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Microbiological Study in the case of Chronic Inflammatory Bowel Disease, Crohn's Disease and Hemorrhagic Ulcerative Colitis

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ABSTRACT The present study focused on the isolation and identification of the intestinal bacterial ecosystem involved in Crohn's disease and Hemorrhagic ulcerative colitis. The patients were male and female with an age range of 18 to 60 years. The isolation carried out after grinding the different biopsies collected by means of low endoscopy, on specific selective culture media, made it possible to select a set of bacteria belonging to the intestinal ecosystem. The data were identified phenotypically through the use of biochemical tests, the various API galleries. On the other hand, the anatomopathological study made it possible to pose and confirm the different diagnoses of the pathologies corresponding to our research study. During Gram staining microscopic observation, in the case of Crohn's disease and Hemorrhagic ulcerative colitis, the identification is mainly represented by the Gram-positive bacilli identified as *Clostridium difficile* and *Bacillus subtilus*. These bacterial strains secrete toxins capable of causing an inflammatory state in the intestinal mucosa and thus inducing their pathogenic powers which can cause an immune disorder in these patients. Nevertheless, the *Bacillus subtilus* strain presents itself as an agent which can induce a defense against the aggressiveness of pathogenic bacteria.

KEY WORDS Biopsies; Culture; Isolation; Identification; Microbiota.

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INTRODUCTION

The gastrointestinal tract is an ecosystem favorable to exogenous microorganisms. It is managed by cooperation between the gastrointestinal epithelium, the immune system, and the microbiota commonly called intestinal flora. Interactions between microorganisms and the host can be of three types: symbiosis, commensalism and pathogenicity (Hooper & Gordon, 2001).

The microbiota of the gastrointestinal tract has been estimated at around 1013-1014 microbial cells

representing 400 to 500 species and subspecies (Moore & Holdeman, 1974; Bjorksten, 2004). The intervention of infectious agents in the pathogenesis of certain gastroduodenal diseases was suspected in 1915 (Berstad, 1993; Vallot, 1994). Chronic Inflammatory Bowel Disease is a group of conditions characterized by chronic inflammation of part of the lining of the digestive tract. This inflammation can be intermittent or continuous, and frequently leads to ulcers of the digestive wall. Inflammatory Bowel Disease evolves by flares interspersed with remission phases (Levy, 2008).

Currently, no etiology of Inflammatory Bowel Disease has been clearly identified. However, there is a large body of data that seems to define them as the result of an exacerbated immune reaction against the intestinal flora in genetically predisposed subjects (Baumgart & Carding, 2007; Kaser et al., 2010).

Chronic Inflammatory Bowel Disease is not a rare disease. The number of patients in Europe is estimated at 1 million patients with Crohn's disease and 1.5 million with Hemorrhagic ulcerative colitis, including nearly 200,000 in France (Chouraki et al., 2011; Shivananda et al., 1996). In the USA, the latest published data estimate that 1.3 million people suffer from Chronic Inflammatory Bowel Disease, posing a real public health problem (Loftus, 2004; Abramson et al., 2010).

These diseases are cryptogenic diseases that result from chronic, intermittent or continuous inflammation of part of the intestinal wall. They begin in young subjects with a peak frequency around 30 years and nearly 10% of new cases in children. Their evolution is due to accelerations interspersed with more or less long periods of remission (Cosnes et al., 2011; Peyrin-Biroulet et al., 2010).

The diversity of digestive manifestations, the presence of extra-intestinal lesions of the ocular, articular and skin type, the inevitable risk of complications linked in particular to fibrosis and cancerous degeneration make them diseases that are difficult to manage (Gillen et al., 1994; Palli et al., 1998).

Their cause is still poorly understood, the current hypothesis is that of an abnormality of the immune response of the intestine towards certain components of the bacterial flora occurring in genetically predisposed individuals (Cho, 2008; Xavier et al., 2008).

This finding therefore points to a participation of the intestinal microbiota in the pathophysiology of Chronic Inflammatory Bowel Diseases (Baumgart & Carding, 2007).

The microbiota is the set of microorganisms made up of bacteria, yeasts, fungi, and viruses, in a specific environment called microbiome. For example, the intestinal microbiota, formerly known as the intestinal flora, constitutes all of the microorganisms living in the intestine that is approximately 100,000 billion, or at least twice the average number of cells in the body (Seksik, 2010).

Crohn's disease is characterized by intermittent transmural inflammation that can affect all segments of the digestive tract, from the mouth to the anus (Marteau & Jian, 2001; Xavier & Podolsky, 2007).

Crohn's disease is linked to a deregulation of the mucosal immune response to an unbalanced intestinal microbiota leading to a dysbiosis situation, under the influence of environmental and genetic factors (Cho, 2008; Macfarlane et al., 2009; Rioux et al., 2007).

The evidence of a bacterial dysbiosis corresponding to an imbalance between pathogenic bacteria and saprophytes constitutes a predictive factor for relapse after the cessation of certain treatments for Crohn's disease and is a strong argument in favor of this hypothesis (Sokol et al., 2008).

Hemorrhagic Ulcerative Colitis is characterized by continuous inflammation of the colonic and rectal mucosa. (Faharat et al., 1999; Xavier & Podolsky, 2007). The disruption of the tolerance balance between the intestinal immune system and the intestinal microbiota led to a dysfunction of the immune response, transforming as an aggressive factor against the non-pathogenic commensal flora. Later, there will be the appearance of precancerous epithelial lesions resulting from epithelial regeneration secondary to chronic inflammatory lesions (Meyer & Treton, 2017).

The objective of this study is to isolate, identify the different microbiota in patients with Crohn's disease and Hemorrhagic ulcerative colitis, and to search for possible anaerobic bacteria and also aerobic bacteria tolerant, and this from the intestinal biopsies of patients, taken at the gastroenterology department.

After an endoscopic examination, one of the biopsy samples is intended to undergo an anatomopathology examination in order to confirm the endoscopic diagnosis of Crohn's disease and Hem-

orrhagic ulcerative colitis. The other samples will be used for the microbiological study.

After the isolation step, biochemical tests intended for bacterial identification, and the use of the different API galleries indicated for each of the isolated bacterial strains were carried out.

MATERIAL AND METHODS

Methods

Endoscopic examination. Colonoscopy of the colon and ileum is essential to visualize and do a biopsy on suspicious lesions (Frexinos & Buscail, 2004). The diagnosis is confirmed by a clinical evaluation and a combination of biological, histological and above all endoscopic examinations (Gersemann et al., 2008; van Assche et al., 2010).

Pathological examination. Pathology allows to objectify in the case of Crohn's disease a granulomatous intestinal discontinuous inflammation (van Assche et al., 2010). On the other hand, the endoscopic aspect characteristic of ulcerative colitis RCH is a continuous attack, starting from the anorectal junction, extending more or less far upstream and ending abruptly (Meyer & Treton, 2017).

Microbiological examinations. For isolation, different intestinal biopsies are crushed in 1 ml of nutrient broth using a sterile mortar to release the bacteria and then seeded in modified Columbia agar medium with 10% blood mutton, and this after successive decimal dilutions. After inoculation, few boxes are immediately incubated in a jar in a microaerobic atmosphere to promote the development in anaerobiosis, and the other boxes are put directly into the incubator to allow growth in aerobic at 37 °C for 5-7 days. The atmosphere is renewed at least every two days for optimal growth. Identification is based on the determination of morphological and biochemical characters (Marchal et al., 1991).

Macroscopic examination. The morphology of the colonies and their size are studied from the cultures obtained on the following media: Colombia agar (Mégraud & Lamouliatte, 1992; Fauchère & Rosenau, 1991).

Microscopic examination. It was performed on a bacterial smear, prepared from suspicious colonies in pure cultures, then fixed and stained by the Gram method (Mégraud, 1994a).

Biochemical tests

Catalase test. Catalase is an enzyme produced in abundance by bacteria with a respiratory metabolism that destroys hydrogen peroxide and releases oxygen (Vezina et al., 1991). The technique involves taking a portion of the colony and emulsifying in a drop of hydrogen peroxide. The release of gas bubbles means that it has catalase production. (Prescott et al., 2003).

Oxidase test. The oxidase assay is based on the bacterial production of an intracellular oxidase enzyme in the presence of atmospheric oxygen and cytochrome C. (Vezina et al., 1991). To determine the oxidase activity, a taken colony is put in a drop of oxidase reagent (Biomerieux France). The development of a purple color means that the test is positive and that the isolate has the oxidase enzyme (Kovács et al., 1995).

Urea rapid test. A biopsy fragment is put using a sterile loop in a tube containing 0.5 ml of urea-indole solution. The reading of the results is interpreted by a turn of the indicator towards red, taking place after 20 min and 24 h after incubation at 37 °C (Cassel-Béraud et al., 1996).

Triple Sugar Iron medium (TSI). It is a sloping and pellet medium by seeding a bacterial colony, the slope is seeded by streaks and the pellet by central bite, then incubation at 37 °C for 18 to 24 hours, and this for the purpose of evaluating the metabolic activity of different bacterial strains, such as, the lactose fermentation on the slope that translates to yellow turn, the fermentation of sucrose which is also visualized by a yellow turn, the presence of gas that is manifested by the detachment of the pellet or the presence of air bubbles, and the production of H2S which is interpreted by a black coloring (Leveau & Bouix, 1980; Marchal et al., 1991).

Mannitol-mobility test. (Le Minor & Richard, 1993). Inoculation of the environment is realized by central sting with the Pasteur pipette buttoned and loaded with a pure bacterial suspension, then put into oven at 37 °C for 24 hours. If the bacterium ferments mannitol, the reaction is called positive (+) mannitol, on the other hand, if the medium turns to red, no fermentation of mannitol, the reaction is called: negative (-) mannitol.

The presence of bubbles indicates a presence of gas, it is said that the reaction is called positive mannitol (+) with gas production. The immobile

germs only grow at the level of the central sting while the mobile germs diffuse in the agar.

Two drops of Nit1 and Nit2 are added in the different tubes, if there is a red ring it is said that we have a positive nitrate reductase (+) reaction, but if there is no reaction, we will proceed to the addition of Zinc powder. If there is no red ring, no reaction, it is the stage of dinitrogen, one says nitrate reductase positive (+). But if there is presence of red ring, it is called nitrate reductase negative (-).

API galleries. The study of biochemical characters is based on the use of API galleries. 20E, 20NE, which will be used to perform specific biochemical tests for each isolated bacterium (Fabre et al., 1994).

Antibiogram

This test makes it possible to study the sensitivity of isolated strains to antibiotics, this sensitivity is tested by the dissemination method (disk method) on Muller-Hinton medium (MH) supplemented with 10% of sheep blood, using several antibiotics. (Lozniewski et al., 1996).

RESULTS AND DISCUSSION

Fig. 1 shows a lesion of Crohn's disease, corresponding to longitudinal ulcers in the right colon giving the appearance of a freshly plowed field.

Fig. 2 gives the appearance of a polyploid lesion at the level of the Coecum, thus making the endoscopic diagnosis of Crohn Coecal.

The histopathological examination carried out on colorectal biopsy microfragements observed under the optical microscope at 20x magnification had revealed acute Hemorrhagic ulcerative colitis in the mucosa where cryptic abscesses are present with the presence of red-colored eosinophils (Figs. 3, 4).

The histopathological examination carried out under an optical microscope at 10x magnification of a colonic biopsy microfragment had objectified lesions extending beyond the mucous membrane thus testifying to Crohn's disease (Figs. 5, 6).

After seeding from different decimal dilutions from the grinding of human intestinal biopsies on different selective isolation media, we noted the appearance of bacterial colonies of different diameters and sometimes rounded shape, light and dark brown in color, creamy , transparent, and shiny. These macroscopic characters are characteristic of different colonies of aerobic and anaerobic bacteria (Fig. 7).

In order to obtain pure bacterial cultures, several successive subcultures were carried out on the different colonies obtained from intestinal biopsies, and subjected to Gram staining. The results of the microscopic examination at 100 × magnification revealed the presence of bacteria in the form of coccis and Gram positive (+) and Gram negative (-) bacilli.

-Isolate C: observation under the microscope revealed the presence of large gram positive bacilli (+) grouped in chains, on which we sometimes find some endospores at the center and at the ends (Fig. 8).

Isolate B: Observation under the microscope revealed the presence of gram positive (+) bacilli on which some endospores are sometimes found, especially at the ends (Fig. 9).

-Isolate D.d: Observation under the microscope revealed the presence of gram-positive (+) bacilli, thick with slightly swollen ends, and spore-forming, isolated and sometimes grouped together (Fig. 10).

-Isolate D: Observation under the microscope revealed the presence of gram-positive (+) bacilli, thick with slightly swollen ends, and spore-forming, isolated and sometimes grouped together (Fig. 11).

Biochemical tests

In the case of aerobic and anaerobic germs isolated from intestinal biopsies, it was found that all the isolates presented a negative urease (-) (Table 1). Reading after 24 hours, after the addition of the Kovacs reagent, showed that all of the aerobic or anaerobic tested isolates degrade tryptophan and produce indole and are therefore indole positive (Table 1).

Table 2 summarizes the results for Crohn's disease and Hemorrhagic ulcerative colitis, catalase and oxidase tests for single organisms, thus showing a positive oxidase for isolates C, B, D, and D.d. The catalase test is positive for isolate C, however isolates B, and D.d have a negative catalase.

For isolates from intestinal biopsies, Table 3 summarizes the results of the mannitol-mobility-nitrate test.

Regarding the isolates from intestinal biopsies,

Table 4 shows the results of the Triple Sugar Iron (TSI) test.

Table 5 summarizes the results of the antibiogram test for anaerobic bacteria isolates, C, B, D, D.d isolated from intestinal biopsies. It was observed in this table that the majority of the isolates, subjected to the study to the sensitivity to the antibiotics usually used in the therapeutic choice by

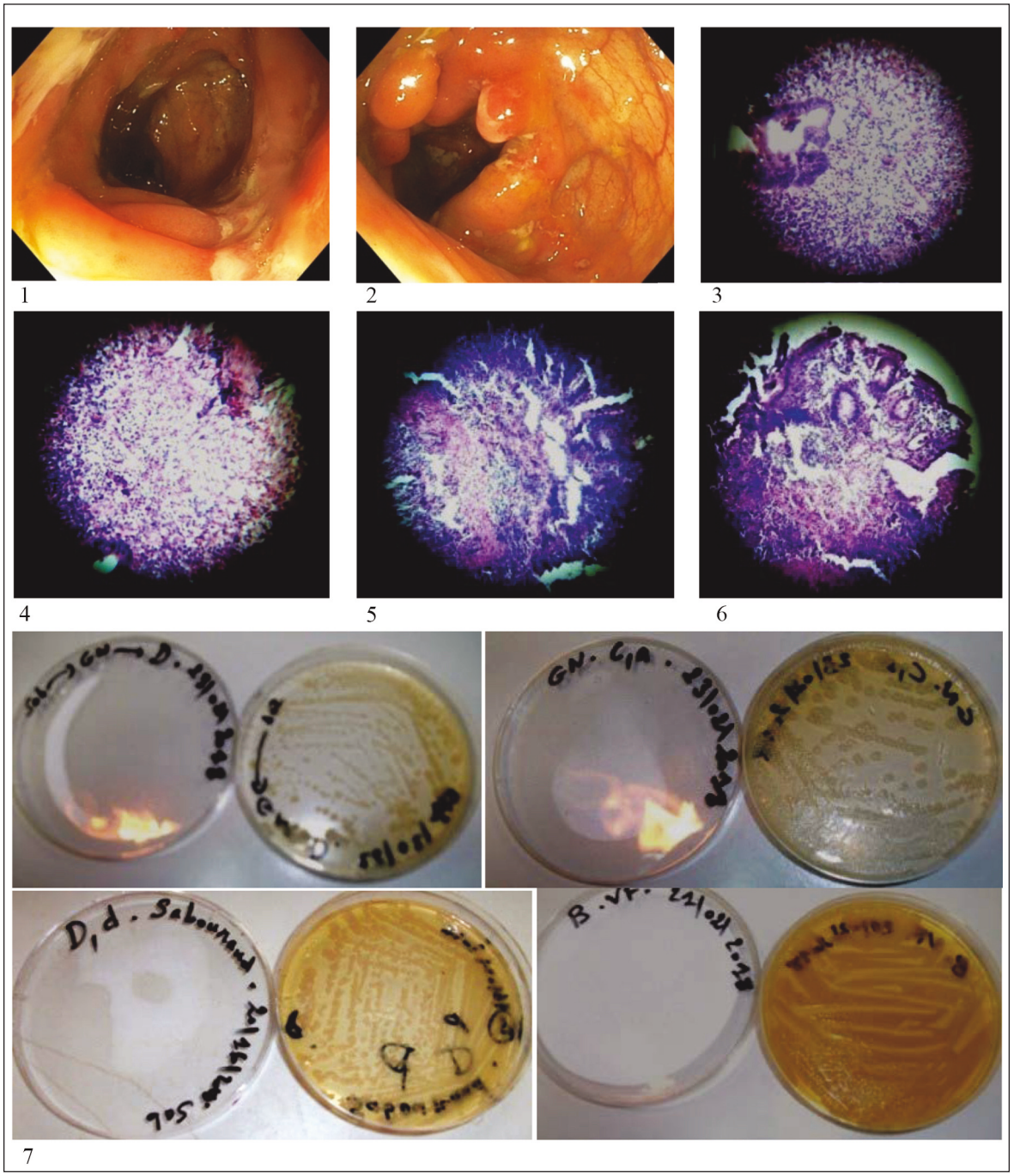


Figure 1. Crohn colic. Figure 2. Crohn Coecal. Figures 3, 4. Pathological observation under an optical microscope of biopsy colorectal microfragment (Gr 20x): Hemorrhagic ulcerative colitis. Figures 5, 6. Pathological observation under an optical microscope of colonic biopsy microfragment (Gr 10x): Crohn. Figure 7. Anaerobic and aerobic culture of bacteria on nutrient agar, sabouraud, and Meat liver. Cases of Crohn's and Hemorrhagic ulcerative colitis.

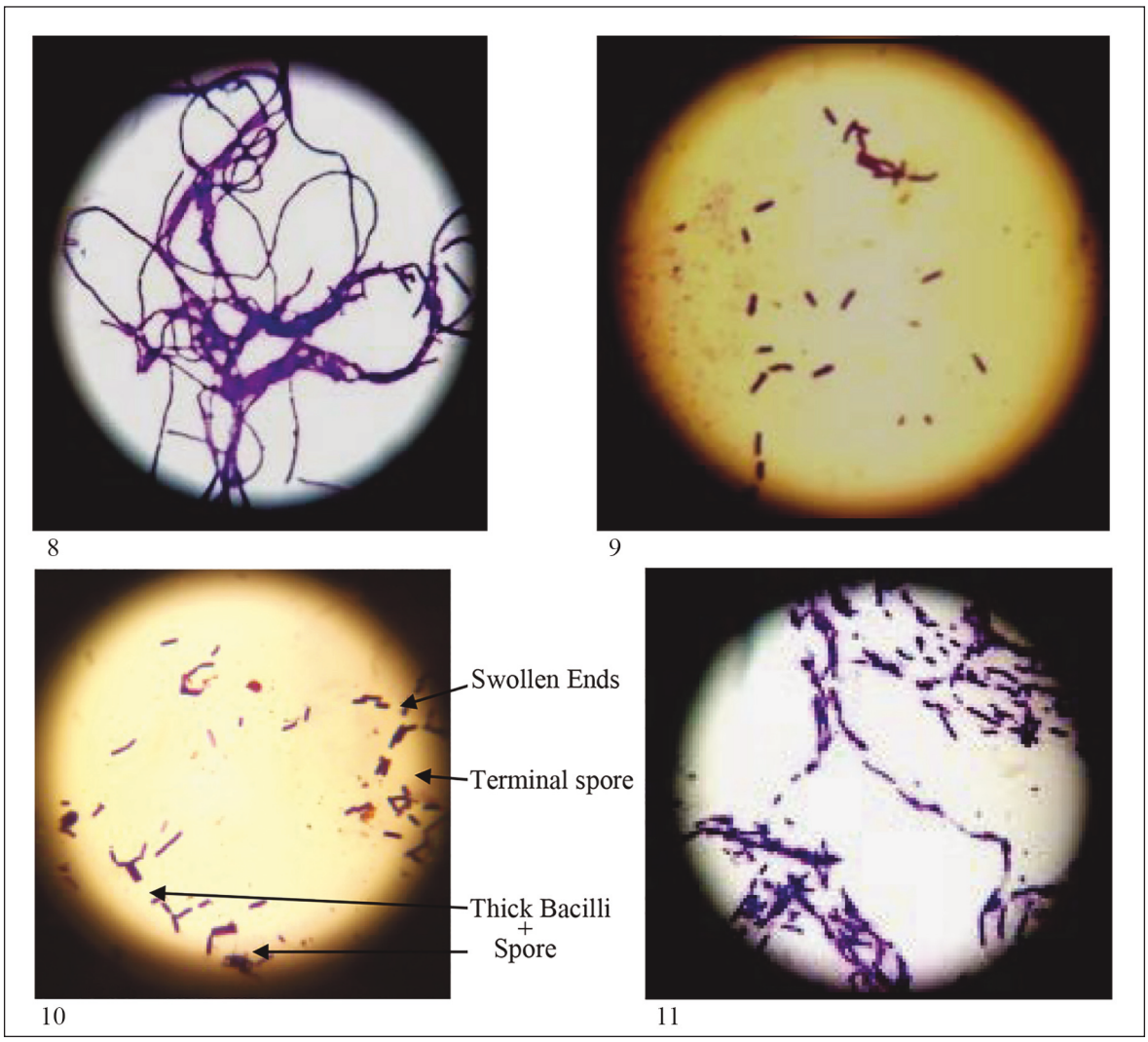


Figure 8. Isolate C. (Gr 100x). Figure 9. Isolate B. (Gr 100x). Figure 10. Isolate D.d. (Gr 100x). Figure 11. Isolate D. (Gr 100x).

the disc diffusion technique, showed the excellent inhibitory activity of the majority of the antibiotics used against these bacteria.

The identification of the germ was confirmed on the basis of the various results obtained, such as the macroscopic appearance of the colony of the bacteria, the staining of gram, the biochemical characteristics and the antibiotic resistance phenotype, the other characters biochemicals are generally studied from API gallery.

The criteria for identifying the isolates found in our research study are shown in Table 6 for each of the two pathologies, Crohn's disease and Hemorrhagic ulcerative colitis.

DISCUSSION

The intestinal ecosystem and in particular the colonic region is considered as the ideal and permanent habitat for the microbial flora with a dense and active population essentially anaerobic, locally producing many metabolites (Cebra, 1999). This flora has the ability to multiply in the environmental conditions of the digestive tract (Mountzouris et al., 2002).

Nevertheless, among this intestinal commensal flora, certain bacterial species can induce their virulent power and become highly pathogenic microbial agents. This transition to pathogenicity is a

Tests / Isolates	Urease	Indole
C	-	+
B	-	+
D	-	+
D.d	-	+

Table 1. Result of the indole urea test for aerobic and anaerobic isolates. Crohn's disease and Hemorrhagic ulcerative colitis.

Tests / Isolates	Mannitole	Gaz	Mobility	Nitrate
C	+	-	Immobile	+
B	+	-	Immobile	-
D	+	- +	Immobile	-
D.d	+	-	Immobile	-

Table 3. Results of the mannitol-mobility test for anaerobic bacteria. Crohn's disease and Hemorrhagic ulcerative colitis.

Tests / Isolates	Catalase	Oxydase
C	+	+
B	-	+
D	+	+ -
D.d	-	+ -

Table 2. Results of the catalase and oxidase test. Crohn's disease and Hemorrhagic ulcerative colitis.

Tests / Isolates	Lactose	Saccha-rose	Glucose	H2S	Gaz
C	+	+	+	-	-
B	+	+	+	+	+
D	+	+	+	-	-
D.d	+	+	+	+	+

Table 4. Results obtained by using the TSI test for anaerobic isolates. Crohn's disease and Hemorrhagic ulcerative colitis.

Antibiotics / Isolates	TIM (mm) 85	CTX (mm) 30	AMC (mm) 30	FOX (mm) 30	AM (mm) 10	VA (mm) 30	TIC (mm) 75	IMP (mm) 10	PRL (mm) 100
C	7	6	11	13	6	43	36	32	13
B	6	12	11	14	6	26	37	25	12
D	12	8	10	14	6	25	30	26	17
D.d	6	26	6	27	6	6	7	7	7

Table 5. Result of the antibiogram of anaerobic bacteria. Crohn's disease and Hemorrhagic ulcerative colitis. Ticarcillin-clavulanic acid (TIM); Cefotaxime (CTX); Amoxicillin-clavulanic acid (AMC); Cefoxitin (FOX); Ampicillin (AM); Vancomycin (VA); Ticarcillin (TIC); Imipinem (IMP); Piperacillin (PRL).

consequence of ill-suited antibiotic therapy or a severe diet during any disease. Pathogenic germs of exogenous origin can also contribute to the genesis of this bacterial dysbiosis in the digestive ecosystem (Mountzouris et al., 2002).

A diet whose nutritional value is low and poor in dietary fiber contributes to the development of a bacterial dysbiosis favoring the presence of pathogenic bacteria, and a decrease in the proportion of bifid (Goulet, 2009).

Clostridium difficile (Clostridioides) are anaerobic and mobile bacteria, ubiquitous and particularly present in the soil, are shaped as sticks, pleomorphic (ability that has an organism or cells of an organism to take different forms in some conditions or under certain influences), and appear in pairs or short chains. Under the microscope, they appear as a stick with a bulge at their terminal end forming subterminal spores. *Clostridium difficile* are Gram positive and, after a strict anaerobic culture, have optimal growth on nutrient agar or blood. *Clostridium difficile* has a negative catalase and produces two types of toxins, enterotoxin A and cytotoxin B, that disrupt cytoskeletal signal transductions in the

host. When *Clostridium difficile* is stressed, it produces spores capable of tolerating extreme conditions that active bacteria can not tolerate and thus become extremophilic bacteria (Ryan & Ray, 2004).

Clostridium difficile coexists in the human colon, accounting for up to 2 to 5% in the adult population. Sometimes long-term antibiotherapy generates an imbalance of the intestinal microbiota. In this case, *Clostridium difficile* can dominate opportunistically, thereby inducing its pathogenicity (Ryan & Ray, 2004).

Pathogenic strains of *Clostridium difficile* produce several toxins (Di Bella et al., 2016). These toxins are considered by enterotoxin and cytotoxin, represented respectively by toxin A and toxin B, which can cause diarrhea and inflammation. Diarrhea can become complicated in a few days to life-threatening pseudomembranous colitis.

Pseudomembranous colitis is associated with intense inflammation of the colon and the formation of pseudomembranes on the surface of the intestinal mucosa (Ryan & Ray, 2004).

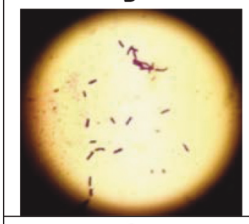
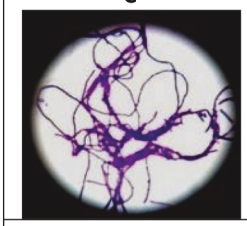
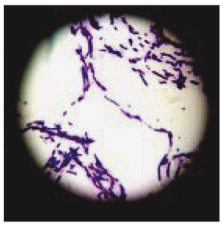
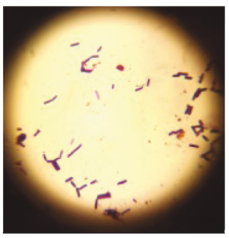
Isolates	Diseases	Macroscopic and microscopic morphology	Microbiological + Biochemical tests	Identification
B 	Hemorrhagic ulcerative colitis	Positive	Positive Meat liver Culture medium positive	*Clostridium difficile*
C 	Hemorrhagic ulcerative colitis	Positive	Positive	*Bacillus subtilus*
D 	Crohn's disease	Positive	Positive	*Bacillus subtilis*
D.d 	Crohn's disease	Positive	Positive Meat liver Culture medium positive	*Clostridium difficile*

Table 6. Result summarizing the criteria for identifying isolates.

Clostridium difficile secretes an adhesion factor called hyaluronidase that allows it to attach to human colon cells (Murray & Mosby, 2005). The bacterium also produces the chemical para-cresol which inhibits the growth of other bacteria in the vicinity and allows it to dominate and be in majority compared to the different germs belonging to the normal human intestinal flora (Calle, 2018).

Clostridium difficile spores are transferred to patients primarily through the hands of health personnel who have touched a contaminated surface or object. *Clostridium difficile* can live on surfaces for a long time (March, 2017). Once the spores are ingested, their resistance to acids allows them to cross the stomach unscathed. They germinate and multiply in the vegetative cells of the colon after exposure to bile acids. As a result, the World Health Organization advocates the use of soap in addition to alcoholic solutions to limit the spread of spores (Didier, 2009).

In patients with Hemorrhagic ulcerative colitis, *Clostridium* with a large population could be involved in intestinal toxicity (Machiels et al., 2014; Vigsnaes et al., 2013). Clostridium difficile generates reactivation of Crohn's disease and causes inflammatory epithelial lesions in vitro (Sartor, 2008). In addition, apoptosis of intestinal epithelial cells contributes to the increased permeability of the intestinal barrier following the release of certain toxins such as *Clostridium difficile* toxin A (Guerra et al., 2011).

Under the microscope, the morphology of *Bacillus subtilis* is in the form of sticks and is approximately 4 to 10 micrometers (μm) long and 0.25 to 1.0 μm in diameter. By belonging to the genus *Bacillus*, it has the ability to form endospores allowing it to survive in extreme environmental conditions of temperature and desiccation (Madigan & Martinko, 2005).

Before the introduction of antibiotics, *Bacillus subtilis* cultures were used as an immunostimulatory agent to assist in the treatment of gastrointestinal and urinary tract diseases. During the 1950, it was used as an alternative drug that, during digestion, significantly stimulated broad-spectrum immune activity, including the activation of the secretion of IgM, IgG and IgA specific antibodies (Ciprandi et al., 1986), and the stimulation of leukocyte and cytokine production activity important in the development of cytotoxicity towards tumor cells (Shylakhovenko, 2003).

Bacillus subtilis has the ability to grow rapidly, and to secrete a large number of molecules with broad spectrum of biological activities, including antibiotics. *Bacillus subtilis* is able to produce peptide molecules that are not derived from the central dogma of molecular biology, that is to say non-ribosomal peptide synthesis (Bolay, 2001). Its ability to produce antibiotics, such as bacitracin, also makes it an organism of interest for the pharmaceutical industry (Kunst et al., 1997).

CONCLUSIONS

This microbiological study is made from biopsies performed by low endoscopic route on patients suspected of chronic inflammatory bowel disease. The pathology examination confirmed the diagnosis of Crohn's disease and Hemorrhagic ulcerative colitis.

The microbiological study based on culture, inoculation of bacteria on culture media, isolation, and biochemical tests made it possible to isolate several isolates which led to a phenotypic identification of several bacterial strains, like *Clostridium difficile* and *Bacillus subtilis*. We found that the isolated bacterial strains are found in the two pathologies.

At the same time, the study of sensitivity to antibiotics aimed to analyze the sensitivity or resistance of certain antibiotics on bacteria such as *Clostridium difficile*, which can become pathogenic when dysbiosis is found in the intestinal microbiota. Nevertheless, the results of this research study have brought back to note that *Bacillus subtilis* presents itself in both pathologies as a force resistance against the pathogenicity of pathogenic bacteria.

It should also be noted that the use of the antibiogram allowed us to know the spectrum of sensitivity and resistance of the isolated bacterial strains vis-à-vis the antibiotics tested and which could be considered as a therapeutic choice.

Concerning the chronic inflammatory pathologies considered especially by Crohn's disease and Hemorrhagic ulcerative colitis, I suggest in these patients to use metagenomics on biopsy tissues to obtain a complete evaluation of the intestinal microbiota in order to confirm the existence of bacterial dysbiosis thus testifying to immune dysfunction.

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