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Effect of *Clostridioides difficile* infection on the structure and functions of the human gut microbiota

Tutor: Prof. Sergio Uzzau

PhD candidate: Laura De Diego

PhD Course Director:

Prof. Leonardo A. Sechi

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MAIN INDEX

Background

The gut microbiota: an overview

The gut microbiota is one of the three core constituents of the gut ecosystem, together with the intestinal barrier, and the enteric nervous structure named as the 'second brain'(Ochoa-Repáraz & Kasper, 2016).

The gut microbiota (GM) is defined as the set of microorganisms - consisting mainly of bacteria, as well as viruses, fungi and parasites - their genes and their metabolites, present in the human gastrointestinal tract, in a number 10 times greater of cells in the human body. The GM represents over than the 70% of the total number of microorganisms colonizing the human organism (Jandhyala, 2015), spreading over a total intestinal area of 300 m^2 (Madara et al., 1990) with both qualitative and quantitative variation along the entire gastro-intestinal (enteric) tract.

In the oral cavity, the microbial population density is high, and is mainly represented by Bacteroides, Peptostreptococci, and members belonging to the genera *Prevotella*, *Fusobacterium*, and *Eubacterium*. In the stomach, given the high acidity of this body district, the bacterial population is less represented and includes members of *Prevotella*, *Streptococcus*, and *Veillonella*. In the duodenum, the microbiota increases its density but is still characterized by a low biomass (around 10^1-10^3 CFU/g), for two main reasons: the transit of chyme and the peristaltic intestinal motility is very rapid, and the bile and pancreatic juices exert a bactericidal action, discouraging a stable colonization of this tract of mucosa. In the rest of the small intestine, the bacterial density tends to increase gradually (10^4-10^7 CFU/g), with a parallel increase in species (richness), especially of gram negatives and obligate anaerobes, including Clostridiales (Ley et al., 2006; Rinninella et al., 2019).

Maximum microbial density is reached in the colon, with approximate values of 10^{11}-10^{12} CFU/g (Bengmark, 2001), consisting mainly of anaerobes especially in the cecum and right colon due to optimal conditions enhancing growth, hence colonization, such as the high availability of fermentable substrates and long residence time (lower enteric motility).

Besides inter-individual variations, 4 phyla characterize the gut microbiota of the adult, averaging in the percentages indicated: Bacillota (synonym Firmicutes) 72%, Bacteroidota (named synonym Bacteroidetes) 20%, Actinomycetota (synonym Actinobacteria), and Pseudomonadota (synonym Proteobacteria) 5-8%. Firmicutes and Bacteroidetes account for about 60-90% of the total. To date, a number of studies have shown the varying relationship between these two phyla as biomarker of a state of dysbiosis, with increase of Firmicutes over Bacteroidetes often correlating with disease, especially not communicable chronic and/or systemic diseases (Magne et al., 2020; Plaza-Diaz, 2020).

The GM consists of autochthonous microorganisms that result from initial colonization and massive changes (ecological succession) in early life. Microbial communities are rapidly acquired at birth (Arrieta et al., 2014), however, while controversial, several studies suggested the presence of bacterial communities in the placenta, amniotic fluid, and meconium (Socha-Banasiak et al., 2021). The origin of the hypothesized pre-birth microbiota is not well known; some studies suggest microbial communities may originate both from the vagina through ascendant colonization and from the maternal oral cavity/gut, through the bloodstream (D'Argenio, 2018; Jiménez et al., 2005; Jones et al., 2009). Other studies did not confirm these data and casted serious doubts on the intra-uterine colonization by microorganisms (Fricke WF, Microbiome 2021). As mentioned above, the GM can be defined as an organ since it is anatomically associated to a human body district, and it is functionally part of it as evolutionary unit (the human holobiont). Further, the GM physiological functions are interconnected and interdependent with those of the human organism. The postpartum period shows significant alterations in the neonatal microbial community structure, driven by an array of influencing factors such as delivery mode and timing, maternal nutritional intake, and the choice of infant nourishment (breastfeeding versus formula feeding). Mode of delivery is one of the first factors that influence the composition of the microbiota in newborns: in vaginal delivery the dominant microbial component derives from that of the mother's vaginal bacterial flora; in caesarean delivery colonization is mostly dependent on skin and upper-respiratory microorganisms from the health care workers attending the newborn delivery and nursery (Dominguez-Bello et al., 2010; Gopalakrishna & Hand, 2020).

Birth is only the beginning of the many factors that influence this complex dynamic system which is influenced in qualitative terms by both microorganism-dependent and host-dependent factors. With regard to the host-dependent influence, this in turn depends on both intrinsic factors, i.e. factors given by the anatomy and physiology of the intestinal tract such as oxygen tension, the abundance and quality of enteral mucus, enteric motility, and extrinsic factors, i.e. all those factors related to the habits of the individual, such as drug, diet, environmental interferents, all of which can be generically encompassed in the macro-definition of 'lifestyle'.

Eubiosis and dysbiosis

The balanced coexistence of the various microbial components ensures a condition defined as eubiosis, in which the various members are functionally connected and in balance with each other and with the gut ecosystem.

Intestinal eubiosis refers to a state of balanced and healthy microbial composition; in a state of eubiosis, the microbial community is stable, diverse, actively cross-feeding, with a proper balance between beneficial and potentially harmful bacteria. In an eubiotic gut, bacteria play crucial roles in supporting various aspects of human health, including:

- digestion and nutrient absorption: bacteria aid in the breakdown of complex food components, facilitating nutrient absorption and supporting optimal digestive function.

- protection against pathogens: a balanced gut microbiota acts as a protective barrier, competing with and inhibiting the growth of harmful pathogens, thereby reducing the risk of infections.
- immune system regulation: the gut microbiota interacts with the immune system, helping to regulate immune responses and maintain immune tolerance to prevent unnecessary inflammation.
- synthesis of beneficial compounds: certain bacteria produce essential vitamins (e.g., B vitamins and vitamin K) and short-chain fatty acids that benefit gut health and overall well-being.
- metabolism and weight management: emerging evidence suggests that the gut microbiota may influence energy metabolism and weight regulation, potentially impacting obesity, and related metabolic disorders. A 2017 study, based on a comparison of the fecal microbiota of obese and lean patients, highlighted an increase in Firmicutes and the ratio of Firmicutes/Bacteroidetes has shown a substantial increase among individuals with obesity, accompanied by a noteworthy decrease in the abundance of Bacteroidetes within these cohorts as opposed to lean individuals (Yun et al., 2017). The identical findings were validated using a mouse model, wherein the microbiota of normal-weight mice was compared with mice exposed to an obesogenic high-fat diet (Ley et al., 2005).

Cho and colleagues' research (Cho et al., 2012) illustrates a consistent pattern when it comes to the effects of antibiotics at the phylum level. Regardless of whether antibiotics are administered in low and continuous doses, they observed an increase in Firmicutes levels and a reduction in Bacteroidetes among treated animals, which aligns with the trend towards obesity. However, what Cho's analysis overlooks is the varying impact of antibiotics based on different segments of the intestinal tract. Specifically, the utilization of antibiotics, even at minimal levels, triggers distinct responses in separate intestinal compartments. This discrepancy results in a more pronounced reduction of the microbial community within the small intestine. Consequently, this reduction prompts a larger influx of dietary fibers and other nutrients into the large intestine. These fibers serve as optimal substrates for bacteria that produce short-chain fatty acids (SCFAs). Paradoxically, this scenario of nutrients escape from the small intestine might lead to an increase in the animals' weight, despite the absence of alterations in caloric intake.

Maintaining intestinal eubiosis is essential for overall health and well-being. However, various factors, as mentioned previously can lead to intestinal dysbiosis, where the gut microbiota is imbalanced.

Promoting intestinal eubiosis involves adopting a balanced and healthy lifestyle, including a diet rich in fiber and diverse plant-based foods, minimizing unnecessary antibiotic use, managing stress, and staying physically active. Additionally, the use of probiotics and prebiotics, which support the growth of beneficial bacteria, may also be beneficial in promoting eubiosis and overall gut health. However, individual responses to interventions may vary, and it is essential to consult with healthcare professionals for personalized guidance on maintaining a healthy gut microbiota. Depending on the type of relationship between the various constituents of the microbiota and the host, a microorganism can be defined as symbiont, commensal or pathobiont. In the first case, both the microorganism(s) and the individual benefit from their association. In the second case, only the microorganisms have benefits from their association with the host. At last, pathobiont bacteria, harmless under balanced conditions, may induce disease when the host is in a state of immune impairment or when the pathobiont colonize districts, moving from the gut to the urogenital or respiratory tracts.

The term 'dysbiosis', in contrast, indicates a qualitative/quantitative change in the gut microbiota and its metabolic activity (Holzapfel et al., 1998), with consequent negative effects on the health of the host. Some of the common factors contributing to intestinal dysbiosis include:

- infections and gastrointestinal diseases: acute gastrointestinal infections and chronic diseases affecting the digestive system can alter the composition of the gut microbiota. Inflammatory conditions and damage to the gut lining can also contribute to dysbiosis.

- stress and lifestyle: chronic stress and an unhealthy lifestyle can influence the gut microbiota. Stress can affect gut motility and the secretion of substances that influence gut bacteria.
- lack of breastfeeding: infants who are not breastfed or receive limited breastfeeding may have an altered gut microbiota, which can impact their health and development.
- age: the composition of the gut microbiota can change with age, leading to differences in the microbial balance in older individuals.
- environmental factors: exposure to environmental pollutants and toxins can affect the gut microbiota composition.
- medications: some medications, such as proton pump inhibitors (PPIs) used to treat acid reflux, can alter the gut microbiota.
- travel and changes in diet: traveling to different regions with diverse diets and environments can temporarily alter the gut microbiota.
- medical procedures: certain medical procedures, such as gastric bypass surgery or bowel resection, can influence the gut microbiota.
- diet: a diet low in fiber and high in processed foods can negatively impact the gut microbiota. The lack of dietary fiber reduces the food source for beneficial bacteria, promoting the growth of less desirable microorganisms. Although it was already known that a low-fiber diet implies a qualitative/quantitative alteration of the gut microbiota, a recent study conducted on a mouse model shows that a low-fiber diet even alters the genetics of the bacteria, determining their ability to utilize the mucins of the intestinal mucosa, thus contributing to a condition of inflammation (Dapa et al., 2022).
- Antibiotics: One of the most significant factors leading to dysbiosis is the use of antibiotics. For this reason, the following paragraph is devoted to it.

Antibiotics are designed to target and eliminate harmful bacteria that cause infections. However, they can also affect the beneficial bacteria in the gut, disrupting the natural balance of the microbiota.

While some of these causes can lead to limited and temporary changes in the gut microbiota, long-term challenge of the GM can overcome its resilience and dysbiosis, in turn may have significant health implications. Functional dysbiosis is accompanied with alterations in bacterial metabolism, as well as the overgrowth of potentially pathogenic microorganisms, resulting in the release of microbial products that represent a risk factor in the pathogenesis of many diseases.

Use of antibiotics and consequences

Antibiotics are among the most prescribed and most sold drugs, particularly by general practitioners to treat gastrointestinal and respiratory infections. The consumption of antibiotics has been growing world-wide for years and continues to grow. The high use increases the incidence of undesirable effects, and the antimicrobial misuse can result in the development of antibiotic resistance.

Antibiotic-associated diarrhea (AAD) is a common side effect of antibiotics that can occur in all populations in up to 40% of cases, both in outpatients and hospitalized patients (Toor et al., 2019). AAD is characterized by the occurrence of at least 3 soft or liquid bowel movements per day for at least 24 hours, either during antibiotic treatment or within 2 months after its discontinuation, with no other identifiable causes (Bartlett, 2002).

AAD can be caused by any type of antibiotic, but the risk is higher with broad-spectrum penicillins, cephalosporins, clindamycin, and fluoroquinolones. Both oral and intravenous antibiotics can lead to AAD. Notably, the onset of AAD may not be immediate, and it can occur several weeks or even months after taking the antibiotic, this phenomenon can last up to 6 months This delayed occurrence makes it important to differentiate between AAD and infectious gastroenteritis. AAD is a consequence of dysbiosis, resulting of antibiotic therapy and can occur in up to

100% of patients. Dysbiosis due to antibiotic treatment can lead to gut inflammation, with clinical evidence (AAD) or subclinical, with loosening or disruption of the intestinal barrier and an ecological succession that promotes the growth of pathogenic bacteria. Gut permeability facilitates the translocation of enteric bacteria or their products in the lamina propria and/or in the liver (throughout the portal system), leading to a strong immune activation and the production of pro-inflammatory cytokines. In severe and prolonged cases, the inflammation become chronic and/or increase susceptibility to the establishment of severe conditions such as *Clostridioides difficile* infection (CDI) with pseudo-membranous colitis and *Klebsiella oxytoca* hemorrhagic colitis (Högenauer et al., 2006). Remarkable shifts in the bacterial community composition inducted by antibiotics are quite similar to those observed in irritable bowel syndrome (IBS). This suggestion is further supported by data from cohort and case-control studies, indicating that antibiotic treatment is associated with an increased risk of IBS (Mamieva et al., 2022). Complications of dysbiosis induced by antibiotics may be more frequent and severe in fragile individuals and/or those with multiple comorbidities, increasing the risk of prolonged hospitalization (Vermeersch et al., 2018).

In addition, the complications lead to an increased prescription of diagnostic tests and therapies and prolonged hospitalization times, with an increase healthcare expenditure.

CDI is one of the most important healthcare-related infections (HCAIs) in industrialized countries, where *C. difficile* is the leading cause of diarrhea in hospital settings. The study 'Global burden of *Clostridioides difficile* infections: a systematic review and meta-analysis', published in 2019 in the *Journal of Global Health*, indicates that the global incidence of CDI is 2.2 per 1,000 hospital admissions per year and 3.5 per 10,000 patient-days per year.

In recent decades, there has been an increase in the number of CDI cases and of the severity of these infections and the associated mortality, especially in elderly patients, with a consequent increase in the length of hospital stay and in direct and indirect healthcare costs. This new epidemiological picture has been associated with the emergence of *C. difficile* strains with characteristics of high virulence and multidrug resistance (MDR), which have international or local spread.

Surveillance therefore assumes a central role in the prevention and control of CDI, enabling the monitoring of the epidemiological trend of the infection and the microbiological characteristics of the strains causing infection, and thus enabling the timely identification of the circulation/emergence of highly virulent strains. In addition, surveillance plays a crucial role in the implementation of CDI intervention programs, providing management and healthcare professionals with up-to-date data on the incidence of infection, the population groups/care settings most affected, the type of circulating strains, and the frequency and characteristics of outbreak events.

According to data estimated by a survey conducted in 14 European countries (European Study Group on *Clostridioides difficile*, ESGCD), the incidence rate of CDI was 2.45 episodes per 10,000 patient-days, whereas the result of an Italian study shows an increase in the incidence of CDI from 0.3 to 2.3 episodes per 10,000 patient-days over a 6-year observation period (2006-2011) (Di Bella et al., 2013).

The economic burden of CDI is considerable: in 2012 a European analysis estimated the annual cost of treating CDI at around EUR 3 billion. In the same year, a retrospective analysis on 133 patients (mean age: 70.2 ± 15.01 years; males: 46.6%) showed an average cost for the treatment of one episode of CDI borne by the hospital of approximately EUR 15,000; the increased length of hospital stay due to the infection represented the main cost item (approximately 90% of the total cost) (S. Magalini, 2012).

Since 2016, the European Centre for Disease Prevention and Control (ECDC) has been coordinating CDI surveillance in acute hospitals in European and European Economic Area (EEA) countries, with an Operational Protocol (OP) produced to implement and harmonize CDI surveillance in Europe.

Only recently (2019), in Italy, the National Surveillance System started the project "Support to the Surveillance of Care-Related Infections also in support of PNCAR", funded by the National Centre for Disease Prevention and Control (CCM) and coordinated by the Istituto Superiore di Sanità (ISS), aiming at setting up a national system dedicated to the surveillance of ICAs, including the surveillance of CDIs. Since 2022 and currently, the pilot phase of CDI surveillance is taking place, based on the EOP proposed by the ECDC, and involves a representative number of Italian public hospital facilities.

Investigating CDI is crucial for several reasons, which can be summed up in eight key points:

- prevalence and incidence: CDI is one of the most common healthcare-associated infections (HAIs) worldwide. It affects individuals in hospitals, long-term care facilities, and community settings. The high prevalence and incidence of CDI put a significant burden on healthcare systems, leading to increased costs, longer hospital stays, and a strain on medical resources.
- antibiotic resistance: *Clostridioides difficile* is often associated with antibiotic use. Broad-spectrum antibiotics can disrupt the normal gut flora, creating an environment where *C. difficile* can flourish and produce toxins that cause the infection. Moreover, *C. difficile* strains have shown resistance to some antibiotics, complicating treatment options and increasing the severity of infections.
- recurrence: CDI has a high recurrence rate, making it a challenging infection to manage effectively. Recurrent episodes can lead to a cycle of repeated treatment, further increasing healthcare costs and impacting patient well-being.
- severity of disease: in severe cases, CDI can lead to life-threatening complications, such as severe colitis, toxic megacolon, and sepsis. Elderly patients and those with underlying health conditions are particularly susceptible to severe outcomes.

- contagious nature: *C. difficile* spores can persist in the environment and spread easily from person to person, making it highly contagious. This can lead to outbreaks in healthcare facilities and other settings, further escalating the public health concern.
- diagnostics and detection challenges: CDI diagnosis can be challenging, as symptoms can overlap with other gastrointestinal conditions. Accurate and timely detection is crucial to initiating appropriate treatment and preventing transmission, but it is not always straightforward.
- limited treatment options: treatment of CDI typically involves specific antibiotics, such as metronidazole, vancomycin, and fidaxomicin. However, with the emergence of antibiotic-resistant strains, treatment options may become limited, and alternative therapies might be needed.
- prevention and infection control: preventing the transmission of *C. difficile* spores in healthcare settings requires strict infection control measures, such as hand hygiene, proper cleaning of surfaces, and isolation precautions. These measures can be challenging since they must be implemented consistently. Lack of strict and consistent adherence contribute to the persistence of CDI as a public health problem.

Overall, CDI poses significant challenges to public healthcare systems due to its high prevalence, recurrence rates, potential for severe outcomes, and the need for robust prevention and control strategies. Efforts to control and manage CDI are essential to reducing its impact on patients, healthcare facilities, and the broader community.

C. difficile: a dangerous pathobiont

Clostridioides difficile, formerly known as *Clostridium difficile*, is a Gram-positive sporigenous bacillus belonging to the Peptostreptococcaceae family. It was first isolated from intestinal tissue in 1935; the noun 'difficile' was chosen to emphasize the difficulty encountered in the isolation process (Hall, 1935).

C. difficile was recognized as a pathogen mainly through the efforts of various researchers and physicians during the 1970/1980s: Edward J. Bartlett and his colleagues published a study linking the use of antibiotics to the growth and toxigenicity of *C. difficile* in the human gut, leading to symptoms of severe diarrhea and colitis (Bartlett et al., 1978).

It is well established that CDI can cause what is now called CDAD (*Clostridium difficile* associated disease), the severity of which varies from mild diarrhea to colitis pseudomembranous colitis, toxic megacolon, and intestinal perforation.

C. difficile has a ubiquitous distribution, it is localized in soil, as well as in the feces of various mammals, including humans where it is part of the intestinal microbial community. Numerous studies show that colonization by this microorganism is present from an early age, probably due to the immaturity of the flora in terms of alpha diversity. Approximately 3-15% of the adult population retains this microorganism in its intestinal microbial flora, without any symptoms. For this reason, *C. difficile* can be defined as a pathobiont microorganism, i.e. a potentially pathogenic microorganism that physiologically colonizes the intestinal tract, but its replication and survival is restricted under eubiotic conditions by co-existing microbiota members and host determinants including bile salts.

The mechanism of colonization of the intestinal mucosa and the adhesion process remain unclear. Some strains of *C. difficile* can express fimbriae (Awad et al., 2014). However, this is not a common feature of all toxigenic strains, and the correlation between fimbriae expression and symptomatic onset of CDI has not been demonstrated. It is well established that the adhesion mechanism occurs in two sequential phases, the first being an adhesion phase with the apical microvilli of the intestinal epithelium cells, followed by the second in which the microorganism produces toxins A and B that compromise the integrity of the intestinal barrier by exposing the epithelial cells. These toxins are two of the main mechanisms of pathogenicity of *C. difficile*. They are exotoxins, i.e. elements that are secreted outside the cell, belonging to the glycotoxins family. The toxins are encoded by the genes known as *tcdA* and *tcdB*, which are located in the same pathogenicity locus (PaLoc) of three further genes: *tcdD* and *tcdC*, respectively positive and negative regulators of *tcdA* and *tcdB*, and *tcdE*, encoding for a protein that enable the secretion of toxins A and B. Variations in the structure of PaLoc underlie the formation of different toxin types.

The toxins A and B secreted by *C. difficile* are commonly known as enterotoxin (toxin A) and cytotoxin (toxin B).

Toxin A binds to the apical side of epithelial cells and, once internalized, it destroys their cytoskeleton and tight intercellular junctions, causing a loss of epithelial barrier function. Dead cells and inflammation mediators released by intoxicated epithelial cells attract neutrophils. Due to the disruption of tight junctions, toxin B is able to cross the epithelium; unlike toxin A, toxin B primarily targets epithelial cells from the basolateral side. Both toxins are cytotoxic and induce the production of TNF-α and pro-inflammatory cytokines, contributing to the initiation and perpetuation of the inflammatory response and the formation of pseudomembranes (Pothoulakis et al., 1998).

An important characteristic of these two toxins is their modularity. They are, in fact, composed of three distinct domains. The N-terminal domain, also known as the A domain, is catalytic in nature and possesses full biological activity. The C-terminal domain is composed of repeated oligopeptides and is responsible for binding to the toxin receptor present on the surface of host intestinal epithelial cells, facilitating toxin endocytosis. The central domain represents the largest part of the two proteins and is characterized by a small hydrophobic segment that appears to mediate membrane insertion during the translocation process (Faust et al., 1998; Jank et al., 2007; von Eichel-Streiber et al., 1991).

The intracellular targets of the glycosyltransferases are small GTPases of the Rho family, which comprises approximately 20 GTP-binding proteins (Just, Selzer, et al., 1995).

Rho proteins function as molecular switches and are involved in numerous cellular signaling processes, including the regulation of actin cytoskeleton, adhesion, and migration. They also control enzymatic activities, gene transcription, cell cycle progression and apoptosis. Toxins A and B catalyze the mono-O-glycosylation of the Rho GTPase at a threonine amino acid residue, which is essential for the GTPase's functions. Inactivating the protein leads to the loss of eukaryotic host cell cytoskeletal structure and, by inducing the production of TNF-α and pro-inflammatory cytokines, contributes to triggering the inflammatory response (Poxton et al., 2001). This response causes the significant tissue damage resulting from bacterial infection, enhancing the cytotoxic mediated mucosal damage.

Another pathogenic mechanism typical of toxins A and B involves the disruption of tight junctions between epithelial cells, resulting in increased epithelial and vascular permeability. In fact, both toxins are associated with the appearance of hemorrhagic phenomena in the intestinal mucosa. Some strains of *C. difficile* have been shown to produce a third toxin, known as binary toxin, composed of two different subunits: an enzymatic subunit called CDTa and a binding subunit called CDTb. This latter toxin is an actin-specific ADP-ribosyltransferase and catalyzes the ADP-ribosylation reaction of actin monomers, leading to cytoskeletal disorganization. It is synthesized from two different genes, *cdtA* and *cdtB*, which are located outside of the PatLoc.

Although the mechanism of action of the binary toxin is well understood, its role as a virulence factor in humans is not yet fully clarified. Animal clinical studies have demonstrated that the binary toxin might cause fluid secretion but not significant damage to epithelial cells (Bongaerts & Lyerly, 1994).

The NAP1/B1/027 strain

In Canada, during the epidemic of the 2000s, a new highly virulent strain identified as NAP1/027 was isolated. From Canada, this strain spread to many European countries, where both epidemic infections (England, Wales, Scotland, Ireland, Belgium, France, Germany, the Netherlands, Switzerland, Denmark) and sporadic cases (Austria, Poland, Spain) were recorded (Kuijper et al., 2006).(Kuijper et al., 2006).(Kuijper et al., 2006).(Kuijper et al., 2006).(Kuijper et al., 2006).(Kuijper et al., 2006).

The NAP1/027 strain of *C. difficile* presents a gene locus known as CdtLoc that encodes for CD196 ADP-ribosyltransferase (CDT) or binary toxin, already described above. The damage caused by CDT promotes bacterial adherence and increased uptake of toxin A and toxin B (Gerding et al., 2014). In addition, this *C. difficile* strain carries a base pair frameshift deletion at nucleotide 117 of the TcdC gene, known to be a negative regulator of toxins, leading to a hyperexpression 16 and 23 times more for toxin A and toxin B, respectively (Warny et al., 2005)(Warny et al., 2005). Åkerlund et al., suggest that increased sporulation by this *C. difficile* strain might contributed to the increased spread of CDI (Åkerlund et al., 2008). To conclude, NAP1 strain has shown a lower sensitivity to metronidazole, rifampicin, moxifloxacin, clindamycin, imipenem, and chloramphenicol (Freeman et al., 2018; Goldstein et al., 2011), contributing to high morbidity and mortality rates compared to other *C. difficile* strains.

Germination of *C. difficile* spores

As mentioned, *C. difficile* is a sporigenous bacterium; the germination process can take place in the intestine, enhanced by specific bile salts.

The bile acids production is one of the functions of liver, using cholesterol as precursor. Primary bile acids – mainly as sodium salts conjugated with the amino acids taurine and glycine, including cholic acid (CA) and chenodeoxycholic (CDCA) – are mostly excreted in the small intestine. Primary bile acids enter the small intestine where they aid in fat emulsification and absorption. Bile acids also restrain the bacterial load in the intestinal mucosa both directly and prompting the activation of antimicrobial peptide expression. This dual action enhances the host's defense mechanism against both commensal microorganisms and specific enteric pathogens (Inagaki et al., 2006). An amount of primary acid is absorbed by passive diffusion, the remaining become substrate of the bacterial bile salt hydrolase (BSH), enzymes involved in the deconjugation reaction, i.e. the removal of taurine and glycine, to contain the reabsorption of primary bile acids. The deconjugated bile acids reach the colon where the final conversion into secondary bile acids [deoxycholic acid (DCA) and lithocholic acid (LCA)] occurs by catalysis of the enzyme 7-α-dehydroxylase. The two key enzymes of this process are produced by members of gut microbiota: while BSHs are predominantly encoded by *Lactobacillus* and *Bifidobacterium*, members of the microbiota known to possess 7-α-dihydroxylation activity belong predominantly to the genus *Clostridium*, clusters XIVa and XI (OHASHI & FUJISAWA, 2019) .

In vivo and in vitro studies show that a loss of members of the gut microbiota involved in bile acid metabolism contributes significantly to the disease process of in CDI. This depletion of gut microbiota members explains the reason of the two key steps in the CDI (Mullish & Allegretti, 2021)

- the enrichment of primary conjugated bile acids (including taurocholic acid, which promotes the germination of *C. difficile* spores);
- loss of secondary bile acids, known to inhibit the growth of *C. difficile*; they might bind and restrict effects of toxins.

Risk factors

The three primary risk factors for the onset of CDI are hospitalization, advanced age, and antibiotic drug treatment. *C. difficile* is found in the gastrointestinal tract of 1-3% of healthy adults, but only 1% of the isolated strains are pathogenic. Colonization can persist for up to 2-3 years without causing any harm to the host. Approximately 60% of healthy adult individuals possess IgG and IgA antibodies against toxins A and B, likely due to exposures occurring in childhood or subclinical infections in adulthood. Noteworthy, exposure in childhood is typically not associated to disease. High rates (50 to 70%) of asymptomatic colonization occur in infants and children younger than 2 years (Director Infection Management and Prevention Service, 2021). Colonization appears to be favored by the immaturity of the intestinal bacterial flora, and the lack of progression to disease seems to be due to the toxin's inability to bind to enterocyte receptors, which are also immature.

This acquired immunity is crucial in preventing the development of *C. difficile*-related conditions or recurrent infections.

Due to a high frequency of environmental contamination with *C. difficile* spores, infection is a problem that currently affects not only hospitals but also other healthcare facilities, including rehabilitation units, long-term care facilities, and elderly care centers. Hospitalized patients have a colonization prevalence of 4-10%, significantly higher than that found in the community.

Old age, considered to be over 60 years, is a risk factor, even more pronounced beyond 80 years. Contrary to what has been published in the literature, recent data from the English surveillance system indicated a 20% increase in infections in the population under 60 years of age. Certain comorbidities such as chronic kidney disease, cystic fibrosis, intestinal and biliary tract surgery, hematologic malignancies, Crohn's disease, and ulcerative colitis can increase the risk of CDI. Antibiotic therapy, especially when combined and/or prolonged with broad-spectrum drugs, disrupts the normal intestinal bacterial flora, reducing resistance to *C. difficile* colonization.

An interesting experience at an Australian hospital showed that modifying the antibiotic usage policy led to a decrease in CDAD incidence from 2.09 to 0.87 cases per 1,000 discharges (Thomas et al., 2002). Virtually every antibiotic can be associated with CDAD, but in practice, some classes (e.g., cotrimoxazole) rarely contribute to the issue. Among antibiotics, those associated with a higher risk of CDI include clindamycin, β-lactams, and cephalosporins (Bignardi, 1998). Fluoroquinolones, although used as antibiotics since 1988, have not only been associated with an increased incidence of CDI (Sunenshine & McDonald, 2006), but they have also been shown to be the primary risk factor in several outbreaks of CD infections, linked to higher morbidity and mortality rates (Chung et al., 2014). Further studies on the effects of different categories of drugs as risk factors for CDAD have revealed that the use of proton pump inhibitors may promote its onset. This is due to the reduction in gastric acidity, the body's natural defense, which can prevent effective inactivation of the bacterial vegetative forms. These forms, once they reach the small intestine, initiate their pathogenicity (Dial, 2005).

In addition to these main drug categories, there are others that appear to influence the course of CDAD, including chemotherapeutics and other immunosuppressants, certain antidepressants, and histamine H2 receptor antagonists in combination with pump inhibitors. Furthermore, a potential correlation between antibiotic use, pump inhibitors, and the incidence of *C. difficile* has been studied, identifying that the combination of fluoroquinolones, cephalosporins, carbapenems, and pump inhibitors increases the risk (King & Lager, 2011).

Clinical presentation/onset

The clinical presentation of CDI can range from mild diarrheal syndrome (watery diarrhea accompanied by abdominal pain, usually in the lower quadrants) to severe (profuse watery diarrhea, abdominal pain, fever, nausea, dehydration) and even progress to pseudomembranous colitis (characterized by epithelial necrosis, ulcerations of the intestinal wall, and pseudomembrane formation), fulminant colitis with toxic megacolon, and intestinal perforation.

The onset of fever, leukocytosis, and abdominal pain in patients who have recently undergone antibiotic treatment, even without the appearance of diarrhea, should raise suspicion of CDI. However, none of these clinical manifestations is specific to this infection, underscoring the importance of an effective and timely diagnosis.

Treatment

The treatment is related to the degree of recurrence and severity of CDI. For the definition of severity, the 2017 ISDA (McDonald et al., 2018) and 2021 ACG guidelines (Kelly et al., 2021a), as well as the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guidelines (van Prehn et al., 2021), distinguish between severe and non-severe CDI by considering blood creatinine values and white blood cell count. Severe CDI is characterized by a white blood cell count ≥15,000 cells/mL or a serum creatinine level >1.5 mg/dL. None of the guidelines define moderate CDI.

Three antibiotics are approved for the treatment an initial CDI episode, each with a different mechanism of action (Bainum et al., 2023):

- metronidazole: is an antibiotic that causes the formation of free radicals that inhibit bacterial DNA synthesis, resulting in a bactericidal effect. It has been the gold standard for three decades, not least because of its lower cost compared to vancomycin, which was chosen in the event of failure of metronidazole therapy or in the event of allergy. Since it is a broad-spectrum antibiotic, especially effective against anaerobic bacteria, its use contributes significantly to the establishment of dysbiosis.
- vancomycin: is a glycopeptide antibiotic, it inhibits cell wall biosynthesis resulting in altered membrane permeability and RNA synthesis. Like metronidazole, it is a broad-spectrum drug, so it also causes an imbalance in the populations of the intestinal microbiota, resulting in dysbiosis.

- fidaxomicin: belongs to the macrolide class, i.e. it has the ability to bind to RNA polymerases and inhibit RNA synthesis. The range of action is narrow, so it is particularly suitable for the treatment of CDI.

In the last decade, fecal microbiota transplantation (FMT) has been successfully adopted in clinical practice following the increased incidence of recurrences of CDI (rCDI) and the failure of antibiotic therapies. The fecal transplantation enables to restore the microbiota, successfully controlling recurrent CDI. The efficacy of FMT has been proven by extensive studies and is therefore now included as a treatment option for rCDI in multiple international guidelines (van Prehn et al., 2021). Although safety has been established, many patients are not suitable for this type of therapy, which is generally administered via a colonoscope, using cryopreserved, homogenized feces from healthy donors. A new preparation consisting of freeze-dried encapsulated stools (cap-FMT) that can be taken orally is currently available, the clinical and adverse outcomes of which were compared with those of conventional FMT. The results show that there were no differences in the efficacy or safety of the two forms of treatment, cap-FMT or conventional-FMT. In addition, cap-FMT mitigated any risk related to colonoscopy. No new safety issues were detected, and no transmission of infectious agents was identified. Thus, cap-FMT may prove to be an easier and safer alternative in the treatment of CDI (Vaughn et al., 2023).

Diagnosis

To limit the spread of CDIs, a prompt and accurate diagnosis is crucial. Radiographs and endoscopies have been largely surpassed by laboratory tests for the detection of *C. difficile*, as they are expensive, unpleasant for patients, relatively insensitive, and not very specific. Laboratory tests are recommended for all adults and children after the first year of age with diarrheal manifestations occurring during or within a few weeks of antibiotic therapy. The diagnosis is based on the detection of the bacterium and/or its antigens, toxins, or nucleic acids in fecal samples. The search for markers of inflammatory reaction in feces (white blood cells, lactoferrin, calprotectin) is often positive but not pathognomonic of CDI. There is no single test or test series that can be universally recommended for indirect non-microbiology diagnosis of CDI. The diagnostic algorithm proposed by SIMPIOS (Italian Multidisciplinary Society for the Prevention of Infections in Healthcare Organizations), based on the most recent guidelines supported by experience and predictive index evaluations, appears to be currently the most rational approach to the microbiological diagnosis of CDI. This process comprises three steps, summarized schematically in the illustrated figure.

The fecal sample should be sent to the laboratory for analysis within one hour of collection, and when this is not possible, the sample can be stored for a maximum of 24 hours at a temperature between 4 and 8°C. This time recommendation is closely linked to the priority of making the diagnosis as soon as possible and to the labile nature of the *C. difficile* toxins. Once the suitability of the sample is confirmed, the *C. difficile* antigen glutamate dehydrogenase (GDH) is searched using immunoenzymatic methodology (EIA), which exhibits a high level of sensitivity. A positive result indicates the presence of *C. difficile*; however, toxigenic nature of the colonizing *C. difficile* is assessed through a highly specific test detecting the presence of toxins A and B (Mylonakis et al., 2001). In contrast, the detection of both toxins A and B using immunoenzymatic methods (EIA) exhibits a moderate sensitivity (75%), contingent on the chosen cut-off, coupled with a robust specificity (90%-100%). Due to its practicality and reliability, this test is currently the most prevalent in laboratories. Unlike the GDH antigen test, it is more influenced by sample storage conditions as toxins degrade relatively rapidly if the sample is not preserved at temperatures between 2°C and 8°C. The detection of toxin A solely, using immunoenzymatic methods, is less sensitive due to the presence of strains producing only toxin B. This approach is diminishing in popularity and is not recommended for use.

PCR-based tests, conducted on the fecal sample, exhibit a higher sensitivity compared to that of the EIA method and features a brief execution time (less than

4 hours) (Peterson et al., 2007). Nevertheless, the sole presence of genes is suggestive but not conclusive and diagnostic of an infection with a *C. difficile* strain colonizing and producing toxins. This step is undertaken when the sample yields a positive result for the GDH antigen but negative results for toxins A and B.

Prevention

Based on the knowledge acquired over the years, it is advisable to follow a few simple rules to reduce the risk of CDI, especially in the hospital environment, where this microorganism is encountered more frequently each year. Best practices include wisely use of antibiotics, especially fluoroquinolones, cephalosporins, and other broad-spectrum antimicrobials; to isolate infected or suspected CDI patients; to pay particular attention to patient's and health worker's hand hygiene, favoring cleansing with soap and water, which can mechanically remove *C. difficile* as opposed to alcoholic gels; to sterilize all reusable instruments that have come into contact with fecal material from infected patients.

In fact, a recent study has shown that between 16% and 80% of *C. difficile* spores present in contaminated hands are transferred during a handshake despite having previously used an alcohol-based skin hygiene gel (Jabbar et al., 2010). In fact, *C. difficile* spores are not killed by alcoholic substances, nor by the acids or ammonia-based substances normally used as disinfectants; the latter as well as being not sporicidal, may trigger *C. difficile* to switch from the vegetative to the more resistant and potentially pathogenic spore form.

The only substances that prove effective in eliminating *C. difficile* spores are solutions of 10% sodium hypochlorite (Wilcox et al., 2003), a concentration normally not used in standard sanitization procedures in hospital inpatient units. It has been shown that *C. difficile* spores survive for years, being continuously moved between various surfaces in the hospital environment (Kim et al., 1981).

Regarding prevention, a thorough review of measures to control of *C. difficile* infections has been developed by the European Centre for Disease Prevention and

Control Disease Control (ECDC), an EU agency with the specific task of identifying, assessing, and communicating threats to human health posed by infectious diseases.

For CDI prophylaxis procedures, pharmacological treatment is strongly discouraged on asymptomatic *C. difficile* carriers. Indeed, it has been shown that if these are treated with vancomycin or metronidazole, they develop a higher risk of re-infection and clinically manifestations than asymptomatic carriers treated with placebo. Not to mention the risk of increasing the bacterium's resistance to the antibiotics commonly used for its eradication (Teasley et al., 1983).

Probiotics have been investigated as a method for preventing CDI in patients receiving antibiotic therapy. One theory behind probiotic efficacy is related to the restoration of the gut microbiome that may be disrupted by antibiotic therapy. Other proposed mechanisms of action include protection against pathogens through competition for resources, maintenance of the epithelial barrier of the gut, the production of compounds that inhibit *C. difficile* growth, and immunomodulation (Liu et al., 2020).

The potential benefits of probiotics have been demonstrated in various meta-analyses and retrospective studies: a meta-analysis comprising 21 randomized, controlled trials evaluating probiotics' role in preventing CDI revealed that four out of five probiotic variants exhibited significant effectiveness for primary prevention (McFarland, 2015). An additional meta-analysis, involving 19 studies and a participant pool of 6000 individuals, highlighted that the probiotic group exhibited a lower occurrence of CDI in comparison to the control group (Shen et al., 2017). Not all studies have shown advantages in preventing CDI upfront: a recent investigation compared CDI risk in propensity-matched patients who were administered probiotics to those who were not, and it revealed no significative distinction between the two groups (Heil et al., 2021).

Although a growing number of studies reported probiotics as promising, most recent version of the Infectious Diseases Society of America (IDSA) and the Society for Healthcare Epidemiology of America (SHEA) CDI guidelines decline to make a recommendation on probiotic use due to insufficient evidence, while the ACG guidelines recommend against the use of probiotics for primary or secondary prevention (Johnson et al., 2021; Kelly et al., 2021b).

The concept of antimicrobial prophylaxis for infectious pathogens is a long-established practice, especially for immunocompromised patients. With regard to CDI, studies are mainly based on high-risk patients, i.e. immunocompromised patients and those on systemic antibiotic therapy. This approach involves the use of an agent that is effective against *C. difficile*, i.e. antibiotics routinely used to treat the infection, for a given period of time while a risk factor persists, in order to prevent an episode of CDI. Currently, there are limited data available on this topic regarding the use of vancomycin and fidaxomicin.

Regarding the vancomycin, a meta-analysis is available that is based on four retrospective studies, two of which were based on pharmacologically immunocompromised subjects and two based on elderly subjects receiving systemic antibiotic therapy. The study showed that the incidence rate of CDI was significantly lower in the groups treated with vancomycin than in the control groups (Maraolo et al., 2022). Data on primary prophylaxis with fidaxomicin are currently based on a randomized, placebo-controlled trial of 600 patients who underwent transplantation and were then rendered pharmacologically immunocompromised. Fidaxomicin prophylaxis led to a reduction in the confirmed rate of CDI compared to placebo at both 30 and 60 days (Mullane et al., 2019).

A previous editorial suggested a direct comparison between vancomycin and fidaxomicin about antimicrobial prophylaxis (Garey, 2020). This is necessary to determine whether an agent is more effective and to identify any differences in antimicrobial resistance developed due to prophylaxis. The narrower spectrum of activity and lower potential damage to the commensal gut microbiota by fidaxomicin compared to vancomycin may make the former a better option for prophylaxis. At present, primary prophylaxis has been consistently shown to be effective in populations with a sufficiently high baseline CDI rate and should therefore be considered for high-risk patients.

Impact of *C. difficile* in the shaping of gut microbiota

The most widely recognized predisposing element for the onset of CDI is the utilization of antibiotics, whether in the short or long term, owing to its influence on the diversity of the gut microbiota. It is worth noting that a thriving and balanced gut microbial ecosystem has the capacity to impede the progression of *C. difficile* spores, thereby not inevitably leading to disease. Nevertheless, any alteration in the microbial milieu has the potential to trigger spore germination, the proliferation of *C. difficile*, and the synthesis of toxins (Seekatz & Young, 2014).

Another established factor contributing to risk is advancing age. In the elderly population, there are observable shifts in the composition of the microbiome, resulting in reduced diversity and a decline in beneficial species like Bifidobacteria and selected Firmicutes. Conversely, there is an increase in Bacteroidetes and Proteobacteria (Claesson et al., 2011).

There are no antibiotics selectively targeting to *C. difficile*. Hence, it is inevitable that any antibiotic taken, necessary for infection treatment, not only leads to an overall reduction in the total intestinal microbial mass, as observed by Thorpe et al. (Thorpe et al., 2018), but also contributes to dysbiosis (Bauer et al., 2009; Gerding et al., 1995).

Research investigating the influence of non-CDI antibiotics on the gut microbiota has been documented since the 1980s. However, information pertaining to antibiotic therapies for CDI, specifically vancomycin and fidaxomicin, remains relatively constrained, with a predominant focus on vancomycin.

Current studies regarding subjects affected by CDI are based on follow-ups of individuals subjected to vancomycin, which results in a decrease in members of Bacteroidaceae and Prevotellaceae families, belonging to the Bacteroidetes phylum, as well as Clostridiaceae, Eubacteriaceae, Lachnospiraceae, and

Ruminococcaceae families belonging to the Firmicutes phylum (Edlund et al., 1997; Louie et al., 2009; Tannock et al., 2010; Vrieze et al., 2014). These authors also reported a simultaneous increase in members of the Enterococcaceae family (Firmicutes), along with an increase in members of the Proteobacteria phylum.

Additional research has been carried out on groups of individuals who were treated with fidaxomicin, a macrolide, already mentioned above. Firstly, reductions in the relative abundance of Bacteroidaceae, Bifidobacteriaceae, Clostridiaceae, and Prevotellaceae observed in patients treated with vancomycin are either not observed or less pronounced in patients treated with fidaxomicin. Furthermore, there was not an increase in the relative abundance of Proteobacteria phylum. Taken together, these data suggest that fidaxomicin induces a dysbiosis with a lower impact compared to vancomycin, which may contribute to the lower rates of CDI recurrence observed in patients treated with fidaxomicin in clinical studies (Guery et al., 2018; Mikamo et al., 2018).

Aim of the research project

This study is focused on a cohort of patients that underwent prolonged antibiotic treatment, leading to intestinal inflammation and diarrhea. Patients were tested for CDI and grouped as CDI positive and CDI negative. While all patients experienced enteric inflammation, we hypothesize that inflammation triggered by *C. difficile* colonization induces a specific reshaping of the GM composition and functions. Therefore, we investigate the GM of patients through a metaproteomic approach, with the following aims:

- to detect any potential taxonomic, functional, and taxon-specific functional differences among CDI+ and CDI- groups.
- to evaluate potential difference in functional dysbiosis in CDI+ patients according to the level of toxins detected in the fecal sample.

Materials and methods

Patients and samples

This study was based on the analysis of fecal leftovers from patients with suspected diagnosis of CDI. Fecal samples routinely arrive at the Clinical Microbiology laboratory of the Azienda Ospedaliero Universitaria of Sassari (AOUSS). All samples met the following characteristics, as set out in a protocol approved by the AOUSS administration:

- patient with ≥ 3 diarrheal discharges in the last 24 hours.
- absence of treatment with laxatives, enemas, suppositories, or other preparations that may have increased intestinal motility in the last 48 hours.
- absence of tests for *C. difficile* in the last 7 days.

Exclusions criteria were as follows:

- unsuitable specimens: rectal swab; formed stools, except in the case of suspected megacolon associated with *C. difficile*.
- samples for with lack of compliance in relation sample management (temperature-controlled storage) and its delivery to the laboratory (within 2 hours from evacuation).

The diagnostic algorithm applied at AOUSS is the one adopted by SIMPIOS, already mentioned above, which consists of several steps. The first step is a screening test with the CLIA method (LIAISON XL, Diasorin, Vercelli, Italy) for the detection of *C. difficile* GDH antigen. The test aims at identifying the enzyme glutamate dehydrogenase in patients' feces. However, a positive test does not distinguish toxigenic from non-toxigenic strains and is therefore immediately followed by the test for toxins A and B, also performed using the CLIA platform. Positivity to this latter test means that the patient is colonized by strains producing toxins A and B. In case of a negative result, the diagnostic algorithm involves molecular tests (PCR) for toxin B (*tcdB*) and binary toxin (*cdt*) genes, assessing the potential toxigenicity of the colonizing strains and to test the presence of the *tcdC* gene deletion at nucleotide 117, that is diagnostic of the 027 ribotype strain, associated with severe CDI and mortality. Positivity for one of these molecular targets confirms colonization by toxigenic *C. difficile*. Clinical results are reported to the hospital ward in order to manage the isolation procedure of the positive patient.

Upon the application of this algorithm, groups of patients were defined:

- GDH-negative patients: samples **n**egative for GDH (this group of patients was named "N" in this study);
- GDH-positive patients not colonized by toxin-producing strains: samples positive for GDH detection and negative for toxins (CLIA) and toxin-coding genes (PCR) (this category is not included in the present study);
- GDH-positive patients colonized by **t**oxin-**p**roducing strains: samples positive for GDH and toxins by CLIA assay (this group of patients was named "Tp" in this study);
- GDH-positive patients colonized by **t**oxi**g**enic (but toxin undetectable) strains: samples positive for GDH detection, negative for toxins (CLIA) and positive for toxin-coding genes (PCR) (this group of patients was named "Tg" in this study).

Following diagnostic investigation and recording of clinical results, the residual fecal samples were frozen at -20°C and then at -80°C until further processing.

The present study involved 80 fecal samples, including 30 negatives (N group), 20 toxigenic (Tg group) and 30 toxin-producing (Tp group).

Metaproteomic processing: protein digestion and FASP

Protein extraction from fecal samples was performed as a short version of an established protocol (Tanca et al., 2014), based on three steps. Extraction buffer (2% SDS, 100 mM DTT, 20 mM Tris-HCl pH 8.5; 100 µl per 50 mg of stool) and a steel bead (5 mm diameter; Qiagen, Hilden, Germany) were added to each sample. Then, samples were processed as follows: incubated at 95°C for 10 min in a thermoblock (FALC, Treviglio, Italy); bead beaten for 10 min (30 cycles/s in a TissueLyser LT mechanical homogenizer, Qiagen) and centrifuged at 14,000 x g for 10 min.

Supernatants were collected and further processed according to a modified filter-aided sample preparation (FASP) protocol (Wiśniewski et al., 2009). Protein extracts (30 µl each) were diluted with 370 µl of UT solution (8M urea, 100 mM Tris-HCl pH 8.5), loaded onto an Amicon Ultra-0.5 filtration device (30 kDa cutoff; Merck, Darmstadt, Germany) and centrifuged at 14,000 x g for 15 min. Then, the following solutions were sequentially added to the samples, which were then centrifuged at 14,000 x g for 10 min: 200 µl of UT solution, 100 µl of 50 mM iodoacetamide in UT solution (followed by 20 min incubation at RT), 100 µl of UT solution, further 100 µl of UT solution, 100 µl of 50 mM ammonium bicarbonate. As a final step, samples were digested overnight at 37°C with trypsin (1 µg per sample, dissolved in 50 mM ammonium bicarbonate solution). A first eluate was collected by centrifugation (14,000 x g for 15 min); then 100 µl of elution solution (20% acetonitrile, 0.2% formic acid) were added to each sample and a second eluate was collected by centrifugation (14,000 x g for 15 min) and merged with the first one. Peptide mixtures were finally concentrated using a Concentrator Plus (Eppendorf, Hamburg, Germany) and sent to an external laboratory for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses.

Offline StageTip purification and nanoLC analysis were performed as described elsewhere (Taverna & Gaspari, 2021). Reconstituted peptide mixtures (approximately 5 µg) were purified by SCX StageTips, eluted in 10 µl of 500 mM ammonium acetate containing 20% acetonitrile, then evaporated to dryness and resuspended in 0.2% formic acid. A 200-ng aliquot of peptide mixture was injected for a preliminary nanoLC-MS/MS analysis. The appropriate injection volume (between 1 and 8 μl) was estimated by analyzing a 1 μl aliquot of the digest using a short LC gradient and by estimating peptide amount based on the overall peptide signal (area under the curve). LC was performed on an EasyLC 1200 instrument (Thermo Fisher Scientific, Waltham, USA). The nanoLC column was a pulled capillary, 0.075x160 mm (i.e. and column length, respectively), in-house packed with C18 silica particles (Dr. Maisch, Ammerbuch, Germany). Peptides were loaded at 500 nl/min in mobile phase A (2% acetonitrile, 0.1% formic acid) and eluted at 300 nl/min by the following gradient: from 0% B to 25% B (80% acetonitrile, 0.1% formic acid) in 60 min, from 25% B to 45% B in additional 10 minutes, then to 100% B in 8 minutes. The column was regenerated for 10 min at 100% B and equilibrated at 0% B for 20 min before the following injection. Two blank injections were performed in between samples. A shorter gradient (45 min) was utilized for blank injections.

Peptides were electrosprayed in positive ion mode using 1800 V as spray voltage into an Orbitrap Exploris 480 (Thermo Fisher Scientific). Internal calibration was automatically performed at the beginning of each run (RunStart Easy IC-on). Full scan MS parameters were as follows: scan range 375-1400 m/z; resolution 60,000; RF lens 40%; AGC target 100%; maximum injection time 50 msec. Data-dependent acquisition was performed using the following parameters: dependent scans 15 (top-15); dynamic exclusion 20 sec; charge states 2-6; intensity threshold $5e^4$. Tandem mass spectrometry scans were acquired as follows: isolation window 1.6 m/z; resolution 30,000; normalized collision energy 30%; AGC target 100%; maximum injection time 120 msec.

Generation of a custom metagenomic database

DNA was extracted from fecal samples with the QIAamp Fast DNA Stool Mini Kit (Qiagen). DNA quantification was performed using a QubitTM Fluorometer with the dsDNA High Sensitivity assay kit (Thermo Fisher Scientific). A DNA pool was prepared by mixing an equal volume of DNA extract from each sample to carry out a shotgun sequencing of the whole gut metagenome. The DNA was subjected to tagmentation and ligation of MiSeq adaptors according to the instructions of the Nextera XT kit (Illumina, San Diego, USA). Libraries (average size of 500 bps) were validated by capillary electrophoresis on a chip using the TapeStation4150 instrument with the D1000 ScreenTape System kit (Agilent Technologies, Santa Clara, USA). Raw reads were filtered and assembled into contigs using MetaVelvet v.1.2.02 (Namiki et al., 2012), by setting 61 as k-mer length, 200 as insert length, and 300 as minimum contig length. Open reading frame (ORF) finding was carried out using FragGeneScan v.1.3(Rho et al., 2010), training for Illumina sequencing reads with about 0.5% error rate. Assembled contigs (amino acid sequences) were included in a fasta file, which was subjected to removal of redundant sequences using CD-HIT (Fu et al., 2012) and used as sequence database for peptide identification.

Bioinformatic analyses

Peptide identification was carried out using the Proteome Discoverer™ software (v.2.5; Thermo Fisher Scientific), with Sequest-HT as search engine and Percolator for peptide validation, setting the false discovery rate (FDR) threshold to 1%. Search parameters were as follows: precursor mass range 350-5000 Da; minimum peak count 6; S/N Threshold 2, enzyme trypsin (full); maximum missed cleavage sites 2; peptide length range 5-50 amino acids; precursor mass tolerance 10 ppm; fragment mass tolerance 0.02 Da; static modification cysteine carbamidomethylation; dynamic modification methionine oxidation. Searches were conducted in parallel against four sequence databases:

- the collection of human gut metagenomes (available at https://ftp.cngb.org/pub/SciRAID/Microbiome/humanGut_9.9M/GeneCatalog/IGC.pep.gz)
- the *Homo sapiens* reference proteome retrieved from UniProtKB/Swiss-Prot (release 2021_04);
- the *C. difficile*, *Candida* and *Saccharomyces* proteome reference retrieved from UniProtKB/Swiss-Prot (release 2021_04);
- custom metagenomic sequences obtained from the same samples, as described above.

Offline mass recalibration and label-free MS1 quantitation were carried out using the Spectrum Files RC and the Minora Feature Detector nodes, respectively. Optimal settings for retention time and mass tolerance windows were calculated by the Minora algorithm based on mass accuracy and retention time variance distribution. A consensus feature list was defined based on Feature Mapper and Precursor Ions Quantifier nodes outputs. MS1 signals of all peptides significantly matching with at least an MS2 spectrum from at least one sample were mapped across runs and quantified by calculating the integrated area of the chromatographic peak.

Unipept Desktop (v.2.0.0) was used to carry out peptide taxonomic annotation, selecting the three available options ("equate I and L", "filter duplicate peptides" and "advanced missed cleavage handling"). Protein sequences were subjected to functional annotation using the eggNOG-mapper web application (v.2.1.9, available at http://eggnog-mapper.embl.de/) (Cantalapiedra et al., 2021), keeping default parameters and then choosing KEGG (Kyoto Encyclopedia of Genes and Genomes) orthology (KO) information as main functional classification. Two more generic functional annotation named as “KEGG module” and “KEGG pathway” were used in various points of this study (Kanehisa et al., 2023).

Host peptides were sorted in master proteins, determined by Proteome Discoverer's protein grouping algorithm, according to the maximum parsimony principle. When an identified peptide could belong to several proteins, these proteins are classified into a protein group, represented by a master protein. As reported by Proteome Discover's manual, we define the master protein as "*the protein most likely present in the sample (usually having the highest number of peptides and highest molecular weight). The master protein is identified by a set of peptides that is not part of another protein group"*.

Meta4P (v.1.2) was used to parse identification, quantification and annotation data and generate aggregated abundance tables (Porcheddu et al., 2023). The abundance of a taxon, a function or a taxon-specific functions was estimated by summing the peak areas associated to all peptides having that feature among their annotations.

Statistical analyses and graph generation

Alpha diversity was calculated based on Shannon's index (Shannon, 1948). The Perseus computational platform (v.1.6.7.0) was used to calculate the beta diversity among the groups by performing the principal component analysis (PCA) on peptide intensity, using the platform as followed: abundance data were uploaded, and missing values were replaced with 0 and using default settings. The same platform was used to carry out the differential analysis on aggregated abundance tables as described: abundance data were subject to binary logarithmic transformation to approximate a normal distribution (checked afterwards using the Kolmogorov-Smirnov test); peptides not reaching 75% of valid values in at least one group (for each comparison) were removed; missing values were replaced with a constant value, corresponding to the binary logarithm of the lowest peptide abundance (approximated to the nearest integer) minus 1; differential peptide abundances between groups were tested with a two-tailed not paired Student's t-test; t-test p-values were corrected for multiple testing by calculating a false-discovery rate (FDR) according to Benjamini and Hochberg (Benjamini & Hochberg, 1995) considering $q = 0.05$ as the significance threshold. Scatter plots were created using GraphPad Prism (v.9).

Results

General metrics

The study involved 80 leftovers of samples collected after the diagnostic activities at the AOUSS from July 2020 to November 2021. Stool samples belonged to three different types of patients as mentioned in the paragraph "Patients and samples". The average age of patients analyzed was 76 years, with an even distribution of the gender, 39 males (49%) and 41 females (51%), as shown in **Table 1**.

Table 1*. Patients' metrics.*

group	age		gender	
	mean	**SD**	**M**	**F**
total	75.68	15.98	39	41
N	75.47	16.61	14	16
Tg	73.85	17.23	12	8
Tp	77.10	14.86	13	17

All stool specimens underwent protein extraction and all peptides obtained via FASP were analyzed by LC-MS/MS and then identified, quantified, and annotated through bioinformatic analysis. A preliminary analysis was carried out against a broad-range, well-annotated database (DB) (UniProtKB/Swiss-Prot, release 2021_04) to figure out whether any samples contained microorganisms usually not included in a 'normal' human gut microbiota. Results (data not shown) highlighted the presence of peptides belonging to eukaryotic genera, namely *Saccharomyces* and *Candida*, in some samples of Tp and Tg groups; accordingly, we added UniProtKB/Swiss-Prot sequences from these two genera as a supplementary DB for peptide identification. Moreover, to be more confident that *C. difficile* sequences were well represented in the DB for peptide identification, we also created a further DB containing *C. difficile* sequences. A custom metagenomic DB was also generated, by sequencing the pool of DNA extracts obtained from the same samples subjected to metaproteomic analysis. This DB was used in parallel with a publicly available collection of human gut metagenomes, with the aim of enhancing the identification yield (Tanca et al., 2016).

A total of 79141 peptides were identified, of which 51358 matched the publicly microbial databases, 10641 matched the human database and 317 matched with both databases (thus classified as 'ambiguous'). Among microbial peptides, 421 peptides matched uniquely the custom metagenomic database and 12858 matched both the custom and the publicly available metagenomic databases. Furthermore, 3391 peptides matched the *Saccharomyces*/*Candida* database and 155 the *C. difficile* database.

Peptide quantification metrics are illustrated in **Figure 1**.

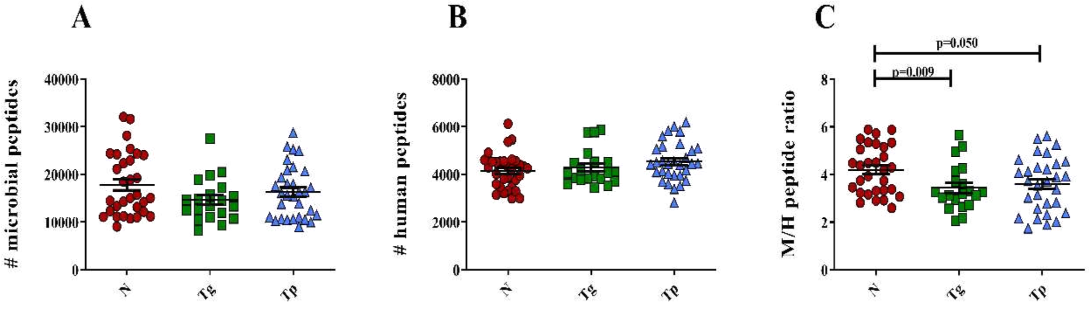

Figure 1. Distribution of the number of quantified peptides among groups. *Each dot represents a different patient; each group is marked by a different combination of shape and color. Black lines indicate mean and standard deviation for each group. P-values are reported above each significant comparison. A positive microbial (M)/ human (H) ratio value indicates a higher amount of microbial peptides; a negative M/H ratio indicates a higher amount of human peptides. A) Scatterplot showing the amount of microbial (M) peptides quantified for each group. B) Scatterplot showing the number of human (H) peptides quantified for each group. C) Scatterplot showing the ratio between the number of M and H peptides quantified for each group.*

The number of microbial (**Fig. 1A**) and human (**Fig. 1B**) peptides quantified did not differ significantly among groups. Furthermore, as shown in **Fig. 1C**, the ratio between the numbers of microbial and host peptides quantified (M/H ratio) was significantly higher in the N group in both comparisons vs Tg (p = 0.009) and vs Tp (p = 0.050).

Abundances of microbial and human peptides, calculated for each sample as the sum of the MS1 peak areas of all quantified peptides, were also evaluated, as detailed in **Figure 2**.

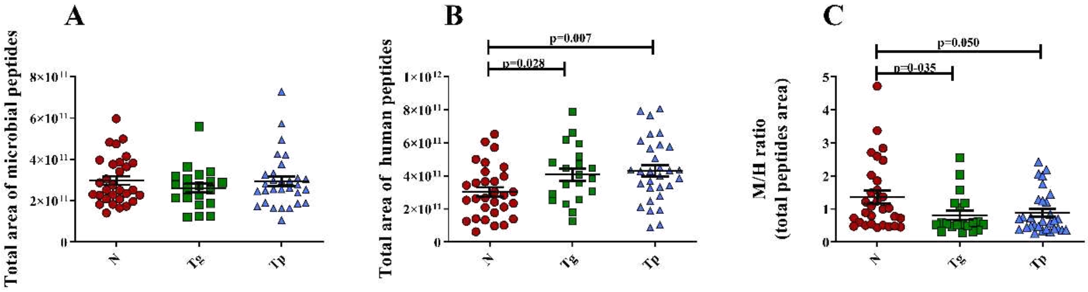

Figure 2. Distribution of peptide abundance among groups. *Each plot represents a different patient; each group is marked by a different combination of shape and color. Black lines indicate mean and standard deviation for each group. P-values are reported above each significant comparison. A positive microbial (M)/ human (H) ratio value indicates a higher amount of microbial peptides; a negative M/H ratio indicates a higher amount of human peptides. A) Scatterplot showing the abundances of microbial (M) peptides for each group. B) Scatterplot showing the abundance of human (H) peptides for each group. C) Scatterplot showing the ratio between the abundances of M and H peptides for each group.*

As shown in **Fig. 2A**, the abundances of the peptides assigned to the GM did not differ significantly between the three groups. On the contrary,

host peptides were significantly less abundant in group N compared with both Tg and Tp groups (p-values 0.028 and 0.007, respectively; **Fig. 2B**). Moreover, as illustrated in **Fig. 1C**, the ratio between microbial and host peptide abundances was significantly higher in the N group in both comparisons vs Tg (p = 0.035) and vs Tp (p = 0.050).

Considering that the abundance of human peptides was significantly higher in the Tg and Tp groups compared to the N group and that, consistently, the M/H peptide abundance ratio was significantly lower in the Tg and Tp groups compared to the N group, a relatively lower amount of microbial proteins appeared to be present in the stool samples of CDI-positive patients compared to those collected from CDI-negative patients. In view of this, before searching for microbial taxa and functions with differential abundance between groups, the effect of the varying relative abundances of the microbial fraction was reduced by filtering out human peptides and performing data normalization. Normalized microbial peptide abundances were then aggregated for each sample based on their assignment to taxonomic and functional features. Abundance values measured for taxa, functions, and taxon-specific functions in the patients of each group were used for all the analyses described below.

Similarly, host protein abundance values (obtained upon the application of Proteome Discoverer's protein grouping algorithm) were also normalized before differential analysis between groups. Results referring to host proteins are detailed in the paragraph *Evaluation of human proteins*.

Evaluation of richness, alpha diversity, and beta diversity of gut microbial communities

Richness was defined for each sample as the number of taxonomic and functional features (at different levels) exhibiting non-zero abundance, as shown in **Fig. 3**.

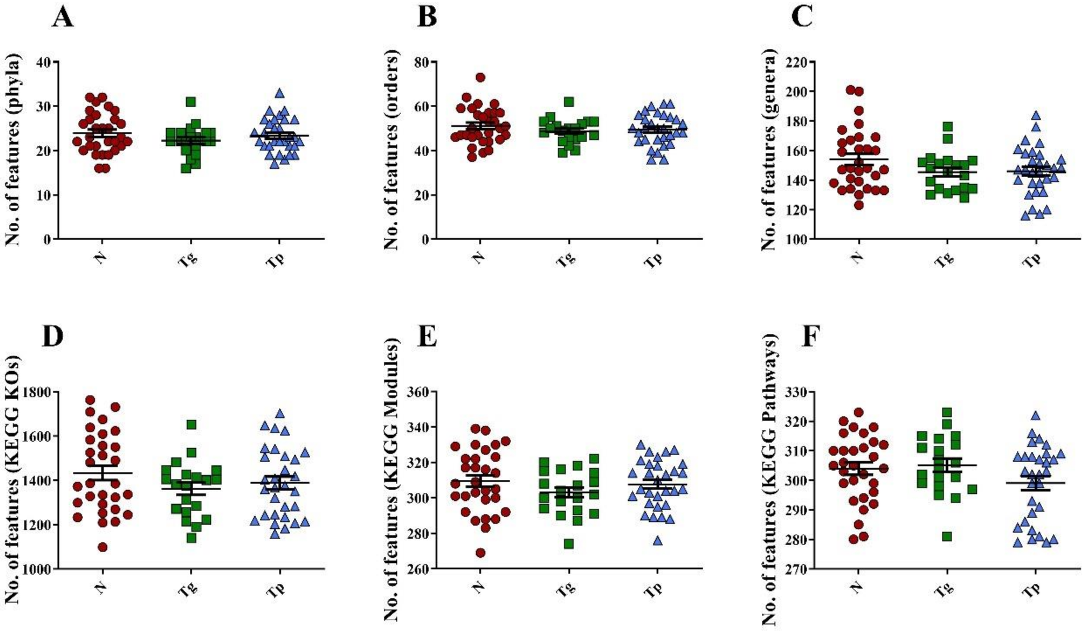

Figure 3. Evaluation of richness at various taxonomic and functional levels. *Scatterplots illustrating the numbers of microbial phyla (A), orders (B), genera (C), KEGG KO functions (D), KEGG modules (E) and KEGG pathways (F) detected in the samples. Each dot represents a different patient; each group is marked by a different combination of shape and color. Black lines indicate mean and standard deviation for each group.*

No statistically significant differences in richness were detected neither at the phylum, order, and genus levels (**Fig. 3A-C**), nor at the different functional levels examined (**Fig. 3D-F**).

Alpha diversity (calculated according to Shannon's index) was also evaluated, by considering peptide abundances aggregated at different taxonomic and functional levels. Scatterplots in **Figure 4** show the distribution of alpha-diversity values in the three experimental groups.

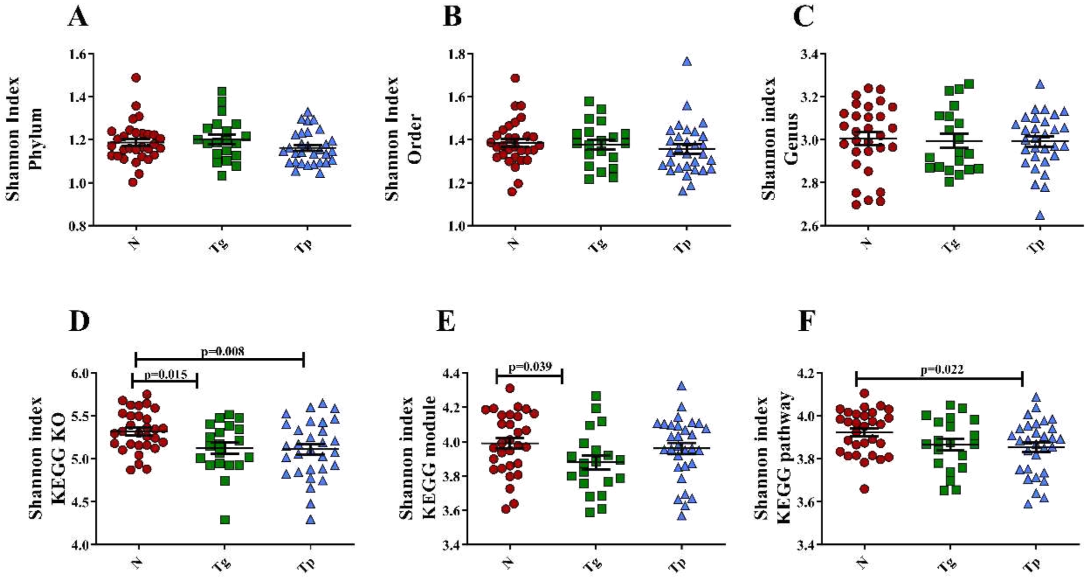

Figure 4. *Alpha diversity (expressed as Shannon's index) calculated based on the abundance distribution of phyla (A), orders (B), genera (C), KEGG KO (D), KEGG modules (E) and KEGG pathways (F). Each dot represents a different patient; each group is marked by a different combination of shape and color. Black lines indicate mean and standard deviation for each group. P-values are shown above each significant comparison.*

As a result, no significant differences were observed for alpha diversity considering taxonomic data. Significant decreases of the Shannon's index in Tg and Tp groups compared to the N group were detected (p-value 0.015 and 0.008, respectively) when considering KEGG KO data. Shannon's index also declined significantly in the Tg group compared to the N group (p-value 0.039) when considering KEGG Module data, as well as in the Tp group compared to the N group (p-value 0.022) when considering KEGG Pathway data.

Abundance data aggregated at different taxonomic and functional levels were also used to assess beta diversity by performing a Principal Component Analysis (PCA), as detailed in **Figure 5**.

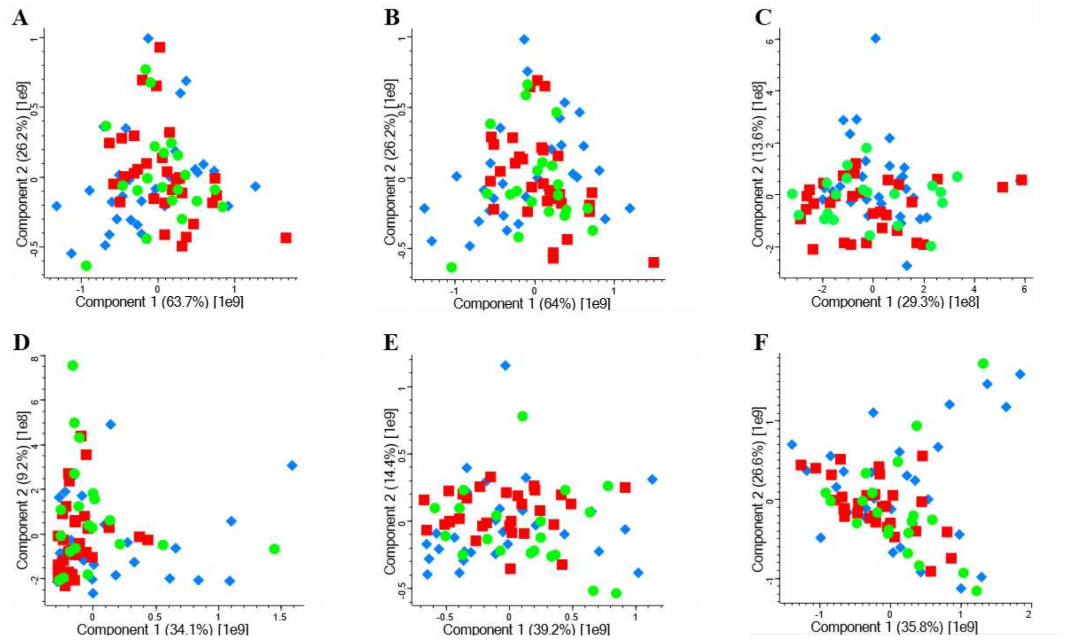

Figure 5. *Principal component analysis (PCA) plots based on phylum (A), order (B) and genus (C), KEGG KO (D), KEGG module (E) and KEGG pathway (F) abundance data. The percentage of variation explained by the first two components are shown in x- and y-axis, respectively. Each dot represents a different patient; each group is marked by a different combination of shape and color.*

As a result, no distinct clustering of any of the three groups was observed at the different levels of aggregation of peptide abundance data.

Distribution of taxonomic and functional features among groups

Peptide abundance data were aggregated at different taxonomic levels. The relative abundance distribution at the phylum level in the three groups is shown in **Figure 6**.

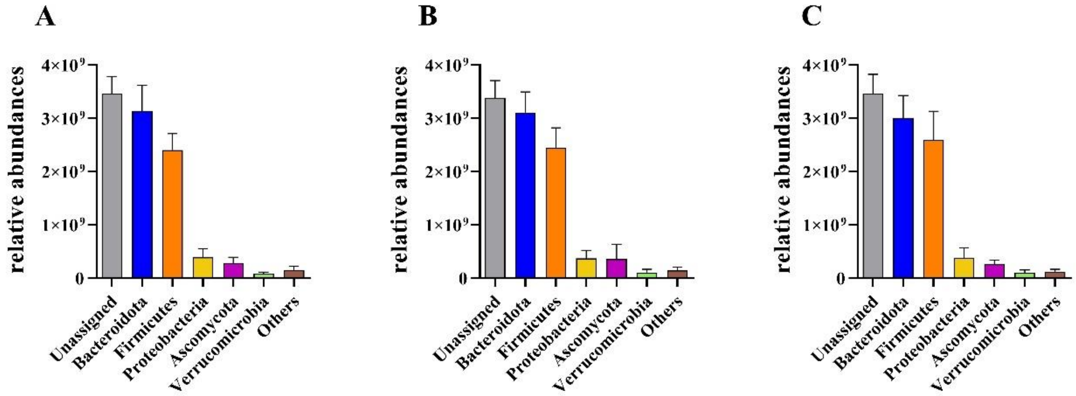

Figure 6. *Relative abundance of the most abundant phyla in groups N (A), Tg (B), and Tp (C). Error bars represent standard of the mean (SEM). The 'unassigned' bar refers to all peptides that could not be assigned to one specific phylum (being their sequence shared between different phyla).*

Bacteroidota resulted as the most abundant phylum in all groups, immediately followed by Firmicutes. Proteobacteria, Ascomycota, and Verrucomicrobia were clearly less abundant compared to the two main phyla.

The most abundant microbial orders and families are provided in **Table 3** and **4**, respectively.

Table 3. *Top ten most abundant microbial orders. Mean abundance and standard deviation (SD) are reported as percentage values.*

Order	**N**		**Tg**		**Tp**	
	Abundance	**SD**	**Abundance**	**SD**	**Abundance**	**SD**
Bacteroidales	49.35%	5.68%	48.33%	5.52%	47.59%	6.99%
Eubacteriales	34.22%	4.88%	34.82%	5.11%	36.34%	8.32%
Saccharomycetales	4.18%	1.68%	5.21%	3.86%	4.05%	1.15%
Enterobacterales	3.58%	1.99%	3.26%	1.62%	3.47%	2.03%
Verrucomicrobiales	1.29%	0.46%	1.61%	0.92%	1.59%	0.90%
Lactobacillales	0.94%	0.41%	0.94%	0.68%	1.27%	1.68%
Acidaminococcales	0.86%	0.42%	0.91%	0.66%	1.36%	1.60%
Burkholderiales	0.69%	0.24%	0.87%	0.58%	0.61%	0.19%
Veillonellales	0.70%	0.34%	0.57%	0.12%	0.74%	0.25%
Bifidobacteriales	0.64%	0.55%	0.55%	0.25%	0.54%	0.32%

Table 4. *Top ten most abundant microbial families. Mean abundance and standard deviation (SD) are reported as percentage values. Peptostreptococcaceae family is also shown, even though it is not included in the ten most abundant families.*

Family	N		Tg		Tp	
	Abundance	**SD**	**Abundance**	**SD**	**Abundance**	**SD**
Bacteroidaceae	22.30%	4.69%	22.88%	5.38%	24.48%	3.67%
Oscillospiraceae	13.13%	3.39%	10.47%	2.95%	11.25%	4.44%
Lachnospiraceae	7.92%	3.97%	9.81%	4.44%	8.66%	3.60%
Rikenellaceae	7.60%	2.37%	7.43%	2.37%	6.68%	1.63%
Clostridiaceae	5.91%	3.27%	6.18%	4.09%	5.33%	2.14%
Tannerellaceae	5.75%	3.79%	4.88%	1.11%	5.66%	2.21%
Prevotellaceae	6.57%	4.63%	5.24%	1.69%	4.36%	1.37%
Saccharomycetaceae	4.62%	2.16%	6.49%	4.97%	5.10%	1.97%
Akkermansiaceae	2.45%	0.80%	3.16%	1.75%	2.98%	1.42%
Eubacteriaceae	1.92%	1.44%	2.18%	1.55%	4.04%	5.43%
Peptostreptococcaceae	0.63%	0.39%	0.60%	0.37%	0.71%	0.35%

Bacteroidaceae, Rikenellaceae, Tannerellaceae, and *Prevotellaceae* - all belonging to the most abundant order, Bacteroidales - resulted as the most represented families, followed by *Oscillospiraceae* and *Lachnospiraceae*, which belong to *Eubacteriales*. *Peptostreptococcaceae*, the family to which *C. difficile* belongs, showed a relative abundance of less than 1% in each of the three groups. The ten most abundant genera are reported in **Table 5**.

Table 5. *Top ten most abundant microbial genera. Mean abundance and standard deviation (SD) are reported as percentage values. Clostridioides is also shown, even though it is not included within the ten most abundant genera.*

Genus	N		Tg		Tp	
	Abundance	**SD**	**Abundance**	**SD**	**Abundance**	**SD**
Bacteroides	25.24%	5.21%	26.21%	5.13%	27.57%	5.37%
Alistipes	8.43%	2.40%	8.57%	2.59%	7.58%	2.60%
Faecalibacterium	7.66%	2.97%	6.39%	3.28%	6.78%	3.31%
Parabacteroides	6.40%	4.01%	5.62%	3.92%	6.36%	3.94%
Prevotella	7.38%	5.38%	5.90%	4.74%	4.75%	4.77%
Clostridium	4.79%	3.12%	5.69%	3.06%	4.49%	2.97%
Nakaseomyces	3.19%	1.67%	4.82%	1.69%	3.62%	1.87%
Ruminococcus	3.98%	1.96%	2.90%	1.97%	2.88%	1.98%
Akkermansia	2.78%	0.86%	3.65%	0.91%	3.37%	0.90%
Eubacterium	2.20%	1.57%	2.51%	1.81%	4.51%	1.77%
Clostridioides	0.44%	0.30%	0.41%	0.27%	0.53%	0.34%

Bacteroides, Alistipes, *Parabacteroides* and *Prevotella,* belonging to the most abundant order (Bacteroidales), are among the most abundant genera. *Faecalibacterium* and *Ruminoccocus*, second and seventh most abundant genus, respectively, belong to the second most abundant order (Eubacteriales). The relative abundance of the genus *Clostridioides*, to which *C. difficile* belongs, is below 1%.

Peptide abundance data were aggregated also at different functional levels. The 10 most abundant KO functions in three groups are reported in **Table 6**.

Table 6. *Top ten most abundant KO functions. Functions are ordered according to their abundance among groups. Abundance (Abd) and standard deviation (SD) are reported for each function.*

KEGG KO	name	N		Tg		Tp	
		Abd	**SD**	**Abd**	**SD**	**Abd**	**SD**
K21572	starch-binding outer membrane protein, SusD/RagB family	4.12%	1.62%	4.28%	1.38%	4.57%	1.79%
K00134	glyceraldehyde 3-phosphate dehydrogenase (phosphorylating)	3.71%	1.89%	3.60%	1.78%	3.98%	2.48%
K13922	propionaldehyde dehydrogenase	1.39%	1.69%	2.75%	3.60%	3.74%	4.26%
K00132	acetaldehyde dehydrogenase (acetylating)	1.31%	1.67%	2.64%	3.57%	3.64%	4.24%
K00262	glutamate dehydrogenase (NADP+)	2.47%	1.18%	1.97%	0.89%	2.60%	1.01%
K01803	triosephosphate isomerase (TIM)	2.02%	2.23%	3.54%	4.26%	1.45%	1.21%
K01715	enoyl-CoA hydratase	1.83%	1.95%	2.52%	1.93%	1.53%	1.36%
K02863	large subunit ribosomal protein L1	2.04%	1.42%	1.60%	1.52%	1.88%	2.28%
K01689	enolase	1.49%	0.71%	2.03%	0.91%	1.71%	1.42%
K00261	glutamate dehydrogenase (NAD(P)+)	1.58%	0.83%	1.22%	0.51%	1.74%	0.77%

Starch-binding outer membrane protein, SusD/RagB family is the most abundant function in all groups.

Taxonomic, functional, and taxon-specific features with differential abundance between groups

Taxonomic, functional, and taxon-specific functional features were then subjected to differential analysis between the three experimental groups. Differences were considered significant when FDR < 0.05.

Among taxa, two genera were found as significantly differentially abundant between groups, as listed in **Table 7**. No significant changes in relative abundance were detected for phyla and orders.

Table *7. Microbial genera with significantly differential abundance between groups. False discovery rate (FDR) and log ratio (LR) are reported. Features are sorted in alphabetical order. Significant FDR values are highlighted in bold (FDR<0.05).*

genus	FDR N/Tg	FDR N/Tp	FDR Tg/Tp	LR N/Tg	LR N/Tp	LR Tg/Tp
Anaerostipes	0.611	**0.043**	0.608	-0.89	-1.65	-0.76
Ruminococcus	0.612	**0.044**	0.924	0.44	0.43	-0.01

Ruminococcus relative abundance was significantly higher in the N group compared to the Tp group. A positive log ratio (LR), labelled in green, indicates a higher abundance of *Ruminococcus* in the N vs Tg group, without reaching significancy. *Anaerostipes*, on the contrary, was significantly more abundant in the Tp group compared to the N group. A negative LR, labelled in red, indicates a higher abundance of *Anaerostipes* also in the Tg vs N group, without reaching significance.

Four KO functions were also found to be differentially more abundant in the Tp group compared to the Tg group, as shown in **Table 8**.

Table 8. *Microbial KO functions with statistically differential abundance among the three comparisons. For each function False Discovery Rate (FDR) and abundance Log Ratio (LR) are reported. Features are ordered based on the KEGG KO number. Significant FDR values are highlighted in bold (FDR<0.05).*

KEGG KO	**KO name**	**FDR N/Tg**	**FDR N/Tp**	**FDR Tg/Tp**	**LR N/Tp**	**LR N/Tp**	**LR Tg/Tp**
K01591	orotidine-5'-phosphate decarboxylase	0.424	0.534	**0.002**	1.07	-0.09	-1.16
K01745	histidine ammonia-lyase	0.595	0.316	**0.046**	0.91	-0.07	-0.98
K02035	peptide/nickel transport system substrate-binding protein	0.413	0.636	**0.029**	0.47	-0.12	-0.60
K03694	ATP-dependent Clp protease ATP-binding subunit ClpA	0.432	0.318	**0.045**	1.18	-1.51	-2.69

These functions include proteins involved in ion and nucleotide transport, in pyrimidine biosynthesis and in histidine degradation.

Among KEGG modules, histidine => N-formiminoglutamate => glutamate (belonging to histidine degradation) was differentially more abundant in the Tp group, as illustrated in **Table 9**.

Table 9. *Microbial KO module with statistically differential abundance among the three comparisons is quoted. False discovery rate (FDR) and log ratio (LR) according to abundance of peptides are reported. Significative FDR values are highlighted in bold (FDR<0.05).*

KEGG module	**module name**	**FDR N/Tg**	**FDR N/Tp**	**FDR Tg/Tp**	**LR N/Tp**	**LR N/Tp**	**LR Tg/Tp**
M00045	Histidine degradation, histidine => N-formiminoglutamate => glutamate	0.919	0.091	**0.008**	0.65	-0.24	-0.89

Combined functional (functions, modules, or pathways) and taxonomic (phylum, order, or genus) annotations are used to investigate taxon-specific functions.

Four phylum-specific KEGG modules resulted as differentially expressed between groups, as detailed in **Table 10**.

Table 10*. Differential phylum-specific KEGG modules. False discovery rate (FDR) and log ratio (LR) are reported for each comparison. Features are alphabetically ordered based on the phyla names, and then on KEGG module number. Significant results (FDR < 0.05) are highlighted in bold.*

Phylum	**module**	**module name**	**FDR N/Tg**	**FDR N/Tp**	**FDR Tg/Tp**	**LR N/Tp**	**LR N/Tp**	**LR Tg/Tp**
Bacteroidota	M00014	Glucuronate pathway (uronate pathway)	0.718	**0.042**	0.697	-1.35	-2.40	-1.05
Bacteroidota	M00307	Pyruvate oxidation, pyruvate => acetyl-CoA	0.626	**0.046**	0.420	-0.93	-1.67	-0-75
Bacteroidota	M00129	Ascorbate biosynthesis, animals, glucose-1P => ascorbate	0.552	**0.042**	0.697	-1.35	-2.40	-1.05
Unassigned	M00045	Histidine degradation, histidine => N-formiminoglutamate => glutamate	0.552	0.684	**0.047**	1.71	0.80	-0.91
Firmicutes			0.733	**0.042**	0.340	-0.27	-0.27	-1.02

Three Bacteroidota-specific KEGG modules (involved in glucuronate pathway, pyruvate oxidation and ascorbate biosynthesis, respectively) were differentially increased in the Tp group when compared to the N group. Bacteroidota-specific functions showed negative LR values in all comparisons. Moreover, a Firmicutes-specific KEGG module related to histidine degradation was found to be significantly more abundant in Tp compared to N. The same module resulted significantly higher in the Tp group compared to the Tg group when combined with the category 'unassigned' (including all peptides that could not be assigned to a specific phylum).

Evaluation of human proteins

After grouping the human peptides into master proteins, 1644 proteins were obtained. Upon differential analysis, no significant differences in abundance among groups were observed.

Discussion

Over the past few years, a considerable amount of research on the GM has focused its attention on medicine and personalized nutrition. A "healthy" gut microbiota is a sign of a healthy individual, as the microbial community has a role in immune system development, metabolism, and protection from enteropathogenic invasion. The identification and characterization of the structure and functionalities of the health-associated gut microbiota is of paramount importance and will be essential for formulating targeted therapeutic interventions. On the other hand, it is also crucial to understand the significant changes linked with gastrointestinal bacterial infections, to optimize management of antibiotic therapy, that should also be paralleled by other measures positively affecting the GM, including dietary interventions. It is well established that CDI is related to a condition of dysbiosis, which is generally determined by long term exposure to antibiotics. Hence, it is extremely difficult to disentangle the effects of antibiotics on the microbial communities from those arising from *C. difficile* toxins activities and enteropathogenic consequences. Further, the effects of *C. difficile* infection on the composition, structure, and functions of the GM have not been thoroughly explored through functional meta-omics methods. More specifically, metaproteomics offers a valuable tool to evaluate the quantity of microbial biomass within the microbiome, analyze its community structure, and assess its functional capabilities. In this study, we chose to employ metaproteomics due to its ability to quantify the bacterial taxa responsible for over 90% of the total microbiota biomass while simultaneously allowing us to investigate changes in both microbial and human functions (X. Zhang & Figeys, 2019).

As the research study focused on the influence of *C. difficile* on the GM, we decided to use all the sequences present on Swiss-Prot related to *C. difficile*, in order to have a good quantification of the associated peptides. We found that *C. difficile*-specific peptides were well represented in the public sequences database.

As previously demonstrated, taxonomic and functional results are highly database-dependent (Tanca et al., 2016); so, for the identification of unique peptide assortment, in addition to the publicly available sequence databases mentioned above, we used another database containing all metagenomic sequences obtained from our experimental groups, allowing us to identify and quantify a large amount of organisms for which no reference genome sequence is available, including members with a low abundance (Cantarel et al., 2011).

Comparing three groups of subjects with a common pre-existing condition - i.e. constant and continuous exposure to antibiotics - thus related to a pre-existing condition of dysbiosis, those who develop CDI presented a different taxonomic-functional picture.

Previous studies on patients exposed to short- or long-term antibiotic treatments showed lower counts of reads (metagenomic studies) and peptides (metaproteomic studies) compared to untreated patients (Abbondio et al., 2023; Wuyts et al., 2023). Lack of difference in the three groups can be explained by the exposure to antibiotic therapy in all patients recruited in this study. Considering the total amount of quantified peptides, human peptides were significantly more represented in both CDI-positive cohorts than in the CDI-negative group. This might be determined by the inflammatory and the diarrheal state of the CDI, enhanced by the presence of toxins in the Tp group. To minimize the effect of variations in the relative abundance of the microbial fraction, we renormalized microbial peptide abundances and aggregated them based on their taxonomic and functional annotations. Summed data were used as input for further analysis.

The alpha-diversity of microbial functions, as measured by mean of Shannon's index, was decreased in the Tg and Tp groups. Regarding the sole richness, no difference was detected. Since the Shannon's index estimates both richness and evenness, it is possible that the main difference between CDI- and CDI+ samples is due to evenness of microbial functions distribution that could be higher in the former. Since patients of all groups are expected to share a dysbiotic condition due to antibiotic exposure, the active colonization of *C. difficile*, as detected by CLIA and molecular tests may be sustained by specific functions encoded by the GM, whose enhancement might explain the evenness reduction in CDI positive samples. While PCAs did not show a clustering of any of the three groups, further differential analysis showed significant variation of GM functions relative abundance between the sample groups.

Focusing on the microbial taxonomy, the alpha-diversity values estimated with the Shannon's index were similar between groups, suggesting less variation at level of taxa than at level of microbial functions. Indeed, differential analysis of aggregated peptides abundance data did not identify any significant variations at the phylum level. While the number of peptides assigned to other taxonomic levels (i.e. genera) is obviously lower, members belonging to the same phylum reciprocally display high diversity in terms of encoded biological functions and their regulation in response to the external stimuli. Specifically, we observed two GM genera with a different response to the *C. difficile* colonization environment, suggesting the existence of a variable degree of vulnerability and/or adaptability to the gut perturbation provoked by *C. difficile* toxins. The genus *Ruminococcus* was significantly decreased in the Tp when compared to N group. Investigating the trend followed by this genus by log ratio values, *Ruminococcus* abundance is decreased also in Tg when compared to N group, although without a statistically significance. This result is consistent with previous findings: in the presence of CDI, it has been seen a decrease in members belonging to the Bacteroidaceae, Lachnospiraceae, and Ruminococcaceae (Han et al., 2019; Vázquez-Cuesta et al., 2023). *Ruminococcus* has been suggested to exhibit a protective role against CDI; this genus is known to produce a bacteriocin known as ruminococcin A, which is known to inhibit the development of various members of the *Clostridium* genus (Gomez et al., 2002). A prospective, observational study reported that subjects who developed CDI following antibiotic therapy showed a microbiota enriched in members of the *Enterococcus* genus with a depletion in *Ruminococcus* (Berkell et al., 2021). This genus could be considered as a predictive marker, protective against CDI (Solbach et al., 2021). *Ruminococcus* has also been associated with the deconjugation of taurocholic acid (Martin et al., 2018), a primary bile acid positively correlated with spore germination, consistently with an inverse correlation between the abundance of this genus and that of *C. difficile.* A limit of this study is the lack of GM data before the administration of antimicrobials. Hence, we could not address the hypothesis that subjects who did not develop CDI in the hospital wards may have had a higher amount of *Ruminococcus* than those belonging to the CDI groups. Previous studies supported the hypothesis that GM composition at baseline might affect susceptibility to infection from this pathogen that is highly transmitted throughout the hospital wards (Solbach et al., 2021) .

The genus *Anaerostipes* showed an opposite variation compared to *Ruminococcus*, with its relative abundance significantly increased in the Tp group compared to N group. Considering the log ratio value, although with no statistically significant difference, *Anaerostipes* abundance was also higher in the Tg group compared to the N group. *Anaerostipes* is able to produce butyrate, a short chain fatty acid that decreases intestinal permeability, thus preventing an inflammatory condition (Rivière et al., 2016; Singh et al., 2023). To this end, this genus has been reported to be decreased in patients with CDI, when compared to a negative group of healthy subjects (Han et al., 2019). On the contrary, consistently with our results, in a comparison of CDI subjects with patients colonized by vancomycin-resistant enterococci (VRE), *Anaerostipes* was found to be increased in the CDI patients (Jeon et al., 2019). While we have no data to explain the increase of *Anaerostipes* in a dysbiotic GM following antibiotic treatment, the study of Jeon et al. and our data suggest that *C. difficile* colonization and growth of *Anaerostipes* might be favored by similar GM and/or host factors during inflammation.

To investigate the GM functional features that might parallel taxonomic variation and to evaluate the potential interdependency between taxonomic and functional variations, the abundance of protein functions was evaluated to investigate the specific differences occurring between groups. Concerning single functions, toxin-producing *C. difficile* colonization was associated to an increased abundance of the complex ATP-dependent Clp protease/ATP-binding subunit ClpA (ClpA), which resulted significantly more abundant in the Tp group, compared to Tg. ClpA belongs to the class I of the regulatory Clp subunit proteins, which is comprised of ClpA, B, C, D, E and L. These energy dependent proteases are classified under the HSP100 family proteins (Doyle & Wickner, 2009). Clps function as both proteases and chaperones (Chastanet et al., 2004), improving the stress tolerance of bacteria under heat and acid conditions causing unfolding and aggregation of proteins. The stress-induced intracellular accumulation of protein aggregates hampers the cell survival and requires the action of these functions. ClpA has been previously described in *Lactobacillus* spp. as one of the few overexpressed proteins during a 12-month adaptive evolution experiment under ampicillin pressure (Cao et al., 2020). Given the well-known importance of bacterial Clp proteases to enable versatile adaptation to diverse environments and efficient stress compensation (Illigmann et al., 2021), ClpA might be expressed as a response to stress strongly provoked by toxin-producing *C. difficile* strains. Recently, similar data were obtained in a cohort of patients treated with metronidazole, tetracycline, and bismuth to eradicate *Helicobacter pylori* infection (Abbondio et al. unpublished data). In that study, increased abundance of ClpB was associated to a single taxon: *Akkermansia muciniphila*. Data obtained with this CDI-/CDI+ cohort extends previous ones and suggest that relative abundance of Clps my serve as biomarker of antimicrobial impact on the GM, regardless the differences in antibiotic regimen prescription.

We report here an increase in the abundance of orotidine-5'-phosphate decarboxylase in patients colonized by toxin-producing *C. difficile* strains. This enzyme is implicated in pyrimidine biosynthesis, including uracil. Interestingly, a previous study in *Drosophila melanogaster* model demonstrated that GM produced uracil can bind and activate dual oxidase dependent (DUOX), a member of the NADPH oxidase family, acting as the first line of defence against enteric pathogens (Lee et al., 2013). DUOX activation leads to production of microbicidal reactive oxygen species (ROS) on gut epithelia, known to boost inflammation condition. While Lee et al. described uracil as produced solely by GM pathobionts, in the present study peptides assigned to orotidine-5'-phosphate decarboxylase were also assigned at level of GM commensal like *Bacteroides*, *Prevotella*, and *Eubacterium*. Kim et al. described the effects of oral vancomycin (VA) on GM and the fecal and host metabolome in a group of healthy men (Kim et al., 2021). After the VA treatment, the study highlights a decrease of relative abundance of members belonging to the phyla Bacteroidota and Firmicutes, and an increase of constituents related to the phylum Proteobacteria, as already reported by Sunwoo et al. (Sunwoo et al., 2020). Concerning metabolomic data, an increase of dihydrouracil (DHU) was detected in fecal specimens after the antibiotic treatment.

Peptide/nickel transport system substrate-binding protein was significantly increased in the Tp subject group compared to the Tg group. This function is related to many different proteins involved in the transport of various molecules and ions, including nickel. It has been already reported the nickel results in changes of the structure and metabolism of the GM (Li & Zamble, 2009) with an increase in ROS. However, focusing on proteins assigned to this KO, we observed that several of the quantified peptides were annotated as uncharacterized proteins or proteins not involved in the nickel transport. Hence, we cannot rule out that the presence of *C. difficile* strains induces a shift in nickel transport, but nickel role in *C. difficile* colonization requires further investigation.

Based on our results, attention should be paid to histidine metabolism. The enzyme histidine ammonia-lyase was found to be more abundant in Tp compared to Tg subjects. Histidine is known to be a co-germinant, i.e. a factor that aids the germination of *C. difficile* spores, indirectly promoting toxin production (Wheeldon et al., 2011). Our data would suggest an increase of histidine catabolism due to higher abundance of histidine in the Tp patients' group, i.e. the patients group with the highest (detectable) toxins productions.

We also found that the Firmicutes-specific proteins belonging to the module histidine => N-formiminoglutamate => glutamate (it belongs to the histidine degradation pathway) was increased in the Tp group compared to the N group. This module consists of 8 KEGG KO functions; we detected 4 of these, and 3 passed the filter set for the differential analysis. One of this function is the enzyme histidine ammonia-lyase (also cited as histidase), already mentioned above. Interestingly, when this module was not assigned to any taxonomic level (indicated in this study as “unassigned”), it was observed as increased in Tp when compared to Tg.

Concurrently, Bacteroidota-specific proteins mapping to the module glucuronate pathway, that belongs to pentose and glucuronate interconversions pathway, resulted increased in Tp compared to N group. This module consists of 12 KEGG KO functions; 7 of these were detected in this study, of which 3 passed the filter we set for differential analysis, including the enzyme uronate reductase. In prokaryotes, histidine is the substrate of histidase producing urocanate, which works as substrate for the prokaryotic enzyme uronate reductase, whose catalytic activity results in the production of imidazole propionic acid (ImP) (Xu et al., 2022). High ImP values are linked to great amount of pro-inflammatory cytokines, due to the fact that ImP is a strong activator of MAP-kinase p38γ (Koh et al., 2018), which has been in turn correlated in a mouse model with heart attacks, and confirmed in humans (Molinaro et al., 2023); this could be one of the reasons why, especially in the case of previous diseases, colonization by *C. difficile* can have a poor prognosis. The same pathway is also activated by *C. difficile* toxin A (P. Zhang et al., 2017). Consistent with our two combined results, high ImP values - a consequence of high catalytic activity of histidine ammonia-lyase and uronate reductase enzymes - were found to be associated with lower alpha diversity values (Molinaro et al., 2020), as we have observed in our study comparing Tp and Tg-patients with N-patients.

Histidine intake from the diet would seem to have no influence on ImP production; however, in this study we did not collect data from the patients' food diary, although a possible imbalance caused by an incorrect diet might be considered negligible compared to that caused by strong antibiotic pressure (Li et al., 2016). In addition, reconnecting with the glucuronate pathway, this also results in the production of xylitol, which appear to inhibit *C. difficile* spores (Rätsep et al., 2017). Furthermore, xylitol can be fermented by *Anaerostipes* producing butyrate (Salli et al., 2019).

The abundance of *Ruminococcus* in group N and *Anaerostipes* in Tp might be considered as indicators of a process aimed at butyrate production. In the case of *Ruminococcus*, this production can be seen as a protective mechanism, while in *Anaerostipes* it might represent an attempt to respond to conditions of increased inflammation caused by the presence of toxins. These toxins are associated with higher levels of ImP (and nickel), and butyrate production by *Anaerostipes* could be a defensive mechanism to mitigate inflammation. However, it must be emphasized that we have not seen differential functional features related to the production of SCFAs, as we have no evidence of nickel enrichment in the fecal sample; these are therefore hypotheses that need further study.

To conclude with taxa-specific functions, our results highlighted an increase of the Bacteroidota-specific module ascorbate biosynthesis, animals, glucose-1P => ascorbate in Tp group compared to N group. This module belongs to ascorbate and aldarate metabolism's pathway and it consists of 6 KEGG KO functions, of which 3 were detected and one passed the filter we set for differential analysis, even if it did not turn out to be differential. Ascorbate cannot be synthesized by humans; thus, it must necessarily be taken with food; however, some GM members, including those belonging to Bacteroidota, are able to produce ascorbate (Arumugam et al., 2011). Ascorbate production could correspond to an attempt to contain the inflammatory response. As demonstrated by Clemetson and colleagues, there is a negative correlation between ascorbate and histamine, due to ascorbate's property to bind and destroy the imidolic ring of histidine, contributing to reduce immune response (Clemetson, 1980).

Conclusion and perspectives

This study was performed to provide insight into the shaping of GM in two groups of patients with vs without *C.* difficile colonization and, further, comparing the impact of toxigenic vs toxin-producing strains of *C. difficile* on the GM of CDI patients.

Our results highlight a CDI-dependent reduction of *Ruminococcus* that reached significance in the toxin-producing (Tp) group, combined with an antiparallel increase of *Anaerostipes* members in the colonized groups.

Regarding functions, *C. difficile* colonization is linked with an increase of histidine degradation, along with an increase of the metabolic pathways "ascorbate synthesis" and "glucuronate pathway" that might concur to mitigate inflammation. Further, in CDI, the degree of mucosa inflammation can be related the production of uracil by the GM members, including commensal.

Although preliminary and regarding small groups of patients, the data obtained from the present study could lay the ground for further in-depth studies on a larger population of CDI patients.

With the aim of fully characterizing the shaping of the structure and functions of the GM in the context of an inflammatory condition as induced/enhanced by the colonization of *C. difficile*, the main challenges that may be addressed in future studies are:

- to design longitudinal studies, including a baseline collection of GM samples at patients admission in the hospital ward, enabling to compared GM taxonomy and functions at time preceding antimicrobial treatment and drug-related dysbiosis. This would enable to identify any potential functional shifts as determined solely by the *C. difficile* colonization.
- to collect food diary, comorbidities, and therapy records for each patient, to figure out the potential role of nickel (or other contaminants), risk factors

due to pathophysiology and patients management, and specific antimicrobials and their dosage, as trigger for *C. difficile* colonization.

Concerning the methodology, this metaproteomics study allowed us to characterize the GM both at taxonomic and functional level, however it has intrinsic limitations, as detailed:

- Limitations in protein identification: protein identification by mass spectrometry may be affected by the availability of reference protein databases. In less studied microbes, there may be a shortage of reference data, making protein identification difficult.
- Protein quantification: quantification of proteins in the GM might be complicated due to the wide range of concentrations and the presence of proteins with similar functions.
- Sample complexity: protein extracts from fecal samples may contain a large amount of human, plant or proteins of unknown origin, making it difficult to concentrate microbial proteins of interest.
- Unknown function of proteins: even if amount microbial proteins are identified, their function may remain unknown. This may limit the understanding of the specific role of microbial proteins in the GM.

Two additional key points, to apply not only to the current study but to all those focused on the GM, concern bioinformatic skills, including:

- Standardized and widely approved pipelines for the analysis of the gut microbiota.
- Sequence reconstruction and functional annotation of a higher number of GM taxa, being currently insufficiently or not characterized.

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