The impact of gut microbiome modulating interventions on fecal metabolome of infants: A systematic review and quality assessment

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Abstract

The development of the gut microbiome in infancy is a vulnerable process that may be perturbed by antibiotics or supported by probiotics. While effects of these “biotics” have been well-studied through DNA sequencing, it remains unclear how the resulting compositional changes affect the microbiome metabolic functions. Additionally, limits in method standardization require careful quality assessment of studies reporting fecal metabolome.

We conducted a systematic search in Embase and MEDLINE for studies describing fecal metabolites from term and near-term infants, together with anti-, pre-, or probiotic intervention. The search identified 680 articles, of which 60 were assessed for eligibility and 21 included. We first developed operational checklists for transparent and reproducible reporting and evaluated the quality of metabolomic methodologies. This analysis supported our aim to summarise changes in the fecal metabolome induced by biotic interventions.

Despite a varying quality of metabolomic methodology, we identified similarities in the fecal metabolome profiles in response to specific biotic interventions. Among the most frequently observed metabolites, which were consistently reported to be altered after biotic interventions, were bile acids, aromatic amino acids, and short-chain fatty acids. We conclude with a discussion on appropriate experimental design, controls, and metabolomics reporting to guide future research permitting meta-analyses.

Introduction

Starting at birth, infants are gradually colonized by microbes in a process that significantly modulates their physiological development (Cryan et al., 2020; Dominguez-Bello et al., 2019; Gensollen et al., 2016). Currently, there is a good understanding of how different perinatal factors affect gut colonization by bacterial taxa, including the effects of the birth mode, gestational age, probiotic and antibiotic use, and feeding type (Esaiassen et al., 2017; Fjalstad et al., 2018; Grech et al., 2021; He et al., 2024; Princisval et al., 2021; Rutayisire et al., 2016). In particular, antibiotic treatment has been associated with reduced gut bacterial diversity, a lower abundance of protective commensal anaerobic bacteria, and an increased susceptibility to colonization by antibiotic-resistant opportunistic pathogens (Dierikx et al., 2020). In contrast, the administration of pre- and probiotics may increase the population of beneficial bacteria, support colonization resistance against taxa with pathogenic potential, enhance immune responses, and strengthen the epithelial cell barrier (Naspolini et al., 2024; Wang et al., 2021; Zimmermann et al., 2019).

Any "biotic" administration leading to an altered gut microbiome will subsequently affect multiple interconnected physiological systems. This effect is facilitated by microbial metabolites that can enter the blood circulation and, together with other small molecules, act as signals to the host physiology (Wikoff et al., 2009). Such systemic effects of an altered gut microbiome are documented in animal models (Antunes et al., 2011; Brown et al., 2023), and their consequences indicated in epidemiological studies (Aversa et al., 2020; Hoskinson et al., 2023). Still, the evidence on the microbiome functional output, *i.e.*, the production of metabolites with the potential to modulate human physiology, is scattered and has not been systematically explored in depth.

Numerous studies have linked the metabolic activity of the gut microbiome to immune system maturation (Donald & Finlay, 2023; Henrick et al., 2021; Hoskinson et al., 2023; Smith et al., 2013), neurodevelopment (Ahrens et al., 2024), epithelial cell homeostasis (Alam & Neish, 2018), and resistance against pathogens (Caballero et al., 2017; Fukuda et al., 2011). The most studied class of microbial metabolites involved in these processes are short-chain fatty acids (SCFA), with a recent systematic review suggesting that early-life SCFA have a protective effect against allergic diseases in childhood (Sasaki et al., 2024). Further, microbially produced aromatic lactic acids have been proposed to impact the immune function in early life (Laursen et al., 2021), and deconjugated bile acids implicated in type 1

diabetes development (Lamichhane et al., 2022). Other microbial metabolites, such as amines, pyruvate, amino acids, fatty acids, and intermediates of the citric acid cycle, also have the potential to modulate host physiology during early life (Roager et al., 2023). However, the roles of these small microbial molecules are difficult to characterize because human cells produce the same or similar metabolites.

Given the essential role of the gut microbiome in human physiology development, understanding its metabolic traits is key for strategies targeting its biological functions. However, the vast number, dynamic range, and chemical diversity make metabolites within a biological sample, such as stool, challenging to analyze and annotate. Consequently, one analytical method alone cannot give a comprehensive picture of the metabolome. Multiple analytical techniques are commonly employed, each with its own advantages and limitations (Danzi et al., 2023; Joshi et al., 2023; Lu et al., 2017). There are two main approaches in metabolome research: targeted- and untargeted metabolomics. In targeted metabolomics, a selection of known metabolites is analyzed using highly pure authentic standards with known concentrations and analogous isotopically labeled internal standards. This approach enables excellent accuracy, precision, and sensitivity, offering absolute quantification of metabolites (Patti et al., 2012). In contrast, untargeted methods do not depend on analytical standards. but employ advanced computational tools and databases. Due to the limited use of standards in untargeted metabolomics, the implementation of robust quality control (QC) and quality assurance (QA) measures is essential to ensure reliable metabolite identification. These measures include, for example, the use of pooled samples, monitoring of the instrument performance, and other QA to ensure data quality and reproducibility (Alseekh et al., 2021).

Following the PRISMA guidelines (Page et al., 2021), we conducted a systematic review of studies that investigated fecal metabolome of term- and near-term infants and reported anti-, pre-, or probiotic intervention. We first assessed the reporting of metabolomic methodology with an emphasis on QA and QC by generating a checklist of reporting requirements. This quality check supported our objective to summarise reported changes in the fecal metabolome induced by different biotic interventions. Lastly, we aimed to determine to what degree fecal metabolite profiles can provide insights into the gut microbiome functions.

Materials and methods

Search strategy and criteria for study selection

Studies were selected through a systematic search conducted in two comprehensive medical research databases, MEDLINE and Embase, via the Ovid Medical Research Platform up until June 28, 2024. Our search strategy considered that MEDLINE and Embase are the two largest biomedical research databases in the world that complement each other (Bramer et al., 2017). The search terms used as index terms or free-text words are shown in **Figure S1**. References from included studies matching the inclusion criteria but not found with the search strategy were also included. Studies were considered eligible for inclusion if they met the following criteria: randomized or observational trials of infants born after 35 weeks gestation and reporting interventions affecting the gut microbiome (*e.g.*, anti-, pre-, or probiotic administration) during the first year of life. Additionally, only studies reporting measurements of fecal metabolites, without discrimination of the metabolomics analysis platform, were included. We excluded studies of infants born before 35 weeks of gestation, written in languages other than English, studies that investigated only the effects of prenatal factors, case reports, case series, and animal and *in vitro* studies. Google Scholar was used for forward citation searching to check for relevant papers that cited the included studies.

Screening, data extraction, and management

The software Covidence (Veritas Health Innovation, Melbourne, Australia, www.covidence.org) was used in the screening and sorting process. Search results were independently screened by two reviewers, each of whom assessed eligible full-text papers. In case of disagreement, a third researcher decided whether an article should be included or not. The following data was extracted: year of study, country, study design, characteristics of the study population, number of participants, delivery mode, feeding strategies, timing and type of anti-, pre-, or probiotic administration, metabolomics approach, metabolomic quality controls, which metabolites were detected, DNA sequencing approach, and which bacterial species were detected. The systematic review has been registered in the International Prospective Register of Systematic Reviews, PROSPERO, with the ID CRD42023459042.

Assessment of metabolomics methodology reporting

The lack of transparent reporting limits the interpretation of results derived from metabolomics experiments. We, therefore, also evaluated metabolomics methodology reporting. As a basis, we took previously developed guidelines and recommendations (Alseekh et al., 2021; Kirwan et al., 2022) and transformed these into a checklist structure inspired by the Joanna Briggs Institute critical appraisal tools (Munn et al., 2020). We created five checklists covering minimum reporting practices in metabolomics for QA and QC, with an emphasis on transparency and reproducibility. We assessed the following: General reporting common for all metabolomics platforms (**Table S1**), reporting of targeted metabolomic analysis (**Table S2**), reporting of QCs used in untargeted metabolomics (**Table S3**), reporting of bioinformatics tools used in untargeted metabolomic data analyses (**Table S4**), and reporting of nuclear magnetic resonance (NMR) analysis (**Table S5**). Two authors independently assessed the studies and filled in the checklists. Any discrepancies in their assessments were resolved through discussion. How well a study scored on the different aspects of the reporting was based on the alignment with reporting requirements (Alseekh et al., 2021; Kirwan et al., 2022), using the percentages of positive answers in our checklists. A score above 80% was defined as excellent reporting, between 50-70% as good reporting, and below 50% as poor reporting (**Table S6**).

Results

The literature search resulted in 680 articles after the removal of duplicates (Embase 551, MEDLINE 129). Sixty studies remained after title and abstract screening. After checking the full text of these studies for eligibility, we included 21 publications that met the inclusion criteria (**Figure 1**). Fourteen of the publications were published after 2020 (**Figure S2**), and only one was published before 2015. The total number of infants included across all studies was 3025, varying from 12 to 575 participants. However, for several studies, only a subset of all collected stool samples was used for metabolomic analyses. Fifteen studies were conducted in Europe, two in North America, three in Asia, and one in Oceania.

The quality of metabolomics methodology reporting varies considerably among studies

Before investigating fecal metabolome changes in response to microbiome-modifying interventions, we wanted to ascertain the credibility of reported metabolomics experiments. To that end, we generated checklists that covered shared and specific requirements for different metabolomics methods (**Figure 2**). First, we evaluated the general reporting of metabolomics experiments, including descriptions of sample pre-processing steps, chromatographic conditions, instrument platforms, biomass normalization, and the accessibility of metabolomics raw data (**Figure 3A**). Out of 21 studies, only one study successfully reported all details necessary for a reproducible metabolomic experiment and also had the raw metabolomic data files accessible, thus fully complying with the minimal reporting requirements we have set. Still, the reporting of seven studies was at an excellent level, only missing the availability of raw metabolomics files or not reporting on biomass normalization (**Table S1**).

Next, we focused on the reporting of targeted metabolomics experiments, including descriptions of method validation for feces according to the guidelines for bioanalytical method validation (ICH M10), the use of internal standards, and the use of authentic internal standards for all targeted compounds (**Figure 3B**). Twelve studies employed targeted metabolomics analysis, but only one paper reported that the targeted analysis was based on a validated method. Three studies did not report using internal standards (*i.e.*, spiked-in standards of known concentrations, most commonly either ^{13}C or D-labeled), and none of the studies described authentic internal standards for all targeted metabolites (**Table S2**).

In reporting of untargeted analysis, we focused on four types of QCs that are commonly employed for monitoring instrumentation performance and other issues that can arise during or between runs (**Figure 3C**): system suitability samples or instrument calibration mixtures, blanks, internal standards (typically authentic isotopically labeled analogous of a subset of, or all metabolites of interest), and pooled aliquots of the targeted samples hereafter referred to as pooled QCs. Pooled QCs can have a wide array of applications but are most commonly used as sample matrix-matched reference material for assessing intra- and inter-batch variation. Additionally, we included chromatographic system conditioning, as this is important for obtaining stable retention times and ionization (Zhou & Yin, 2016). Overall, we graded the reporting of QCs in untargeted metabolomics as excellent in one study, good in four, and poor in four studies (**Table S3**). Further, from the nine studies that employed untargeted metabolic profiling, three studies fully met all the listed reporting requirements for reporting untargeted metabolomic data analyses (**Figure 3D)**. At the same time, six had good reporting, and one study failed to describe the methodology details (**Table S4**).

Finally, for studies that analyzed metabolites using NMR, we required the descriptions of suitability tests, quality controls, blanks, the chemical shift reference compound, and the peak processing (**Figure 3E**). None of the four studies reported system suitability checks, and three studies partly described the use of quality controls or blanks. On the other hand, all the studies clearly described the sample solvent and post-acquisition processing steps (**Table S5)**. Taken together, among the 21 evaluated studies, only one had an overall score of more than 80% for complying with the reporting requirements, and we therefore graded its reporting as excellent (**Figure 3F)**. Eleven studies were graded as good, and nine studies failed to report their metabolomics methodology sufficiently (**Table S6)**. Keeping this evaluation in mind, we moved on to summarise the findings on the infant fecal metabolome.

Studies describing the effects of antibiotic use on the infant fecal metabolome

In our search, we identified seven studies that presented their findings on fecal metabolome in relation to antibiotic use and did not have an intervention with other biotics (**Table S7)**. We noted that three studies reported the exact dosing and type of antibiotics (Frayman et al., 2024; Li et al., 2022; Strasser et al., 2020), and all studies used DNA sequencing for microbiota profiling except for one (Łoniewska et al., 2023).

Two studies had antibiotic prophylaxis as the primary intervention. Li et al. (2022) used untargeted metabolomics (LC-MS) to compare the fecal metabolome in the first week of life

of antibiotic-treated infants from parturient women with and without intrauterine infection and antibiotic-untreated neonates born to healthy parturients. N-formyl-L-methionine was the most discriminant metabolite between the intrauterine infection group and the control group. Further, metabolites connected to primary and secondary bile acid biosynthesis, bile secretion, arginine biosynthesis, and cholesterol metabolism pathways were altered in the intrauterine infection group as compared to the control group. On the other hand, Strasser et al. (2020) used targeted gas chromatography (GC)-MS-based metabolomics to study how low-dose antibiotic prophylaxis affected levels of SCFA in infants with urogenital tract malformations. The authors did not detect any differences in SCFA profiles during the first 70 days after starting the antibiotic treatment between infants treated with a second-generation cephalosporin long-term prophylaxis (n=7) and those not receiving the prophylaxis (n=5). However, one limitation of the study was the low number of patients. In addition, although the infant population was homogenous (vaginally and term-born, breastfed, no previous antibiotic exposure, and with urogenital malformation), the ages of the participants upon enrolment varied between 21 and 289 days of life, further complicating the interpretation of the results.

Five studies emphasized that antibiotic use was essential to consider when addressing their research objectives. However, in two studies investigating the fecal metabolome in infants with cystic fibrosis (Eng et al., 2021; Frayman et al., 2024), the study designs did not allow to examine the relationship between fecal metabolites and antibiotic use, which was reported for 77% of the infants with CF by (Frayman et al., 2024). Three other studies focused on different features of gut microbiome development during early life. Lowienska et al. (2023), Wu et al. (2023), and Martin et al. (2016) used targeted metabolomics for analyses of SCFA or fecal organic acids by GC-flame ionization detector (FID) (Łoniewska et al., 2023), GC-MS (Wu et al., 2023), or high-performance liquid chromatography (HPLC) (Martin et al., 2016). Both Martin *et al*. (2016) Wu *et al*. (2023) aimed to determine how delivery mode and feeding pattern influence the variation of fecal microbial metabolites. Although antibiotic use was reported for a portion of infants in both studies, the authors did not make any direct conclusion on whether antibiotics had any effect on fecal metabolites. Focusing on antibiotics, Loniewska *et al*. (2023) showed that the use of antibiotics in children had no impact on how SCFA concentrations changed over time. However, this association was derived statistically by using information on antibiotic use obtained from interviews. In this way, the authors could investigate the long-term effect of antibiotic use but did not cover its potential short-

term effects. On the other hand, they reported higher concentrations of acetate, propionate, and total SCFAs in the meconium of infants born to mothers who had taken antibiotics during pregnancy.

Studies describing the effect of prebiotic, postbiotics, or food supplements on the infant fecal metabolome

Five studies described interventions with prebiotics (**Table S8**), either alone or in combination with postbiotics, the latter defined as bioactive compounds produced by food-grade microorganisms. The prebiotic was a mixture of short-chain galactooligosaccharides (scGOS) and long-chain fructooligosaccharides (lcFOS), administered with whey- or cow's milk-based formula. Two studies (Béghin et al., 2021; Rodriguez-Herrera et al., 2022) used a postbiotic mixture (FERM) derived from the fermentation process of *Bifidobacterium breve* strain C50 and *Streptococcus thermophilus* strain O65, which, among others, results in the production of 3'-galactosyllactose, an oligosaccharide found in human milk. Beghin *et al*. (2021) used targeted metabolomics (GC-FID) to determine SCFA levels in stool samples of term infants who were fed four types of infant formulas. However, due to the limited sensitivity of the method used, the authors could not compare the actual SCFA levels and instead performed presence-absence analysis of a small number of samples, preventing any statistical analysis.

Rodriguez-Herrera et al. (2022) also compare the fecal metabolome of infants receiving cow's milk-based formula with the prebiotic/postbiotic mix (FERM/scGOS/lcFOS) and a control infant formula by using a combination of targeted (GC-MS) and untargeted (LC-MS) metabolomics. The results from LC-MS indicated significant differences between metabolite abundances of breastfed and formula-fed infants at baseline, which disappeared at 17 weeks of life. The targeted GC-MS analysis suggested higher levels of acetate and L-lactate and lower levels of propionate, D-lactate, butyrate, and valerate in the intervention group compared with the control formula group at week 17. Similarly, Wopereis et al. (2017) applied targeted metabolomics (GC-FID) on stool samples of infants receiving whey-based formula with prebiotics (scGOS, lcFOS, and pectin-derived acidic oligosaccharides), standard cow's milk formula, or breastmilk. The infants fed a formula containing prebiotics appeared to have higher levels of lactate and lower levels of propionate, butyrate, and branched-chain fatty acids at 26 weeks of life.

Two studies investigated fecal metabolome in infants receiving food supplements (**Table S8**) (Francavilla et al., 2012; Zhao et al., 2023). Zhao *et al*. (2023) used targeted NMR analysis to

evaluate stool samples from 3-month-old infants and reported positive associations between vitamin D supplementation and increased levels of acetate and 1,2-propanediol. Another study by Francavilla *et al*. (2012) used targeted (GC-MS) and untargeted (NMR) metabolomics to determine the fecal metabolome of infants with cow's milk allergy receiving an extensively hydrolyzed whey-based formula with no lactose for two months, followed by an identical lactose-containing formula for an additional two months. The lactose supplementation appeared to lead to higher levels of SCFA (particularly acetate and butyrate), lactate, and certain amino acids after the intervention period.

Studies describing the effect of probiotics or synbiotics on the infant fecal metabolome

In the last result chapter, we focus on nine studies that used probiotics as the main intervention (**Table S9**). Four studies used a single strain as the probiotic, with two having only the probiotic strain (Henrick et al., 2021; Li et al., 2023), while another two used a synbiotic combination of a probiotic strain and prebiotic oligosaccharides (Lagkouvardos et al., 2023; Sjödin et al., 2023). Three studies included various species of *Bifidobacterium* (Bazanella et al., 2017; Heppner et al., 2024; Sillner et al., 2021), one combined two *Bifidobacterium* species with *Lactococcus lactis* (Kim et al., 2015), and one used three species of *Bifidobacterium* together with four species of *Lactobacillus* and *Streptococcus thermophilus* (Baldassarre et al., 2018).

Henrick et al. (2021) investigated the fecal metabolome of exclusively breastfed term infants supplemented, or not, with *Bifidobacteium infantis* strain EVC001 optimized for human milk oligosaccharide utilization. Samples from the control (n=20) and probiotic (n=20) groups sampled at 21 days postnatal were analysed using a combination of GC- and LC-MS untargeted metabolomics. The authors identified 564 biochemical features that were significantly different between the intervention and control groups. Specifically, tryptophan metabolism was enriched, with indole-3-lactic acid as one of the metabolites altered in the EVC001-supplemented group.

Sjodin et al. (2023) used a combination of targeted (GC-MS) and untargeted (LC-MS) metabolomics to investigate fecal metabolome of term infants who were weaned from breast milk and randomized to receive a prebiotic formula (FOS/GOS) or the same prebiotic formula with *Lactobacillus paracasei* ssp. *paracasei* strain F19 (synbiotic) from 1 to 6 months of age. Similar to Henrick *et al*. (2021), this study reported higher production of aromatic amino acid metabolites in the synbiotic group, particularly the antimicrobial metabolite 3-phenyl lactic

acid, although samples were analyzed at a later time points than in the study by Henrick (2021). They also reported alterations in pectin metabolism where galacturonic acid levels were increased in the synbiotic group. Metabolite profiles at 12 months showed age-related differences but no significant impact of the interventions.

A study by Li et al. (2023) decribed untargeted LC-MS-based metabolomics to compare the fecal metabolome of 40 late-preterm infants treated or not with probiotic *Clostridium butyricum* strain MIYARI 588. All the late preterm infants had mandatory antibiotic treatment lasting an average of 12 days, followed by probiotic administration in half of them (n=20). The authors showed a clear separation of fecal metabolite profiles between the two infant groups, with an increase in metabolites involved in vitamin digestion and absorption, and the metabolism of glycerolipids, lysin, and biotin significantly increased in the probiotic-treated group. However, the authors did not report on any effects of antibiotics on the fecal metabolome, nor did they have aged-matched sampling between the groups.

Lagkouvardos et al. (2022) employed targeted metabolomics (GC-FID) to analyze stool samples from a randomized, controlled intervention study of infants receiving or not a synbiotic intervention formula with *Limosilactobacillus fermentum* CECT5716 and GOS. The authors reported higher acetate and lower butyrate in the synbiotic group at four months of age. They further state that the fecal microbiomes of the synbiotic group resembled breastfed infants more closely than controls.

Sillner et al. (2021) conducted a randomized, placebo-controlled trial to investigate the impact of a probiotic mix *(B. bifidum, B. breve, B. longum* ssp*. infantis, B. longum)* in formula compared to control formula and breast milk within the first year of life. By using untargeted metabolomics (LC-MS), they found minimal metabolic response, with 1% of the difference between the groups attributed to the intervention. Although they did not see significant differences between probiotic supplementation and control formula, they found alterations of several bile acids in response to formula feeding, including lower intensities of glychochenodeoxycholic acid and glycocholic acid (conjugated bile acids) in the intervention group at 1 and 3 months, which the authors hypothesize to be a sign of probiotic activity. The metabolites 4-hydroxyphenyl lactic and indoleacetic acid showed no significant differences but were higher in the probiotic group at 3 and 5 months, which resembled exclusively breastfed controls. These differences almost disappeared after weaning.

Heppner et al. (2024) used untargeted metabolomics (LC-MS) to compare fecal metabolome across feeding modes. The authors studied five infant groups, including four receiving

formula (with a combination of *B. breve* and *B. longum* ssp. *infantis* probiotic strains, a prebiotic GOS mixture, a synbiotic mixture of GOS and the probiotic strains, or a placebo) and breastmilk. At 3 months, the infants showed apparent differences in fecal metabolite profiles between formula-fed and breastfed infant groups, with the latter having higher levels of sugar metabolites. In contrast, infants fed formula containing pre-, pro, or synbiotic had higher levels of indoles, such as indolelactic acid. Although they reported differences between breastfed and formula-fed infants, no clear differences were observed in the formula groups where the infants were fed with either pre-, pro-, or synbiotics.

In another randomized controlled trial, Bazanella et al. (2017) investigated the effects of a probiotic mixture (*B. bifidum*, *B. breve*, *B. longum* ssp. *infantis, B. longum*) added to infant formula by a combination of untargeted and targeted metabolomics (LC-MS). At 1 month, the metabolic profile in response to the probiotic supplementation was significantly different from the control formula, but not at later time points. Although the authors analyzed SCFA, no significant differences were detected between the probiotic and control formula groups.

Kim et al. (2015) performed untargeted NMR to analyze the fecal metabolome of infants with a family history of allergy who received probiotic supplementation (*B. bifidum* W23, *B. animalis* subsp. *lactis* W52, and *Lactococcus lactis* W58) during the first 12 months of life. The authors observed higher levels of lactate and SCFAs (butyrate, propionate, acetate) and lower lactose and succinate in the probiotic group at three months of age. Finally, Baldassarre *et al.* (2018) investigated whether a multistrain probiotic mixture in breastfed infants can modulate colic symptoms. Using untargeted NMR, the authors reported minor differences where metabolites such as acetate, alanine, hydroxy isovalerate, and oxo isocaproate involved in amino acid metabolism were increased in the placebo group, whereas propylene glycol was higher in the probiotic group after 21 days of intervention (age 51-111 days).

Discussion

In this systematic review, we evaluated the methodological quality of studies reporting the infant fecal metabolome in response to interventions that modify the gut microbiome, and the biological significance of reported findings. Typically, microbiome research links microbial taxa profiling by DNA sequencing to other omics technologies, with the taxonomic composition serving as the foundation for predicting association and causation. Given that metabolite levels can change due to changes in microbial composition, we have hypothesized that metabolites will provide essential biological insights into alterations caused by different biotic interventions, even in the absence of detectable changes in microbial composition. Twenty-one studies met the inclusion criteria, most of which were published after 2020, reflecting a growing interest in this field of research (**Figure S2**).

Seven studies received excellent scores for general reporting (**Table S1**), which included details on sample preparation, instrumentation, biomass normalization, and raw data accessibility. A common shortcoming was the failure to describe metabolite quenching, a crucial step achieved by using organic solvent-based solutions during fecal sample collection (Lu et al., 2017). This is important, as certain metabolites, such as SCFAs, can change significantly within hours, even at 4ºC (Liebisch et al., 2019). In general, some metabolites can have turnover times of a few seconds, highlighting that quenching is essential to preserve the metabolic fingerprint of a sample (Lu et al., 2017). Several of the studies analyzed fecal samples that were transported to freezers without reporting prior quenching, potentially allowing enzymatic activities to persist and alter metabolite levels. Further, biomass normalization techniques in fecal metabolomics lack standardization, and we found their transparent reporting in only 12 studies, compromising the comparability of reported results across studies.

More than half of the included studies used MS-based metabolomics. In general, variations in stool sample processing might lead to differences in the final metabolite extracts injected into the instruments. More specifically, for MS-based targeted metabolomics, inclusion of authentic internal standards for all targeted compounds is crucial for reliable identification and quantification. Surprisingly, none of the 11 studies using targeted measurements employed authentic internal standards for all targeted compounds, raising concerns about the accuracy of metabolite quantification in these analyses (**Table S2**).

On the other hand, pooled QCs have become standard in untargeted metabolomics methods and were used in all the untargeted studies we inspected (**Table S3**). However, pooled QCs can serve multiple purposes, such as batch normalization, drift correction, and system conditioning (Kirwan et al., 2022). Unfortunately, most studies failed to report these details, potentially hindering reproducibility. Of the nine studies conducting untargeted analysis, five reported using internal standards, enhancing the reliability of untargeted analysis by allowing correction for instrumental drift and monitoring metabolite retention times and potential signal drift (Gertsman & Barshop, 2018). Finally, annotation of putative metabolites in untargeted analysis remains a significant challenge (Alseekh et al., 2021). Because of that, reporting annotation levels and the number of unknown features, which may represent false positives, provides an additional confidence level in the presented results. In cases where annotation levels are not provided, we emphasize the importance of making raw data accessible, allowing others to re-analyze and verify the integrity of the results.

By assessing the quality of metabolomics methodology reporting, we wanted to stress the necessity of appropriate experimental and technical controls when comparing metabolite levels between diseased and healthy states or an intervention product and a placebo. Besides different perinatal factors, such as gestational age, feeding type, and medication use, which are known to affect the gut microbiome and, consequently, the fecal metabolome, differences in collection, transport, and storage of samples, together with inconsistent methodology, can introduce unwanted variability. We found missing descriptions and low reporting quality for nine studies (reporting score below 50%, **Table S6**). In several cases, these studies did not report any significant results. Specifically, Wu et al. 2022, Strasser et al. 2020, Bazanella et al. 2017, and Eng et al. 2021 did not identify any significant impact of biotic interventions on the fecal metabolite profiles (**Table 1**). Because of the limitations in methodology reporting, rendering a low confidence in the reproducibility of the results, we highlight in the discussion the 12 studies with a reporting score greater than 50%. Our checklists (**Table S1-S6**) provide an operational structure for evaluating the quality of metabolomic experiments as well as guidelines for future studies.

Among seven studies examining the effects of antibiotics on the fecal metabolome, one included healthy, term infant cohorts, while the others involved infants with CF, urogenital tract malformations, or late preterm births. Fecal metabolites serve as a proxy for metabolic activity of gut microbes but do not necessarily account for variations in metabolite absorption, bowel movement frequency, or other physiological phenomena that might be different

between disease and healthy states, and which may thus also influence levels of microbial metabolites. For example, given that CF patients have a higher incidence of constipation compared to healthy individuals (Stefano et al., 2022) and that prolonged fecal transit time has been associated with changes in fecal metabolite pools (Roager et al., 2016), the tracked metabolite alterations over time in CF infants can be due to the pathophysiology of the disease. In the two studies with CF cohorts, none of them had healthy controls in their metabolomic analysis, preventing comparison with a general population.

S-sulfo-cysteine and N-formyl-methionine were significantly increased after antibiotic treatment in predictive models (Eng et al., 2021) and measured in the study by Li *et al*. (2022). Although these metabolites belong to different pathways, both are involved in sulfur metabolism, which has been linked to inflammatory bowel disease (IBD) in children (Kushkevych et al., 2020). Kronman et al. (2012) conducted a retrospective cohort study and found associations between antibiotic exposure and IBD development (Kronman et al., 2012). Future research targeting sulfate metabolism might elucidate potential links with disease progression. In summary, the studies by Li *et al.* (2022) and Lowienska *et al*. (2022) have provided the most relevant, although yet incomplete, information on how antibiotic use might affect the levels of fecal metabolites during early life and thus alter the metabolic function of the gut microbiome. Notably, both studies had a relatively good level of reporting their metabolomics methodology, further augmenting the confidence in their findings.

Two studies investigating the effects of postbiotics were included in this review. Both ranked poorly in metabolomic reporting, raising questions about the results' reliability. On the other hand, only one out of nine studies describing probiotic use received a poor rating. Interestingly, several studies observed elevated levels of metabolites linked to aromatic amino acid metabolism following probiotic or synbiotic intervention (Henrick et al., 2021; Heppner et al., 2024; Sillner et al., 2021). Meanwhile, Frayman *et al.* (2024), whose study had excellent metabolomic reporting, observed lower levels of indoleacetate correlating with higher carriage of *Pseudomonas* in their CF cohort. Commensal bacteria are known to metabolize aromatic amino acids through pathways related to immune modulation and brain health via neurotransmitter production (Chen et al., 2021; Roager et al., 2023). For example, *Lactobacillus* species are key in converting tryptophan into indoles, which have also been shown to strengthen the epithelial barrier and reduce inflammation (Bansal et al., 2010). Comparably, Henrick et al. (2021) demonstrated that indole-3-lactic acid has an anti-inflammatory effect, regulating T-cell formation *in vitro*.

Among the probiotic-focused studies, several reported an increase in SCFA after the probiotic intervention (Francavilla et al., 2012; Kim et al., 2015), although the findings were inconsistent. Notably, butyrate, propionate, and lactate were commonly reported across the studies. Most showed no effect or reduced levels of butyrate and propionate following various interventions, while lactate was elevated in several studies following pre- or probiotic administration. In a study by Tsukuda et al. (2021), *Bifidobacteria* in early life were correlated with higher formate and lactate levels, whereas butyrate and propionate were more elevated later in infancy, especially after weaning (Tsukuda et al., 2021). This pattern was also observed in one of the studies (Zhao et al., 2023), where 1,2-propanediol was negatively correlated with butyrate and propionate. There are four main pathways for butyrate production: the acetyl-CoA, glutarate, 4-aminobutyrate, and the lysine pathway, which converge at a step where crotonyl-CoA is converted to butyryl-CoA (Vital et al., 2014). Low butyrate in early life may be associated with the inability of *Bifidobacterium* spp., the dominant colonizers in breastfed infants, to produce butyrate. Thus, butyrate production relies on cross-feeding interactions with other bacterial species, particularly members from the *Bacillota* phylum. This phylum includes bacteria that possess butyryl-CoA CoA-transferases or butyrate kinase, which are essential for the last step of butyrate synthesis. One notable example is *Eubacterium hallii*, which has been demonstrated to produce butyrate from 1,2-propanediol, acetate, and lactate (Rivière et al., 2016; Schwab et al., 2017; Vital et al., 2014). Hence, elevated 1,2-propanediol levels, along with its inverse correlation with butyrate, may indicate a lack of cross-feeding between *Bifidobacterium* spp. and other commensals, such as *E. hallii* (Bunesova et al., 2018), and absence or domination of certain gut bacterial species.

Two studies provided the most comprehensive insight into bile acid metabolism. Li *et al.* (2022) focused on antibiotics as the intervention, while Sillner *et al.* (2021) investigated the effects of *Bifidbacterium*-based probiotics. The dynamics of bile acid metabolism are complex; however, we speculate that the detection of specific bile acids could predict the presence of gut bacterial taxa due to enzymatic requirements for bile acid transformation. Primary bile acids are produced in the liver, where they are conjugated and stored in the gallbladder before being released into the intestine (Chiang, 2013). Two key enzymes modulate bile acid transformation: bile salt hydrolases (BSH), responsible for deconjugation, and α-7-dehydroxylase, which converts primary bile acids to their secondary forms. In early life, the gut is typically dominated by *Bifidobacterium* species exhibiting BSH activity (Turroni et al., 2012), which has been linked to the regulation of inflammation and prevention

of metabolic disorders (Bourgin et al., 2021). However, neither *Lactobacillus* nor *Bifidobacterium* possesses α-7-dehydroxylase, an enzyme present in species like *Clostridium,* which expands after weaning (Chiang & Ferrell, 2020; Takahashi & Morotomi, 1994). In a cohort of healthy, term infants, Xiong *et al*. (2021) found that primary bile acids increased steadily after birth (Xiong et al., 2021). In contrast, secondary bile acids only began to rise around six months of age, coinciding with weaning and subsequent alterations in the gut microbiome (Pantazi et al., 2023). Li et al. (2022) detected elevated levels of conjugated bile acids (such as taurocholate and glycochenodeoxycholate) and some secondary bile acids after antibiotic treatment, which could suggest heightened α-7-dehydrogenase activity and a greater prevalence of *Clostridum* species. Additionally, higher levels of conjugated bile acids indicate lower BSH activity, suggesting reduced *Bifidobacterium* and *Lactobacillus* populations. Sillner *et al*. (2021) found lower levels of the conjugated bile acids glycocholate and glycochenodeoxycholate in the probiotic group, which could indicate increased BSH activity in the gut of infants receiving probiotics. Taken together, reduced levels of conjugated bile acids may signal probiotic activity, whereas elevated levels of secondary and conjugated bile acids in early life may reflect diminished *Bifidobacterium* levels and increased *Clostridium* metabolism.

In conclusion, among the 21 studies included in this systematic review, nine were rated as poor, 11 as good, and only one achieved an excellent score for metabolomic reporting (**Table 1**). This comparative QA analysis highlights that there remains substantial work in terms of standardization and adherence to best practices with regard to reporting fecal metabolite profiling. As a result, many of the findings reported by studies that we graded as poor on the reporting cannot be trusted entirely, and any comparative analysis remains a challenge. We stress that future research employing metabolomics of fecal samples should adhere to the QA and QC recommendations proposed by us and others (Alseekh et al., 2021; Kirwan et al., 2022; Lu et al., 2017) as a minimum reporting practice.

Despite these limitations, some consistent biological patterns emerged across the studies. By focusing on the results from the studies with a score above 50 %, we found more consistency in the reported metabolic outcome (**Figure 4**). The fecal metabolome was notably impacted by anti-, pre-, and probiotics, and in particular, alterations of metabolites belonging to the pathways of secondary and conjugated bile acids and general amino acid metabolism were evident after antibiotic intervention. Pre- and probiotic intervention had overlapping effects, possibly driven by increased abundance and subsequent metabolism of the probiotic bacteria,

with evident changes in aromatic amino acids, central carbon metabolism (including SCFA), and conjugated bile acids. The dynamics of these metabolite classes, as exemplified above for bile acids, could serve as predictors of the presence or even abundance of specific microbial genera in the gut microbiome during early life.

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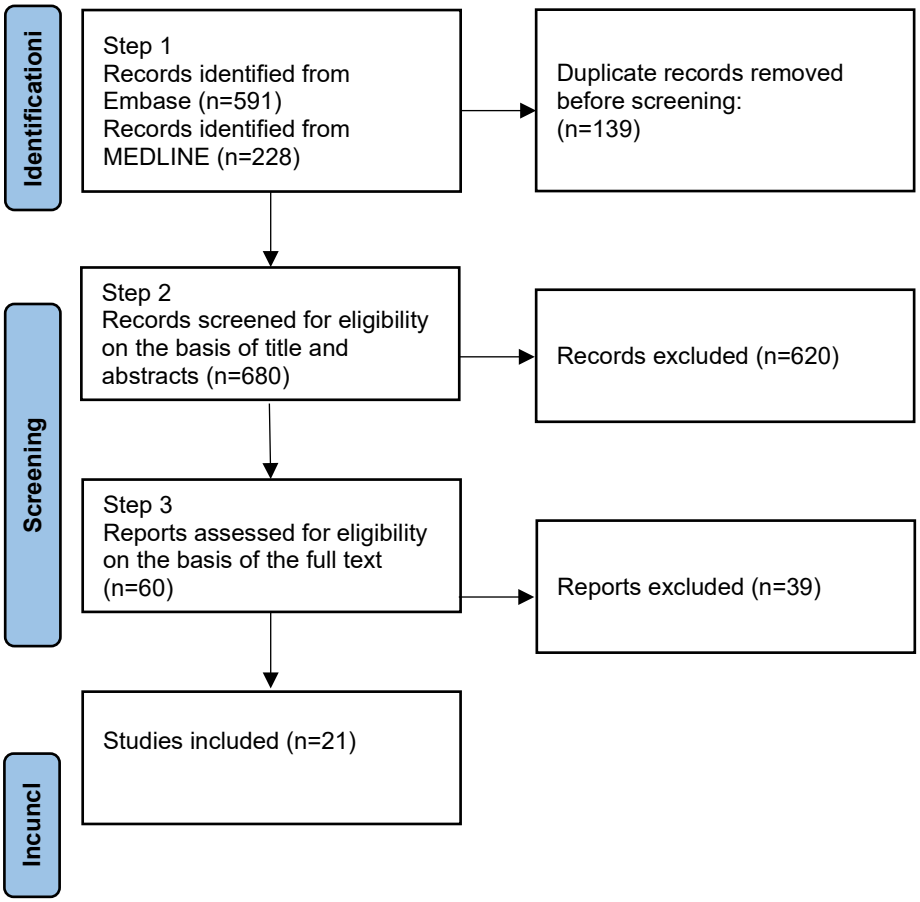

Figure 1: Study selection process using the PRISMA 2020 flow diagram

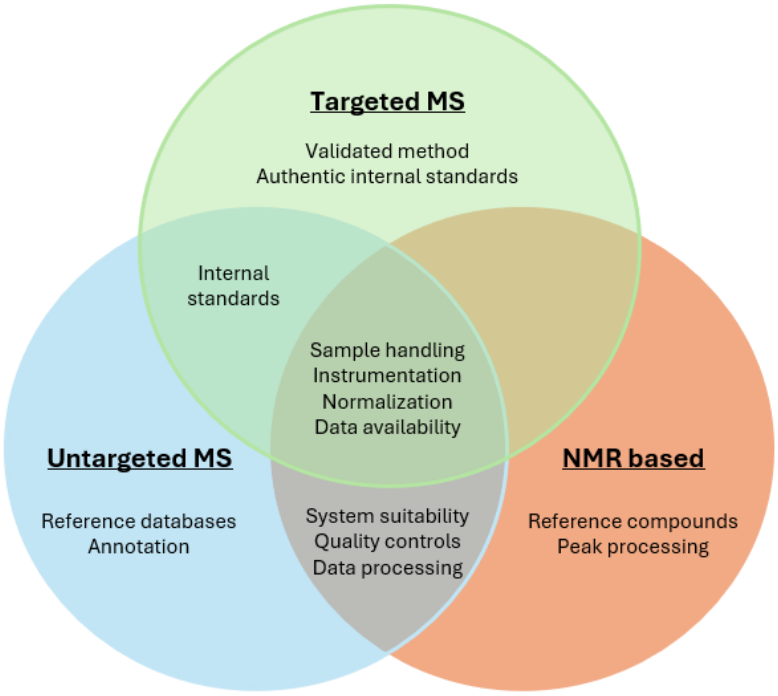

Figure 2 Shared and specific reporting requirements for targeted MS-, untargeted MS-, and NMR-based metabolomics methodology.

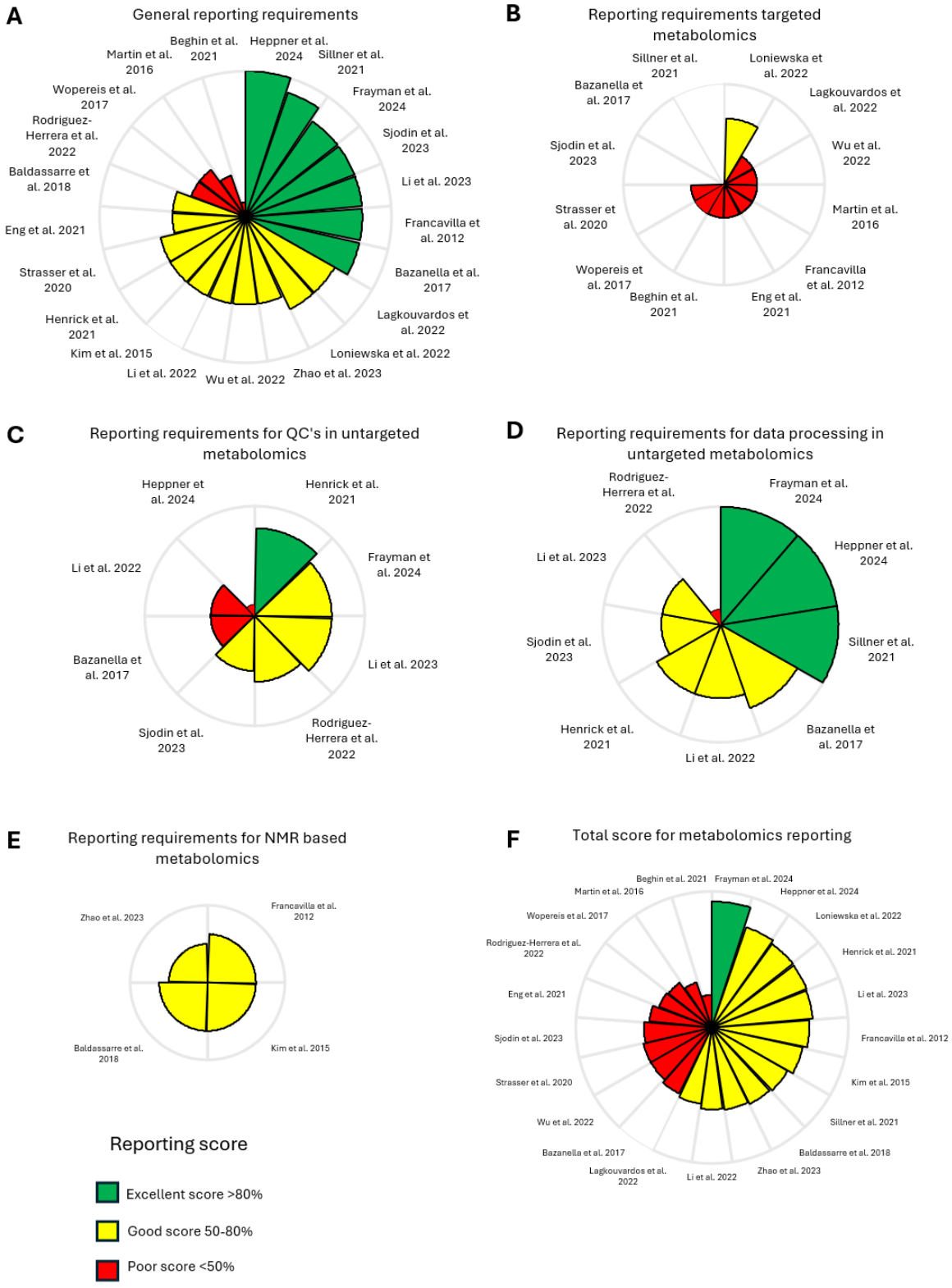

Figure 3: Score (%) of studies based on A) general reporting requirements common for all metabolic platforms, B) for targeted MS-based metabolomics, C) for quality control (QC) reporting in untargeted MS-based metabolomics, D) for data processing in untargeted MS-

based metabolomics, E) for NMR based metabolomics, and F) the total score for each study. A score > 80% is labelled green, 50-80% yellow and <50% red.

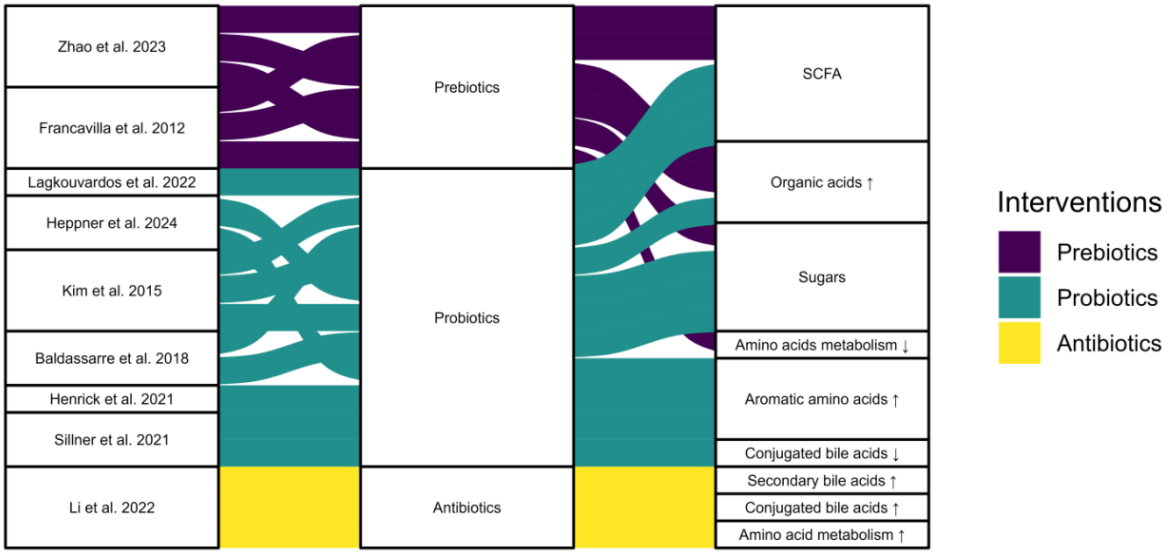

Figure 4: The relationship between studies with a clear intervention outcome and a reporting score above 50%, their corresponding interventions and the reported metabolomic outcome. SCFA – short-chain fatty acids.

Table 1: Biological effects of antibiotic, probiotic or prebiotic interventions on bacterial and metabolic abundance.

Studies	Intervention	Modulations in response to intervention				Comments	Reporting score
		Bacteria ↑	Bacteria ↓	Metabolites ↑	Metabolites ↓		
Frayman et al. 2024	Antibiotic	Pseudomonadota (phylum)		Quinate and shikimate	Butyrate, indole and indoleacetate	The participants were cystic fibrosis infants treated with antibiotics.	Excellent
Loniewska et al. 2022	Antibiotics					SCFA no difference	Good
Wu et al. 2022	Antibiotics					No clear effect of antibiotics	Poor
Li et al. 2022	Antibiotics	Bacillota (phylum)	Pseudomonadota (phylum)	Secondary BA - TCA, GCDCA, N-formyl-methionine, conjugated bile acids - TLC, GDC, TDC	Secondary BA - GCA		Good
Strasser et al. 2020	Antibiotics					SCFA no difference	Poor
Martin et al. 2016	Antibiotics		*Bifidobacterium* (genus) and *Staphylococcus* (genus)				Poor
Eng et al. 2021	Antibiotics					No significant difference in metabolite profile	Poor
Lagkouvardos et al. 2022	Probiotics	*Bifidobacterium* (genus)	*Clostridioides difficile* (species)		Butyrate		Good

Heppner et al. 2024	Probiotics	*Bifidobacterium* (genus)		Indolelactic acid	Sugars		Good
Kim et al. 2015	Probiotics			SCFA and lactate	Lactose and succinate		Good
Baldassarre et al. 2018	Probiotics			1,2-propanediol	Acetate, alanine, isovalerate		Good
Henrick et al. 2021	Probiotics			Tryptophan metabolism, indole-3-lactate			Good
Sillner et al. 2021	Probiotics			4-hydroxyphenyllactic acid and indolelactic acid	Conjugated BA - GCDCA, GCA		Good
Bazanella et al. 2017	Probiotics					SCFA no difference	Poor
Sjodin et al. 2023	Probiotics	*Bifidobacteriaceae* (family). *Bifidobacterium breve* (species)	*Eubacteriaceae* (family), *Lachnospiracea* (family), *Erysipelotrichaceae* (family), *Klebsiella* (genus)	3-phenyllactic acid, galacturonic acid			Poor
Li et al. 2023	Probiotics		The species *Staphylococcus aureus*, *Sphingomonas echinoides, Pseudomonas putida*		Biotin metabolism, glycerolipid, lysine and glutathione		Good
Zhao et al. 2023	Prebiotics	*Bifidobacteriaceae* (family), *Lactobacillaceae* (family), *Enterobacteriaceae* (family), *Staphylococcaceae* (family)	*Lachnospiraceae* (family), *Ruminococcaceae* (family), *Veillonellaceae* (family)	1.2-propanediol, lactate, acetate, formate	Butyrate and propionate		Good
Francavilla et al. 2012	Prebiotics	*Bifidobacterium* (genus)	*Bacteroides* (genus) , *Prevotella* (genus) and *Clostridia* (class)	SCFA, lactate, threonine	Amino acids		Good

Beghin et al. 2021	Prebiotics	*Bifidobacterium* (genus)	The species: *Parabacteroides distasonis*, *C. lituseburense*, *C. histolyticum*, *B. coccoides*	Lactate			Poor
Rodriguez-Herrera et al. 2022	Prebiotics	*Bifidobacterium* (genus)	*Clostrodioides difficile* (species)	Acetate, L-lactate	Propionate, D-lactate, butyrate, and valerate		Poor
Wopereis et al. 2017	Prebiotics	*Bifidobacterium* (genus)	*Clostridium* (genus)	Lactate	Propionate, butyrate, and branched chain-SCFA		Poor

* SCFA – short-chain fatty acids; BA – bile acids; TCA – taurocholic acid; GCDCA – glycochenodeoxycholic acid; GCA – glycocholic acid; TLC – taurolithocholic acid; GDC – glycodeoxycholic acid; TDC – taurodeoxycholic acid. The study name labelled green received excellent reporting score, yellow good and orange poor.