JOSÉ JOAQUIM SOARES CURADO DE MATOS

BACTEROIDES SPECIES IN CHILDREN WITH TYPE 1 DIABETES

Faculdade de Ciências e Tecnologia
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Mestrado em Biologia Molecular e Microbiana
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Leonor Faleiro

Faculdade de Ciências e Tecnologia
2018

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Declaração de autoria de trabalho

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

“And once the storm is over, you won’t remember how you made it through, how you managed to survive. You won’t even be sure, whether the storm is really over. But one thing is certain. When you come out of the storm, you won’t be the same person who walked in. That’s what this storm’s all about.”

—Haruki Murakami

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Abstract

Type 1 diabetes *mellitus* is an autoimmune disease, characterized by the destruction of the pancreatic beta cells leading to insufficient insulin production. Gut dysbiosis, which is associated with unbalanced diversity of microbiota has been linked with type 1 diabetes (T1D).

High population levels of bacteria that belong to the phylum *Bacteroidetes* have been reported in children with T1D, in contrast to healthy children which show higher levels of *Firmicutes*. In Finnish children, particularly from the city of Turku, at early-onset of seroconversion it was reported a predominance of *Bacteroides dorei* in their guts.

The main objective of this study was to analyse the *Bacteroides* population of children with T1D from the Algarve region. To achieve this the *Bacteroides* spp., including *B. dorei* were isolated and quantified in faecal samples collected from children with established T1D and control children including their healthy siblings. The isolates of *B. dorei, B. uniformis* and *B. xylanisolvens* were genotyped by rep-PCR. The levels of *Firmicutes, Bacteroidetes, Bifidobacterium* spp. and *Lactobacillus* spp. were determined by real time PCR. Despite the low amount of *B. dorei* and *Lactobacillus* found in the Algarve children, there is a similarity between the children in the study groups, although there is some difference in the amount of *Lactobacillus*.

Key words: Diabetes *mellitus* type 1, microbiota, *Bacteroides* spp., *Bacteroides dorei*, children

Resumo

A diabetes é uma doença crónica, cuja sua principal característica é a deficiência na produção ou atividade 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 tratamento da doença não forem realizados atempadamente, 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* gestacional, ocorre unicamente durante a gestação, adquirindo as mesmas características da diabetes tipo 2 sendo que logo após o nascimento do bebé, os problemas associados à doença desvanecem. A diabetes *mellitus* tipo 2 surge maioritariamente numa idade mais adulta e tem, como característica principal, a resistência das células à ação da insulina, na qual se verifica uma diminuição da resposta dos recetores da glicose. Esta ocorre geralmente em pessoas com excesso de peso ou numa idade mais avançada. 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 disbiose leva ao aparecimento de respostas inflamatórias que têm sido associadas a diferentes patologias como a síndrome do intestino inflamado e até mesmo o cancro do colon e reto. Por conseguinte é de todo o interesse académico estudar as alterações da microbiota intestinal e as suas interações com o hospedeiro.

A microbiota intestinal das crianças com DT1 tem despertado muito interesse não só devido ao aumento de casos em crianças cada vez mais novas, bem como ao aumento de relatos de uma associação entre a doença e a própria microbiota. Tem sido reportado 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* Nas crianças Finlandesas, da cidade de Turku em risco de desenvolverem a DT1 foi reportado a

predominância de *Bacteroides dorei* no seu intestino. Contudo o seu papel no desenvolvimento da doença ou na sua manutenção não foi ainda esclarecido.

As bactérias do género *Bacteroides* são Gram negativas, anaeróbias, mutualistas que existem em grandes quantidades no intestino humano. São muito importantes 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, por parte destas bactérias, advém da fermentação de uma grande variedade de açúcares, estando também associadas ao mecanismo de degradação do glúten. A bactéria *B. dorei* foi descrita apenas no ano de 2006, tendo sido considerada até então na espécie *B. vulgatus*, devido a toda a sua similaridade morfológica e metabólica. Este trabalho teve como principal objetivo identificar, caracterizar e quantificar as bactérias do género *Bacteroides* spp., incluindo *B. dorei* em amostras fecais de crianças com DT1,bem como em crianças saudáveis, incluindo os seus irmãos saudáveis residentes na zona do Algarve. A análise das relações filogenéticas dos isolados de Bacteroides spp foi realizada por rep-PCR. A avaliação das populações de *Firmicutes*, *Bacteroidetes*, *Bifidobacterium* spp. e *Lactobacillus* spp. foram realizadas através de qPCR.

Neste estudo foram recolhidas amostras de 17 crianças com diabetes tipo 1 e 17 crianças controlo, das quais 10 provieram de irmãos saudáveis de crianças com DT1

O isolamento das bactérias pertencentes ao género *Bacteroides* foi realizado com a utilização do meio de cultura *Bacteroides Vulgatus* Selective Agar, as colónias caracteristicas foram selecionadas e transferidas para o meio de cultura Brain Heart Infusion suplementado com hemina (0,1%, p/v) onde se procedeu à sua purificação. A identificação bacteriana foi realizada através da sequenciação do gene *16S rRNA* que foi amplificado com os primers *27F* e *1492R*. Para além de B. dorei foram isoladas outras espécies, nomeadamente *B. xylanisolvens, B. thetaiotaomicron, B. vulgatus* e *B. ovatus*. Isolados *Parabacteroides distasonis*, foram encontrados dois grupos de crianças, mas com maior frequência em crianças com DT1.

A análise dos elementos BOX conseguiu discriminar melhor os isolados por espécie em comparação com a análise dos elementos ERIC. Contudo análise de agrupamento dos elementos BOX ou ERIC não diferenciou nenhum dos isolados de *Bacteroides* spp. de acordo com a origem das amostras.

Palavras chave: Diabetes *mellitus* tipo 1, microbiota, *Bacteroides* spp., *Bacteroides dorei*, crianças

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Glossary

BC – Before Christ

***Bft* –** *Bacteroides fragilis* toxin

BHI – Brain heart infusion medium

BHI+H – Brain heart infusion plus hemin medium

BVSA – Bacteroides vulgates selective media

DNA – Deoxyribonucleic acid

gDNA – genomic Deoxyribonucleic acid

LB – Luria Broth medium

PBS – Phosphate buffer saline

PCR – Polimerase chain reaction

PS A – Capsular polysaccharide PS A

qPCR – Quatitative polimerase chain reaction

T1D – Type 1 diabetes

1. Introduction

1.1. Diabetes *mellitus*

1.1.1. Historical context of diabetes

The first reference given to the diabetes disease dates back to the Ebers papyrus of the year 1500 BC, being characterized by extreme urge to urinate and it was possible to be treated by plants (Ghalioungui, 1987). Although recent studies already consider that there is a description prior to this, in the Kahun papyrus of 2000 AC this only had the title of "Treatment of a thirsty woman" but due to the lack of text and details in any of the papyri, it cannot be affirmed that the ancient Egyptians are referring to diabetes (Ghalioungui, 1987). In the fifth century BC a famous Indian named Sushruta, in his published work, Samhita, describes diabetes as madhumeha, *i.e.* honey-like urine. Indicating already some symptoms, of which the sweet taste of urine as well as grunting (Peumery, 1987). In the second century BC, a Greek physician named Aretaus Cappadocian, was the first person to give a detailed clinical description of the disease, indicated that diabetes provoked extreme thirst polyuria of the human body being able to kill (Lakhtakia, 2010). The first name to be accepted clinically was *diarrhea of the urine*, later recognized only by diabetes. Despite the studies all performed by him, he stated that the intake of cereals, milk and wine helped in the cure treatment. Around the year 160-219 in ancient China, they already referred to diabetes as a disease polyuria, polydipsia and weight loss (Peumery, 1987), and in the seventh century AC has already added a few more symptoms and "treatments" possible for its cure. (Savona-Ventura, 2002) In the 8 century several physicists observed the tendency of development of infections in the skin like boils, ulcers and problems in the cornea (Lakhtakia, 2010).

In the 11th century an Arab scholar, Avicenna in his book (El-Kanin) described the diabetes and mentions some complications, such as gangrene and sexual dysfunction. A few years later Moises Maimonides described in more detail the diabetes including the symptom of acidosis. (Savona-Ventura, 2002).

The English physician Thomas Willis (1621-1675) re-introduced the concept of honey-like urine, previously described by Sushruta (Furdell, 2009), and hence began to call this type of diabetes, diabetes *mellitus* (honey) (Lakhtakia, 2010). Johann Peter Frank (1745-1821) identified the differences between diabetes *mellitus* and diabetes *insipidus.* (Nabipour, 2003). In 1776, Matthew Dobson confirmed that the honey taste he had once identified came from a type of sugar that it is in excess in urine and blood. (Dobson, 1968). In the early

nineteenth century scientists began to hypothesize that pancreas may have some role in the pathophysiology of the disease (Lakhtakia, 2010). Starting from this principle, Claude Bernard (1812-1878) start to investigate and discovered that there was a glycogen action in the liver tissues, causing the glucose to not be eliminated. This study began to open doors for the discovery of the nature of this disease (Holmes, 1997). Interested in the studies of Claude Bernard, Joseph von Mering (1849-1908) and Oskar Minkowski (1858-1931), who discovered in 1889 the importance of the pancreas in the disease, for which they tested on dogs the excision of pancreas and as a result all developed the symptoms of diabetes and died within a short time (Holmes, 1997).

In 1910, Sir Edward Albert Sharpey-Schafer began to argue that the problem associated with the pancreas was due to a deficiency in a single chemical produced by it. He named this insulin substance, the Latin insula, meaning island, and in order to refer to the islets of Langerhans existing in the pancreas and insulin producers (Himsworth, 2011). In 1923 Frederick Banting (1891-1941), Charles Best (1899-1978) and John Macleod (1876-1935) finally discovered human insulin. Only Frederick Banting and John Macleod have won the Nobel Prize for Medicine in 1923 (Karamanou, 2016). The distinction of the two types of diabetes, type 1 diabetes and type 2 was established by Sir Harold Percival Himsworth in January 1936 (Karamanou, 2016).

1.1.2. Definition, Causes and Consequences

According to the World Health Organization (WHO) Diabetes is a chronic disease that occurs when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces (WHO, 2017). Insulin is a hormone that regulates the levels of the sugar in the blood (Park *et al.*, 2018). Hyperglycaemia, or raised blood sugar, is a common effect of uncontrolled diabetes and over time leads to serious damage to many body's systems, especially the nerves and blood vessels. For several decades several attempts to discover and associate the true causes of this disease have been done, but to date they are still to be elucidated (Villanueva-Millán, *et al.*, 2015). It is known that the genetic factor is quite present and is pointed out as one of the main causes for the onset of the disease (Davis-Richardson *et al.*, 2014), that is, there may be a genetic predisposition of the person for the malfunction of β cells of the pancreas, MODY: ”Maturity onset diabetes of the young”, or even genetic defects (e.g., some mutation of the gene that decreases insulin production) or its

activity (for example, misrecognition of the insulin molecule in β- cells of the pancreas), but it is not only due to genetic factors, such as exocrine pancreatic diseases (pancreatitis, neoplasia, hemochromatosis, cystic fibrosis, etc.) or by some type of induced defects or chemicals (diuretics, corticoids, beta-blockers, contraceptives, etc.) are associated with of the disease (WHO 2017).

Diabetes besides being a disease for which the triggers of the disease are still to be identified the co-morbities are plenty, identified as being several organs affected, such as the heart, eyes, kidneys, blood vessels and nerves. In diabetes, the risk of having some type of cardiovascular disease or strokes (myocardium, brain) is greatly increased. There is also the possibility of having other types of cardiovascular diseases, as angina or arteriosclerosis. Due to the decrease in blood flow to the extremities of the body, especially in the posterior zone, there is the possibility of the development of gangrene, better known as diabetic foot disease, if not treated in due time can have very serious consequences on physical and psychological health of the patient. In addition to poor circulation, the high levels of blood sugar, causes neuropathies, nephropathy, retinopathies, which is due to the fact that in the smaller capillaries the sugar causes damages in the wall, deficiency of the nutrition of neuronal cells, the liver or retina, leading to serious co-morbidities. (WHO 2017).

1.1.3. Diagnosis and types of diabetes

The method of diagnosis of diabetes is related to the level of glucose in the blood as well as the presence or absence of symptoms characteristic of the disease. **Table 1.1.1** lists the diabetes assessment criteria described by the general direction of health (Direção geral da saúde 002/2011).

Table 1.1.1. Evaluation criteria of diabetes *mellitus* (adapted from (Direção geral da saúde 002/2011).

Criteria	Evaluation
1	The value of fasting blood glucose must be ≥ 126 mg / dl (or ≥ 7.0 mmol / l
2	Classical symptoms of disease and glycemia occasionally greater than ≥ 200 mg / dl (or ≥ 11.1 mmol / l
3	The glycemia value ≥ 200 mg / dl (or ≥ 11.1 mmol / l) 2 hours after the start of the oral glucose tolerance test (OCTG) with 75 g of glucose
4	Glycated hemoglobin A1c (HbA1c) ≥ 6.5%

The main diagnostic symptoms of diabetes *mellitus* are polyuria (i.e. a large need to urinate) polyphagia (i.e. a large increase in appetite) and polydipsia (i.e. increased thirst and fluid intake). There are also other symptoms, such as tiredness, weight loss, blurred vision. There is also the possibility of some kind of infections, such as urinary tract infections, candidiasis as well as the appearance of diabetic ketoacidosis (Portal da Saúde and Banco da Saúde, 2012).

There are 3 types of diabetes *mellitus* type 1, type 2 and gestational. It has recently been suggested by De La Monte (2008) that there is a "type 3" of diabetes *mellitus*.

Type 2 diabetes is the most common type found in about 90 to 95% of cases diagnosed with this type of disease. It arises essentially in people with a more advanced age but the number of cases has been diminishing, however the number of cases in children and adolescents, due to their bad eating habits is increasing (Portal da Saúde and Banco da Saúde, 2012). This type of diabetes is characterized by a decrease in glucose-insulin receptors, which consequently leads to insulin resistance, causing more insulin to be produced. If not treated in time, it can lead to the failure of β-cells in the pancreas causing serious problems similar to the type 1 diabetes or worse since the time of diagnosis in most cases is delayed (Portal da Saúde and Banco da Saúde, 2012).

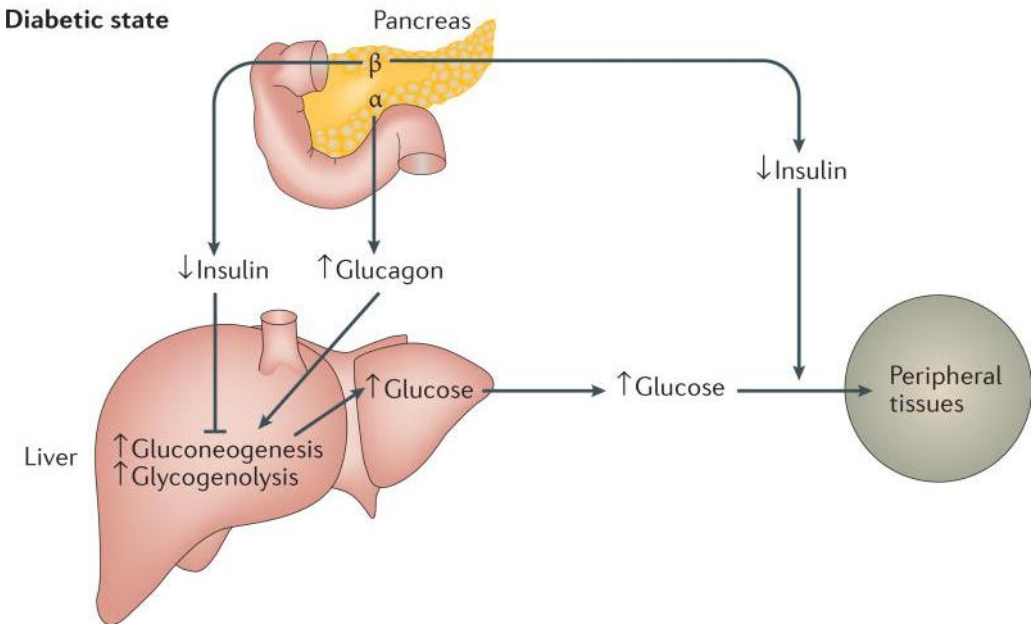


Figure 1.1.1 – Schematic of type 2 diabetes *mellitus.* Type 2 diabetes develops by reduce of insulin action than leaves the glucose mechanism without a effect control (Source: adapted from (Rines *et al.*, 2016))

Gestational diabetes *mellitus* shares some similarities with type 2 diabetes. This result from the insufficient amount of insulin produced for the needs of the body. This type is diagnosed during pregnancy and may disappear soon after childbirth (about 90%), but in some cases the disease may progress to type 2 or type 1. This type of diabetes occurs in about 3-9% of pregnancies and can lead to some types of consequences if not treated in time for both the mother and the baby, such as the mother may develop type 2 diabetes, the need for caesarean section, complications during childbirth, among others and for the child, high birth weight, abnormalities in the kidneys, heart or spine, possible development of type 1 diabetes (Portal da Saúde & Banco da Saúde, 2012).

Another type recently described is "type 3 diabetes", which has been related to Alzheimer's disease evidencing insulin resistance in the brain. Studies by a research team from the Brown University's Warren Alpert Medical School proposed the possibility of a new form of diabetes after discovering that insulin resistance may occur in the brain (De La Monte, 2008). The researchers described that insulin resistance as well as insulin rise as being a key part of the progression of Alzheimer's disease. The authors exclude the Alzheimer's patients with type 1 or type 2 diabetes in order to characterized type 3 diabetes. However also found that both patients with type 2 diabetes and with Alzheimer's disease have a deposition of a protein (beta amyloid) in the pancreas and as well as in the brain (De La Monte, 2008).

Type 1 diabetes *mellitus,* also known as insulin-dependent diabetes it is one of the most well-known autoimmune diseases and is characterized by lymphocytic infiltration or inflammation of pancreatic islets, also known as insulitis (Lee, 2011).

It is not known which mechanism drive the disease, but it is known to have a great genetic predisposition in its essence, but several other environmental factors have to be associated with the disease, such as intestinal microbiota, diet and stress (Vaarala, 2008; Atkinson and Neu, 2008; Davis-Richardson *et al.*, 2014).

The vast majority of people, who have this disease, display the destruction of the beta cells of the pancreatic islets, that is driven by T cells. Thus only when 90% of the pancreatic beta cells are destroyed the disease begins to manifest (Craig, 2009).

Serologic markers of an autoimmune pathological process, including islet anti-cell antibodies, such as, GAD, IA-2, IA-2α or anti-insulin autoantibodies (IAA) are present in 85 to 90% of individuals when hyperglycemia is detected. Susceptibility to autoimmune type 1 diabetes is determined by multiple genes, and a recent analysis has shown 40 different sites in the human genome that may be associated with type 1 diabetes *mellitus*. HLA (Human Leukocyte Antigen) genes are those that demonstrate a greater association with the disease,

and there are also links with specific combinations of alleles in the DRB1, DQA1 and DQB1 loci with both sensitive or protective haplotypes (Craig, 2009).

Associated with type 1 diabetes there may be many other serious diseases, such as Hashimoto's thyroiditis, Addison's disease, Celiac disease, Pernicious anemia (Kahaly and Hansen, 2016). One of the reasons for this onset of these diseases and many other complications as is the case of some infections, is due to the fact that diabetes *mellitus* in general and not just type 1 have a "defective" immune system (Turnbaugh *et al.*, 2007).

Cellular studies on innate immunity demonstrated a decrease in the functions of chemotaxis, phagocytosis and death by the polymorphonuclear and monocyte / macrophage diabetic cells relative to the control cells. Better regulation of diabetes *mellitus* leads to an improvement in these cellular functions. It is known that some microorganisms become more virulent in a high glucose environment. Another mechanism that may lead to increased prevalence of infections in diabetic patients is an increase in the adherence of microorganisms to diabetic cells compared to non-diabetic cells. This phenomenon has already been described for *Candida albicans* (Hostetter, 1990). It is thought that the existence of carbohydrates in the receptor can trigger this phenomenon (Geerlings and Hoepelman, 1999).

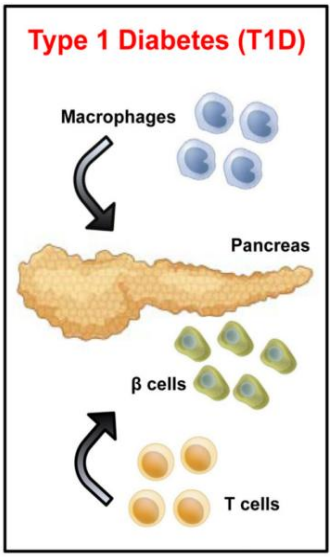


Figure 1.1.2 – Schematic drawing of type 1 diabetes *mellitus.* T1D develops as a result of the immune attack carried out by immune cells, such as macrophages and T cells. (Source: adapted from (Teo, 2014))

1.1.4. Epidemiology of Diabetes in Portugal and in the world

According to the annual report of the National Diabetes Observatory the prevalence of diabetes in 2015 was 13.3% of the Portuguese population aged between 20 and 79 years, corresponding to about 1,024 million individuals. In that same year, the prevalence rate of Diabetes was 56% of individuals would have already been diagnosed, and 44% were not diagnosed. There was also a significant difference in prevalence between men and women (**Fig 1.1.3**) (OND 2015).

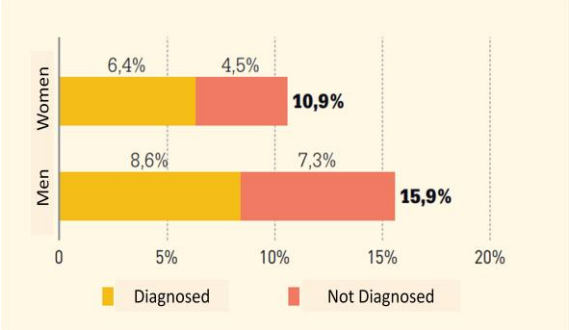


Figure 1.1.3. Prevalence of diabetes in Portugal by genera in 2015
(Source: adapted from PREVADIAB - SPD; treatment – OND 2015)

There has been a marked increase in new cases diagnosed annually in Portugal in the last 4 years, these values are approaching the maximum values recorded in the years 2010 and 2011 (OND 2015).

Table 1.1.2. Incidence of diabetes in Portugal (Adapted from: DOCE Register (DGS); OND 2015)

	2010	2011	2012	2013	2014	2015
Nº of new cases per 100 000 persons	625.3	651.8	500.9	557.1	522.1	591.5

Regarding type 1 diabetes, its prevalence in children and young people aged 0-19 is tending to remain around 3300 individuals in recent years, which corresponds to approximately 0.16% of the population within this age group (OND 2015).

Table 1.1.3. Prevalence of type 1 diabetes in children and young people in Portugal (Adapted from: DOCE Register (DGS); OND 2015)

	2010	2011	2012	2013	2014	2015
Number total cases (0-14 years)	1816	1856	1918	1945	1940	1828
Prevalence rate of Type 1 Diabetes (0-14 years)	0.11%	0.12%	0.12%	0.13%	0.13%	0.13%
Number of total cases (0-19 years)	3085	3206	3292	3361	3393	3327
Prevalence rate of Type 1 Diabetes (0-19 years)	0.14%	0.15%	0.16%	0.16%	0.17%	0.16%

If we look at the incidence in 2015, 11.5 new cases were detected per 100,000 young people in the age group (0-19 years), which is much lower than in recent years (OND 2015).

Table 1.1.4. Incidence of diabetes type 1 diabetes in children and young people in Portugal (Adapted from: DOCE Register (DGS); OND 2015)

	2010	2011	2012	2013	2014	2015
Number total cases (0-14 years)	338	281	320	319	265	195
Number of cases per 100,000 individuals (0-14 years)	21.2	17.9	20.6	21.0	17.8	13.3
Number of total cases (0-19 years)	405	332	374	362	308	233
Number of cases per 100,000 individuals (0-14 years)	18.7	15.1	17.8	17.5	15.1	11.5

According to the International Diabetes Federation (IDF, 2015), there are about 425 million people with Diabetes, and it is estimated that this number will rise significantly in the coming years, with an estimated 626 million people having diabetes in 2045, mainly type 2 diabetes. Type 1 diabetes is highly variable depending on the country, zone or even ethnic group. In Europe the incidence is closely associated with the frequency of the HLA gene. The frequency of the gene differs from one ethnic population to another, as for example the Chinese and the Japanese, which have an incidence of 0.1 per 100,000 and 2.4 per 100,000, respectively, a much lower incidence compared to Caucasians. This has to do with the unique combination of HLA.

The incidence of the disease and its increase are linked with the low-risk HLA gene in certain populations. The change in seasonal values at the onset of diabetes is described and its peak has been observed in the winter months. Diabetes has a high genetic pressure and despite the family genetic inheritance, which corresponds to approximately 10% of cases, there is still no recognizable pattern of this inheritance. Type 1 diabetes is 2 to 3 times more common in

children from diabetic father (3.6 to 8.5%) in comparison to children from diabetic mother (1.3 to 3.6%) (Craig, 2009).

As a result of this contribution, both genetically and food-related, more and more is recognized the role of environmental factors, including the diet type which affects the organ in which the absorption of nutrients occurs and an active microbial community works, the intestine.

1.2. Human intestine

1.2.1. Microbial composition of the gastrointestinal tract

From the beginning of life, the gastrointestinal tract is colonized by various microorganisms (Schwarzer *et al.*, 2018). The gastrointestinal tract is a specialized organ, to convert the ingested food into components that can be absorbed by the body. In the gastrointestinal tract there is a community of microorganisms (Klaassens *et al.*, 2011) that are physically and chemically related to the host (Klaassens *et al.*, 2011; Shoaie and Nielsen, 2014). The microbiota is greatly affected by external and internal factors of the body, such as age, infections, diet, immunological status and pH of the gastrointestinal tract. Over the years the intestinal microbiota has proved to be of great importance for large-scale studies, leading to more in-depth knowledge on this subject, such as the Human Microbiome Project and MegaHIT (Clemente *et al.*, 2012). With this, it has become an interesting area of research and awareness for public health, contributing to the development of several metagenomics platforms.

The microbiota is associated with every multicellular organism on Earth (Quigley, 2013). The human being has about 10^{14} microorganisms mirrored by the whole body, both on the outside (skin) and inside (respiratory, digestive and urinary tract) (Aroniadis and Brandt 2014; Prakash *et al.*, 2011).

The gastrointestinal tract is the zone of the human body that has more microorganisms, but throughout its structure a very large variation of chemical environments is observed which leads to a greater increase of diversity of the microbiota, with each zone having a microbiota different from another (Berg, 1996; Berrilli *et al.*, 2012).

This microbiota is formed from the beginning of life, soon after the passage of the foetus through the birth canal is exposed to an enormous complexity of existing organisms. One of the first factors to affect the intestinal microbiota that the new being will have is the

way the baby is born, i.e. if the baby is born by normal birth (eutocic), it will pass through the mother's vagina, which has a microbiota that will influence the microbiota of the baby's intestinal tract, with a microbiota similar to that of the mother (Drell *et al.*, 2017). The same is no longer the case with infants who are born by caesarean-section, they will have a composition of the microbiota different from babies born by eutocic delivery (Drell *et al.*, 2017). Another important factor that will influence the microbiota in the initial phase of life will be the baby's feeding. If breastfed, babies will show a very different intestinal microbiota from babies who are fed by powdered milk. It is also in this early stage of life, more properly in the first year of age that the intestinal microbiota varies from baby to baby as well as over time, being the composition of this microbiota less diverse. Over time, the microbiota become more complex (Sherrill-Mix *et al.*, 2018).

The great majority of the bacteria residing in the intestine are in the last section of the digestive tract, about 10^{10}-10^{11}, that is in the large intestine, since it is in this zone where there is the least influence of the released acids during the digestion process, such as bile pancreatic secretions, among others, these acids being quite harmful to microorganisms (Walter and Ley, 2011).

The intestinal microbiota consists essentially of 5 phyla, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Proteobacteria,* and *Verrucomicrobia*, and small portions of *Fusobacteria, Tenericutes*, *Spirochaetes* and *Cyanobacteria* (Hillman *et al.*, 2017; Sherrill-Mix *et al.*, 2018). As the main and most important phyla present are *Bacteroidetes* and *Firmicutes*, in contrast to a very small percentage, about 0.1% of organisms of the *Fungi* phylum, the *Archea* domain and some Viruses (Annalisa *et al.*, 2014; Jandhyala *et al.*, 2015).

The upper gastrointestinal tract, which is composed of the stomach and duodenum, contains organisms that are more resistant to low pH being the predominant genera *Lactobacillus* and *Streptococcus* (Hooper and MacPherson, 2010). The small intestine, although it is the zone where there is a greater amount of nutrients, is also present a great quantity of glycoproteins of which one of the most important is mucin, and facultative anaerobic bacteria, such as *Escherichia*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Lactobacillus* and *Proteus* are present. The terminal part of the gastrointestinal tract, the large intestine, is a zone densely populated by anaerobes, the dominant genera being *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Clostridium*, *Peptococcus*, *Peptostreptococcus* and *Ruminococcus* (Lozupone *et al.*, 2012; Hajela *et al.*, 2015; Lu and Ni, 2015). Their functions in health and disease are under intensive research.

1.2.2. Main functions of the intestinal microbiota

Humans carry 2 types of genomes within it, their own genome and the genome of the microbiota they own. That in total there are about 23,000 genes in their human cells and 3 million genes in their microbiota which represents 150 times more microbial genes than humans. Due to this huge variety of microbial genes, there is a profound influence of these on the human organism. The intestinal microbiota plays a key role in various mechanisms of the human body, such as metabolic processes, nutrition, physiological and immune responses (Gerritsen *et al.*, 2011).

The main functions of the intestinal microbiota are the production of vitamins, synthesis of amino acids and biotransformation of bile acids (Prakash *et al.*, 2011a).

The ability to ferment carbohydrates is a focal point for obtaining human energy. For this, the human microbiota produces enzymes that will help in the degradation of complex chains of polysaccharides so that they can be digested or absorbed in the form of monosaccharides or short-chain fatty acids. The main short-chain fatty acids produced are acetate, propionate and butyrate, with the first two being absorbed into the portal circulation and the third used by colocytes as energy source. Other products resulting from the fermentation of some amino acids are alkyl carboxylic acids, such as valerate and caproate (Prakash *et al.*, 2011).

In addition to carbohydrates, lipid metabolism is also highly influenced by the microbiota. It is possible for certain microorganisms to suppress inhibition of lipoprotein lipase (LPL) activity in adipocytes, or even increase the efficiency of lipid hydrolysis by regulating the expression of the required colipase by pancreatic lipase so that lipid digestion is achieved (Sekirov, *et al.*, 2010).

The microbiota is also involved in the ability to metabolize proteins. The intestinal microbiota helps in the breakdown of proteins producing proteinases and peptidases, which together with human proteinases will result in amino acids. These amino acids can be transported to the cell wall of the bacteria giving rise to signal metabolites thus doubling the human peptide production function as well as the bacteria producing the necessary bacteriocins to regulate the intestinal microbiota (Prakash *et al.*, 2011).

If it were not the bacteria present in the microbiota, certain compounds, such as vitamin K or vitamin B, were not present in the gut, which are responsible for their production (Clarke *et al.*, 2014).

The intestinal microbiota has a symbiosis relationship with the human intestinal mucosa producing metabolic, immunological and protective substances to proportional well-being and functioning of the human being (Round and Mazmanian, 2009).

1.2.3. Intestinal microbiota present in health and disease

Humans and their microbiota have evolved over time, making their symbiotic relationship extremely close (Gerritsen *et al.*, 2011). Due to this close connection, and with the amount of substances produced by the microbiota, it is extremely important that from a young age the microbiota can be established providing a healthy life for its host (Prakash *et al.*, 2011a).

The shaping of this complex environment between microbiota and host is largely dependent on the history of transmission of microbes, and it is inevitable that the characteristics of the modern lifestyle, such as antibiotics, caesarean section, hygiene, artificial feeding, introduce obstacles in symbiont transmission with consequences for the functional development of microbiota. The stability of this core composition is crucial since any minor change causing dysbiosis, which can lead to various problems (Sekirov *et al.*, 2010).

Dysbiosis can be taken into account as a biomarker to gauge one's health status. It is a factor that leads to the appearance of inflammatory responses, in humans, or in any animal (David and David, 2010).

More and more studies are trying to associate the microbiota to the development of certain diseases and in many cases, if this is happening, there are diseases that are associated with dysbiosis and when trying to understand where and what changes one can try to prevent or make some kind of treatment so that it can restore normality. Some of the disorders associated with dysbiosis are allergies (David and David, 2010), autoimmune diseases, obesity, type 1 and type 2 diabetes (Sekirov *et al.*, 2010), Crohns disease, and different types of cancer (Prakash *et al.*, 2011a).

In the microbiota composition there are certain bacteria that can cause a pro inflammatory response. They can thus produce cytotoxins, genotoxins and immunotoxins, Gram negative bacteria release an endotoxin that can elicit an inflammatory response, thereby aggravating insulin resistance (Hara *et al.*, 2013). Thus the microbiota is able to potentiate both pro-and anti-inflammatory responses, but not only the composition of the bacterial

communities in the gut is crucial, it is their action on nutrient metabolism, capacity to metabolize xenobiotics and drugs, antimicrobial protection activity and their contribution to immunomodulation that can assure the gut integrity and promote the human health (Jandhyala *et al.*, 2015).

Although the immune system is thought to be responsible for recognizing, and responding to an endless number of foreign molecules in the sense of protecting the human body against infections, over time it has been observed that in humans there is a complex coexistence between the microbiota and the host immune system interacting extensively in order to protect each other (Craig, 2009; Sekirov *et al.*, 2010; Hara *et al.*, 2013; Jandhyala *et al.*, 2015).

1.2.4. Intestinal microbiota and type 1 diabetes

The intestinal microbiota of children at risk of developing type 1 diabetes has been studied (Brown *et al.*, 2011; Giongo *et al.*, 2011; Davis-Richardson *et al.*, 2014; Endesfelder *et al.*, 2014; Kemppainen *et al.*, 2015). Several studies have been carried out in order to understand how the microbiota of these patients varies in relation to healthy people and in what way this may or may not influence the disease (Hamari *et al.*, 2015).

The studies that have been carried out showed that there is a difference at phylum level, namely between phylum *Bacteroidetes* and the phylum *Firmicutes* (Brown *et al.*, 2011; Davis-Richardson *et al.*, 2014; Leonard *et al.*, 2014). The population of *Bacteroidetes* increases in children at risk in comparison to healthy children. The opposite is observed with the phylum *Firmicutes*, diabetic children show less quantity than control children (Kemppainen et al. 2015; Knip and Honkanen 2017).

The genus from *Bacteroidetes* phylum that was the most abundant in children at risk of autoimmunity in Finland was the genus *Bacteroides* (Brown *et al.*, 2011; Murri *et al.*, 2013), particularly the specie *B. dorei* and it is possible that some of the species belonging to this genus may be important for the appearance of this disease.

Recently it has been demonstrated through comparative studies of children at high risk of developing autoimmunity in Nordic countries, Finland and Sweden, compared to two states of the United States of America that the composition of the microbiota of children at risk is influenced by the geographic region (Kemppainen *et al.*, 2015). Finland is one of the European countries with the highest T1D rate, followed by Sweden and Norway (IDF, 2015).

In the study conducted by Kemppainen *et al.*, (2015) the intestinal microbial diversity was more similar between the state of Colorado and Finland in comparison to Finland and Sweden that are neighbouring countries. The composition of the intestinal microbiome of the Swedish autoimmune children was more similar to that of the Washington State, thus verifying that the study of the microbiota has to be performed locally to try to discover microbial patterns of that location. It is important to note that in that study the genus *Bacteroides* was the predominant genus in any region, but the state of Colorado was the one with the highest *Bacteroides* abundance (Kemppainen *et al.*, 2015).

According to (Murri *et al.*, 2013), studies carried out in Spain with children with established T1D concluded once again that children suffering from T1D showed higher populations of *Clostridium*, *Bacteroides* and *Veillonella* in contrast to control children that showed higher populations of *Bifidobacterium*, *Lactobacillus*, *Blautia coccoides* / *Eubacterium rectale* group and *Prevotella*.

A recent study carried out in the Algarve region (Portugal) (Pinto *et al.*, 2017) have analysed the intestinal microbial proteome of T1D children and have observed a different protein pattern between T1D and control proteome, in particular the T1D proteome was rich in proteins originated from *Eubacterium rectale*, *Faecalibacterium prausnitzii*, *B. dorei* and *B. uniformis* in contrast to the healthy children that showed a proteome rich in proteins originated from *Bifidobacterium adolescentis*, *Bifidobacterium longum infantis*, *Ruminococcus*, *Collinsella aerofaciens*, *Coprococcus comes* and *Clostridium* spp. It is worth to note that some proteins produced by the bacteria of T1D children were proteins related to combat stress conditions, which can translate a unfavourable intestinal environment for them.

1.2.5. Intestinal Microbiota and the immune system

As mentioned above a leaky gut has been correlated with T1D, and an aberrant gut microbiome was proposed as the factor that results in leaky gut followed by altered immune responses leading to disease (Zipris, 2013; Geuking *et al.*, 2015; Mejía-León and Calderón de la Barca, 2015; Blandino *et al.*, 2016).

According to the hygiene hypothesis, this suggests that the reduction of exposure to both pathogenic and symbiotic microorganisms during early life changes the “normal” development of the immune system, which leads us to believe that the maturation and development of the immune system is deeply connected with the microbiota carried by

humans. This lead to the conviction that altered microbiota may be a predisposing factor for the development of several diseases, in this case inflammatory and autoimmune diseases, such as type 1 diabetes *mellitus* (Geuking *et al.*, 2015).

Several studies reinforce the idea that a disturbed intestinal microbiota participates on the formation of the dysbiosis state altering the intestine permeability and modifying the immune regulatory processes leading to the autoimmune state that will culminate on the development of T1D (Vaarala *et al.*, 2008; Sekirov *et al*, 2010; Brown *et al.*, 2011; Giongo *et al.*, 2011; Murri *et al.*, 2013; Davis-Richardson *et al.*, 2014). Since the drastic growth of the onset of the disease cannot be justified just by genetic susceptibility, it is presumed that environmental factors also have influence, such as microorganisms leading to inflammatory responses, as well as the appearance of leaky gut suggesting that the mucosal surface of the intestine has greater permeability, which leads to the surface of the intestine being subject to food antigens, commensal and pathogenic microorganisms, which can induce a pro-inflammatory reaction, and gut associated lymphoid tissues (GALT) which makes the interconnection between all these external agents and the microbiota associated with the host immune system (Kabat *et al.*, 2014).

The incidence of T1D has been also associated with exposure to viral agents (Cinek *et al.*, 2017; Rodriguez-Calvo *et al.*, 2016; Federico *et al.*, 2018; Zhao *et al.*, 2017;). The association of viruses with the development of T1D was also demonstrated in animal models (Coleman *et al.*, 1973; Oldstone *et al.*, 1991).

Studies have shown that the type of birth (vaginal delivery or caesarean section) as well as breastfeeding or artificial milk may in the presence of an altered microbiota lead to T1D, due to the perturbation, at more early ages, of the intestinal microbiota (Neu and Rushing, 2011; Geuking *et al.*, 2015).

Animal models to study the influence of the microbiota on the disease were developed, namely, the Biobreeding mice, from which two groups were created, one prone to diabetes (BB-DP) and the second resistant to diabetes (BB-DR). Analysis of the intestinal microbiota of these mice allowed the identification of alterations in the presence of *Lactobacillus* in the faeces of these animals, in particular the species, *L. johnsonii* and *L. reuteri* (Walter and Ley, 2011). There was a decrease in the incidence of type 1 diabetes and its progression, when animals prone to diabetes were exposed to these two bacterial species (Walter and Ley, 2011).

It was reported that the damage to the pancreas can be initiated by a cross-reaction of the immune system to antigens from the diet (Antvorskov *et al.*, 2014; Mejía-León and Calderón de la Barca, 2015), and the association with the microbiota may arise due to

intestinal cells expression microbe-associated molecular pattern (MAMP) receptors, mainly stemming from the Toll-like receptors (TLRs) (Hara *et al.*, 2013). TRLs are transmembranal proteins that allow the adaptation of the immune system, being responsible for the recognition of pathogenic lines. These, in the gut ,when activated together with the microbiota can induce the production of cytokines, chemokines and antibacterial products (Masanta *et al.*, 2013; Antvorskov *et al.*, 2014). One of the molecules to which this recognition is attached is MyD88 (myeloid differentiation primary response gene 88), which once activated can prevent the onset of T1D (Lee, 2011; Jandhyala *et al.*, 2015). When inactivated MyD88 (knock out) may leads to dysbiosis, which will in turn will contribute to the onset of T1D (Lee, 2011).

Many bacterial communities can induce the production of T-helper cells in an inflammatory response, cells that in turn will develop and originate regulatory T cells and secrete immunoglobulins A, which then lead to the characteristic autoimmunity of T1D (Zipris, 2013).

Virtanen *et al.*, (2000) hypothesised that Finnish children, due to high intake of bovine dairy products, were at increased risk of development of autoimmune β-cells and subsequent progression to T1D. In fact, the presence of high concentrations of anti-α-casein at the time of T1D diagnosis suggests that the antibody response to this protein may be relevant for autoimmune diabetes (Birgisdottir *et al.*, 2006). In addition, it was proposed that diets with a high gluten content could radiate the main drivers of intestinal dysbiosis associated with the development of T1D (Virtanen *et al.*, 2000; Davis-Richardson and Triplett, 2015).

1.2.6. Milk and gluten implications in the development of Type 1 diabetes

The consumption of bovine milk has been very present in the human diet. From a few years to now the dilemma of whether or not the consumption of cow's milk is harmful to humans remains. Since is recognized that T1D does not depend only on genetic factors for the onset, associations with animal milk consumption, particularly cow's milk, have been made (Monetini *et al.*, 2002). The major component of cow's milk, which is hypothesized to be present in the development of the disease is β-casein and A1 bovine protein (Monetini *et al.*, 2002). A study conducted in Iceland (Birgisdottir *et al.*, 2006) compared milk from this area with milk from the Scandinavian area and claim that the quantity of these two proteins present in Icelandic milk is much lower in comparison with the milk produced in Scandinavia, which

has a high rate of children with T1D. In this study the authors hypothesized that these proteins may somehow be related to the onset of the disease in children with a high genetic predisposition to it.

In the study of Monetini et al (2002) it was also be observed that healthy siblings of children with diabetes and also with a high milk intake were also associated with the development of the disease, especially if they carried a moderate or high genetic risk by the HLA DQB1 genotype.

Another food protein which may be implicated with the onset of T1D type 1 is gluten. Although numerous studies have suggested that there is a potential pathogenic role of gluten has not yet been fully realized why can constitute a trigger of T1D. It is understood that there are some gluten peptides that have resistance to the enzymatic process originated in the intestine, leading to an impact on the microbiota that may degradate this protein in peptides with inflammatory action causing dysbiosis (Antvorskov *et al.*, 2014; Hamari *et al.*, 2015; Serena *et al.*, 2015). The stimulation emanating from cereal proteins showed increased proliferative response of T cells in comparison with control patients. The peripheral blood mononuclear cells (PBMC) of these patients produced significantly more proinflammatory cytokines compared to controls (Hamari *et al.*, 2015).

The **Figure 1.1.4.** illustrates the interaction of the potential factors on the development of T1D.

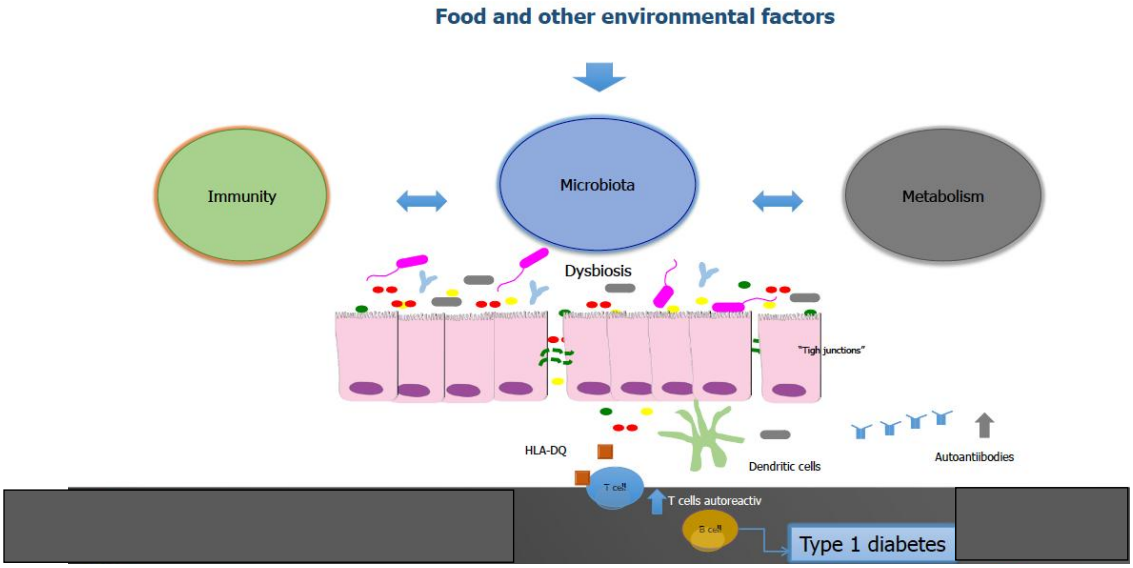


Figure 1.1.4. Illustration of the interaction between environmental factors including diet components on the composition of the intestinal microbiota, and their influence on the immune system and metabolism. The immune regulatory mechanisms modified by the intestinal dysbiosis and higher gut permeability seem to initiate the autoimmune response that leads to the injury of β-cells in the pancreatic islets (Adapted from Sanz et al. 2015).

As mentioned above *Bacteroides* spp. including *B. dorei* have been implicated on the development of the disease. Following a brief description of this genus and the evidences suggesting the association of *B. dorei* to the development of T1D will be discussed.

1.3. *Bacteroides* spp. and its association with Type 1 Diabetes

Bacteroides spp. are Gram negative, rod-shaped anaerobic bacteria, not motile that are included in the phylum *Bacteroidetes*. One particular feature of this bacterial group is the existence of sphingolipids in their membranes, as well as the presence of meso-diaminopimelic acid in its peptidoglycan layer (Wexler, 2007). *Bacteroides* spp. are a mutualistic group that is present in high quantities in the gut (10^{10}-10^{11} cells per gram of human faeces) (Wexler, 2007). *Bacteroides* spp. play a very important role in the supply of nutrients to humans since have great capacities of degradation of plant carbohydrates, (Martens *et al.*, 2009).

The major source of energy used by this group of organisms comes from the fermentation of a large variety of sugars held in human intestines derived from plants, and some of these components are highly harmful to the humans (Martens *et al.*, 2009). With the elimination of these toxic compounds, the fermentative processes produced by these bacteria are quite beneficial to humans, generating metabolites, such as acetic acid and succinic acid. *Bacteroides* also have a great ability to remove bile acid side chains, causing them to return to the hepatic chain (Slonczewski and Foster, 2009).

The *Bacteroides* genus is also associated with resistance to a wide range of antibiotics, such as beta-lactams, aminoglycosides and erythromycin and tetracycline but varies according to species and regions (Karlowski *et al.*, 2012; Boyanova *et al.*, 2015; Ho *et al.*, 2017; Snydman *et al.*, 2017).

It has been observed that many *Bacteroides* species have several impacts at clinical level as is the case of *B. fragilis*, being responsible for a large number of anaerobic infections in the human gut (Li *et al.*, 2017). The pathogenicity is due to the fact of its capacity to produce capsular polysaccharide, which is protective against phagocytosis and stimulates abscess formation (Rashidan *et al.*, 2018). Although it is the specie most frequently detected in clinical samples, its population in the gut is not significant, reaches about 0.5% of the total population of *Bacteroides* (Virtanen *et al.*, 2000).

Another highly reported species in the gut is *B. thetaiotaomicron* because of its importance in the study of symbiotic bacterial host relationships in the human intestine, as well as its metabolic processes in the digestion to facilitate the absorption of nutrients by the host, it also contributes to the development of the postnatal intestine and the physiology of the host. However, it is also an important bacterial pathogen, causing potential interest because of its resistance to antibiotics (Cho *et al.*, 2001; Ho *et al.*, 2017; Shipman *et al.*, 1999; Teng *et al.*, 2004).

Bacteroides xylanisolvens was only isolated from samples of human faeces. A recent study tried to prove whether *B. xylanisolvens* may possess probiotic qualities (Brodmann *et al.*, 2017). In order to be a probiotic the bacterial strain has to be considered ambiguously safe. *B. xylanivsolvens* is free of genes that biosynthesis *Bacteroides fragilis* enterotoxin (*bft*) and capsular polysaccharide PS A (PS A) (Ulsemer *et al.*, 2012). These are two factors that contribute to the development of the pathogenecity of this organism, since these compost PS A contributes positively to the immunomudularity being an important factor for the development and abscesses (Eradi *et al.*, 2018), since the *bft* gene produces a toxin that is to be expressed in some pathologies including colon cancer (Ulger Toprak *et al.*, 2006; Boleij *et al.*, 2015). *B. xylanivsolvens* is also unable to adhere to the intestinal epithelial cells, which eliminates a successful colonization of the intestine (Atherly and Ziemer, 2014). It also has no plasmid material in the genome of the cell, making it a safer probiotic option. All of these qualities of *B. xylanivsolvens* contribute to its potential use as probiotic (Chassard *et al.*, 2008).

Bacteroides dorei, in turn has been associated with the development of the autoimmune state in children at risk of T1D (Davis-Richardson *et al.*, 2014). However its association with T1D risk was not found in the study conducted by Cinek et al. (2017).
B. dorei was first isolated in 2006 by (Bakir *et al.*, 2006) from human gut. Its characteristics are similar to other *Bacteroides* spp., and the appearance of the colonies in the culture medium OF *Bacteroides vulgatus* selective media (BVSA) are of black colonies of about 2 mm, convex, bright, and with a halo of degradation of the esculin after 48 h incubation at 37ºC. In Brain Heart Infusion agar medium supplemented with hemin, the colonies show a very smooth and tenuous blue colour achieving 4-5 mm (Bacic and Smith, 2008).

Until 2006 *B. dorei* was included in the species of *B. vulgatus* since they are morphologically and genetically very similar. Studies prior to the separation of these two species indicated that *B. vulgatus* was present in some pathologies, such as ulcerative colitis and Crohn's disease (Bakir *et al.*, 2006).

A recent study have shown the DNA methylation (Dam methylation) in *B. dorei*, particularly the motif GATC, which means that, when methylation of the gene occurs, there will be difficulties in the expression of many genetic processes of chromosome replication, mismatch repair and nucleoid structure (Leonard *et al.*, 2014). This possible problem, along with its antibiotic resistance, can cause serious problems.

When Giongo et al. (2011) started the study of T1D in Finland and analysed the microbiome of 4 children at risk of developing T1D concluded that children a risk have a very unstable microbiota but the number of *Bacteroidetes* was increasing whereas the number of *Firmicutes* was decreasing along the sampling period. The dominance of the microbiome by *B. dorei* in children at risk of autoimmunity in Finland was reported by Davis-Richardson et al. (2014).

As stated above in the study of Kemppainen et al. (2015) the intestinal microbiome of children at risk of T1D from Germany, Sweden, Finland and USA was compared. At all sampling sites *Bacteroides* was the prevailing genus. The authors found surprisingly differences between the neighbouring countries Finland and Sweden, namely the microbiome of Finnish children showed a significantly higher abundance of *Bacteroides* and *Veillonella*, and was depleted of *Bifidobacterium*, *Akkermansia* and *Ruminococcus*. The microbiome of Finnish children was more similar to the Colorado state, in contrast the Swedish children microbiome was more similar to the Washington state. Data from this study evidenced that children at high risk for T1D possess location-specific microbiome patterns of the gut with intercontinental resemblances but intracontinental dissemblance (Kemppainen *et al.*, 2015). This study stressed the importance of carring studies on the impact of the microbiome of children who are genetically predispose to T1D taking in account the geographical location.

The microbiome of Spanish children with established diabetes also evidenced higher *Bacteroidetes* in their gut and a lower content of *Bifidobacterium* spp. (Murri *et al.*, 2013). The metaproteomic study carried with children with established diabetes from the Algarve region (Portugal) evidenced an abundance of proteins produced by *B. dorei* and *B. uniforms* along with the higher production of proteins from the *Clostridial* groups XIVa and IV (Pinto *et al.*, 2017). However, in contrast to the study of Murri *et al.*, (2013) the population of *Bacteroides* was not higher in children with T1D in comparison to control children, so the authors hypothesized that the intestinal T1D milieu was not affecting the number of *Bacteroides*, but instead was modulating their proteome (Pinto *et al.*, 2017).

Overall the studies evidenced the significant prevalence of *Bacteroides* in either the development of T1D or in established disease, and *B. dorei* seem to stand out from this group.

How this bacterial group, and in particular *B. dorei* impact on the development of T1D is not yet clarified. In the study of Vatanen et al. (2016) the lippolysaccharides (LPS) from *B. dorei* showed a lower immune stimulatory activity in comparison to the LPS from *E. coli*. However the *B. dorei* strain tested in the study of Vatanen et al. (2016) was a strain isolated from a healthy adult individual.

A series of questions need to be answered, in particular how the bacterium interacts with the intestinal epithelial cells and the immune system, and how food components (e.g., milk and gluten) impact its activity? In such approach isolates from T1D patients and controls, including their healthy siblings must be tested.

1.3.1. *Bacteroides* spp. genotyping

Typing is a process that allows the characterization of microorganisms existing in nature, contributing to their classification and to discrimination between individuals of the same species (Dawkins, 1978; Johannsen, 2014).

There is phenotypic and genotypic typing (Dolka *et al.*, 2016). Phenotyping is a typing method based on morphological and biochemical tests, phage sensitivity, sensitivity to bacteriocins, immunological profiles and susceptibility profiles to antimicrobial agents. Although many tests are performed on the same organism, it is very useful if necessary to characterize the genus or species, and, in many cases, it is not possible to distinguish strains of the same species for which genotyping will be very helpful (Dawkins, 1978).

Genotyping is a process of determining the differences in the genetic makeup of a microorganism using molecular methods for this division. Usually in this type of analysis part or all of the genome is used for this differentiation. This method is based essentially on the size polymorphism of the restriction fragments generated from the genome (Johannsen, 2014). Genotypic methods are crucial in phylogenetic classification and identification at different taxonomic levels, and as well have a relevant role in diversity analyses of prokaryotic taxa (Glaeser and Kämpfer, 2015). Since the introduction of the small subunit ribosomal RNA (rRNA) gene base phylogeny by Woese and Fox (1977), the Sanger sequencing technique (Sanger *et al.*, 1977) and the polymerase chain reaction (Saiki *et al.*, 1988) enormous advances have been observed in microbial taxonomy. With the advent of next-generation sequencing approaches (Snyder *et al.*, 2013; Veenemans *et al.*, 2014; Deng *et*

al., 2015; Thorburn *et al.*, 2015; Dominguez *et al.*, 2016) more impact changes in microbial taxonomy and epidemiological studies are expected.

There are several methods for genotyping including restriction fragment length polymorphism (RFLP), random amplified polymorphic detection (RAPD), amplified fragment length polymorphism detection (AFLPD), Ribotyping, multilocus sequence typing (MLST), repetitive based sequence PCR (rep-PCR), pulsed-field gel electrophoresis (PFGE) (Ishii and Sadowsky, 2009). All these methods have advantages and disadvantages (Johannsen, 2014). The rep-PCR was used in *Bacteroides* spp genotyping (Atherly and Ziemer, 2014). The rep- PCR technique is based in the use of oligonucleotide initiator sequences that are complementary to the high conserved repetitive DNA sequences, which are present in high copy numbers in the genomic DNA of the majority of bacteria, mainly Gram negative but also Gram positive (Versalovic *et al.*, 1994). Multiple repetitive sequences have been identified in many microbial species by whole genome sequencing (Tobes and Ramos, 2005; Land *et al.*, 2015; Dominguez *et al.*, 2016). There are three families of repetitive sequences REP (Repetitive Extragenic Palindromic elements) with 35-40 base pairs, which are conserved in several bacterial species, the ERIC sequences (Enterobacterial Repetitive Intergenic Consensus elements) with 124-127 bp that contain a central repetitive element highly conserved and are found in the extragenomic regions of the bacterial genome, and the BOX element with 154 bp. Knowing that each bacterial strain or isolate has repetitive sequences distributed along different regions of their genome it is possible by amplification of these repetitive sequences, using appropriate primers to target these sequences generating multiple amplicons. These amplicons will diverge in size directly related to the genomic distance between the binding sites of contiguous repetitive elements. The pattern of the amplicons, will correspond to the genomic DNA fingerprinting of each isolate or strain, which can be analyzed by electrophoresis and compile the similarity group in the form of a dendogram (Versalovic *et al.*, 1994).

2. Objectives

The main objective of the current study is to analyse the *Bacteroides* population of T1D children in the Algarve region. To achieve this our specific objectives, include:

1) Isolate and quantify *Bacteroides* spp., including *B. dorei* from children with established T1D and control children including their healthy siblings.

2) Genotyping the isolates of *Bacteroides* spp.

3) Determine the ratio of *Firmicutes* / *Bacteroidetes* and quantify *Bifidobacterium* sp. and *Lactobacillus* sp. population in T1D and control children.

3. Material and Methods

3.1. Material

• AnaeroGen 2.5 L, Thermo Scientific (UK)

• Autoclave Uniclave 88 AJC (Lisbon, Portugal)

• Analytical balance AE 200, Mettler (USA)

• Analytical balance XS-410, Fisher Scientific (Portugal)

• Multiplaces dry heating bath, Selecta (Spain)

• Bio48 Laminar Flow Chamber, Faster (Italy)

• Mini-V / PCR PCR camera, Telstar (Spain)

• Mikro 22R Centrifuge, Hettich Zentrifugen (UK)

• Ultra low temperature freezer freezer -80ºC U725, Innova New Brunswick Scientific (USA)

• Electrophoresis Power Supply - EPS 301 (USA)

• Binder Incubator (Germany)

• Anaerobic jar, Difco Laboratories (Detroit Michigan)

• Kodak EDAS 290 (USA)

• pH meter GLP21, Crison (Spain)

• ATC 2000 Microscope, Leica (Portugal)

• Heating and agitation plate, Selecta, Agimatic-E (Spain)

• Serum type reaction vials 10 mL (Supelco)

• Serum type reaction vials 30 mL (Supelco)

• Sterile syringes of 5 mL (Pico)

• Agarose gel trough for PCR, Pharmacia Biotech GNA100, (USA)

• Thermocycler T-Gradient (Biometra, Germany)

•Thermocycler T-personal (Biometra, Germany)

•Thermocycler T1 (Biometra, Germany)

• Ultrassons P, Selecta (Spain)

• Vortex L46, Labinco (The Netherlands)

3.1.1. Culture media

• Brain Heart Infusion (VWR) prepared according to the manufacturer's instructions, supplemented with hemin (0.1%, v / v, Fluka) and L-cysteine (0.1%, w / v, Sigma-Aldrich) (BHI+ H), pH 7.1. When required to obtain solid medium agar at 1.5% (Invitrogen) was added.

• Luria Broth base, Miller (LB) prepared according to the manufacturer's instructions. When required was supplemented with Ampicillin (0.1%, w / v) (Sigma), X-Gal (5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside) (0.1%, w / v) (VWR) and agar (1.5% w / v) (Invitrogen).

• *Bacteroides Vulgatus* Selective Agar (BVSA) 30g / L Trypticase soy broth (TSB) (Biokar), 1 g / L esculin (Sigma-Aldrich), 2 g / L yeast extract (Biokar), 2 g / L dehydrated ox-bile (Sigma-Aldrich), 0.5 g / L ferric ammonium citrate (Merck) supplemented with kanamycin (0.2%, w / v) (Sigma-Aldrich), vancomycin (0.075%, w / v) (Sigma-Aldrich), colistin (0.015%, w / v) (Sigma-Aldrich), vitamin K1 (1%, v /v) and 1.5% (w / v) agar (Invitrogen) pH 7 (Bacic and Smith, 2008).

• Garche's broth peptone, 20 g / L; yeast extract (Biokar), 2 g / L; lactose (Merck), 10 g / L; L-cysteine hydrochloride (Fluka), 0.4 g / L; sodium acetate (Merck), 6 g / L; $MgSO_4$ x 7 H_2 O (Baker), 0.12 g / L; KH_2PO_4 (Merck), 2 g / L; Na_2 HPO_4 x 12 H_2O (Merck), 2.5 g / L; pH 6.4 (Jarocki *et al.*, 2016). When required agar was added at 1.5% (w / v).

3.1.2. Solutions

The solutions used in the present study were as follows:

• Phosphate Buffered Saline (PBS) - 8 g /L NaCl, 0.2 g / L KCl, 1.44 g / L Na_2HPO_4, 0.24 g / L KH_2PO_4

• Lysis Buffer - 1 mL of 1M Tris HCl, pH 9 to 2.5 mL 1M KCl, 0.1 mL Triton-X, 93.9 mL of MilliQ water.

• Guanidine, EDTA, Sarcosil (G.E.S.) - 60 g of guanidine thiocyanate (Promega), 20 mL of 0.5 M EDTA solution pH8 (Sigma), 5 mL of sarcosyl, 100 mL of distilled water.

• Ammonium acetate solution 10 M - 77.05 g of ammonium acetate (Merck) in 100 mL of distilled water.

• Chloroform: Isoamyl alcohol (24: 1) (v / v) - 24 mL of chloroform (LAB-Scan) and

1 mL of isoamyl alcohol (Merck).

• Tris-Acetate-EDTA buffer (TAE) 50x - 242 g / L Tris base (Sigma), 57.1 mL Glacial acetic acid (Panreac), 100 mL 0.5 M EDTA pH 8.

• Buffer 1M Tris HCl pH 9 to 2 - 121.11g Tris (Sigma) 1L of distilled water, set at selected pH value with 1M HCl.

• 0.005 M NaOH solution - 0.019 g NaOH in 100 mL distilled water.

• Glucose-Tris-EDTA (GTE) buffer - 4.505 g glucose, 12.5 mL 1M Tris HCl, pH 7.5, 10 mL 0.5 M EDTA pH 8 in 500 mL distilled water.

• Ethylenediamine tetraacetic acid (EDTA) 0.5 M pH 8 - 18.6 g EDTA in 100 mL distilled water.

• 0.2 M NaOH / SDS solution - 0.08 g NaOH, 0.1 g SDS (Merck) in 10 mL of distilled water.

• 3 M solution of potassium acetate - 29.45 g potassium acetate in 100 mL of distilled water.

• Calcium chloride 50mM - 0.55g of Calcium chloride (Merck) in 100 mL of distilled water.

• Lysozyme solution (100 mg / mL) (Merck).

• Hemin solution 0.1% - 0.1 g hemin, 2 mL 1M NaOH in deionized water to 100 mL – requires protection from light since is light sensitive.

• Vancomycin solution (7.5 mg / mL) (Sigma). The antibiotic was dissolved in distilled water.

• Kanamycin solution (50 mg / mL) (Sigma). The antibiotic was dissolved in distilled water.

• Colistin solution (0.015 mg / mL) (Sigma). The antibiotic was dissolved in distilled water.

• Ampicillin solution (100 mg / mL) (Sigma). The antibiotic was dissolved in distilled water.

• X-Gal solution (50 mg / mL) (Sigma). The compound was dissolved in DSMF.

• Vitamin K1 solution (1%). The vitamin was dissolved in 96% alcohol (Pancear)

3.1.3. Biological samples

The bacteria used in the current study are indicated in **Table 3.1.1**

Table 3.1.1. Bacteria used in this study

Bacteria	Source
***Bacteroides dorei* DSM 17885**	DSMZ
***Bacteroides thetataiomicron* DSM 2079**	DSMZ
***Bifidobacterium longum* subsp. *infantis* DSM 20088**	DSMZ
***Enterococcus faecalis* NCTC 775**	NCTC
***Lactobacillus casei* DSM 20011**	DSMZ

DSMZ- Deutsche Sammlung von Mikroorganismen und Zellkulturen.

NCTC- National Collection of Type Cultures, Public Health England.

3.1.4. *Primers*

The primers used in the present study are described in **Table 3.1.2.** The primers were purchased from Sigma.

Table 3.1.2. Primers used to amplify the different targets

Primer	5'-3' sequence	Target	Reference	Melting temperature (°C)	Amplicon (bp)
27F	AGAGTTTGATC MTGGCTCAG	Universal 16S	(Weisburg *et al.*, 1991)	50	1400-1600
1492R	CGGTTACCTTGTTACGACTT	Universal 16S	(Turner *et al.*, 1999)	50	
799F	AACMGGATTAGATACCCKG	Universal 16S	(Chelius and Triplett, 2001)	58	
BOXA1R	CTACGGCAAGGCGACGCTGACG		(Versalovic *et al.*, 1991)	76.5	

ERIC1R	ATGTAAGCTCCTGGGGATTCAC		(Versalovic *et al.*, 1991)	65.1	
ERIC2	AAGTAAGTGACTGGGGTGAGCG		(Versalovic *et al.*, 1991)	66.4	
BactesF	CATGTGGTTTAATTCGATGAT	*Bacteroidetes*	(Murri *et al.*, 2013) (Guo *et al.*, 2008)	58.8	126
BactesR	AGCTGACGACAACCATGCAG	*Bacteroidetes*	(Murri *et al.*, 2013) (Guo *et al.*, 2008)	66.5	
FtesF	ATGTGGTTTAATTCGAAGCA	*Firmicutes*	(Guo *et al.*, 2008)	59.6	126
Firm1060R	AGCTGACGACAACCATGCAC	*Firmicutes*	(Guo *et al.*, 2008)	66.5	
LbF	AGCAGTAGGGAATCTTCCA	*Lactobacillus* spp.	(Rinttilä *et al.*, 2004)	55	341
LbR	CACCGCTACACATGGAG	*Lactobacillus* spp.	(Rinttilä *et al.*, 2004)	55	
g-Bifid-F	CTCCTGGAAACGGGTTGG	*Bifidobacterium* spp.	(Matsuki *et al.*, 2004)		550
g-Bifid-R	GGTGTTCTTCCCGATATCTACA	*Bifidobacterium* spp.	(Matsuki *et al.*, 2004)		
BdRTiF	TTGACTAGGTCGGCCGTTACCC	*Bacteroides dorei*	This study		120
BdRTiR	GAAGATTAATCCAGGATGGGAT	*Bacteroides dorei*	This study		

3.2. Methods

3.2.1. Study participants

The current study included 17 Caucasian children with T1D, ages varying between 6 and 12 years old, and 17 healthy children including healthy siblings of T1D children, ages varying between 2 and 12 years old. In order to avoid confusing factors, such as climate, drinking water, health practices and air quality all children that participated in the study were from Algarve region (Davis-Richardson and Triplett, 2015). Excluding criteria included antibiotic treatment, hospitalization and diagnosis of infectious diseases three months before and at the study entry. A brief questionnaire regarding children's diet, lifestyle and medication was conducted among parents. The stool samples were collected by parents at home and delivered to the laboratory within 2 h in refrigerated bags.

3.2.2. Isolation of *Bacteroides dorei*

In order to isolate *Bacteroides dorei* and other *Bacteroides* species from faeces samples the culture medium *Bacteroides Vulgatus* Selective Agar (BVSA) was used. For the isolation of *Bacteroides*, stool specimens at arrival to the laboratory a sample of about 0.2-0.3 g was collected to a sterile 2 mL Eppendorf, and the faeces were resuspended in 1 mL of PBS. The sample was homogenised by vortexing. Afterwards the sample was centrifuged at low speed, 700 xg for 5 minutes, so the solid particles precipitated. After centrifugation 500 μL of the supernatant was collected to a new Eppendorf and serial dilutions were prepared to the dilution 10^{-4}, the remaining supernatant was transferred to another Eppendorf tube. Each stool sample was kept in BHI+H supplemented with 25% (v / v) glycerol. A volume of 100 μL of the dilutions 10^{-3} and 10^{-4} were inoculated in BVSA plate. Two replicates of each dilution were inoculated. The inoculated plates were incubated in an anaerobic jar with an anaerobic sachet (Oxoid) at 37ºC for 3-4 days. Afterwards, the characteristic colonies (black, shiny colonies with a dark halo zone) were counted, and representative colonies of each morphological group were transferred to BHI + H agar medium for obtain pure cultures. The incubation was done at 37ºC for 48 h. Pure cultures were maintained in BHI + H supplemented with 25% (v / v) glycerol at -80ºC.

3.2.3. DNA extraction

DNA extraction was performed for bacterial cultures and stool samples. The procedure for each is described below.

3.2.4. DNA extraction from Stool samples

The extraction of DNA from stool samples was performed using the kit QIAmp DNA Stool Mini Kit (QIAGEN) according to the manufacturer's instructions. The stool quantity used for DNA extraction was 220 mg. The extracted DNA was eluted in 200 µL of AE buffer and maintained at -20ºC until use.

3.2.5. DNA from bacterial cultures

The DNA from bacterial cultures was extracted using the GES method (Pitcher et al. 1989) or using the Wizard Genomic DNA Purification Kit (PROMEGA).

For the GES method, the bacterial culture was grown on BHI + H broth at 37ºC for 48 h days under anaerobic conditions. The bacterial cells were collected by centrifugation at 5000 xg for 10 min. The supernatant was discarded and glass beads (425-600 µm Sigma) were added in the same proportion as the pellet obtained. A volume of 500 µL of lysis buffer was then added and the bacterial suspension was sonicated (Sonorex) for 10 min. Afterwards to improve bacterial lysis cells a vortexing step for 1 min was carried out. A volume of 35 µL of lysozyme (100 mg / mL) was added and the mixture was incubated at 37°C for 1 h 30 min. The liquid phase was collected to a new Eppendorf, to which was added 500 µL of the GES buffer and placed on ice for 10 min. To the previous mixture 250 µL of ammonium acetate (10 M) was then added and the tube was maintained on ice for 20 min. After this time interval 500 µL of a mixture of Chloroform: Isomyl alcohol (24:1) was added and the mixture was homogenized by inversion. Afterwards the tube was centrifuged (16000 xg), 10 min at 4°C). The aqueous phase was collected into a new Eppendorf tube and ½ of the volume of isopropanol (Fluka) was added. The mixture was done by inversion. To collect the isolated DNA a new centrifugation step (13000 rpm, 5 min at 4°C) was done. The isolated DNA was washed with 800 µL of cold alcohol 70%. The washing process was repeated 3 times. The isolated DNA pellet was air-dried and then resuspended in nuclease-free water. DNA quantification was performed on the Nanodrop 2000c spectrophotometer (Thermo Scientific,

Waltham). The integrity of the genomic DNA was verified by running each sample in 1% (w / v) agarose gel electrophoresis (Generose). The agarose gel was prepared with the addition of 5 μL of the dye "Green Safe" at a concentration of 1: 100. In the wells of the gel were loaded 2 μL of DNA and 2 μL of Loading Buffer 6x concentrate (VWR). The marker “GeneRuler ™ DNA Ladder Brews 1 kb" was used. The gel electrophoresis was run in 1x Tris-Acetate EDTA buffer at 120V for 30-40 min.

The DNA samples were aliquoted and maintained at -20°C until use.

3.2.5.1.DNA purification

The extracted DNA from the GES method showed frequently to be contaminated with RNA, and in order to eliminate this contamination after hydration of the DNA sample using nuclease free water, a centrifugation step (3000 rpm, 20 min at 4°C) was done. The supernatant was transferred to a new 2 mL Eppendorf. Following 3 μL of RNAse (10 mg / mL) was added. The tube was maintained at room temperature for 15 min. After this time interval, 1/10 volume of 3 M sodium acetate and 2 volumes of 100% ethanol was added. The tube was incubated in the freezer (-20°C) for 30 min. Afterwards the mixture was centrifuged (3000 rpm, 10 min at 4°C), and the precipitated DNA was washed twice with 70% ethanol following each a centrifugation step (3000 rpm, 5 min at 4°C). The ethanol was allowed to evaporate and 20 μL of nuclease free water was added to re-hydrate the DNA.

3.2.6. Amplification of 16S gene by polymerase chain reaction (PCR)

The gene 16S was amplified by polymerase chain reaction (PCR) in order to identify the isolates of *Bacteroides,* and as well to confirm the identification of the reference bacteria. A final reaction volume of 25 μL was used. The composition of the PCR reaction according to the target is indicated **Table 3.2.1.**

The PCR reactions were performed in the thermocycler T-personal or T1 (Biometra) according to the conditions indicated in **Table 3.2.1**

The amplicon of each reaction was visualized through gel electrophoresis as described in section 3.2.5.

The amplicons of the appropriate size were quantified and sent for sequencing at the Molecular Biology Laboratory of the Centro Ciências Mar (CCMar) using standard Sanger sequencing procedures using the *1492R* primer. The 16S sequences were analysed and those ≥1200 bp were selected for analysis. The edition of the sequences was done using Bioedit. The identification of the closest matches to sequence queries was performed using the Blast algorithm of the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The Ribosomal Database Project (Cole *et al.*, 2014) (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) was also used for the determination of the isolates at the species/strain level. Phylogenetic analysis and tree construction were conducted with the software MEGA 5 (Tamura *et al.*, 2011). A dendogram was generated using standard pairwise alignment and underweighted pair group method UPGMA clustering.

Table 3.2.1. Composition of the PCR reaction and amplification conditions

Organism	Reagent	Volume (µL)	Conditions		
			Cycles	Temperature (ºC)	Time
Bacteroides dorei/ Bacteroidetes/ Lactobacillus casei/ Bifidobacterium longum infantis	Buffer	2.5	1	94	7 min
	$MgCl_2$	2.0			
	DNTP's	0.5	35	94	1 min
	27F	0.5		59	1 min
	1492R	0.5		72	1 min 30 s
	Taq	0.125	1	72	5 min
	DNA	17.875			
Enterococcus faecalis	Buffer	2.5	1	94	7 min
	$MgCl_2$	2.0			
	DNTP's	0.5	35	94	1 min
	799F	0.5		59	1 min
	1492R	0.5		72	1 min 30 s
	Taq	0.125	1	72	5 min
	DNA	17.875			
Bacteroides dorei	Buffer	2.5	1	94	5 min
	$MgCl_2$	0.5	30	94	1 min
	DNTP's	0.5			
	Bd F	0.5		60	45 s
	Bd R	0.5			
	Taq	0.125		72	1 min
	DNA	19.375			
Bacteroidetes	Buffer	2.5	1	94	3 min
	$MgCl_2$	1	30	94	1 min
	DNTP's	0.5			
	Bactes F	0.5		60	45 s
	Bactes R	0.5			
	Taq	0.125		72	45 s
	DNA	18.875			
Bacteroides dorei	Buffer	2.5	1	94	10 min
	$MgCl_2$	0.5			
	DNTP's	0.5	30	94	40 s
	BdRTi F	0.5		59	45 s
	BdRTi R	0.5		72	30 s
	Taq	0.125	1	72	5 min
	DNA	19.375			

Table 3.2.1. (continued)

Organism	Reagent	Volume (µL)	Conditions		
			Cycles	Temperature (ºC)	Time
Firmcutes	Buffer	2.5	1	95	5 min
	$MgCl_2$	0.5	30	95	15 s
	DNTP's	0.5			
	Ftes F	0.5		60	1 min
	Firm 1060R	0.5			
	Taq	0.125		72	30 s
	DNA	19.375			
Lactobacillus casei	Buffer	2.5	1	95	3 min
	$MgCl_2$	2.5	40	94	15 s
	DNTP's	0.5			
	Lb F	0.5		58	20 s
	Lb R	0.5			
	Taq	0.125		72	30 s
	DNA	17.375			
Bifidobacterium longum infantis	Buffer	2.5	1	94	5 min
	$MgCl_2$	0.5			
	DNTP's	0.5	30	94	40 s
	g-Bifid-F	0.5		50	40 s
	g-Bifid-R	0.5		72	30s
	Taq	0.125	1	72	5 min
	DNA	19.375			

3.2.7. Characterization of the isolates of *Bacteroides* by repetitive sequence-based PCR

The *Bacteroides* isolates, including *B. dorei*, *B. xylanisolvens* and *B. uniformis* were analysed by rep-PCR with *BOXA1R* primer and *ERIC1R* and *ERIC2* primers (Versalovic *et al.*, 1991; Atherly and Ziemer, 2014). The composition of the PCR reactions is indicated in **Table 3.2.2.** The PCR reactions were performed with Platinum Taq polymerase (Invitrogen). The PCR reaction composition and conditions are indicated in **Table 3.2.2.** The PCR amplicons (25 µL) were separated by gel electrophoresis on 1.5% Agarose (Generose) gel in TAE 1x buffer at 100 V during 6 h. The 100 bp and 500 bp markers (Invitrogen) were used. Gels were stained with ethidium bromide for 30 min and visualized under UV light using the DC 290 Kodac camera.

Analysis of the gel banding patterns was done using GelCompare II (Biomérieux) and dendograms were generated using Pearson correlation similarity coefficient with optimization of 1% and the cluster analysis was performed using the Unweighted Pair Group Method using Arithmetic Averages (UPGMA) clustering method.

Table 3.2.2. Composition of the PCR reactions and the conditions of amplification.

Reagent	Amount per sample (µL)
Buffer	**2.5**
$MgCl_2$	**1**
DNTP's	**0.5**
BOXA1R	**1**
Taq	**0.125**
H_2O MilliQ	**18.725**
DNA	**1.0**

Reagent	Amount per sample (µL)	Conditions for both reactions
Buffer	**2.5**	94 ºC 10min
$MgCl_2$	**1**	94 ºC 40s
DNTP's	**0.5**	30x 59 ºC 45s
ERIC1R	**1**	72 ºC 30s
ERIC2	**1**	72 ºC 5min
Taq	**0,125**	
H_2O MilliQ	**17,875**	
DNA	**1.0**	

3.2.8. Insertion of the fragment of interest (16S gene) into the cloning vector

Genomic DNA (gDNA) or vectors carrying the cloned target sequences are commonly used as standards in quantitative PCR (qPCR). In this study the use of vectors containing the 16S gene of each target bacterial group to be quantified by real-time PCR was selected. The amount of insert to be used was calculated according with the following formula:

$$\frac{ng\ of\ vector\ \times kb\ size\ of\ insert}{kb\ size\ of\ vector} \times molar\ ratio\ of\ \frac{insert}{vector} = ng\ of\ insert$$

For cloning the 16S gene of *L. casei* was done using the vector pNZY28 (NZYTech). However, the cloning process using this vector for the other 16S genes was unsuccessful. So, the cloning of the 16S gene of *B. dorei*, *B. uniformis*, *E. faecalis*, and *B. infantis* was performed using the vector pCR ™ 2.1-TOPO (Invitrogen).

For the ligation of the 16S gene into the vector the amplified PCR product (25 µL) was run in a gel electrophoresis and after recovery from the agarose gel was purified using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). The components of the ligation reactions for each vector are presented in **Table 3.2.3.** and **Table 3.2.4.**

Table 3.2.3. Components of the ligation reaction for the vector pNZY28.

Components of the reaction	Volume	
	Sample	**Control**
DNA	X µL corresponding to 78.13ng	----
Ligase buffer (4x)	2.5 µL	2.5 µL
pNZY28 (50 ng/µl)	1 µL	1 µL
Speedy ligase (5U/µl)	1 µL	1 µL
H_2O sterile	Up to 10 µL	Up to 10 µL

Table 3.2.4. Components of the ligation reaction for the vector pCR ™ 2.1-TOPO.

Components of the reaction	Volume	
	Sample	**Control**
DNA	X µL corresponding to 78.13ng	----
Salt solution (1.2M NaCl and 0.06M $MgCl_2$	1 µL	1 µL
H_2O sterile	Up to 5 µL	Up to 5 µL
pCR ™ 2.1-TOPO (10 ng/µl)	1 µL	1 µL

3.2.9. Generation of Competent cells

The bacterial transformation process includes the conversion from one genotype to a different genotype through the reception of exogenous DNA. This process was firstly identified in the bacterium *Streptococcus pneumoniae* in 1928 (Robyt and White, 1990). However, not all bacterial species can capture external DNA from their environment, and the ones that are able to do it are designated by competent. Nevertheless, natural transformation is not a common process. To proceed with bacterial transformation it is necessary to use a suspension of rapidly dividing bacteria in a cold "transformation buffer" that is usually a cold

calcium chloride solution and then expose the bacterial cells to a very short thermal shock (40s) in the presence of the DNA fragment to be incorporated. This transformation technique allows an efficiency of 10^5-10^7 transformants per μg of DNA.

So, in order to generate competent cells for transformation with pNZY28 and pCR ™ 2.1-TOPO the bacterial cells of *E. coli* DH5α and MACH1 (Invitrogen) were grown in *Luria Bertani* (LB) agar (Oxoid). An isolated colony was selected and transferred to 10 mL of LB broth. The incubation occurred overnight in the water bath at 37°C with agitation (120 rpm). Afterwards a volume of 200 μL of the previous culture was transferred to a new tube with 10 mL of LB broth. The culture was grown under the same conditions until an absorbance at 550 nm (A_{550nm}) was 0.5. Meanwhile several sterile 1.5 mL Eppendorfs were refrigerated at -20ºC. This step is very important since assures that the competent bacteria that are fragile will lose their state of competence. The bacterial culture was then centrifuged (2790 xg) during 5min at 4ºC. The supernatant eliminated and the bacterial pellet was resuspended in 5 mL of (15% glycerol, 5 mM Tris-HCl pH 7.5, 5 mM magnesium chloride and 100 mM calcium chloride). The bacterial suspension was maintained on ice for 5 min and then centrifuged (2790 g, 5 min at 4ºC). The supernatant was eliminated and the cells were again resuspended in 50 mM $CaCl_2$ placed and kept on ice for 20 min. Afterwards were centrifuged (2790 xg, 5 min at 4ºC) and the pellet was resuspended in 1 mL of 50 mM $CaCl_2$ and placed again on ice for 1h 30 min. Finally, 1 mL of 50% LB + glycerol was added and 100 μL was distributed through the refrigerated Eppendorf tubes. The tubes were maintained at -80°C until use.

3.2.10. Transformation of Competent cells

Using the transformation technique is possible to generate billion of copies of DNA fragment of interest by growing the transformed cells in the appropriate culture medium.

To transform *E. coli* DH5α 5 μL of the ligation reaction was transferred to 100 μL of competent cells. The mixture was maintained on ice for 30 min. Afterwards a thermal treatment was done (42ºC for 40 s) in order to the cell membrane become permeable to the entry of the vector. After that it was put the cells in ice for 2 min, and add 900 μL of LB.

To transform MACH1 2 μL of the ligation reaction were transferred into the tube with competent MACH1 cells previously turned competent as described above, and placed on ice

for 30 minutes. Afterwards the thermal treatment was done (42ºC for 30 s) and after that was placed immediately on ice for 2 min and then 250 µL of LB was added.

The two different bacterial cultures were incubated at 37ºC with agitation (180 rpm) during 90 min in order to allow the sufficient replication of the vector. Afterwards the bacterial cells were inoculated in LB agar supplemented with 100 mg/mL of ampicillin and 40 µL of X-Gal **(**5-bromo-4-cloro-3-indolil-beta-D-galacto-piranoside) (50 mg / mL) (Fermentas). After 16-18 h the transformed cells were counted and a few were selected for examination by PCR colony. It is convenient to stress that the transformed cells are able to growth on the presence of the antibiotic and have a white appearance once the insertion of the exogenous DNA fragment on the vector inactivates the synthesis of the β-galactosidase, not allowing the degradation of the X-Gal generating, this way, white colonies.

3.2.11. Extraction and purification of plasmid DNA ("Miniprep")

Once the transformed clones are selected is necessary to isolate and purify the vector with the insert of interest. To achieve such a process designated by Miniprep is being used. This process is divided in three phases: i) growth of the selected clones in culture medium (liquid), ii) collection and lysis of the bacterial cells, and iii) purification of the plasmid DNA by precipitation. So, in order to extract and purify the plasmids from our selected clones a selected colony was inoculated into 5 mL broth of LB supplemented with 100 µg / mL ampicillin. The bacterial culture was grown in a water bath overnight with shaking (120 rpm) at 37°C. Afterwards 2 mL of this culture was transferred to an Eppendorf which was centrifuged (16000 xg at 4ºC for 2 min). The supernatant was removed, and the bacterial pellet was resuspended in 200 µL of GTE buffer and 10 µL of RNAse A (10 mg / mL) (Thermo Scientific). The mixture was homogenized by vortexing and left at room temperature for 10 min. Afterwards 400 µL of a freshly prepared solution of 0.2 M NaOH/SDS was added and homogenized by inversion (5-6 times) and placed on ice for 5 min. At the end of the time interval 300 µL of 3M potassium acetate (cold) was added and thoroughly homogenized by inversion. The mixture was incubated for 30 min on ice following a centrifugation step (16000 xg, 15 min). The supernatant was transferred to a new Eppendorf where 1 mL of 100% ethanol was added in order to precipitate the DNA. The mixture was homogenized by vortexing and it was incubated at room temperature for 2 min. Following the tube was

centrifugated (16000 xg, 15 min at 4°C), and the precipitated DNA was washed twice with cold 70% ethanol. Finally, the plasmid DNA was air-dried until the ethanol evaporated and the DNA was dissolved in nuclease free water. The plasmid DNA was maintained at -20°C until use.

3.2.12. qPCR

In order to perform the quantification of the bacterial groups of interest, a quantitative real-time PCR (qPCR) was performed.

In the current study the SsoFastTM kit Eva Green Supermix (BioRad) was used. The PCR reactions contained 5 μL of the mixture, 0.5 μL of each primer, 3 μL of water and 1 μL of the DNA sample. For each bacterial group a standard curve was constructed which was obtained by amplifying the 16S rRNA gene target. The qPCR reaction for the construction of the standard curve included 5 μL of the mixture, 0.5 μL of each primer, 1.5 μL of water and finally 1.0 μL of the plasmid DNA sample. The standard curves were generated according to the average number of copies of the 16S rRNA gene for each bacterium. Four replicates of the plasmid DNA dilutions in order to obtain the number of copies that ranged from 10^{10}-10 were prepared according to the formula.

$$Plasmid\ concentration\ =$$
$$number\ of\ copies \times \left((bp\ of\ the\ insert + bp\ of\ the\ vector) \times 1.096^{-21}\right) \times$$
$$volume\ of\ the\ plasmid\ need\ in\ the\ PCR$$

The number of copies of each bacterial group in the samples was calculated from each standard curve.

Finally, the efficiency of each pair of primers was calculated using the following formula

$$Efficiency = 10^{\left(-\frac{1}{slope}\right)} - 1$$

It is very important to calculate the efficiency of the primers, since not all sequences are amplified due to several factors, such as inhibitors, poor primer delineation, or even human error.

The qPCR reaction for the amplification of the faecal samples was similar to the above but was used 2.5 μL of the DNA sample.

4. Results

4.1. Isolation and identification of *Bacteroides* spp.

Our main goal was to evaluate the intestinal *Bacteroides* population, and particularly the isolation of *B. dorei* in T1D children in the Algarve region, according to this it was possible to collect 17 feces samples from T1D children and 17 samples from Control children, including 10 samples from T1D healthy siblings and 7 samples from non-T1D children to proceed with our aim. The isolation of *B. dorei* was performed by analyzing between 30 to 40 characteristic colonies. A characteristic plate of BVSA medium with potential *Bacteroides* colonies is illustrated in **Fig. 4.1.1**. Representatives of the different colony patterns, namely the size of the colony, degree of esculin hydrolysis, and the form and margin of the colony were isolated. An isolated colony of *B. dorei* strain PtF D1P5 in BHI agar is illustrated in **Fig 4.1.2**. The samples from which no *B. dorei* was isolated were evaluated a second and even a third time. In this study over 3000 isolates were studied.

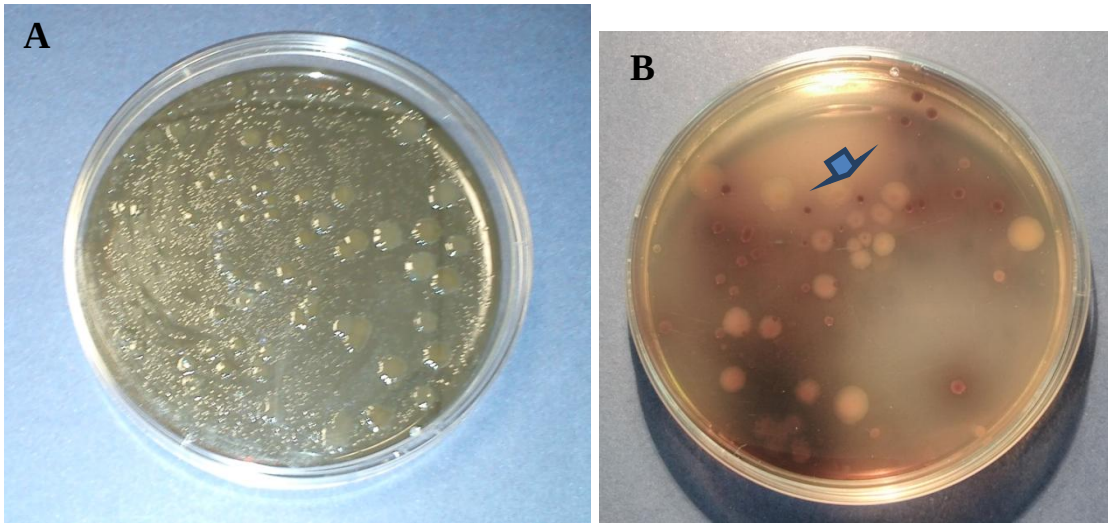


Fig. 4.1.1. A representative plate of the isolation of *Bacteroides* spp. in BVSA medium from a fecal sample (dilution 10^{-2}) (A), a characteristic colony of *Bacteroides* sp. is indicated by and arrow (blue) (B).

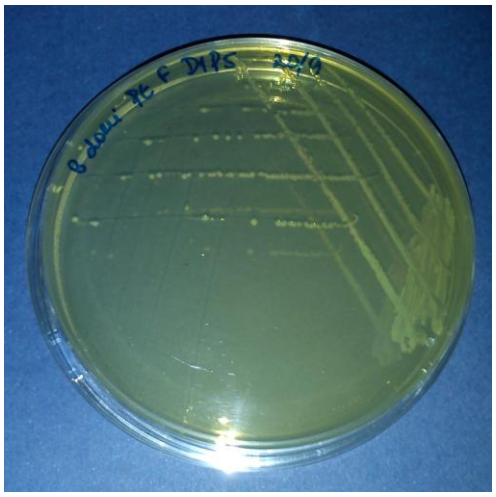


Fig 4.1.2. The isolate of *B. dorei* PtF D1P5 in BHIagar.

The population of *Bacteroides* spp. (Log $_{10}$ CFU / g feces) in the feces samples is indicated in **Table 4.1.1**. The mean of the counts of *Bacteroides* spp. in the T1D group was 6.60±0.1 Log_{10} CFU /g feces. The T1D sample that showed the lowest number of *Bacteroides* was D14 (Log_{10} CFU / g feces 4.91±0.01) whereas the D1 and D20 samples showed the highest counts (Log_{10} CFU / g feces 7.93±0.01 and 7.73±0.03, respectively). In the Control samples the lowest number of *Bacteroides* was observed on the sample Sb6 and C3 (5.64±0.04 and 5.46±0.04, respectively) whereas the samples Sb15 and C6 showed the highest number, 7.29±0.07 and 7.25±0.07 Log_{10} CFU / g feces, respectively.

Table 4.1.1. Determination of *Bacteroides* spp. counts in fecal samples of T1D and Control children

Sample	Log_{10} CFU / g feces	Sample	Log_{10} CFU / g feces
	T1D		**Control**
D1	7.93±0.01	**Sb1**	6.72±0.01
D2	7.42±0.05	**Sb2**	6.30±0.07
D3	6.26±0.46	**Sb3**	6.15±0.04
D4	6.14±0.15	**Sb6**	5.64±0.04
D5	6.44±0.23	**Sb7**	6.51±0.05
D6	6.24±0.12	**Sb8**	6.49±0.07
D7	6.56±0.04	**Sb9**	6.64±0.05
D8	6.48±0.23	**Sb13**	6.96±0.19
D9	6.94±0.06	**Sb15**	7.29±0.01
D10	6.13±0.01	**Sb19**	6.09±0.01
D13	6.06±0.17	**C1**	5.28±0.10
D14	4.91±0.01	**C2**	6.71±0.06
D15	7.08±0.02	**C3**	5.77±0.11
D16	6.98±0.06	**C4**	5.46±0.04
D17	7.39±0.02	**C5**	6.64±0.02
D19	6.15±0.02	**C6**	7.25±0.07
D20	7.73±0.03	**C7**	6.79±0.05
Mean	6.64±0.74[a]	**Mean**	6.40±0.06[a]

Data represent the mean ± standard deviation of 2 replicates. The mean values with the same superscript letter are not significantly different (P>0.05).

The identification of the *Bacteroides* isolates representative of the observed colonies in the T1D and Control samples was done by sequencing the *16S* gene. In order to screening the potential *B. dorei* isolates a PCR reaction with the primers *BdF / R* primers was performed. These primers target the gene that codifies β-galactosidase of *B. dorei*, and were validated using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against several *Bacteroides* genomes, and particularly attention was given to *B. vulgatus* results since these two species are very similar. No overlapping with other *Bacteroides* species was observed. However, through our work we observed that several isolates give an amplicon similar to the expected but were not identified as *B. dorei*. The amplification of the gene that codifies the β-galactosidase of *B. dorei* is illustrated in **Fig. 4.1.3** as observed from the eight amplicons seven showed the expected size (445 bp). However, just two of them were identified as *B. dorei* by sequencing the *16S* gene.

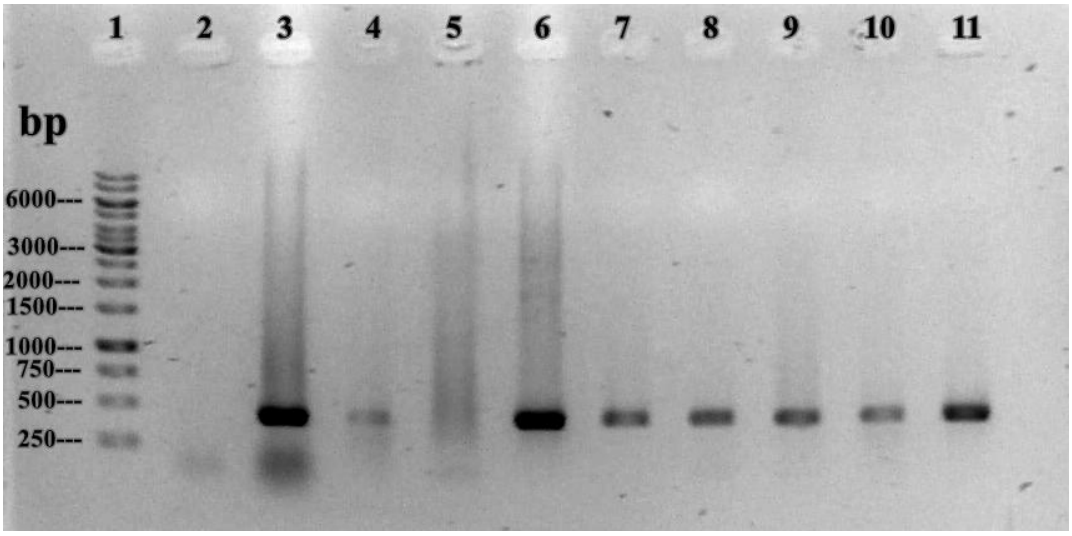


Fig. 4.1.3. Amplicons of the PCR reaction with the *BdF / R* primers.1 - Ladder (GeneRuler ™) 2 – Control reaction (no DNA added), 3 - *Bacteroides dorei* DSM 17855, 4 - 11 – Potential isolates of *B. dorei* from the T1D sample D1. The expected size of the amplified fragment is 445 bp. Only the amplicons of the well 6 and 11 were identified as *B. dorei* in the further sequencing of the *16S* gene.

To overcome this problem a new pair of primers were designed in order that could also be used in real time PCR. The new primers target the *16S rRNA* gene of *B. dorei*. The primers were validated through Ribosomal Database Project (http://rdp.cme.msu.edu/probematch/search.jsp), and were also tested by real time PCR using DNA of different species of *Bacteroides*. The amplification reaction by real time PCR is illustrated in the **Fig. 4.1.4**. The Ct (cycle threshold) values for *B. dorei* 17855 was 6.6 and 9.3 for *B.dorei* D1P5, whereas for *B. uniformis* was 22.5, *B. xylanisolvens* 30.2 and for *B. vulgatus* and *B. thetaiotaomicron* no Ct signal was observed whereas for *Bifidobacterium longum* (an outsider gut bacterium) was 43.8. The selected isolates that showed a characteristic Ct value in real time PCR were subjected to sequencing of the *16S rRNA* gene, and all were identified as *B. dorei*. The **Fig. 4.1.4.1** illustrates the melting curve of the qPCR amplicons shown on **Fig. 4.1.4**. and in the **Fig. 4.1.4.2** is the agarose gel electrophoresis of the qPCR amplicons. The follow up analysis of the melting curve and the agarose gel electrophoresis reveals that the Ct value for *B. uniformis* corresponds to an amplicon that has a higher size (>250 bp) than the expected (120 bp) and none of the other *Bacteroides* spp showed any amplification product. The melting curve of *B. uniformis* amplicon showed a value of dRFU / dT lower in comparison to the *B. dorei* (**Fig. 4.1.4.1**).

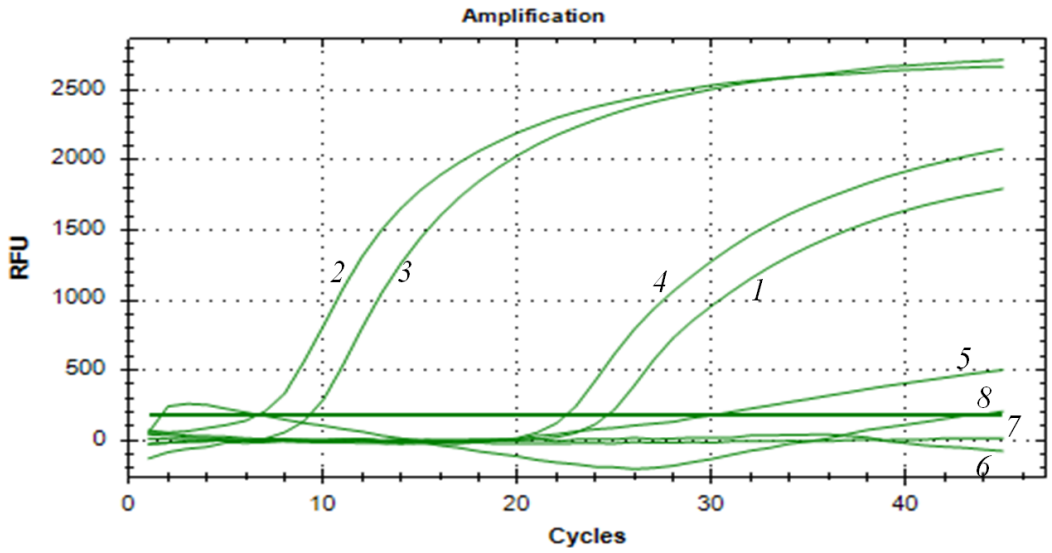


Fig. 4.1.4. Real time PCR using the primers *BdRTiF / R*. The amplification of the *16S rRNA* amplicon relative to 1) No DNA added (Control), 2) *B. dorei* DSM 17855, 3) *B. dorei* PtF D1P5, 4) *B. uniformis*, 5) *B. xylanisolvens*, 6) *B. vulgatus*, 7) *B. thetaiotaomicron*, 8) *Bifidobacterium longum* subsp. *longum*.

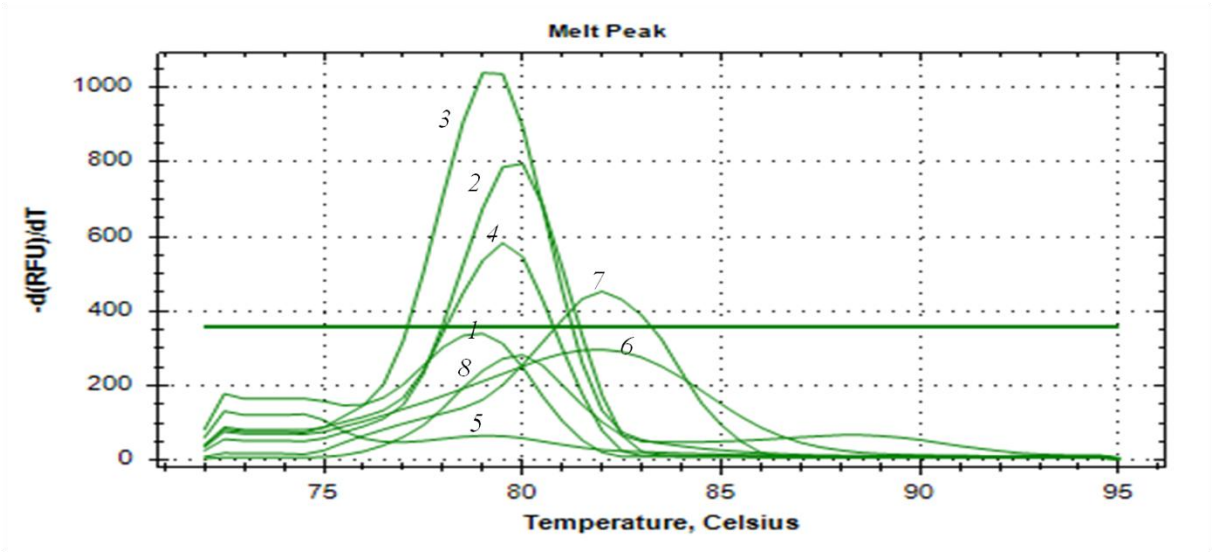


Fig.4.1.4.1. Melting curves of the amplicons using the primers *BdRTiF / R* represented in Fig. 4.1.4. The melt curve of the amplicons of 1) No DNA added (Control), 2) *B. dorei* DSM 17855, 3) *B. dorei* PtF D1P5, 4) *B. uniformis*, 5) *B. xylanisolvens*, 6) *B. vulgatus*, 7) *B. thetaiotaomicron*, 8) *Bifidobacterium longum* subsp. *longum*.

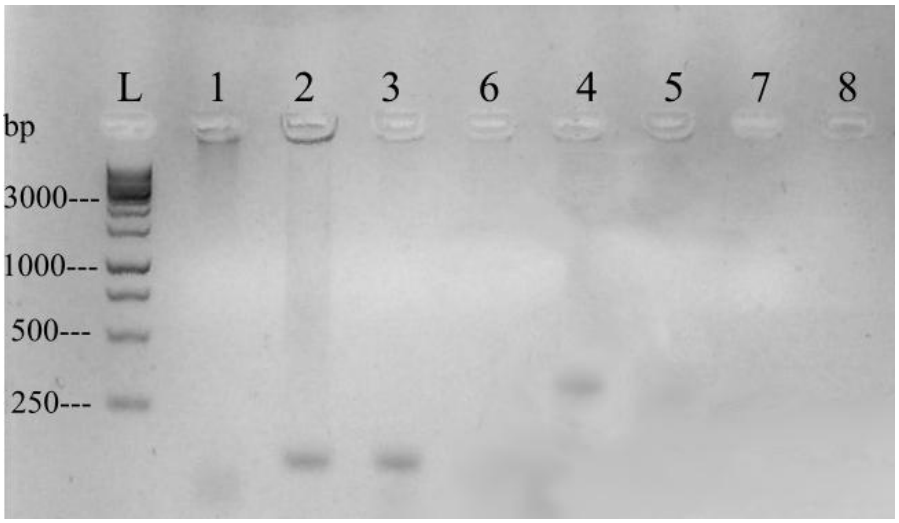


Fig.4.1.4.2. Agarose gel electrophoresis of the amplicons using the primers *BdRTiF / R* represented in Fig 4.1.4. The melt curve of the amplicons of 1) No DNA added (Control), 2) *B. dorei* DSM 17855, 3) *B. dorei* PtF D1P5, 4) *B. uniformis*, 5) *B. xylanisolvens*, 6) *B. vulgatus*, 7) *B. thetaiotaomicron*, 8) *Bifidobacterium longum* subsp. *longum*. L) Ladder (GeneRuler ™).

The **Fig. 4.1.5** illustrates the amplification of the *16S rRNA* gene with an expected size of about 1500 bp. All bacteria isolated in this study showed an approximately 1.5 Kb to the relative DNA size marker (**Fig. 4.1.5**).

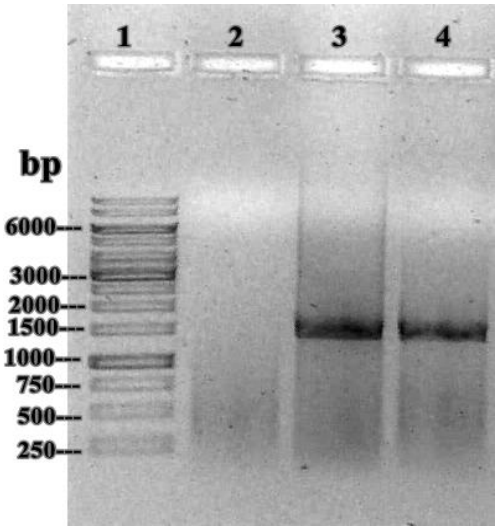


Fig. 4.1.5. Agarose gel of the amplicons produced using the primers *27F* and *1492R* 1 - Marker 1 Kb (GeneRuler ™), 2 - negative control (no DNA added), 3 – DNA *Bacteroides dorei* DSM 17855, 4 – DNA of the isolate from T1D sample D1. Expected size 1500 bp.

Table 4.1.2 summarizes the identified species of *Bacteroides* in T1D and Control samples and **Fig. 4.1.6** illustrates the% of identification of each *Bacteroides* specie in each group. It was only possible to recover *B. dorei* in culture from the T1D samples D1, D8 and D16, reaching 10.34% whereas in Controls *B. dorei* was recovered from the samples Sb6, Sb8, Sb19 and C1 achieving 18.18%. In T1D samples several *Bacteroides* species were recovered at similar percentages to *B. dorei*, such as *B. uniformis*, *B. vulgatus* and *P. distasonis* (10.34%) (**Fig. 4.1.6**). The species *B. ovatus* and *B. xylanisolvens* were recovered at analogous percentage (13.79%) (**Table 4.1.2, Fig. 4.1.6**). *B. thetaiotaomicron* was retrieved from T1D samples at 6.90%, in contrast to Control samples from which was recovered at 9.09% (**Fig. 4.1.6**). The species *B. graminisolvens*, *B. cellulosilyticus, B. finegoldii*, *B. stercoris*, *B. eggerthii* and *B. fragilis* were just isolated from one T1D sample (3.45%) and none from Controls (**Table 4.1.2**, **Fig. 4.1.6**).

In Control samples the specie *B. uniformis* was recuperated in culture at analogous percentage to *B. dorei*, 18.18%, and the species *B. ovatus*, *B. vulgatus* and *B. xylanisolvens* were recovered from these samples at the same percentage, 13.64%. The specie *B. caccae* was recovered at 9.09%, in contrast to the T1D samples that achieved 3.45%. *P. distasonis* just achieved 4.55% in Control samples, in contrast to T1D samples that was recovered at 10.34%.

Overall the core of *Bacteroides* species identified in the two groups besides *B. dorei* includes the species *B. ovatus*, *B. vulgatus*, *B. uniformis* and *B. xylanisolvens*.

Table 4.1.2. *Bacteroides* isolates identified in T1D and Control samples by sequencing the *16S* rRNA *gene*

Sample	Identification	Sample	Identification
D1	*Bacteroides ovatus* *Bacteroides graminisolvens* *Parabacteroides distasonis* *Bacteroides dorei* *Bacteroides vulgatus*	Sb1	*Bacteroides caccae* *Bacteroides vulgatus*
D2	*Parabacteroides distasonis* *Bacteroides cellulosilyticus* *Bacteroides thetaiotaomicron* *Bacteroides vulgatus*	Sb2	*Bacteroides xylanisolvens* *Parabacteroides distasonis* *Bacteroides vulgatus*
D3	*Bacteroides ovatus* *Bacteroides uniformis* *Bacteroides xylanisolvens*	Sb3	*Bacteroides uniformis* *Bacteroides xylanisolvens*
D4	*Bacteroides thetaiotaomicron* *Bacteroides xylanisolvens*	Sb6	*Bacteroides dorei*
D5	*Bacteroides caccae*	Sb8	*Bacteroides dorei*
D6	*Bacteroides xylanisolvens* *Bacteroides uniformis*	Sb9	*Bacteroides caccae*
D7	*Bacteroides finegoldii* *Bacteroides ovatus*	Sb13	*Bacteroides uniformis* *Bacteroides xylanisolvens*
D8	*Bacteroides dorei* *Bacteroides stercoris*	Sb19	*Bacteroides dorei*
D10	*Bacteroides eggerthii*	C1	*Bacteroides dorei*
D13	*Bacteroides xylanisolvens* *Bacteroides fragilis*	C2	*Bacteroides thetaiotaomicron*
D14	*Parabacteroides distasonis*	C3	*Bacteroides uniformis* *Bacteroides ovatus*
D15	*Bacteroides ovatus*	C4	*Bacteroides ovatus*
D16	*Bacteroides dorei*	C5	*Bacteroides ovatus*
D17	*Bacteroides vulgatus*	C6	*Bacteroides vulgatus* *Bacteroides thetaiotaomicron*
D20	*Bacteroides uniformis*	C7	*Bacteroides uniformis*

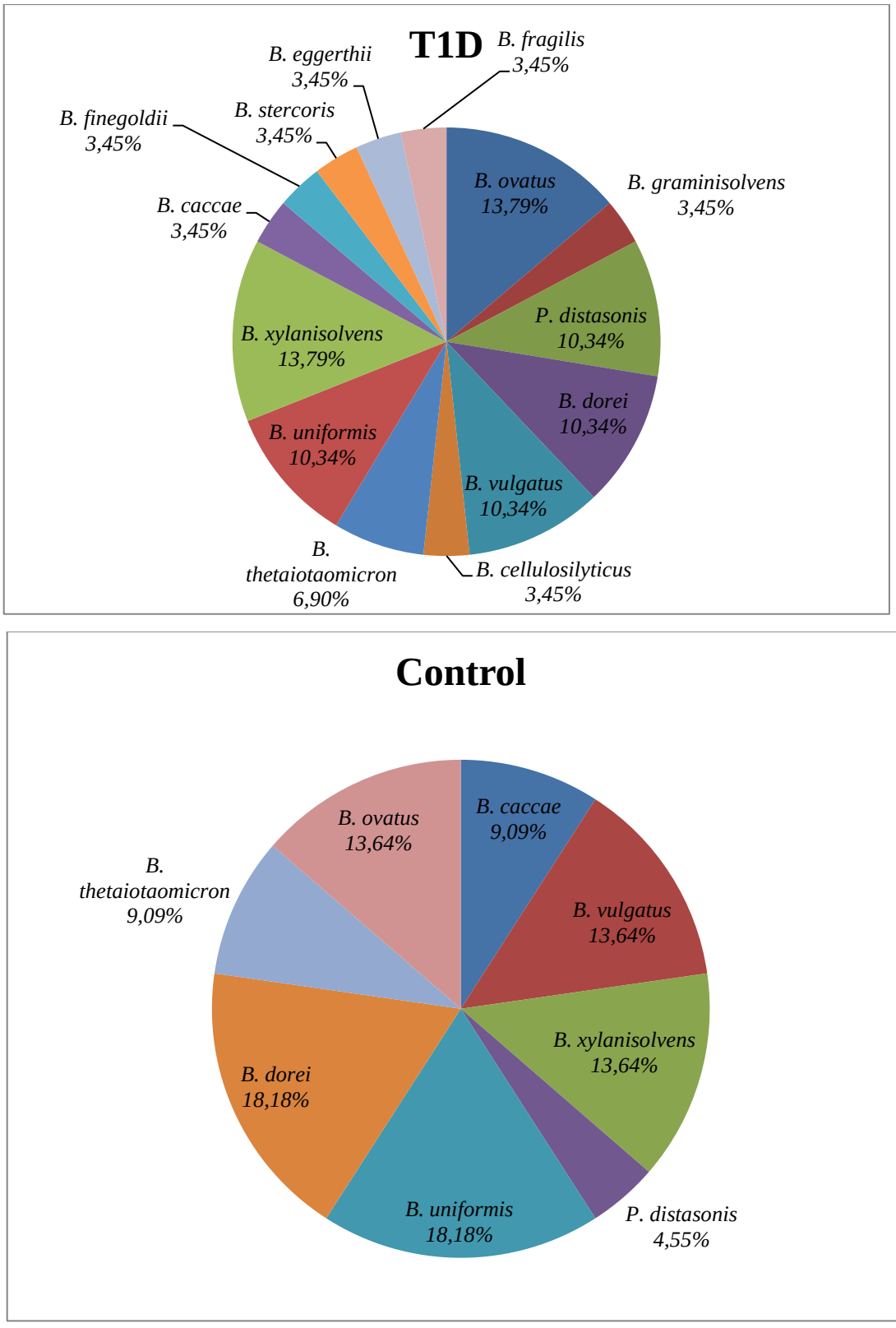


Fig. 4.1.6. *Bacteroides* spp. identified in fecal samples from T1D (A) and Control children (B).

4.2. Phylogenetic analysis

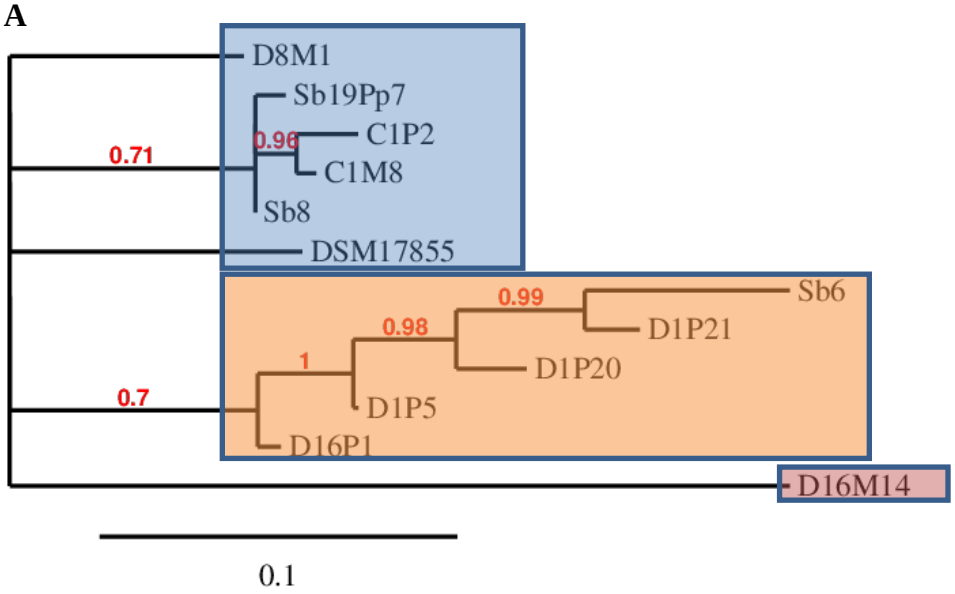
4.2.1. *16S rRNA* phylogeny

The phylogenetic analysis based on the partial *16S rRNA* gene sequencing of the isolates of the species *B. dorei*, *B. uniformis* and *B. xylanisolvens* from T1D and Control samples was able to discriminate several groups of isolates according to the *Bacteroides* species using the Maximum Likelihood tree program, PhyML (**Fig 4.2.1 A, B and C**). Within the sequences of the *B. dorei* isolates (11) the D16M14 formed a branch that was separated from the remaining associated sequences (**Fig 4.2.1 A**). The Maximum Likelihood analysis disclosed the presence of three well resolved lineages according to *16S rRNA* analysis. The similarity between them was higher than 99%. No separation of the isolates recovered from T1D and Control samples was observed. The *16S rRNA* sequence of the reference strain is within the cluster that includes the isolates from the T1D D8 (D8M1) and the isolates from Control samples (Sb19Pp7, C1P2, C1M8 and Sb8). The second cluster is formed by the isolates from the T1D sample D1 and D16 (D1P5, D1P20, D1P21 and D16P1) and also the isolate Sb6 from the Control sample. The third cluster includes just the *B. dorei* isolate from T1D sample D16 (D16M14) (**Fig. 4.2.1 A**).

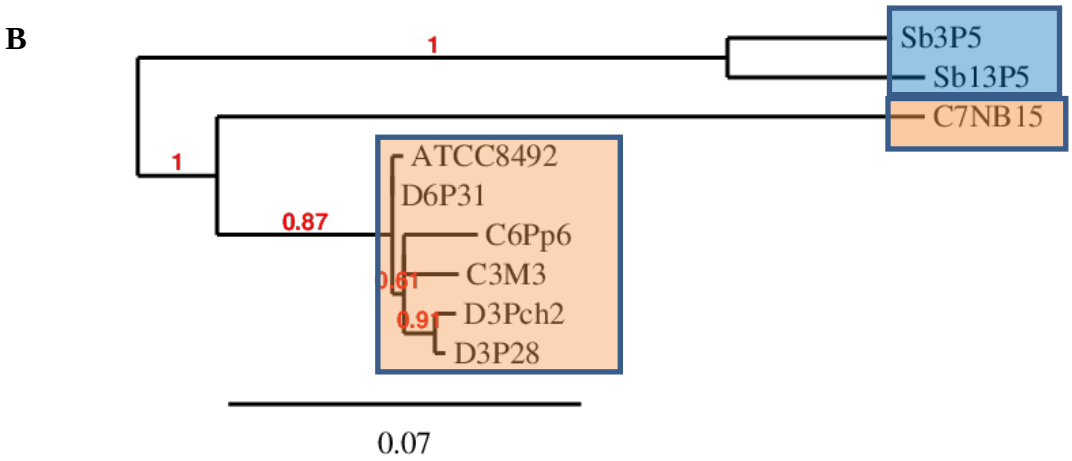
Regarding the 16S rRNA sequence analysis of the *B. uniformis* isolates two resolved lineages were revealed (Fig 4.2.1 B). The first cluster includes the isolates from Control samples Sb3 and Sb13 (Sb3P5 and Sb13P5). The second cluster includes the isolates from T1D samples D6 and D3 (D3Pch2, D3P28, D6P31) and from the Control sample C6 (C6Pp6) and as well the reference strain *B. uniformis* ATCC 8492. As observed with the *B. dorei* isolates also the *B. uniformis* isolates were not clustered according the sampling groups (**Fig. 4.2.1 B**).

The 16S rRNA sequence analysis of the *B. xylanisolvens* isolates from T1D and Control samples showed that these isolates just form a unique cluster with some of them showing higher branches, namely the isolates from the T1D samples D4 and D6 (D4P14 and D6P5) and from the Control sample Sb2 (Sb2PH), and as well the reference strain *B. xylanisolvens* XBA1 (**Fig. 4.2.1 C**). As perceived in the other *Bacteroides* species, *dorei* and *uniformis* also the isolates of the specie *xylanisolvens* did not clustered in agreement with the sampling groups.

A


D8M1
Sb19Pp7
C1P2
C1M8
Sb8
DSM17855
Sb6
D1P21
D1P20
D1P5
D16P1
D16M14
0.71
0.96
0.99
0.98
1
0.7
0.1

B


Sb3P5
Sb13P5
C7NB15
ATCC8492
D6P31
C6Pp6
C3M3
D3Pch2
D3P28
1
1
0.87
0.91
0.07

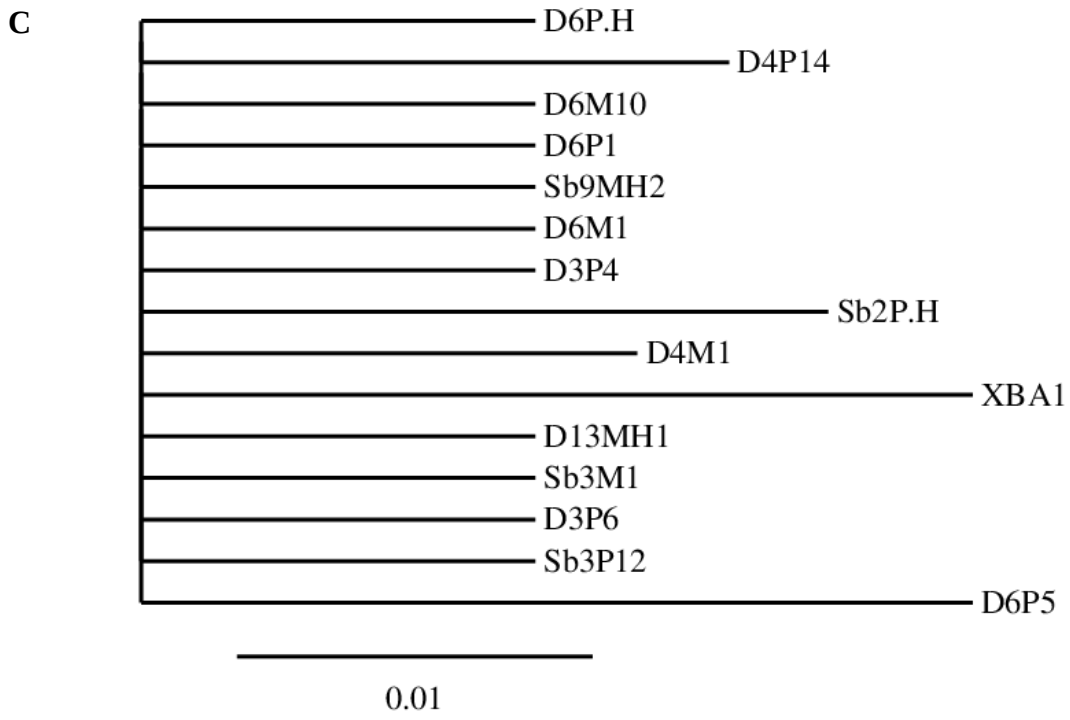


Fig. 4.2.1. Dendogram of the different isolates and reference strains of the *Bacteroides* species, A) *B. dorei* (reference strain DSM 17855), B) *B. uniformis* (reference strain ATCC 8492) and C) *B. xylanisolvens* (reference strain XBA1[=DSM 18836]) based on 16S rRNA gene from T1D (samples indicated with the letter D) and Control samples (samples indicated with Sb or C). The identified clusters within each specie group are highlighted by color boxes. The bar represents the amount of genetic changes and the value in red indicates the bootstrap values.

4.2.2. Rep-PCR analysis

The PCR fingerprints either with the *BOX* primer (BOX-PCR) or the *ERIC* primers (ERIC-PCR) showed band patterns for the different *Bacteroides* spp. isolates (**Fig. 4.2.2** and **4.2.3**). Both BOX and ERIC-PCR generated multiple distinct bands of sizes ranging from approximately 75 to 3500 bp and their number varied between 0 and 13 according to the *Bacteroides* spp. and the rep-PCR (**Fig. 4.2.2** and **4.2.3**).

The cluster analysis of BOX-PCR fingerprints of the isolates identified as belonging to the species *B. dorei*, *B. uniformis* and *B. xylanisolvens* were grouped according to the specie, except the *B. uniformis* D3Pch2 isolate that showed a singleton pattern with 18.6% similarity to *B. dorei* isolates (**Fig. 4.2.4**). The *B. dorei* isolates were divided in three clusters, A, B and C. The reference strain DSM 17855 clustered together with the Control isolate Sb8 with 80.7% similarity forming the cluster A (**Fig. 4.2.4)**. The cluster B included the main *B. dorei* isolates (D16P1, D16M14, Sb19Pp7, Sb6, D1P5, D1P20, D1P21 and D8M1). The cluster C

was formed by the isolates from the C1 sample (C1P2 and C1M8). Overall the BOX-PCR patterns of *B. dorei* isolates were found to be highly related clustering with 60.7% similarity (**Fig. 4.2.4**).

The *Bacteroides* isolates that were identified as belonging to the specie *B. xylanisolvens* formed two main clusters; D and E. In cluster D there were two sub-clusters which included in D1the isolates of the T1D sample D3 and the respective healthy sibling isolates, Sb3 (D3P4, D3M6, D3P6, Sb3P12 and Sb3P12) and D2 was formed by the isolate D13MH1. In cluster E there were four sub-clusters which included in E1 the isolates D4M1 and Sb9MH2. Interesting the E2 sub-cluster was formed by all five *B. xylanisolvens* isolates recovered from T1D sample D6 (D6 P hid, D6M10, D6P1, D6P5, and D6M1) achieving about 99% similarity indicating that they constitute a possible unique strain. The E3 sub-cluster included the isolate D4P14 and the E4 the isolate Sb2Phid. Together the BOX-PCR patterns of *B. xylanisolvens* showed a high similarity (59.4%) (**Fig. 4.2.4**).

Regarding the *B. uniformis* isolates it was observed that the cluster analysis based on the BOX-PCR identified the cluster F that was subdivided in three sub-clusters (F1, F2 and F3). The F1 sub-cluster included the isolates Sb3P5, Sb13P5, D3P28, D6P31, F2 included the isolates from the Control samples C3 and C6 (C3M3, C6Pp6) and the F3 sub-cluster was just formed by the isolate from the Control sample C7 (C7NB15). The% similarity between the *B. uniformis* isolates belonging to the F1 and F2 sub-clusters was very high (67.3%). However, the% similarity displayed by the isolate C7NB15 from the F3 sub-cluster showed a low relatedness with the F1 and F2 sub-clusters (37.5%) (**Fig. 4.2.4**).

It is important to stress that no discrimination of the *Bacteroides* isolates by BOX-PCR of the genotyped species *B. dorei*, *B. uniformis* and *B. xylanisolvens* according to the sampling groups was observed.

The cluster analysis of ERIC-PCR fingerprints of the *Bacteroides* isolates identified four main clusters, A, B, C and D. The cluster A was formed by the isolates of *B. uniformis*, D3p28, sb3P5 and Sb13P5 (**Fig. 4.2.5**). The cluster B grouped the majority isolates of *B. xylanisolvens*, whereas the cluster C was formed by the majority of *B. dorei* isolates but was divided into two sub-clusters; C1 and C2 (**Fig. 4.2.5**). The C1 sub-cluster included eight *B. dorei* isolates; D1P20, D1P21, Sb8, C1M8, D16P1, D16M14, D1P5 and Sb6 whereas the C2 sub-cluster included the reference strain DSM 17855, D8M1 and Sb19Pp7 (**Fig. 4.2.5**). The

cluster D was formed by the *B. dorei* isolate C1P2 and the *B. uniformis* isolates C3M3 and C7NB15 that evidenced a very reduced number of ERIC elements (**Fig. 4.2.5**).

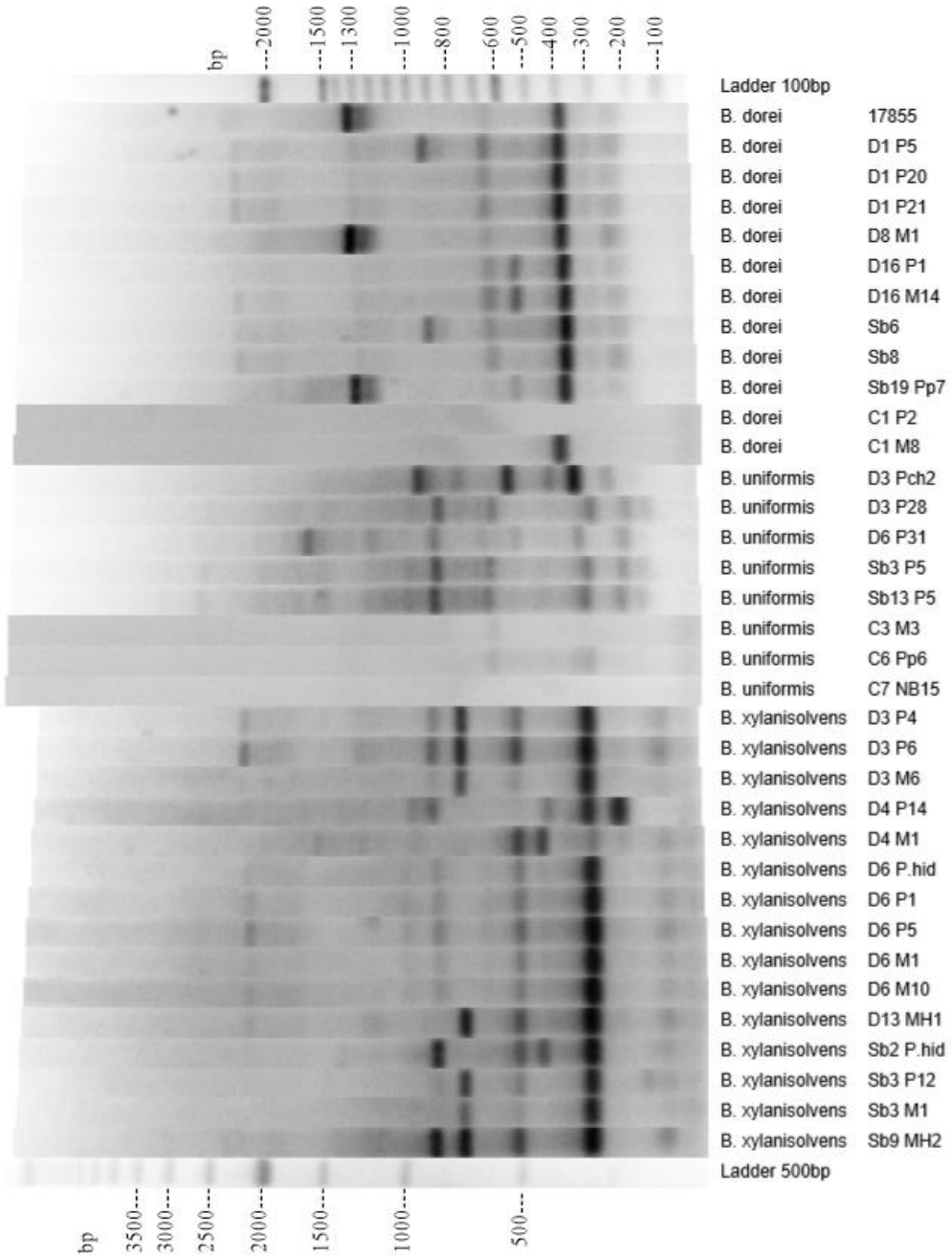


Fig. 4.2.2. BOX-PCR DNA profiles obtained for the *Bacteroides* isolates identified as belonging to the species of *B. dorei*, *B. uniformis* and *B. xylanisolvens* that were collected from T1D and Control feces samples.

Five ERIC-PCR singletons were identified, two of them were isolates of *B. xylanilsolvens*, the D4P14 and D4M1 isolates and three belong to the specie *B. uniformis*, the

D6P31, C6Pp6 and D3Pch2 isolates (**Fig. 4.2.4**). Interesting the *B. uniformis* isolate D3Pch2 showed a singular singleton in both BOX-PCR and ERIC-PCR (**Fig. 4.2.3 and 4.2.4**).

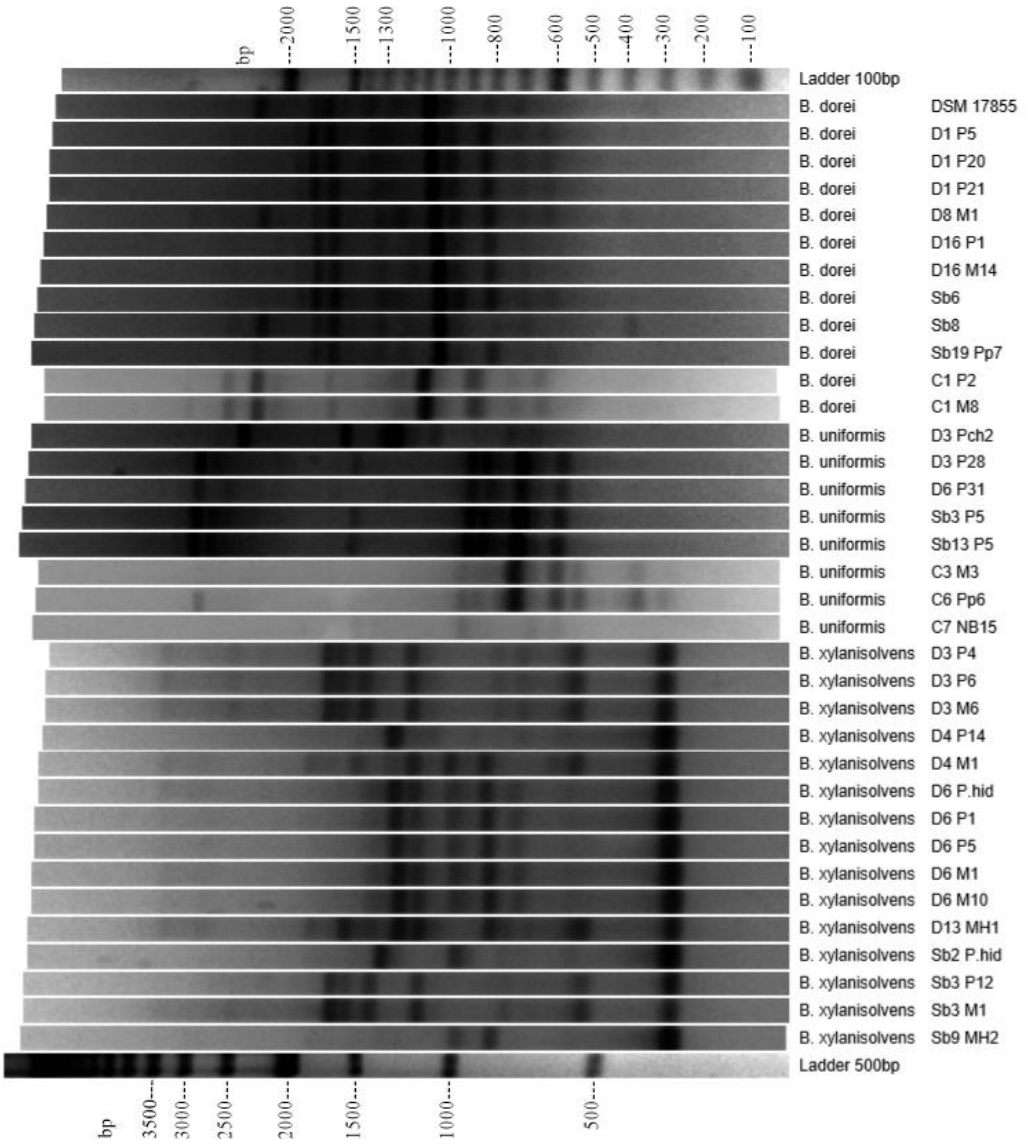


Fig. 4.2.3. ERIC-PCR DNA profiles obtained for the *Bacteroides* isolates identified as belonging to the species *B. dorei*, *B. uniformis* and *B. xylanisolvens* that were collected from T1D and Control feces samples.

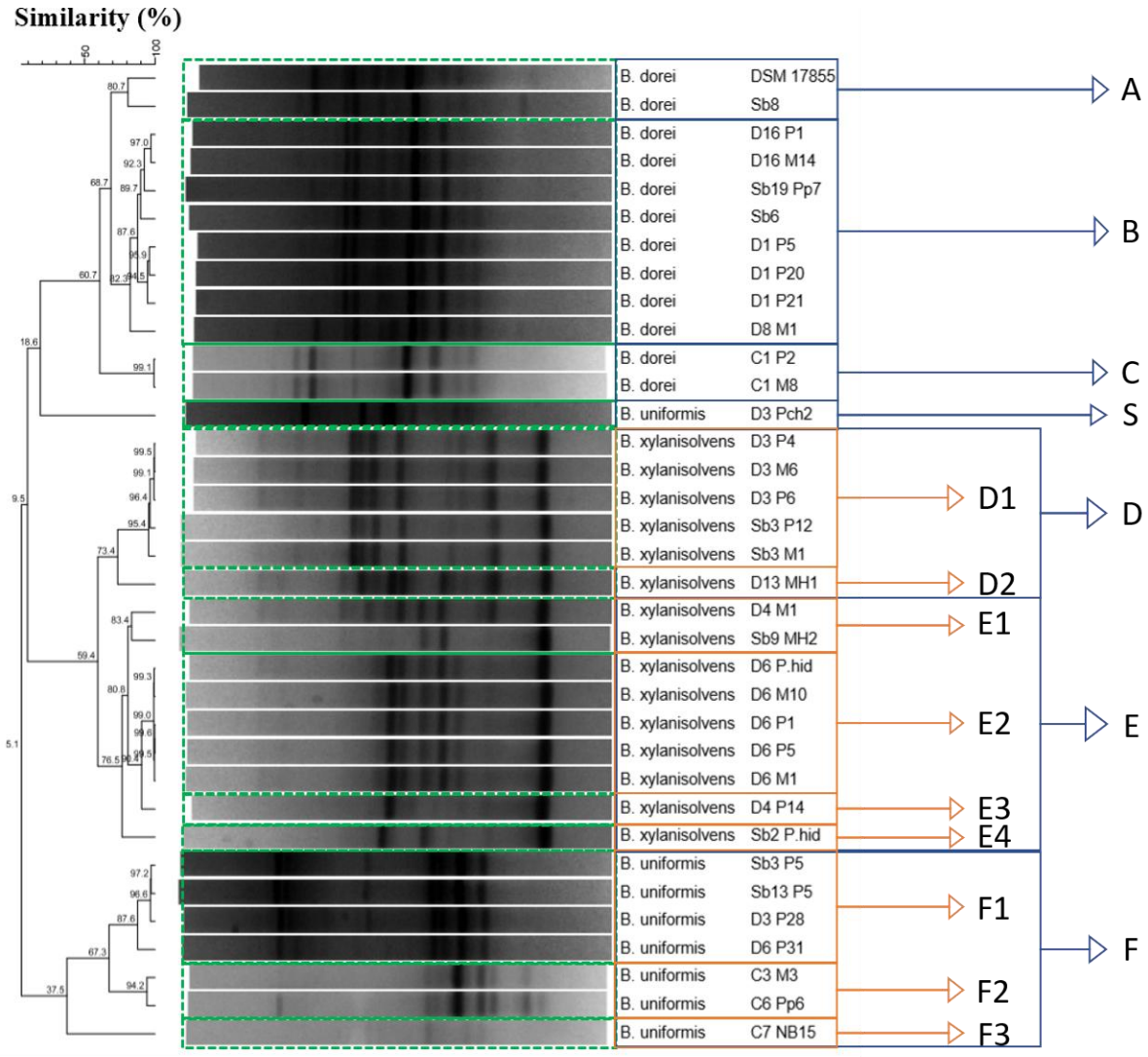


Fig. 4.2.4. Cluster analysis of BOX-PCR genotyping of *Bacteroides* isolates identified as belonging to the species *B. dorei*, *B. uniformis* and *B. xylanisolvens*.

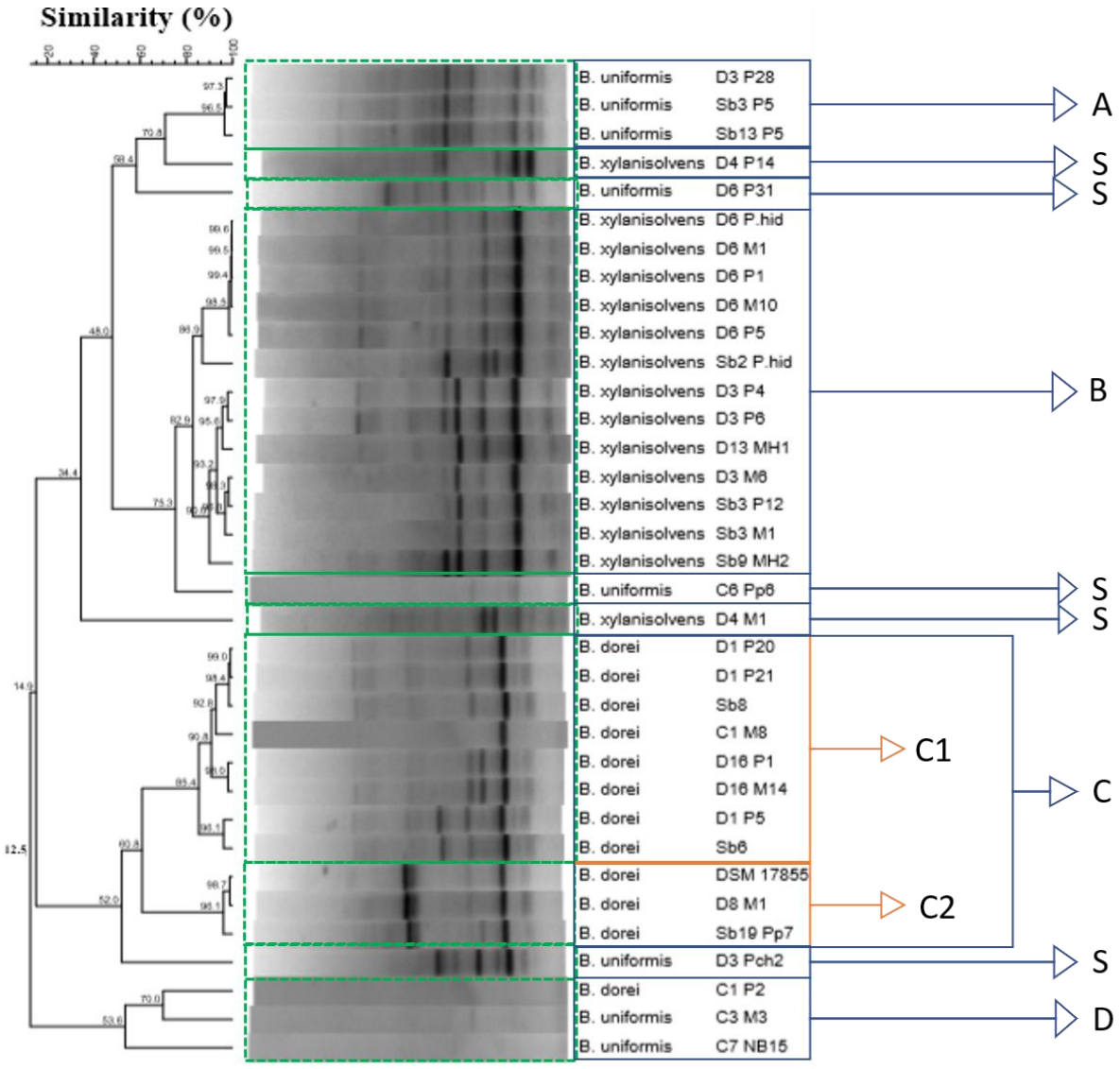


Fig. 4.2.5. Cluster analysis of ERIC-PCR genotyping of *Bacteroides* isolates identified as belonging to the species *B. dorei*, *B. uniformis* and *B. xylanisolvens*.

4.3. Real time PCR Quantification (qPCR) of intestinal microbiota phyla and genera in T1D and Control children

4.3.1. Cloning the *16S rRNA* gene of each target group and efficiencies of the primers

The real-time PCR quantification (qPCR) was performed by using the cloned *16S rRNA* gene of each target group in a plasmid for constructing the standard curve. The transformed *E. coli* cells (white colonies) (**Fig. 4.3.1.1**) were validated by PCR (**Fig.4.3.1.2 - 4.3.1.5**).

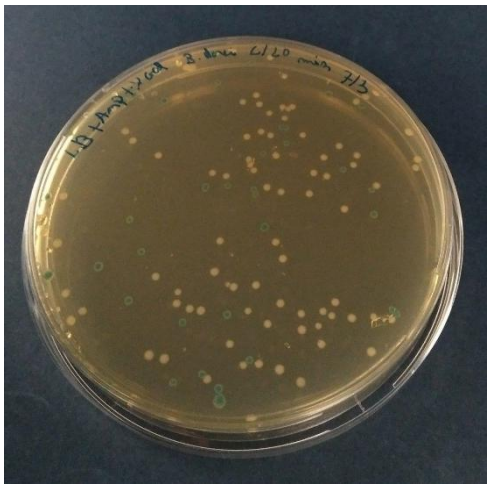


Fig. 4.3.1.1 A representative Petri dish with *E. coli* MACH1 colonies potentially transformed with a *16S rRNA* target gene (white colonies) and not transformed (blue colonies).

The **Figures 4.3.1.2-4.3.1.6** illustrate the PCR reactions performed to validate the transformation of *E. coli* cells with the *16S rRNA* target gene. A representative validated transformed colony was selected for further use in the following qPCR reactions.

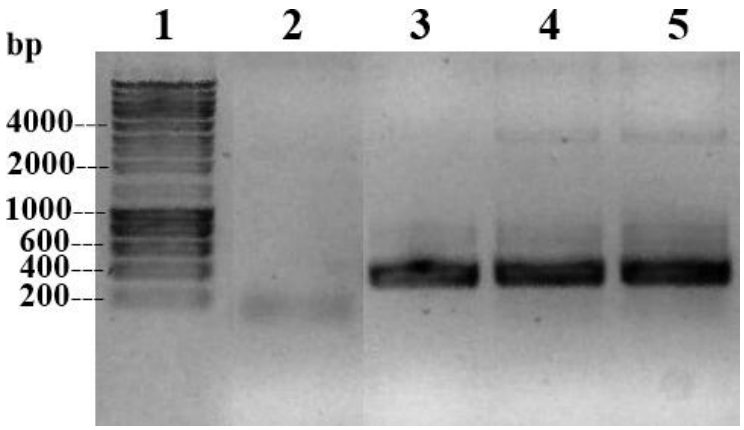


Fig 4.3.1.2. PCR reaction for trial the potential transformed *E. coli* DH5α cells with the *16S rRNA* gene of the *Lactobacillus casei*. 1 - Ladder (GeneRuler ™) 2 – Control reaction (no DNA added), 3 - DNA *Lactobacillus casei* DSM 20011, 4 - 5 – Potential transformants of *E. coli.* The expected size of the amplified PCR product was 341 bp.

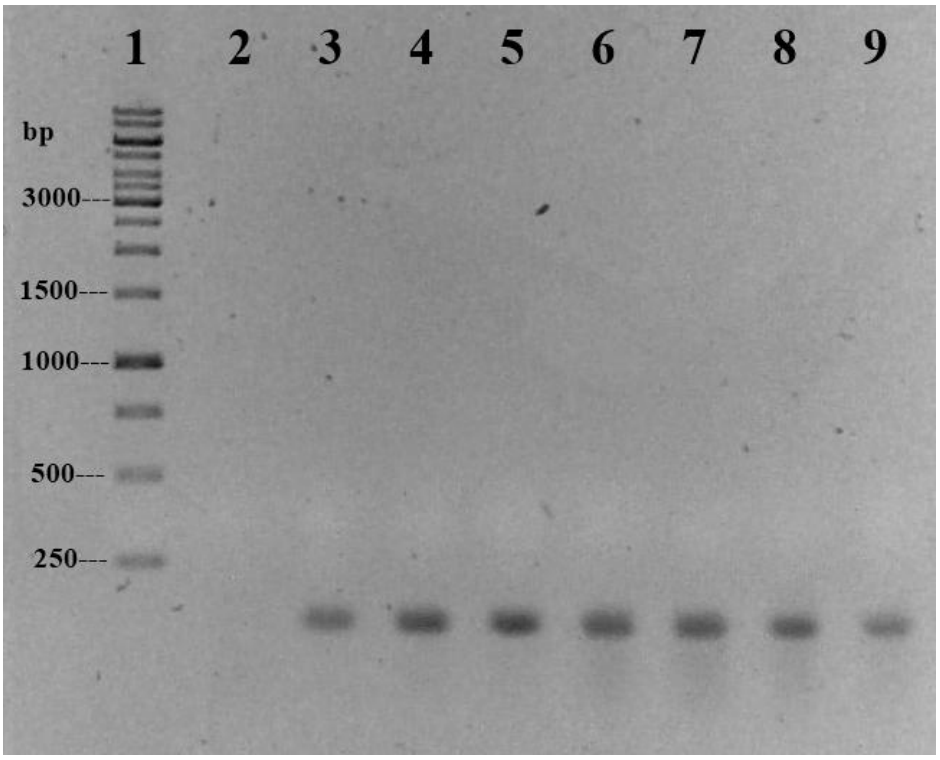


Fig 4.3.1.3. PCR reaction for trial the potential transformed *E. coli* MACH1 ™ cells with the *16S rRNA* gene of the *Bacteroides dorei*. 1 - Ladder (GeneRuler ™) 2 – Control reaction (no DNA added), 3 – DNA of *Bacteroides dorei* DSM , 4 - 9 – Potential transformants of *E. coli*. The expected size of the amplified PCR product was 120 bp.

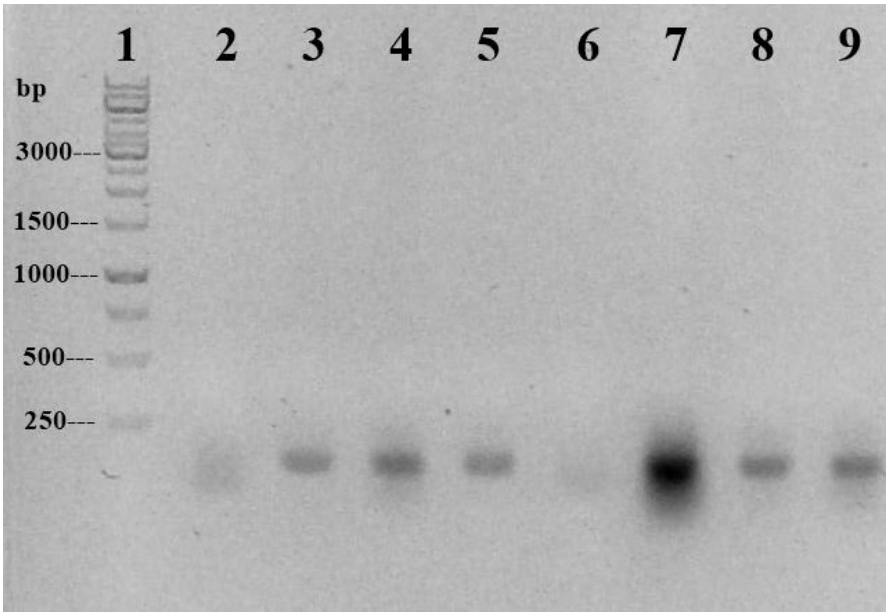


Fig 4.3.1.4. PCR reaction for trial the potential transformed *E. coli* MACH1 ™ cells with the *16S rRNA* gene of the *Enterococcus faecalis.* 1 - Ladder (GeneRuler ™) 2 – Control reaction (no DNA added), 3 – DNA of *Enterococcus faecalis* NCTC 775, 4 - 9 – Potential transformants of *E. coli*. The expected size of the amplified PCR product was 126 bp.

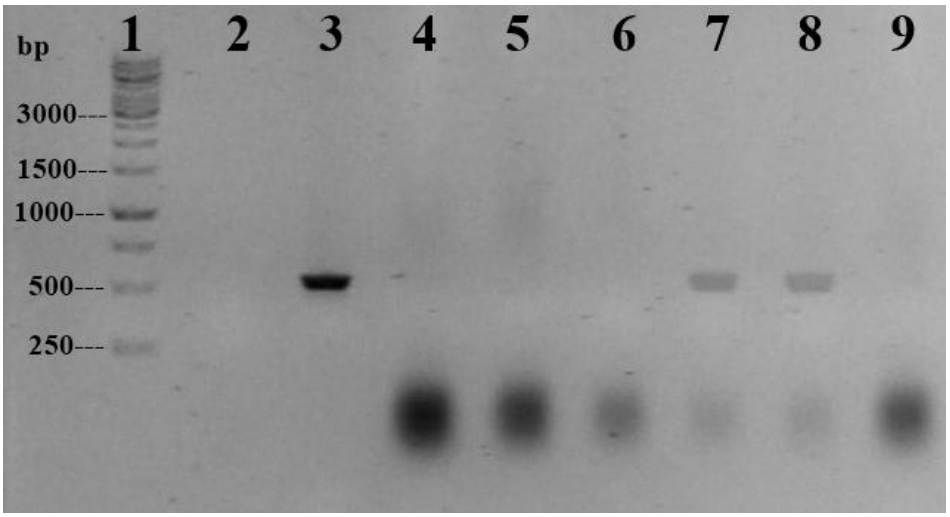


Fig 4.3.1.5. PCR reaction for trial the potential transformed *E. coli* MACH1 ™ cells with the *16S rRNA* gene of the *Bifidobacterium longum* subps *infantis.* 1 - Ladder (GeneRuler ™) 2 – Control reaction (no DNA added), 3 – DNA of *Bifidobacterium longum* subps *infantis* DSM 20088, 4 - 9 – Potential transformants of *E. coli*. The expected size of the amplified PCR product was 550 bp.

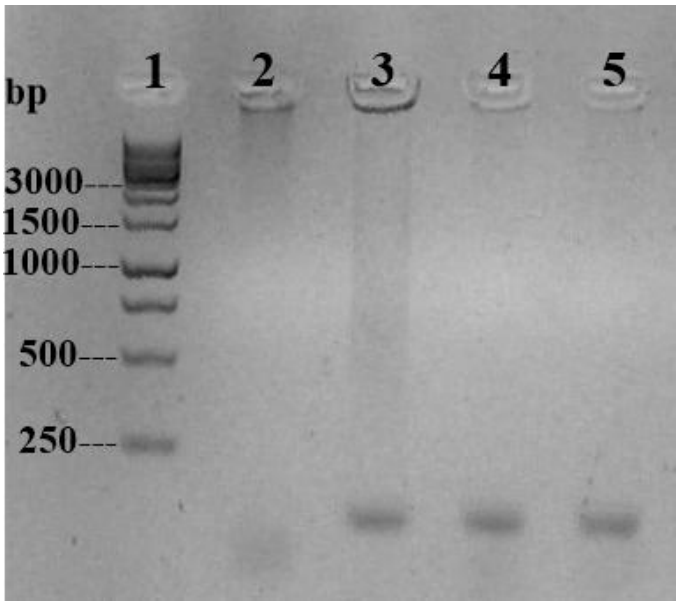


Fig 4.3.1.6. PCR reaction for trial the potential transformed *E. coli* MACH1 ™ cells with the *16S rRNA* gene of the *Bacteroides thetaiotaomicron.* 1 - Ladder (GeneRuler ™) 2 – Control reaction (no DNA added), 3 – DNA of *Bacteroides thetaiotaomicron* DSM 2079, 4 - 8 – Potential transformants of *E. coli*. The expected size of the amplified PCR product was 126 bp.

The calculated efficiencies of the primers used to quantify the different phyla and genera is indicated in **Table 4.3.1**. The used primers showed efficiencies values between 90 to 110%.

Table 4.3.1. Efficiency data of primers used to qPCR the microbiota phyla and genera.

Target group	R^2	Slope	Efficiency (%)
Firmicutes	0.994	-3.27	102
Bacteroidetes	0.998	-3.34	99
***Lactobacillus* spp.**	0.997	-3.32	100
B. dorei	0.997	-3.10	110
***Bifidobacterium* spp.**	0.994	-3.60	90

4.3.2. qPCR of intestinal microbiota phyla and genera

The qPCR data of the different intestinal microbiota and genera of T1D and Control children is summarized in the **Table 4.3.2**. No significantly differences (P>0.05) were found for the examined phyla and genera except for the population of *Lactobacillus* spp. that showed a slightly higher value in T1D children (P<0.05). In fact, the amounts of *Lactobacillus* spp. and *B. dorei* were the lowest in both groups. The *Bifidobacterium* spp. amounts achieved approximately 6 Log_{10} copies/g feces in both groups. Also, no significantly differences (P>0.05) were observed for the ratio of *Firmicutes* to *Bacteroidetes* between the groups. In the Control group the ratio was 1.61±0.41 and in the T1D was 1.72±0.38 (**Table 4.3.2)**.

Correlations between the amounts of each specific microbial group and glycated hemoglobin values (HbA1c) were not performed since no microbial phyla or genus showed significant differences between T1D and Control samples.

Table 4.3.2. Real time PCR quantification of intestinal microbiota and genera in T1D and Control children

Target Group	Control	T1D	*P*
Firmicutes	8.56 ± 1.05	8.35 ± 0.93	0.384
Bacteroidetes	5.15 ± 1.05	5.40 ± 1.05	0.496
***Bifidobacterium* spp.**	5.94 ± 0.61	5.87 ± 0.51	0.581
***Lactobacillus* spp.**	3.98 ± 0.34	3.80 ± 0.39	0.047
B. dorei	4.38 ± 1.69	4.78 ± 1.99	0.158
***Firmicutes* to *Bacteroidetes* ratio**	1.61 ± 0.41	1.72 ± 0.38	0.105

Values represent the mean ± SD and are expressed in Log_{10} copies/g feces. N=17 participants per group. Differences between the two groups were analyzed using the Mann-Whitney U test. Values are significantly different at P<0.05

5. Discussion

The cause for the development of Type 1 diabetes (T1D) in children is still not clarified despite the significant efforts towards the role of the different factors, such as the microbiome, viruses and vitamin D deficiency in triggering the disease (Brown *et al.*, 2011; Giongo *et al.*, 2011; Murri *et al.*, 2013; De Goffau *et al.*, 2013, 2014; Soyucen *et al.*, 2014; Davis-Richardson *et al.*, 2014; Endesfelder *et al.*, 2014; Kemppainen *et al.*, 2015; Kostic *et al.*, 2015; Cinek *et al.*, 2017; Zhao *et al.*, 2017; Federico *et al.*, 2017) From these studies it is evident the disruption of the balance between the host and the microbial community (also known by disbiosis) in children at risk of developing T1D, which is also characteristic of other autoimmune and inflammatory conditions (Willing *et al.*, 2009; Vieira *et al.*, 2015). However, the "healthy" microbial gut composition is still not clearly defined (Schnorr *et al.*, 2014; De Filippo *et al.*, 2017), whatever will be this definition the microbiota action on the digestion of nutrients, shaping the immune system and in the establishment of the bidirectional communication between the gut microbiota and the brain is vital (Sonnenburg and Bäckhed, 2016; Geva-Zatorsky *et al.*, 2017; Malan-Muller *et al.*, 2017).

Across several regions the intestinal microbiome of children at risk of T1D did not showed an overlap, instead some regions were more similar than others, such as the observed between the microbiome patterns of Finnish children and the microbiome of the children from Colorado state that were at risk of T1D (Kemppainen *et al.*, 2015). In this study was observed that *Bacteroides* was the predominant genus among all sites analyzed (Colorado, Georgia/Florida, Washington state, Finland, Germany and Sweden), and children from Colorado showed particularly high abundances (Kemppainen *et al.*, 2015). The high abundance of *Bacteroides* in Spanish children with established T1D was also reported (Murri *et al.*, 2013). In contrast the T1D children from Algarve showed no significantly different amounts of this bacterial genus and the recovered species were similar between T1D and the Control group (the main core was constituted by the species *B. dorei*, *B. uniformis*, *B. ovatus*, *B. vulgatus* and *B. xylanilsolvens*). Our results are consistent with those reported by Pinto *et al.*, (2017) in which although the higher protein abundance originated from *Bacteroides* (*B. dorei* and *B. uniformis*) in T1D fecal samples in comparison to Control samples (N=3) the authors did not observed a higher amount of *Bacteroides* in T1D samples. In addition, our study shows that in T1D children from Algarve the identified *Bacteroides* spp. do not diverge from the ones found in the Control samples, except some individual species (*B.*

graminisolvens, *B. cellulosilyticus*, *B. finegoldii*, *B. stercoris*, *B. eggerthii* and *B. fragilis*) that were not recovered from Control samples. Furthermore, in Mexican T1D children (treated for ≥2 years) the levels of *Bacteroides* in feces samples was similar to Control samples (Mejia-Léon et al., 2014). The discrepancies between the studies can be explained by geographical factors (Davis-Richardson *et al.*, 2014; Kemppainen *et al.*, 2015; Cinek *et al.*, 2017).

The effect of geographical location factors on association between the abundance of a particular *Bacteroides* specie and the onset of islet autoimmunity is particularly evidenced in the studies conducted by Davis-Richardson *et al.*, (2014) and Cinek *et al.*, (2017). Davis-Richardson *et al.*, (2014) reported the intestinal dominance of *B. dorei* at early-onset islet autoimmunity in children from the Finnish city of Turku. In contrast Cinek *et al.*, (2017) although had observed an imbalance within the genus *Bacteroides* did not found an increase on the amounts of *B. dorei* in the feces samples from children at early-onset islet autoimmunity from the Finnish cities of Tampere and Oulu, instead the authors reported an association to islet autoimmunity with a decrease on the abundance of *B. vulgatus* and *B. caccae*.

In our current study the recovery of *B. dorei* from the feces of T1D or Control children was very low (3 T1D samples and 4 Control). Moreover, the qPCR data for the quantification of *B. dorei* showed similar amounts of this bacterium in both groups (4.38±1.69 and 4.78±1.99 Log_{10} copies/g feces in T1D and Control samples, respectively). Furthermore, the *BOX* or *ERIC* elements did not differentiate the *B. dorei* T1D isolates from Controls. However, the 16S rRNA sequence analysis suggest that the T1D D16 children seems to be colonized by two different *B. dorei* strains regardless these two isolates have clustered together either by BOX-PCR or ERIC-PCR. Through our approach we are not able to know if T1D children from Algarve displayed at early-onset islet autoimmunity an imbalance in the *Bacteroides* population, particularly a high or low abundance of *B. dorei* in the gut. Nevertheless, the low amounts of *B. dorei* observed in the feces samples of both groups is important to stress that *B. dorei* isolates may exhibit different metabolic functions depending on their methylation patterns as reported previously by Leonard *et al.*, (2014). Another metabolic feature that can differentiate the *Bacteroides* population between T1D and Control samples is the contribution of *Bacteroides* activity in mucin synthesis and degradation that can cause a diminish mucus layer favoring the increase of gut permeability and inflammation in T1D children (Tlaskalová-Hogenová *et al.*, 2011). The process is drived by butyrate

producers in the gut (such as *Faecalibacterium prausnitzii*) through the use of lactate (Rios-Covian *et al.*, 2015) inducing the synthesis of mucins avoiding the contact of pathogens and toxins with the epithelial cells by keeping a good layer of mucus. In contrast the *Bacteroides* use lactate to produce short chain fatty acids (SCFAs), such as acetate, succinate and propionate, which do not contribute to the synthesis of mucins leading to a thinner mucus layer and promoting the disruption of the tight junctions (Brown *et al.*, 2011). Such functional differences may explain the autoimmunity development in T1D patients. So, the fact that the T1D children from Algarve showed similar levels of *Bacteroides* and share analogous species in their gut their metabolic functionality may be different.

In our study it was interesting to observe that the recovery of the *Bacteroides* related genus *Parabacteroides* (Sakamoto and Benno, 2006), particularly the specie *P. distasonis* was recuperated at a higher percentage in T1D samples in comparison to Control samples. *P. distasonis* in a mice model for studying Inflammatory Bowel Disease (IBD) using dextran sulphate sodium (DSS) (a compound that damages the intestinal epithelium) evidenced the ability to promote the development of colitis in combination with *P. falsenii* and *B. eggerthii* (Dziarski *et al.*, 2016). However, the role of *P. distasonis* on the gut inflammatory diseases is still controversial, for instances in a similar DSS-induced colitis mice model the oral administration of membrane fractions collected from *P. distasonis* decreased the disease indicators (Kverka *et al.*, 2011) and recently was reported that *P. distasonis* ATCC 8503 (DSM 20701) in a obesity mice model of colorectal cancer (Apc^{1638N}) was able to completely blocked the tumor formation in high-fat diet azoxymethane treated mice (azoxymethane is a common compound used to drive colon cancer, which in the intestine is metabolized into methylazoxymethanol by CYP2E1 that in turn produces DNA mutations (Chen and Huang, 2009; Koh *et al.*, 2018). The proposed protection mechanism of *P. distasonis* against colorectal cancer is based on its ability to promote apoptosis that will be associated with a cell surface factor and not a secretion element that demands the activity of viable cells since the metagenomic data using *16S rRNA* gene showed no recovery of *P. distasonis* in the feces of mice suggesting the inability of the bacteria to colonize these mice Koh *et al.*, (2018). Usually such differences between the studies can be associated with different sample sources (feces/tissues) and approaches used. A whole genome analysis of *P. distasonis* ATCC 8035 (previously *B. distasonis*) has revealed important features in comparison to the other common gut *Bacteroides*, namely it was considered a gut specialist in comparison to *B. thetaiotaomicron* in virtue of display the smallest genome in comparison to the other

sequenced *Bacteroides* species and evidenced a very limited collection of genes belonging to the GO categories related with environmental sensing and gene regulation, and as well a well restricted number of genes linked with carbon degradation and a significant amount of laterally transferred genes (70% versus 30-35% for *B. fragilis*, *B. vulgatus* and *B. thetaiotaomicron*) and high DNA methylation proteins (Xu *et al.*, 2007). All together these characteristics may play a crucial role for *P. distasonis* accomplish its success in such competitive ecosystem, as the gut. We are not aware of any previous reports about the high prevalence of *P. distasonis* in T1D children. The reason why in our current study we were able to recover a higher number of *P. distasonis* isolates from T1D children in comparison to Control samples needs further investigation.

The T1D children from Algarve also showed a similar ratio of *Firmicutes* to *Bacteroidetes* in comparison with Control children, diverging from the findings of Murri *et al.*, (2013) that observed a significant higher ratio of *Firmicutes* to *Bacteroidetes*. Also, the divergence of data regarding this parameter can be justified by differences in geographical location factors. The study reported by Endesfelder *et al.*, (2014) reinforces the message that besides no significant differences in the microbial composition between T1D samples and Controls the microbial interactions differ between the two groups. In their study they compared the bacterial composition (targeting 16S rRNA) of the gut between seropositive or seronegative European children that have a first degree relative with T1D. Their findings evidenced the absence of differences between autoantibody positive and negative children considering microbial diversity and composition and also did not found any particular bacterial genus which abundance was changed. However, the authors observed major alterations in the interaction networks of bacterial populations in children who became anti-islet autoantibody positive. So, this study highlight that the trigger of anti-islet cell autoantibodies may not lay on specific microbiota but on their interconnection.

The *Lactobacillus* genus was impaired in T1D children from Algarve, which is in line with the reported by Murri *et al.*, (2013), and also with findings observed in bio-breeding diabetes prone mice by Roesch *et al.*, (2009). However, this is in conflict with the results reported by Brown *et al.*, (2011) that observed a higher abundance of *Lactobacillus*, *Lactococcus*, *Bifidobacterium* and *Streptococcus* in four Finnish autoantibody positive children. The beneficial role of *Lactobacillus* on the decreasing or delaying the development of T1D in mice has been reported (Matsuzaki *et al.*, 1997; Valladares *et al.*, 2010). In the study conducted by Matsuzaki *et al.*, (1997) the authors observed that the administration of a

diet supplemented with 0.05% of heat killed cells of *L. casei* resulted in a lower incidence of diabetes in non-obese diabetic mice in comparison with the control group. Furthermore, the performed pathological analysis reveal that the mice group feed with *L. casei* exhibited diminished damage signs of the insulin secreting β cells. It is important to stress that the authors tested the action of the bacterial surface elements and not any factor that requires active viable cells. In contrast Valladares *et al.*, (2010) administrated different strains of *Lactobacillus* (*L. reuteri* TD1 or *L. johnsonni* N6.2) ($1x10^8$ CFU/ animal) collected from bio-breeding diabetes resistant mice in either 1-day old bio-breeding diabetes prone mice during mother feeding and also at post-weaning to 21 day old. The authors observed strain dependent results and time of weaning; the incidence of T1D was not changed when *Lactobacillus* was administrated during the pre-weaning time interval but when the strain *L. johnsonni* N6.2 was administrated the incidence of T1D diminished in contrast to the feeding with *L. reuteri* TD1 that did not show any impairment in the disease incidence. Both studies evidenced using mice models for T1D that *Lactobacillus* have a positive effect on the incidence and ameliorate the disease signs. Several strains of *Lactobacillus* together with other bacterial genera, including *Bifidobacterium* have recognized probiotic proprieties (beneficial impact on the health of the host) (Douillard *et al.*, 2013; Liévin-Le Moal and Servin, 2014). It is also known that several surface proteins are key for the interaction of the bacterium with the host epithelial cells, particularly the mucus binding factor (MBF), the surface protein MabA and the mucus binding pili (Vélez *et al.*, 2010; Von Ossowski *et al.*, 2010; von Ossowski *et al.*, 2011). The interaction of *Lactobacillus* with the intestinal epithelial cells may be key along with their capacity to produce lactate that serves butyrate producers stimulating the synthesis of mucins resulting in a healthier gut environment. As mentioned above the abundances of specific bacterial communities are not sufficient to infer about their role on triggering or maintaining the disease a more integrated approaches, namely the investigations of how the bacterial interconnections are compromised in disease versus health status is crucial. In this perspective it is important to examine the impact of the lower amounts of *Lactobacillus* in T1D children.

6. Conclusion

The increased abundance of *Bacteroides* population in the gut of children at onset or with established T1D has been reported, and the metaproteome of T1D children from the Algarve region has shown to be enriched with *Bacteroides* proteins, particularly *B. dorei* and *B. uniformis* in contrast to Control samples that were enriched with *Bifidobacterium* proteins. Therefore, in this study it was proposed to isolate *Bacteroides* spp. and particularly *B. dorei* from fecal samples from T1D and healthy (including their healthy siblings) children and perform their genomic characterization using rep-PCR together with the quantification of the two major phyla in the gut, *Firmicutes* and *Bacteroidetes* and also the two well-known genera for their beneficial roles to the host, *Bifidobacterium* and *Lactobacillus*.

The results on the determination of the *Bacteroides* population evidenced that T1D children are colonized with similar amounts to Control children. The identification of the *Bacteroides* isolates by 16S rRNA sequencing evidenced that both groups share a core of *Bacteroides* species, namely *B. dorei*, *B. ovatus*, *B. vulgatus*, *B. uniformis* and *B. xylanisolvens*. However, it was observed a higher percentage of isolates that were identified as *P. distasonis* in T1D samples in comparison to Control samples.

The number of *B. dorei* isolates recovered from T1D or Control samples was very limited and the quantification of *B. dorei* by qPCR showed that both groups are colonized with similar and low amounts of this *Bacteroides* specie (about 4 Log_{10} copies/g feces).

Regarding the rep-PCR analysis through the *BOX* and *ERIC* elements patterns on the isolates identified as *B. dorei*, *B. uniformis* and *B. xylanisolvens* was possible to distinguish these *Bacteroides* species apart from some individual singletons. The *Bacteroides* isolates clustered according to the species, particularly in the BOX-PCR, evidencing to be a good tool to differentiate the species of this genus in comparison to the ERIC-PCR results. Interesting a *B. uniformis* isolate (D3 Pch2) in both BOX and ERIC-PCR clustered together with *B. dorei* isolates evidencing possible coevolution within the host that approximates this *B. uniformis* isolate to the *B. dorei* genetic signature. Nevertheless, it was not possible to distinguish the isolates according to the target group. Another interesting observation is that the 16S sequence analysis of the two *B. dorei* isolates from the D16 children (D16P1 and D16 M14) suggest that D16M14 has diverge implying that this T1D child may be colonized by more than one *B. dorei* strain.

Regarding the qPCR data for *Firmicutes*, *Bacteroidetes*, *Bifidobacterium* and *Lactobacillus* levels the T1D fecal samples showed similar amounts to Control samples of *Firmicutes*, *Bacteroidetes* and *Bifidobacterium* spp., except *Lactobacillus* spp. for which the T1D samples were depleted.

Taken together, the data collected in the current study, it is possible to conclude that the T1D children in the Algarve region evidence a similar quantity and diversity of *Bacteroides*, in comparison to Control children but are impaired with *Lactobacillus*. In virtue of the results observed in the current study it will be interesting to explore several other approaches to help identify unbalances in their intestinal microbiota.

7. Future perspectives

Nowadays we are assisting to a rapid and significant improvement in sequencing technologies and their costs are suffering substantial reduction allowing their access to get a full picture of the human gut microbiota and as well their functionality. The previous metaproteomic data point out for differences in the intestinal bacterial functionality between T1D and Control children. The current data evidences and agrees with other studies that the abundances of key intestinal bacterial groups are similar to the healthy control children. However, to get a more complete picture of the intestinal bacterial community of T1D children from Algarve is necessary to use new tools, such as metagenomics. Our set of data evidence no differences in the *Bacteroides* population together with other bacterial groups. However, is important to stress, as mentioned above that besides the microbial community seems not being affected in their composition, their interconnections may be compromised. At least one study already has stated differences in the bacterial interaction networks between T1D and Controls.

The fact that we have collected a significant number of *Bacteroides* isolates from T1D and Control samples it will be interesting to investigate the interaction of these isolates with the intestinal epithelial cells, particularly if they affect their tight junctions favoring the intestinal leaking. Another aspect that can be studied is to analyze the *Bacteroides* genomes looking for prophages, since they can alter the phenotype of their hosts, designated lysogenic conversion, to directly impact the intercommunication between their bacterial hosts and the immune system.

It is important to retain that children with T1D do not show yet co-mordities (retinopathy, nephropathy, and neuropathy), however these children are at risk of long-term complications of diabetes. Therefore, all approaches that can provide new strategies to improve their health status are important and the improvement of their intestinal bacterial community can constitute an important tool.

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