ISABEL CRISTINA SOARES CURADO DE MATOS

INTERACTIONS OF *BACTEROIDES DOREI* WITH INTESTINAL EPITHILIAL CELLS

Faculdade de Ciências e Tecnologia

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INTERACTIONS OF *BACTEROIDES DOREI* WITH INTESTINAL EPITHILIAL CELLS

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Faculdade de Ciências e Tecnologia

2019

INTERACTIONS OF *BACTEROIDES DOREI* WITH INTESTINAL EPITHILIAL CELLS

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And remember that life’s A Great Balancing Act.
And will you succeed? Yes! You will, indeed! (98 and ¾ percent guaranteed).
Kid, you’ll move mountains.”

– Dr. Seuss, Oh, The Places You’ll Go!

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Abstract

Type 1 diabetes (T1D) is an autoimmune disease characterized by the destruction of pancreatic beta cells, leading to insufficient insulin production. Intestinal dysbiosis, (unbalanced microbiota composition), has been associated with T1D.

High population levels of bacteria belonging to the phylum *Bacteroidetes* have been reported in children with T1D, in contrast to healthy children. The intestinal metaproteome of children with T1D from the Algarve region has shown to be enriched with proteins originating from *Bacteroides* spp., including *B. dorei* and *B. uniformis*. Faecal analyzes allowed the isolation of these species from T1D and Control children of Algarve.

The main goal of the current study was to evaluate the interactions between *Bacteroides* spp. isolates, including *B. dorei*, and the intestinal epithelial cells HT29-MTX-E12.

All tested *Bacteroides* spp. were able to adhere to HT-29-MTX-E12 cells, ranging from 75.43 ± 2.98% to 83.06 ± 1.29%. *B. dorei* strains were able to invade HT-29-MTX-E12 ranging from 70.11 ± 5.66% to 85.25 ± 5.42%. In contrast, the strains of *B. uniform*is were not able to invade. *Parabacteroides distasonis* PtF D14MH1 showed the highest invasion ability (97.38 ± 1.62%). Disruption of the tight junctions of HT29-MTX-E12 cells was evident after exposure to *B. dorei* and *P.* distasonis isolates in contrast to slightly impaired after exposure to *B. uniformis* isolates. The viability of HT29-MTX-E12 cells decreased after exposure to the bacterial supernatant, and it was most evident when the medium was supplemented with 25 and 50% (v/v) of bacterial supernatant. The invasive ability of *Bacteroides* spp. significantly decreases in the presence of *Lactobacillus casei* DSM 20011. Cytokine production by HT29-MTX-E12 cells exposed to *Bacteroides* spp. was not noticeable and only induced the production of IL-8 and MIP-1β.

The damage caused by *Bacteroides* spp. to the intestinal epithelial cells (disruption of cell junctions, decreased cell viability, adherence and invasive ability of the isolates) may contribute to dysbiosis in children with T1D.

Key words: Type 1 Diabetes, *Bacteroides* spp., *Lactobacillus casei*, HT29-MTX cells, Invasion, Tight junctions.

Resumo

A diabetes é uma doença crónica caracterizada pela deficiência na produção ou ineficiente uso da insulina produzida pelo pâncreas. Essa deficiência na produção de insulina a longo prazo irá provocar um desequilíbrio no metabolismo da glicose. Os sintomas clássicos da doença são a poliúria, polidipsia e polifagia. Se o diagnóstico e / ou o tratamento da doença for tardio, inúmeras complicações graves e crónicas poderão surgir.

Existem 3 tipos de diabetes *mellitus*, confirmados, a diabetes *mellitus* gestacional, a diabetes *mellitus* tipo 1 e a diabetes *mellitus* tipo 2. A diabetes *mellitus* tipo 1 (DT1) também conhecida como diabetes insulino-dependente, é uma doença autoimune, caracterizada pela destruição das células beta dos ilhéus pancreáticos, efetuada pelas células T. Só quando 90% das células beta pancreáticas são destruídas é que tem inicio a manifestação da doença. Alterações na microbiota intestinal (disbiose) têm sido referidas quer em crianças no período antecedente ao desenvolvimento da autoimunidade, quer em crianças com DT1 estabelecida.

A microbiota intestinal das crianças com DT1 tem despertado muito interesse não só devido ao aumento de casos em crianças cada vez mais novas, mas também devido ao aumento de relatos sobre a associação entre a doença e a microbiota do trato gastrointestinal que manifesta um desequilíbrio na sua composição (disbiose). Uma disbisose pode resultar no aparecimento de respostas inflamatórias associadas a diferentes patologias como a síndrome do intestino inflamado ou cancro coloretal.

Uma maior abundância de bactérias pertencentes ao Filo *Bacteroidetes* quer em crianças em seroconversão, quer em crianças com DT1 estabelecida, em contraste com crianças saudáveis que são portadoras de uma maior população de bactérias pertencentes ao Filo *Firmicutes* tem sido reportada. Um estudo metabólico sobre a microbiota intestinal de crianças diagnosticadas com DT1 e crianças controlo da região do Algarve revelou que crianças diagnosticadas com DT1 apresentam um proteoma mais abundante em proteínas com origem em *Bacteroides* spp., incluindo *B. dorei* e *B. uniformis*, quando comparados com o proteoma de crianças controlo. Uma análise fecal a crianças DT1 e controlo, também do Algarve, permitiu o isolamento de *Bacteroides* spp. de ambos os grupos de crianças, diferenças na recuperação de algumas espécies de *Bacteroides*.

Bacteroides são bactérias Gram negativas, anaeróbias, mutualiastas que colonizam, em quantidades significativas, o intestino humano. Este género é de extrema importância para a nutrição humana, pois possuem uma grande capacidade de degradação de hidratos de carbono provenientes das plantas e uma das principais fontes de energia provém da fermentação de uma grande variedade de açúcares, estando também associadas ao mecanismo de degradação de mucinas (constituintes do muco que reveste o trato gastrointestinal humano). Pouco se sabe sobre as interações de bactérias, como *B. dorei* e *B. uniformis*, com as células epiteliais intestinais. A ligação de *Bacteroides* à DT1 tem vindo a ser cada vez mais explorada, assim como a sua ligação, se existir, com doenças associadas à DT1. Por conseguinte é de todo o interesse estudar as interações de *Bacteroides* spp. com o hospedeiro.

O principal objetivo deste estudo foi avaiar a capacidade de estirpes de *B. dorei*, *B. uniformis* e *Parabacteroides distasonis* de interagir com células epiteliais intestinais, nameadamente HT29-MTX-E12. Para atingir este objetivo foram estabelecidos os seguintes objetivos específicos:

1) Avaliar a capacidade de adesão e invasão das estirpes de *B. dorei* (DSM 17855, PtF D1P5, PtF D8M1, PtF D16P1, PtF D16M14, PtF Sb6, PtF Sb8, PtF C1P2), *B. uniformis* (PtF Sb3P5 e PtF D3Pch2) e *P. distasonis* PtF D14MH1.
2) Examinar o efeito da exposição das células HT29-MTX-E12 aos diferentes isolados nas ligações celulares.
3) Determinar a viabilidade das linha celular após exposição ao metaboloma bacteriano.
4) Avaliar a indução de uma resposta inflamatória após a exposição da linha celular às células bacterianas.

A linha celular HT29-MTX foi isolada da linha HT-29 (diferenciação é irreversível) devido à resistência das células a altas concentrações de metatrexato (MTX) após adaptação gradual a este fármaco anticancerígeno. Este clone é capaz de diferenciar espontaneamente células produtoras de muco. Têm sido utilizadas como modelo para estudar a adesão celular de bactérias lácticas a células epiteliais intestinais.

A linha celular foi cultivada no meio in Dulbecco's Modified Eagles Medium High Glucose (Sigma) suplementado com soro fetal bovino inativado (10 %, v/v), amino ácidos (1% , v/v) e os antibióticos penicilina e estreptomicina (1 %, v/v). A incubação da linha celular decorreu a 37ºC numa atmosfera húmida com 5 % (v/v) CO_2.

A linha celular foi utilizada nos ensaios com 21 dias de diferenciação. A exposição da linha celular aos diferentes isolados decorreu em condições de anaerobiose.

O meio de cultura Brain Heart Infusion (Biokar, France) suplementado com hemina (0.1%, v/v) e L-cisteina (Sigma-Aldrich, Madrid) foi utilizado para crescer as diferentes espécies de *Bacteroides*. A estirpe *L. casei* DSM 20011 foi cultivada no meio de cultura Man Rogosa and Sharpe (Biokar, France).

Os resultados obtidos evidenciaram que todas as espécies de *Bacteroides* testadas têm capacidade de aderir às células HT-29-MTX-E12, variando de 75,43 ± 2,98 % a 83,06 ± 1,29 %. Os isolados de *B. dorei* apresentaram diferentes capacidades de invasão das células epiteliais intestinais, variando entre 70,11 ± 5,66 % e 85,25 ± 5,42 %. Em contraste, as estirpes de *B. uniformis* demonstram não possuir capacidade invasiva para estas células. O isolado que apresentou a maior capacidade invasiva para as células HT29-MTX-E12 foi *P.distasonis* PtF D14MH1 (97,38 ± 1,62%).

A disrupção das *tight junctions* das células HT29-MTX-E12 foi evidente após a exposição aos isolados de *B. dorei* e *P. distasonis,* pelo contrário as células expostas aos isolados de *B. uniformis* apresentaram um leve dano em comparação com as células controlo.

A viabilidade das células HT29-MTX-E12 diminuiu após a exposição ao sobrenadante bacteriano, tendo esta diminuição sido mais evidente quando o meio foi suplementado com 25 e 50 % (v/v) de sobrenadante bacteriano.

A capacidade invasiva de *Bacteroides* spp. diminuiu significativamente na presença de *Lactobacillus casei* DSM 20011.

A produção de citocinas pelas células HT29-MTX-E12 expostas a *Bacteroides* spp. não foi notável tendo apenas induzido a produção de IL-8 e MIP-1β.

Os danos causados pela exposição das células epiteliais intestinais a *Bacteroides* spp. (aderência e capacidade invasiva dos isolados, rutura das *tight junctions* e diminuição da viabilidade celular) podem contribuir para a disbiose em crianças com DT1.

Palavras chave: Diabetes tipo 1, *Bacteroides* spp., *Lactobacillus casei*, HT29-MTX cells, Invasão, Junções celulares

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Glossary

Btf – *Bacteroides fragilis* toxin

BHI – Brain heart infusion

BHI+H – Brain heart infusion supplemented with L-cystein and hemin

BSAPs – Bacteroidales species secreted antimicrobial proteins

CDC – Centre for disease control

CFU – Colony forming units

DAPI – 4’,6’-diamidine-2-phenylindole

EG – Eggerth Gagnon

FRA – Enterotoxigenic fragilisin

HLA – Human leukocyte antigen

HPLC – High performance liquid chromatography

LC-MS – Liquid Chromatography-Mass Spectrophotometry

LPS - Lipopolysaccharide

MAMPs- Microbe associated molecular patterns

MPII – Metalloprotease II

MRS – Man Rogosa and Sharp

MTT – 3-(4,5-dimethyllthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MTX – Metatrexate

MUB - Mucus-binding proteins

PBS – Phosphate buffered saline

PS A – Capsular polysaccharide PS A

SCFA – Short-chain fatty acids

TER – Transepithelial electrical resistance

TLR – Toll-like receptors

TRITC-phalloidin – Tetramethylrhodamine B isothiocyanate-phalloidin

T1D – Type 1 diabetes

T2D – Type 2 diabetes

UV – Ultra violet light

1. Introduction

1.1. Intestinal Microbiota

All multicellular organisms of the Earth are colonized with microorganisms and humans are the host of about 10^{14} microbial cells (Aroniadis et al., 2014; Jakobsen et al., 2018; Prakash et al., 2011; Quigley, 2013). These microorganisms colonize the mucosal surfaces of the human body playing important roles in several physiological processes that are crucial for the health of the host health (Lim et al., 2016; Zhuang et al., 2019).

The gut microbiota (microbial community that colonize the gastrointestinal tract) is a dynamic system that can be affected by several factors, such as the host age, infections, antibiotic treatment, diet and immune status (Berg, 2004; Berrilli et al., 2012; Tun et al., 2018; Wampach et al., 2018).

Humans and their intestinal microbiota evolve dynamically over time making their symbiosis extremely intimate (Endesfelder et al., 2016; Kostic et al. 2015).

1.1.1. The impact of the gut microbiota from birth to childhood

The intestinal microbiota, that is established early in human life is of extreme importance due to its close association with the host and the diversity and quantity of substances of microbial origin in the human intestine are crucial for the survival and maintenance of a healthy status of the host (Del Chierico et al., 2015; Gerritsen et al., 2011; Prakash et al., 2011).

The human gut is initially colonized *in utero* by *Proteobacteria* (Kostic et al. 2015; Pearson et al. 2018) and the first years of human life are important periods in the establishment and shaping of the intestinal microbial community (Del Chierico et al., 2015). Even though there are other factors that modulate the gut microbiota right after birth contributing for differences in the microbial composition, namely state of the immune system, hygiene and access to medical care (Endesfelder et al., 2016), the initial gut microbiota is characterized by instability and low diversity (Jakobsen et al., 2018).

At the moment of birth, the mode of delivery, eutocic (vaginal) or dystocic (caesarean), will impact the composition of the microorganisms that will colonize the new-born (Del Chierico et al., 2015). Other factors also may influence this microbial colonization, such as the use of antibiotics, feeding and hygiene (Bokulich et al., 2016; Kostic et al., 2015; Livanos et al., 2016). In the case of an eutocic mode of delivery the new-born passing through the vaginal canal of the mother will be exposed to the vaginal microorganisms. Since this will be the first microbial contact at birth it will influence the baby's microbiota being more similar to that of the mother. In case of a dystocic delivery its microbiota will be different since the neonate is not exposed to the vaginal microbiota of the mother (Drell et al., 2017). The differences between the vaginally delivery and caesarean delivery microbiomes are also at the level of microbial functionality, namely the functionality of the vaginally delivery microbiome shows enrichment in several functional pathways including lipopolyssacharide (LPS) biosynthesis that are depleted in the caesarean neonates microbiome (Wampach et al., 2018). This LPS biosynthesis is of particular relevance in virtue of the stimulation of the primary immune system (Wampach et al., 2018).

Feeding also exerts an important influence on the establishment of the intestinal microbiota at the initial phase of the child's life. If the baby was breastfed it will show a different intestinal microbiota in comparison with children feed with formula milk (Sherrill-Mix et al., 2018). At this stage, more precisely until the first year of the child's life, the intestinal microbiota varies from child to child and according to age, feeding and mode of delivery (Sherrill-Mix et al., 2018). In this first year of the child’s life, the microbiota seems to have the main functions of that of an adult microbiota should have, however, the microbiota seems to diversify further until the age of 3 years old, when it begins to stabilize (Harmsen et al., 2016). From this point on the microbiota suffers a slow differentiation, in infancy and adolescence the intestinal microbiota, although stable, still differs from that of the adult stage (Harmsen et al., 2016).

The intestinal microbiota, even while going through composition developments during the childhood and adolescence, have 5 main phyla that can be distinguished, namely *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* (Hillman et al., 2017; Hollister et al., 2015; Sherril-Mix et al., 2018). The most abundant phyla are *Bacteroidetes* and *Firmicutes*, contrasting with *Archea* and viruses that constitute the lower abundant groups (only 0.1% of the intestinal microbiota) (Annalisa et al., 2014; Jandhyala et al., 2015).

1.1.2. Distribution of the microbiota in the gastrointestinal tract

The 5 main phyla that constitute the microbiota of the gastrointestinal tract aren't all in the same locations of this body system (Jandhyala et al., 2015). All the organs that integrate the digestive system have in their physiognomy different physical-chemical environments that promote the colonization of this system by different microorganisms (Jandhyala et al., 2015). The gastrointestinal tract is, therefore, the area of the human body where there are more microorganisms with a massive diversity (Jandhyala et al., 2015). The main genera that colonize each part of the gastrointestinal system are depicted in **Figure 1.1.2.1**.

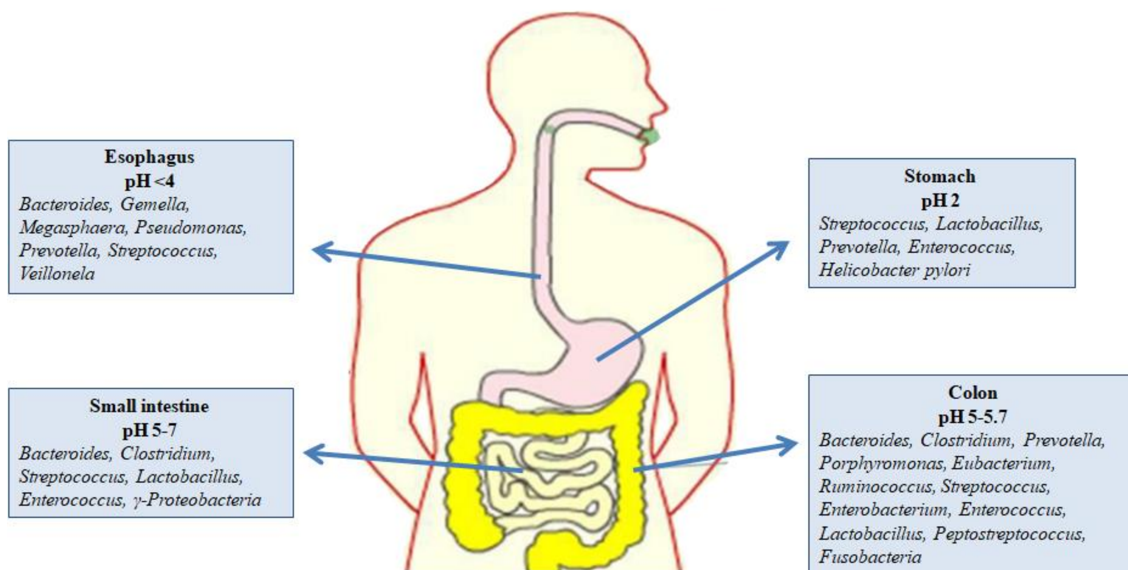

Figure 1.1.2.1 – Distribution of the normal human gut microbiota. Adapted from Jandhyala et al. 2015 and Paul et al. 2015.

The human digestive system, besides being responsible for digest the food, is also responsible for acquiring the nutrients necessary for the different functions of the organism, such as absorption of water, electrolytes and other nutrients (Seeley et al., 2003). The gastrointestinal system consists essentially of the digestive tract (mouth, pharynx, oesophagus, stomach, small and large intestine and anus) and accompanying glands. It is divided into the upper gastrointestinal tract (from the mouth to the stomach) and the lower gastrointestinal tract (from the small intestine to the anus) (Seeley et al., 2003).

All the organs that integrate the digestive system have in their physiognomy different physical-chemical environments that promote the microbial colonization of

this system. The gastrointestinal tract is, therefore, the area of the human body where there are more microorganisms with an enormous diversity (Berrilli et al., 2012; Hajela et al., 2015; Lu et al., 2015).

The upper gastrointestinal tract contains the more resistant organisms to low pH, being the predominant genera *Lactobacillus* and *Streptococcus* (Alves et al., 2017; Hooper et al., 2010; Jandhyala et al., 2015).

The large intestine, the terminal area of the lower gastrointestinal tract, is densely populated by anaerobes, namely the genera *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Clostridium*, *Peptococcus*, *Peptostreptococcus* and *Ruminococcus* (Alves et al., 2017; Biedermann et al., 2015; Hajela et al., 2015; Lozupone et al., 2012). It is in the large intestine, that the highest amount of bacteria is found, about 10^{10}-10^{11} cells per gram of faeces can be detected. This remarkable bacterial content occurs since there is a reduced influence of the acids released during the digestion process that can be harmful to the microorganisms, nevertheless these microorganisms are resilient to bile and a high content of salt (Walter et al., 2010).

1.1.3. Commensal and opportunistic gut microorganisms

With so many microorganisms (10^{14}) living within the human body using it as their host, the gastrointestinal system is the most colonized area of the body. It is crucial that pathogenic and non-pathogenic organisms are kept under vigilance (Soyucen et al., 2014) accepting the commensal simbionts, that maintain intestinal epithelial homeostasis, by controlling inflammation (Soyucen et al., 2014; Vaarala et al., 2008).

Each individual hosts an unique microbial community in his gastrointestinal system, although the previous mentioned 5 main phyla are present (Pearson et al., 2018). The definition of what constitutes an healthy microbiota is still in debate even though guidelines of what constitutes a microbiota that is the “healthiest” have been proposed (Endesfelder et al., 2016; Leiva-Gea et al., 2018; Pearson et al., 2018; Soyucen et al., 2014; Zhuang et al., 2019). The most crucial functions of a “healthy” microbiota include digestion of nutrients, development of mucosal immunity and supporting gut-brain communications (Pearson et al., 2018).

As mentioned above the establishment of this complex environment between the microbiota and its host is dependent on the mode of the transmission of microbes, the

lifestyle, use of antibiotics, feeding and hygiene and access to modern medicine (Bokulich et al., 2016; Del Chierico et al., 2015; Drell et al., 2017; Jakobsen et al., 2018; Kostic et al., et al., 2015; Livanos et al., 2016). Many of the commensal intestinal microbiota has been coevolving with their host having gain specialized mechanisms of interaction (Endesfelder et al., 2016). To prevent the onset of diseases due to the presence of the microorganism in the gastrointestinal tract this system is armed with a high density of immune cells (Endesfelder et al., 2016). This endless interactions between the gut microbiota and the immune system of the host has caught the attention of the researchers (Endesfelder et al., 2014; Endesfelder et al., 2016; Vatanen et al., 2018).

1.1.4. Gut microbiota and the intestinal mucus

Intestinal homeostasis and avoidance of anomalous immune responses are vital to ensure a healthy host, so there are numerous mechanisms to control the microbial gut colonization. The first mechanism, and the first barrier of defence of the gut against foreign microorganisms, is the mucus synthesized and secreted by the goblet cells that line the epithelial tissue of the gut (Sicard et al., 2017). To produce the mucus, the goblet cells produce mucins, structural glycoproteins in complex clusters of specific O-linked glycans (Forstner, 1995). The human mucin family consists of members (MUC1 to MUC21) that have been sub-classified into secreted and transmembrane mucins (Kufe, 2008). The expression of the secreted mucins genes (for example, *MUC2, MUC5AC, MUC5B* and *MUC6*) are found in diverse animals like chimpanzees, chickens, rats, zebrafish, as well as humans, but they do not only exist in the colon (van Tassell et al., 2011). Mucins can be also found in lungs and trachea, pancreas and small intestine, basically any organ that possesses a mucosa layer (van Tassell et al., 2011). The predominant genes that express membrane-related mucins in human goblet cells are *MUC1, MUC3A / B, MUC4* and *MUC12;* but *MUC2* is the major secretor mucosal gene expressed in the colon, comprising most of the mucous gel that protects the underlying tissue (van Tassell et al., 2011).

This mucus layer varies in thickness through the gut, the thickest layer being in the colon, the highest gastrointestinal zone colonized by microorganisms (Sicard et al., 2017). In this organ the mucus is composed of two layers, one connected to the

epithelium and an outer one exposed to the lumen, being the outer layer the most vulnerable to the bacterial proteolytic activity (Ponce de León-Rodríguez et al., 2018; Sicard et al., 2017). This external layer of mucus has numerous O-glycans that bacteria use as adhesion sites as well as are used for nutrients by the gut microbiota while the inner layer of mucus is less perturbed or invaded by the microbiota present in healthy hosts (Sicard et al., 2017). The microorganisms that colonize the gut compete for the adhesion sites located in the mucus most external layer (Li et al., 2015).

One of the groups of intestinal bacteria that colonize the colon is able to degrade mucin oligosaccharides. This group of mucin-degrading specialist bacteria include *Akkermansia muciniphila*, *Bacteroides thetaiotaomicron*, *Bacteroides fragilis* and *Bifidobacterium bifidum*. They degrade mucin oligosaccharides that afterwards can be metabolized by other gut bacteria (Sicard et al., 2017).

B. thetaiotaomicron possess various glycosidase enzymes that are suitable to different glycans (Marcobal et al., 2013; Xu et al., 2003). Mucin degradation, however, is only completed after the activity of a combination of enzymes from various mucinolytic bacteria (Derrien et al., 2010; Marcobal et al., 2013). Thus, mucin glycans are nutrient sources for bacteria that can use mucus-derived sugars but lack these enzymes, that are required to cleave the sugar bonds (Arike et al., 2016; Johansson et al., 2015). As a result products derived from O-glycan mucus are made available to other bacteria by mucinolytic bacteria (Sicard et al., 2017).

The O-glycans of the mucus, besides being a source of energy, are also used as ligands for bacterial adhesion process (Arike et al., 2016). The carbohydrates that form the mucins provide an initial binding site for bacteria that may facilitate epithelial cell invasion (Derrien et al., 2010). There are no specific adhesion sites for microorganisms that have been identified, however there is evidence that bacteria can bind directly to mucins, for example, *Bifidobacterium longum* subsp. *infantis* uses the Family 1 Protein Binding Adhesin to adhere to mucin oligosaccharides (Sicard et al., 2017).

Different bacteria use different strategies to adhere to mucus. Mucus-binding proteins (MUB), used by lactic acid bacteria, such as *Lactobacillus reuteri*, are cell-surface proteins with repeated domains (MacKenzie et al., 2010; van Tassell et al., 2011). Gram positive adhesins specific to sialylated mucin glycans share structural and functional homologies with MUBs (Etzold et al., 2014). *Bacteroides fragilis* is found in the intestinal mucus whereas the commensal *Escherichia coli* is only found in the intestinal lumen. This difference is due to the ability of *B. fragilis* to specifically bind to

highly purified mucins constituting the mechanism by which *B. fragilis* colonizes the human intestine (Huang et al., 2011).

Bacterial adhesion to mucins can lead to the growth of microcolonies that can develop further in biofilms (Sicard et al., 2017). Biofilms are a complex and self-produced polymeric matrix where microorganisms can attach to each other and be attached to the mucosal surface. Biofilms can also be formed on the surface of the intestinal epithelium and interact with the secreted or attached mucins since the biofilm is originated from bacteria that adhered to mucin (de Vos, 2015). The biofilm formation at the intestinal epithelium is illustrated in **Figure 1.1.4.1**.

Secretory IgA (SIgA) and mucins, mucus layer components might have a role in biofilm formation. Studies *in vitro* have shown that these components can modulate biofilm formation (Bollinger et al., 2006; Slízová et al., 2015). The ability to form biofilms coupled with the heavy presence of the groups *B. fragilis* and *Enterobacteriaceae* in the gut may justify the onset of inflammatory bowel diseases (Sicard et al., 2017).

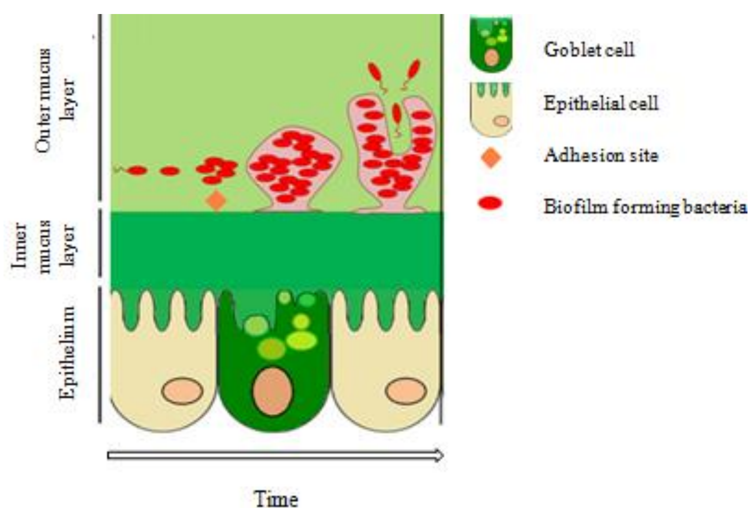

Figure 1.1.4.1 - The intestinal mucus layer with biofilm. The colonic epithelium is covered by a mucus layer consisting of mucins. These consist of a protein nucleus and a high number of O-linked glycans that are secreted by goblet cells. Mucins form a network structure of dense layers firmly attached to the cells. The mucus O-linked glycans can act as bacterial adhesion sites, facilitating colonization and even allowing bacteria to form biofilms. The bacterial surrounding zone (pink) is the exopolysaccharide matrix. Adapted from Sicard et al. 2017.

Alterations of the integrity of the intestinal mucosa are associated with gastrointestinal diseases, such as Crohn's disease and inflammatory bowel disease in which there is an abnormal expression glycosylation of the mucin or modulation of the mucus component (Sicard et al., 2017). The alterations in the mucosa integrity are also

linked or caused by dysbiosis, and once the mucosa is compromised, the bacteria can access the epithelium causing inflammation (Antoni et al., 2014). Dysbiosis can be triggered by several factors, such as lifestyle, diet or use of antibiotics (Pearson et al., 2018; Sicard et al., 2017). Thus, the integrity of the mucus layer plays a key role in the maintenance of the balance between the intestinal microbiota and its host.

1.1.4.1. Microbiota and the immune system

Although some bacteria are able to adhere to the gastrointestinal mucus, the majority of the intestinal bacteria are non-adherent and do not cross the dense mucous layer that covers the epithelium, the balance between the intestinal microbiota and its host is largely conducted independently of contact through bacterial metabolites, (Pedicord et al., 2015; Sicard et al., 2017). Such communication occurs, for example, by short-chain fatty acids (SCFA) produced by several gut bacteria present in the colon lumen that diffuse rapidly through epithelial cell membranes and are used directly by the epithelial cells themselves or to instruct differentiation of the intestinal immune cells, such as regulatory T cells (Smith et al., 2013). The microbiota that colonizes the human gastrointestinal tract has then developed specialized interaction mechanisms with its host (Endesfelder et al., 2016). Commensal microorganisms colonizing the gut are required for the maturation of the immune system leading to its ability to differentiate commensal bacteria from the pathogenic (Lazar et al., 2018; Nakanishi et al., 2015). Toll-like receptors (TLRs) from the membrane of the epithelial and lymphoid cells are involved in the differentiation of the immune system playing a role in the development of the intestinal mucosal immune system, by suppressing the inflammatory response and stimulating tolerance to the incoming microorganisms (Lazar et al., 2018; Pearson et al., 2018). The TLRs recognize microbe-associated molecular patterns (MAMPs) triggering the innate intestinal immunity when no recognition occurs (Gülden et al., 2015; Lazar et al., 2018). When the microorganism are not recognized, a cascade of signals are activated leading to the activation of the transcription of genes that encode the chemokines, cytokines, acute phase proteins, and other effectors of the humoral immune response (Lazar et al., 2018). To allow the development of a proper microbiota in the gut, the activity of TRLs is decreased in the first few weeks of the new-born (Lazar et al., 2018). To guarantee the intestinal homeostasis, the inhibition of the

inflammatory reaction is required, so the TLR activation by antigens belonging to the normal microbiota activates this inhibition (Lazar et al., 2018).

The gut microbiota can modulate neutrophil migration and function as well, by interfere in the differentiation of T cell population into helper cells or regulatory T cells (Lazar et al., 2018; Owaga et al., 2015). This can be an issue since some types of T helper cells, such as Th17 are responsible for the secretion of cytokines, that have a huge role on the immune homeostasis and inflammation (Lazar et al., 2018). It has been shown that the introduction of a purified capsular polysaccharide of the commensal bacterium *B. fragilis* suppresses the production of IL-17 and protects the colonic mucosa against inflammatory reactions initiated by bacterial antigens (Mazmanian et al., 2008). However, cytokines produced due to the presence of bacteria do not just prevent inflammatory reactions, they also can induce quite opposite responses. Cytokines, such as IL-6 and TNF-α can increase the secretion of MUC2, MUC5A, MUC5B and MUC6 by the intestinal cell line LS180 (Enss et al., 2000).

The communication between the host and the commensal microbiota can trigger an antimicrobial response from the epithelium to release, for example, antibacterial lectins (Lazar et al., 2018). When released antibacterial lectins can reduce the amount of potentially pathogenic microbes and provide protection against subsequent abnormal immune responses. The commensal *B. thetaiotaomicron* can initiate the production of antimicrobial peptides that target other intestinal bacteria (Lazar et al., 2018).

Microbial products may induce chronic stimulation of immune responses, leading to chronic, non-resolving inflammation and tissue damage, particularly after mucosal injury (Endesfelder et al., 2016; Lazar et al., 2018; Pearson et al., 2018). For example, adherent-invasive *E. coli* LF82 strain is capable of altering T84 colonic cell mucin and IL-8 gene expression which can also lead to a defective mucus layer (Elatrech et al., 2015).

Different metabolites, such as SCFA, are the result of the primary and secondary fermentation of the undigested food consumed by the host, which intestinal microbiota uses as primary source of energy. Thus, the gut metabolome (the collection of all low-molecular-weight metabolites) is strictly dependent on the metabolic pathways leaded by members of the intestinal microbiota (Vogt et al., 2015). The disturbance in the composition and functionality of the gut microbiota causes dysbiosis (unbalanced composition of the intestinal microbiota) which in turn can lead to various health problems, namely autoimmune diseases, such as allergies, autism, lupus and Type 1

Diabetes (T1D) (Brown et al., 2011; Caminero et al., 2018; Endesfelder et al. 2014; Feehley et al., 2019; Finegold et al., 2010; Soyucen et al. 2014; Vatanen et al. 2018; Vieira et al., 2018).

1.2. Intestinal Microbiota and Type 1 Diabetes

1.2.1. Diabetes *mellitus*

Diabetes is defined by the World Health Organization (2019) as a chronic metabolic disease characterized by elevated levels of blood glucose. The insulin hormone, required to regulate the sugar levels in the blood, is not produced or not recognized by the body regarding this disease. The first symptoms of this disease are usually polyuria, polyphagia and polydipsia; however, other symptoms may also be associated, such as weight loss, blurred vision and fatigue (World Health Organization, 2019).

To date, 3 types of diabetes *mellitus* are reported and accepted by the World Health Organization, namely Diabetes *mellitus* type 1, type 2 and gestational. There is also a new type of diabetes, type 3 diabetes, recently reported but not yet clinically confirmed (de La Monte et al., 2008).

Type 2 Diabetes (T2D) is characterized by a decrease in glucose-insulin receptors causing insulin resistance which results in an increased amount of the glucose-regulating hormone in the blood. If untreated T2D can lead to the failure of β cells in the pancreas causing serious problems similar to those associated with T1D, and since the diagnosis of T2D is usually delayed, the comorbidities of the disease can be aggravated (Portal da Saúde and Banco da Saúde, 2012). T2D is the most common type contributing to 90-95% of the diagnosed diabetes cases, affecting mainly people in adulthood. Interesting, the incidence of T2D in children and adolescents has been increasing due to changes in lifestyle and poor eating habits (Portal da Saúde and Banco da Saúde, 2012).

Similar to T2D, gestational Diabetes *mellitus* is characterized by insufficient insulin production in relation to the requirements of the body. This condition is usually solved after delivery (in about 90% of cases), but in some cases, it may progress to T1D or T2D (World Health Organization, 2019). This type of diabetes is diagnosed in 3-9%

of pregnancies and comorbities arise if not treated, either for the mother as for the fetus. The mother can develop one of the other mentioned type of diabetes as well as have complications during childbirth, the new-born may show overweight, suffer from deficiencies in the kidneys, heart, and spine, and as well as develop T1D (Portal da Saúde and Banco da Saúde, 2012).

T1D is also known as insulin-dependent diabetes. It is characterized by lymphocyte infiltration or inflammation of the pancreas islets (insulitis). This type of diabetes is one of the most well known and studied autoimmune diseases. Most people diagnosed with this pathology have no symptoms until 90% of the β cells in the pancreas are destroyed by the T cells (Lee, 2011) (**Figure 1.2.1.1**).

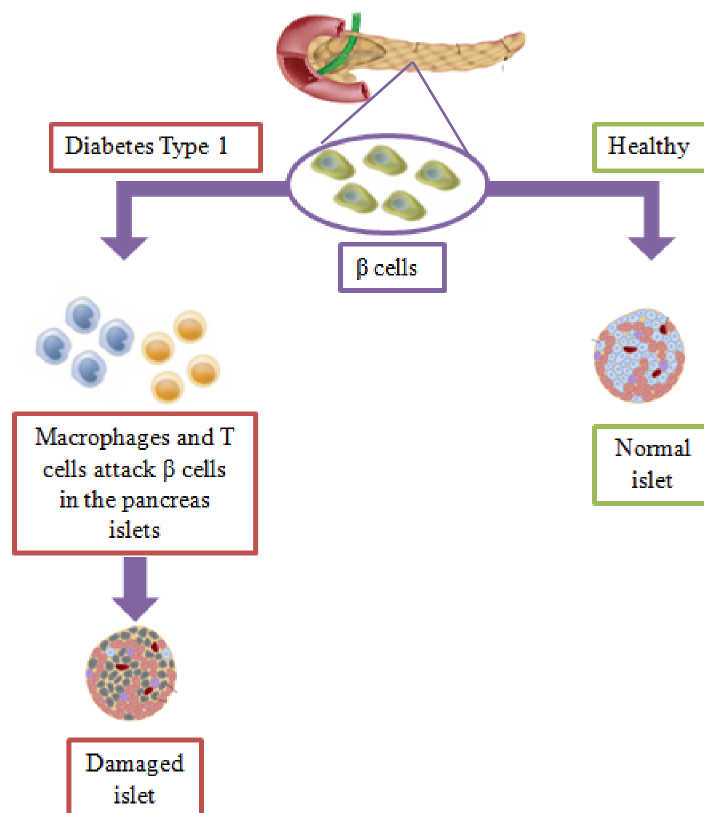

Figure 1.2.1.1 – β cells that produce insulin in the pancreatic islets. Type 1 diabetes is a disease that is characterized by the attack and destruction of the pancreatic islets by macrophages and T cells. Source: Teo et al., 2013 and U.S. National Institute of Diabetes and Digestive and Kidney diseases, 2019.

After decades of research the causes for the onset of this disease are not yet clarified. However, it is known that the genetic factor is present and associated with the onset of T1D, the genetic predisposition of the patient to the malfunction of the β cells of the pancreas, genetic mutations in the production of insulin or activity may be interconnected with the disease (Davis-Richardson et al., 2014; Vaarala et al., 2008).

Serological markers of an autoimmune pathologic process, including islet cell, GAD, IA-2, IA- 2β, or insulin autoantibodies, are present in 85-90 % of individuals when fasting hyperglycaemia is detected (Kostic et al. 2015). Human Leukocyte Antigen (HLA) genes are those that demonstrate a greater association with the disease, being that the genetically susceptible individuals (around 95% of patients) harbour either human leukocyte antigen DR3-DQ2 or DR4-DQ8 haplotypes, or have the UBASH3A mutation (chromosome 21), which are also linked with other autoimmune diseases, such as celiac disease (Lazar et al., 2018).

It is not known which mechanism drives T1D onset, but it is known that the disease has a large genetic predisposition and in general is considered a complex and multifactorial disease (Teo et al., 2013). However, the genetic predisposition by itself does not justify the increasing cases of T1D worldwide. Environmental factors, such as intestinal microbiota, diet and stress have been proposed to constitute a trigger factor in T1D (Kostic et al. 2015). The events associated with dysbiosis triggering autoimmune diseases including T1D are illustrated in **Figure 1.2.1.2**.

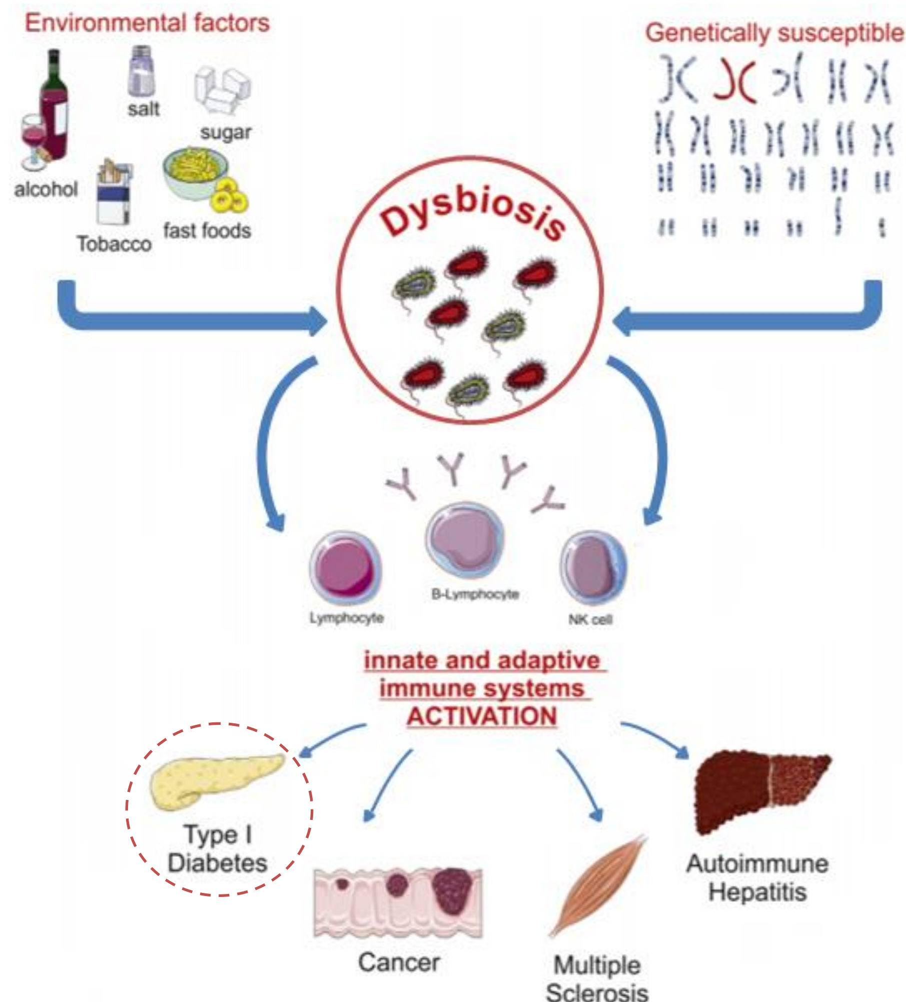

Figure 1.2.1.2 - Summarized factors that may cause a dysbiosis in the intestinal microbiome. Internal factors (genetic susceptibility) and or external factors (diet, lifestyle and use of antibiotics) can facilitate an imbalance of the gut homeostasis resulting in dysbiosis. Dysbiosis can then cause self-antigen immune activation, resulting in diseases, such as cancer, multiple sclerosis, autoimmune hepatitis and Type 1 diabetes. Adapted from Szychlinska et al., 2019.

1.2.2. Dysbiosis and Type 1 Diabetes

The intestinal microbiota of children at risk of developing T1D has been investigated, and the aim of the majority of the studies is to understand how the microbiota of patients differs from healthy individuals and how these differences may contribute to the development of the disease (Davis-Richardson et al., 2014; de Goffau et al., 2014; Endesfelder et al., 2014; Gavin et al., 2018; Kemppainen et al., 2015; Kostic et al., 2015; Leiva-Gea et al., 2018; Livanos et al., 2016; Pinto et al., 2017; Soyucen et al., 2014; Vatanen et al., 2018). In several studies carried in children at risk of developing autoimmunity is reported an increase in the *Bacteroides* population and

also a decrease in SCFA-producing bacteria (Davis-Richardson et al., 2014; de Goffau et al., 1982; Endesfelder et al., 2016; Mejía-León et al., 2014). Other studies reported that after islet autoimmunity but before T1D onset occurs an increase in the intestinal permeability and a decrease in the microbial diversity (Kostic et al., 2015; Maffeis et al., 2009).

The study of Giongo et al., (2011) that was conducted to explore the relationship between the intestinal microbiome and the development of T1D using a pyrosequencing approach showed that children with islet-positive autoantibodies showed higher *Bacteroidetes*/*Firmicutes* ratio and a lower diversity in the intestinal microbiome. Additional studies revealed that *Bacteroides dorei* and *Bacteroides vulgatus* had a marked abundance in Finnish children (Turku city) at high risk of T1D and associated with autoantibody positivity (Davis-Richardson et al., 2014; de Goffau et al., 2014). The abundance of SCFAs acid producing bacteria and lactate producing bacteria was reduced in patients with T1D (de Goffau et al., 2014, 2013). In the study conducted by de Goffau et al., (2014) when the intestinal microbiota of newly diagnosed T1D children from seven different European countries, like France, Greece, Estonia, Lithuania and Finland, showed an higher abundance of the bacteria of the phylum *Bacteroidetes* in comparison with the Control children that showed an higher abundance of bacteria of the genus *Bifidobacterium* (de Goffau et al., 2014). It is also showed that newly diagnosed T1D patients show a bigger abundance of *Bacteroides* while control subjects display a higher abundance of *Prevotella* (Alkanani et al., 2015). In virtue of the abundance of *B. dorei* in Finnish children from the city of Turku that were at risk of autoimmunity the evaluation of *B. dorei* population was proposed as possible predictor for T1D in Finland (Davis-Richardson et al., 2014). However, the gut microbiota can be influenced by several factors, namely the geographic location, eating habits, and sanitary conditions. Therefore, the study of Kemppainen et al., (2015) showed that according to the geographic region the children at risk of autoimmunity display a different microbiome.

A study conducted in Spain with children with established T1D reported an increase in *Bacteroides* in T1D in comparison with Control children along with a higher abundance of *Clostridium* and *Veillonella* and a reduction in the populations of *Bifidobacterium*, *Lactobacillus*, *Blautia coccoides/Eubacterium rectale* group, and *Prevotella* (Murri et al., 2013). Using a metaproteomic approach Pinto et al., (2017) reported the abundance of proteins originated from *Bacteroides* and *Clostridium* cluster

XVa and cluster IV concurrently with a reduction in proteins originated from *Bifidobacterium* in children with established T1D, in contrast the Control children that showed a metaproteome enriched with proteins originated from *Bifidobacterium*. However, the numbers of *Bacteroidetes* were similar between cases and controls.

1.2.3. *Bacteroides* spp.

The intestinal microbiota of healthy adults is constituted by about 20 to 80 % of bacteria of the phylum *Bacteroidetes* including the genus *Bacteroides*, *Parabacteroides*, *Prevotella* and *Alistipes* (Huttenhower et al., 2012). The order *Bacteroidales* is the most abundant group of Gram-negative bacteria in the human intestine reaching densities of 10^9-10^{11} colony forming units per gram of faeces (CFU/g faeces) (Naamah et al., 2011). *Bacteroides* is considered one of the main genus of the gut microbiota, whose species, with relative abundance greater than 1 %, refer to *B. uniformis*, *B. vulgatus*, *B. caccae* and *B. thetaiotaomicron*, but this genus also displays different diversity among different individuals (Jeffery et al., 2016; Kurokawa et al., 2007; Qin et al., 2010; Tap et al., 2009).

Bacteroides are obligate anaerobes and are non-spore-forming, non-motile, rod-shaped with round ends (Krieg et al., 2007). One of the well-acknowledged characteristics of *Bacteroides* species is the strong ability to degrade both plant and host polysaccharides (Marcobal et al., 2013; Xu et al., 2003). These microorganisms produce SCFA by consuming non-digestible plant or animal based glycans, including the host intestinal mucus (Arike et al., 2016; Bolam et al., 2012; Derrien et al., 2010; Johansson et al., 2015; Marcobal et al., 2013). Despite this characteristic, intestinal mucin will not be digested by the majority of the *Bacteroides* species unless it is the only nutrition source available (Zitomersky et al., 2013).

The *Bacteroides* genus is also associated with resistance to a wide range of antibiotics, such as beta-lactams, aminoglycosides, such as erythromycin and tetracycline but varies according to species and region of isolation (Boyanova et al., 2015; Ho et al., 2017; Nagy et al., 2015; Nguyen et al., 2000; Snydman et al., 2017).

Regarding the clinical impact of *Bacteroides* species the specie *B. fragilis*, for example, is responsible for a large number of anaerobic infections in the human gut (Li et al., 2017). The pathogenicity is due to its capsular polysaccharide, which protects the

bacterium against phagocytosis and stimulates abscess formation (Rashidan et al., 2018). *B. fragilis* has a pathogenicity island encoding secretory metalloprotease II (MPII), which together with one of the three homologous enterotoxigenic fragilisin (FRA) isoenzymes may have pathological importance in the gut leading to a weakening of cell-cell contacts as well as their cling joints. The MPII toxin may further induce IL-8 production by intestinal epithelial cells (Wu et al., 2004).

Bacteroides xylanisolvens, so far, has only been isolated from human faecal samples. Brodmann et al., (2017) evaluated the possibility of *B. xylanisolvens* possess probiotic proprieties (a probiotic has to be a bacterium ambiguously safe) since *B. xylanivsolvens* is free of genes associated with the biosynthesis of enterotoxin of the type produced by *B. fragilis* (*bft*) and capsular polysaccharide PS A (PS A) (Brodmann et al., 2017; Ulsemer et al., 2012). These components are important for the *B. fragilis* virulence, namely PS A contributes positively to the immunomodulatory activity, being an important factor in the formation of abscesses (Eradi et al., 2018), and the *bft* gene is responsible for the production of a toxin associated with pathologies, such as the colon cancer (Boleij et al., 2015; Ulger et al., 2006). *B. xylanivsolvens* is also unable to adhere to the intestinal epithelial cells, which eliminates a successful colonization of the intestine (Atherly et al., 2014). All this characteristics of *B. xylanivsolvens* combined with the absence of plasmid material in its genome supports the potential use of *B. xylanivsolvens* as probiotic (Brodmann et al., 2017; Chassard et al., 2008; Tan et al., 2019).

The other abundant *Bacteroides* specie in the gut, the *B. thetaiotaomicron* revealed its importance in the study of symbiotic bacterial host relationships in the human intestine (Xu et al., 2003), as well as its metabolic processes in the digestion process to facilitate the absorption of nutrients by the host (Wrzosek et al., 2013), and it also contributes to the development of the postnatal intestine and the physiology of the host (Xu et al., 2003). However, in virtue of its antibiotic resistance may constitute a source of resistant elements through the gut microbiota what is a significant concern (Cho et al., 2001; Ho et al., 2017; Shipman et al., 1999; Tan et al., 2019; Teng et al., 2004).

As mentioned above *B. dorei* has been associated with the development of the autoimmune state in children at risk of T1D. Vatanen et al., (2018) analysed the potential of the LPS produced by *B. dorei* strains to induce an immune response in comparison with *E. coli* and observed the LPS produced by *B. dorei* display a lower

immune response, which may result in a more unprepared immune system to combat foreign microorganisms. However, the number and origin of strains cannot be considered significant to generalise these results (Davis-Richardson et al., 2014; Murri et al., 2013; Pinto et al., 2017).

1.2.3.1. *Bacteroides dorei* and the link with Type 1 Diabetes

B. dorei was first isolated in 2006 by Bakir et al. from the human gut. The colonies are usually circular, white, raised and convex when in EG agar after an incubation of 48 h at 37ºC in anaerobic conditions. Colonies usually reach a diameter of 2.0 mm (Bakir et al., 2006; Song et al., 2015). In Brain Heart Infusion agar medium supplemented with hemin, the colonies show a very smooth and tenuous blue colour achieving 4-5 mm (Bacic et al., 2008).

As previously mentioned *Bacteroides* can be found in the mucus and in conditions of nutrient depletion they display their mucinolytic activity. Taking that into account and the recent studies linking *B. dorei* to T1D and the coeliac disease and some of illnesses associated to this disease like gastroparesis (Bharucha et al., 2017) seems reasonable to hypothesize that *B. dorei* may have a more significant impact on dysbiosis of children at risk and with established T1D than just a lower capacity to induce immune responses. Studies that explore the interactions of *B. dorei* with the intestinal epithelial cells and their ability to disturb the integrity of the cell junctions will be helpful to clarify its possible roles in these diseases.

1.2.4. The use of cell lines to study interactions between bacteria and the intestinal epithelium

As described above, the intestinal epithelium is a permeable barrier that plays an important role in regulating the exchange of solutes and fluids. Different types of cells form the intestinal epithelium, and good cooperation between all these cells maintains the integrity of the mucosa, which is challenged daily by external factors in the lumen environment. Loss of intestinal barrier function has clinical and nutritional consequences, including chronic disorders, inflammation and malnutrition. Any intestinal inflammation is often associated with other pathological conditions, such as

metabolic syndromes and some non communicable diseases (e.g obesity) (Ding et al., 2011; Esser et al., 2014; Walters et al., 2014).

In recent decades, *in vitro, ex vivo* and *in vivo* models have been explored in order to study different functions and metabolism of the intestinal epithelium, particularly in the inflamed state, and extrapolate those studies for the human being (Ponce de León-Rodríguez et al., 2018).

Although animal models, also known as *in vivo* models, can simulate the physiology of an entire organism, variation in responses due to species differences and difficulty in extrapolating results to humans are the disadvantages of these models (Fitzgerald et al., 2015).

The use of *in vitro* models of cell culture has increased due to increased regulation and ethical issues involved in the use of animals. Improved *in vitro* models using human cells have the advantage of reducing animal experimentation and enabling the study of molecular mechanisms in a simple and reproducible manner. For example, molecular study of bioactive food compounds and their interactions with the intestinal epithelial barrier and microbiota has become possible (Ponce de León-Rodríguez et al., 2018).

Cell culture has become an essential tool in toxicology and nutrition studies since the establishment of *in vitro* animal and human cell lines. The use of primary cell cultures is limited because of their availability, repeatability and their use in long term studies due to their short lifespan, but transformed cell lines are widely used, like Caco-2 and HT-29 cell lines (Langerholc et al., 2011; Zucco et al., 2005).

1.2.4.1 Caco-2 Cell Line

The human epithelial cell line Caco-2 has been the most widely used cell line as an intestinal model for absorption, transport and bioavailability studies (Cheng et al., 2008; Hidalgo et al., 1989; Hillgren et al., 1995). This cell line was isolated from a human colorectal adenocarcinoma and established by Fogh and Trempe in 1974 (Fogh et al., 1975). It is well characterized as an enterocyte model, thanks to its morphological and functional characteristics expressed after differentiation. It takes these cells about five days to reach confluence and spontaneously begin to differentiate during 30 days of culture. Once differentiated, they form a monolayer of polarized cells with apical and

basolateral membranes, a junction complex, and a brush border with microvilli on the apical side typical of human enterocytes (Chantret et al., 1988; Hidalgo et al., 1989; Pinto et al., 1983).

However, the Caco-2 parental lineage has certain limitations, including the formation of a heterogeneous monolayer linked to culture time and number of passages as well as the presence of multilayer zones (Artursson et al., 2001; Briske-Anderson et al., 1997). Cultivation conditions may also select cell subpopulations. Also, this cell line cannot differentiate into goblet cells, so an important limitation is its inability to produce mucus (Verhoeckx et al., 2015).

1.2.4.2. HT-29 Cell Line

HT-29 is another intestinal cell line, established by Fogh and Trempe. This cell line was also derived from a colon adenocarcinoma (Fogh et al., 1975) and has been used as an enterocyte model in bioavailability and cell mechanism studies (Andoh et al., 2001; Hagesaether, 2011; Yi et al., 2016). The HT-29 cell line is considered a pluripotent intestinal cell line because changes in culture media may lead to different pathways of enterocytic differentiation (Ponce de León-Rodríguez et al., 2018). Unlike the Caco-2 cell line, HT-29 differentiation is not spontaneous, but depends on nutritional and culture conditions (Huet et al., 1987; Viallard et al., 1986; Zweibaum et al., 2011). In glucose free medium this cell line differentiates into enterocyte cells expressing hydrolases, such as sucrose isomaltase, which are restricted to the border of the intestinal brush, while when grown in the presence of glucose remain undifferentiated. In addition, differentiation in HT-29 cells can be inhibited and reversed by adding glucose to the medium (Zweibaum et al., 1985). Other differences between the Caco-2 and HT-29 lines are a longer period of differentiation and the absence of lactase expression in the HT-29 cell line (Ponce de León-Rodríguez et al., 2018). However, the main difference between HT-29 and Caco2 cell lines is that under certain culture conditions HT-29 can differentiate into goblet-like cells and thus has the ability to produce mucus (Laburthe et al., 2011). The mucus produced by these goblet cells is a water-insoluble gel composed mainly of glycoprotein oligomers and related monomers that form a protective layer in the intestine (Béduneau et al., 2014). When treated with sodium butyrate, for example, HT-29 cells differentiate into distinct

phenotypes some of which are mucus-secreting clones analogous to intestinal goblet cells (Augeron et al., 1984).

HT29-derived cell lines are a valuable tool related to strengthening the studies of intestinal mucus barrier as well as the mucin stimulating activity of food compounds. Stable clone HT29-MTX was isolated from HT-29 (this differentiation is irreversible) due to the cells resistance to high Metatrexate (MTX) concentrations following gradual adaptation to this anticancer drug. This clone is capable of spontaneously differentiating goblet-like mucus-producing cells (Lesuffleur et al., 1990), that have been used as a model to study lactic acid cell adhesion to intestinal epithelial cells (Turpin et al., 2012).

2 Objectives

The main objective of the current study is to evaluate the ability of the *Bacteroides* spp. to interact with intestinal epithelial cells, namely HT29-MTX-E12. To achieve this goal the following specific objectives were included:

- Evaluate the adhesion and invasion ability of several isolates of *Bacteroides* spp. including *B. dorei* to HT29-MTX-E12 cell line.
- Examine the effect on the integrity of HT29-MTX-E12 cell lines exposed to *Bacteroides* spp.
- Determine the viability of the cell lines after the exposure to the bacterial metabolome.
- Evaluate the induction of an inflammatory response after the exposure of the cell line to the bacterial cells.

3 Material and Methods

3.1 Equipment

- AE2000 Inverted Microscope, Motic (Hong Kong)
- Anaerobic jar, Difco Laboratories (Detroit Michigan)
- Analytical balance AE 200, Mettler (USA)
- Analyticall balance XS-410, Fisher Scientific (Portugal)
- ATC 2000 Microscope, Leica (Portugal)
- Autoclave Uniclave 88 AJC (Lisbon, Portugal)
- Bio48 Laminar Flow Chamber, Faster (Italy)
- Binder Incubator (Germany)
- Cryogenic ULT freezer -150°C MDF-C2156VAN (Sanyo, Japan)
- Faster BH-EN (TECHLAB, France)
- Heating and agitation plate, Selecta, Agimatic-E (Spain)
- HeraCell CO_2 Incubator Heraeus, Thermo Fisher Scientific (UK)
- Mikro 22R Centrifuge, Hettich Zentrifugen (UK)
- Vortex L46, Labinco (The Netherlands)
- Ultra low temperature freezer freezer -80ºC U725, Innova New Brunswick Scientific (USA)
- Puradisc 30 syringe filters 0.2µm, Whatman (Germany)
- Plate reader Infinite M200, Tecan (Switzerland)
- Sterile syringes of 10 mL, Terumo (USA)
- SW20 Shaking water bath, Julabo (Germany)
- SZ Stereo Microscope, Olympus (Japan)

3.2 Cell culture medium and reagents

The culture medium and solutions used for cell culture are indicated in **Table 3.2.1**.

Table 3.2.1 – Components of cell culture media and solutions used in the study

Solutions and medium	Volume	Supplier
Cell Culture maintenance		
Dulbecco's Modified Eagles Medium High Glucose	500 mL	Sigma, Germany
Dulbecco's Modified Eagles Medium/ Nutrient Mixture F12 Ham	500 mL	Sigma, Germany
Heat inactivated fetal bovine serum (FBS)	500 mL	Sigma, Germany
Penicillin/Streptomycin (PEN-STREP), 5000 units/mL Penicillin + 5000 µg/mL Streptomycin	100 mL	Gibco, USA
Non essential amino acids 100x	100 mL	Sigma, Germany
L-Glutamine, 200 mM	25 g	Sigma, Germany
Freezing solution		
Dulbecco's Modified Eagles Medium High Glucose	500 mL	Sigma, Germany
Heat inactivated Fetal Bovine Serum (FBS)	500 mL	Sigma, Germany
Penicillin/Streptomycin (PEN-STREP), 5000 units/mL Penicillin + 5000 µg/mL Streptomycin	100 mL	Gibco, USA
Non-essential amino acids 100x	100 mL	Sigma, Germany
Dimethyl sulphoxide (DMSO)	50 mL	Sigma, Germany
Trypsin-EDTA		
Phosphate Buffered Saline (PBS) 137 mM/L NaCl, 2.7 mM/L KCl, 10.1 mM/L Na_2HPO_4, 1.8 mM/L KH_2PO_4		
Trypsin-EDTA 10x	100 mL	Sigma, Germany
Disposable pipettes: 1, 5, 10, and 25mL		Greiner Bio-one, Austria

3.3 Cell line used in the study

The cell line, HT29-MTX-E12 (ECACC 12040401) (Sigma-Aldrich, Germany) was used in the present study. The cell line was grown in Dulbecco's Modified Eagles Medium High Glucose, supplemented with 10 % heat-inactivated foetal bovine serum, 1 % of non-essential amino acids and 1 % of penicillin and streptomycin (hereafter called DMEM complete). In the viability assays the Dulbecco's Modified Eagles Medium/ Nutrient Mixture F12 Ham, supplemented with the same components and measurements as DMEM complete, here after called DMEM complete without phenol red, was used in order to eliminate any interference of the phenol red with the MTT used in the cell viability assay.

All the cell culture work was performed under sterile conditions inside a laminar airflow, (Faster BH-EN,TECHLAB, France). Prior to start the flow cabinet was sterilized by UV for 20 min and after that all the working surfaces and equipment were disinfected with 70 % ethanol.

The growth of the cell line was performed using 75 cm^2 filtered cap culture flasks (Sarstedt, USA) and the growth medium was changed at least twice a week, or when required.

When the cell reached 85-95% confluence they were passaged. For this the old-growth medium was removed, and the cells were washed with 3 mL PBS. The last PBS volume was then removed and 3 mL Trypsin-EDTA was added into the flask. After 10 min (interval of time required for the cells being detached from the flask surface) 3 mL of DMEM complete was added in order to neutralize the effects of the Trypsin-EDTA. One millilitre of the cell suspension was then transferred to a new 75 cm^2 growth flask and 9 mL of DMEM complete was added.

The incubation of the HT29-MTX cells took place in a HeraCell CO_2 incubator (Heraeus Thermo Fisher Scientific, UK) at 37ºC with 5 % (v/v) CO_2 in a humidified atmosphere.

3.4 Determination of the number of Cells

It is crucial to work with the correct quantity of epithelial cells when performing any assay as well as to freeze cells for maintenance. Therefore, each experiment started by determining the number of cells in the culture flasks. For this the cells in the 75 cm^2 sized flasks were trypsinized with 3 mL Trypsin-EDTA for 10 min, to detach the cells from the flask, followed by its neutralization by adding 3 mL of DMEM complete into the culture flask. One millilitre of the cell suspension was then transferred into sterile conical centrifuge 1.5 mL tube (Sarstedt, USA). The cell count was determined using the Neubauer chamber (Hirschmann laborgeraete, Germany) into which 20 μL of cell suspension was loaded and the cells were counted using the ATC 2000 Microscope (Leica, Portugal). If needed the cell suspension was diluted in PBS (1:10). All cell counts were done in duplicate. The scheme and the direction of the cells counts using the Neubauer chamber are illustrated in **Figure 3.2.1**.

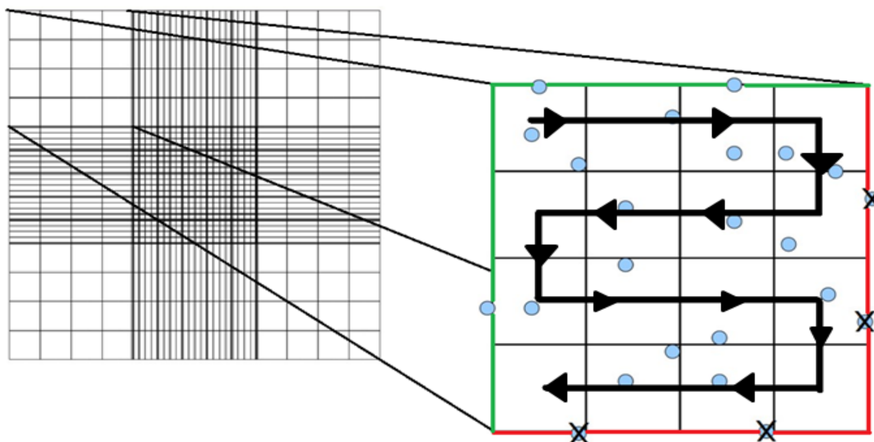

Figure 3.2.1 - Grid layout of the Neubauer chamber and schematic direction of the cells counts. The green sides are the outer limits considered whereas the red outer limits are the ones discarded during cell counting. The arrows represent the flow of the cell count. The considered area for counts was 1 mm^2. The depth of the chamber was of 0.1 mm.

3.5 Preservation of HT29-MTX-E12 Cells

The cell line HT29-MTX-E12 (ECACC 12040401) was used from passage 52 to 60 to minimize any potential physiological differences between passages. Therefore, several samples of these passages were frozen to ensure access to new and viable sets.

For the preparation of the preservation samples the cells grown on 75 cm^2 flasks were trypsinized, detachment of the cells from the flask, after which DMEM complete was added to neutralize the trypsin. The cells were then counted to guarantee that about $3.0x10^6$ cell/mL were achievable for cryopreservation. Thereafter the cell suspension was transferred into 15 mL tubes (VWR, USA) and centrifuged for 5 min at 5000 xg. The supernatant was discarded and the freezing medium was added in order to achieve a cell suspension of about $3.0x10^6$ cell/mL. The cells were gently re-suspended in the freezing medium. One millilitre of this cell suspension was transferred into each 1.6 mL CryoPure tubes (Sarstedt, USA). The composition of the freezing solution was described in **Table 3.2.1**.

The Cryotubes were retained in a -20ºC freezer overnight after which they were transferred to a Ultra low temperature freezer -80ºC (U725, Innova New Brunswick Scientific, USA). Half the samples that were 72 h in the -80ºC freezer were then transferred to a Cryogenic ULT freezer -150°C MDF-C2156VAN (Sanyo, Japan) for long term storage. All these steps were performed to freeze cells slowly and avoid any excessive temperature differences.

3.6 Bacterial strains and growth conditions

The strains of *Bacteroides* spp. were collected from T1D and Control children from the Algarve region within the Health Marathon project (Matos, 2018). The bacteria used in the current study are listed in **Table 3.6.1**.

Table 3.6.1 - List of bacteria used in the study

Bacteria	Source
***Lactobacillus casei* DSM 20011**	German Type Culture Collection
***Bacteroides dorei* DSM 17855**	German Type Culture Collection
***Bacteroides dorei* PtF D1P5**	Health Marathon Project (isolated from faeces of children from the Algarve region (Matos, 2018))
***Bacteroides dorei* PtF D8M1**	
***Bacteroides dorei* PtF D16P1**	
***Bacteroides dorei* PtF D16M14**	
***Bacteroides dorei* PtF Sb6**	
***Bacteroides dorei* PtF Sb8**	
***Bacteroides dorei* PtF C1P2**	
***Bacteroides uniformis* PtF Sb3P5**	
***Bacteroides uniformis* PtF D3Pch2**	
***Parabacteroides distasonis* PtF D14MH1**	

The strains of *B.* dorei, *B. uniformis* and *P. distasonis* used in this study were grown using the Brain Heart Infusion medium (BHI), (Biokar, France) supplemented with hemin (0.1 %, v/v) and L-cysteine (Sigma-Aldrich, Madrid) (BHI+H). The strain of *L. casei* DSM 20011 was grown on Man Rogosa and Sharpe (MRS), (Biokar, France). When required agar was added at 1.5 % (w/v).

The bacteria were recovered from cryopreservation at -80ºC using the culture medium BHI+H agar under anaerobic conditions by using an Anaerobic jar (Difco Laboratories, USA) with an AnaeroGen sachet (Thermo Scientific, Oxoid UK), at 37°C for 48 hr. After this the purity of the bacterial cultures was examined by observation of the cultures characteristics (small to medium blue colonies with a smooth surface) under a magnifier glass (SZ Stereo Microscope, Olympus Japan). An isolated colony was transferred to 5 mL of BHI+H and covered with 1 mL of sterile paraffin to create anaerobic conditions. The cultures were grown during 24 h at 37ºC.

Lactobacillus casei DSM 20011 was grown in MRS medium and instead of anaerobic it was grown in microaerophilic conditions, atmosphere low in O_2, at 37ºC during 24 h.

The number of bacterial cells was determined by the Miles and Misra technique (Miles et al., 1938).

3.7 Evaluation of the bacterial adherence ability

For the bacterial adhesion assay the cell line HT29-MTX-E12 (ECACC 12040401) was grown in DMEM complete and incubated at 37°C in the presence of 5 % (v/v) CO_2 in a humidified atmosphere. The cells were seeded in 24-well microplates (Greiner bio-one, Germany) at a concentration of $4x10^4$ cells/well. The culture medium, DMEM complete, was changed every two days and medium without antibiotics was used for the last change of medium. After the HT29-MTX-E12 cell differentiation period of 21 days the adherence assay was performed as described by Gagnon et al. (2013), and started by inoculating the bacterial cells at 10^7 colony forming units/mL (CFU/mL) using three biological replicates and two technical (n=6). Thereafter the cell line was exposed to bacteria during 30 min at 37°C under anaerobic conditions. After this time interval the non-adherent bacteria were removed by washing with 1 mL of PBS. Subsequently the cell line with adherent bacteria were treated with 250 μL Trypsin-EDTA and incubated at 37°C for 10 min. To inactivate the Trypsin-EDTA, 250 μL of DMEM complete was added to each well. From this suspension, serial decimal dilutions were performed in PBS and then each dilution was inoculated on BHI+H agar. The inoculated plates were incubated under anaerobic conditions at 37°C for 48 h.

The adhesion results were expressed as the percentage of the number of adherent bacterial cells in relation to the total number of bacteria used on the assay.

3.8 Evaluation of the bacterial invasion ability

As described above for the determination of the bacterial adherence capacity also for the evaluation of the bacterial invasion ability the HT29-MTX-E12 cell were allowed to differentiate for a period of 21 days and after that the cell line was infected with 10^7 CFU/mL (n=6).

The invasion ability was determined as described by Gagnon et al. (2013). The inoculated cell lines were incubated at 37°C for 4 h under anaerobic conditions. The cell lines were washed with 1 ml of PBS and exposed to 250 μL of DMEM medium containing 150 μg/mL of gentamicin (Sigma-Aldrich, Germany) for 1 h at 37°C to eliminate any non invasive bacteria. A new washing step was carried out with 1 mL

PBS and 250 µL of Trypsin-EDTA to detach the cells from the bottom of the wells. The cells were incubated at 37°C for 10 min. The cell lines were then exposed to 0.1 % (v/v) Triton X-100 (Merk, Germany) for 10 min at 37°C to permeabelize the cells enabling the count of invasive bacteria. From this suspension, serial decimal dilutions were done in PBS and then each one was inoculated on BHI+H agar. The inoculated plates were incubated under anaerobic conditions at 37° C for 48 h.

The results of the invasion ability were expressed as the percentage of the number of invasive bacteria in relation to the total bacteria used in the assay.

3.9 Evaluation of the ability of *Bacteroides* spp. to disrupt the tight junction's

To evaluate the capacity of the different strains of *Bacteroides* spp. to damage the tight junctions of the cell line HT29-MTX-E12 the cells were seeded in wells containing a sterile a coverslip, Ø 13 mm and 1.5 thickness (VWR, USA). Bacterial suspensions at a concentration of 10^7 CFU/mL (n=6) were transferred to the epithelial intestinal cells after the differentiation period of 21 days. The cell line was exposed to the bacterial cells during 4 h at 37°C under anaerobic conditions. After this incubation period the supernatant was collected, centrifuged (3000 xg, 10 min) and maintained at -80°C until use. The infected cells were then washed with 1 mL of PBS 3 times and fixed with 100 µL of 3.7 % (v/v) formaldehyde (Labscan Analytical Sciences, Thailand) for 15 minutes. Three new washing steps with 1 mL of PBS were performed. The cell line were then permeabilized with 100 µL of 0.5 % Triton X-100 (Merk, Germany) for 10 min followed by 3 washing steps with 1 mL of PBS. The cells were then blocked for non-specific binding with 150 µL of 3 % (w/v) bovine serum albumin (Merk, Germany) at 4°C during 1 h. Thereafter the cells were incubated during 20 min with 100 µL tetramethylrhodamine B isothiocyanate-phalloidin (TRITC-phalloidin) (Sigma-Aldrich, Germany) (diluted 1:200 from an initial solution of 0.1 mg/ml). The cells are then exposed for 3 min to 100 µL of 4',6'-diamidine-2-phenylindole (DAPI) (Sigma-Aldrich, Germany) (diluted 1:200 of an initial solution of 14.3 mM). Two final washes were carried out with 1 mL of PBS.

Each coverslip was then mounted (inverted so that the cells lie between the coverslip and the microscope slide) on a previously identified microscope slide (VWR,

Germany) with a drop of Fluromount (Sigma-Aldrich, Germany). The observation of the cell lines was done using the microscope Axio Imager Z2 (Zeiss, Norway).

3.10 Evaluation of the effect of the metabolome of *Bacteroides* spp. on the intestinal epithelial cells

The evaluation of the effect of the bacterial metabolome on the cell line viability after exposure to it was performed using the Vibrant MTT cell proliferation assay kit (Molecular Probes, Germany).

The bacterial cells were grown in BHI+H at 37ºC during 24 h under anaerobic conditions. Thereafter the bacterial suspensions were centrifuged (5000 xg, 10 min, 4ºC) and the supernatant was collected and filtered (0.2 µm diameter of pore, Whatman, Germany). After filtration the supernatant was distributed by sterile conical centrifuge 2 mL tubes (Sarstedt, USA) with 1 mL each. The supernatant samples were stored at -20ºC until use.

According to the recommendation of the Vibrant MTT cell proliferation assay kit the number of HT29-MTX-E12 cells required were about 8000 cells per well and seeded in a 96-well microplate (Sarstedt, USA). To reach an optimal population density the cells were incubated during 48 h at 37ºC in a 5 % (v/v) CO_2 humidified atmosphere.

Afterwards the DMEM complete used in the previous incubation was discarded and replaced with DMEM complete supplemented with the bacterial supernatant different percentages (10, 25 and 50 % (v/v)) (n=12). The pH value of the different combinations was 7.8 ± 0.11 for the 10 % (v/v) mix, 7.53 ± 0.12 for the 25 % (v/v) and for the 50 % (v/v) mix the pH was 7.21 ± 0.26. The cell line was exposed to the bacterial supernatant during 24 h. After exposure, the DMEM medium with the supernatant was replaced with DMEM complete and the MTT solution was added to each well. The cell lines were then incubated at 37°C in a 5% (v/v) CO_2 humidified atmosphere for 4 h. Thereafter the SDS-HCl solution was gently mixed into each well followed by another incubation of 4 h at 37ºC in a 5% (v/v) CO_2 humidified atmosphere. Finally, each well was homogenized to eliminate any precipitate and the absorbance value was determined at 570 nm using a microplate reader (Tecan Infinite, M200 Switzerland).

3.11 Evaluation of the impact of *Bacteroides* spp. on cytokine production

The evaluation of cytokine production by the cell line HT29-MTX-E12 under exposure to the bacterial supernatant was performed using the Multi-Analyte ELISArray, Human TLR-induced Cytokine II Kit (Qiagen, USA).

The bacterial samples used in this assay were the supernatant from the invasion assays (**section 3.8**). The samples were recovered from -80ºC by slowly defrosting, centrifuged (3000 xg, 10 min) and filtered (0.2 µm diameter of pore, Whatman, Germany) . The samples were kept on ice until use.

All reagents were prepared according to the instructions of the manufacturer. The assay buffer was added to each well of the ELISArray plate and each sample and controls were added to the corresponding wells. The incubation was at room temperature during 2 h. Thereafter the wells were washed 3 times. After that the detection antibody solution was added, followed by 1 h incubation at room temperature. After this time interval the wells were washed 3 times and the Avidin-HRP reagent was added. The incubation period was performed at room temperature during 1 h. After that the wells were washed 4 times following the addition of the development solution. The incubation was performed at room temperature for 15 min in the dark. Thereafter the stop solution was added and the optical density was determined at 450 nm using the microplate plate reader (Tecan Infinite M200, Switzerland).

3.12 Statistical analysis

All statistical analyses were performed using the statistic software IBM SPSS 25.0 for Windows. All statistical analysis were performed using one way analysis of variance (ANOVA) with a Post-Hoc Tukey HSD test for each assay. Means were compared and statistical significance differences were established at $P < 0.05$.

4. Results

4.1.Evaluation of the adherence ability

The results of the adherence ability of the *Bacteroides* spp. to the intestinal epithelial cells HT29-MTX-E12 cell line (with 21 days of differentiation) is summarized in **Table 4.1.1**. All *Bacteroides* spp. tested, namely *B. dorei*, *B. uniformis* and *Parabacteroides distasonis* were able to adhere to the HT29-MTX cell line.

The *Bacteroides* isolates that showed the lowest adhesion capacity ($P < 0.05$) were the *B. dorei* PtF D16P1 and PtF Sb6 (75.43 ± 2.98 % and 75.54 ± 2.38 %, respectively)

All the other *Bacteroides* and the *P. distasonis* isolates showed similar ability ($P > 0.05$) to adhere to HT29-MTX-E12 (**Table 4.1.1**). No discrimination of the isolates according to their origin (T1D or Control children) was observed.

Table 4.1.1 - Adherence ability (%) of the *Bacteroides* spp. tested with the HT29-MTX-E12 cell line.

Bacteroides spp.	Strain	Adherence (%)*
B. dorei	DSM 17855	83.07 ± 1.29^{b}
	PtF D1P5	81.12 ± 2.39^{b}
	PtF D8M1	78.79 ± 3.07^{b}
	PtF D16M14	82.46 ± 1.15^{b}
	PtF D16P1	75.43 ± 2.98^{a}
	PtF Sb6	75.54 ± 2.38^{a}
	PtF Sb8	81.18 ± 2.56^{b}
	PtF C1P2	81.85 ± 2.05^{b}
B. uniformis	PtF D3Pch2	81.60 ± 3.70^{b}
	PtF Sb3Pch2	81.42 ± 1.25^{b}
P. distasonis	PtF D14MH1	80.90 ± 3.98^{b}

*- Data is the mean ± standard deviation. Data with the same lowercase are not statistically different ($P > 0.05$). (n=6)

4.2. Evaluation of the invasion ability

The results of the ability of *Bacteroides* isolates to invade the HT29-MTX-E12 cells are summarized in **Table 4.2.1**. The invasion capacity of the *Bacteroides* isolates can be differentiated by species and strains, namely among the *B. dorei* isolates. The *B. dorei* PtF D16P1 isolate showed the highest invasion capacity (85.25 ± 5.42 %) together with the isolates *B. dorei* PtF Sb6 and PtF Sb8 (76.13 ± 2.24 %, 82.72 ± 1.58 %, respectively). The remaining *B. dorei* isolates showed similar ($P > 0.05$) ability to invade the cells tested (**Table 4.2.1**). In contrast the *B. uniformis* isolates, PtF D3Pch2 and PtF Sb3P5, were not able to invade the HT29-MTX-E12 cells, but the isolate *P. Distasonis*, PtF D14MH1, was the one that showed the highest ($P < 0.05$) invasion capacity (97.38 ± 1.62 %).

As for the adherence ability, after the evaluation of the invasion ability it was not possible to discriminate the isolates by their origin (from T1D or Control children).

Table 4.2.1 - Invasion ability (%) of the *Bacteroides* spp. tested with the HT29-MTX-E12 cell line.

Bacteroides spp.	Strain	Invasion (%)*
B. dorei	DSM 17855	71.09±4.93^{a}
	PtF D1P5	70.11±5.66^{a}
	PtF D8M1	70.66±7.89^{a}
	PtF D16M14	74.81±3.44^{a,b}
	PtF D16P1	85.25±5.42^{c}
	PtF Sb6	76.13±2.24^{a,b,c}
	PtF Sb8	82.72±1.58^{b,c}
	PtF C1P2	72.02±8.76^{a}
B. uniformis	PtF D3Pch2	N.I.
	PtF Sb3Pch2	N.I.
P. distasonis	PtF D14MH1	97.38±1.62^{d}

*Data represent the mean ± standard deviation. Data with the same lowercase are not statistically different ($P > 0.05$). N.I.- Non Invasion. (n=6)

4.3. Tight junction's disruption by *Bacteroides* spp.

The *Bacteroides* spp. tested, except *B. uniformis*, were able to adhere and invade HT29-MTX-E12 cells further the effect of infection on tight junction integrity was investigated. The HT29-MTX-E12 cells were stained with TRITC-phalloidin, a phallotoxin that binds to actin filaments. This binding enables the possibility to observe any damage that has occurred in the network of tight junctions as a result of the action of the *Bacteroides* spp. impairing the normal functioning of paracellular communication.

The damage on the tight junction of HT29-MTX-E12 cells exposed to all *B. dorei* strains and *P. distasonis* isolate was very evident and similar, in contrast with the HT29-MTX-E12 cells exposed to *B. uniformis* that were slight affected.

A representative image of the disturbed tight junction's by the infection of HT29-MTX-E12 cells with the strain *B. dorei* PtF D16P1 and *B. uniformis* PtF D3Pch2 is illustrated in **Figure 4.3.1**. As can be observed the control cells (not exposed to the isolates) show a strong TRITC-phalloidin signal indicating the abundance of F-actin present in the tight junction's evidencing the complexity and the intricate cellular communication network. In contrast, the observed signal emitted by TRITC-phalloidin in the intestinal epithelial cells exposed to *B. dorei* PtF D16P1 is weaker indicating a decrease of active F-actin in the tight junction's evidencing damaged tight junction's compromising their integrity, which seems to be proportional to the invasive capacity of this strain. The intestinal epithelial cells exposed to the *B. uniformis* isolate showed a TRITC-phalloidin signal similar to the control cells, not exposed to bacteria, only displaying a slight disruption of the tight junctions.

The integrity of cells tight junctions was also tested when in the presence of *L. casei* and *Bacteroides* spp. in equal portions (10^7 CFU / mL). In this case, it was not possible to present fluorescence images since the mucus production by the epithelial intestinal cells exposed to this combination of bacteria was high which prevented the binding of phallotoxin to the active actins of the cells. This impediment of the biding made it impossible to evaluate the impact of *L. casei* and *Bacteroides* spp. in the integrity of the tight junctions of the cells.

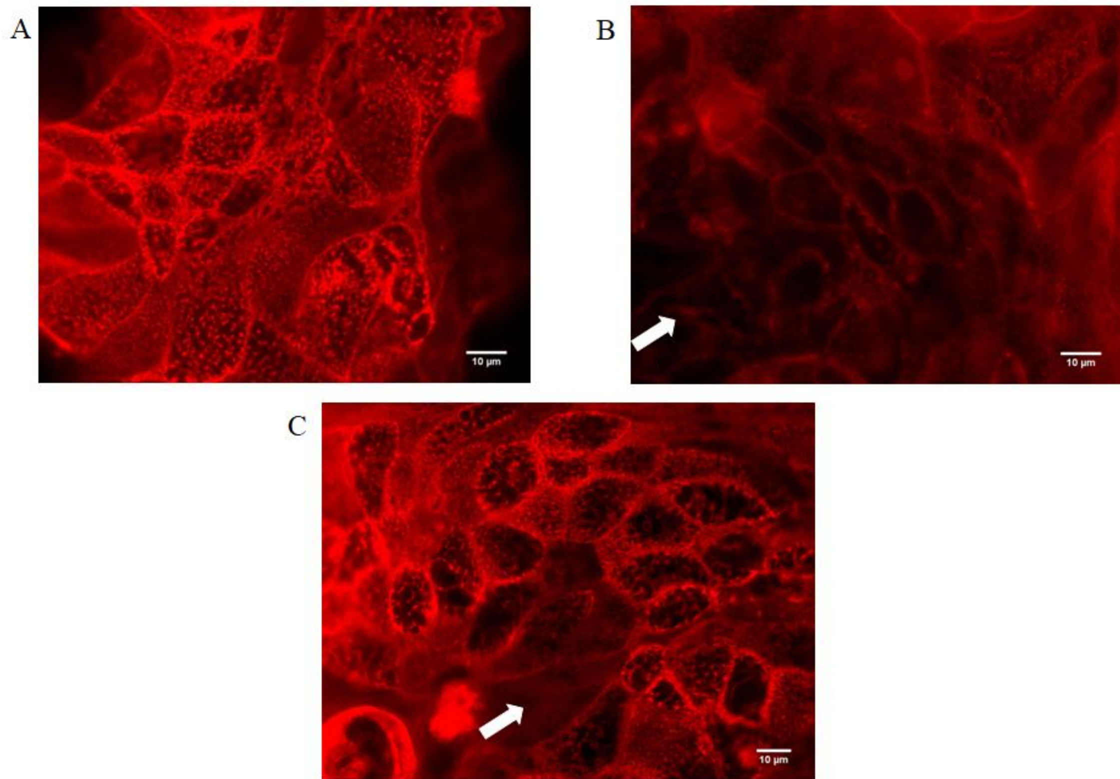

Figure 4.3.1 - TRITC-phalloidin staining (red) of cell junction's of the cell line HT29-MTX-E12. Uninfected cells (A) and after infection (4 hours) of the isolate *Bacteroides dorei* PtF D16P1 (B) and *B. uniformis* PtF D3Pch2 (C) Arrow evidences the cell junction's disruptions after *B. dorei* PtF D16P1 and *B. uniformis* PtF D3Pch2 infection in comparison with the undisrupted cell junctions of the control monolayers, respectively.

4.4. Effect of the metabolome of Bacteroides spp. on intestinal epithelial cells

The *Bacteroides* spp. may produce metabolites that can compromise the viability of the intestinal epithelial cells. In order to investigate this, the HT29-MTX-E12 cells were exposed to culture medium supplemented with different percentages (10, 25 and 50 % (v/v)) of the supernatant from the bacterial cultures during 24 h. The results are summarized in **Table 4.4.1**.

The viability of the HT29-MTX-E12 cells exposed to culture medium supplemented with 10 % (v/v) of the bacterial culture supernatant is similar ($P > 0.05$) for the majority of the *Bacteroides* ssp. in comparison to control cells that were exposed to only 10 % (v/v) of culture medium. However, the viability of HT29-MTX-E12 cells exposed to culture medium supplemented with 10 % (v/v) of the bacterial culture supernatant of *B. dorei* PtF D1P5 and PtF Sb6 was the most affected showing a percentage of viable cells of 75.54 ± 5.3 % and 84.50 ± 8.5 %, respectively. At percentages of 25 % (v/v) of the supernatant from *B. dorei* PtF Sb6, *B. uniformis* PtF Sb3P5 and *P. distasonis* PtF D14MH1 the impaired viability of the intestinal cells was evident achieving 54.86 ± 9.01 %, 66.42 ± 10.37 % and 50.07 ± 2.25 %, respectively (**Table 4.4.1**). The viability of the intestinal epithelial cells was similar ($P > 0.05$) for the remaining samples. The viability of HT29-MTX-E12 cells exposed to 50 % (v/v)of the supernatant from *B. dorei* PtF D16P1, PtF Sb6 and *P. distasonis* PtF D14MH1 was affected, namely the values observed were 48.42 ± 6.15 %, 40.60 ± 5.92 % and 52,36 ± 2,37 %, respectively (**Table 4.4.1**).

The increase of the control medium to 25 and 50 % (v/v) significantly diminished the viability of HT29-MTX-E12 cells (**Table 4.4.1**). The same was observed for some of the supernatant samples of the bacterial cultures, namely the supernatant of *B. dorei* PtF D16P1 at 50 % (v/v) ($P < 0.05$) and the supernatant of *B. dorei* PtF Sb6, PtF Sb8, *B. uniformis* PtF D3Pch2, PtF Sb3P5 and *P. distasonis* PtF D14MH1 at 25 and 50 % (v/v) ($P < 0.05$) (**Table 4.4.1**).

Table 4.4.1 Viability of the HT29-MTX-E12 cells when exposed to different percentages of the supernatant of the different *Bacteroides* strains. The addition of only bacterial culture medium (BHI) at the concentrations tested was used as control metabolites

Bacteroides	Strain	Supernatant (%, v/v)*		
		10	25	50
	Control	$90.83 \pm 11.55^{a,A}$	$87.23 \pm 12.57^{b,A}$	$79.28 \pm 10.29^{b,c,A}$
B. dorei	DSM 17855	$82.02 \pm 4.80^{a,A}$	$83.53 \pm 7.14^{a,A}$	$86.58 \pm 4.11^{a,A}$
	PTF D1P5	$75.54 \pm 5.3^{a,B}$	$78.03 \pm 4.13^{a,A}$	$82.62 \pm 8.04^{a,A}$
	PTF D8M1	$85.17 \pm 13.34^{a,A}$	$96.24 \pm 11.29^{a,A}$	$83.58 \pm 8.51^{a,A}$
	PTF D16M14	$82.32 \pm 7.6^{a,A}$	$89.57 \pm 7.34^{a,A}$	$73.82 \pm 7.17^{a,A}$
	PTF D16P1	$84.50 \pm 8.5^{a,A}$	$93.00 \pm 8.68^{a,A}$	$48.42 \pm 6.15^{b,B}$
	PTF Sb6	$72.59 \pm 3.77^{a,B}$	$54.86 \pm 9.01^{b,B}$	$40.60 \pm 5.92^{b,B}$
	PTF Sb8	$87.26 \pm 8.9^{a,A}$	$74.96 \pm 7.50^{b,A}$	$68.87 \pm 4.26^{b,A}$
	PTF C1P2	$85.42 \pm 13.95^{a,A}$	$83.28 \pm 9.31^{a,A}$	$78.92 \pm 6.71^{b,A}$
B. uniformis	PTF D3Pch2	$91.77 \pm 2.63^{a,A}$	$73.99 \pm 8.34^{b,B}$	$70.04 \pm 4.68^{b,A}$
	PTF Sb3P5	$87.90 \pm 7.19^{a,A}$	$66.42 \pm 10.37^{b,B}$	$65.88 \pm 4.50^{b,A}$
P. distasonis	PTF D14MH1	$79.06 \pm 2.05^{a,A}$	$50.07 \pm 2.25^{b,B}$	$52.36 \pm 2.37^{b,B}$

*Data represent the mean ± standard deviation. Data with the same lowercase are not statistically different across the different concentrations of metabolites of the same isolate ($P > 0.05$). Data with the same capital letter are not statistically different compared to the control within the same concentration of metabolites ($P > 0.05$). (n=12).

4.5. The impact of *Lactobacillus casei* on the invasion of *Bacteroides* spp.

Children with T1D from Algarve region showed significantly less *Lactobacillus* spp. in comparison to Control children (Matos, 2018). This fact may influence the invasive capacity of *Bacteroides* spp. To evaluate the possible effect of *Lactobacillus* on internalization of *Bacteroides* spp. representative isolates of the different invasive groups were selected, in particular *P. distasonis* PtF D14MH1 as representative of the highest invasive group, *B. dorei* PtF D16P1 and PtF Sb8 as representative of the intermediate group and as representative of the lowest invasive group *B. dorei* PtF D1P5.

The results of the impact of *L. casei* DSM 20011 on the invasion ability of the selected strains of *B. dorei* and *P. distasonis* PtF D14MH1 is summarized in **Table 4.5.1**.

In the presence of *L. casei* DSM 20011 there was a significant ($P < 0.05$) decrease in the invasion ability for all strains, except for *B. dorei* PtF D16P1 (**Table 4.5.1**) The isolate *B. dorei* PtF Sb8 showed the smallest difference in the reduction of the invasive capacity in the presence of *L. casei* achieving a decrease of approximately 25 %. *B. dorei* PtF D1P5 and *P. distasonis* PTF D14MH1 showed a decrease in invasive capacity in the presence of *L. casei* of approximately 52 % and 72 %, respectively.

Table 4.5.1 – Effect of *Lactobacillus casei* DSM 20011 on the invasion ability of the selected strains of *B. dorei* and *P. distasonis*. Invasion capacity is expressed as percentage. In order to facilitate the analysis of the data the results of the invasion of the strains in the absence of *L. casei* are here included (from **Table 4.2.1**)

Bacteria		**Invasion (%)***	
Species	**Strain**	In the absence of *L. casei*	In the presence of *L. casei*
B. dorei	PtF D1P5	70.11 ± 5.66^{a}	33.74 ± 4.75^{b}
	PtF D16P1	85.25 ± 5.42^{a}	77.09 ± 4.87^{a}
	PtF Sb8	82.72 ± 1.58^{a}	61.67 ± 6.86^{b}
P. distasonis	PtF D14Mh1	97.38 ± 1.62^{a}	27.38 ± 4.72^{b}

*Data represent the mean±standard deviation. Data with the same lowercase are not significantly different ($P > 0.05$). (n=6)

4.6. Impact of *Bacteroides* spp. on cytokine production

The evaluation of the cytokines production by the intestinal epithelial cells after exposure to *Bacteroides* spp. was performed to evaluate the potential immunological response. For this purpose, six strains were selected: *B. dorei* PtF D1P5 and *B. dorei* PtF C1P2 both showed a low invasive capacity, as well as one of them was isolated from a T1D child (*B. dorei* PtF D1P5) and the other was isolated from a Control (*B. dorei* PtF C1P2). *B. dorei* PTF Sb6 was selected as it presents an intermediate invasive capacity and was isolated from a healthy sibling. *B. dorei* PtF D16P1 showed a high invasive capacity and was isolated from a T1D child. *B. uniformis* PtF D3Pch2 was included as it showed no-invasion ability and was isolated from a T1D child. *B. dorei* DSM 17855 was tested as reference strain.

The system used (**section 3.11**) allowed to evaluate the production of 12 cytokines that are microbial induced (TNFα, IL-1β, IL-6, IL-12, IL-17A, IL-8, MCP-1, RANTES, MIP-1α, MIP-1β, MDC and Eotaxin). The results are summarized in **Table 4.6.1**. It was observed that only two cytokines revealed signal after exposure the intestinal cells to the *Bacteroides* isolates. *B. dorei* PTF D1P5 induced the lowest ($P < 0.05$) IL-8 production in contrast with *B. dorei* PtF Sb6 and *B. uniformis* PtF D3Pch2 that showed the highest ($P < 0.05$) induction. The strains *B. dorei* PtF D16P1, PtF C1P2 showed an induction of IL-8 similar ($P > 0.05$) to the reference strain *B. dorei* DSM 17855. However, all the IL-8 induced values with the tested strains were lower ($P < 0.05$) in comparison to the control of the reaction (**Table 4.6.1**). No significantly differences ($P > 0.05$) between the tested strains was observed for the induction of the MIP-1β cytokine and all observed values were lower ($P < 0.05$) in comparison to the positive control of the reaction.

Table 4.6.1 - Cytokine production by intestinal epithelial cells after exposure to the *Bacteroides* isolates.

Bacteroides spp.	Strain	IL-8	MIP-1β
B. dorei	DSM 17855	0.70 ± 0.33[a,b]	0.23 ± 0.00[a]
	PtF D1P5	0.34 ± 0.04[a]	0.21 ± 0.01[a]
	PtF D16P1	0.61 ± 0.01[a,b]	0.21 ± 0.01[a]
	PtF Sb6	0.97 ± 0.89[b]	0.35 ± 0.01[a]
	PtF C1P2	0.59 ± 0.29[a,b]	0.34 ± 0.01[a]
B. uniformis	PtF D3Pch2	0.81 ± 0.03[b]	0.36 ± 0.00[a]
Control	Positive Control	2.34 ± 0.03[c]	1.08 ± 0.45[b]

Data represent the mean ± standard deviation. Data with the same lowercase in the column are not statistically different ($P > 0.05$).

5. Discussion

Our study aimed to evaluate the interactions of *Bacteroides* spp. isolated from the feces of children with T1D or healthy children with intestinal epithelial cells, namely HT29-MTX-E12. *In vitro* cell culture models have been used to study interactions between bacteria and epithelial cells. The selection and development of the HT29-MTX-E12 cell line after a 21 day differentiation demonstrated increased trans-epithelial electrical resistance, which is indicative of tight junction formation and cell polarization (Dolan et al., 2012). This cell line has been shown to differentiate into mature mucus-secreting cells which simulate the environment of the human colon allowing the evaluation of bacterial interactions with its host intestinal cells in a more reliable way simulating the human intestinal melieu (Dolan et al., 2012; Ponce de León-Rodríguez et al., 2018).

The studies on the interactions of *B. dorei* with intestinal epithelial cells are very limited. At our best knowledge the current study is the first report on these interactions.

Microbial adhesion to hosts cells is the first step in colonization of the gut (Sicard et al., 2017). The intestinal lumen is in constant movement making the colonization of the intestines only possible if the bacteria are able to adhere to the mucus coating the epithelial intestinal cells. Bacteria usually adhere to mucins which can lead to the growth of microcolonies that can develop further in biofilms (Sicard et al., 2017). In the current study all *Bacteroides* isolates showed to be able to adhere to the mucus secreting cells, HT29-MTX-E12. Furthermore no differences in adhesion ability were found correlated with the origin of the isolate or the *Bacteroides* species (*B. dorei*, *B. uniformis* or *P. distasonis*). It is know that the species *B. thetaiotamicron* and *B. fragilis* are able to adhere to mucus secreting and non mucus secreting intestinal epithelial cells and even are able to adhere to highly purified mucins (Ferreira et al., 2002; Huang et al., 2011; Altamimi et al., 2016). The mechanism by which the *Bacteroides* spp. adhere to the intestinal epithelial cells still remains unclear. However, there are some studies that have demonstrated that some species from the order Bacteroidales secrete antimicrobial proteins (BSAPs) which contain membrane attack complex / perfornin helping the gut bacteria effectively compete with BSAP-sensitive strains allowing BSAPs-resisting bacteria to adhere and colonize the human gut. The presence of BSAPs was reported in *B. uniformis* and also reported in *B. fragilis*

(Roelofs et al., 2016). Isolates of *Bacteroidetes* collected from adults with Chron's disease have been shown to possess PepD, a protein that is involved in human gut adhesion and colonization (Juste et al., 2014). There are other proteins that may be linked to the adhesion ability of *Bacteroides* ssp. to intestinal epithelial cells, namely Natarajan et al., 2015 reported that *B. vulgatus* and *B. fragilis* carry proteins with N-terminal domains BVU_4064 and BF1687 that have been associated with interaction of these *Bacteroides* species with host cell surfaces allowing their adhesion. Although further studies are required to determine the mechanisms by which our tested *Bacteroides* species were able to adhere it is entirely possible that *B. dorei* also possess these domains since shares a great number of characteristics with *B. vulgatus* (Bakir et al., 2006).

After adherence to the host's cells, pathogenic bacteria usually will disrupt normal cell function and then disturb or rearrange the cell cytoskeleton (Sicard et al., 2017). Proteins in the bacterial cell surface may interact with the host's protein receptors affecting signal transduction within the cell (Ribet et al., 2015). In the present study only the strains of *B. dorei* and *P. distasonis* were able to invade HT29-MTX-E12 in contrast with *B. uniformis* that showed no ability to invade these intestinal cells. As in the adherence assay, also in the invasion assay no differences were observed regarding the origin of the isolate. Although the mechanism by which *Bacteroides* spp. are capable to invade the intestinal epithelial cells the mechanisms used by known pathogenic bacteria have been described (Ribet et al., 2015). For example, *Salmonella* Thyphimurium, a Gram negative bacteria that can cause severe gastroenteritis (Gagnon et al., 2013), can invade intestinal epithelial cells by inducing its internalization into non-phagocytic cells via a trigger mechanism. Using this mechanism *Salmonella* Thyphimurium directly injects in the host cell cytoplasm a set of sophisticated bacterial effectors that activate cellular responses. Some of these effectors activate host cells Rho GTPases that stimulate actin cytoskeleton rearrangements that allow membrane ruffling and posteriorly the internalization of the bacteria (Ribet et al., 2015).

Shigella flexneri¸ another Gram negative bacteria that causes bacilliary dysentery, was found to get in contact with the epithelial cell layer via filopodial-like extensions emanating from the hosts cells (Romero et al., 2011). Upon this contact these extensions retract and bring *S. flexneri* in contact with the cell body where invasion occurs in a Zipper like mode (Ribet et al., 2015). Although these two intestinal pathogens invade using a trigger and zipper mechanism, respectively, to invade

intestinal epithelial cells, one given pathogen can be internalized by different cell types and express different sets of virulence genes which can lead to different diseases progressions (Ribet et al., 2015).

The exposure of infected HT29-MTX-E12 cells to the tested *Bacteroides* spp. showed that these species are able to disrupt the tight junction complexes. Visualization of the tight junction disruptions was consistent with the invasion ability of the *Bacteroides* isolates. The impact of *B. uniformis* on the tight disruption was minor in comparison with the *B. dorei* PtF D16P1. This correspondence, between the damaged sustained by the intestinal epithelial cells after exposure to the bacteria and the invasion ability of the bacteria, was also found in an interaction study with *Salmonella* (Gagnon et al., 2013). The mechanism by which *Bacteroides* spp. can damage the tight junctions is not acknowledge. However, it is known that *B. fragilis* is able to produce izoenzimes that can weaken cell-cell contacts (apical junction complex that encloses tight junctions) (Bischoff et al., 2014).

Exposure of intestinal epithelial cells to the metabolome of *Bacteroides* spp. caused a decreased on the viability of the HT29-MTX-E12 cells. *B. dorei*, *B. uniformis* and *B. distasonis* (presently *P. distasonis)* share several metabolic characteristics, such as acid production from, for example, maltose, mannose and xylose, as well as enzymatic activities of α and β galactosidase, α and β glucosidase, α -arabinosidase and glutamyl glutamic acid arylamidase (Song et al., 2015). *P. distasonis* also has the ability to metabolize more sugars than the other two species, which in turn results in the production of a greater diversity of acids in comparison with *B. dorei* and *B. uniformis*. The majority of the metabolic products of *P. distasonis* and *B. uniformis* are acetic acid, succinic acid and propionic acid (Song et al., 2015). These acids alone are not considered to be very toxic. However, at high doses, acetic acid has been shown to decrease the viability of cervical cancer cells and malignant fibroblasts, both from humans, as well as to induce cell death in rat gastric epithelial cells (Okabe et al., 2014). The American Centre for Disease Control (CDC, 2019) reported that propionic acid at 45 mmol / L / 24h is lethal for liver tumor cells, as well as impairs human skin cells at 0.08 mL / well / 3M. It is expected that the tested bacterial supernatant will not have such high content on these acids, so the probable cause of the decreased cell viability after exposure of the intestinal epithelial cells to the bacterial supernatant will be due to a combination of specific metabolic products and their content since the increase of the

percentage of the supernatant resulted in a lower viability of the intestinal epithelial cells.

The potential immunological response by the cell line HT29-MTX-E12 after exposure to the tested *Bacteroides* spp. was examined by testing 12 common cytokines that are microbial induced. The results showed that only IL-8 and MIP-1β were induced by the tested *Bacteroides* isolates. IL-8 is a cytokine that mediates the inflammatory response that guides the neutrophils to the site of infection. *E. coli* LF82 has been shown to induce the production of this chemoatractant and consequently lead to a defective mucus layer (Elatrech et al., 2015). The other cytokine found to be produced by the cells in the presence of the our isolates was MIP-1β. This molecule acts as a chemoatractant for natural killer cells and T cells. It is important to highlight that the induction of the mentioned cytokines was slight. The absence of the induction of the other cytokines may be of some mechanisms, like the one found in *B. fragilis* since it was reported that this specie can suppress some immune responses (Mazmanian et al., 1976). The cytokines IL-6 and TNF-α, were not induced by *Bacteroides* spp. isolates alone but should be considered in further studies testing the combination of *Bacteroides* spp. with *L. casei.* These cytokines have been shown to induce the production of mucus (Enss et al. 2000) and since the visualization of the cell tight junctions after exposure to *Bacteroides* spp. and *L. casei* was not possible due to a high amount of mucus production, it would be of interest to identify if these cytokines are involved in this phenomenon.

The children with T1D from Algarve are depleted of *Lactobacillus* spp. (Matos, 2018) a finding that was also reported for Spanish children with T1D (Murri et al., 2013).

The decrease in invasion ability of *Bacteroides* spp. in the presence of *L. casei* was prominent, just the strain *B. dorei* PtF D16 P1 maintained the ability to invade in the presence of *L. casei* and the most noticeable reduction on the ability to invade HT29-MTX-E12 cells (3.5 x less) was observed with *P. distasonis* PtF D14MH1. The genus *Lactobacillus* has been shown to protect the integrity of the intestinal epithelial barrier (Yu et al., 2015). Our finding is in accordance with previously published studies that reported the ability of *L. casei* to inhibit the internalization of *Staphylococcus aureus* into bovine mammary epithelial cells (Bouchard et al., 2013). It was also reported that the specie *L. fructosus* can inhibit the adherence of *E. coli* and *S.* Thyphimurium as well as diminish immunological responses (decreased IL-8

production) (Yu et al., 2015). Our data and others show that *Lactobacillus* are able to inhibit the bacterial invasion. This same study (Yu et al., 2015) also showed a reduction on the damaged to the tight junctions when the cells were exposed to *E. coli* and *S.* Thyphimurium, in the presence of *L. fructosus*. Despite in our current study we were not able to properly observe the tight junctions of the intestinal epithelial cells exposed to both *L. casei* and *Bacteroides* spp. it was noted an increased production of mucus as stated before. There is no reports about the interactions between *L. casei* and *Bacteroides* spp. but based on previous studies with other bacteria it is reasonable to anticipate that *L. casei* might hamper the damage caused by *Bacteroides* spp to the intestinal epithelial.

6. Conclusion

In this study the interactions of *Bacteroides* spp. isolated from T1D and Control children and with the intestinal epithelial cells, HT29-MTX-E12 was evaluated. Regarding the adhesion ability all *Bacteroides* spp. isolates were able to adhere to HT29-MTX-E12 cells and this ability did not allowed to distinguish the strains according to their origin.

The results of the invasion ability of *Bacteroides* spp. isolates evidenced differences between the tested species, namely *B. uniformis* showed no capacity to invade the HT29-MTX-E12, in contrast the *B. dorei* strains showed different ability to invade these intestinal epithelial cells. However, it was not possible to discriminate the *B. dorei* isolates according to the origin. The isolate *P. distasonis* PtF D14MH1 showed the highest invasive capacity.

The evaluation of the impact of the exposure of the the HT29-MTX-E12 cells to *Bacteroides* spp. showed that the strains of *B. dorei* and *P. distasonis* isolates caused a noticeable disruption of the tight junctions. In contrast the tight junctions of the intestinal epithelial cells exposed to *B. uniformis* isolates were slightly impaired.

The HT29-MTX-E12 cells exposed to the bacterial supernatant at different concentrations caused a significant decrease on their viability . The greater impact on the HT29-MTX-E12 viability was more evident when the cell medium was supplemented with 25 and 50% (v/v) of bacterial supernatant.

The invasion approach using the combination of *L. casei* with the *B. dorei* strains and *P. distasonis* showed that the presence of *L. casei* significantly reduced the invasion ability of these strains, except for *B. dorei* PtF D16P1.

The analysis of the induction on cytokines production by the HT29-MTX-E12 cells exposed to the *Bacteroides* spp. showed that the tested strains did not induce a noticeable cytokine production.

In conclusion the results of the present study evidence that *B. dorei* and *P. distasonis* strains are able to invade the intestinal epithelial cells and disrupt the tight junctions. However, the presence of *L. casei* limits their invasion capacity. This finding coupled with the reported early that children with T1D from Algarve are depleted of *Lactobacillus* spp. evidences that this bacterial unbalance may contribute to dysbiosis.

7. Future perspectives

Although the present study showed the damage that *Bacteroides* spp. can cause to the tight junctions of intestinal epithelial cells, the method used by staining the tight junctions and visualization on the microscope, was merely qualitative. A Transepithelial electrical resistence (TER) study on the intestinal epithelial cells during the exposure to *Bacteroides* spp. isolates will provide a more quantitative view on the damage caused by these bacteria.

To the best of our knowledge the current study is the first one that has examined the impact of the metabolites produced by *B. dorei, B. uniformis* and *P. distasonis* on the viability of the intestinal epithelial cells. In order to identify the possible metabolites that had affected the viability of the HT29-MTX-E12 it will be useful the use of High Performance Liquid Chromatography (HPLC) or Liquid Chromatography-Mass Spectrophotometry (LC-MS).

The insights on the interactions of these bacteria with the human gut will contribute to develop approaches to restore and improve the gut microbiota of these children that at long term will suffer from comorbidities of T1D that largely impact their health.

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