124	EOS: PEN + GEN, LOS: (1) TZP + VAN or (2) FLU + GEN	**Yes vs. no:** No significant difference **Broad vs. narrow:** ↑ MDR Enterobacteriaceae colonization
Noy et al., 1974 (UK)	Prospective cohort	584	NDA	**Yes vs. no:** ↑ Antibiotic-resistant *E. coli* & *Klebsiella* spp. colonization
Parm et al., 2010 (Estonia)	RCT	276	EOS: (1) PEN + GEN or (2) AMP + GEN	**Broad vs. narrow:** ↓ Ampicillin-resistant *Acinetobacter* spp. colonization
Pessoa-Silva et al., 2003 (Brazil)	Prospective cohort	379	EOS: AMP + GEN, LOS: Varying antibiotics	**Yes vs. no:** ↑ ESBL-producing *K. pneumoniae* colonization
Raz et al., 1987 (Israel)	Before-after study	118	(1) AMP + GEN or (2) AMP + AMK	**Broad vs. narrow:** ↑ Gentamicin-resistant GNB and *E. cloacae*
Rettedal et al., 2013 (Norway)	Case-control	99	NDA	**Yes vs. no:** ↑ ESBL-producing *K. pneumoniae* colonization
Sehgal et al., 2007 (India)	Case-control	63	EOS: AMP + GEN, LOS: 3rd gen. cephalosporin + AMK	**Yes vs. no:** ↑ ESBL-producing GNB blood stream infection
Thatrimontrichai et al., 2013 (Thailand)	Case-control	96	EOS: AMP + GEN, LOS: 3rd gen. cephalosporin + AMK	**Broad vs. narrow:** ↑ CRAB blood stream infection
Thatrimontrichai et al., 2016 (Thailand)	Case-control	101	EOS: AMP + GEN, LOS: varying antibiotics	**Broad vs. narrow:** ↑ odds of CRAB ventilator associated pneumonia
Toltzis et al., 2001 (USA)	Prospective cohort	1180	NDA	**Long vs. short:** ↑ antibiotic resistant GNB colonization

Categories: **Yes vs. no;** compares neonates exposed to antibiotics with non-exposed neonates, **Long vs. short;** compares long and short treatment durations, and **Broad vs. narrow;** compares broad spectrum antibiotic treatment to narrow spectrum treatment. RCT; randomized controlled trial, NDA; no data available, EOS; early onset sepsis, AMX; amoxicillin, CTX; cefotaxime, LOS; late onset sepsis, NET; netilmicin, AMP; ampicillin, GEN; gentamicin, CRO; ceftriaxone, OXA; oxacillin, TOB; tobramycin, FLU; flucloxacillin, KAN; kanamycin, SAM; ampicillin/sulbactam, CXM; cefuroxime, TZP; piperacillin/tazobactam, CREC; cephalosporin-resistant *Enterobacter cloacae*, GNB; Gram-negative bacteria, CRAB; carbapenem-resistant *Acinetobacter baumannii*