C. difficile may be overdiagnosed in adults and is a prevalent commensal in infants

Pamela Ferretti[1], Jakob Wirbel[1,x], Oleksandr M Maistrenko[1,2,x], Thea Van Rossum[1,x], Renato Alves[1], Anthony Fullam[1], Wasiu Akanni[1], Christian Schudoma[1], Anna Schwarz[1], Roman Thielemann[1], Leonie Thomas[1], Stefanie Kandels[1], Rajna Hercog[3], Anja Telzerow[3], Ivica Letunic[4], Michael Kuhn[1], Georg Zeller[1], Thomas SB Schmidt[1] and Peer Bork[1,5,6,7]

Affiliations:

1 European Molecular Biology Laboratory, Structural and Computational Biology Unit, 69117 Heidelberg, Germany

2 Present address: Royal Netherlands Institute for Sea Research (NIOZ), Department of Marine Microbiology & Biogeochemistry, 1797 SZ, 't Horntje (Texel), Netherlands

3 Genomic Core Facility, European Molecular Biology Laboratory, Heidelberg, Germany

4 biobyte solutions GmbH, Bothestr 142, 69126 Heidelberg

5 Max Delbrück Centre for Molecular Medicine, Berlin, Germany

6 Yonsei Frontier Lab (YFL), Yonsei University, Seoul 03722, South Korea

7 Department of Bioinformatics, Biocenter, University of Würzburg, Würzburg, Germany

x These authors contributed equally to this work.

*Correspondence: sebastian.schmidt@embl.de and Bork@embl.de

Abstract

Clostridioides difficile is an urgent threat in hospital-acquired infections world-wide, yet the microbial composition associated with *C. difficile*, in particular in *C. difficile* infection (CDI) cases, remains poorly characterised. To investigate the gut microbiome composition in CDI patients, we analysed 534 metagenomes from 10 publicly available CDI study populations. We then tracked *C. difficile* on a global scale, screening 42,900 metagenomes from 253 public studies. Among the CDI cohorts, we detected *C. difficile* in only 30% of the stool samples from CDI patients. However, we found that multiple other toxigenic species capable of inducing CDI-

like symptomatology were prevalent. In addition, the majority of the investigated studies did not adhere to the recommended guidelines for a correct CDI diagnosis.
In the global survey, we found that *C. difficile* prevalence, abundance and biotic context were age-dependent. *C. difficile* is a rare taxon associated with reduced diversity in healthy adults, but common and associated with increased diversity in infants. We identified a group of species co-occurring with *C. difficile* exclusively in healthy infants, enriched in obligate anaerobes and in species typical of the healthy adult gut microbiome. *C. difficile* in healthy infants was therefore associated with multiple indicators of healthy gut microbiome maturation.
Our analysis raises concerns about potential CDI overdiagnosis and suggests that *C. difficile* is an important commensal in infants and that its asymptomatic carriage in adults depends on microbial context.

Keywords: *Clostridioides difficile*, metagenomics, CDI, antibiotic-associated diarrhoea, infant microbiome, overdiagnosis

Relevance of this study

To our knowledge, this is the first large-scale metagenomic meta-analysis on *Clostridioides difficile*. We investigated *C. difficile* prevalence in 42,900 metagenomic samples from 86 countries and used machine learning to identify a global CDI-specific microbial signature. Our findings suggest that *C. difficile* may be overdiagnosed in the clinical context, with other enteropathogens able to induce CDI-like symptomatology, erroneously contributing to the *C. difficile* burden estimation. Disentangling the real burden of *C. difficile* from other pathogens is a fundamental step towards the development of adapted therapeutic interventions. Furthermore, this study provides new insights into the gut microbiome composition in presence of *C. difficile,* not only in CDI patients, but also across other diseases and in healthy subjects. In particular, we found that *C. difficile* colonisation was associated with multiple indicators of a healthy gut microbiome development in infants, an age group in which *C. difficile* has been so far understudied. This study also represents a blueprint for future large-scale species-specific metagenomic meta-analyses.

Introduction

*Clostridioides difficile (*previously *Clostridium* or *Peptoclostridium difficile)* was first isolated in 1935 from the stools of healthy infants (Hall and O'toole 1935), but later identified as a causative agent of pseudomembranous colitis (Bartlett et al. 1978), a severe condition with potentially life-threatening outcomes. While not all *C. difficile* strains are toxigenic, *C. difficile* toxin A (TcdA) and toxin B (TcdB) can increase intestinal permeability and promote intense inflammation (Voth and Ballard 2005), a condition referred to (Bartlett et al. 1978)as *C. difficile* infection (CDI). CDI is typically characterised by diarrhoea and/or pseudomembranous colitis and often unresponsive to repeated antibiotic treatments. Known risk factors include hospitalisation, age (≥65 years of age), and broad-spectrum antibiotic therapy (Eze et al. 2017).

CDI has an increasing clinical relevance (at an estimated annual economic burden of US$6.3 billion in the US alone (Bartlett et al. 1978)) and is considered one of the most urgent threats in hospital-acquired infections(CDC, 2019).

C. difficile has primarily been studied as a pathogen, with a strong focus on CDI aetiology (Smits et al. 2016), mechanisms of *C. difficile* toxicity (Voth and Ballard 2005) or efficacious therapies (Kociolek and Gerding 2016). However, an increasing number of other enteropathogens has been linked to antibiotics-associated diarrhoea (AAD) with similar, and sometimes indistinguishable, symptomatology to CDI (Tang et al. 2016; Chia et al. 2018; Larcombe et al. 2018), making the correct diagnosis, estimation and management of CDI particularly challenging.

Far less is known about asymptomatic *C. difficile* carriage among the healthy population, as sentinel studies are often limited in sample size and geography (Galdys et al. 2014; Kato et al. 2001; Cui et al. 2021; Mani et al. 2023) (Galdys et al. 2014; Kato et al. 2001). Gut microbial composition associated with *C. difficile* presence in healthy subjects, in particular in infants where *C. difficile* is highly prevalent (Rousseau et al. 2012), remains poorly understood.

Here we leverage public shotgun metagenomic data to characterise gut microbial signatures associated with CDI in adult and elderly patients and to quantify other AAD-associated species with similar signatures. We then contextualise our (Rousseau et al. 2012) analysis by tracing *C. difficile* in a broad multi-habitat collection of 42,900 metagenomes. We investigate *C. difficile* prevalence, relative abundance and its associated microbial community structure and composition in the human gut over lifetime, with particular focus on healthy infants, where several lines of evidence point to *C. difficile* being a commensal and a hallmark species of healthy infant gut microbiome maturation.

Results

C. difficile is metagenomically detectable in only 30% of patients with a CDI diagnosis

We conducted a metagenomic meta-analysis of the gut microbiome associated with CDI, analysing 534 fecal samples across 10 geographically diverse study populations (Vincent et al. 2016; Smillie et al. 2018; Podlesny and Florian Fricke ; Kim et al. 2020; Langdon et al. 2021; Monaghan et al. 2020; Stewart et al. 2019; Kumar et al. 2017; Watson et al.), including patients diagnosed with CDI, diseased control subjects without CDI (D-Ctr, see Methods), and healthy subjects (H-Ctr) (**Figure 1A** and **Supplementary Table 1**).

Using mOTUs-based species-level taxonomic profiling, *C. difficile* was detectable in just 30% of CDI samples at established metagenomic detection limits (Milanese et al. 2019) (**Figure 1A**), with considerable variance across study populations (9.2%-92.3%), and with much lower prevalence among diseased (2.6%) and healthy (1.1%) controls (**Supplementary Figure 1A**). *C. difficile* toxin genes *tcdA* and *tcdB* were detectable in about half of *C. difficile*-positive CDI metagenomes, but not in any *C. difficile*-negative CDI samples nor controls, indicating a lower sensitivity of toxin-based detection (**Figure 1A**, see Methods). We confirmed that marker gene-based *C. difficile* detection was not limited by sequencing depth (linear mixed-effect model p=0.35, ANOVA, adjusted p=0.54; R^2=0.01), and that detection was not skewed towards metagenomic detection limits (Parks et al. 2021). Neither species richness (**Figure 1B**) nor composition (**Supplementary Figure 2**) differed between *C. difficile*-positive and -negative CDI samples, suggesting that *C. difficile* presence was not associated with characteristic community-level shifts in these generally disbalanced microbiomes.

The microbiome of CDI patients is characterised by an enrichment of enteropathogens beyond *C. difficile*

Several other species were enriched in prevalence or abundance in CDI patients relative to controls (**Figure 1C, Supplementary Figure 3**). Among these, we identified several opportunistic enteropathogens known to induce antibiotic-associated diarrhoea (AAD), resulting in a CDI-like diarrhoeal symptomatology (Chia et al. 2018; Chia et al. 2017; Kiu and Hall 2018; Zollner-Schwetz et al. 2008; Larcombe et al. 2016; Larcombe et al. 2018; Högenauer et al. 1998; Fakhkhari et al. 2022), such as *Staphylococcus aureus*, *Klebsiella oxytoca*, *Enterococcus faecalis*, *Enterobacter cloacae*, *Citrobacter amalonaticus*, *Clostridium innocuum*, *Clostridium perfringens*, and *Pseudomonas aeruginosa*. In particular, *C. innocuum* was highly prevalent

(63.2%) in CDI samples, and significantly enriched relative to both healthy and diseased controls (**Figure 1C**, Wilcoxon test $p=8.2x10^{-16}$ and $p=4.3x10^{-3}$, respectively). 94% of all CDI samples contained at least one of these other AAD species (*C. difficile* excluded) with variable prevalence across study populations (Fisher's test, $p=2.2x10^{-16}$, OR=29.62; **Supplementary Figure 1A** and **1B**). Presence and potential toxigenicity of several enteropathogens was further confirmed by the detection of characteristic toxin genes that were likewise enriched in CDI samples (**Figure 1D**), in particular enteropathogenic *E. coli* and *S. flexneri* toxin genes (see Methods for full list). Overall, CDI samples, independently of *C. difficile* presence, were significantly enriched ($p=2.2x10^{-16}$) in species carrying toxin genes in terms of cumulative relative abundance, indicating that CDI symptomatology could be entirely or partially driven by other enteropathogens than *C. difficile*.

To integrate these univariate associations of individual species into characteristic and predictive multi-species signatures of CDI, we next trained a series of LASSO-regularised logistic regression models in a leave-one-study-out validation approach (see Methods). As expected, *C. difficile* was the most predictive species for CDI samples, although the enrichment of other putative AAD enteropathogens (such as *S. aureus* or *C. perfringens*) was also highly characteristic, in particular in CDI samples where *C. difficile* was not detectable (**Figure 2A**). Moreover, several oral species (such as *Veillonella parvula, Veillonella atypica* or *Rothia dentocariosa*) were enriched in CDI gut metagenomes, implying increased oral-intestinal microbial transmission(Schmidt et al., 2019) among these patients. Enrichment of common probiotics (*Lactobacillus casei*, *L. plantarum* or *L. fermentum*) in CDI is likely due to the widespread use of probiotic therapy among these patients (Golić et al. 2017; Na and Kelly 2011).

Using these models, CDI samples were distinguishable from diseased and healthy controls with high accuracy, at an area under the receiver operating characteristics curve (AUROC) of 0.78, ranging between 0.56 and 0.98 depending on the study population (**Figure 2B**), in line with a previous 16S-based investigation (Schubert et al. 2014). Model performance moderately improved when considering only healthy controls (overall AUROC = 0.81) (**Figure 2B**). To assess the importance of *C. difficile* to predicting CDI state, we next re-trained models under explicit exclusion of *C. difficile*. Interestingly, this did not lead to a noticeable reduction in accuracy (**Figure 2B**), indicating that the presence or enrichment of *C. difficile* was indeed not an essential defining feature of CDI samples. Rather, *C. difficile* appeared to be just one of

several AAD species characterising the CDI-associated microbiome in varying constellations, forming a loose clique instead of fixed co-occurring species groups.

Extensive asymptomatic carriage of *C. difficile* across age and geography

Given that *C. difficile* appears to have a much lower prevalence in CDI patients than anticipated, we next studied its prevalence in an extended collection of 253 publicly available studies, including a novel cohort of 447 samples from the citizen science project MyMicrobes, for a total of 42,900 shotgun metagenomic samples (**Supplementary Table 2).** This collection included healthy and diseased subjects of all ages, with samples from multiple body sites, hosts, environments and geographic locations (**Figure 3A and 3B**). Statements from this point onwards refer to this extended collection of samples.

C. difficile was detected almost exclusively in human and animal feces (8.7% and 1.7% prevalence, respectively) with sporadic detection in hospital surfaces (0.2%), the respiratory tract of diseased patients (0.4%) and freshwater (0.9%) (**Figure 3C** and **Supplementary Table 3**). *C. difficile* was also detectable in the colon, ileum and cecum lumen, and biopsies from the colon and rectal mucosa of healthy subjects. We again confirmed in this larger dataset that there was no relationship between *C. difficile* detection and sequencing depth (linear mixed-effect model p=0.36; logistic regression, ANOVA adjusted p=0.52, **Supplementary Figure 4A** and **4B**). Nevertheless, we adjusted all subsequent analyses for total sequencing depth.

In healthy human populations, both *C. difficile* prevalence and relative abundance in fecal samples varied considerably across age ranges. Infants aged one year or younger showed by far the highest *C. difficile* carriage, up to 76.5% prevalence at the age of 8 to 10 months (**Figure 4A**), at an average relative abundance of 1.1% (and up to 50% in a two-weeks-old healthy preterm infant). *C. difficile* prevalence remained elevated (43.4%) between 1 and 4 years of age, in contrast to previous reports (Jangi and Thomas Lamont 2010; Lees et al. 2016). With increasing age, *C. difficile* was increasingly rare, found in less than 1% of the healthy population (6.5% in adolescents, 0.8% in adults) (**Figure 4A** and **Supplementary Table 3**) at average abundances of 0.2%, suggesting a considerably lower asymptomatic carriage among adults than previously estimated (up to 15% (Crobach et al. 2018)). *C. difficile* carriage varied across geography (**Supplementary Figure 5A**) and was lower in non-westernised populations, at 4.8% and 0.4% in infants and adults, respectively.

C. difficile is enriched upon antibiotic treatment

Diseased or antibiotics-treated subjects exhibited consistently increased *C. difficile* carriage rates across all age groups (**Figure 4A**). Prevalence was highest among infants taking antibiotics (81.1%, relative abundance $2.2x10^{-2}$) and infants suffering from cystic fibrosis (78.7%, $4.0x10^{-3}$) or neonatal sepsis/necrotizing enterocolitis (53.7%, $3.2x10^{-2}$; **Supplementary Figure 5B**). An elevated *C. difficile* asymptomatic carriage rate in cystic fibrosis patients is well documented (Deane et al. 2021) and may be the result of long-term antibiotics usage and frequent exposure to the hospital environment (Bauer et al. 2014) . Among adolescents, adults and elderly, *C. difficile* prevalence was highest in patients suffering from CDI (31%, see above) or undefined diarrhoea (25.9%), followed by subjects taking antibiotics (on average 25% across age groups, relative abundance $4.6x10^{-3}$). Inflammatory bowel diseases, liver diseases, diabetes, and various forms of cancer were likewise associated with an increased *C. difficile* carriage relative to the healthy background population (**Supplementary Figure 5B**).

C. difficile is a frequent commensal in the healthy infant gut

C. difficile was among the most prevalent species in infants during the first year of life. Carriage was increased in infants born via C-section (28.7%; logistic regression, adjusted ANOVA $p=3.0x10^{-3}$, $logOR=2.0x10^{-5}$) compared to age- and health status-matched vaginally born infants (19.6%) (**Figure 4B** and **Supplementary Figure 6**). Likewise, prevalence was moderately increased among pre-term (39.14%; $p=1.7x10^{-1}$, $logOR=1.1x10^{-5}$) compared to full-term healthy infants (23.8%) (**Figure 4C** and **Supplementary Figure 6**). Exclusively formula-fed infants were more frequently colonised by *C. difficile* (43.2%; $p=5.6x10^{-6}$, $logOR=3.1x10^{-5}$), compared to partially or exclusively breastfed infants during the first 6 months of life (14.2% and 8.6% respectively) (**Figure 4D**), in line with previous reports (McDonald et al. 2018; Drall et al. 2019).

C. difficile was not maternally acquired at birth: across 829 mother-infant pairs included in the dataset, we found no single case of vertical transmission of *C. difficile* from any of the maternal body sites investigated (gut, vagina and oral cavity). Rather, *C. difficile* was likely sourced from the social environment (i.e. other infants, family members or pets) during early life, in line with previous observations on *Clostridia* (Korpela et al. 2018). Leveraging longitudinal data, we observed that the first appearance of *C. difficile* was not arbitrarily distributed over the first year

of life, but instead concentrated in two defined time windows, between the 2nd-4th and 8th-10th months of age (**Supplementary Figure 7**). The first interval coincided with the increased strain influx from environmental sources to the infant gut reported in a previous study (Ferretti et al. 2018), probably due to the start of mouthing. The second interval, dominated by samples from the UK, overlaps with the paid maternity leave length in that country (Thevenon et al. 2016), suggesting that it could be due to the start of day care. C-section and premature birth were associated with increased rate of *C. difficile* appearance, compared to vaginally born and full-term infants (**Supplementary Figure 7**). Together these results suggest that *C. difficile* is acquired from the environment, and that increased exposure to novel environmental sources increases the chances of *C. difficile* acquisition. In addition, during the first year of life, the gut microbiome of healthy infants colonised with *C. difficile* was significantly more similar, in terms of composition, to that of their mothers (**Supplementary Figure 8**).

These vast differences in *C. difficile* carriage across age did not translate into differential toxin burdens. *C. difficile* toxin genes were detectable in metagenomes of healthy *C. difficile*-positive infants (22.4%) and adults (18.6%) at similar rates (**Supplementary Figure 9**).

C. difficile occurs in distinct biotic and physiological contexts in infants and adults

The *C. difficile*-associated microbiome varied considerably across age ranges. *C. difficile* carriage was associated with a significantly decreased overall species richness in subjects above 1 year of age, independently of the health status (**Figure 4E,** see Methods for the definition of diseased). However, in healthy infants we observed the opposite trend: species richness was higher in subjects carrying *C. difficile* (**Figure 4E** and **Supplementary Figure 10A**), irrespective of delivery mode, gestational age or geography (**Supplementary Figure 10B** and **10C**). Species richness was also elevated in *C. difficile*-positive diseased infants, albeit to a lesser degree. In line with these trends, *C. difficile* carriage was associated with higher community evenness (lower dominance of individual species) in infants, but a more uneven community structure in adults (**Supplementary Figure 10D**).

To further characterise how the biotic context of *C. difficile* in the gut changes over lifetime, we studied its co-occurrence with other intestinal species, stratified by age group and health status. We broadly distinguished two groups of species based on their co-occurrence relationships with *C. difficile* (**Figure 5**), independently of the presence of its toxin genes. The first set of species

("Group 1") consistently co-occurred with *C. difficile* across all age groups. Part of this group are *Clostridium paraputrificum* and *Clostridium neonatale* (**Supplementary Figure 11A**), in line with previous reports(Daquigan et al., 2017), an association that was conserved also in animal hosts (**Supplementary Figure 11B**). Group 1 was enriched in facultative anaerobic or aerobic species and comprised several known AAD enteropathogens, such as *K. oxytoca*, *C. perfringens*, *C. amalonaticus* or *C. innocuum*. A second set of species ("Group 2") co-occurred with *C. difficile* exclusively in healthy infants, but showed strong avoidance patterns in later life stages and, to a lesser extent, in diseased infants. Group 2 was characterised by obligate anaerobic species typical of the healthy adult gut microbiome and included several common butyrate producers (such as *Roseburia sp.*, *Faecalibacterium prausnitzii* or *Anaerostipes hadrus*) (**Figure 5** and **Supplementary Figure 12**).

To investigate whether healthy infants carried specific *C. difficile* strains, we performed an analysis of metagenomic single nucleotide variants (SNVs). Despite the distinct biotic context associated with *C. difficile* presence in healthy infants at the species level, *C. difficile* strain populations found in this group did not cluster separately from those identified in other age groups and health status, with consistent patterns of toxin-gene carriage (**Supplementary Figure 13**). Therefore, the general asymptomatic nature of *C. difficile* carriage in healthy infants is likely determined by its surrounding microbial community and host age, rather than specific strain groups.

Discussion

C. difficile is predominantly studied in context of its pathogenicity, yet its role in the human gut is arguably much more nuanced. Our data suggests that *C. difficile* may be both overestimated as a pathogen among the elderly and underestimated as a commensal among infants, and that its ambivalent role in the gut is malleable throughout the host's lifespan depending on external triggers (like antibiotics treatment) and biotic context.

We detected fecal *C. difficile* in just 30% of cases diagnosed with CDI, consistent with previous smaller-scale reports based on 16S rRNA (Daquigan et al. 2017; Seekatz et al. 2016) and WGS metagenomics (Vincent et al. 2016; Zhou et al. 2016), even when combined with laboratory-based tests (Seekatz et al. 2016). This surprisingly low prevalence was neither due to

metagenomic detection limits, as sequencing depth was not associated with *C. difficile* prevalence, nor could it be explained by (temporary) reduction of *C. difficile* abundance below detection limits by antibiotics, as stricter inclusion criteria for samples from active and symptomatic CDI cases did not change the observation. A more likely explanation for the under-detection of *C. difficile* among CDI patients is a lack of diagnostic specificity. Challenges in CDI diagnosis are further complicated by the vast array of protocols and diagnostic algorithms available in clinical practice (Jo et al. 2022). Diarrhoeal symptoms, often the initial diagnostic prompt, are not specific to CDI (Bartlett and Gerding 2008; Polage et al. 2012; Jackson et al. 2016; Reich et al. 2019) and pseudomembranous colitis, long thought to be characteristic (and often eponymously referred to as "*C. difficile* colitis"), may indeed be caused by other enteropathogens, such as *C. innocuum* or *E. coli* *(Gateau et al. 2018; Humphries et al. 2013; Burnham and Carroll 2013; Lee et al. 2021; McDonald et al. 2018; Crobach et al. 2016; Tenover et al. 2011; Rodriguez et al. 2016; Martínez-Meléndez et al. 2017; Litvin et al. 2009)*. Additional challenges include the large variability in sensitivity and specificity in diagnostic laboratory tests (Gateau et al. 2018; Humphries et al. 2013; Burnham and Carroll 2013; Lee et al. 2021; McDonald et al. 2018; Crobach et al. 2016; Tenover et al. 2011; Rodriguez et al. 2016; Martínez-Meléndez et al. 2017; Litvin et al. 2009; BBL™ Clostridium difficile Selective ...), contradicting results between cultivation and toxin detection ass(Polage et al. 2015)ays (Parks et al. 2011) and an excessive reliance on single molecular tests (Polage et al. 2015) Recommended guidelines discourage stand-alone tests for CDI diagnosis and recommend a two-step procedure (Crobach et al. 2016; Gateau et al. 2018), but due to costs, test availability, capacity and turnaround times, diagnostic algorithms vary between hospitals and often deviate from recommended procedures (Tenover et al. 2011). Despite the recent improvements in the CDI diagnosis guidelines awareness, low compliance is still reported (Viprey et al. 2023). In our meta-analysis, almost two thirds (62.5%) of studies deviated from recommended best practices for CDI diagnostic protocols.

Moreover, *C. difficile*'s role in causing antibiotics-associated diarrhoea (AAD) or pseudomembranous colitis (PMD) may be overstated, as simultaneous colonisation of multiple enteropathogens is very common among CDI patients (Hensgens et al. 2014; Spina et al. 2015). Enteropathogens like *C. perfingens, C. innocuum, S. aureus, E. coli* or *S. flexneri*, among others, are known to induce AAD or PMC (Chia et al. 2018; Kiu and Hall 2018; Zollner-Schwetz et al. 2008; Larcombe et al. 2018; Högenauer et al. 1998; Larcombe et al. 2016) and were prevalent and enriched among CDI samples, as were their characteristic toxin genes – in

particular also in samples where *C. difficile* was not detectable. Indeed, while *C. difficile* was the most individually characteristic species of CDI, its presence was not an essential input to microbiome-based classification models accurately predicting CDI-diagnosed samples. Taken together, this suggests that *C. difficile* is often not the most parsimonious explanation for clinical manifestations of CDI that may instead be driven by alternative enteropathogens, implying mis- or over-diagnosis in practice and an overestimation of the real burden of *C. difficile* relative to other AAD species. Differential diagnosis against multiple enteropathogens may therefore stratify patients with CDI-like symptoms, towards adapted therapeutic interventions.

Widespread asymptomatic carriage in healthy individuals is a further indicator that *C. difficile* plays a complex role in the gut, beyond pathogenicity. We tracked *C. difficile* across an extensive set of 42,900 metagenomes sampled from multiple habitats and hosts, includingn=28,423 (66.3%) human gut samples which covered a wide range of subject ages, disease states and geographic origins. *C. difficile* was particularly common among infants, peaking at >75% prevalence at 8-10 months of age. On average *C. difficile* was present in 25% of healthy infants aged 1 year or younger, in line with previous studies (Lees et al. 2016; Jangi and Thomas Lamont 2010).

In healthy infants, the occurrence of *C. difficile* coincided with an increased resemblance to the maternal gut microbial community, general increase in microbiome richness and with several obligate anaerobic commensals, butyrate producers and a generally more adult-like microbiota, indicating that *C. difficile* may be a transient hallmark of healthy gut microbiome maturation (Bäckhed et al. 2015; Yassour et al. 2016; Ferretti et al. 2018; Chu et al. 2017; Rodríguez et al. 2015). In contrast, *C. difficile* presence in adulthood was associated with decreased microbiome richness and an enrichment in facultative and obligate aerobes, generally associated with dysbiosis (Rigottier-Gois 2013; Rivera-Chávez et al. 2016). We found multiple lines of evidence suggesting that the transition between these apparent dichotomic states (commensal in infancy, pathobiont in adulthood), begins at around 10 (±2) months of age. We hypothesise that *C. difficile* could be an indicator species of a larger-scale and fundamental change taking place in the microbial ecology of the gut.

C. difficile toxin genes were likewise prevalent among infants, in line with previous reports (Rousseau et al. 2012; Kubota et al. 2016; Mani et al. 2023), indicating asymptomatic carriage of toxigenic strains. Indeed, *C. difficile* testing is generally discouraged by paediatric guidelines

even in presence of diarrhoeal symptoms (McDonald et al. 2018; Schutze et al. 2013) and toxin concentration may not be a reliable proxy for inferring disease and its severity (Davis et al. 2016; Kubota et al. 2016). The apparent protection from CDI symptoms has been ascribed to a lack of toxin receptors in the immature infant gut, although this early hypothesis (Chang et al. 1986) has since been called into question (Keel and Songer 2007; Eglow et al. 1992). The biotic context of *C. difficile* suggests additional or alternative explanations. We observed a significant enrichment in butyrate producers co-occurring with *C. difficile* in healthy infants, but not later in life nor in diseased infants. High levels of butyrate have been linked to inflammation inhibition, regulation of cell-to-cell tight junctions and increased mucin production (and therefore mucosal layer thickness and integrity) (Cornick et al. 2015; Willemsen et al. 2003), all considered protective against *C. difficile* toxins (Pruitt and Lacy 2012).

Our analyses are descriptive and arguably inherently limited by the underlying metagenomic data, such as DNA extraction bias against endospores (Felczykowska et al. 2015) or general detection limits due to finite sequencing depth. Moreover, although some longitudinal data was available, time series were too sparse to infer causal relationships, such as e.g. the possible role of *C. difficile* in a partially deterministic community succession in the infant gut. To our knowledge, ours is the largest single-species metagenomic survey to date, demonstrating the utility of metagenomic meta-studies as a nuanced approach to gut microbial ecology, beyond *C. difficile*. Overall, our results suggest that the ability of *C. difficile* to induce disease may be context-dependent and multifactorial (i.e influenced by the rest of the microbiome composition, toxin presence and host age). Further study of *C. difficile*'s association with health outcomes should therefore adopt a more holistic view of the ambivalent role of this species over the human lifespan.

Methods

Human study

We compiled a metagenomic dataset of both public (see below) and 447 newly generated samples. The novel cohort is part of the citizen science-based my.microbes project (http://my.microbes.eu/) (Voigt et al. 2015), conducted in accordance with the WMA Declaration of Helsinki and with the approval of the EMBL Bioethics Internal Advisory Board. Sample collection, processing and sequencing were performed as previously described in (Voigt et al.

2015; Costea et al. 2017; Hildebrand et al. 2021). This cohort includes human fecal (n=437) and oral (n=10) samples, from a total of 142 subjects (aged 0-97 years). Self-reported subject metadata are included in **Supplementary Table 4**.

Public data overview

CDI meta-analysis

In the CDI meta-analysis (see **Supplementary Table 1)**, out of 534 samples in total (from as many subjects, with an average age of 60±19.9 years), 234 were identified as CDI, 114 as diseased controls and 186 as healthy controls. Samples from subjects diagnosed with CDI in the original study population were classified as CDI in our meta-analysis. Samples were identified as controls in any of the following cases: (i) H-Ctr: healthy subject, reported as "control" in the metadata of the original study population; or (ii) D-ctr: diseased subject, that either had diarrhoeal symptoms but negative CDI diagnostic outcome, or was diarrhoea-free but diagnosed for a disease other than CDI. The majority of the samples in the latter group (D-Ctr) belong to hospitalised subjects. Study-specific CDI diagnostic criteria are indicated, when published in the original studies, in Supplementary Figure 1.

Global meta-analysis

We analysed a total of 42,900 publicly available metagenomic samples (collectively >220Tbp) from 253 different studies, including the novel cohort of 447 samples from the citizen science project MyMicrobes described above (see **Supplementary Table 2**). The collection includes samples from 86 countries, 35 animal species and 6 different human body sites: gastrointestinal tract (stools, rectal swabs and biopsies), vagina, skin, oral cavity, respiratory tract and human milk. The selected environmental samples come from potentially faecally contaminated habitats, such as wastewater, freshwater, indoor surfaces, soil and polluted harbor marine waters.

Public data download

Metagenomes publicly available on the 22nd of April 2021 were downloaded using fetch-data (Coelho et al. 2021). The collection process aimed at leveraging the largest possible set of samples and to maximise the number of covered environments, with the only inclusion criteria being samples sequenced with whole genome sequencing on Illumina platforms. No minimum number of samples per study or minimum sequencing depth threshold was applied at this stage.

Metadata curation

Manually curated metadata for each dataset include: health status, age, geography (country and continent name), westernised lifestyle or not, diagnosis for *C. difficile* infection (CDI), use of antibiotics, delivery mode, premature birth (pre-term or full-term), and sex. Subjects were categorised by age group, defined as follows: 0 ≤ infant ≤ 12 months, 1 year < child ≤ 10 years, 10 years < adolescent ≤ 18 years, 18 years < adult ≤ 65 years, and elderly > 65 years of age. If a specific age was not available, a range of age was provided in alternative. Given the broad range of perturbations in the gut microbiome associated with antibiotics intake, we ad-hoc defined "diseased" samples as any sample taken from a subject with any medically diagnosed disease or syndrome and/or intake of one or more antibiotics at the time of the sampling.

Filtering on read count

Two consecutive read count filtering steps were performed on all samples: (i) samples with zero reads mapping to mOTU marker genes (Milanese et al. 2019) were discarded (n=1,091) and (ii) samples with less than 59 reads mapping to mOTU marker genes, corresponding to 95%ile calculated on the remaining samples, were discarded (n=2,075). An additional third read count filtering was performed only on human gut stool samples, corresponding to 99%ile, removing samples with less than 100 reads (n=132 samples). The resulting filtered dataset included 39,338 samples, of which 26,610 were human fecal metagenomes.

Identification of timeseries-representative samples

Out of 26,610 samples, 24,690 had subject-level metadata and were associated with 12,352 unique subjects. For 3,573 subjects, multiple timepoints were available. The mean number of timepoints per subject was 2 (3.2 for infants, 1.7 for children, 3.5 for adolescents, 1.7 for adults and 1.3 for elderly), with a maximum of 205 time points per subject.

In order to avoid under- or over-estimating *C. difficile* prevalence, only one sample per time series was used in cross-sectional analyses. We distinguished three cases for each time series:

(i) *C. difficile* observed in all samples. In this case, the sample with the highest *C. difficile* read count is selected as representative and the corresponding subject was considered *C. difficile* positive.

(ii) *C. difficile* observed in none of the samples. In this case, the sample of the first time point was selected as representative and the corresponding subject was considered *C. difficile* negative.

(iii) *C. difficile* observed in only some samples. In this case, the sample with the highest *C. difficile* read count was selected as representative and the corresponding subject was considered *C. difficile* positive.

1,920 samples had no subject identifier. In this case, we assumed one sample per subject. One representative sample for each time series was used for all downstream analyses, if not specified otherwise. The timeseries dereplication procedure described above has also been applied to the CDI study populations before downstream analysis.

Metagenomic data processing

Taxonomic profiling

Metagenomes were taxonomically profiled at the species level with mOTUs v2.0 (Milanese et al. 2019), requiring the confident detection of at least two taxonomic marker genes. All data analyses were conducted in the R Statistical Computing framework v3.5 or higher. Only *C. difficile* positive samples were considered for *C. difficile* relative abundance estimation.

Microbiome diversity

Local community diversities ('alpha' diversities) of human gut samples were estimated by iteratively rarefying taxonomic count tables to 100 marker gene-mapping reads and computing average Hill diversities at q=0 (species richness), q=1 (exponential Shannon entropy) and q=2 inverse Simpson index), as well as evenness measures as ratios thereof. Unless otherwise stated, results in the main text refer to taxa richness. Differences in alpha diversity were tested using ANOVA followed by post hoc tests and Benjamini-Hochberg correction, as specified in the main text.

Prevalence and abundance estimations of C. difficile

Prevalence estimations of *C. difficile* over life time were based on human gut samples (stools, rectal swabs and biopsies) where the precise subject age in months was available (samples with missing or too broad or age ranges were discarded). Both *C. difficile* mOTUs2.0 ("ref_mOTU_0051" and "ref_mOTU_0052") were considered in downstream analysis. Abundance estimations included only *C. difficile* human stool samples with precise age metadata.

Species co-occurrence analysis

Human gut stool samples, with at least 100 reads per sample and with known age group and health status were considered for this analysis. One representative sample per time series was considered. Fisher's exact test, followed by Benjamini-Hochberg correction, were applied to identify co-occurring species. Meaningful positive association was identified for species with adjusted p-value <0.05 and logarithm of the odds ratio >1, while meaningful negative association was identified for species with adjusted p-value <0.05 and logarithm of the odds ratio < -1.

Machine learning modelling

L1-regularised LASSO logistic regression models to predict CDI status were built using the SIAMCAT R package (Wirbel et al. 2021) with 10-fold cross-validation. For this analysis, we focused on the subset of 10 CDI or diarrhoea-associated datasets (**Supplementary Table 1**) and then trained two different sets of models: one set of models to distinguish CDI samples and samples from healthy controls (excluding controls from diseased subjects) and another set of models to distinguish CDI samples and any type of control samples. In order to minimise overfitting and to counter batch effects (Wirbel et al. 2021), we pooled datasets across studies in a leave-one-study-out approach. In short, all except one study were jointly processed to train a LASSO model that was then used to predict the left-out study. Additionally, to check if the microbial signature for CDI was independent of *C. difficile*, we trained another set of models with the same cross-validation splits but excluded *C. difficile* from the feature table. Feature weights were extracted from the models, normalised by the absolute sum of feature weights, and averaged across cross-validation folds. For the heatmap in Figure 2, all microbial species that were assigned non-zero weights in at least 80% of cross-validation folds were included.

Linear mixed-effect model

To test for differential abundance of microbial species between CDI and non-CDI samples while taking into account possible confounding factors, we employed linear mixed-effect models as implemented in the lmerTest package (Kuznetsova et al. 2017). After filtering for prevalence (prevalence of at least 5% in three or more studies), we tested the log-transformed abundance of each microbial species using a linear mixed-ffect model with "CDI status" as fixed and "Study" and "Age group" as random effects. Effect size and p-values were extracted from the model and p-values were corrected for multiple hypothesis testing using the Benjamini-Hochberg procedure. In addition, we used a linear mixed-effect model to test for a relationship between sequencing depth and the ability to detect *C. difficile* in CDI study populations as well

as on the wider global set of samples (considering only dereplicated samples from time series). In this analysis, study was considered as a random effect.

Pathogenic toxin genes detection in metagenomes

Identification of toxin-coding genes and their assignment was performed using the VFDB database (Liu et al. 2019), downloaded in March 2021. In our analysis we did not investigate the presence of the *C. difficile* binary toxin gene, since this toxin alone has not been associated with disease severity (Goldenberg and French 2011). Mapping against VFDB was performed via BWA (Li and Durbin 2009) and filtering via NGLess (Coelho et al. 2019), using 99% as minimal alignment percentage identity threshold and 75bp as minimal read length match. Toxin gene profiles were computed with gffquant (https://github.com/grp-bork/gff_quantifier, version 1.2.3) using the 1overN count model. Toxin genes included in VFDB refer to the following strains: *C. difficile* 630, *E. coli* CFT073, O44:H18 042 and O157:H7 str. EDL933, *S. flexneri* 2a str.301, *P. aeruginosa* PAO1, *C. perfringens* str.13 and SM101, *S. aureus* RN4220, subsp. aureus MW2 and N315. The comprehensive list of virulence-related genes for each of these strains is available on the VFDB website (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi).

Subspecies analysis

C. difficile subspecies detection was performed on all *C. difficile* positive samples with MetaSNV2 (Van Rossum et al. 2021). Reads from 2,428 samples were mapped against proGenomes v1 (Mende et al. 2017) species representatives genomes for 3 species in the Clostridium genus: i) specI_v2_0051 (NCBI taxonomy ID 272563, PRJNA78) *Clostridioides difficile*, ii) specI_v2_0052 (NCBI taxonomy ID 1151292, PRJNA85757) *Clostridioides difficile* and iii) specI_v2_1125 (NCBI taxonomy ID 1408823, PRJNA223331) *Clostridioides mangenotii.* By mapping to multiple genomes and only using the uniquely mapped reads, we focused on the species-specific core genomic regions. Mappings that had at least 97% identity and a match length of at least 45bp were kept. Mapping and filtering was performed using BWA (Li and Durbin 2009) and NGLess (Coelho et al. 2019). Reads that mapped uniquely across the 3 reference genomes were used to call single nucleotide variants (SNVs) using metaSNV2 with default parameters. Of the initial 2383 samples, 197 passed the requirements for robust SNV calling (8%) and 179 could be used to detect subspecies presence. Substructure within the population was assessed in the resultant SNV profiles according to a previously reported approach (Li and Durbin 2009; Costea et al. 2017). Briefly, dissimilarities between samples

were calculated based on SNV abundance profiles and the resultant distance matrix was tested for clusters using the Prediction Strength algorithm (Yuan et al. 2020). Distance matrix is plotted using R and the pheatmap package with average clustering. Six samples with extreme dissimilarity to all other samples were removed from the distance matrix for illustrative purposes (SAMN08918181, SAMN09980608, SAMN10722477, SAMN13091313, SAMN13091317, SAMN13091322).

Building SNP-tree

SNPs obtained via metaSNV2 were arranged into an alignment PHY input format file suitable for IQ-TREE2 tree-builder (Minh et al. 2020). Sites which had multiple variants/alleles (in this case only SNPs) were replaced with alternative non-reference allele if they were supported by >50% of reads mapped against specI_v2_0051 (NCBI taxonomy ID 272563, PRJNA78), see 'Subspecies analysis' section for mapping details. This results in forced fixing of alleles across polymorphic sites within each sample. To include specI_v2_0052 (NCBI taxonomy ID 1151292, PRJNA85757) *Clostridioides difficile* and specI_v2_1125 (NCBI taxonomy ID 1408823, PRJNA223331) *Clostridioides mangenotii* into SNP input file for IQTREE2 we used Mauve(Darling et al., 2004) to 1) order contigs of both genomes against specI_v2_0051 genome, 2) align ordered contigs, 3) export SNP profile from Mauve and further merged alignment SNP profile on metaSNV2 output file using matching positions. Missing coverage was kept as gaps in the alignment and tree construction. Following command was used to build phylogenetic tree: iqtree -s <input snp file> -m GTR+ASC -B 1000.

Availability of data and materials

This study did not generate new unique reagents. Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contacts, Sebastian Schmidt (sebastian.schmidt@embl.de) and Peer Bork (bork@embl.de). Novel raw metagenomic sequencing data have been uploaded to the European Nucleotide Archive under the accession number PRJEB50977. The accession numbers relative to the other public studies included in this meta-analysis are available in Supplementary Table 2. The filtered taxonomic profiles and associated metadata used for the analyses are available in Supplementary Tables 4-6. The analysis code and commands used in the project are deposited in the GitHub repository at https://github.com/grp-bork/cdifficile_Ferretti_2022.

Acknowledgements

We are thankful to the members of the groups of Peer Bork, Georg Zeller, Thomas Dandekar (University of Würzburg) and Dr. Athanasios Typas (EMBL) for fruitful discussions. We are also grateful to Lia Oken for her continuous support and her availability for brainstorming sessions. We acknowledge funding from EMBL and the European Research Council (MicrobioS grant no. ERC-AdG-669830 to P.B.). This work comprises results from Pamela Ferretti's doctoral thesis.

Author contributions

Conceptualization, P.F., T.SB.S. and P.B.; data collection, P.F., T.SB.S., R.A., A. F., W.A. and M.K.; metadata curation, P.F., T.SB.S., R.A., A.S., R.T., L.T, I.L. and M.K.; novel samples collection and processing, S. K., R.H. and A.T.; data analysis, P.F., J.W., O.M.M., T.VR., R.A., A. F., W.A., C.S. and T.SB.S.; writing – original draft, P.F. and T.SB.S.; writing – review & editing, P.F., J.W., O.M.M., T.VR, C.S., M.K, G.Z. T.SB.S. and P.B.; supervision, T.SB.S., G.Z. and P.B.; funding acquisition, P.B.

Declaration of interests

The authors have no conflicts of interest to declare.

Figure Legends

Figure 1. Metagenomic meta-analysis of the gut microbiome associated with CDI

(A) On top, fecal metagenomes used in the meta-analysis from 10 public CDI or diarrhoeal study populations, divided by groups: subjects diagnosed with CDI (CDI, n=234), subjects diagnosed with other diseases than CDI (D-Ctr, n=114), and healthy subjects (H-Ctr, n=186) samples. See Methods for further group description. At the bottom, fraction of *C. difficile* positive samples per group. *C. difficile* toxin genes were found only among CDI *C. difficile* positive samples. (B) Rarefied species richness across the three groups. (C) Prevalence, relative abundance of species that induce antibiotic-associated diarrhoea (AAD) among CDI and controls. (D) Prevalence of single and multiple toxigenic species found among *C. difficile* positive and *C. difficile* negative samples, divided by sample group. Mean comparison p-values calculated using Wilcoxon-test, * for $P \leq 0.05$, ** for $P \leq 0.01$, *** for $P \leq 0.001$, **** for $P \leq 0.0001$, empty when non significant.

Figure 2. A CDI-specific microbial signature

(A) Microbial species signature associated with CDI, healthy controls and diseased controls, as seen by LASSO model and (B) its associated AUC values for all samples as well as for single study populations, when comparing CDI to healthy and diseased controls combined (left) and CDI with healthy controls only (right). In the latter, the AUC value of Vincent_2016 is left intentionally blank as only diseased controls (D-Ctr) are available for this study population (see **Supplementary Table 1**).

Figure 3. *C. difficile* tracking in a global dataset collection

(A) Overview of the global dataset collection composed of 42,900 samples, from 253 publicly available studies, including host-associated and environmental samples (internal ring), and their subcategories (external ring). The human gut was the most represented environment (66.3%, n=28,423) in our collection. (B) Stratification of the human gut samples, divided by health status and age group (see Methods for detailed age group description, numbers refer to the number of samples prior to read filtering). (C) Prevalence of *C. difficile* positive samples per category. Total values refer to the number of samples after the initial read filtering and before time series dereplication (see Methods). From this point onwards, results refer to this extended collection of 42,900 samples.

Figure 4. *C. difficile* prevalence and associated microbial diversity

(A) *C. difficile* prevalence in stool samples in healthy and diseased human (left) and animal (right) hosts over lifetime. *C. difficile* prevalence divided by (B) health status and delivery mode, (C) health status and prematurity and (D) feeding mode within the first semester of life. For animal samples, only species with at least ten total samples are shown. (E) Species richness of human stool samples, with and without *C. difficile,* across age groups in healthy (left) and diseased subjects (right). See Methods for details on disease and age group definitions. For samples belonging to time series, only the representative samples are included in the estimations (see Methods). Mean comparison p-values calculated using t-test, * for $P \leq 0.05$, ** for $P \leq 0.01$, *** for $P \leq 0.001$, **** for $P \leq 0.0001$, empty when non significant.

Figure 5. Biotic context associated with *C. difficile*

Species associated with *C. difficile*, divided by age group and health status: significant positive associations are shown in dark orange, significant negative ones in dark blue (p-values adjusted using BH). Lighter shades indicate non significant associations. Only species significantly associated with *C. difficile* in at least one age group are shown. Number of samples per each category shown in the lower part. See Methods for details on age group definitions. Species group separation was not affected by presence of *C. difficile* toxin genes (data not shown). On the right, per species annotation of their i) oxygen requirement (see also Supplementary Figure 12 for the differential enrichment across the two groups) and ii) trend over lifetime. Positive Spearman's rho values indicate species more commonly found in the gut microbiome of healthy adults, negative values the opposite trend.

Supplementary Figure Legends

Supplementary figure 1. (A) Prevalence of *C. difficile* and other antibiotic-associated diarrhoea (AAD) species, divided by study. For *C. difficile* only, the portion of samples with toxigenic *C. difficile* is shown (dotted segments). CDI diagnosis procedure is shown on top of each study. Each row represents the diagnostic algorithm (combination of tests) used to diagnose CDI. Tests performed are indicated with black dots (white if not). For example, in "Langdon et al. 2021", CDI was diagnosed if symptoms were present and toxigenic culture for *C. difficile* was positive, or if symptoms were present and the enzymatic immunoassays for *C. difficile* was positive, or if pseudomembranous colitis was identified. Diagnostic protocols abbreviations: "EIA": Enzymatic ImmunoAssays, "GDH": glutamate dehydrogenase, "NAAT" nucleic acid amplification test (including PCR), "PMC": pseudomembranous colitis. (B) Prevalence of the number of AAD species (*C. difficile* not included) identified in each CDI sample, divided by *C. difficile* positivity.

Supplementary Figure 2. Species-level composition for CDI, diseased control and healthy control samples as seen by mOTUs v2.0. Species with minimum relative abundance ≥ 0.01 and prevalence ≥ 0.1 are shown.

Supplementary figure 3. Species significantly enriched (in yellow) or depleted (in blue) in terms of relative abundance in CDI compared to diseased and healthy controls, as identified by linear mixed-effect model analysis. Species known to cause antibiotic-associated diarrhoea (AAD) are highlighted in red. *C. difficile* is not included in the analysis. Study and age group were considered as nuisance variables (and modelled as random effects). To be noted that *Enterobacteriaceae* sp. includes *Shigella flexneri* and *Escherichia coli*.

Supplementary figure 4. (A) To check the influence of sequencing depth on the detection of *C. difficile*, the marker gene coverage values across all samples were binned into ten quantiles. The proportion of *C. difficile*-positive samples is shown in the barplot. A possible association between coverage quantile and *C. difficile* detection was tested using a linear mixed-effect model with Study as random effect (P-value=0.63). (B) Adjusted p-values for ANOVA analysis aiming to explain variance in *C. difficile* presence by the available metadata, either as single factors (two top rows) or by a combination of multiple factors (sequential, bottom rows, in the

exact order shown along the X axis), in data sets that were broken down by age group (infants versus adults).

Supplementary figure 5. (A) Geographical distribution across continents and countries for *C. difficile* prevalence in the stools of healthy infants (left) and adults (right). Only countries with *C. difficile* prevalence above 1% are shown. Prevalence was calculated based on presence/absence of *C. difficile* from mOTUs v2.0[30] taxonomic profiles. (B) Prevalence of *C. difficile* across subjects diagnosed with specific diseases (left) and subjects taking antibiotics (right). Average *C. difficile* prevalence is shown with grey bars, age-group specific prevalence is highlighted with colored dots. Number of datasets used for prevalence estimations is reported in the lower part. Abbreviations: NEC: "Necrotizing enterocolitis"; ASCVD: "atherosclerotic cardiovascular disease"; T2D: "Type 2 diabetes"; CRC: "Colorectal cancer"; ADA: "advanced adenoma"; NAA: "non-advanced adenoma". See Methods for details on age group definitions.

Supplementary figure 6. *C. difficile* prevalence in infants and children, divided by possible combinations of health status, prematurity and delivery mode.

Supplementary figure 7. Time of the first appearance of *C. difficile* in infants and children timeseries. The grey line indicates the total number of samples per age interval, considering all samples independently of health status, delivery mode, gestational age and diet.

Supplementary figure 8. Community similarity (Bray-Curtis index) of healthy infant-mother pairs in presence or absence of *C. difficile* in stools across the first four years of life. Only full-term infant samples were included. Mean comparison p-values calculated using t-test, * for $P \leq 0.05$, ** for $P \leq 0.01$, *** for $P \leq 0.001$, **** for $P \leq 0.0001$, empty when non significant.

Supplementary figure 9. Prevalence of *C. difficile* toxin genes in the stools of healthy and diseased subjects over lifetime, based on the detection of either one or both of *C. difficile* Toxin A (*tcdA*) or Toxin B genes (*tcdB*) (see Methods for details). Prevalence is calculated on the number of *C. difficile* positive samples. *C. difficile* toxin genes were exclusively found among human stool samples. See Methods for details on age group definitions.

Supplementary figure 10. Alpha diversity calculations in *C. difficile* positive and negative samples. (A) Species richness in healthy infants with detailed age resolution. (B-C) species

richness in infant samples divided by delivery mode, gestational age and health status. (D) Species evenness (as the inverse Simpson index divided by Richness) across age groups and health status. Species richness for continents with at least five *C. difficile* positive samples are shown. The number of samples per group is shown under each boxplot. Mean comparison p-values calculated using t-test, * for P ≤ 0.05, ** for P ≤ 0.01, *** for P ≤ 0.001, **** for P ≤ 0.0001, empty when non significant. See Methods for details on age group definitions.

Supplementary figure 11. Species with co-occurrence (logOR>0) or co-exclusion (logOR<0) pattern with *C. difficile* across gut stool samples from humans (A) and animals (B). Labelled species are highlighted in aquamarine and violet, for humans and animals respectively. *Clostridium paraputrificum* and *Clostridium neonatale* were among the very few species significantly associated with *C. difficile* in both human and animal stools.

Supplementary Figure 12. Spearman's Rho values for the species listed in Figure 5, across the two groups. Mean comparison p-values calculated using t-test, * for P ≤ 0.05, ** for P ≤ 0.01, *** for P ≤ 0.001, **** for P ≤ 0.0001, empty when non significant.

Supplementary Figure 13. SNV similarity across *C. difficile* positive samples from our global metagenomic survey. Tree rooted with *Clostridioides mangenotii* as outgroup. Missing metadata are shown in white. All metagenomes have ANI similarity >95%. See Methods for detailed analysis description.

Supplemental Table Legends

Supplementary Table 1. Overview of the public metagenomic CDI study populations included in the CDI meta-analysis, divided by sample group.

Supplementary Table 2. Overview of the public metagenomic studies included in the global meta-analysis.

Supplementary Table 3. Mean *C. difficile* prevalence and relative abundance estimation across age groups and health status.

Supplementary Tables 4. Samples metadata before read filtering and timeseries dereplication steps.

Supplementary Table 5. Samples taxonomic profiles, as provided by mOTUs v2.0.

References

Bäckhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., Li, Y., Xia, Y., Xie, H., Zhong, H., Khan, M. T., Zhang, J., Li, J., Xiao, L., Al-Aama, J., Zhang, D., Lee, Y. S., Kotowska, D., Colding, C., … Wang, J. (2015). Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. *Cell Host & Microbe*, *17*(6), 852.

Bartlett, J. G., & Gerding, D. N. (2008). Clinical recognition and diagnosis of Clostridium difficile infection. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, *46 Suppl 1*, S12–S18.

Bartlett, J. G., Moon, N., Chang, T. W., Taylor, N., & Onderdonk, A. B. (1978). Role of Clostridium difficile in antibiotic-associated pseudomembranous colitis. *Gastroenterology*, *75*(5), 778–782.

Bauer, M. P., Farid, A., Bakker, M., Hoek, R. A. S., Kuijper, E. J., & van Dissel, J. T. (2014). Patients with cystic fibrosis have a high carriage rate of non-toxigenic Clostridium difficile. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *20*(7), O446–O449.

BBL™ Clostridium difficile Selective Agar. (n.d.). Retrieved October 21, 2021, from https://www.bd.com/resource.aspx?IDX=20564

Burnham, C.-A. D., & Carroll, K. C. (2013). Diagnosis of Clostridium difficile infection: an ongoing conundrum for clinicians and for clinical laboratories. *Clinical Microbiology Reviews*, *26*(3), 604–630.

CDC. (2019). *Antibiotic resistance threats in the United States, 2019*. https://doi.org/10.15620/cdc:82532

Chang, T. W., Sullivan, N. M., & Wilkins, T. D. (1986). Insusceptibility of fetal intestinal mucosa and fetal cells to Clostridium difficile toxins. *Zhongguo Yao Li Xue Bao = Acta Pharmacologica Sinica*, *7*(5), 448–453.

Chia, J.-H., Feng, Y., Su, L.-H., Wu, T.-L., Chen, C.-L., Liang, Y.-H., & Chiu, C.-H. (2017). Clostridium innocuum is a significant vancomycin-resistant pathogen for extraintestinal clostridial infection. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *23*(8), 560–566.

Chia, J.-H., Wu, T.-S., Wu, T.-L., Chen, C.-L., Chuang, C.-H., Su, L.-H., Chang, H.-J., Lu, C.-C., Kuo, A.-J., Lai, H.-C., & Chiu, C.-H. (2018). Clostridium innocuum is a vancomycin-resistant pathogen that may cause antibiotic-associated diarrhoea. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *24*(11), 1195–1199.

Chu, D. M., Ma, J., Prince, A. L., Antony, K. M., Seferovic, M. D., & Aagaard, K. M. (2017). Maturation of the infant microbiome community structure and function across multiple body sites and in relation to mode of delivery. *Nature Medicine*, *23*(3), 314–326.

Coelho, L. P., Alves, R., Del Río, Á. R., Myers, P. N., Cantalapiedra, C. P., Giner-Lamia, J., Schmidt, T. S., Mende, D. R., Orakov, A., Letunic, I., Hildebrand, F., Van Rossum, T., Forslund, S. K., Khedkar, S., Maistrenko, O. M., Pan, S., Jia, L., Ferretti, P., Sunagawa, S., … Bork, P. (2021). Towards the biogeography of prokaryotic genes. *Nature*. https://doi.org/10.1038/s41586-021-04233-4

Coelho, L. P., Alves, R., Monteiro, P., Huerta-Cepas, J., Freitas, A. T., & Bork, P. (2019). NG-meta-profiler: fast processing of metagenomes using NGLess, a domain-specific language. *Microbiome*, *7*(1), 84.

Cornick, S., Tawiah, A., & Chadee, K. (2015). Roles and regulation of the mucus barrier in the gut. *Tissue Barriers*, *3*(1-2), e982426.

Costea, P. I., Coelho, L. P., Sunagawa, S., Munch, R., Huerta-Cepas, J., Forslund, K., Hildebrand, F., Kushugulova, A., Zeller, G., & Bork, P. (2017). Subspecies in the global human gut microbiome. *Molecular Systems Biology*, *13*(12), 960.

Crobach, M. J. T., Planche, T., Eckert, C., Barbut, F., Terveer, E. M., Dekkers, O. M., Wilcox, M.

H., & Kuijper, E. J. (2016). European Society of Clinical Microbiology and Infectious Diseases: update of the diagnostic guidance document for Clostridium difficile infection. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *22 Suppl 4*, S63–S81.

Crobach, M. J. T., Vernon, J. J., Loo, V. G., Kong, L. Y., Péchiné, S., Wilcox, M. H., & Kuijper, E. J. (2018). Understanding Clostridium difficile Colonization. *Clinical Microbiology Reviews*, *31*(2). https://doi.org/10.1128/CMR.00021-17

Daquigan, N., Seekatz, A. M., Greathouse, K. L., Young, V. B., & White, J. R. (2017). High-resolution profiling of the gut microbiome reveals the extent of Clostridium difficile burden. *NPJ Biofilms and Microbiomes*, *3*, 35.

Darling, A. C. E., Mau, B., Blattner, F. R., & Perna, N. T. (2004). Mauve: multiple alignment of conserved genomic sequence with rearrangements. *Genome Research*, *14*(7), 1394–1403.

Davis, M. Y., Zhang, H., Brannan, L. E., Carman, R. J., & Boone, J. H. (2016). Rapid change of fecal microbiome and disappearance of Clostridium difficile in a colonized infant after transition from breast milk to cow milk. *Microbiome*, *4*(1), 53.

Deane, J., Fouhy, F., Ronan, N. J., Daly, M., Fleming, C., Eustace, J. A., Shanahan, F., Flanagan, E. T., Dupont, L., Harrison, M. J., Haworth, C. S., Floto, A., Rea, M. C., Ross, R. P., Stanton, C., & Plant, B. J. (2021). A multicentre analysis of Clostridium difficile in persons with Cystic Fibrosis demonstrates that carriage may be transient and highly variable with respect to strain and level. *The Journal of Infection*, *82*(3), 363–370.

Drall, K. M., Tun, H. M., Morales-Lizcano, N. P., Konya, T. B., Guttman, D. S., Field, C. J., Mandal, R., Wishart, D. S., Becker, A. B., Azad, M. B., Lefebvre, D. L., Mandhane, P. J., Moraes, T. J., Sears, M. R., Turvey, S. E., Subbarao, P., Scott, J. A., & Kozyrskyj, A. L. (2019). Clostridioides difficile Colonization Is Differentially Associated With Gut Microbiome Profiles by Infant Feeding Modality at 3–4 Months of Age. In *Frontiers in Immunology* (Vol. 10). https://doi.org/10.3389/fimmu.2019.02866

Eglow, R., Pothoulakis, C., Itzkowitz, S., Israel, E. J., O'Keane, C. J., Gong, D., Gao, N., Xu, Y. L., Walker, W. A., & LaMont, J. T. (1992). Diminished Clostridium difficile toxin A sensitivity in newborn rabbit ileum is associated with decreased toxin A receptor. *The Journal of Clinical Investigation*, *90*(3), 822–829.

Felczykowska, A., Krajewska, A., Zielińska, S., & Łoś, J. M. (2015). Sampling, metadata and DNA extraction - important steps in metagenomic studies. *Acta Biochimica Polonica*, *62*(1), 151–160.

Ferretti, P., Pasolli, E., Tett, A., Asnicar, F., Gorfer, V., Fedi, S., Armanini, F., Truong, D. T., Manara, S., Zolfo, M., Beghini, F., Bertorelli, R., De Sanctis, V., Bariletti, I., Canto, R., Clementi, R., Cologna, M., Crifò, T., Cusumano, G., … Segata, N. (2018). Mother-to-Infant Microbial Transmission from Different Body Sites Shapes the Developing Infant Gut Microbiome. *Cell Host & Microbe*, *24*(1), 133–145.e5.

Galdys, A. L., Nelson, J. S., Shutt, K. A., Schlackman, J. L., Pakstis, D. L., Pasculle, A. W., Marsh, J. W., Harrison, L. H., & Curry, S. R. (2014). Prevalence and duration of asymptomatic Clostridium difficile carriage among healthy subjects in Pittsburgh, Pennsylvania. *Journal of Clinical Microbiology*, *52*(7), 2406–2409.

Gateau, C., Couturier, J., Coia, J., & Barbut, F. (2018). How to: diagnose infection caused by Clostridium difficile. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *24*(5), 463–468.

Goldenberg, S. D., & French, G. L. (2011). Lack of association of tcdC type and binary toxin status with disease severity and outcome in toxigenic Clostridium difficile. *The Journal of Infection*, *62*(5), 355–362.

Golić, N., Veljović, K., Popović, N., Djokić, J., Strahinić, I., Mrvaljević, I., & Terzić-Vidojević, A. (2017). In vitro and in vivo antagonistic activity of new probiotic culture against Clostridium difficile and Clostridium perfringens. *BMC Microbiology*, *17*(1), 108.

Hall, I. C., & O'toole, E. (1935). INTESTINAL FLORA IN NEW-BORN INFANTS: WITH A

DESCRIPTION OF A NEW PATHOGENIC ANAEROBE, BACILLUS DIFFICILIS. *American Journal of Diseases of Children* , *49*(2), 390–402.

Hensgens, M. P. M., Dekkers, O. M., Demeulemeester, A., Buiting, A. G. M., Bloembergen, P., van Benthem, B. H. B., Le Cessie, S., & Kuijper, E. J. (2014). Diarrhoea in general practice: when should a Clostridium difficile infection be considered? Results of a nested case-control study. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *20*(12), O1067–O1074.

Hildebrand, F., Gossmann, T. I., Frioux, C., Özkurt, E., Myers, P. N., Ferretti, P., Kuhn, M., Bahram, M., Nielsen, H. B., & Bork, P. (2021). Dispersal strategies shape persistence and evolution of human gut bacteria. *Cell Host & Microbe*, *29*(7), 1167–1176.e9.

Högenauer, C., Hammer, H. F., Krejs, G. J., & Reisinger, E. C. (1998). Mechanisms and management of antibiotic-associated diarrhea. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, *27*(4), 702–710.

Humphries, R. M., Uslan, D. Z., & Rubin, Z. (2013). Performance of Clostridium difficile toxin enzyme immunoassay and nucleic acid amplification tests stratified by patient disease severity. *Journal of Clinical Microbiology*, *51*(3), 869–873.

Jackson, M., Olefson, S., Machan, J. T., & Kelly, C. R. (2016). A high rate of alternative diagnoses in patients referred for presumed Clostridium difficile infection. *Journal of Clinical Gastroenterology*, *50*(9), 742–746.

Jangi, S., & Thomas Lamont, J. (2010). Asymptomatic Colonization by Clostridium difficile in Infants: Implications for Disease in Later Life. In *Journal of Pediatric Gastroenterology & Nutrition* (Vol. 51, Issue 1, pp. 2–7). https://doi.org/10.1097/mpg.0b013e3181d29767

Jo, H. B., Ham, S. Y., Jung, J., Moon, S. M., Kim, N.-H., Song, K.-H., Park, J. S., Park, K. U., Kim, E. S., & Kim, H. B. (2022). Prevalence of and factors associated with inappropriate Clostridioides difficile testing in a teaching hospital in Korea. *Antimicrobial Resistance and Infection Control*, *11*(1), 70.

Kato, H., Kita, H., Karasawa, T., Maegawa, T., Koino, Y., Takakuwa, H., Saikai, T., Kobayashi, K., Yamagishi, T., & Nakamura, S. (2001). Colonisation and transmission of Clostridium difficile in healthy individuals examined by PCR ribotyping and pulsed-field gel electrophoresis. *Journal of Medical Microbiology*, *50*(8), 720–727.

Keel, M. K., & Songer, J. G. (2007). The distribution and density of Clostridium difficile toxin receptors on the intestinal mucosa of neonatal pigs. *Veterinary Pathology*, *44*(6), 814–822.

Kim, J., Cho, Y., Seo, M.-R., Bae, M. H., Kim, B., Rho, M., & Pai, H. (2020). Quantitative characterization of Clostridioides difficile population in the gut microbiome of patients with C. difficile infection and their association with clinical factors. In *Scientific Reports* (Vol. 10, Issue 1). https://doi.org/10.1038/s41598-020-74090-0

Kiu, R., & Hall, L. J. (2018). An update on the human and animal enteric pathogen Clostridium perfringens. *Emerging Microbes & Infections*, *7*(1), 141.

Kociolek, L. K., & Gerding, D. N. (2016). Breakthroughs in the treatment and prevention of Clostridium difficile infection. *Nature Reviews. Gastroenterology & Hepatology*, *13*(3), 150–160.

Korpela, K., Costea, P., Coelho, L. P., Kandels-Lewis, S., Willemsen, G., Boomsma, D. I., Segata, N., & Bork, P. (2018). Selective maternal seeding and environment shape the human gut microbiome. *Genome Research*, *28*(4), 561–568.

Kubota, H., Makino, H., Gawad, A., Kushiro, A., Ishikawa, E., Sakai, T., Akiyama, T., Matsuda, K., Martin, R., Knol, J., & Oishi, K. (2016). Longitudinal Investigation of Carriage Rates, Counts, and Genotypes of Toxigenic Clostridium difficile in Early Infancy. *Applied and Environmental Microbiology*, *82*(19), 5806–5814.

Kumar, R., Yi, N., Zhi, D., Eipers, P., Goldsmith, K. T., Dixon, P., Crossman, D. K., Crowley, M. R., Lefkowitz, E. J., Martin Rodriguez, J., & Morrow, C. D. (2017). Identification of donor microbe species that colonize and persist long term in the recipient after fecal transplant for recurrent Clostridium difficile. In *npj Biofilms and Microbiomes* (Vol. 3, Issue 1).

https://doi.org/10.1038/s41522-017-0020-7

Kuznetsova, A., Brockhoff, P. B., & Christensen, R. H. B. (2017). lmerTest Package: Tests in Linear Mixed Effects Models. In *Journal of Statistical Software* (Vol. 82, Issue 13). https://doi.org/10.18637/jss.v082.i13

Langdon, A., Schwartz, D. J., Bulow, C., Sun, X., Hink, T., Reske, K. A., Jones, C., Burnham, C.-A. D., Dubberke, E. R., Dantas, G., & CDC Prevention Epicenter Program. (2021). Microbiota restoration reduces antibiotic-resistant bacteria gut colonization in patients with recurrent Clostridioides difficile infection from the open-label PUNCH CD study. *Genome Medicine*, *13*(1), 28.

Larcombe, S., Hutton, M. L., & Lyras, D. (2016). Involvement of Bacteria Other Than Clostridium difficile in Antibiotic-Associated Diarrhoea. *Trends in Microbiology*, *24*(6), 463–476.

Larcombe, S., Hutton, M. L., Riley, T. V., Abud, H. E., & Lyras, D. (2018). Diverse bacterial species contribute to antibiotic-associated diarrhoea and gastrointestinal damage. *The Journal of Infection*, *77*(5), 417–426.

Lee, H. S., Plechot, K., Gohil, S., & Le, J. (2021). Clostridium difficile: Diagnosis and the Consequence of Over Diagnosis. *Infectious Diseases and Therapy*, *10*(2), 687–697.

Lees, E. A., Miyajima, F., Pirmohamed, M., & Carrol, E. D. (2016). The role of Clostridium difficile in the paediatric and neonatal gut - a narrative review. *European Journal of Clinical Microbiology & Infectious Diseases: Official Publication of the European Society of Clinical Microbiology*, *35*(7), 1047–1057.

Li, H., & Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* , *25*(14), 1754–1760.

Litvin, M., Reske, K. A., Mayfield, J., McMullen, K. M., Georgantopoulos, P., Copper, S., Hoppe-Bauer, J. E., Fraser, V. J., Warren, D. K., & Dubberke, E. R. (2009). Identification of a pseudo-outbreak of Clostridium difficile infection (CDI) and the effect of repeated testing, sensitivity, and specificity on perceived prevalence of CDI. *Infection Control and Hospital*

Epidemiology: The Official Journal of the Society of Hospital Epidemiologists of America, *30*(12), 1166–1171.

Liu, B., Zheng, D., Jin, Q., Chen, L., & Yang, J. (2019). VFDB 2019: a comparative pathogenomic platform with an interactive web interface. *Nucleic Acids Research*, *47*(D1), D687–D692.

Martínez-Meléndez, A., Camacho-Ortiz, A., Morfin-Otero, R., Maldonado-Garza, H. J., Villarreal-Treviño, L., & Garza-González, E. (2017). Current knowledge on the laboratory diagnosis of Clostridium difficile infection. *World Journal of Gastroenterology: WJG*, *23*(9), 1552–1567.

McDonald, L. C., Gerding, D. N., Johnson, S., Bakken, J. S., Carroll, K. C., Coffin, S. E., Dubberke, E. R., Garey, K. W., Gould, C. V., Kelly, C., Loo, V., Shaklee Sammons, J., Sandora, T. J., & Wilcox, M. H. (2018). Clinical Practice Guidelines for Clostridium difficile Infection in Adults and Children: 2017 Update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, *66*(7), e1–e48.

Mende, D. R., Letunic, I., Huerta-Cepas, J., Li, S. S., Forslund, K., Sunagawa, S., & Bork, P. (2017). proGenomes: a resource for consistent functional and taxonomic annotations of prokaryotic genomes. *Nucleic Acids Research*, *45*(D1), D529–D534.

Milanese, A., Mende, D. R., Paoli, L., Salazar, G., Ruscheweyh, H.-J., Cuenca, M., Hingamp, P., Alves, R., Costea, P. I., Coelho, L. P., Schmidt, T. S. B., Almeida, A., Mitchell, A. L., Finn, R. D., Huerta-Cepas, J., Bork, P., Zeller, G., & Sunagawa, S. (2019). Microbial abundance, activity and population genomic profiling with mOTUs2. *Nature Communications*, *10*(1), 1014.

Minh, B. Q., Schmidt, H. A., Chernomor, O., Schrempf, D., Woodhams, M. D., von Haeseler, A., & Lanfear, R. (2020). IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. *Molecular Biology and Evolution*, *37*(5), 1530–1534.

Monaghan, T. M., Sloan, T. J., Stockdale, S. R., Blanchard, A. M., Emes, R. D., Wilcox, M., Biswas, R., Nashine, R., Manke, S., Gandhi, J., Jain, P., Bhotmange, S., Ambalkar, S., Satav, A., Draper, L. A., Hill, C., & Kashyap, R. S. (2020). Metagenomics reveals impact of geography and acute diarrheal disease on the Central Indian human gut microbiome. *Gut Microbes*, *12*(1), 1752605.

Na, X., & Kelly, C. (2011). Probiotics in clostridium difficile Infection. *Journal of Clinical Gastroenterology*, *45 Suppl*, S154–S158.

Parks, D. H., Rigato, F., Vera-Wolf, P., Krause, L., Hugenholtz, P., Tyson, G. W., & Wood, D. L. A. (2021). Evaluation of the Microba Community Profiler for Taxonomic Profiling of Metagenomic Datasets From the Human Gut Microbiome. *Frontiers in Microbiology*, *0*. https://doi.org/10.3389/fmicb.2021.643682

Parks, T., Chapman, T., Culver, E., & Scarborough, M. (2011). Persistent diarrhoea after presumed Clostridium difficile infection at Oxford Radcliffe Hospitals NHS Trust: an unrecognised entity. *Gut*, *60*(Suppl 1), A72–A72.

Podlesny, D., & Florian Fricke, W. (n.d.). *Microbial Strain Engraftment, Persistence and Replacement after Fecal Microbiota Transplantation*. https://doi.org/10.1101/2020.09.29.20203638

Polage, C. R., Gyorke, C. E., Kennedy, M. A., Leslie, J. L., Chin, D. L., Wang, S., Nguyen, H. H., Huang, B., Tang, Y.-W., Lee, L. W., Kim, K., Taylor, S., Romano, P. S., Panacek, E. A., Goodell, P. B., Solnick, J. V., & Cohen, S. H. (2015). Overdiagnosis of Clostridium difficile Infection in the Molecular Test Era. *JAMA Internal Medicine*, *175*(11), 1792–1801.

Polage, C. R., Solnick, J. V., & Cohen, S. H. (2012). Nosocomial diarrhea: evaluation and treatment of causes other than Clostridium difficile. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, *55*(7), 982–989.

Pruitt, R. N., & Lacy, D. B. (2012). Toward a structural understanding of Clostridium difficile toxins A and B. *Frontiers in Cellular and Infection Microbiology*, *2*, 28.

Reich, N., Payne, M., Sharma, A., Champagne, S., Romney, M. G., Hinch, M., Leung, V., & Lowe, C. F. (2019). Prospective Review of Clostridioides difficile Testing Indications to Inform Local Laboratory Stewardship Initiatives. *Infection Prevention in Practice*, *1*(2), 100017.

Rigottier-Gois, L. (2013). Dysbiosis in inflammatory bowel diseases: the oxygen hypothesis. *The ISME Journal*, *7*(7), 1256–1261.

Rivera-Chávez, F., Zhang, L. F., Faber, F., Lopez, C. A., Byndloss, M. X., Olsan, E. E., Xu, G., Velazquez, E. M., Lebrilla, C. B., Winter, S. E., & Bäumler, A. J. (2016). Depletion of Butyrate-Producing Clostridia from the Gut Microbiota Drives an Aerobic Luminal Expansion of Salmonella. *Cell Host & Microbe*, *19*(4), 443–454.

Rodriguez, C., Warszawski, N., Korsak, N., Taminiau, B., Van Broeck, J., Delmée, M., & Daube, G. (2016). Laboratory identification of anaerobic bacteria isolated on Clostridium difficile selective medium. *Acta Microbiologica et Immunologica Hungarica*, *63*(2), 171–184.

Rodríguez, J. M., Murphy, K., Stanton, C., Ross, R. P., Kober, O. I., Juge, N., Avershina, E., Rudi, K., Narbad, A., Jenmalm, M. C., Marchesi, J. R., & Collado, M. C. (2015). The composition of the gut microbiota throughout life, with an emphasis on early life. *Microbial Ecology in Health and Disease*, *26*, 26050.

Rousseau, C., Poilane, I., De Pontual, L., Maherault, A.-C., Le Monnier, A., & Collignon, A. (2012). Clostridium difficile carriage in healthy infants in the community: a potential reservoir for pathogenic strains. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, *55*(9), 1209–1215.

Schmidt, T. S., Hayward, M. R., Coelho, L. P., Li, S. S., Costea, P. I., Voigt, A. Y., Wirbel, J., Maistrenko, O. M., Alves, R. J., Bergsten, E., de Beaufort, C., Sobhani, I., Heintz-Buschart, A., Sunagawa, S., Zeller, G., Wilmes, P., & Bork, P. (2019). Extensive transmission of microbes along the gastrointestinal tract. *eLife*, *8*. https://doi.org/10.7554/eLife.42693

Schubert, A. M., Rogers, M. A. M., Ring, C., Mogle, J., Petrosino, J. P., Young, V. B., Aronoff,

D. M., & Schloss, P. D. (2014). Microbiome data distinguish patients with Clostridium difficile infection and non-C. difficile-associated diarrhea from healthy controls. *mBio*, *5*(3), e01021–14.

Schutze, G. E., Willoughby, R. E., Committee on Infectious Diseases, & American Academy of Pediatrics. (2013). Clostridium difficile infection in infants and children. *Pediatrics*, *131*(1), 196–200.

Seekatz, A. M., Rao, K., Santhosh, K., & Young, V. B. (2016). Dynamics of the fecal microbiome in patients with recurrent and nonrecurrent Clostridium difficile infection. *Genome Medicine*, *8*(1), 47.

Smillie, C. S., Sauk, J., Gevers, D., Friedman, J., Sung, J., Youngster, I., Hohmann, E. L., Staley, C., Khoruts, A., Sadowsky, M. J., Allegretti, J. R., Smith, M. B., Xavier, R. J., & Alm, E. J. (2018). Strain Tracking Reveals the Determinants of Bacterial Engraftment in the Human Gut Following Fecal Microbiota Transplantation. *Cell Host & Microbe*, *23*(2), 229–240.e5.

Smits, W. K., Lyras, D., Lacy, D. B., Wilcox, M. H., & Kuijper, E. J. (2016). Clostridium difficile infection. *Nature Reviews. Disease Primers*, *2*, 16020.

Spina, A., Kerr, K. G., Cormican, M., Barbut, F., Eigentler, A., Zerva, L., Tassios, P., Popescu, G. A., Rafila, A., Eerola, E., Batista, J., Maass, M., Aschbacher, R., Olsen, K. E. P., & Allerberger, F. (2015). Spectrum of enteropathogens detected by the FilmArray GI Panel in a multicentre study of community-acquired gastroenteritis. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *21*(8), 719–728.

Stewart, D. B., Wright, J. R., Fowler, M., McLimans, C. J., Tokarev, V., Amaniera, I., Baker, O., Wong, H.-T., Brabec, J., Drucker, R., & Lamendella, R. (2019). Integrated Meta-omics Reveals a Fungus-Associated Bacteriome and Distinct Functional Pathways in Clostridioides difficile Infection. In *mSphere* (Vol. 4, Issue 4).

https://doi.org/10.1128/msphere.00454-19

Tang, D. M., Urrunaga, N. H., De Groot, H., von Rosenvinge, E. C., Xie, G., & Ghazi, L. J. (2014). Pseudomembranous Colitis: Not Always Caused by Clostridium difficile. *Case Reports in Medicine*, *2014*, 812704.

Tang, D. M., Urrunaga, N. H., & von Rosenvinge, E. C. (2016). Pseudomembranous colitis: Not always Clostridium difficile. *Cleveland Clinic Journal of Medicine*, *83*(5), 361–366.

Tenover, F. C., Baron, E. J., Peterson, L. R., & Persing, D. H. (2011). Laboratory diagnosis of Clostridium difficile infection can molecular amplification methods move us out of uncertainty? *The Journal of Molecular Diagnostics: JMD*, *13*(6), 573–582.

Thevenon, O., Adema, W., & Clarke, C. (2016). *Background brief on fathers' leave and its use*. OECD. https://doi.org/10.13140/RG.2.2.27717.24808

Van Rossum, T., Costea, P. I., Paoli, L., Alves, R., Thielemann, R., Sunagawa, S., & Bork, P. (2021). metaSNV v2: detection of SNVs and subspecies in prokaryotic metagenomes. *Bioinformatics* . https://doi.org/10.1093/bioinformatics/btab789

Vincent, C., Miller, M. A., Edens, T. J., Mehrotra, S., Dewar, K., & Manges, A. R. (2016). Bloom and bust: intestinal microbiota dynamics in response to hospital exposures and Clostridium difficile colonization or infection. *Microbiome*, *4*, 12.

Voigt, A. Y., Costea, P. I., Kultima, J. R., Li, S. S., Zeller, G., Sunagawa, S., & Bork, P. (2015). Temporal and technical variability of human gut metagenomes. *Genome Biology*, *16*, 73.

Voth, D. E., & Ballard, J. D. (2005). Clostridium difficile toxins: mechanism of action and role in disease. *Clinical Microbiology Reviews*, *18*(2), 247–263.

Watson, A. R., Fuessel, J., Veseli, I., DeLongchamp, J. Z., Silva, M., Trigodet, F., Lolans, K., Shaiber, A., Fogarty, E., Runde, J. M., Quince, C., Yu, M. K., Soylev, A., Morrison, H. G., Lee, S. T. M., Kao, D., Rubin, D. T., Jabri, B., Louie, T., & Murat Eren, A. (n.d.). *Adaptive ecological processes and metabolic independence drive microbial colonization and resilience in the human gut*. https://doi.org/10.1101/2021.03.02.433653

Willemsen, L. E. M., Koetsier, M. A., van Deventer, S. J. H., & van Tol, E. A. F. (2003). Short chain fatty acids stimulate epithelial mucin 2 expression through differential effects on prostaglandin E(1) and E(2) production by intestinal myofibroblasts. *Gut*, *52*(10), 1442–1447.

Wirbel, J., Zych, K., Essex, M., Karcher, N., Kartal, E., Salazar, G., Bork, P., Sunagawa, S., & Zeller, G. (2021). Microbiome meta-analysis and cross-disease comparison enabled by the SIAMCAT machine learning toolbox. *Genome Biology*, *22*(1), 93.

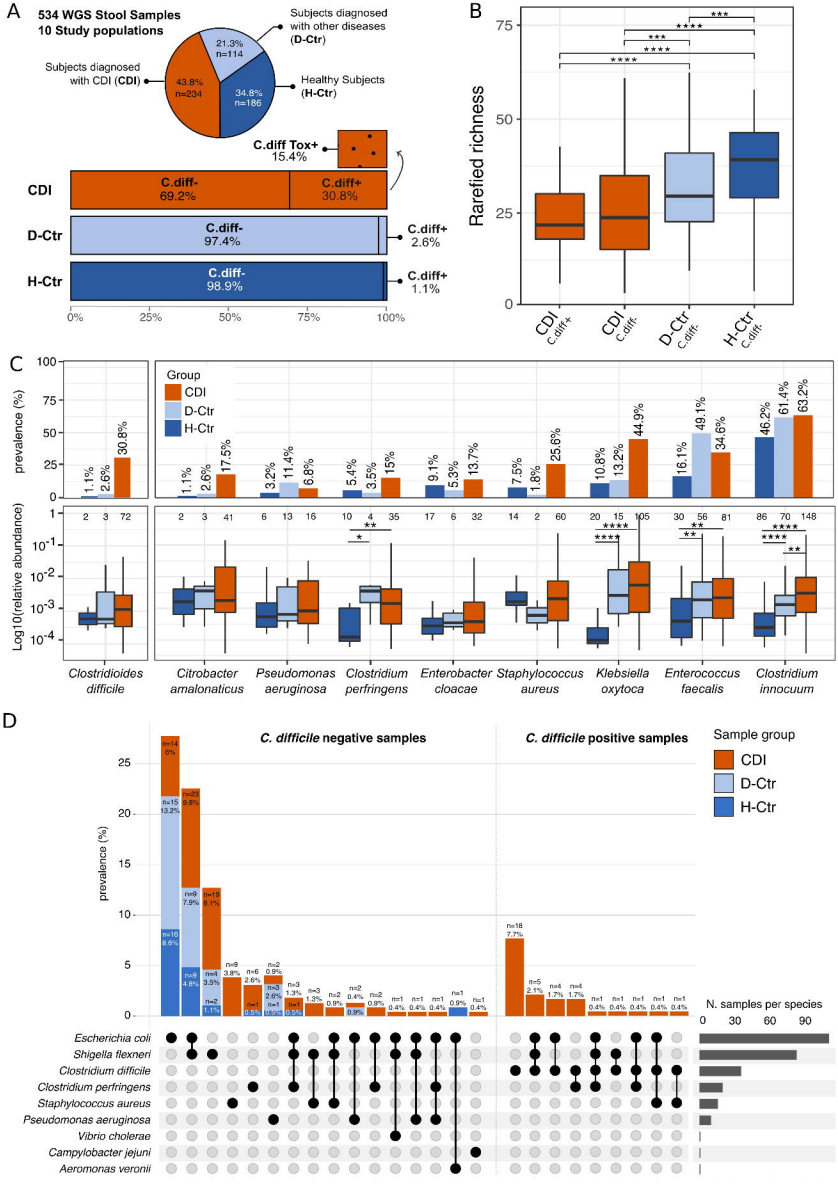
Yassour, M., Vatanen, T., Siljander, H., Hämäläinen, A.-M., Härkönen, T., Ryhänen, S. J., Franzosa, E. A., Vlamakis, H., Huttenhower, C., Gevers, D., Lander, E. S., Knip, M., DIABIMMUNE Study Group, & Xavier, R. J. (2016). Natural history of the infant gut microbiome and impact of antibiotic treatment on bacterial strain diversity and stability. *Science Translational Medicine*, *8*(343), 343ra81.

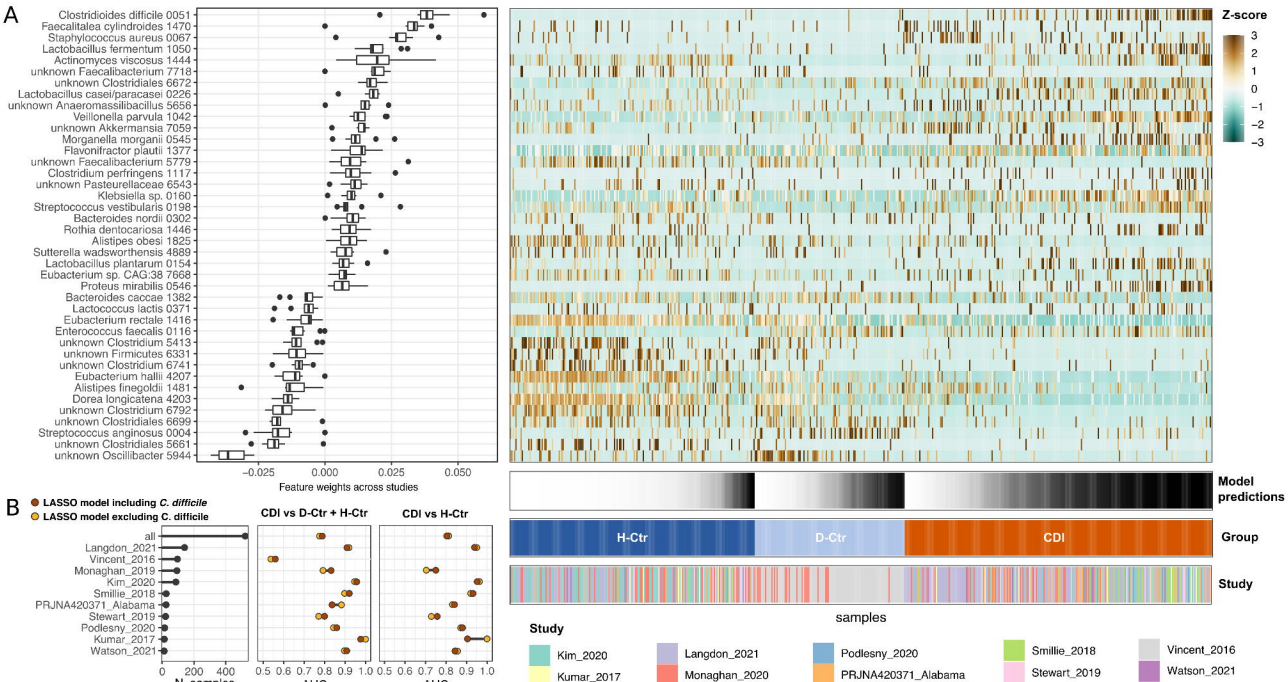
Yuan, Q., Guo, X., Ren, Y., Wen, X., & Gao, L. (2020). Cluster correlation based method for lncRNA-disease association prediction. *BMC Bioinformatics*, *21*(1), 180.

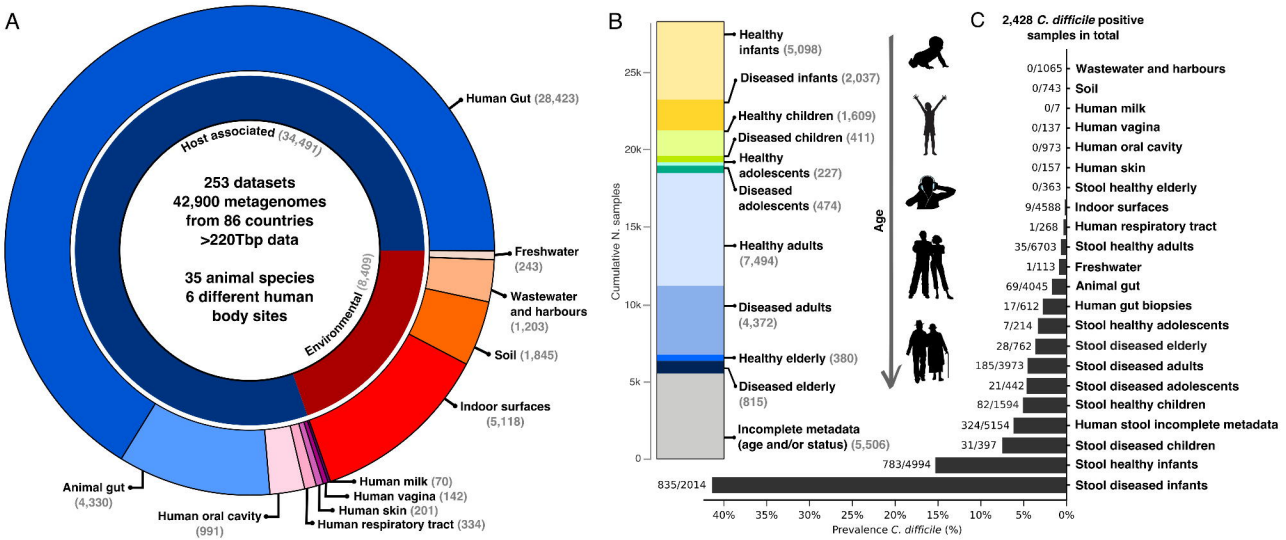
Zhang, S., Palazuelos-Munoz, S., Balsells, E. M., Nair, H., Chit, A., & Kyaw, M. H. (2016). Cost of hospital management of Clostridium difficile infection in United States-a meta-analysis and modelling study. *BMC Infectious Diseases*, *16*(1), 447.

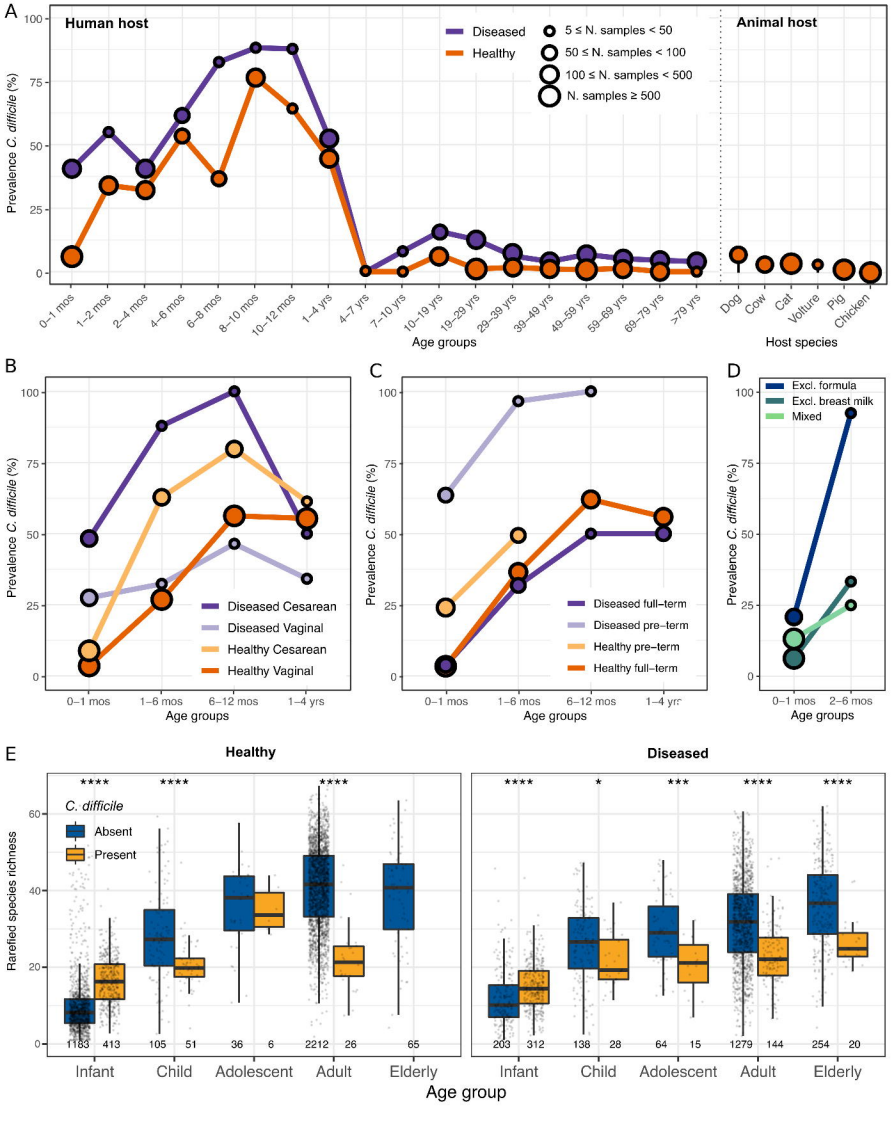
Zhou, Y., Wylie, K. M., El Feghaly, R. E., Mihindukulasuriya, K. A., Elward, A., Haslam, D. B., Storch, G. A., & Weinstock, G. M. (2016). Metagenomic Approach for Identification of the Pathogens Associated with Diarrhea in Stool Specimens. *Journal of Clinical Microbiology*, *54*(2), 368–375.

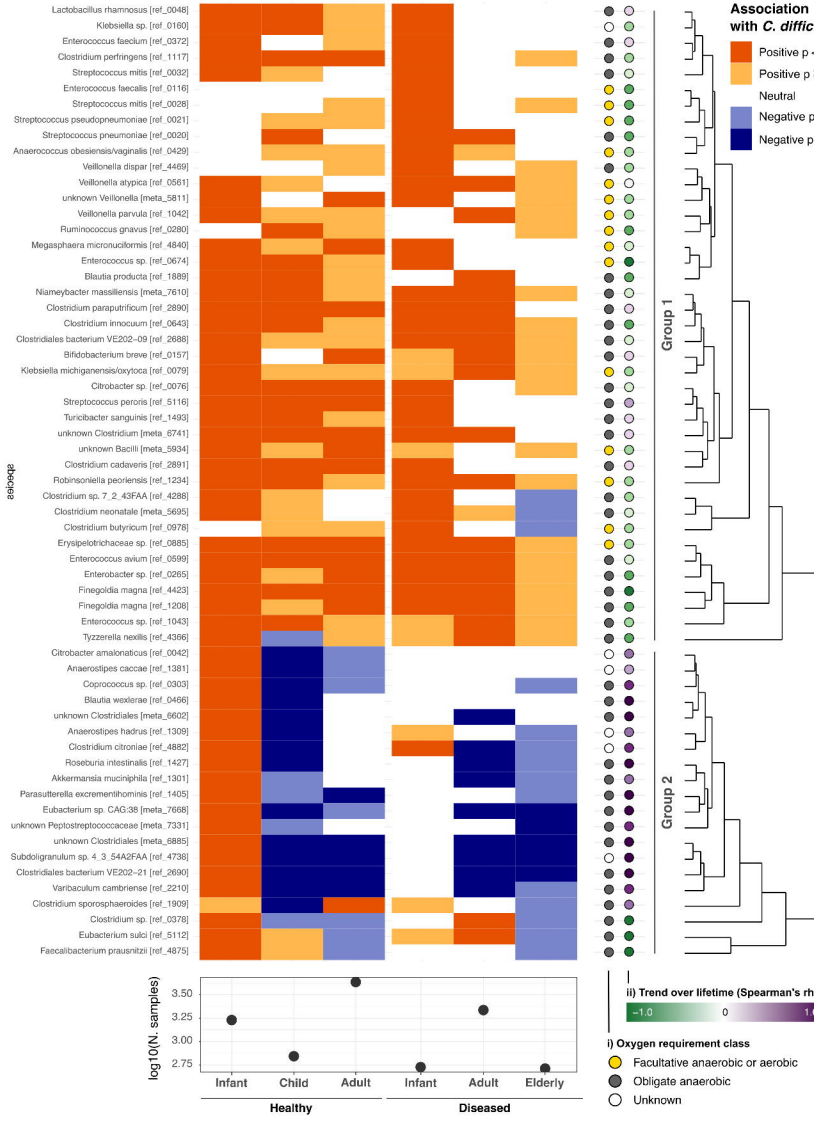
Zollner-Schwetz, I., Högenauer, C., Joainig, M., Weberhofer, P., Gorkiewicz, G., Valentin, T., Hinterleitner, T. A., & Krause, R. (2008). Role of Klebsiella oxytoca in antibiotic-associated diarrhea. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, *47*(9), e74–e78.


A
534 WGS Stool Samples
10 Study populations
Subjects diagnosed with other diseases (D-Ctr)
21.3% n=114
Subjects diagnosed with CDI (CDI)
43.8% n=234
Healthy Subjects (H-Ctr)
34.8% n=186
C.diff Tox+ 15.4%
CDI
C.diff- 69.2%
C.diff+ 30.8%
D-Ctr
C.diff- 97.4%
C.diff+ 2.6%
H-Ctr
C.diff- 98.9%
C.diff+ 1.1%
0%
25%
50%
75%
100%
B
Rarefied richness
CDI C.diff+
CDI C.diff-
D-Ctr C.diff-
H-Ctr C.diff-
C
Group
CDI
D-Ctr
H-Ctr
prevalence (%)
Log10(relative abundance)
Clostridioides difficile
Citrobacter amalonaticus
Pseudomonas aeruginosa
Clostridium perfringens
Enterobacter cloacae
Staphylococcus aureus
Klebsiella oxytoca
Enterococcus faecalis
Clostridium innocuum
D
C. difficile negative samples
C. difficile positive samples
Sample group
CDI
D-Ctr
H-Ctr
prevalence (%)
N. samples per species
Escherichia coli
Shigella flexneri
Clostridium difficile
Clostridium perfringens
Staphylococcus aureus
Pseudomonas aeruginosa
Vibrio cholerae
Campylobacter jejuni
Aeromonas veronii


A
Clostridioides difficile 0051
Faecalitalea cylindroides 1470
Staphylococcus aureus 0067
Lactobacillus fermentum 1050
Actinomyces viscosus 1444
unknown Faecalibacterium 7718
unknown Clostridiales 6672
Lactobacillus casei/paracasei 0226
unknown Anaeromassilibacillus 5656
Veillonella parvula 1042
unknown Akkermansia 7059
Morganella morganii 0545
Flavonifractor plautii 1377
unknown Faecalibacterium 5779
Clostridium perfringens 1117
unknown Pasteurellaceae 6543
Klebsiella sp. 0160
Streptococcus vestibularis 0198
Bacteroides nordii 0302
Rothia dentocariosa 1446
Alistipes obesi 1825
Sutterella wadsworthensis 4889
Lactobacillus plantarum 0154
Eubacterium sp. CAG:38 7668
Proteus mirabilis 0546
Bacteroides caccae 1382
Lactococcus lactis 0371
Eubacterium rectale 1416
Enterococcus faecalis 0116
unknown Clostridium 5413
unknown Firmicutes 6331
unknown Clostridium 6741
Eubacterium hallii 4207
Alistipes finegoldii 1481
Dorea longicatena 4203
unknown Clostridium 6792
unknown Clostridiales 6699
Streptococcus anginosus 0004
unknown Clostridiales 5651
unknown Oscillibacter 5944
−0.025
0.000
0.025
0.050
Feature weights across studies
Z-score
3
2
1
0
−1
−2
−3
Model predictions
H-Ctr
D-Ctr
CDI
Group
Study
samples
Study
Kim_2020
Kumar_2017
Langdon_2021
Monaghan_2020
Podlesny_2020
PRJNA420371_Alabama
Smillie_2018
Stewart_2019
Vincent_2016
Watson_2021
B
LASSO model including C. difficile
LASSO model excluding C. difficile
all
Langdon_2021
Vincent_2016
Monaghan_2019
Kim_2020
Smillie_2018
PRJNA420371_Alabama
Stewart_2019
Podlesny_2020
Kumar_2017
Watson_2021
0
200
400
N. samples
CDI vs D-Ctr + H-Ctr
CDI vs H-Ctr
0.5
0.6
0.7
0.8
0.9
1.0
AUC


A
Host associated (34,491)
253 datasets
42,900 metagenomes
from 86 countries
>220Tbp data
35 animal species
6 different human
body sites
Environmental (8,409)
Human Gut (28,423)
Freshwater (243)
Wastewater and harbours (1,203)
Soil (1,845)
Indoor surfaces (5,118)
Animal gut (4,330)
Human oral cavity (991)
Human respiratory tract (334)
Human skin (201)
Human vagina (142)
Human milk (70)
B
Cumulative N. samples
25k
20k
15k
10k
5k
0
Healthy infants (5,098)
Diseased infants (2,037)
Healthy children (1,609)
Diseased children (411)
Healthy adolescents (227)
Diseased adolescents (474)
Healthy adults (7,494)
Diseased adults (4,372)
Healthy elderly (380)
Diseased elderly (815)
Incomplete metadata (age and/or status) (5,506)
Age
C
2,428 C. difficile positive samples in total
0/1065 Wastewater and harbours
0/743 Soil
0/7 Human milk
0/137 Human vagina
0/973 Human oral cavity
0/157 Human skin
0/363 Stool healthy elderly
9/4588 Indoor surfaces
1/268 Human respiratory tract
35/6703 Stool healthy adults
1/113 Freshwater
69/4045 Animal gut
17/612 Human gut biopsies
7/214 Stool healthy adolescents
28/762 Stool diseased elderly
185/3973 Stool diseased adults
21/442 Stool diseased adolescents
82/1594 Stool healthy children
324/5154 Human stool incomplete metadata
31/397 Stool diseased children
783/4994 Stool healthy infants
835/2014 Stool diseased infants
40% 35% 30% 25% 20% 15% 10% 5% 0%
Prevalence C. difficile (%)


A
Human host
Animal host
Diseased
Healthy
5 ≤ N. samples < 50
50 ≤ N. samples < 100
100 ≤ N. samples < 500
N. samples ≥ 500
Prevalence C. difficile (%)
0–1 mos
1–2 mos
2–4 mos
4–6 mos
6–8 mos
8–10 mos
10–12 mos
1–4 yrs
4–7 yrs
7–10 yrs
10–19 yrs
19–29 yrs
29–39 yrs
39–49 yrs
49–59 yrs
59–69 yrs
69–79 yrs
>79 yrs
Age groups
Dog
Cow
Cat
Volture
Pig
Chicken
Host species
B
Diseased Cesarean
Diseased Vaginal
Healthy Cesarean
Healthy Vaginal
C
Diseased full–term
Diseased pre–term
Healthy pre–term
Healthy full–term
D
Excl. formula
Excl. breast milk
Mixed
2–6 mos
E
Healthy
Diseased
C. difficile
Absent
Present
Rarefied species richness
Infant
Child
Adolescent
Adult
Elderly
Age group


Association with C. difficile
Positive p < .05
Positive p ≥ .05
Neutral
Negative p ≥ .05
Negative p < .05
Group 1
Group 2
species
Lactobacillus rhamnosus [ref_0048]
Klebsiella sp. [ref_0160]
Enterococcus faecium [ref_0372]
Clostridium perfringens [ref_1117]
Streptococcus mitis [ref_0032]
Enterococcus faecalis [ref_0116]
Streptococcus mitis [ref_0028]
Streptococcus pseudopneumoniae [ref_0021]
Streptococcus pneumoniae [ref_0020]
Anaerococcus obesiensis/vaginalis [ref_0429]
Veillonella dispar [ref_4469]
Veillonella atypica [ref_0561]
unknown Veillonella [meta_5811]
Veillonella parvula [ref_1042]
Ruminococcus gnavus [ref_0280]
Megasphaera micronuciformis [ref_4840]
Enterococcus sp. [ref_0674]
Blautia producta [ref_1889]
Niameybacter massiliensis [meta_7610]
Clostridium paraputrificum [ref_2890]
Clostridium innocuum [ref_0643]
Clostridiales bacterium VE202-09 [ref_2688]
Bifidobacterium breve [ref_0157]
Klebsiella michiganensis/oxytoca [ref_0079]
Citrobacter sp. [ref_0076]
Streptococcus peroris [ref_5116]
Turicibacter sanguinis [ref_1493]
unknown Clostridium [meta_6741]
unknown Bacilli [meta_5934]
Clostridium cadaveris [ref_2891]
Robinsoniella peoriensis [ref_1234]
Clostridium sp. 7_2_43FAA [ref_4288]
Clostridium neonatale [meta_5695]
Clostridium butyricum [ref_0978]
Erysipelotrichaceae sp. [ref_0885]
Enterococcus avium [ref_0599]
Enterobacter sp. [ref_0265]
Finegoldia magna [ref_4423]
Finegoldia magna [ref_1208]
Enterococcus sp. [ref_1043]
Tyzzerella nexilis [ref_4366]
Citrobacter amalonaticus [ref_0042]
Anaerostipes caccae [ref_1381]
Coprococcus sp. [ref_0303]
Blautia wexlerae [ref_0466]
unknown Clostridiales [meta_6602]
Anaerostipes hadrus [ref_1309]
Clostridium citroniae [ref_4882]
Roseburia intestinalis [ref_1427]
Akkermansia muciniphila [ref_1301]
Parasutterella excrementihominis [ref_1405]
Eubacterium sp. CAG:38 [meta_7668]
unknown Peptostreptococcaceae [meta_7331]
unknown Clostridiales [meta_6885]
Subdoligranulum sp. 4_3_54A2FAA [ref_4738]
Clostridiales bacterium VE202-21 [ref_2690]
Varibaculum cambriense [ref_2210]
Clostridium sporosphaeroides [ref_1909]
Clostridium sp. [ref_0378]
Eubacterium sulci [ref_5112]
Faecalibacterium prausnitzii [ref_4875]
log10(N. samples)
3.50
3.25
3.00
2.75
Infant
Child
Adult
Infant
Adult
Elderly
Healthy
Diseased
ii) Trend over lifetime (Spearman's rho)
−1.0
0
1.0
i) Oxygen requirement class
Facultative anaerobic or aerobic
Obligate anaerobic
Unknown