Gut Microbiota and Clostridiodes difficile

Subjects: Microbiology

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Clostridiodes difficile can lead to a range of situations from the absence of symptoms (colonization) to severe diarrhea (infection). Disruption of gut microbiota provides an ideal environment for infection to occur. Comparison of gut microbiota of infected and colonized subjects could provide relevant information on susceptible groups or protectors to the development of infection, since the presence of certain genera could be related to the inhibition of transition from a state of colonization to infection.

Keywords: Clostridiodes difficile ; Clostridiodes difficile infection ; Clostridiodes difficile colonization ; gut microbiota ; 16S ribosomal RNA ; dysbiosis

1. Introduction

Clostridioides difficile is a Gram-positive bacillus that is strictly anaerobic and spore-forming [1]. It is one of the main causes of nosocomial diarrhea in hospitalized patients. Its pathogenicity is associated with the use of antibiotics and a decreased immune response, as well as with advanced age, hospitalization, and greater severity of underlying disease [2] [3]. Clinical issues are due to production of the toxins TcdA and TcdB of cytotoxic action [4]. However, intestinal colonization of *C. difficile* can lead to a range of situations such as an absence of symptoms (colonized subjects) to severe diarrhea or fulminating pseudomembranous colitis (infected subjects) [5][6].

C. difficile infection (CDI) is an especially important problem in terms of mortality, morbidity, and associated costs. In addition, the risk of recurrence is extremely high. It has also been shown that the epidemiology of the CDI has changed since the beginning of the 21st century. Apart from an increase in mortality and morbidity generated by the CDI, existence of many cases from the community has begun to be described [7].

Disruption of endogenous gut microbiota (dysbacteriosis) provides the ideal environment for infection to occur; however, a healthy intestinal microbiota can prevent it. This phenomenon is called resistance to colonization [8]. A corrupted mechanism of resistance to colonization by *C. difficile* key is concern to the metabolism of bile acids [9]. It has been shown that primary bile acids have a germinating activity on spores of *C. difficile*, while secondary bile acids have an inhibitory activity on germination [10]. The gut microbiota provides enzymes responsible for transforming primary bile acids into secondary bile acids (mainly Bile Acid 7α Dehydroxylase or BaiCD.) [11]. The members of the gut microbiota that contribute these enzymes produce an inhibitory effect on germination of *C. difficile*. These genera are present in an extremely low quantity and their loss is associated with CDI [11][12]. Other characteristic alterations of gut microbiota of patients with CDI are a depletion of butyrate producing families such as Lachnospiraceae and Ruminococcaceae [13] and an increase of opportunistic pathogens, mainly from the phylum Proteobacteria [14].

On the other hand, the rate of carriers or colonized subjects by *C. difficile* is variable and dependent on age. It is higher in those subjects with certain concomitant situations that favor colonization as in patients with cystic fibrosis, with inflammatory bowel disease, or with repeated contact with hospital environments [15][16].

Comparison of gut microbiota of individuals infected and colonized by *C. difficile* has been minimally studied, and it is important to know what occurs in the gut microbiota of colonized subjects by *C. difficile* so that individuals do not develop clinical signs. This approach could provide relevant information on susceptible groups or protectors to the development of CDI. This approach will also provide information on pathogenetic mechanisms underlying CDI and to design more focused treatments (including probiotics to modulate gut microbiota) in the future.

2. Discussion

Results show a loss of alpha diversity and richness in infected and colonized subjects with respect to healthy controls. No statistically significant differences were observed between these two groups. This is a frequent finding in patients with CDI

[17] and has also been observed in colonized subjects by *C. difficile* [18] and even in patients with nosocomial diarrhea not due to *C. difficile* [13]. This finding does not differentiate the state of colonization from that of infection. Administration of antibiotics has been considered one of the main triggers of dysbacteriosis with loss of alpha diversity, which is an essential risk factor for the development of CDI [19]. In addition, as has been observed in some of the few prospective studies in this regard, loss of alpha diversity is not an inexcusable development factor towards CDI [20]. In a group of patients with CDI, 93% (14 of 15) had received antibiotics in the previous 3 months, mainly cephalosporins, beta-lactamics, and fluoroquinolones, which are considered of high risk. In colonized subjects, the rate of previous antibiotic administration was 53% (8 of 15), although in the remaining 27%, these data were unknown (4 of 15). Therefore, we have a cause that largely explains the loss of alpha diversity in both groups. There are other factors different from administration of antibiotics that can lead to a loss of alpha diversity, such as liver disease, inflammatory bowel disease, and malignant blood diseases [21]. The presence of these situations occurred in 47% (7 of 15) of the patients in the CDI group, while they were not observed in any of the colonized individuals and healthy controls.

The statistical tool we used to assess beta diversity in gut microbiota of study groups was the UniFrac analysis, the objective of which is to assess whether there is any factor that explains most of the variability of data. We observed that structure of gut microbiota of healthy controls is completely different from that of infected and colonized subjects, which would overlap. Therefore, we could not use beta diversity as a notable differentiator of the state of colonization and infection by *C. difficile*. This finding is in accordance with the bibliography in this regard [18]. Another finding that we can extrapolate from the UniFrac model is the greater interindividual variability in infected and colonized subjects than in healthy controls where the samples are grouped in a more specific region. This finding is linked to the fact demonstrated in prospective studies that gut microbiota, in the context of infection or colonization by *C. difficile* in a hospital setting, is subject to a dynamic of changes [19], which would lead to the greater variability observed in these study groups. This factor has not been found to be significant in colonized subjects from the community setting, such as that which occurs in most of the subjects included in group P of the present study (87%); therefore, we do not know if this factor could influence this group. A total of 47% of subjects of CDI group came from the hospital environment compared to the remaining 53% that came from the community; therefore, this could have an influence in some way.

Loss of genera belonging to the main butyrogenic families Lachnospiraceae and Ruminococcacaceae in infected and colonized subjects is particularly significant and deep. This finding is very characteristic of patients with CDI [13][14] but has also been observed in colonized subjects [18] and in patients with nosocomial diarrhea not due to *C. difficile* [13]. Regarding the genera, at the expense of which the decreases in these families occur, greater variability appears in the bibliography. This may be since gut microbiota forms a dynamic ecosystem with physiological, metabolic, immunological, and protection functions against pathogens, which is stable on the basis that there is a degree of functional redundancy of its members, that is, of a core functionality. Therefore, the relative decrease in the genera of these majority families compared to cohorts of reference subjects can be variable since a few genera would exercise the same functions. However, in previous studies [13][14][18], there are decreases in genera that appear in this study such as *Faecalibacterium*, *Subdoligranulum*, and *Roseburia*. Regarding butyrate as a key metabolite in intestinal homeostasis, it only comes from bacterial anaerobic fermentation and is the colonocyte's main source of energy. Butyrate is absorbed and oxidized, which reduces the osmotic load in the colon due to the presence of indigestible carbohydrates and, as the main anion in the colon, they form an acidic environment that would prevent the proliferation of intestinal pathogens such as *C. difficile*. In addition to these properties, butyrate is a powerful anti-inflammatory with the ability to regulate the secretion of pro-inflammatory cytokines in different intestinal immune cells through different mechanisms. They even have an influence on regulatory T lymphocytes, which decrease Th17 lymphocytes, whose activation stimulates granulopoiesis and the recruitment of neutrophils at the site of infection [22]. Therefore, we observed that in infected and colonized subjects a gut microbiota has been established with a very reduced capacity to synthesize butyrate, which would generate a pro-inflammatory environment but not enough to differentiate the state of colonization and infection.

Another important alteration in the composition within the phylum Firmicutes in this study was the greater conservation of genera with activity to biotransform primary bile acids into secondary bile acids in colonized subjects compared to infected subjects, where we have observed greater eradication, with moderate statistical significance (**Table 1**). The gut microbiota provides the enzyme BaiCD that transforms primary bile acids into secondary acids; however, this capacity is limited to a small group of bacteria [12]. Bacteria whose genome encodes these enzymes would produce an inhibitory effect on the germination of *C. difficile* spores. Losing these genera would mean a loss of this mechanism of resistance to colonization of intestinal pathogens. These specific species with BaiCd enzymatic capacity are mainly found in the genus *Clostridium* (cluster *Clostridium* XIVa), *Blautia*, and *Eubacterium* [12], although the activity is highly variable between species [23]. A recent study quantified the *BaiCD* gene cluster as a measure of the levels of intestinal bacteria with BaiCD activity, and a strong negative correlation with CDI was found [11]. The results of this study suggest that the relationship between bile acid metabolism by members of gut microbiota and its relationship with the pathogenesis of CDI is an essential

mechanism, and the approach of the present study through a group of patients with CDI and a group of colonized subjects show this. However, it is suggested that in the future we will resort to a combination of metabolomic, functional genomics, and metagenomics studies, together with the study of the *16S rDNA* gene to consolidate this evidence. It is possible that, the total number of members of gut microbiota with BaiCD activity may be unknown. Metabolism of bile acids by gut microbiota differentiates the state of colonization from that of infection in this study.

Table 1. Composition analyses of genera of phylum Firmicutes with activity BaiCD in study groups.

Family	Genus	Group CDI	Group P	Group CTRL	CDI versus CTRL	P versus CTRL	CDI versus P
Lachnospiraceae	***Blautia***	**0.5904**	**1.5385**	**0.6994**	**0.1831**	**0.1829**	**0.7395**
Lachnospiraceae	***Eubacterium ventriosum***	**0.0000**	**0.0032**	**0.7224**	**<0.0001**	**0.0001**	**0.3506**
Lachnospiraceae	***Eubacterium eligens***	**0.0063**	**0.1217**	**0.3189**	**0.0260**	**0.1126**	**0.5383**
Lachnospiraceae	***Eubacterium xylanophilum***	**0.0000**	**0.0010**	**0.1578**	**0.0001**	**0.0003**	**1.0000**
Lachnospiraceae	***Eubacterium ruminantium***	**0.0000**	**0.0000**	**0.0420**	**0.1498**	**0.1498**	**1.0000**
Lachnospiraceae	***Eubacterium fissicatena***	**0.0082**	**0.0485**	**0.0053**	**0.5155**	**0.6324**	**0.2879**
Lachnospiraceae	***Eubacterium hallii***	**0.0011**	**0.0056**	**0.0040**	**0.0740**	**0.2220**	**0.5772**
Lachnospiraceae	***Eubacterium oxidoreducens***	**0.0000**	**0.0000**	**0.0001**	**0.3340**	**0.3340**	**1.0000**
Eubacteriaceae	***Eubacterium***	**0.0011**	**0.0118**	**0.0003**	**0.5634**	**0.0790**	**0.2401**
Clostridiales family XIII	***Eubacterium brachy***	**0.0000**	**0.0051**	**0.3334**	**0.0004**	**0.0036**	**0.3506**
Clostridiales family XIII	***Eubacterium nodatum***	**0.0239**	**0.1099**	**0.0196**	**0.0261**	**0.1174**	**0.0040**
Ruminococcaceae	***Eubacterium coprostanoligenes***	**0.0939**	**0.1774**	**1.3705**	**0.0001**	**0.0001**	**0.9617**

Composition analyses of genera of phylum Firmicutes with activity BaiCD in study groups. Columns Group CDI, Group P, and Group CTRL are the mean of relative abundance (%). Columns CDI versus CTRL, *P* versus CTRL, and CDI versus *P* are the *p*-value of the Wilcoxon test.

We observed an increase in lactic acid-producing bacteria such as *Streptococcus*, *Enterococcus*, and *Lactobacillus* in infected and colonized subjects compared to healthy controls, with greater statistical significance in the case of *Enterococcus*. We consider this expansion as an "effect" and not as a "cause" of dysbacteriosis in infected and colonized subjects, because butyrate oxidation occurs in the colonocyte, mainly contributed by members of the phylum Firmicutes. Butyrate is transformed to CO_2 by consuming O_2. The dysbacteriosis observed in CDI and P groups would cause metabolic reorientation towards anaerobic glycolysis, lower O_2 consumption, and increased oxygenation of the colonocyte surface, with the consequent expansion of facultative anaerobes such as these lactic acid-producing bacteria [24]. The literature has not emphasized much on the importance of CDI pathogenesis in these genera. *Streptococcus* and *Enterococcus* are opportunistic pathogenic bacteria, and *Lactobacillus* has potential probiotic effects. On the other hand, lactic acid in vitro reduces TcdA in a dose-dependent way and the bacterial load of *C. difficile* in a dose-independent way [25]. These genera present a duality of protective and predisposing effects of CDI and future studies should be used, for example to determine *Enterococcus* species and virulence factors such as cytosilin that are well established. However, presence of these genera does not differentiate the state of colonization from that of infection. Perhaps the genus that has been most affected that increases in patients with CDI is *Enterococcus* [13][14][26]. However, this increase has also been evidenced in colonized subjects [18][27].

Genus *Clostridiodes*, which is equivalent to *C. difficile* since it only contains that species, presented the same relative abundance in infected and colonized subjects compared to non-presence, as expected in healthy controls. Therefore, the load of *C. difficile* did not differentiate the state of colonization from that of infection in this study. Furthermore, the results show that the positivity of *tcdB* gene also does not distinguish between colonization and infection, since 53% of the group of colonized individuals presented toxigenic strains (**Table 2**). This is in line with other works in this regard [28]. In addition, it has also been seen that the toxin load of *tcdB* gene (colonized and infected subjects with high load *tcdB* gene *versus*

low high *tcdB* gene) did not distinguish between colonization and infection [27]. Data of this study point to importance of evaluating virulence factors of *C. difficile* in the future, together with studies of *16S rDNA* gene and metagenomic studies. That is why factors concerning *C. difficile* should be considered in the differentiation between infection and colonization. For example, *tcdA* and *tcdB* genes are located at *PaLoc* or locus of pathogenicity. Interestingly, changes can occur in coding region of *PaLoc* as insertions, deletions, and point mutations that make up genetic heterogeneity, giving rise to several different toxinotypes. This means that there could be strains with different activity and specificity of their toxins with respect to the reference strain of *C. difficile* VPI 10463 [29]. Therefore, toxinotypes are important because they show functional properties of the *C. difficile* toxin variants, that is, greater or lesser activity and greater or less production. However, the correlation between the different toxinotypes that allows us to discriminate infection from colonization, as well as the severity of CDI, has not been clarified.

Table 2. Clinical and demographic characteristics of the study groups.

Clinical and Demographic Characteristics	Group CDI	Group P	Group CTRL
Sex, number (%)			
Men	**4 (26%)**	**10 (66%)**	**7 (46%)**
Women	**11 (74%)**	**5 (34%)**	**8 (54%)**
Age (mean ± SD)	**69 ± 19**	**51 ± 26**	**44 ± 12**
Antibiotics last three months, number (%)	**14 (93%)**	**8 (53%)**	**-**
Cephalosporins	**5 (33%)**	**2 (13%)**	**-**
Fluorquinolones	**4 (27%)**	**2 (13%)**	**-**
B-Lactamics	**5 (33%)**	**3 (20%)**	**-**
Others	**5 (33%)**	**4 (27%)**	**-**
Without antibiotics	**1 (7%)**	**2 (13%)**	**-**
Unknown	**0 (0%)**	**4 (27%)**	**-**
Strain Type, number (%)			
Toxigenic	**15 (100%)**	**8 (53%)**	**-**
Non-toxigenic	**0 (0%)**	**7 (47%)**	**-**
Comorbidities			
Hepatic disease	**1 (7%)**	**0 (0%)**	**-**
Crohn's disease	**1 (7%)**	**0 (0%)**	**-**
Malignant blood disease	**2 (13%)**	**0 (0%)**	**-**
Other intestinal disease	**3 (20%)**	**0 (0%)**	**-**
Other comorbidity	**13 (87%)**	**6 (40%)**	**-**
Previous CD, number (%)	**2 (13%)**	**0 (0%)**	**-**
Origin, number (%)			
Hospital	**7 (47%)**	**2 (13%)**	**-**
Community	**8 (53%)**	**13 (87%)**	**-**
Resolution, number (%)			
Complete	**11 (73%)**	**-**	**-**
Exitus letalis	**3 (20%)**	**-**	**-**
Recurrence	**1 (7%)**	**-**	**-**

Clinical and demographic characteristics of the study groups. Group CDI: Subjects infected by *C. difficile*, Group P: Subjects colonized by *C. difficile*, Group CTRL: Healthy controls. SD: Standard deviation.

The increase of *Bacteroides* (and of phylum Bacteroidetes) in infected and colonized subjects could be surprising from the point of view that it is not a finding typically presented in previous studies, wherein decreases of this genus are normally observed [9][18]. However, some important studies have shown an increase in *Bacteroides* in patients with CDI compared to healthy controls [13]. Through murine models, an ability to mitigate CDI has been evidenced in some species [30] and it has an immunomodulatory activity in intestinal inflammatory processes that could limit the exacerbated immune response observed in patients with CDI [31]. On the other hand, *Bacteroides* has an unusual ability to recognize and metabolize a large quantity of polysaccharides from the diet and from the host itself. However, since competition for nutrients between members of gut microbiota is greater when groups of bacteria are more phylogenetically related [32], it would imply that the depletion of genus *Bacteroides* evidenced in patients with CDI and colonized subjects by many authors [9][18], although not observed in this study, could have less a priori influence on the loss of resistance to colonization of intestinal pathogens by nutrient competition mechanisms, since this would be more accused with loss of members of the phylum Firmicutes, especially of Clostridia class, which is phylogenetically closer to *C. difficile*.

An increase in Enterobacteriaceae at the expense of *Escherichia-Shigella* is another common finding in patients with CDI [14]. In this study, we also observed an increase in colonized subjects. This result has also been evidenced in recent studies [18][27]. We assume that the increase in *Escherichia-Shigella* occurs at the expense of *Escherichia coli* species in the majority, as has been evidenced by metagenomic studies [33]. There are different *E. coli*-producing diarrhea species such as enteropathogenic *E. coli*, enterotoxigenic *E. coli*, enteroinvasive *E. coli*, enteroaggregative *E. coli*, Shiga toxin-producing *E. coli*, verocytotoxigenic *E. coli*, and diffusely adherent *E. coli* [34]. Therefore, since similar pronounced increases of *Escherichia-Shigella* were found in patients with CDI and colonized subjects in this study, where *E. coli* would probably be a majority component of this cluster and knowing that there are several strains of enteropathogenic *E. coli*, it would be interesting to know the presence of these strains in both study groups. That is why we formulate the new hypothesis that there is a colonization of pathogenic strains in the CDI group that could enhance the effects of *C. difficile* toxins. On the other hand, this colonization would not occur in colonized subjects. This fact could partly explain the different clinical expressions in infection with respect to colonization of *C. difficile*.

The practical eradication of *Bifidobacterium* in infected subjects and the greater conservation in colonized subjects that we found in this study is an important point that differentiates the state of colonization and infection. Few microbiota studies have valued the importance of *Bifidobacterium* and its potential protective role in CDI. This genus has been seen to decrease in patients with CDI [14], and inconclusive results have been found in colonized subjects [18][27]. *Bifidobacterium* is a beneficial genus of antimicrobial and anti-inflammatory properties [35] whose decrease has been correlated with intestinal pathogen overgrowth [36], in vivo and in vitro inhibition of growth of *C. difficile* and reduced production [37] and neutralization of its toxins [38], and decreased tissue damage and mortality in infected mice [39]. We consider that a genus of beneficial properties of *Bifidobacterium* is eradicated in CDI patients and preserved in colonized subjects, an important result. This implies better control of *C. difficile* in colonized subjects. To our knowledge, it is the first time that this conclusion has been reached in microbiota studies.

The increase of *Akkermansia* in infected subjects and decrease in colonized subjects with respect to healthy controls is of special importance. The genus *Akkermansia* contains the species *Akkermansia muciniphila* (the only species isolated in humans) that has a highly effective capacity to ferment the mucin of the intestinal mucosa layer [40]. Regarding CDI, only two relatively recent studies have emphasized the importance that it could present in its pathogenesis. Sangster et al. observed an increase in *A. muciniphila* in 12 patients with CDI compared to 12 healthy controls. The authors highlighted that due to the ability of *A. muciniphila* to degrade mucin, and since *C. difficile* by itself is also capable of degrading mucin, it would provide it with a selective advantage of expanding, since it is able to adhere to a layer altered mucosa with better efficacy than other members of gut microbiota [26]. Another work that was published on the same date also evidenced an increase in *A. muciniphila* of 3.6% in patients with CDI, compared to 0.6% that was observed in subjects who did not receive antibiotic treatment. These authors pointed out that, although *A. muciniphila* has beneficial properties, its expansion in patients with CDI could be related to the modification of the intestinal microenvironment and could reflect the inflammation of the mucosa layer [14]. For first time, our results show an increase of *A. muciniphila* in patients with CDI and a decrease in colonized subjects. Since one of functions of the intestinal mucosa layer is protection against intestinal pathogens, an alteration in the integrity means that it is more permeable and allows greater access to the epithelium and this fact could generate inflammation. On the other hand, intestinal mucosa layer is also a potential source of nutrients for intestinal pathogens. This fact is evident in antibiotic treatment, since it disturbs gut microbiota and the availability of fucose and sialic acids in mucin, which facilitates expansion of *C. difficile* [41]. Therefore, the increase in patients with CDI reflects a greater degradation of intestinal mucosa and the decrease in colonized subjects would show a greater integrity. This finding differentiates the state of colonization from that of infection and would imply a greater control of *C. difficile* in colonized subjects.

We consider that the present study has two main limitations. The first limitation is the sample size. Each study group is made up of 15 subjects. This sample size has allowed us to find statistically significant differences in terms of diversity, richness, and composition in infected and colonized subjects with respect to healthy controls. However, the differences in the composition of colonized subjects with respect to those infected are in many cases not statistically significant. We think that with a larger sample size the differences discussed would have greater statistical significance due to the low statistical power of this study. The main obstacle to increasing the sample size has been the inclusion of colonized subjects by *C. difficile*, since they have been difficult to include and locate. The second limitation that we consider for interpretation of the results is diet. Diet is a factor that modulates the composition of gut microbiota, and in this study, no variables have been collected in this regard. We also think that this could have a greater influence on the gut microbiota of healthy controls.

3. Conclusions

On the basis of the main objective of the study, which was the comparison of gut microbiota of patients with CDI and colonized subjects by *C. difficile* with respect to a group of healthy controls, we can conclude that infected and colonized subjects present a gut microbiota with a diversity, richness, structure, and composition completely different from that of healthy controls. However, gut microbiota of infected and colonized subjects showed great similarities in terms of diversity, richness, and structure. It is in composition where we find that colonized subjects, especially in minority genera, present differences with respect to those infected. This fact explains, at least in part, the state of colonization by *C. difficile.*

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