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Characterization of gut microbiome composition in T2D patients under SGLT2i and GLP1RA treatment

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1. Characterization of gut microbiome composition in T2D patients under SGLT2i and GLP1RA treatment

Abstract:

Type 2 Diabetes (T2D) is a globally recognized health concern that has garnered significant research attention. This thesis work focuses on gut microbiome analysis in T2D patients who start taking two medications antidiabetic drugs: Empaglifrozin (SGLT2i) and Semaglutide (GLP1RA). Microbiome composition was analyzed using 16S rRNA sequencing. The research aims to enhance our understanding of how the gut microbiome is related to SGLT2/GLP1 drug intake, potentially leading to the development of personalized treatment approaches for improved patient outcomes.

Keywords:

Type 2 Diabetes, SGLT2i, GLP1RA, gut microbiome, Empaglifrozin, Semaglutide

CERCS: 3.3, Medical Biotechnology

Lühikokkuvõte:

Tüüpi diabeet (T2D) on maailmas tunnustatud terviseprobleem, mis on saanud märkimisväärset uurimistähelepanu. Käesolev lõputöö keskendub T2D patsientide soole mikrobiomi analüüsile, kes alustavad kahe antidiabeetilise ravimi võtmist: empaglifrotsiin (SGLT2i) ja semaglutiid (GLP1RA). Mikrobiomi koostist analüüsiti 16S rRNA järjestamise abil. Uurimistöö eesmärk on suurendada meie arusaamist soole mikrobiomi seosest SGLT2/GLP1 ravimite tarbimisega, võimaldades potentsiaalselt arendada personaliseeritud ravistrateegiaid paremate patsiendi tulemuste saavutamiseks.

Võtmesõnad:

Tüüpi diabeet, SGLT2i, GLP1RA, soole mikrobioom, Empaglifrotsiin, Semaglutiid.

CERCS: 3.3 Medical Biotechnology

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INTRODUCTION

Type 2 diabetes (T2D) is a prevalent metabolic disease affecting a substantial portion of the diabetic population. It is characterized by elevated blood sugar levels resulting from either insulin resistance or inadequate insulin secretion by the pancreas. The management of T2D entails medication and dietary modifications aimed at normalizing and stabilizing blood sugar levels. Recent advancements in T2D treatment, such as the introduction of medications like GLP1 receptor agonists and SGLT2 inhibitors, have shown promising results in improving glycemic control and reducing the risk of associated cardiovascular diseases (Cresci et al., 2015).

However, it has been observed that the composition of the gut microbiota can significantly influence the efficacy and side effects of drugs. Consequently, in this study, we aimed to investigate the alterations in the gut microbiome of T2D patients undergoing treatment with the GLP1 receptor agonist Semaglutide and the SGLT2 inhibitor Empagliflozin. We collected samples from these patients both before initiating the treatment and within a year of commencing it.

To analyze the gut microbiome, we employed the widely used 16S ribosomal RNA technique, which involves sequencing and analyzing the V3-V4 region of the 16S rRNA gene. This technique provides valuable information regarding the diversity and composition of bacterial communities in the gut. By implementing this approach, we could identify and classify different bacterial species present in the microbiome and determine their proportional changes during the course of treatment (Barak et al.,2023).

We collected samples for this study from T2D patients at the University of Tartu Clinic. The research was conducted within the microbiome research group of the Estonian Genome Centre, Institute of Genomics, under the Genetics and Biotechnology field of the University of Tartu as part of the genetic technology Bachelor's curriculum. The experiments and methodology were carried out in the Biotechnology department of the Institute of Molecular and Cell Biology at Tartu University.

1 LITERATURE REVIEW

1.1 Type 2 Diabetes: Statistical and Descriptive Overview

Type 2 diabetes (T2D) is a chronic disease that is considered one of the major worldwide problems caused by chronic hyperglycemia, impaired carbohydrates, lipids, and partially insufficient insulin secretion (Reed et al., 2021). T2D is the most common form among the two forms of diabetes, accounting for approximately 90% of all cases, with an estimated 462 million T2D patients worldwide.

In Europe in 2019, the prevalence rate of T2D was 8529 per 100 000 cases. Over the years, the number of T2D cases has been increasing, as reported by the World Health Organisation (WHO) (Sharma and Tripathi, 2019). From 1980 until 2014, T2D cases increased from 108 to 422 million and it is expected to increase by 55% by 2035 (Heerspink et al.,2018). T2D can be a major risk for death in some patients and is considered a main reason for nephropathy(renal disorder), neuropathy(nerve damage), retinopathy(retinal damage), and cardiovascular diseases(heart disorders), although some may have inadequately controlled diabetes for a long time without developing these complications (Zinman et al., 2015). According to WHO statistics, 50% of diabetics are dying from cardiovascular diseases and 10-20% of people with diabetes die of kidney failure. After approximately 10 years of disease, 2% of diabetics lead to retinopathy, about 10% lead to visual impairment, and 50% cannot avoid neuropathy (World Health Organization, n.d., 2022). The complications of T2D impair the quality of life and impose a significant financial burden on healthcare systems globally, with the main complications including retinopathy, neuropathy, kidney disease, and cardiovascular disease (Heerspink et al., 2016). Poor management of T2D can lead to serious complications. T2D is associated with various chronic and acute complications, which can significantly impair the quality of life and lead to a higher mortality rate (American Diabetes Association, 2019).

1.1.1 Etiology and Pathogenesis: The Role of Glucose Metabolism and Pancreatic β-cell.

In the context of eating, carbohydrates are broken down into glucose, which is transported into the bloodstream and stored as glycogen in the liver (Pagliuca et al., 2014). The pancreas

then detects the high glucose levels in the blood and produces insulin to regulate glucose homeostasis. However, an inadequate response by pancreatic islet β-cells can result in glycemic load, insulin resistance, and obesity, which are hallmark signs of T2D (Ichikawa et al., 1999) (Pandey et al., 2015). Insulin secretion by β-cells in the islets of Langerhans is primarily regulated by glucose entry via its transporter (Wu et al., 2014). When pancreatic β-cells fail to deliver sufficient insulin, honest diabetic hyperglycemia progresses from normal glucose resistance to impaired glucose resilience(Pagliuca et al., 2014; Echouffo-Tcheugui&Garg, 2017).

In the natural course of T2D, insulin resistance leads to compensatory insulin oversecretion, although β-cells can become unresponsive to glucose at an early stage of the disease, and later on, there can be a reduction in β-cell mass (Ichikawa et al., 1999). β-cell dysfunction can result from either qualitative or quantitative changes in molecules regulating insulin synthesis or secretion. Genetically programmed apoptosis may also be responsible for β-cell loss, as well as β-cell exhaustion from long-term hypersecretion (Pick et al., 1998).

1.1.2 Risk Factors and Effects of Obesity

Type 2 diabetes (T2D) is a multifactorial disease caused by a complex interaction between environmental and genetic factors(Prasad and Groop, 2015). Multiple risk factors have been identified, including obesity, low physical activity, smoking, poor diet, age, and gut dysbiosis (Wu et al., 2014). Obesity is one of the main risk factors for T2D, occurring in 80% of T2D patients (Barnes, 2011). Excess adipose tissue storage leads to disorders such as cardiovascular disease, insulin resistance, and T2D. The influence of increased adipose tissue mass on the development of insulin resistance is not yet fully understood, but it has been suggested that interference of free fatty acids (FFAs) with hepatic clearance of insulin may lead to increased insulin concentration in systemic circulation (Kissebah and Krakower, 1994) (Björntorp, 1990)(Randle et al., 1963). Adipocytes, skeletal muscle cells, pancreatic β-cells, and hepatocytes are among the cells responsible for energy homeostasis, and high serum FFA concentration causes oxidative stress in these cells.

1.1.3 Assessing T2D: Diagnostic Criteria and Testing Methods

T2D is characterized by elevated plasma glucose and glycosylated hemoglobin levels (HbA1C) levels. To diagnose T2D, blood glucose level tests such as Fasting Plasma Glucose (FPG), oral glucose tolerance test (OGTT), or measuring HbA1C are conducted. These tests are performed in the morning after an overnight fast, and patients are advised to avoid eating, smoking, drinking (except water), chewing gum (regular or sugar-free), or doing exercises for at least 10-16 hours before the FPG test to have blood drawn. OGTT is used to measure how well the body can process a larger amount of sugar and glucose intolerance is defined as an impaired ability for glucose disposal. If the blood sugar measured in the test is above a certain level (Table 1), this could be a sign that sugar is not being absorbed enough by the body's cells. Maintaining normal glucose levels is important for overall health and well-being. Normal glucose tolerance is a term used to describe the body's ability to keep blood glucose levels within a healthy range (Table 1) after consuming a meal containing carbohydrates. A typical OGTT involves fasting for at least 8 hours, ingesting a standardized amount of glucose (usually 75 grams), and measuring blood glucose levels at 1 and 2 hours after ingestion. In a person with normal glucose tolerance, blood glucose levels should increase after ingestion and return to normal levels of 120-140 mg/L (6.7-7.8 mmol/L) (Table 1) within 2 hours (Association Professional Practice Comitee, 2022, p.S17-S38).

Impaired glucose tolerance (IGT) occurs when blood glucose levels remain elevated for longer than 2 hours after glucose ingestion, but do not meet the criteria for a diabetes diagnosis, but considers pre-diabetics. Specifically, IGT is defined as a blood glucose level between 140 and 199 mg/dL 2 hours after glucose ingestion. IGT is considered a precursor to type 2 diabetes, as individuals with IGT are at an increased risk of developing diabetes in the future (Petersen and McGuire, 2005).

The HbA1c level measures glucose levels of individuals with T2D, every 3 to 4 months and is used to evaluate the risk of complications associated with T2D (Lind et al., 2009). However, patients diagnosed with prediabetes should be checked at least once a year, as it's a critical stage with the potential for developing the disease. The main priority in T2D management is to reduce blood sugar levels and avoid further complications and disease-related death. The FPG test has one number that is taken during the period of overnight fasting making it less expensive and easy to perform. The HbA1c test is useful in identifying prediabetes in patients and helps screen glucose action with taken glucose (Mitchai et al., 2021).

Table 1. Comparative Blood Glucose chart: Pre and Postprandial Diabetics, and Normal Individuals.

Blood Glucose chart			
	Fasting	After Eating	2-3 hours after eating
Normal	80-100 mg/l (4.4-5.6 mmol/L)	170-200 mg/l (9.4-11.1 mmol/L)	120-140 mg/l (6.7-7.8 mmol/L)
Impared glucose (pre-diabetic)	101-125 mg/l (5.6-6.9 mmol/L)	190-230 mg/l (10.6-12.8 mmol/L)	140-160 mg/l (7.8-8.9 mmol/L)
Diabetic	126+ mg/l (7.0+ mmol/L)	220-300 mg/l (12.2-16.7 mmol/L)	200+ mg/l (11.1 mmol/L)

In order to avoid diabetic complications, it's critical to maintain appropriate blood glucose levels. To maintain healthy glucose levels and lower their chance of acquiring type 2 diabetes, people with normal glucose tolerance and impaired glucose tolerance should be more careful mostly with lifestyle and weight management. A nutritious diet, frequent physical exercise, weight management, and regular blood glucose monitoring are all crucial for obtaining and sustaining healthy glucose levels. (Garber et al., 2017).

In conclusion, diagnosing T2D requires blood glucose level tests such as FPG, OGTT, or measuring HbA1c levels. Regular monitoring and screening for prediabetes are essential to prevent the progression of T2D.

1.2 Approaches for T2D Treatment Management

While dietary and activity modifications are crucial in the therapy of T2D, medication is frequently required to get the best blood sugar control. One of the most commonly prescribed medications for T2D is metformin. Metformin is considered the first medication to be prescribed for the management of Type 2 Diabetes (T2D). Metformin works by lowering glucose levels, which prevents hyperglycemia, helps to lose weight and minimizes the risk of cardiovascular mortality. Initially, it was used for the treatment of chronic kidney diseases (Wang et al., 2017). Metformin also declines the secretion of β-cells that reduces the amount of glucose produced by the liver and improved body sensitivity to insulin and prevents

complications associated with diabetes such as heart disease, kidney damage, and nerve damage, which makes it a very effective drug for T2D treatment (Danaei et al., 2011)(Rena et al., 2017),(Barzilai et al. 2016),(Danaei et al., 2011),(Rena et al., 2013),(El-Mir et al., 2000). However, not all patients can resist the effects of metformin, even in minimum doses of 500 mg twice a day or 850 mg once a day (Wang et al., 2017). Metformin may cause side effects such as diarrhea, nausea, or abnormal discomfort in 50% of patients (National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), 2022).

In addition to metformin, other drugs such as Sodium-Glucose Co-transporter inhibitors (SGLT2i) and Glucagon-like Peptide-1 receptor agonist (GLP1-RA) are also used for T2D treatment. When used in conjunction with SGLT2 inhibitors and GLP1RA medications, it can provide even more effective results.

1.2.1 GLP1RA. Semaglutide Medication

GLP1 receptor agonists are effective treatments for type 2 diabetes (T2D), as they regulate postprandial blood sugar levels (Donnelly, 2012). GLP1RA is a 30-residue peptide released by intestinal L-cells and regulates insulin secretion and glucose regulation in the body (Nauck and Meier 2018). (Figure 1) GLP1 mechanisms prevent the liver from producing an excess of insulin, inhibit β-cell apoptosis, promote β-cell neogenesis, delay gastric emptying, promote satiety, and increase peripheral glucose disposal (Donnelly, 2012). Semaglutide is a recently modified analog of the GLP1RA, with a tiny sequence difference and covalently linked to a C-18 acyl chain. Semaglutide has a C-18 fatty acid modification, with 94% of its binding to albumin, which prolongs its plasma half-life (Donnelly, 2012)(Nauck and Meier, 2018). GLP1 receptor agonists raise insulin and lower glucagon secretion after activating in the pancreas. The disadvantage of these agonists is that they are degraded by the liver enzyme, dipeptidyl peptidase-4 (DPP4), in minutes and are mainly eliminated by the kidney. Semaglutide is a long-acting GLP-1 receptor agonist with more stable drug exposure, with effective GLP-1 receptor agonist concentrations remaining constant throughout 24 hours and/or 1 week, even though the interval between injections was 1 day or 1 week (Nauck et al., 2011) (Umapathysivam et al., 2014) (Linnebjerg et al., 2008). Rapid responses can be observed within hours or days when the glycemic profile of patients treated with long-acting GLP1 receptor agonists shows a more pronounced increase in postprandial blood glucose concentrations than patients treated with short-acting GLP1, and become receptor agonists within weeks (Drucker et al. 2008;Sjöholm 2010).

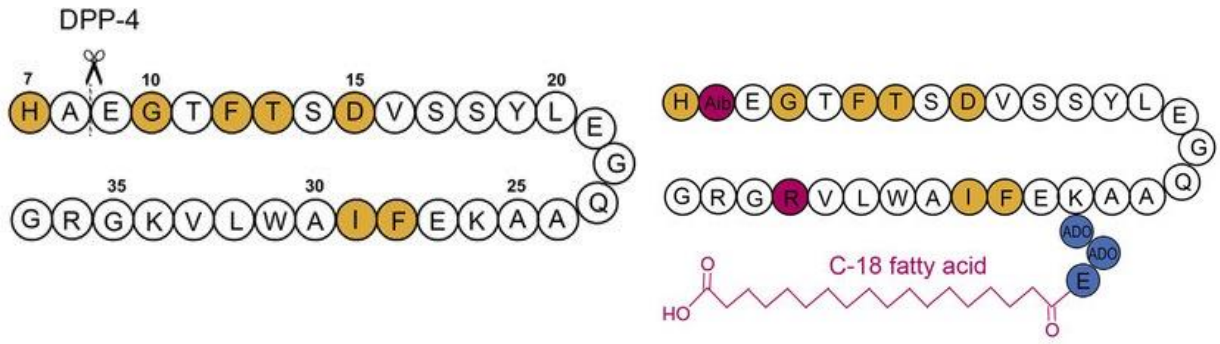

Figure 1. Native GLP1(Left) and Semaglutide (Right) structures. Semaglutide has a similar structure to GLP1, except 31 amino-acid peptides bind to albumin(Fig. 1, ResearchGate).

1.2.2 SGLT2i. Empaglifrozin Medication

Sodium-glucose co-transporter 2 inhibitors (SGLT2i) medications are designed for the treatment of type 2 diabetes (T2D) (Figure 2). They lead to a modification in fuel substrate consumption, resulting in an increase in fat oxidation and ketogenesis, and a reduction in carbohydrate metabolism, which accounts for 90% of glucose reabsorption. SGLT2i reduces blood glucose concentrations through urinary glucose excretion and stimulates lipolysis, which drives ketone production in the liver. Hyperosmolar hyperglycemic state (HHS) is increased by circulating concentrations of counterregulatory hormones. However, the ketone effect on the body has protective functions in cardiovascular disease (CVD) (Heerspink et al., 2018; Heerspink et al., 2016; Lee et al., 2018).

SGLT2 inhibitors have been shown to reduce cardiovascular mortality by 38%, heart disease by 35%, and immediate death by 31%(Heerspink et al. 2018; Kashihara, Kidokoro, and Kanda 2020; Lee et al. 2018a). Empagliflozin is one such inhibitor that reduces blood glucose, increases urinary glucose excretion, improves β-cell function and insulin sensitivity, reduces body mass, and expresses cardioprotective and renoprotective effects. It is taken once a day and does not associate with other drug interactions, except UGT enzymes should be avoided due to the expected reduction of efficiency. Empagliflozin is also used for patients carrying chronic kidney disease (CKD) with max 3rd stage, with hypertension, and who are obese(Frampton, 2018)

SGLT2 inhibitors have a significant impact on reducing cardiovascular mortality and heart disease in T2D. Empagliflozin, in particular, provides a wide range of benefits, including

reduced blood glucose, increased urinary glucose excretion, improved β-cell function and insulin sensitivity, reduced body mass, and cardioprotective and renoprotective effects. The drug is suitable for patients with T2D who have CKD with max 3rd stage, hypertension, and obesity (White Jr, 2015).

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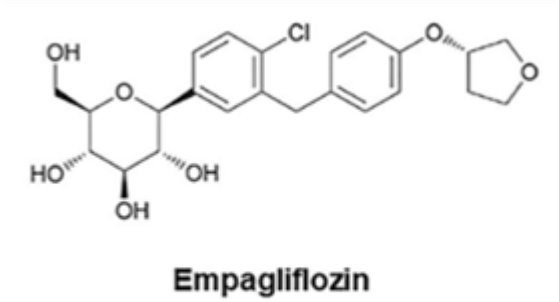


Figure 2. Chemical structure of Empagliflozin (White Jr, 2015).

1.3 The Human Gut Microbiota

The human body is home to a diverse community of microorganisms, including bacteria, archaea, fungi, microbial eukaryotes, and viruses. This collection of microbes is collectively called the human microbiota, and their genes are called the microbiome(Liu et al., 2019). The colonization of the microbiome occurs in the early years of life when newborns are exposed to maternal and environmental microbes that initiate gut microbiome establishment (Strandwitz et al. 2019; Murray, 2018, p.19). The human gut has the greatest diversity of bacterial species, with an estimated 500-1000 different species present. The gut microbiome is vital for human health and is responsible for various functions, including digestion, metabolism, immune system regulation, and protection against pathogens (Schmidt et al., 2018).

The human microbiome is influenced by several factors, including diet, age, gender, medications, stress, and physical activity. Consuming a diet high in fiber and plant-based foods promotes diversity, while a diet high in processed foods and sugar decreases diversity (David et al., 2014). The gut microbiota changes as we age, with a decrease in beneficial bacteria and an increase in potentially harmful bacteria. Studies have shown differences in the gut microbiota between males and females, and hormonal differences may play a role. (O'Toole and Jeffery, 2015; Markle et al. 2013) In addition, many medications, antibiotics, and human-targeted drugs like proton pump inhibitors, and non-steroidal anti-inflammatory

drugs, have also been shown to affect the microbiota composition. (Francino, 2015) Therefore, maintaining a healthy gut microbiome is crucial for overall health. (Imhann et al. 2016; Foster et al., 2017)

1.3.1 Microbiota in Human Health

Microorganisms, which include bacteria, viruses, fungi, and archaea, are ubiquitous and play crucial roles in maintaining human health. The human microbiota consists of these microorganisms, which are present in various parts of the body, including the gut, skin, mouth, and respiratory tract. Among these, the gut microbiota is considered to have the most significant impact on human health. The gut microbiota performs various essential functions, such as aiding in the digestion of food, producing vitamins, modulating the immune system, and protecting against pathogens (Hill et al., 2014).The microbiota's balance and diversity are critical for maintaining a healthy gut environment and preventing diseases such as inflammatory bowel disease (IBD), obesity, and metabolic disorders. Some microorganisms that colonize the gut are harmless, some are potentially harmful, and some are helpful mutualistic symbionts, which benefit the host. A mutualistic relationship is when the microbe and host benefit from one another. Commercial microbes are those that live in the human body without causing harm or giving any benefits to the host. Microbiota dysbiosis, which is an imbalance in the composition of the microbiota, can lead to various diseases. Thus, understanding the role of microbiota in human health is crucial for the development of new therapies and treatments. ("Sender, 2016; Clemente et al., 2012; Flint et al., 2012)

1.3.2 Role of Gut Microbiome in Human Health

The gut microbiota plays a crucial role in human health by performing various essential functions, such as aiding in the digestion of food, producing vitamins, modulating the immune system, and protecting against pathogens. The microbiota's balance and diversity are critical for maintaining a healthy gut environment and preventing diseases such as inflammatory bowel disease (IBD), obesity, and metabolic disorders(Sharma and Tripathi 2019;Kasper , 2014; Vrieze et al. 2012).

According to investigations of 3000 samples, 17 bacteria were identified as the main microbiome present and presented in 95% of samples. The human gut contains up to 100

trillion bacterial cells, which is more than the human cells in the body(Heintz-Buschart and Wilmes, 2018).

Microbes that colonize the gut can be categorized as commercial, opportunistic, or mutualistic symbionts. Commensal microbes exist within the human body without causing harm or providing direct benefits to the host. Opportunistic microbes, on the other hand, can cause disease in healthy individuals. Mutualistic symbionts, however, establish a mutually beneficial relationship with the host, offering advantages to both parties involved.(Volk, 1978, p.340). An imbalance in the gut microbiota (dysbiosis), can cause various diseases, such as obesity, diabetes, affective disorders, cardiovascular disease, inflammatory bowel disease (IBD), and neurodegeneration (Qin et al., 2010).In summary, the gut microbiota is essential for maintaining human health by performing various functions and preventing diseases. The balance and diversity of the microbiota are critical for a healthy gut environment, and dysbiosis can lead to various diseases (Agus, 2018).

1.3.3 Methods of Analyzing the Human Microbiome

The study of the human microbiome involves the use of various methods to identify and analyze the microbial communities present in different body sites. These methods have evolved rapidly over the past decades with the advent of culture-independent next-generation sequencing technologies. Two of the most commonly used culture-independent sequencing methods are 16S rRNA sequencing and shotgun metagenomic sequencing. 16S rRNA sequencing is a targeted approach that amplifies and sequences a specific gene 16S ribosomal RNA (rRNA) found in all prokaryotes, while shotgun metagenomic sequencing is an untargeted approach that sequences all DNA fragments present in a sample. Both methods have their own advantages and limitations and are often used in combination to provide a comprehensive understanding of the microbial communities and their functions (Heintz-Buschart and Wilmes, 2018; Ley et al., 2006; Quince et al., 2017; Gilbert et al., 2014; Huttenhower et al., 2012).

The 16S rRNA gene contains both conserved regions, which are highly similar among different species, and nine hypervariable regions (V1-V9), which show more variation and can be used to differentiate between closely related species. This makes it a useful target for compositional analysis in microbiome studies. By randomly sequencing fragmented DNA, it generates a large dataset of short reads that represent the genetic content of all the organisms in the sample. This provides not only compositional information but also

additional data on functional capabilities and community interactions. By analyzing these reads and comparing them to known sequences in databases, it is possible to identify the types of microorganisms present in the sample, as well as their functional capabilities and interactions within the community (Upadhya et al. 2011; Lozupone et al. 2012; Qin et al. 2010; Quince et al. 2017).

1.3.4 The Gut Microbiota in Metabolic Diseases: Type 2 Diabetes and Obesity

The gut microbiota is associated with various metabolic diseases such as type 2 diabetes (T2D) and obesity. The gut microbiota acts as an endocrine organ, metabolizing nutrients in the diet and producing metabolites and microbial products that can influence metabolic diseases, including T2D, obesity, cardiovascular disease, and liver steatosis.

A core gut microbiome in individuals with T2D has been identified across multiple studies, which includes 17 bacterial taxa that were consistently enriched or depleted compared to non-diabetic controls. The authors found that these bacterial taxa were present in at least 50% of individuals with T2D and concluded that they may play a role in the pathogenesis of T2D (Vrieze et al. 2012).

Fabien Magne Researcher in his paper explains, that various factors such as low physical activity and excessive food intake, including genetic factors, influence obesity. The gut microbiota has been suggested as an additional factor contributing to weight gain, fat storage, and insulin resistance. His researches show, that *Firmicutes* and *Bacteroidetes* are associated with obesity, especially, when *Firmicutes* are increased, and *Bacteroidetes* decreased in an abundance of obese individuals. *Firmicutes* might be more efficient at extracting energy from food by causing a gain in weight (Magne et al., 2020). Moreover, *Firmicutes* have been associated with an increased extraction and storage of energy from the diet, potentially contributing to obesity and metabolic disorders. And *Bacteridetes* have been linked to the degradation of complex carbohydrates and the production of SCFAs, which have beneficial effects on gut health and metabolic processes.

Previous research provided, that *Actinobacteria* in T2D were decreased by ≈11%, comparing healthy individuals, which means dysbiosis or imbalance, of the gut microbiome with T2D, while *Proteobacteria* increases by 18% and here we can assume, that *Actinobacteria* and *Proteobacteria* alter in T2D carriers. This shows, that The dysbiosis of *Actinobacteria* in T2D suggests that alteration in the gut microbiota composition may contribute to the

development and progression of T2D. Lower level of *Actinobacteria* may relate to metabolic dysbiosis (Ullah Goraya et al., 2023). However, an association between T2D and *Actinobacteria,* and *Proteobacteria* is not fully discovered yet.

1.3.4.1 Gut Microbiome in T2D Medications

In the context of type 2 diabetes (T2D) medications, the investigation of phylum-level microbes has provided valuable insights into the specific bacteria affected by the response two other T2D medications, such as incretin-based therapies and sodium-glucose cotransporter-2 (SGLT2) inhibitors(Wu et al., 2017). For example, the efficacy of GLP-1 receptor agonists, which stimulate the secretion of GLP-1, may depend on the composition of the gut microbiome, as the gut microbiota plays a role in the fermentation of non-digestible carbohydrates that stimulate GLP-1 secretion(Dao et al., 2016). Similarly, SGLT2 inhibitors have been shown to alter the gut microbiome composition, and this may contribute to their glucose-lowering effects (Zelniker, 2019; Chávez-Carbajal, 2020). Studies have suggested that GLP-1 agonists, which stimulate the secretion of GLP-1, may be associated with changes in the gut microbiota composition at the phylum level. Similarly, SGLT2 inhibitors have also been linked to potential alterations in the gut microbiome at the phylum level. However, the precise mechanisms and specific bacterial taxa affected by these medications need to be investigated in more detail.

While metformin's mechanism of action is not fully understood, recent research has highlighted the potential role of the gut microbiome in its therapeutic effects (Forslund et al., 2015; Wu et al., 2017; Miura et al., 2019; Gérard, 2016; Huang et al., 2021). Changes in the gut microbiome may contribute to the glucose-lowering effects of metformin by improving intestinal barrier function, reducing inflammation, and altering the production of gut-derived hormones(Wu et al. 2017).

Overall, these findings suggest that the gut microbiome plays an important role in the management of T2D medications and their therapeutic effects. Further research is needed to better understand the underlying mechanisms and potential for targeting the gut microbiome as a therapeutic strategy for T2D.

2 THE AIMS OF THE THESIS

This study aimed to investigate the effects of drug treatment of type 2 diabetes (T2D) patients on gut microbial composition by characterizing taxonomic composition and diversity analysis. We compared microbial profiles of T2D patients who started to receive two types of T2D medications: GLP-1 receptor agonist (semaglutide) and SGLT-2 inhibitor (empagliflozin).

The specific aims of the study were:

- To characterize the taxonomic composition of the gut microbiota in T2D patients before and after treatment with SGLT2 and GLP1 treatment using 16S rRNA sequencing
- To analyze microbial diversity (alpha and beta) in T2D patients before and after treatment with SGLT2i and GLP1RA drugs

3 EXPERIMENTAL PART

The experimental part of this study is focused on the analysis of the microbial community in faecal samples using 16S rRNA sequencing analysis. These samples were collected from patients participating in a one-year screening program conducted by the Tartu University Clinic in Estonia. By examining these faecal samples, we aim to gain insights into the composition and dynamics of the microbial communities present in the gastrointestinal tracts of the individuals under study.

3.1 Materials and Methods

3.1.1 Sample population and collection:

In this study, 15 T2D patients (7 females and 8 males) were enrolled from the University of Tartu clinic by endocrinologist Dr. Ingrid Reppo. Subjects were indicated for enhancement of diabetes treatment with a GLP-1 receptor agonist or SGLT-2 inhibitor and had no contraindications to a GLP-1 receptor agonist or SGLT-2 inhibitor. All subjects who participated in the study were previously taking the diabetes medication metformin 1.5 grams per day and had a body mass index (BMI) of at least 30. The study inclusion criteria required that they didn't have any progression or change of disease during 90 days and used antibiotics during the last 60 days, do not carry severe heart failure, severe liver disease, active malignancy, and immunomodulation treatments.

Subjects were divided into two groups according to whether the GLP-1 receptor agonist semaglutide or the SGLT-2 inhibitor Empagliflozin was prescribed for the treatment of diabetes (Table 2). GLP1 receptor agonist Semaglutide (GLP-1-RA) medication was taken by 8 patients (number of samples = 29) and 7 patients were taking SGLT2 inhibitor Empagliflozin (number of samples = 23). For each individual, stool samples were collected at different timepoints and stored in 200 ul RNAlater solution in a -80C freezer. Stool samples were taken for most individuals at 4 different timepoints, once before starting to take GLP1 or SGLT2 medications and 3 times after, 1 month, 3 months, and a year later (total of 52 stool samples (Figure 3).

For each individual, stool samples were collected at different timepoints and stored in 200 ul RNAlater solution in a -80C freezer. Stool samples were taken for most individuals at 4 different timepoints, once before starting to take GLP1RA or SGLT2i medications and 3

times after, 1 month, 3 months, and a year later (total 52 stool samples) (Figure 3**)**. Stool samples were stored in 200 ul RNAlater solution in a -80C freezer.

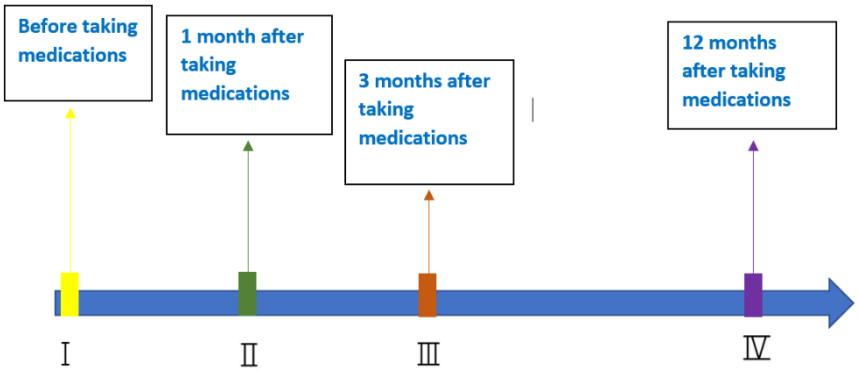


Figure 3. Stool sample collection timepoints

At the beginning of the study, patients were prescribed long-term medical treatment with a GLP-1 receptor agonist or an SGLT-2 inhibitor. From the GLP-1 agonist drug group, patients were prescribed semaglutide, according to the regimen of which the drug needed to be administered once a week as a subcutaneous (subcutaneous) injection. The initial dose of the drug was 0.25 mg, after four weeks the dose was increased to 0.5 mg, and from the 9th week of treatment, the prescribed dose was 1 mg.

There were a total of seven subjects in the group who used SGLT-2 inhibitors (7 women/8 male). As an SGLT-2 inhibitor, participants assigned to the control group used empagliflozin, administered orally once daily in the morning. The dose of the drug was 10 mg from the beginning of the treatment. Informed consent was obtained from all the patients, and the study followed sampling protocols approved by the Ethics Committee of the University of Tartu (No: 290/T-20)

In total 52 stool samples were collected from 15 patients. Table 2 characterizes samples collected in this study.

Table.2 Characteristics of the study samples.

Sample	Timepoint	Medication	Gender	DNA_concentration ng/ul	DNA_260-230 nm/nm	DNA_260-280 ratio nm-nm
Patient 1	I	GLP-1-RA	Female	79.6	1.39	0.14
	II			42.6	1.87	0.19
	III			92.4	1.87	0.22
	IV			38	1.82	0.22
Patient 2	I	GLP-1-RA	Female	17	1.8	0.09
	II			95.5	1.84	0.97
	III			42.6	1.82	0.48
	IV			9.3	1.5	0.21
Patient 3	I	GLP-1-RA	Male	7.8	2.38	0.24
	III			15.9	2.03	0.18
Patient 5	I	GLP-1-RA	Female	30.1	1.78	0.31
	II			6.1	1.65	0.09
	III			14.2	1.59	0.12
	IV			4.3	1.56	0.03
Patient 6	I	GLP-1-RA	Female	7.4	1.4	0.04
	II			6.9	1.84	0.02
	III			8.1	1.69	0.05
	IV			21.7	1.77	0.13
Patient 7	I	GLP-1-RA	Male	19.7	1.96	0.14
	II			22.2	1.85	0.21
	III			28	1.85	0.19
	IV			22.2	1.86	0.8
	I	SGLT-2	Male	5.4	1.91	0.12

Patient 9	II			6.3	1.86	0.06
	III			9.8	1.84	0.17
	IV			8.9	1.92	0.1
Patient 10	I	GLP-1-RA	Male	48.3	1.9	0.58
	II			39.6	1.85	0.32
	III			25.5	1.96	0.13
	IV			82.1	1.85	0.96
Patient 11	I	SGLT-2	Female	3.6	2.71	0.03
	III			20.3	1.75	0.54
	IV			3	3.15	0.03
Patient 12	I	SGLT-2	Female	100.9	1.87	0.25
	II			120.4	1.86	0.84
	III			94.7	1.88	1.86
	IV			81.4	1.43	1.86
Patient 13	I	SGLT-2	Male	38.6	0.16	1.88
	II			55.1	0.69	1.85
	III			116.2	1.9	0.41
Patient 14	I	SGLT-2	Male	45.4	1.86	0.15
	II			36.9	1.82	1.02
	III			20.8	1.85	0.36
Patient 15	I	GLP-1-RA	Female	78.8	1.88	1.94
	II			137.9	1.87	1.82
	III			39.7	1.8	1.31
Patient 16	I	SGLT-2	Male	16.1	1.83	0.18
	II			95.8	1.89	0.46
	III			33.6	1.87	0.78

Patient 17	I	SGLT-2	Male	28.1	1.89	0.1
	II			14.3	2.13	0.02
	III			11.9	1.9	0.67
NEG_control				-0.8	-0.03	0.52

Some patients did not have samples from all four-timepoints for analysis. For example, patient 3 had missing samples from I and II timepoints, the second timepoint from patient 9, and patients 11-15 didn't send their 4th samples yet. Samples 4 and 8 were missing.

3.1.2 Bacterial DNA Extraction

DNA extraction from all samples was performed using a Qiagen DNeasy PowerSoil PRO kit (catalog number 47014),(Qiaqen, Venio, The Netherland). For each sample 100ul stool samples stored in RNAlater solution was used as the starting material following the DNA extraction kit manufacturer's instructions, except that the samples were incubated for an additional 10 min at 65 °C after adding solution CD1 to ensure the proper lysis of difficult-to-lyse bacterial cells. The cell disruption step was performed using a Precellys 24 tissue homogenizer (Bertin Instruments, Montigny-le-Bretinneux, France) and parameters: 2500 rpm for 2×30 s duration, 30 s pause between cycles. This homogenization and lysis procedure helps to disperse the soil particles, begin to dissolve humic acids, and protect nucleic acids from degradation. The rest of the procedure was followed by the manufacturer's instructions provided.

After bacterial DNA isolation, DNA concentration was measured in 2 μL using NanoDropTM 2000/2000c spectrophotometer (Thermo Fisher Scientific, Waltham, USA). DNA concentrations are shown in (Table 2). The extracted DNA samples were stored in a freezer at – 20C before sequencing.

3.1.3 Sequencing Data Analysis

Amplicon sequencing was conducted in the Institute of Genomics Core Faculty at the University of Tartu. The extracted bacterial DNA samples were quantified with Qubit® 2.0 Flu-rometer (Invitrogen, Grand Island, USA), by a standard protocol. Bacterial DNA was

amplified using the primers 16S_F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′) and 16S_R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-GACTACHVGGGTATCTAATCC-3′) for the amplification of approximately 460 bp region within the hypervariable (V3-V4) region of the prokaryotic 16S ribosomal RNA gene, At the end sequencing was carried out on an Illumina MiSeq System using Miseq Reagent Kit v2, using a second-generation Illumina. PCR amplification, library preparation, and quality control was done at the Institute of Genomics Core Facility and sequencing was carried out on an Illumina MiSeq System using a MiSeq Reagent Kit v3 in paired-end 2 x 300 bp mode. A negative control sample (milli-Q water, Millipore Q-POD) was amplified and sequenced following the same protocol.

3.1.4 Data analysis

Bioinformatics analysis were performed by using Qiime2 (version 2021.11.0) using the Paire-dEndSequencesWithQuality import type. The majority of samples in qza/qzv format were visualized web-based tools available at https://view.qiime2.org/.

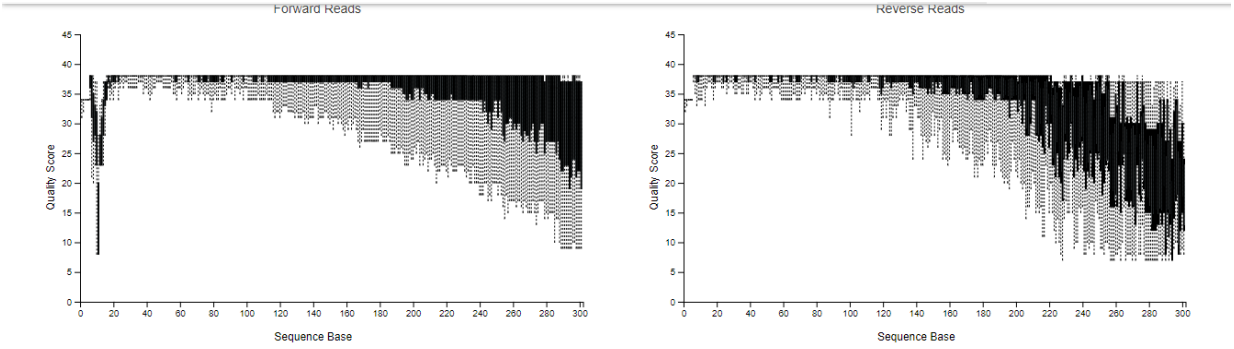


Figure 4: Forward and Reverse reads.

The quality of the samples was assessed using demultiplex function in QIIME2. DADA2 software, implemented in Qiime2, was applied for quality control to evaluate the quality scores of the reads and identify regions, that required clipping due to unsatisfactory quality. The trimming and truncation of reads were performed using the q2-dada2-denoise-paired script, considering the quality scores associated with each read. According to the findings, illustrated in (Figure 4), it's apparent, that forward reads with length ranging from trimming

at position 30 and truncated at position 220 demonstrated higher accuracy, while the remaining section appears to be more chaotic and less reliable. However, reverse reads were removed due to unappropriated quality scores.

After preprocessing and quality control steps, the taxonomic classification of ASVs (amplicon sequence variants) was performed using a q2-feature-table-classifier script in the QIIME2 program. A Bayesian classifier was utilized, which operated based on SILVA 16S V3-V4 v132_99 reference sequencing database with a 99% similarity threshold. The quality check and classifier used in this analysis were prepared by Annabel Raudsep, a master's student at the University of Tartu. The FASTTREE script was utilized to generate a phylogenetic tree. Most samples successfully passed the quality control step, except sample 5 (3 reads) and sample 6 (133 reads), both collected at the second timepoint and sample 5 at the first timepoint (0 reads). A negative control, as expected, showed 0 ASV after the quality control steps.

3.1.5 Statistical Analysis

The gut microbiome of T2D patients was analyzed using Qiime, a widely used bioinformatics tool for microbiome analysis. A total of 52 stool samples + Negative control were collected from T2D patients.

"qiime diversity alpha-group-significance" plugin, specifies the diversity metric and the group column. This will generate statistical results, including p-values, using methods like the t-test (Ostertagova et al., 2014). A p-value threshold of <0.05 was considered statistically significant.

To illustrate the beta diversity component analysis, Principal Component Analysis (PCA) was also performed using Qiime2.

3.2 RESULTS

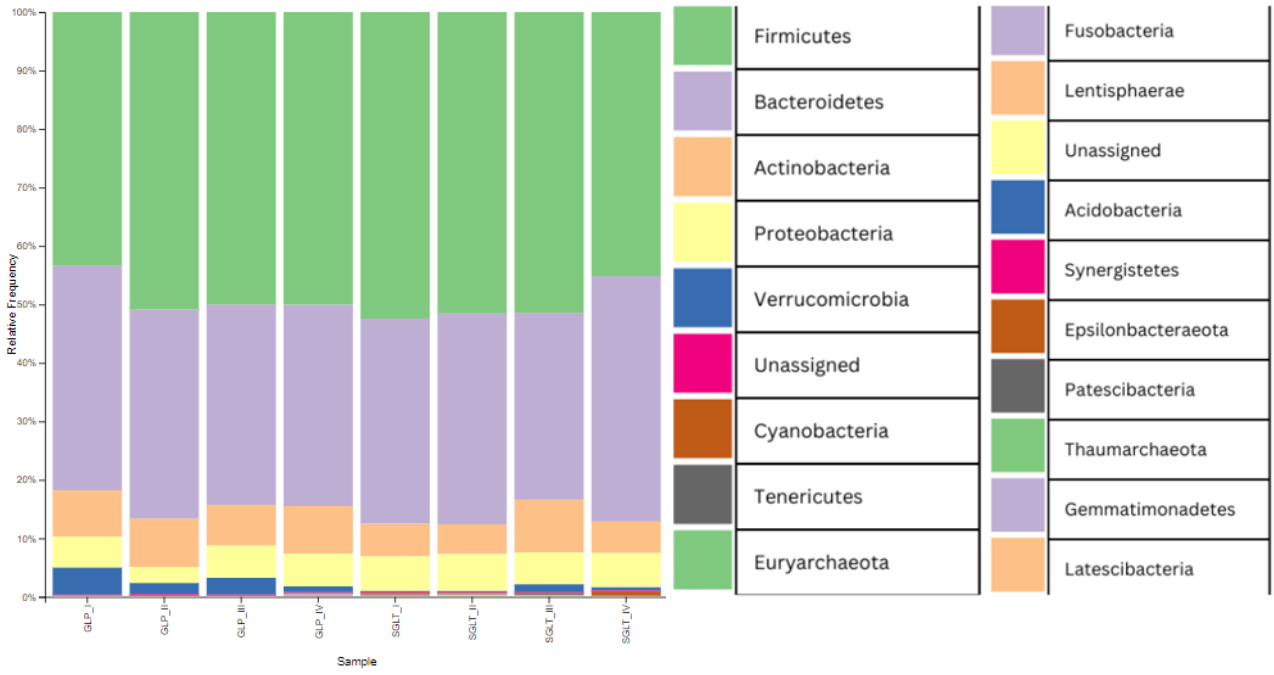
3.2.1 Characterizing Gut Microbiome Composition in different study groups

A total of 15 T2D patients were included in the analysis, 8 patients started GLP-1 receptor agonist treatment, and 7 patients SLTG- 1 inhibitor treatment. In total 52 stool samples were collected from 4 different timepoints (before the study, 1 month, 3 months, and 1 year later) from these patients (29 samples from GLP-1 and 23 samples from SLTG-1). The average bacterial DNA concentration in patients receiving GLP-1 receptor agonist was 37.362 ng/µl (range 4.3 -137.9 ng/µl, SD 33.449), and in patients with SGLT-2 inhibitor was 42.065 ng/µl (range 3 -120.4 ng/µl, SD 38.356). The lower DNA concentrations can be affected by the sample's dilution with RNAlater reagent.

A total of 3 745 144 reads (mean 70 663, range 0-225908) were present in all sequenced samples (52 stool samples, negative control). There were 1 511 439 reads (mean 52 119, range 0 - 225908) in 29 samples from 8 subjects taking a GLP-1 receptor agonist. A total of 2 233 705 reads (mean 97 117,6, range 45188 - 93425) were found in 23 samples from 7 subjects taking SGLT-2 inhibitors. First, we analyzed the taxa-plot.qza file in Qiime2, which represents the taxonomic composition of samples at a specific taxonomic level.

A total of 19 phylum, 26 classes, 45 order, 84 families, 330 families were found in all samples. However, we only focused on phylum-level taxa. In total, across all samples, we detected 19 phyla (Figure 5). We observed a typical Western diversity profile for gut microbiota, where Firmicutes (43.092%) and Bacteroidetes (32.176%) were the dominant phyla, followed by, Actinobacteria (7.053%), Proteobacteria (5.281%) and Verrucomicrobia (12,381%).

Figure 5. Taxonomy bar plots give taxonomy information at a phylum level comparing both drugs at all timepoints.



Firmicutes and *Bacteriotides* are mostly present in all samples. According to the relative abundances, the *Firmicutes* frequencies were before starting treatment 43.316% in GLP1_I and 52.424% in SGLT2_I. One year later, the relative abundance of *Firmicutes* was for GLP1_IV 50.02%, indicating an increase of frequency of 6.704%, and the abundance was reduced to 7,282% in SGLT2 (SGLT2_IV = 45.142%). Comparing *Bacteriotides*, another prevalent bacterium in the gut, the frequency decreased from 38.484% to 34.453% in GLP1 and increased from 35.077% to 42.00% in SGLT2, over one year period. Furthermore, while considering the additional timepoints in between (I and II) we don't observe substantial variations in the abundance of *Firmicutes* and *Bacteriodetes*.

Actinobacteria and *Proteobacteria* were the next most abundant genera, but their abundance was relatively lower compared to *Firmicutes* and *Bacteroides.* Around 8% of *Actinobacteria* could be found in all timepoints of patients before and after GLP receptor agonist treatment. In the same samples, *Proteobacteria* account for 5%, which decreased to 2.7% at the second GLP-1-RA timepoint, while remaining at a similar level for SGLT2 (approximately 5%-6%). *Actinobacteria* frequency increased up to 9% from 5% at timepoint III when taking the SGLT2 inhibitor.

3.2.2 Microbial Diversity Analysis

3.2.2.1 Alpha Diversity Analysis

Next, we calculated the alpha and beta diversity indices. Alpha richness we looked Shannon index which takes into account the relative abundances of different taxa. Shannon diversity showed similar values for both treatments and no differences between different timepoints in both drugs ($p_{\text{Shannon}} = 0.3274$) (Figure 6). Analyzing the alpha diversity graph of the samples collected over the course of one year at four different timepoints (before, after one month, after six months, and after one year), we observed a slightly lower Shannon index in GLP1 samples compared to SLTG 1 samples. Also, it seems that the Shannon index increases after GLP1 treatment, starting at 0.1791 at the I timepoint and reaching to 2.841 level a year later. For SLTG samples, no changes in Shannon index values can be seen between different timepoints (Figure 6).

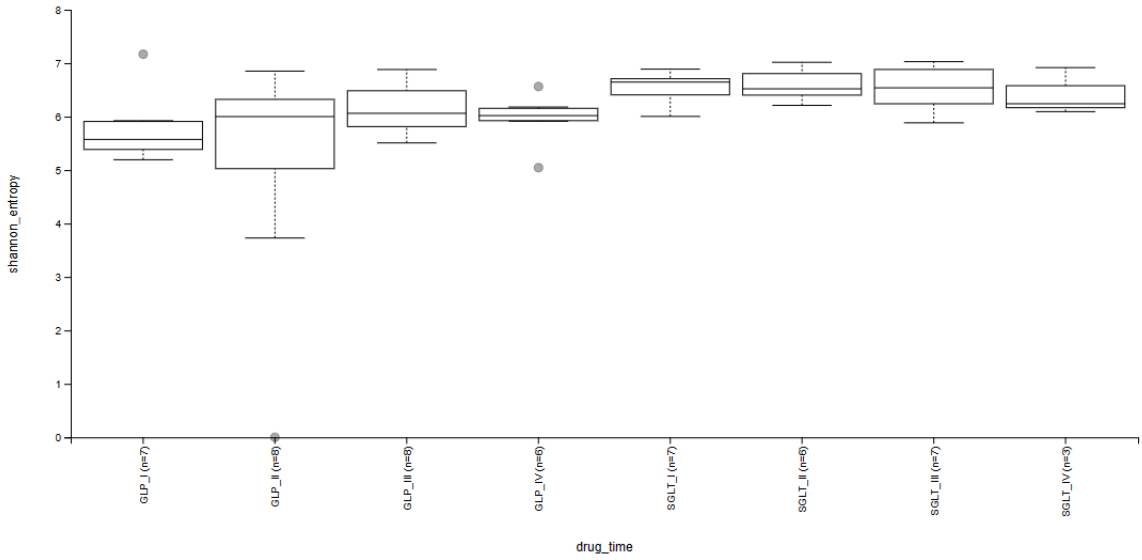


Figure 6. Boxplots of alpha diversity values (Shannon index) in T2D patients taking GLP1 (N=29) and SGLT2 (N=23).

3.2.2.2 Beta Diversity Analysis

The assessment of beta diversity was conducted next. When evaluating beta diversity, which represents how much the community changes between different timepoints, we observed that for both drugs, the samples of the same individuals grouped together regardless of the timepoints (Figures 7 and 8). This indicates that the differences between the subjects were greater than the differences between the timepoints.

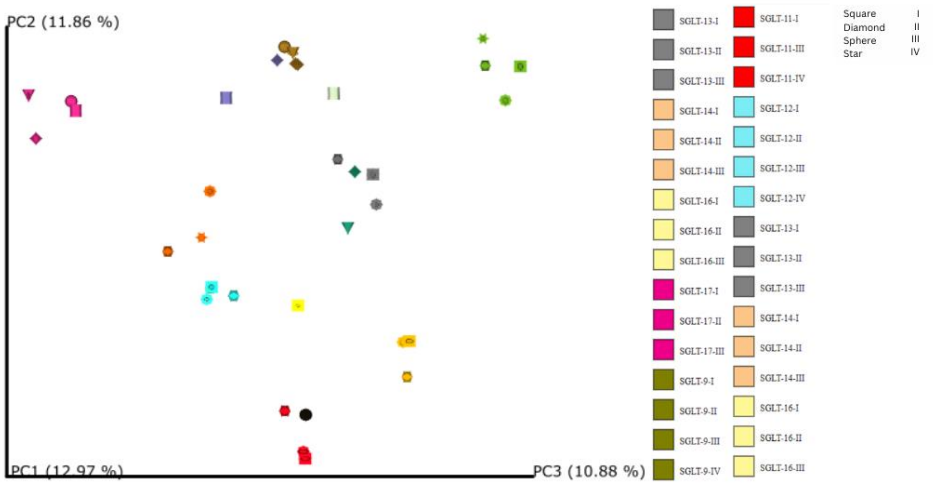


Figure 7. Principal component analysis(PCA) of β-diversity between timepoints in patients taking SGLT2 inhibitor. Samples are colored on the individual's ID.

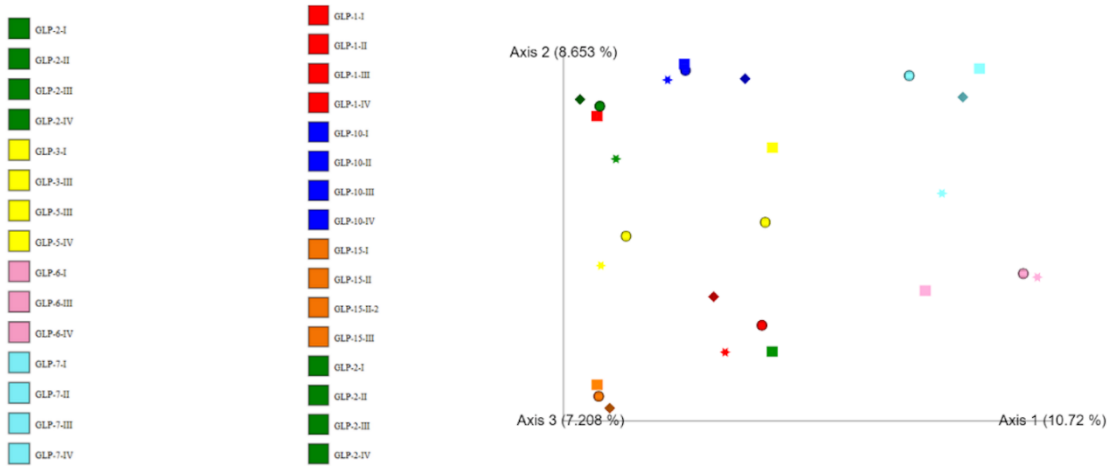


Figure 8. Principal component analysis(PCA) of β-diversity between timepoints in patients taking GLP-1. Samples are colored on the individual's ID.

3.3 DISCUSSION

The objective of this study was to investigate the interplay between medications used in T2D and the gut microbiome, with a particular focus on characterizing microbiome composition and diversity in T2D patients who start treatment with new medications.

According to the taxonomy plot, as expected 90% of bacteria in the human gut belonging to the phyla *Firmicutes* and *Bacteroides,* followed by the phyla *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia*. This is typical for a Western microbial community structure (Iglesias-Vazquez L, 2020). According to the investigations *Firmicutes* and *Bacteroides*, as two major phyla that dominate the gut microbiota, these two phyla tend to be in an inverse ratio with each other and have an impact on fatty acid metabolism. They can influence the metabolism of dietary fats and the production of short-acids(SCFAs), through their enzymatic activities. Several studies have shown, that healthy individuals have a higher abundance of *Bacteroides*, compared to those with T2D. As we described, the percentage of the abundance of phyla-level taxonomic group *Bacteroides* decrease in patients who take Semaglutide medication, which shows aims to elucidate its precise role in the progression or worsening of the patient's condition. Compared to Emphaglifrozin medication taken by patients, it's completely opposite(Magne et al. 2020; Dowis and Banga 2021).

Actinobacteria and *Proteobacteria* are two phyla that exist in the gut but are present in much lower frequencies, than dominant phyla *Firmicutes* and *Bacteroides*. (Hills et al. 2019) In this case, we didn't observe large differences between different timepoints. However, it is noteworthy, that the second timepoint of GLP1 receptor agonist medication treatment showed a small decrease in *Proteobacteria.* According to previous research small decrease may indicate a favorable change or improvement in the gut composition (Liu et al., 2018). In the context of SGLT2 inhibitor treatment, the observed increase in *Actinobacteria* percentage during the third timepoint can be regarded as a favorable outcome. Such growths may be considered positive or beneficial in relation to previous research findings.(Dowis and Banga 2021).

Figure 5 results show that there might be some notable differences in the observed genera among samples, however, proper statistical analysis and investigation of a larger number of samples are needed to fully understand the changes in microbiome composition after treatment with GLP1RA and SLTG2 drugs. Analysis of beta diversity clearly showed that

differences between individuals were greater than differences between timepoints of drug intake.

Comparing the results obtained from different studies in the context of the microbiome and type diabetes (T2D) is challenging due to various factors that can influence the outcomes. These factors include the source of the samples taken from the faecal specimens, the methods used for bacterial DNA extraction, the specific region of the 16rRNA gene analyzed, and the analytical techniques employed. Each of these factors holds the potential to substantially affect the outcomes, impeding direct comparisons between studies.

This study serves as a small practice, examing initial samples collected for a larger research initiative. In this thesis, very preliminary microbiome analyses were performed and therefore for the final result, it is necessary to carry out analyses at other taxonomic levels and to perform statistical analyzes to evaluate the significance of the results. To elucidate the influence of the microbiome on T2D treatment, future investigations should involve sampling a larger number of patients at different treatment stages together with important clinical outcome parameters. Such studies could help to understand T2D treatment strategies in the future and identify the influence of the microbiome on the effects of drugs.

SUMMARY

The results of our study provide a small glimpse into the complex interactions between the composition of the gut microbiome and the drugs used to treat T2D. We started to evaluate the role of the gut microbiome in T2D treatment, specifically, we analyzed T2D patients' gut microbiome profiles before and after treatment with GLP1RA and SGLT2 medications. We characterized microbial communities in the gut by the examination of taxonomic composition and microbial alpha and beta diversity.

The results of the whole project might have a number of applications in clinical practice. First, this project in general provides necessary information on how the gut microbiome could influence T2D treatment. Second, the effects of various drugs on the gut microbiota highlight the need for customized methods of medicine selection that take into consideration each person's particular gut microbial makeup. This customized strategy may be able to improve treatment response and reduce side effects.

The results show a taxonomic difference in diabetic patients compared to four timepoints. Four common gut microbiome phyla, *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* were detected in all samples. This thesis contributed to the understanding the relationship between the gut microbiome and drugs used to treat type 2 diabetes. Analysis of microbial diversity (alfa and beta) with samples taken from different timepoints enables the detection of microbiome change over time in response to GLP1 and SGLT2 drugs. Alpha diversity was not significantly different between timepoints in SGLT2i drug, however, it has significant change between 1^{st} and 4^{th} timepoint in GLP1RA. Beta diversity, indicated, that the microbiome can be different between study groups.

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