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Master thesis

Development of an Antidiabetic Functional Product from Beetroot by Lactofermentation

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ABBREVIATIONS

16s rRNA – 16s ribosomal RNA

AGE – advanced glycation end products

AMPK – AMP-activated protein kinase

ANOVA – Analysis of variance

BRJ – beetroot juice

DAG – diacylglycerol

DNA – deoxyribonucleic acid

DPP-IV – dipeptidyl peptidase- 4

FFA – free fatty acid

GI – glycemic index

GLP-1 and 2 – Glucagon like petide-1 and 2

Glut-4 – glucose transporter-4

GM – glucose measure

GTT – glucose tolerance test

GWASs – Genome-Wide Association Studies

HbA1c – haemoglobin A1c

HPLC-MS - High Performance Liquid Chromatography – Mass Spectometry

IRS – insulin receptor substrate

IFG –impaired fasting glucose

IGT –impaired glucose tolerance

LAB – lactic acid bacteria

LCD – Low-carbohydrate diet

LPS - liposaccharides

MRS – de Man, Rogosa and Sharpe

OD – optical density

ROS – reactive oxygen species

SCFA – short chain fatty acid

SGLT-2 – sodium glucose co-transposter-2

SNP – single-nucleotide poltmorphism

STZ – streptozotocin

T1DM or **T1D** – type 1 diabetes

T2DM or **T2D** – type 2 diabetes

WGS – whole genome sequencing

INTRODUCTION

Diabetes refers to a group of metabolic disorders characterized by persistent high blood glucose levels. Diabetes increases the risk of developing other chronic conditions such as hypertension, and cardiovascular diseases which reduce quality of life and increase mortality (Baena-Díez et al. 2016). A sustained high blood glucose level leads to generalized vascular damage which affects many vital organs. The global prevalence of diabetes in adults has increased exponentially due to urbanization, drastic change in diet and lifestyle. It is estimated that over 400 million people are living with diabetes all over the world and over 54 000 in Lithuania. The prevalence of diabetes in adults is predicted to be about 9.9 % by 2045 (Cho et al. 2018) which indicates the need for designing strategies for combating the diabetes.

In recent years, many studies have reported that diabetics have significantly altered gut microbiota (Sircana et al. 2018). Knowing that the gut microbiota can be manipulated by diet, designing functional foods that reduce hyperglycemia is imperative. Over the years, several functional foods that positively influence postprandial glycaemia have been developed. These foods are usually rich in polyphenolic compounds which reduce hyperglycemia by inhibiting carbohydrate digestion, reducing glucose absorption in the gut, stimulating insulin release from the pancreas, activating insulin receptors, or modulating glucose uptake in insulin-sensitive cells (Sawicki and Wiczkowski 2018). It is also possible that these polyphenols affect the quality and levels of gut bacteria to play a role in the blood glucose reduction. This is because over 90 % of dietary polyphenols escape absorption and end up in the large intestine where they are metabolized by the gut microbiota and absorbed into circulation (Pasinetti et al. 2018).

Interestingly, beetroot has received much scientific attention because it is rich in phenolic and other bioactive compounds. Yet since less than 10% of its polyphenols may be absorbed in the upper intestinal tract, fermenting the vegetable with lactic acid bacteria (LAB) (which are generally regarded as safe) could be helpful in converting the bioactive compounds into readily absorbable activated forms. Studies of the effects of beetroot consumption were carried out and showed beneficial effect on glucose metabolism and other metabolic markers (Aliahmadi et al. 2021) but full beetroot potential as new functional food product after lacto-fermentation and its effects during diabetes remains unknown.

In this study, we will develop a fermented product from beetroot that reduces hyperglycemia in diabetic mice model.

Goal:

Development of a lacto-fermented product from beetroot that reduces hyperglycemia in diabetic mice model.

Obejctives:

- To isolate LAB from Lithuanian fermented tomatoes, pears, sauerkraut, pickles, kefir, kombucha, kvass and yogurt.
- To screen for LAB with β-Glucosidase activity.
- To ascertain the total antioxidant activity, DPP-IV inhibitory ability, α-Amylase inhibitory activity and α-Glucosidase inhibitory ability of the fermented samples.
- To ferment beetroot with LAB and establish the optimized conditions for fermentation.
- To identify potential anti-diabetic bioactive compounds generated in the fermented samples.
- To identify the potent LAB strain using 16S rRNA and later confirming by whole genome sequencing.
- To ascertain the antimicrobial susceptibility and antimicrobial resistant genes.
- To induce diabetes in C57BL/6 mice by streptozotocin injection.
- To study the effects of the lacto-fermented beetroot product in C57BL/6 diabetic mice.

1. LITERATURE REVIEW

1.1. Diabetes and its types

Diabetes mellitus belongs to a group of metabolic disorders characterized by persistent high blood glucose levels. There are two types of diabetes: insulin-dependent and insulin-independent. Insulin-dependent or type 1 diabetes (T1D) is an autoimmune disease that leads to insulin-producing pancreatic β-cell destruction (Lucier and Weinstock 2022). This essential anabolic hormone (insulin) has multiple effects on growth as well as metabolism of minerals, lipids, protein, and glucose. Insulin signals muscle and adipose cells to take up glucose, stimulate liver to store it as glycogen and stimulate the uptake of amino acids and potassium (Lucier and Weinstock 2022). People who cannot produce insulin have to undertake the perpetual insulin replacement treatment (Saxby et al. 2020; Lucier and Weinstock 2022). Insulin-independent or type 2 diabetes (T2DM) does not show any particular signs but can cause health issues in long term.

1.1.1. Pathophysiology of Type 2 diabetes

T2DM is an endocrine disease, which is characterized by impaired insulin secretion and/or decreased response of the body to an insulin (insulin resistance). This impairs metabolism of carbohydrates, fats, and proteins ("Diagnosis and Classification of Diabetes Mellitus" 2009). Pancreatic β cells that secret insulin during hyperglycaemia promotes oxidative stress which leads to reactive oxygen species (ROS) generation. This stops Ca^{2+} circulation and induces proapoptotic signals (Galicia-Garcia et al. 2020). Oxidative stress increases proinsulin formation leading to further ROS generation and β-cell damage (Galicia-Garcia et al. 2020; DeFronzo et al. 2015) leading to a decrease in insulin production. Several factors can lead to T2DM. For instance, high fat diet consumptions could change the lipid layer of tissues to change their insulin receptor availability (Kahle et al. 2014). Lipid metabolites such as diacylglycerol (DAG) affects protein kinase C and phosphorylated insulin receptors. This modification impacts signals to glucose transporter 4 (Glut-4) and cells lose the ability to get glucose (DeFronzo et al. 2015). Furthermore, insulin sensitivity could be lost in muscles, liver tissues, kidneys, gastrointestinal tract, brain and fat cells or pancreas itself due to multiple abnormalities (Fig.1.1). For instance, muscle tissue could have defective insulin receptors that would not interact with insulin. Supressed insulin signalling pathways in muscle tissues can inactivate glucose transport, deactivate receptor auto-phosphorylation of tyrosine residues of the insulin receptor substrates (IRS), PI3K, Akt, and protein kinase C resulting in decreased glucose transport and its use (Ormazabal et al. 2018). In adipocytes, inflammation and insulin resistance provoke free fatty acids (FFA) and pro-inflammation

cytokine release. This increases DAG and ROS formation and signals from TLR4 and TNFR receptors further aggravating insulin resistance (Krüger et al. 2008). When the pancreas cannot produce enough insulin to reduce blood glucose levels glucose deficiency in liver causes promotes gluconeogenesis and the accumulation of glucose in the blood results hyperglycaemia (DeFronzo et al. 2015). This state triggers pro-inflammatory cytokine production and leads to immune system dysregulation and formation of advanced glycation end products (AGEs) which damage DNA and the nervous system (Kubis-Kubiak, Rorbach-Dolata, and Piwowar 2019; Hulkower, Pollack, and Zonszein 2014). Hyperglycaemia decreases blood vessel elasticity and creates functional changes in cellular permeability, inflammation, angiogenesis, and cell growth. Diabetes associated endothelial dysfunction and platelet aggregation increases atherothrombosis formation that can affect all organs (Paneni et al. 2013). Haemoglobin A1c (HbA1c) is a strong biomarker of T2DM. HbA1c levels give an indication of the average glycemia levels over a period and is therefore important factor for assessing the effectiveness of diabetes treatment and the risk of complications. HbA1c tests measure the amount of sugar attached to haemoglobin in the blood and high levels of HbA1c can cause stroke, cardiovascular diseases, metabolic syndrome, neuropathy, eye and kidney diseases (Sherwani et al. 2016; Aljenaee et al. 2019).

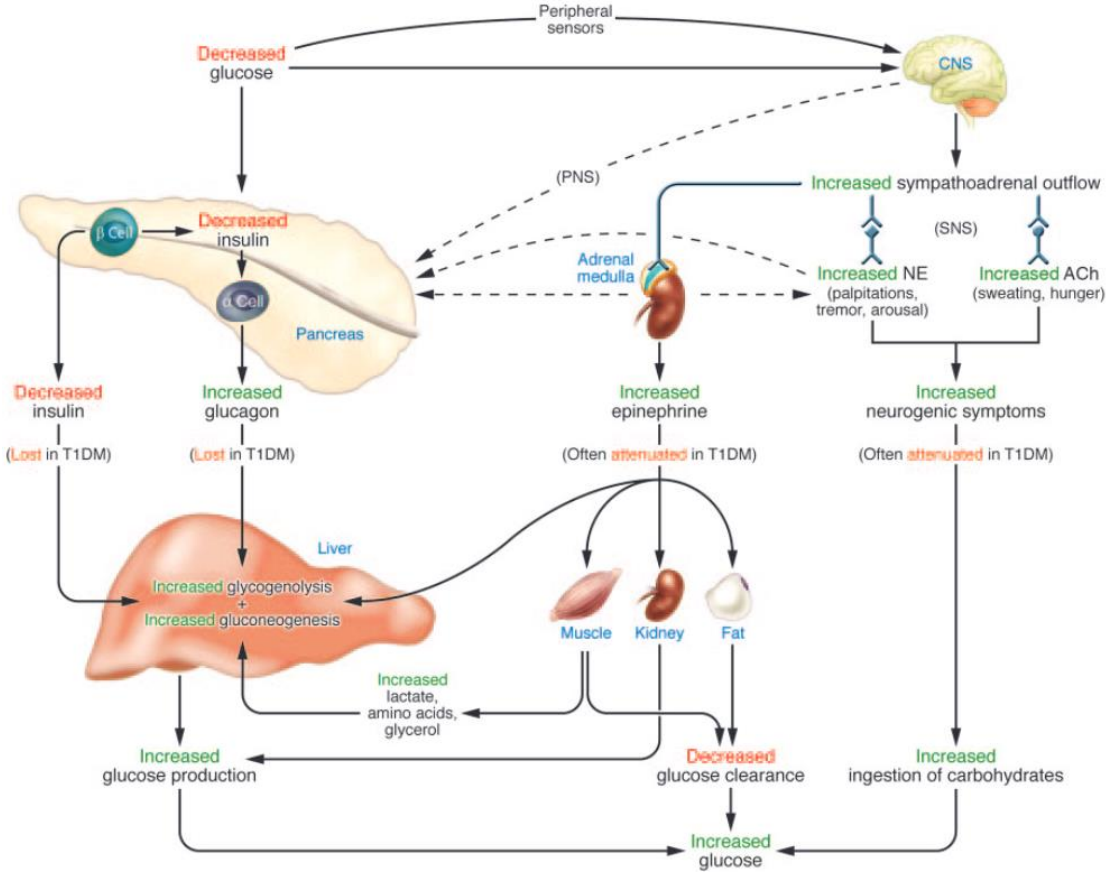


Fig.1.1. Physiological and behavioral defenses against hypoglycemia. Decrements in insulin and increments in glucagon are lost and increments in epinephrine and neurogenic symptoms are often

attenuated in insulin deficient T1DM and advanced T2DM. SNS, sympathetic nervous system; PNS, parasympathetic nervous system; NE, norepinephrine; ACh, acetylcholine; α cell, pancreatic islet α cells; β cell, pancreatic islet β cells. Adapter from Philip E Cryer (Cryer 2009).

1.1.2. Risks factors for diabetes

Risk factors that induce diabetes are obesity, physical inactivity, sedentary lifestyle, old age, urbanization or even smoking, some medications, sleep deprivation and low socioeconomic status (Park et al. 2003; DeFronzo et al. 2015). Genetic predisposition and obesity are thought to cause hyperglycaemia and diabetes. In addition, diabetics with complications do not only have high glucose levels (more than 125 mg/dL or 7 mmol/L), but also symptoms such as feeling tired, fatigue, thirst, frequent urination, delayed wound healing, blurred vision or limb numbness (Baena-Díez et al. 2016). Diabetes can cause nervous system and cardiovascular system damage as well as an increase the risk of cancer development, blindness, organ failure, lower limbs amputation and overall reduced life quality (Baena-Díez et al. 2016).

The global prevalence of diabetes in adults has increased exponentially due to genetics and environmental factors. For scientists it has been a challenge to identify genes that are responsible for this disease development due to the influence of many factors. Genome-Wide Association Studies (GWASs) shows that single-nucleotide polymorphism (SNP) in TCF7L2 could affect T2DM development, with possibly more than 100 combinations. Genetic analysis identified that 13 of 37 variants associated with T2DM could be responsible for fasting glucose regulation (Morris et al. 2012). In other regions such as the Icelandic population, TCF7L2 locus was most influential and common in T2DM variants (Cauchi et al. 2007). Other genes: SL20A8 – encodes zinc transporter that required to store insulin, KCNJ11 – encodes an ATP-dependent potassium channel, GCKR – encodes glucokinase regulatory proteins, have also been shown to be linked to T2DM (Sladek et al. 2007). unfortunately, these gene clusters could be transfer to offspring more likely from T2DM mothers than from fathers (DeFronzo et al. 2015).

Studies show that gut microbiome composition may influence the development of diabetes. Greater microbiome diversity, along with better number of butyrate-producing gut bacteria, was associated with lower diabetes and insulin resistance appearance and development among non-diabetic individuals (Z. Chen et al. 2021).

1.1.3. Global prevalence of diabetes

Globally, 2010 statistics show that 285 million people had diabetes and by 2030 it is projected to be 439 million (L. Chen, Magliano, and Zimmet 2012). So far, T2DM ranks as ninth leading cause of

mortality. Aging appears to have a huge influence on T2DM development. Gender comparison indicates that men are affected by this disease faster and more often than women (Khan et al. 2020; Oluyombo et al. 2015). Even though woman during pre and post-menopausal stages increase chances of metabolic abnormalities and T2DM development (Oyewande et al., n.d.).

T2DM used to be relatively rare in developing countries, for example, in 1980 in China less then 1 % had the prevalence of the disease (Chan et al. 2009). However, higher rates observed in Mauritius (Dowse et al. 1990) and Asian immigrants in Western countries (McNeely and Boyko 2004) allowed to predict epidemic of T2DM that now appearing in India and China (Fig.1.2). Unfortunately, diabetes mellitus more often takes place in developing than in developed countries. Worldwide, less developed countries have 80% of cases of diabetes mellitus (Shaw, Sicree, and Zimmet 2010). Due to the rapid economic development, urbanization and dietary transition, Asia is considered to be the world's „diabetes epicenter" (Chan et al. 2009). Asia has 5 out of 10 countries that are predicted to have highest numbers of people diagnosed with diabetes by 2030 (Shaw, Sicree, and Zimmet 2010). Statistic show that between 2007 and 2008 China has overtaken India and become the global epicenter of the diabetes with more than 92 million adults who have diabetes mellitus and 148.2 million adults who are prediabetes, including people with impaired fasting glucose (IFG) and/ or defective glucose tolerance (Wenying et al. 2010).

Similarly to Asia, other areas like Middle East (Shaw, Sicree, and Zimmet 2010) and Africa (Mbanya et al. 2010) have shown to be the hot spots for diabetes mellitus. Scientists identified that immigration, for example immigrants from the Middle East living in Sweden has higher prevalence of diabetes then comparing to local Sweden's (Wändell et al. 2008). Developed countries have lower proportion young to middle-aged people that are effected by T2DM then developing countries (Shaw, Sicree, and Zimmet 2010). Moreover, it was believed that T2DM less prevalent in rural than in urban areas, and it is not fully true. In India a study showed that between 2000 to 2006 there was a significant increase in diabetes mellitus prevalence in both areas: urban – from 13.9 % to 18.2 % and rural – from 6.4 % to 9.2 % (Ramachandran et al. 2008). Similar effect has been seen between 2001 and 2006 study among Chinese individuals. In rural area occurrences of diabetes mellitus in men increased from 5.3 % to 14.2 % and from 8.9 % to 13.8 % in women, parallel to urban areas from 11.3 % to 19.2 % in man and from 11.3 % to 16.1 % in woman (Wang et al. 2022).

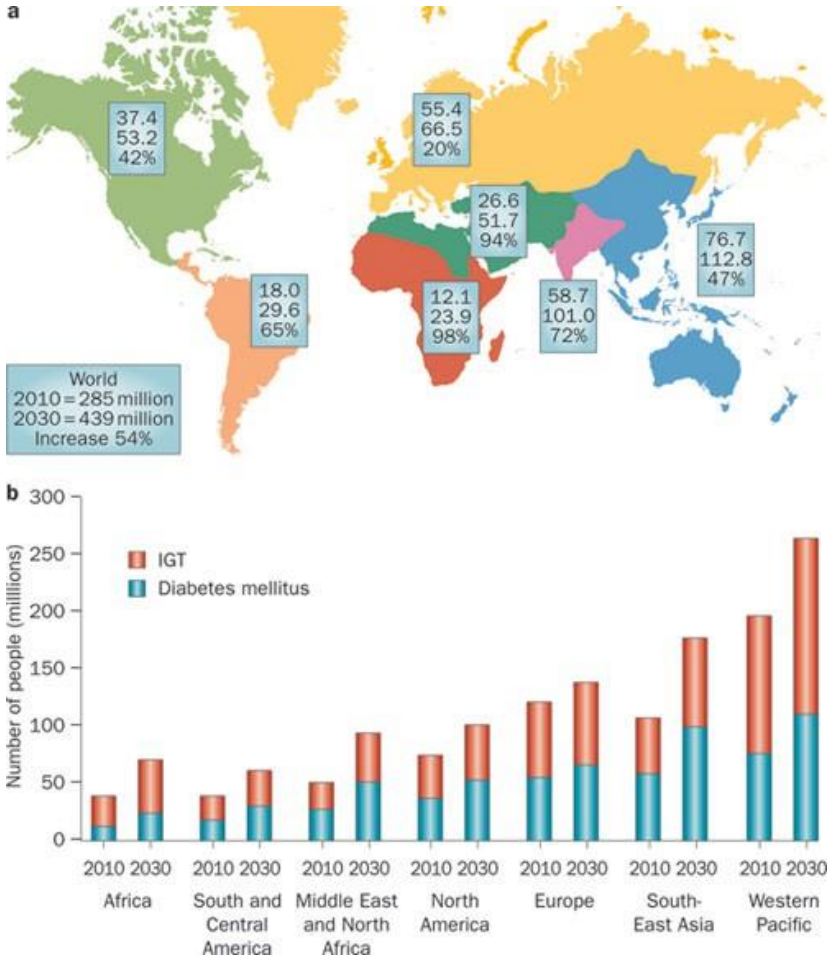


Fig.1.2. a) In each box, the top and middle values represent the number of people with diabetes mellitus (in millions) in each of seven world regions (depicted with different colors) for 2010 and 2030, respectively; the bottom value is the percentage increase from 2010 to 2030. The number of people globally with diabetes mellitus is projected to rise from 285 million in 2010 to 439 million by 2030, a 54% increase. **b)** The number of people with diabetes mellitus and IGT (in millions) by region among adults aged 20–79 years for the years 2010 and 2030. Data courtesy of the International Diabetes Federation Diabetes Atlas. Adopted from (Chen, Magliano et al. 2012).

1.2. Diabetes treatment

There are several treatment options for diabetes: medication, lifestyle changes or dietary changes. Drugs can be classified as enzyme inhibitors or other type drugs (*Medication for Type 2 Diabetes* 2020).

Chemical enzyme inhibitors (medications/ drugs)

- Gliptins like sitagliptin, vildagliptin saxagliptin are known as Dipeptidyl peptidase-4 (DPP-4) inhibitors. DPP-4 is an adenosine deaminase complexing protein 2 that in the

human body is associated with immune regulation, signal transduction, and apoptosis. It could be expressed in different human tissues like placenta, lung, liver, gut, or kidney. DPP-4 cleaves a wide range of substrates, including growth factors, chemokines, and peptides. Enzymatic activity increases blood glucose level by degradation of GLP-1 or -2 and inactivating glucose dependent insulinotropic peptide (GIP). That way pancreatic β cells do not produce enough insulin that is needed for signalling cells to use glucose and store blood sugar in the liver. DPP-4 inhibitors increase levels of incretins, enhance hormone GLP-1 and other peptide activity, help the body to produce more insulin only when its needed to lower the amount of glucose being produced by the liver when it is not needed (Seshadri and Kirubha 2009).

- Gliflozins are sodium glucose co-transporter-2 (SGLT2) inhibitors. SGLT is a co-transporter that can be found in the kidney, small intestine. SGLT is found in the proximal tubule. Its main feature is to reabsorbed glucose back to a system. Inhibitors like dapagliflozin, canagliflozin, empagliflozin inhibit glucose absorption, and increase its extortion in the urine (Hummel et al. 2011).That way, decreasing blood glucose level. It results in the amelioration of glucotoxicity, with improved β-cell function. However, this drug can stimulate endogenous glucose production, with side effects such as genital mycotic infections in females and increase in urinary tract infections. Due to the mode of action, this inhibitor can only be used on patients with normal glomerular-tubular function (Marín-Peñalver et al. 2016)
- Acabose, voglibose, miglitol are inhibitors of α-Glucosidase (Joshi et al. 2015). α-Glucosidase is an enzyme associated with carbohydrate digestion in the gut. It cleaves terminal non-reducing (1→4)-linked α-glucose residues from dietary carbohydrates and starch that way, producing a single α-glucose molecule that could be easily absorbed in the system. This enzyme could be produced by gut bacteria or work actively on the intestinal epithelial surface. Acabose competes with oligosaccharides to attach to enzyme reactive centre that way reducing starch degradation and glucose absorption. Its inhibition shows that it decreases glucose uptake that way lowering blood glucose level. Some studies show that natural flavonoids could have inhibition effects (Proença et al. 2017) which broadens horizons and helps to find new substances that could be used as drugs.
- α-Amylase inhibitors chalcones, flavones, benzothiazoles could be used as anti-diabetic drug. Amylase is an enzyme that's main function is to cleave glycosidic bonds in starch

molecules and turn long and complex carbohydrates to oligosaccharides like maltose, maltotriose or dextrin. There are three known classes of enzyme: α, β and gamma. A-amylase can be found in microorganisms, plants and animals, while β in plants, microbes, last but not lease, gamma can be found in plants and animals (Akinfemiwa and Muniraj 2022). Human secret α-amylase from salivary glands in the mouth, is first step of chemical food digestion. A amylase randomLy hydrolyze α (1-4) glycosidic linkages to produce small molecules that that are easier to absorb in the bloodstream. However the inhibitors prevent the digestion and absorption of glucose and lower blood glucose level (Bashary et al. 2020). These drugs are still in testing trials and not used for real treatment.

- Other type of drug includes metformin. It is the best studied drug that is known to have the best tolerance. It reduces insulin resistance by increasing glucose transporter effectivity and signals pancreatic β cells to produce more insulin. Metformin also inhibit their apoptosis through AMPK signalling pathway (Cravalho et al. 2020). The drug further inhibits DPP-4 and GLP-1 metabolism (Thondam et al. 2012) and increases its enteroendocrine secretion by stimulating microbial SCFA production (Wu et al. 2017). Long term use of metformin reduces micovascular complications (Nathan et al. 2015). The possible side effects include diarrhea and in rare cases, acidosis that could lead to breathing problems, nausea or circulatory shock (Strack 2008).
- Sulfonylureas is another orally administered antidiabetic drug. They can be classified in to 2 groups: 1 (olbutamide and chlorpropamide) and 2 (gliclazide, glipizide, glibenclamide and glimepiride) generation. Drugs cannot be prescribed if patients are overweight, have metformin or do not have enough adequate glycemic control ("Standards of Medical Care in Diabetes—2013" 2013). The main effect of the drug is to rise plasma insulin concentration. It decreases hepatic clearance of insulin and stimulate insulin secretion by pancreatic β cells. They act as depolarizing β-cell, attaching to K^+ channels and blocking K^+ flow. It increases Ca^{2+} flow in the cell that causes the contractions of the filaments of actomyosin that is responsible for exocytosis, therefore promptly secreting insulin in larger amounts (Sola et al. 2015). That's why this drug is used then there are still functioning β cells.

Lifestyle changes

Making lifestyle changes for people with diabetes can be challenging. However, to achieve metabolic control many people with diabetes see changes in nutrition and physical activity as an essential

part of their treatment. Other method that is used for diabetes treatment besides lifestyle changes such as modified diet or increased exercise and stress management. Urbanization, the growing and shifting economy changed people's work style from hard physical labour to sedentary occupations. Lack of exercise causes muscle cells to lose sensitivity to insulin, weakens blood flow and vascular system. This inhibits the use of glucose for energy production but converted to fat and stored increasing obesity risk. Exercise plays an important role in the prevention and control of insulin resistance, prediabetes, T2DM, and diabetes-related health complications. Both resistance and aerobic training improve insulin action and can help to control blood glucose levels, lipids, blood pressure, cardiovascular risk, or mortality. To have real beneficial effect training should be planned out to include regular exercise and be undertaken regularly. Most people that have diabetes can perform exercise safely if certain precautions are taken. Increasing overall physical activity is critical for optimal health in individuals with T2DM (Colberg et al. 2010; Scheurink et al. 1999; Pan et al. 1997).

Dietary changes

A diabetes diet simply means eating the healthiest foods in moderate amounts and sticking to regular mealtimes. Economic growth and environmental factors change because of food production, processing and accessibility to unhealthy, fast-food options. Nowadays food has higher calorie content due to increased serving portions, carbohydrate and fat content, sugary beverages and additional food flavourings (Popkin, Adair, and Ng 2012). A diabetes diet is a healthy-eating plan that is naturally rich in nutrients and low in fat and calories. Key elements are fruits, vegetables and whole grains (Ley et al. 2014; Sami et al. 2017).

- Foods high in carbohydrates are an important part of a healthy diet. however, foods with high glycemic index (GI) can cause hyperglycaemia, resulting in diabetes (Atkinson, Foster-Powell, and Brand-Miller 2008) (Fig.1.3). Some studies have showed that a low-carbohydrate diet (LCD), can reduce weight, improve blood glucose levels and regulate blood lipids in patients with T2DM mellitus (Ley et al. 2014; Sami et al. 2017).

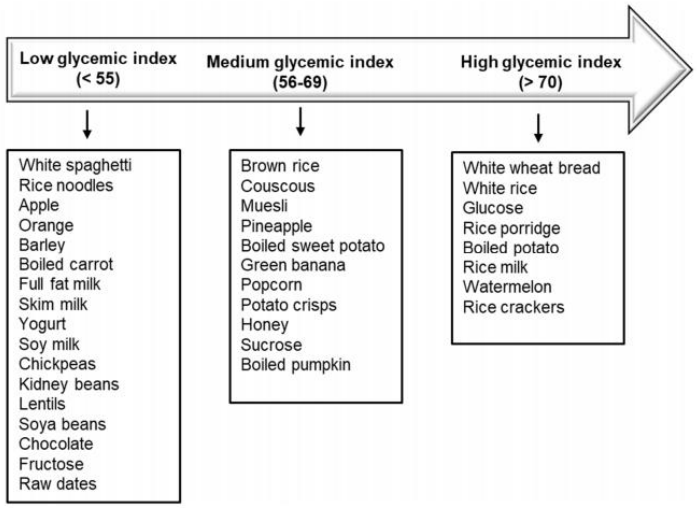


Fig.1.3. Glycemic index of different foods. Adapted from (Bonsembiante, Targher, and Maffeis 2021).

- Fats are essential to our everyday diet. Low-fat diet without calorie intake could reduce the risk of obesity, reducing insulin resistance and reducing the incidence in people with diabetes (Pan et al. 1997). However, some studies have shown that even a low-fat diet can help lose weight, they may have no effect on glycemic control (Gerhard et al. 2004).
- Another important macronutrient is protein. Protein consumption increases satiety thereby lowering post-prandial glycemic markers and increasing thermogenesis. Improved body consumption increases insulin release and sensitivity while accelerating fat oxidation (Beaudry and Devries 2019).

1.2.1. Side effects of antidiabetic medications and the new therapeutic strategies

Over the years, many synthetic drugs have been used for managing and preventing T2D, however, these are not without side effects. Some side effects include Vitamin B12 deficiency, which may cause anemia and neuropathy, pancreatitis, upper respiratory tract infection, ketoacidosis, genital mycosis, increased low-density lipoprotein cholesterol, bone fractures, lipoatrophy and lipohypertrophy among others. Therefore, there is a need to find antidiabetic therapies that are not only safe and effective in controlling blood glucose levels, but that also lack serious negative side effects. The consumption of various foods has also been shown in animal and human studies to have positive impacts on blood glucose regulation. Over the last few decades, numerous studies have been carried out to try and identify the antidiabetic constituents in these food products and their mechanisms of action. Dietary components, including soluble fiber, phenolic compounds, and peptides, have been shown to display various antihyperglycemic properties, such as inhibitory activity against digestive enzymes, insulin

secretagogues effects and activation of receptors involved in glucose metabolism. Although not as potent as synthetic drugs, the antidiabetic constituents from food products could have the advantage of causing less undesirable side effects and thus, could represent an interesting complementary approach in the management of diabetes.

1.3. Role of the gut microbiota and diabetes

Gut microbiome modulation could be a new and alternative treatment of diabetes. Scientists compare it as another human's digestive system "organ".

Disruption of the commensal relationship between the gut microbiota and the host can lead to an imbalance in the bacterial population, resulting in pathogenic bacteria becoming the predominant gut population (Fig.1.4). Gut microbiota dysbiosis likely promotes diet-induced obesity and metabolic complications through multiple mechanisms, including immune dysregulation, altered energy regulation, altered gut hormone regulation, and pro-inflammatory mechanisms. Type 2 diabetics have shown that they have significantly different gut bacteria compared with healthy people. Impaired Bacteroidetes/Firmicutes ratio has been associated with increased intestinal permeability, where bacterial by-products pass through the leaky intestinal barrier, triggering the subsequent inflammatory responses characteristic of diabetes (Iatcu, Steen, and Covasa 2021). Experiments in animal and human studies have produced growing evidence for the causality of the gut microbiome in developing obesity and T2DM (Meijnikman, Gerdes et al. 2017). It is evident that the levels of some bacteria involved in SCFA production were significantly lower in people with T2DM (Salamone, Rivellese et al. 2021). Microbial SCFAs adhere to G-protein coupled receptors to trigger secretion of GLP-1, an important incretin hormone, which is made by enteroendocrine L cells (Fig. 4) (Zhang, Sun et al. 2019). GLP-1 impedes secretion of glucagon, hampers gluconeogenesis in the liver, improves insulin sensitivity and augments central satiety, thereafter resulting in bodyweight loss (Ayala, Bracy et al. 2010). Furthermore, SCFAs can directly hinder the low-grade inflammatory response caused by bacteria migration from the intestines into the mesenteric adipose tissue and the blood (Brahe, Astrup et al. 2013).

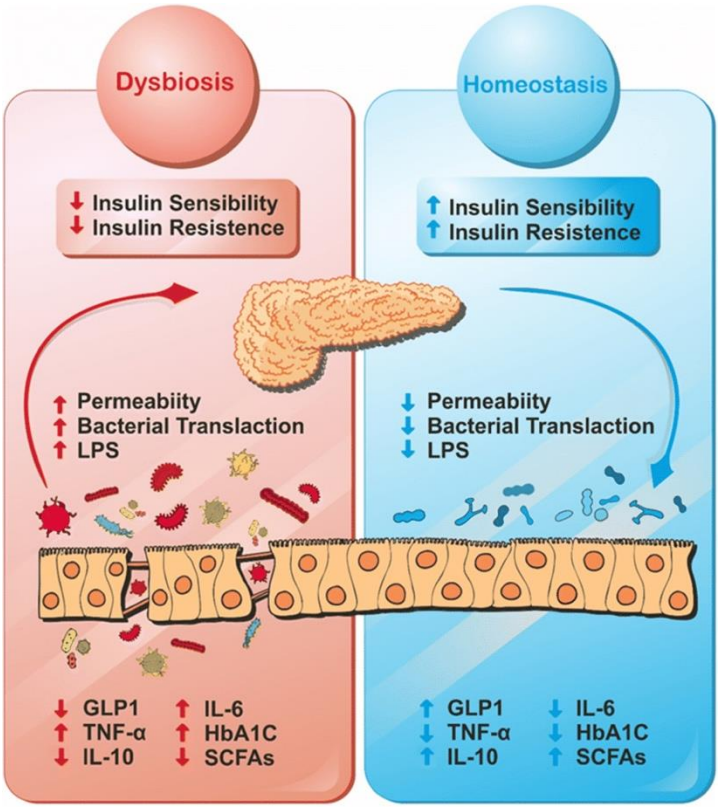


Fig.1.4. Intestinal microbiota in homeostasis and dysbiosis promoted by T2DM and consequent impact on the development or prevention of T2DM. Intake of probiotics can positively modulate the intestinal microbiota, resulting in increased production of saccharolytic fermentation SCFA, and improved function of the intestinal barrier. Increased SCFAs are implicated in the release of GLP-1, which have an important impact on satiety, hunger, insulin sensitivity, and improve intestinal barrier function. Consequently, increased bowel barrier function may reduce translocation of bacteria and LPS, and thus reduce pro-inflammatory markers (interleukin-6 (IL-6), tumor necrosis factor (TNF), and increase anti-inflammatory markers (interleukin-10 (IL-10)), as well as increase glycosylated hemoglobin A1c (Salgaço, Oliveira et al. 2019).

1.3.1. Gut microbiota modulation strategies

Gut microbiota modulation aims to treat microbial dysbiosis that is associated with disease development. Gut dysbiosis could influence glucose metabolism, chronic inflammatory disease, and digestive system cancer. Studies have shown that functional materials such as probiotics, prebiotics and functional foods have significant modulatory abilities on the gut.

- Probiotics are live-microorganisms that offer a health benefit to the host when consumed in adequate quantities (Cordaillat-Simmons, Rouanet et al. 2020). Probiotic use has been applied to T2DM patients. Meta-analysis showed a significant effect of probiotics in reducing fasting plasma glucose and insulin levels that are associated with hyperglycaemia. In addition, it lowers triglyceride, cholesterol levels, both systolic and diastolic blood pressure that reduce risk of cardiovascular disease (Kocsis et al. 2020; Sanders et al. 2019). Other studies have shown that probiotic consumption can reduce HbA1c, fasting blood glucose and homeostatic model assessment for insulin resistance in diabetic patients (Tao, Gu et al. 2020). Mechanisms through which probiotics improve glucose homeostasis likely stem from changing the composition of the host gut microbiota. Altering the gut microbiota can improve intestinal barrier integrity to reduce circulating bacterial endotoxin, and ultimately, reduce systemic inflammation (Harkins, Kong et al. 2020). Also, probiotics may alter microbiota-derived metabolites, such as butyrate and acetate, which have been associated with changes in glucose and lipid metabolism as well as appetite signaling (Tolhurst, Heffron et al. 2012).

- Prebiotics are known as food components used by host microbes. It includes high fiber food such as fruits, vegetables, legumes, and grains that are not broken down by human digestive enzymes. Prebiotics can be either naturally extracted from non-digestible carbohydrate materials or synthetically produced. They don't work directly on a person's organism but help existing microorganisms to grow and function in specific genera or species. Microbial growth improves bowel movement, fecal bulking. Immune regulation may be affected by increased bacterial biomass and cell wall components. Metabolic products include organic acids that lower the pH of the gut while simultaneously affecting microbial pathogens and minerals absorption. Metabolic products can also affect epithelial integrity and hormone regulation. Bacteria that react to prebiotic treatment may affect the composition of the microbiota due to antimicrobial agents (e.g., peptides) and competitive interactions possibly reducing infections and bacteria containing LPS. Increased metabolic effect in the gut increases defence against pathogens, strengthens the inner lumen cell wall. Decrease LPS amount in blood system decrease inflammation possibility has been suggested to be a causative factor in diabetes (Sanders et al. 2019). Consumption of prebiotics for modulating the gut microbiota results in the production of microbial metabolites such as SCFA that play essential roles in reducing blood glucose

levels, mitigating insulin resistance, reducing inflammation, and promoting the secretion of GLP-1 in the host, and this accounts for the observed remission of metabolic diseases (Megur, Daliri et al. 2022).

1.4. Functional foods

"Functional foods" are also known as designer foods or nutraceuticals. They don't have a universal definition, but in the context of benefits extend beyond their nutritional value.

Treatment for diabetes focuses more on food rich in fiber, non-digestive carbohydrates and richness in bioactive compounds (Mirmiran, Bahadoran, and Azizi 2014). Whole grains are made of non-digestible polysaccharides like insoluble and soluble fiber, β-glucan, even insulin and non-carbohydrate functional components: phenolic acids, carotenoids, phytates and others. These compounds could regulate insulin sensitivity, increase insulin secretion by improving pancreatic β cells function (Borneo and León 2012). Soluble and fermented fiber could act as prebiotic in the gut and modulate gut microbiota leading to better metabolic response (Martínez et al. 2013). Bioactive compounds like peptides could have an α-amylase inhibitory effect, that way reducing digestion and absorption of dietary carbohydrates (Duranti 2006; Khang et al. 2016). Nuts have high-biological value proteins, bioactive peptides, functional fatty acids, fiber, phytosterols, polyphenols, tocopherols, and other antioxidant vitamins. They can normalize lipid and lipoprotein levels and improve insulin resistance. Anti-obesity effects could be induced by creating satiety (modulating regulatory appetite neurotransmitters), lowering dietary fat absorption, and increased fat excretion (inducing fatty acid β-oxidation) (Jenkins et al. 2008; Tey et al. 2011). Fruits and vegetables vitamins, and various phytochemicals that by food pigment colour could be reflected as predominant. They have a lot of antioxidants that could enhanced antioxidant defence system, decrease oxidative stress and inflammatory markers, while lowering HbA1c and triglyceride levels (Shashirekha, Mallikarjuna, and Rajarathnam 2015; Hegde et al. 2013). Overall functional food identification and well-planned diet could be important in health promotion, treatment and prevention of diabetes.

1.4.1. Developing functional foods from beetroot

Beetroot (lat. *beta vulgaris*) is a common and cheap vegetable that is prevalent all around the world. It is grown in temperate climate zones and main producers are China, USA, and Europe where it has long been used as a traditional cuisine. The edible part of beetroot is underground and aboveground. Young leaves contain a lot more of protein, minerals, and vitamins than roots. The root contains protein, fiber, vitamins (C, A, B1), organic acids (citric, oxalic, malic, vinous), folic acid and many minerals

(including manganese, iron, potassium, magnesium, cobalt). Studies have shown that have biologically active phyto-nutritions not only vitamins but also variety of polyphenols, phenolic, saponins, betains, betalains that could have anti-oxidant, anti-depressant, anti-microbial, anti-fungal, anti-inflammatory together with inhibition of peroxidation (Jasmitha, Shenoy, and Hegde 2018; Ks et al. 2019). For instance, betanins most known and studied compound (Fig.1.5). This tyrosine-derived pigment not only gives red colour to beetroots but also has antitumor and antioxidant activities. Betanin's ability to neutralize free radicals and high antioxidant activity are related to the presence of phenolic hydroxyl groups in the structure (Vieira Teixeira da Silva et al. 2019). These properties indicate that betalains reduce the risk of liver and kidney damage, cardiovascular and cerebrovascular diseases (Ravichandran et al. 2013). Hydrolysis of β-glucose molecule allows the formation of new bioactive compounds (Fig.1.6) that also has health promoting effects. There are two main groups: red betacyanins (betanin, prebetanin, isobetanin and neobetanin), yellow betaxanthins (containing vulgaxanthin I and II, and indicaxanthin) and they both belong to betalains class. Red pigments are more sensitive to temperature changes, oxygen and ph, so betanins can degraded that results cyclo-DOPA and betalamic acid formation. However, this process is reversible.

Dietary betaine prevents gut dysbiosis by increasing strains such as *Akkermansia muciniphila*, *Lactobacillus,* and *Bifidobacterium.* While *Akkermansia muciniphila* is an important to improve microbiome divercity it also increase strains that produce SCFAs that further more preventing the development of obesity and glucose intolerance. (Du et al., n.d.). On the other hand, due to the anti-microbial activity betanin and vulagaxanthin could inhibit gram-positive bacteria growth such as *Staphylococcus aureus* and *Bacillus sp.* (Wijesinghe and Choo 2022). Consumption beetroot products could improve health starting with anti-inflammation, antioxidant atherosclerosis or T2DM (Edziri et al. 2019). Researchers have found a betanin and its derived compounds effect T2DM prevalence but how modified structures work and effect T2DM gut microbiota are still unknown.

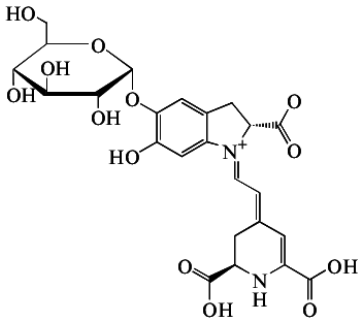


Fig.1.5. Betanin structure: betanidin 5-O-β-D-glucoside. Base on National Center for Biotechnology Information (2022).

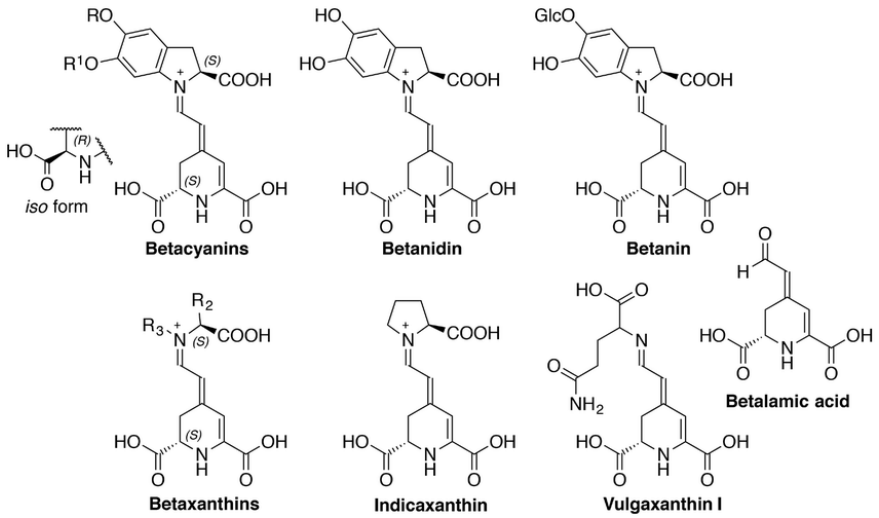


Fig.1.6. Generic structures of betacyanins and betaxanthins. Adapted from (Quina and Bastos 2018).

There are few ways how people process and use beet root as functional food. Josef V. study made in 1973 have shown that 100-200 g daily consumption of beetroot can have- antidiabetic effect for diabetes patients. Due to the big number of bioactive compounds scientists prefer to use beetroot juice (BRJ) and its extracts. For successful batalains extraction methods like pulsed electric fields, microwaving, microwaving coupled with enzyme treatment, aqueous 2-phase technique, thermoultrasonication have been used (Kannan, n.d.; Cardoso-Ugarte et al. 2014; Cruz-Cansino et al. 2015; Khang et al. 2016; Nemzer et al. 2011). Experiments show that at high temperature leads to product color changes from red to brown and long periods of time on room temperature changes product color to yellow (Shynkaryk, Lebovka, and Vorobiev 2008) indicating changes of bioactive compounds composition. Other common processing techniques to get bioactive compound concentrates are air-drying, freeze-drying and spray-drying (Nemzer et al. 2011). This method not only gives wide variation of compounds composition but also measure and prepare suitable dosages (Nemzer et al. 2011). Extracts form beetroots have shown to be effective treatment for diabetics. Anti-diabetic potential has been seen then 2 g extract/kg of bodyweight was used in animal study. It decreased blood glucose level, non-enzymatic glycation and lipid peroxidation (Ozsoy-Sacan et al. 2004). Treatment with betavulgaroside I, II, III, and IV compounds from beetroot after animal consumed glucose have shown hypoglycemic activity (Yoshikawa et al. 1996). Extracted flavone quercetin have shown that after 6 weeks consumption can lower blood glucose levels and sugar extraction in urine in streptozotocin (STZ) induces diabetic mice (Vessal, Hemmati, and Vasei 2003).

Beetroot are high in fiber that have also shown a potential anti-diabetic properties. In intestine it interacts with gut microenvironment. Dietary fiber lower the uptake of nutrients while soluble fiber effect slower and steady absorption of nutrients (Brownlee 2011). High fiber consumption especially rich in soluble fiber can improve glycemic control and decrease hyperinsulinemia (Chandalia et al. 2000) in addition working as prebiotic for gut bacteria.

1.4.2. Fermentation as a method of improving the antidiabetic functions of beetroot

To increase the amount and diversity of compounds in the biotechnology industry uses fermentation with microorganisms (Klewicka et al. 2012; Kumar, Manoj, and Giridhar 2015). This process not only weakens beetroot sturdy cell wall and release more pigments, with bacteria contribution, pigments could be modified and give a new characteristic to a product (Casciano et al. 2022). Using LAB fermentation process to concentrate and get betalain rich-extracts have been determined to be safe (Klewicka et al. 2012). Scientists like apply and use probiotic bacteria, specifically LAB, that could be extracted from other fermented products, because they have lack of toxins, many characteristics are known and they safe to use in industry (Casciano et al. 2022). Sometimes bacteria can be used because of its specific enzyme activity that would allow to get wanted product like LAB involved in the fermentation of plant foods. For example, for olive fermentation *Lactobacillus plantarum, L. pentosus, L. brevis* species have been used to hydrolyze oleuropein thought β-Glucosidase activity (Ghabbour et al. 2011). Similar enzyme use have been shown in soy milk fermentation with *Streptococcus thermophilus, L. acidophilu* and *Bifidobacterium* species to increase the amount of genistein and daidzein (Rekha and Vijayalakshmi 2011) Bacteria that have β-Glucosidase activity (Michlmayr and Kneifel 2014) could be used for beetroot fermentation and to create new and high levels of bioactive compounds. Even spontaneous lactic acid fermentation of a BRJ have different chemical properties (Kazimierczak et al. 2014). Using beetroot and specifically selected probiotic bacteria betanin can be bioconverted to betalains or other analogues (Fig.1.7) that could have similar or stronger biological effect: stability, antioxidant, ROS, anti-inflammation activity, inhibit lipid peroxidation ant others (Sadowska-Bartosz and Bartosz 2021), then the parent compounds. After identifying new compounds and their properties, beetroot could be stated as excellent raw material for fermentation and allow to create new functional products.

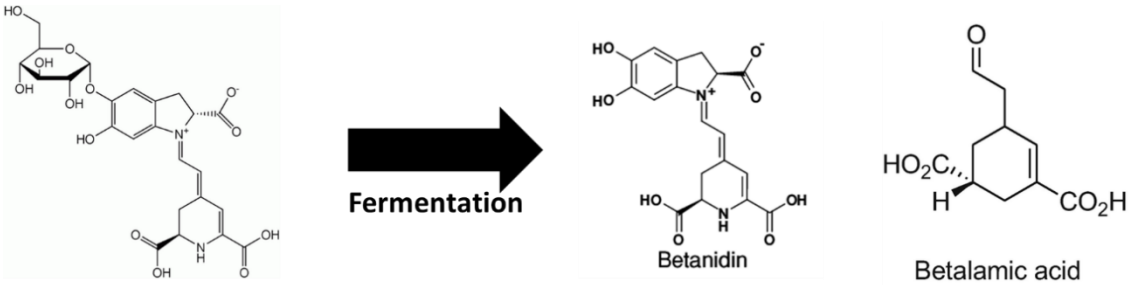


Fig.1.7. Bioconversion of betanin to be it metabolites by fermentation. (Made by author).

1.5. Implication to treat diabetes

To sum up, diabetes is a serious problem that is growing rapidly all over the world. High sugar levels are not only caused by genetics, but also by the impact of our surrounding environment and our own lifestyle decisions. There are widely known typical treatment methods in the world: drugs, lifestyle changes such as sports, getting rid of harmful substances, stress management. However, changes in diet have the greatest influence and have the best effects of diabetes mellitus treatment or prevention. The development of functional food could be a great aim to improve people's health. Using beetroot fermentation for functional product creation, bacteria are capable to extract and covert new compounds that could not only help in the treatment of hyperglycemia, but also modulate intestinal bacterial composition.

2. MATERIALS AND METHODS

2.1 Equipment, media, buffer solutions, animals and other reagents used in the work

2.1.1. Chemicals and reagents

- α-Glucosidase inhibitor screening kit – Abcam (Cambridge, UK)
- α-Amylase inhibitor screening kit – Abcam (Cambridge, UK)
- Animal chew - Maintenance diet for rats and mice – 10 mm pellets (altromin, Germany)
- Antibiotics discs (Erythromycin (E), Vancomycin (VA), Kanamycin (K), Penicillin (P), Ampicillin (AMP), Gentamicin (GEN), Streptomycin (S), Tetracycline (TE), Rifampicin (RIF)) – Carl Roth (Karlsruhe, Germany)
- β-Glucosidase assay kit – Abcam (Cambridge, UK)
- De Man, Rogosa and Sharpe (MRS) agar and broth – Sigma-Aldrich Sp. z o.o. (Poznań, Poland)
- DPP-IV inhibitor screening kit – Abcam (Cambridge, UK)
- Glycerol – Carl Roth (Germnay)
- Glucose (20 % conc.) – Sigma Aldrich (Germany)
- Insulin – Lantus, Biopharma (Belgium)
- Peptone buffer Sigma-Aldrich Sp. z o.o. (Poznań, Poland)
- Streptozotocin – Sigma Altrich (Germany)
- Total antioxidant capacity assay kit – Sigma-Aldrich Sp. z o.o. (Poznań, Poland)

2.1.2. Equipment

- Blender – Philips H3664/90
- Centrifuge 5424 (Eppendorf)
- Glucose meter – CONTOUR®PLUS
- Glucose meter strips – CONTOUR®PLUS
- Laminar – MARS 1200, II safety class (ScanLAF)
- Microplate reader – SpectraMax i3x Multi-Mode Microplate Reader (REUZEit, LLC, Temecula)
- pH meter – (Mettler Toledo® FiveEasy,)
- Scales – (KERN ADB)
- Scales – A&D weight (Labochema)_
- Thermostat – (Binder)
- Thermoshaker – (CERTOMAT U)

- Vortex – VORTEX-GENE 2 T (Scientific Industries)

2.1.3. Animals

- Mice C57BL/6 J line (Charles River, France),

2.2. Isolation of microorganisms from the fermented food products

Beetroot (herein referred to as beetroot), locally fermented tomatoes, pears, sauerkraut, and pickles were bought from Kalvariju market (Kalvarijų turgus, Vilnius- Lithuania) and homemade kefir, kombucha, kvass, yogurt. All the samples were placed on ice and transported to the laboratory. Each sample paste (1 g) was transferred aseptically into separate test tubes containing 9 mL (or 1 mL) of sterile peptone water (0.1 % w/v) and 100 μL of the diluted sample was spread on De Man, Rogosa and Sharpe (MRS) agar and incubated aerobically at 35 °C for 48 h. Bacteria colonies were separated based on their morphological differences and single colonies were inoculated in MRS broth and incubated at 35 °C for 48 h under aerobic conditions. An aliquot (100 μL) of the overnight cultures were spread on MRS agar and incubated at 35 °C for 48 h under aerobic conditions. Pure isolates were identified by microscopic and phenotypic tests. The bacteria strains were tested for their ability to grow in the presence of BRJ using the agar well diffusion test. Cultures that grew and had no inhibition zones were used in further reasearch (Fig.2.2.). In all, 22 out of 160 isolates were resistant to BRJ. These strains were selected, and stock cultures were prepared in MRS broth containing 20 % glycerol (v/v). The bacteria stocks were maintained at −80 °C deep freezer (Fig.2.1).

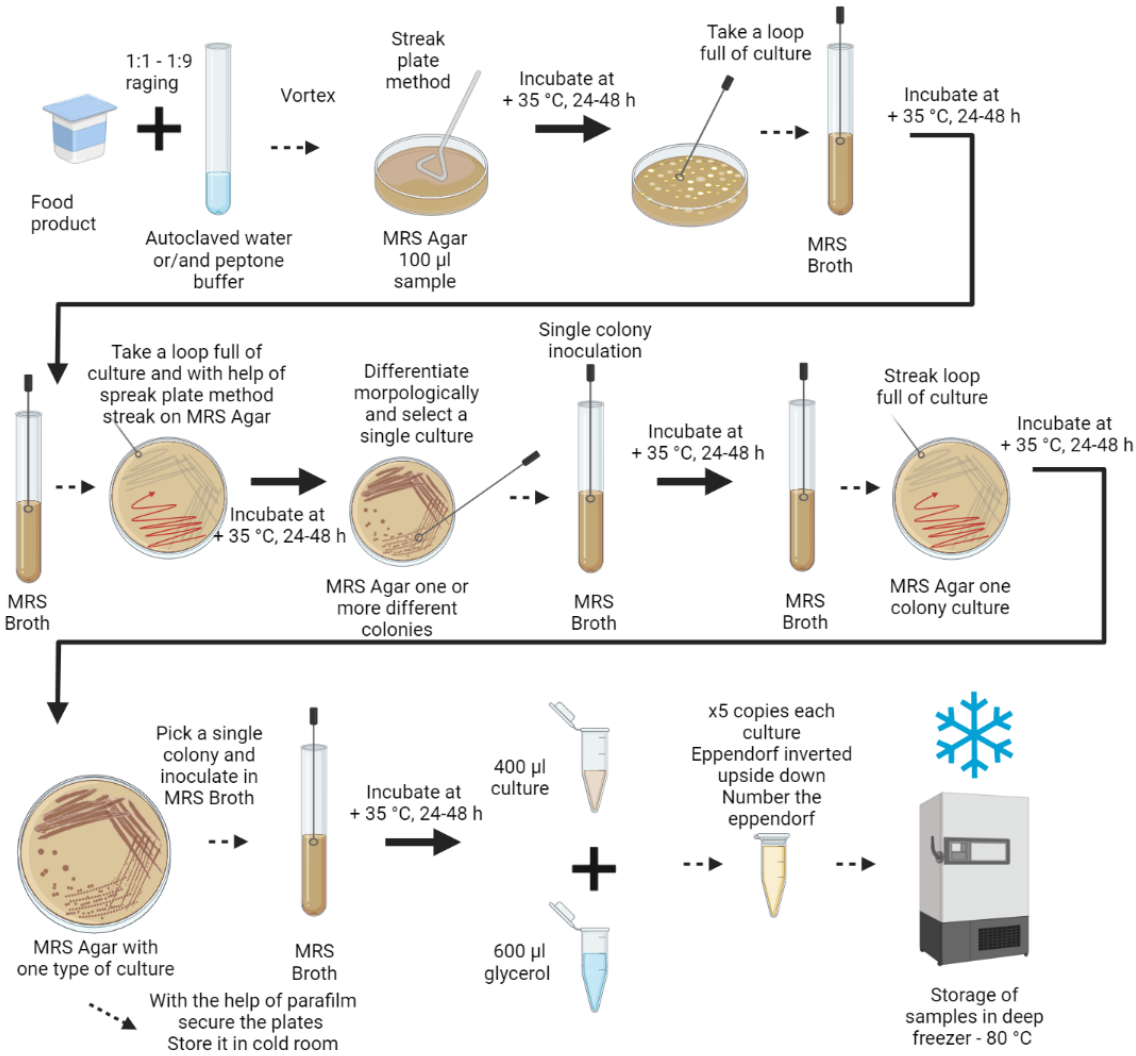


Fig.2.1. Microorganism isolation and storage. Scheme made by (author with Biorender program).

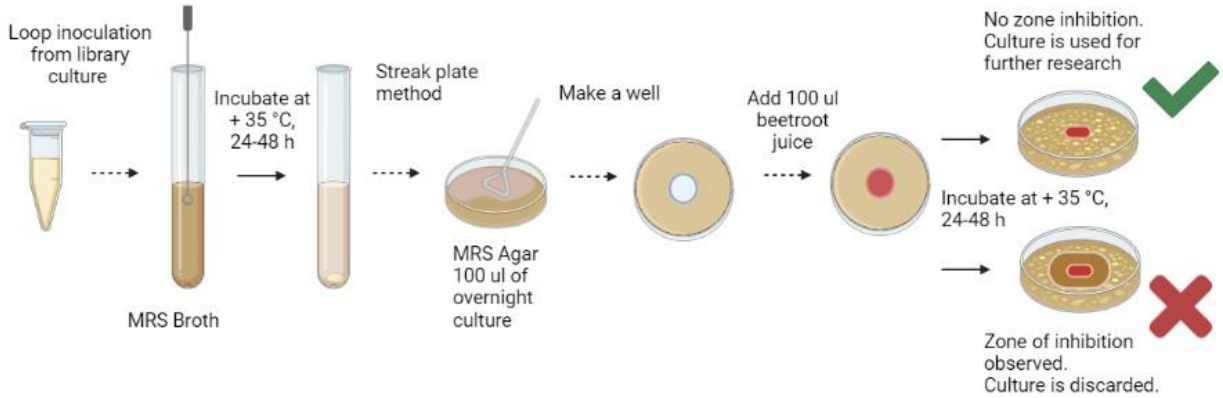


Fig.2.2. Microorganism selection using agar well diffusion test with BRJ. Scheme made by (author with Biorender program).

2.3. Selection of LAB based on their β-Glucosidase activities

Frozen bacteria cultures were revived by streaking on MRS agar and incubated at 35 °C for 24 h. MRS broth was inoculated with single colonies of bacteria, incubated at 35 °C and harvested at the exponential phase of growth. Twenty-two bacteria were screened for their β-Glucosidase activity using a β-Glucosidase assay kit according to the manufacturer's instructions. Briefly, by protocol, prepared water, and calibrator wells. Overnight cultures of each strain were vortexed and 20 µL were added to 200 µL of working reagent containing p-nitrophenyl-β-D-glucopyranoside. The optical density (OD) was read at 405 nm with microplate reader and the mixture was incubated at 35 °C for 30 min. After incubation the final absorbance measurement was taken at 405 nm. Bacteria that showed β-Glucosidase activity above 1000 U/L were selected and used for further studies.

β-Glucosidase activity of the sample (U/L) was calculated as:

$$\text{β-Glucosidase Activity} = \left(\frac{OD8h - OD0h}{ODcalibrator - ODH20}\right) \times 250(U/L)$$

Where OD_{0h} = OD at 405 nm at time 0 h

OD_{8h} = OD at 405 nm at time 30 min

$OD_{calibrator}$ = OD at 405 nm at time 30 min

OD_{H20} = OD at 405nm at time 30 min

2.4. Beetroot preparation and fermentation

The beetroots were washed with distilled water to remove surface dirt. To inactivate present microbes and enzyme activity they were blanched in hot water at 100 °C for 5 min. Adding distilled water and making 1 g of beetroot to 1 mL ratio (1g/mL), the sample were blended using a Philips H3664/90 blender.

For beetroot compound, activity identification and bacteria culture inhibition - blended samples were centrifuged at 4000 rpm for 5 min and the supernatant was stored at −80 °C deep freezer for further studies.

Fermentation was carried out as described by Czyżowska et al. (Czyżowska et al., 2006) with modifications. Selected LAB cultures were inoculated into BRJ (2 % of culture ratio) as a main source of energy and incubated at 35 °C for 48 h at 180 rpm agitation. The fermented sample was centrifuged at 4000 rpm for 5 min and the supernatant was stored at −80 °C deep freezer for further studies.

2.5. *In vitro* antidiabetic activity of fermented beetroot

2.5.1 DPP-IV inhibitory ability

DPP-IV inhibitory ability was assessed using a DPP-IV inhibitor screening kit according to the manufacturer's instructions. Briefly, 10 µL of diluted, fresh, and fermented beetroot samples were mixed with 10 µL of diluted DPP-IV and transferred to wells containing 50 µL of diluted DPP-IV substrate while adding 30 µL of buffer to bring volume up to 100 µL. In similar perspective, controls of enzyme activity and stigaliptin positive control inhibitor wells were prepared. The plate was incubated at 37 °C for 30 min and fluorescence was measured at excitation wavelength of 350-360 nm and an emission wavelength of 450-465 nm. Sitagliptin was used as a positive control inhibitor. Samples whose inhibitory abilities were stronger than fresh beetroot were chosen for further studies.

DPP-IV inhibitory ability was calculated as:

$$\% \text{ Inhibition} = \left(\frac{Activity\ (without\ inhibitor) - Activity\ (with\ inhibitor)}{Activity\ (without\ inhibitor)}\right) \times\ 100\%$$

2.5.2. Total antioxidant capacity

Total antioxidant activity was analyzed using a total antioxidant capacity assay kit according to the manufacturer's instructions. Briefly, trolox standards were prepared by adding 0, 4, 8, 12, 16, and 20 µL of the 1 mM (1 nmol/µL) Trolox standard solution into a 96 well plate and the volume was brought to 100 µL with distilled water. T2DM and fermented beetroot samples were diluted with distilled water in a ratio 1:4. 100 µL of dilute samples were mixed with 100 µL of the Cu^{2+} working solution and incubated at room temperature (~25 °C) for 90 min. Due to the fact that beetroots large amounts of pigments and phenolic compounds that can affect colorimetric measurements, an absorbance was measured at 570 nm before (time 0) and after incubation (time 90 min). Samples with total antioxidant concentrations greater than fresh beetroot were chosen for further studies. Concentration of antioxidants in each sample was calculated as Trolox equivalents as $S_a/S_v = C$

Where S_a = Trolox equivalent of unknown sample well (nmol) from standard curve

S_v = Sample volume (µL) added into the wells

C = Concentration of antioxidant in sample (mM Trolox equivalents)

2.5.3. α-Amylase inhibitory ability

The α-Amylase inhibitory assay was carried out using the α-Amylase inhibitor screening kit according to the manufacturer's instructions. Briefly, enzyme active control was prepared by adding 50

µL of assay Buffer and 50 µL of enzyme solution, while inhibitor control added 10 µL of inhibitors, 40 µL of buffer and 50 µL of enzyme solution. 50 µL of diluted, fresh, and fermented samples were mixed with 50 µL of diluted α-Amylase enzyme. Plate was incubated at room temperature for 10 min to let inhibitors to connect with enzymes. Diluted α-Amylase substrate (50 µL) was added to all the wells and mixed thoroughly. The OD was measured at 405 nm under room temperature in kinetic mode for every 3 min for 30 min. Relative percentage inhibition was calculated as shown below:

$$\% \text{ Relative inhibition} = \left(\frac{Slope\ of\ (EC) - Slope of\ (T)}{Slope\ of\ (EC)}\right) \times\ 100\%$$

Where EC= Enzyme control

T = Test sample

2.5.4. α-Glucosidase inhibitory ability

Fermented samples with α-Glucosidase inhibitory ability were identified using the α-Glucosidase inhibitor screening kit according to the manufacturer's instructions. Briefly, 10 µL of α-Glucosidase enzyme was mixed with 10 µL of 5 time diluted fresh and fermented samples and incubated in the dark at room temperature for 30 min. An aliquot (20 µL) of α-Glucosidase substrate was added and mixed. Acabose was used as the standard α-Glucosidase inhibitor and absorbance was measured at 410 nm in the kinetic mode at room temperature for 1 h. Samples whose inhibitory abilities were stronger than fresh beetroot was chosen for further studies. Relative percentage inhibition was calculated as shown below:

$$\% \text{ Relative inhibition} = \left(\frac{Slope\ of\ (EC) - Slope of\ (T)}{Slope\ of\ (EC)}\right) \times\ 100\%$$

Where EC= Enzyme control

T = Test sample

2.6. Effect of temperature and fermentation time on antioxidant capacity and DPP-IV inhibition

The influence of fermentation temperature and fermentation time on antioxidant capacity, DPP-IV was performed as reported by Yang et al. (Yang et al., 2018) with modifications. Briefly, beetroot samples were fermented either at 30, 37 or 45 °C for 24, 48 or 72 h using the selected lactic acid bacterium. The bacteria growth kinetics was measured at 600 nm using a spectrophotometer and the changes in pH were measured periodically with a pH meter. The fermented sample that showed the strongest antioxidant capacity was tested for DPP-IV inhibitory abilities using the methods earlier described.

2.7. Identification of selected bacterium

The molecular identification of LAB strains was conducted by 16s rRNA and later confirmed by whole genome sequencing (WGS) analysis.

For 16sRNA sequencing the strains were sent to Microgen, Netherlands. Sequence amplicon was BLAST® analyzed and aligned with the National Center for Biotechnology Information (NCBI) Sequence comparison database (www.ncbi.nlm.nih.gov) to determine the sequence identity and GenBank accession number.

For the WGS the strains were sent to Cosmos, USA. Briefly, following the manufacturer's instructions, QIAGEN DNeasy PowerSoil Pro Kit was used to extract DNA from the samples. DNA samples were measured using the QuantiFluor® dsDNA System (Promega) chemistry and the GloMax Plate Reader System from Promega. The Nextera XT DNA Library Preparation Kit (Illumina) and IDT Unique Dual Indexes were used to create DNA libraries with a total DNA input of 1ng. An equal amount of Illumina Nextera XT fragmentation enzyme was used to lyse genomic DNA. Each sample received distinct dual indexes, and then 12 cycles of PCR were used to build libraries. DNA libraries were cleaned using Beckman Coulter AMpure magnetic beads and rinsed with QIAGEN EB buffer. Qubit 4 fluorometer and Qubit dsDNA HS Assay Kit were used to quantify DNA libraries. Illumina NovaSeq platform 2x150bp was then used to sequence the libraries. With a read quality trimming theshold of 22 for isolates, raw paired end reads were trimmed and processed using BBDuk. Using SPAdes and the -careful parameter, the trimmed fastqs were put together. CheckM's lineage_wf function was used to assess how complete the built isolate was. In order to assess the phylogenetic placement and single-nucleotide polymorphism (SNP) differences for useful epidemiological conclusions, the assembled contigs were subsequently processed though the CosmosID core genome SNP typing pipeline. Parsnp was used as the core genome aligner in the CosmosID SNP typing pipeline to align the core genomes of various microbial genomes. The phylogenomic link among the genome was then constructed by Parsnp utilizing FastTree2 from the remaining set of core-genome SNPs.

2.8. HPLC-MS analysis of beetroot samples

Beetroot and fermented samples were analyzed in Vilnius university laboratory, Lithuania. Chomatographic analysis was carried out as reported by Slatnar et al. (Slatnar, Stampar, Veberic, & Jakopic, 2015) using an UltiMate™ 3000 HPLC (Thermo Scientific, San Jose, Calif., U.S.A.) and a Gemini 3 µm C18 column (4.6 × 150 mm; Phenomenex, CA 90501-1430, U.S.A) kept at 35 °C. Briefly, solvent A consisted of acetonitrile and solvent B was 1% formic acid in double distilled water. An

injection volume of 10 µL was used and a flow rate of 0.5 mL/min was kept. Detection was performed with a diode array detection system at 480 nm. The compounds were identified by a Finnigan LCQ Duo LC-MS detector (Thermo/Finigan, Califonia, U.S.A.) with electrospray interface set in positive ion mode. MS data was analyzed by scanning m/z from 110 to 1500. Compounds were confirmed by comparing retention times, fragmentation, and previous reports in literature.

2.9. Safety assessment of selected bacteria

2.9.1. Antibiotic susceptibility test

The selected cultures were tested against 15 µg Erythromycin (E), 30 µg Vancomycin (VA), 5 µg Kanamycin (K), 10 U Penicillin (P), 10 µg Ampicillin (AMP), 10 µg Gentamicin (GEN), 10 µg Streptomycin (S), 20 µg Tetracycline (TE), 5 µg Rifampicin (RIF) using disc diffusion method. After incubation ant 35C^{o} for 24 h the agar plates were examined for the absence or presence of zone of inhibitions.

2.9.2. Search for antimicrobial resistance gene, virulence factors and plasmid

The bacteria genomes were screened against two antimicrobial resistance gene databases; the ResFinder server 4.1 (https://cge.food.dtu.dk/services/ResFinder/) and ResFinderFG 2.0 server (https://cge.food.dtu.dk/services/ResFinderFG/). Identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial genome was performed using the antiSMASH 7.0 server (https://antismash.secondarymetabolites.org/#!/start).

2.10. Experimental animal model creation and procedures

40 female C57BL/6 mice weighing 18-22 g at the beginning of the experiment were used in this study. Mice were housed in groups of 5, in controlled laboratory conditions with the temperature maintained at 21 °C ± 1 °C, humidity at 55 % ± 10 %, 12-hour day/night regime at animal research facility in Vilnius University, Life Science center. Animal procedures and interventions were conducted in strict accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and were approved by the local ethical committee (license No. G2-239). All animal experiments were performed under blinded conditions.

2.10.1. Beetroot ferment administration

Animals were maintained on normal mice chow diet and water *ad libitum*. Mice that were treated with fermented PN39 beetroot product had 5-time dilution with water solution that was changed every 2-3 days. Insulin was injected (0.02 U/mL) to the animals every day for 21 days.

2.10.2. Induction of Diabetes

To induce diabetes in animals, STZ injections were injected by using recommendations from Furman B. L. (Furman 2021). Briefly, mice were fasted for 4 hours (only water) and injected with STZ injections at 40 mg/kg (1 mL/100 g). Diabetes was induced at 6 to 8 weeks of age. After each injections mice had free access to normal food and 10% sucrose water. Injections procedures were repeated for 5 days (Fig.2.3). Diabetes was confirmed 11 days post STZ injections using strip-operated glucose meter on blood sample obtained via tail prick in the study.

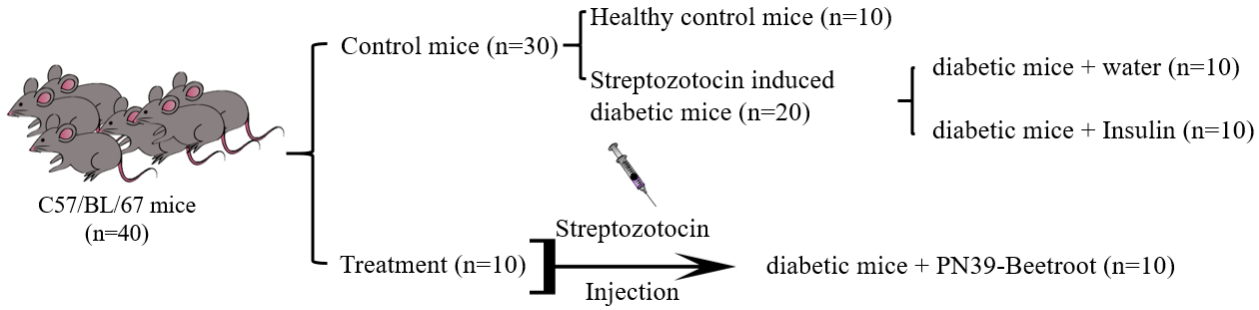


Fig.2.3. Scheme of experimenal diabetic animal model and mice distribution. Scheme made by (author).

2.10.3. Glucose level measurements

Mice were weighted using scales (A&D weight) every week during the study. Every few weeks blood glucose was measured using tail prick method and glucose meter after 6 h fasting. Glucose tolerance test (GTT) was conducted at the proceeded after mice fasted for 12 h. and had 20 % glucose solution (10µL/1g) injections. At the end of the study, the animals were consecutively sacrificed. The pancreases, colon, cecum and blood were collected. Cecum was weighted, plasma collected from centrifuged blood and all organs stored at − 80 °C for future research.

2.11. Statistical analysis

All fermented product measurements were performed with 3 replicates (n = 3). In the animal experiments, there were 10 animals in each group (n =10). GraphPad Prism 5 was used for data analysis and graphing. For determine differences between products and groups One-Way ANOVA, Post Hoc Tukey HSD tests were used. Results are expressed graphs as mean ± SD.

3. RESULTS AND DISCUSSION

3.1. Screening for LAB with β-Glucosidase activity

To improve functional activities during functional food development, deglucosylation during fermentation is a well-known and broadly used mechanism (Ávila et al., 2009; Michlmayr & Kneifel, 2014). To select LAB for beetroot fermentation, their ability to hydrolyze β-glycosidic bonds were tested. All the 22 bacteria (Supplementary table 1) tested showed β-D-glucosidase activity to various extents and this agrees with earlier studies that reported that the enzyme is widespread in LAB (Michlmayr & Kneifel, 2014). Meanwhile, the differences in enzyme activities among the strains could be due to strain specificity and differences in bacteria metabolism. To select only bacteria with the highest β-D-glucosidase activities among the 22 isolates, strains that exhibited enzyme activities less than 1000 U/L were excluded from further studies (Fig.3.1). The remaining 18 bacteria were used for beetroot fermentation and further analysis.

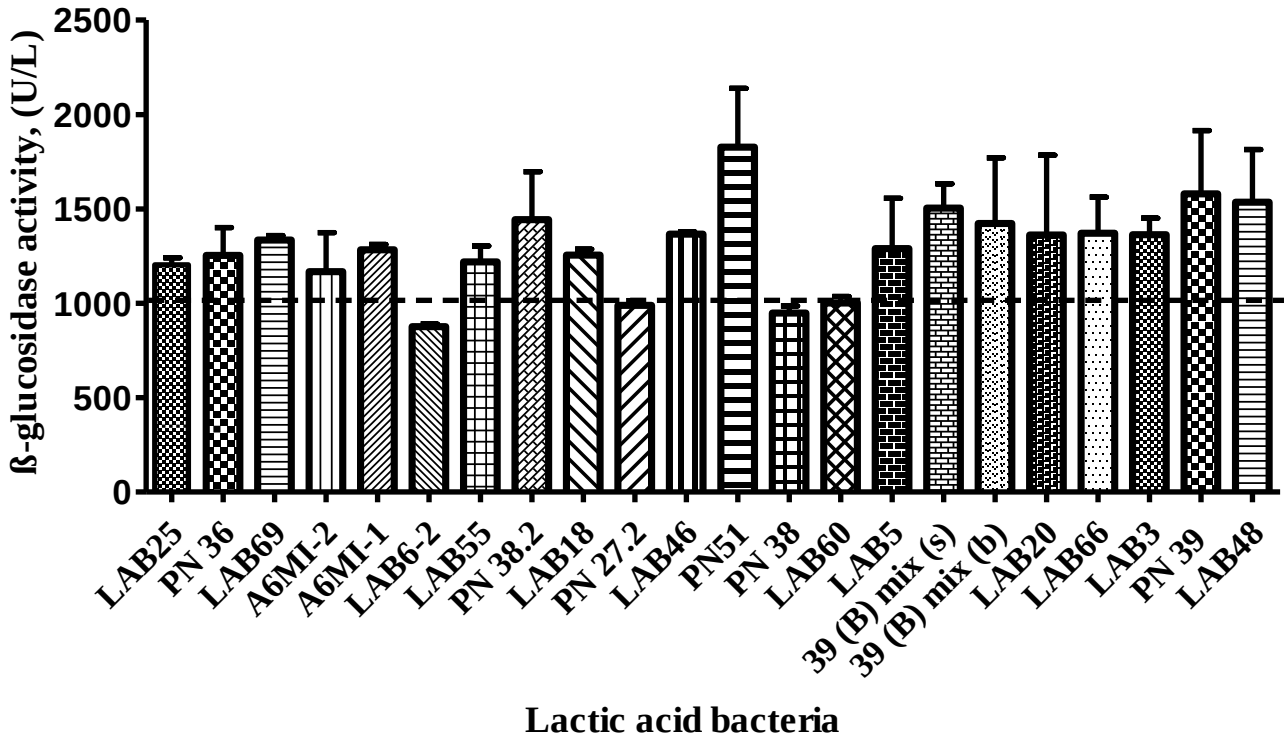


Fig.3.1. β-D-glucosidase activity of LAB isolated from fermented foods. Each bar represents the means of three replicates (n = 3) ± S.D. Strains below the dotted line were eliminated from further studies.

3.2. Effects of beetroot fermentation on DPP-IV inhibitory ability

Diabetes patients have high plasma DPP-IV activity which hydrolyze GLP-1 (an insulinotropic and glucose-lowering molecule) resulting in impaired glucose metabolism and hyperglycemia (Sarkar, Nargis, Tantia, Ghosh, & Chakrabarti, 2019). Therefore, fermented foods that inhibit DPP-IV activity have potential antidiabetic abilities. Out of the 18 fermented samples tested against DPP-IV, only those fermented with PN36, PN39 and PN51 demonstrated inhibitory abilities stronger than that exhibited by fresh BRJ (Fig.2). Specifically, samples fermented with PN39 demonstrated the strongest DPP-IV inhibitory ability of 48.5 % ± 3.5% while samples fermented with PN36 and PN51 had inhibitory abilities of 43.8 % ± 1.6 % and 43.1 % ± 0.6% respectively. However, the DPP-IV inhibitory ability of fresh BRJ was only 37.5 % ± 0.3%. This indicates that fermentation with these bacteria significantly improved the DPP-IV activity of beetroot. The samples fermented with these 18 bacteria were therefore subjected to further analysis.

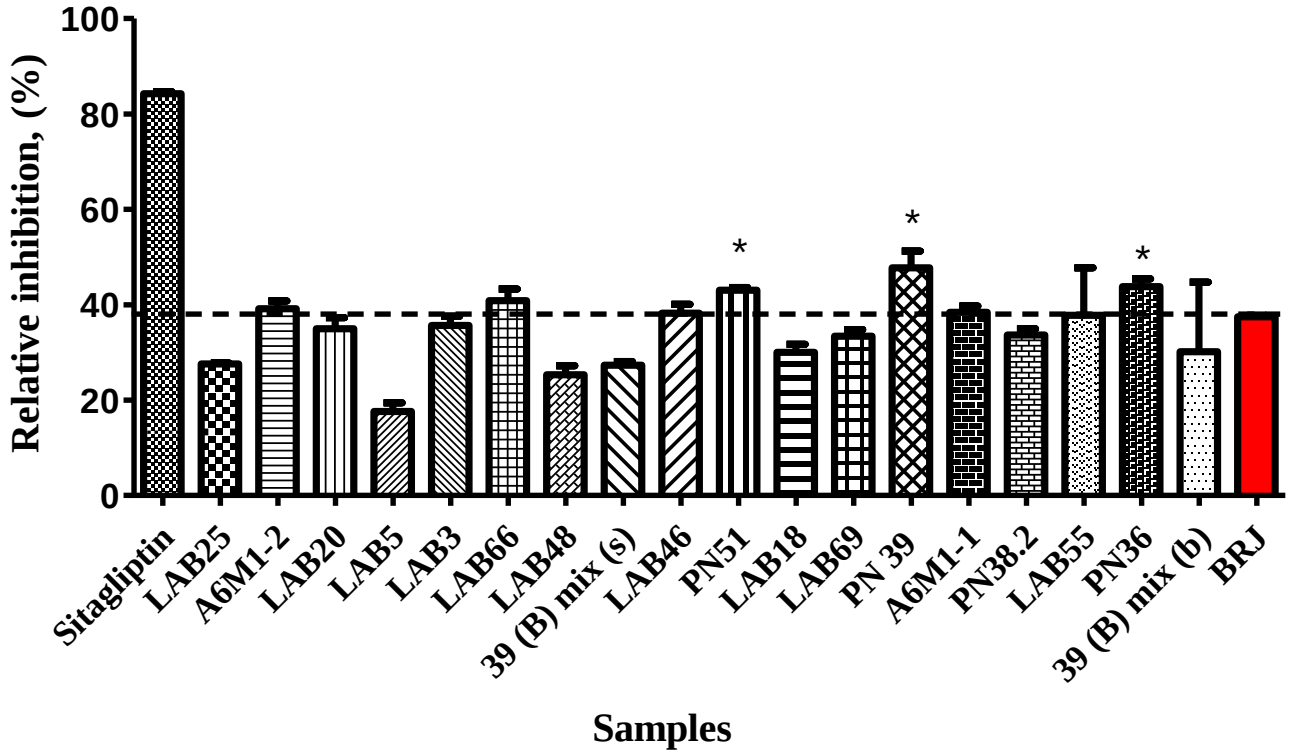


Fig.3.2. DPP-IV inhibitory activity of beetroot samples fermented with LAB compared with the inhibitory ability of fresh beetroot. Each bar represents the means of three replicates (n = 3) ± S.D. Red bar indicates fresh BRJ DPP-IV inhibitory. * indicates significant difference ($p < 0.05$).

3.3. Effects of beetroot fermentation on carbohydrate hydrolyzing enzymes (α-Amylase and α-Glucosidase)

Diabetes is particularly characterized by carbohydrate metabolic disorders and hence modulating dietary carbohydrate digestion effectively regulates blood glucose levels (Mills et al., 2022). In the gut, α-amylases hydrolyze α-1,4 glycosidic bonds of starch into shorter glucose chains during digestion (Kaur et al., 2021). For this reason, inhibiting α-Amylase activity decreases the rate of starch digestion and reduces postprandial hyperglycemia. In this study, among 22 cultures there were 5 LAB fermented samples that did not have the α-Amylase inhibitory abilities. However, culture LAB25 had 32.21 % inhibition ability (Fig.3.3A). Indeed, some studies have demonstrated the ability of certain LAB to improve α-Amylase inhibition after fermentation of food materials (Klongklaew et al., 2022; Ujiroghene et al., 2019), yet the functional activity of a fermented sample would depend on the bacteria species and the substrate used. Meanwhile, since the disaccharides and oligosaccharides released by α-Amylases must be further cleaved to release glucose, further studies to test the ability of the fermented samples to inhibit α-glucosidase activity needed to be proceeded.

α-Glucosidase hydrolyses α (1→4) glycosidic bonds at the non-reducing terminal of carbohydrates to yield α-glucose molecules which increase blood glucose levels after intestinal absorption (Akmal & Wadhwa, 2022). Inhibiting the enzyme activity would therefore delay glucose absorption thereby moderating blood glucose levels. Among the 18 fermented beetroot samples tested, only the sample fermented with 39 (b) mix (s) demonstrated α-Glucosidase inhibitory ability of 18.64 % and PN36 culture 20.41 % (Fig.3.3B). Takács-Hájos and Vargas-Rubóczki (Takács-Hájos & Vargas-Rubóczki, 2022) have shown that beetroot contains polyphenolic compounds which are strong α-Glucosidase inhibitors (Cenobio-Galindo et al., 2019). The ability of fermented samples to strongly inhibit α-Glucosidase agrees with a study by Zahid et al. (Zahid et al., 2022) However, this test showed that activity of LAB may reduce the α-Glucosidase inhibitory ability of polyphenol containing foods. This might have happened because during the fermentation process compounds might have been used by bacteria or converted into other molecules that have lower inhibition effect. In addition, environmental factors such as aerobic environment, temperature and time may have contributed to the alteration or degradation of the compounds. For a further study, only 5 cultures that had best abilities for DPP-IV inhibition – PN39, PN51, PN36, α-Amylase inhibition – LAB25 and α-Glucosidase inhibition - 39 (b) mix (s) were used to measure antioxidant capacity.

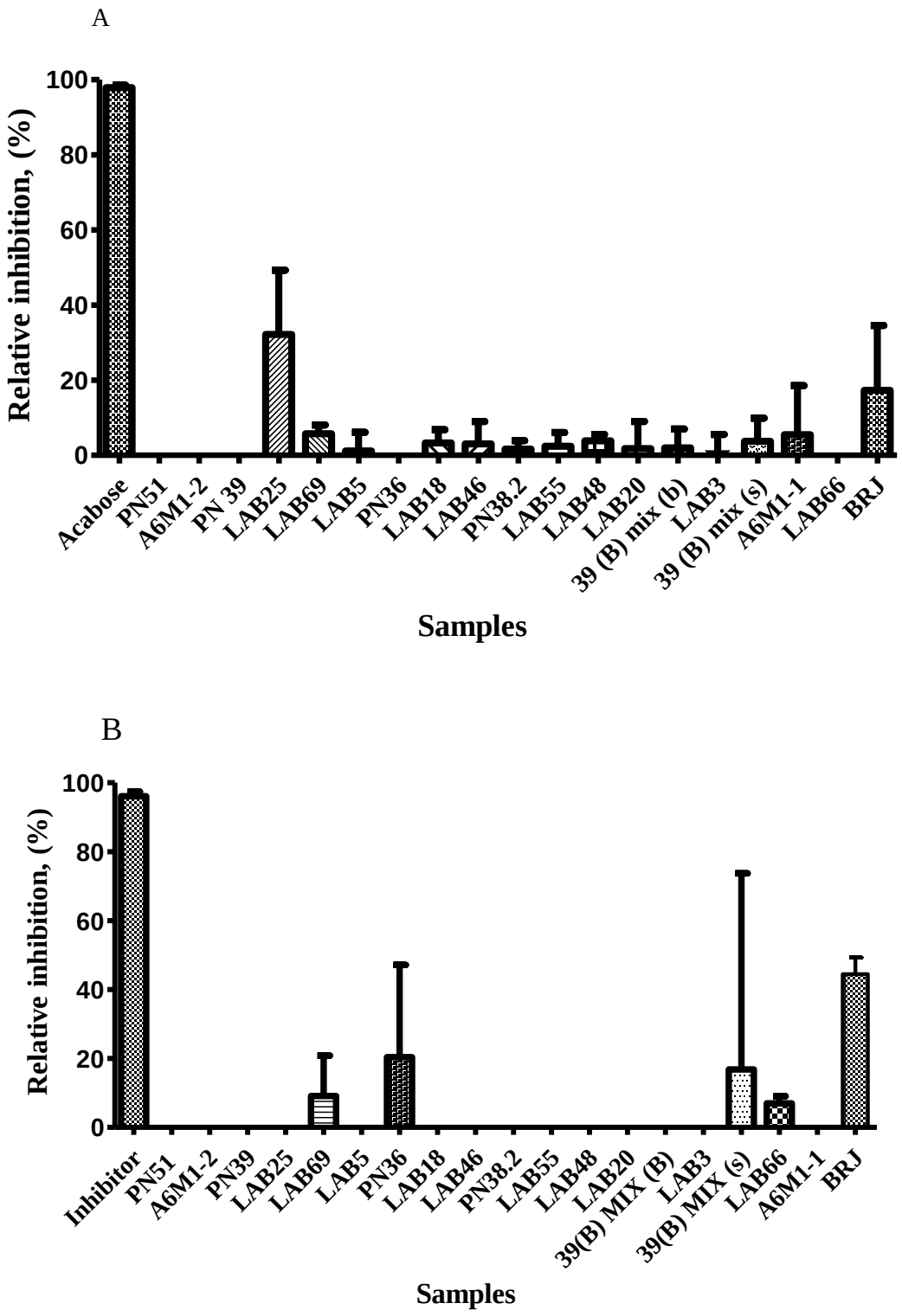


Fig.3.3. Inhibition of carbohydrate hydrolyzing enzymes by fermented and unfermented beetroot samples. (**A**) α-Amylase inhibitory activity of fresh beetroot compared with inhibitor and fermented beetroot samples. (**B**) α-Glucosidase inhibitory activity of fresh beetroot compared with fermented beetroot.

3.4. Effects of beetroot fermentation on antioxidant capacity

During diabetes, hyperglycemia initiates the activation of the electron transport chain, thereby resulting in the production of substantial quantities of ROS. This process is known to cause adverse effects on β-cell functionality, as well as an increase in insulin resistance. (Ayer, Fazakerley, James, & Stocker, 2022). However, antioxidants can reduce oxidative stress caused by ROS to alleviate their harmful effects. For this reason, improving the antioxidant capacity of functional foods is imperative for mitigating the disease. In this study, the antioxidant capacity of fresh beetroot was increased from 2.20 mmol/L ± 0.11 mmol/L to 2.85 mmol/L ± 0.07 mmol/L, 3.42 mmol/L ± 0.12 mmol/L, 2.65 mmol/L ± 0.07 mmol/L, 3.65 mmol/L ± 0.02 mmol/L and 2.98 mmol/L ± 0.07 mmol/L and when fermented with PN51, PN39, PN36, LAB25, 39 (b) mix (s) respectively (Fig.3.4). The increased antioxidant capacity was possibly due to the ability of the bacteria to release bound antioxidant compounds and also to generate bioactive compounds from beetroot during the fermentation process (Y. S. Zhao et al., 2021). Our results agree with recent studies that have reported that LAB fermentation of food can improve their antioxidant capacities (Madjirebaye et al., 2022; Sandez Penidez, Velasco Manini, LeBlanc, Gerez, & Rollán, 2022). After identifying that culture PN39 had best activity in inhibition of DPP4 and antioxidant capacity, further on study proceeded to test effects of temperature and fermentation time for beetroot fermentation with PN39 culture.

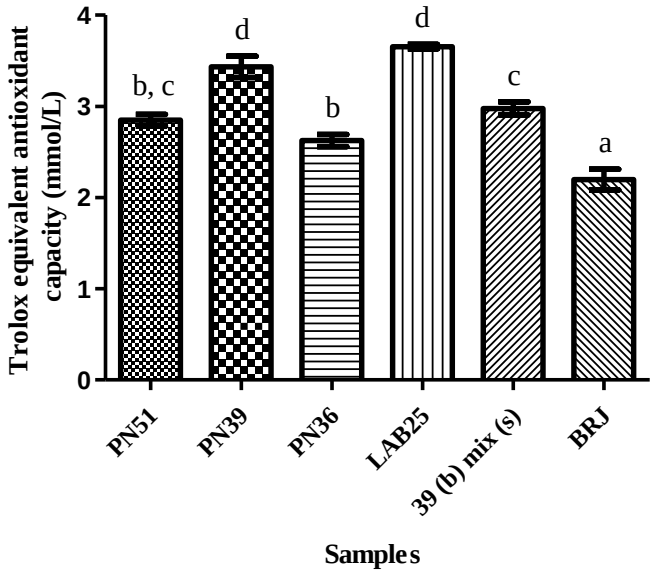


Fig.3.4. Total antioxidant capacity of beetroot fermented with LAB compared with fresh BRJ. Each bar represents the means of three replicates (n = 3) ± S.D. Bars with different alphabets are significantly different ($p < 0.05$).

3.5. Effects of temperature and fermentation time on antioxidant capacity, DPP-IV inhibition

Bacteria growth and metabolism are influenced by fermentation temperature and most LAB show optimum growth at temperatures between 30 °C to 45 °C (Yang et al., 2018). Also, most studies that have investigated the effects of fermentation time on the antidiabetic potentials of artificially inoculated food samples have done so within a timeframe ranging from 24-72 hours (Fujita, Sarkar, Genovese, & Shetty, 2017; Klongklaew et al., 2022; Ramakrishna, Sarkar, Dogramaci, & Shetty, 2021). Fallowing tests investigated how these fermentation conditions impact the antioxidant capacity and DPP-IV inhibition of the fermented sample. As shown in Fig.3.5A1, the bacterium showed similar growth kinetics and reached stationary phase after 18 h when grown at 30 °C or 37 °C. At 45 °C however, stationary phase was reached after 8 h of fermentation. To survive heat stress, LAB has thermosensors such as CtsR that detect temperature changes and can help to regulate microbial replication leading to slow growth during high temperatures (Darsonval, Julliat, Msadek, Alexandre, & Grandvalet, 2018). Also, they excrete catabolic intermediates such as organic acids into the environment to reduce heat stress (Frank & Evolution, 2020). These factors may account for the growth kinetic of the bacterium at 45 °C and the continuous reduction in sample pH (Fig.3.5A2) from 6.3 pH to 5.5 pH (at the 24th hour) though stationary phase was reached at the 8th hour. Fermentation at 30 °C significantly increased the antioxidant capacity of beetroot only when the sample was fermented for 72 h (Fig.3.5B). On the other hand, samples fermented at 37 °C increased antioxidant capacity from 2.13 mmol/L ± 0.01 mmol/L (in fresh beetroot) to 2.30 mmol/L ± 0.05 mmol/L after 48 h of fermentation. However, the antioxidant capacity slightly decreased to 1.91 mmol/L ± 0.12 mmol/L when fermentation time was increased to 72 h. The decrease in antioxidant capacity could be because some of the antioxidant compounds generated at the 48th h served as energy sources for microbial growth or as substrates for generating new compounds. Fermenting beetroot at 45 °C increased the antioxidant capacity to 4.15 mmol/L ± 0.56 mmol/µL after 24 h. The antioxidant content further increased to 9.06 mmol/L ± 0.94 nmol/µL and 15.44 mmol/L ± 0.94 mmol/µL after fermentation for 48 h and 72 h respectively. Our results agree with earlier studies that reported that fermentation temperature and time have significant effects on antioxidant activities of the final product (Liu et al., 2020). Interestingly, the antioxidant capacity of samples fermented at 45 °C for 24 h were significantly different from those fermented at 37 °C for 24 h ,48 h, 72 h and 30 °C for 24 h and 48 h indicating that increasing fermentation temperature increased antioxidant activity and decreased fermentation time.

Since fermentation at 45 °C demonstrated the highest antioxidant capacity, we maintained the fermentation temperature but varied the fermentation time to determine the impact of fermentation time

on DPP-IV inhibitory activities. As shown in Fig.3.5C, DPP-IV inhibition increased with fermentation time when temperature was held at 45 °C. Inhibition increased from 37.4 % (at time 0 h) to 66.3 % after 24 h of fermentation and further increased to 87.5 % and 89.3 % after 48 h and 72 h.

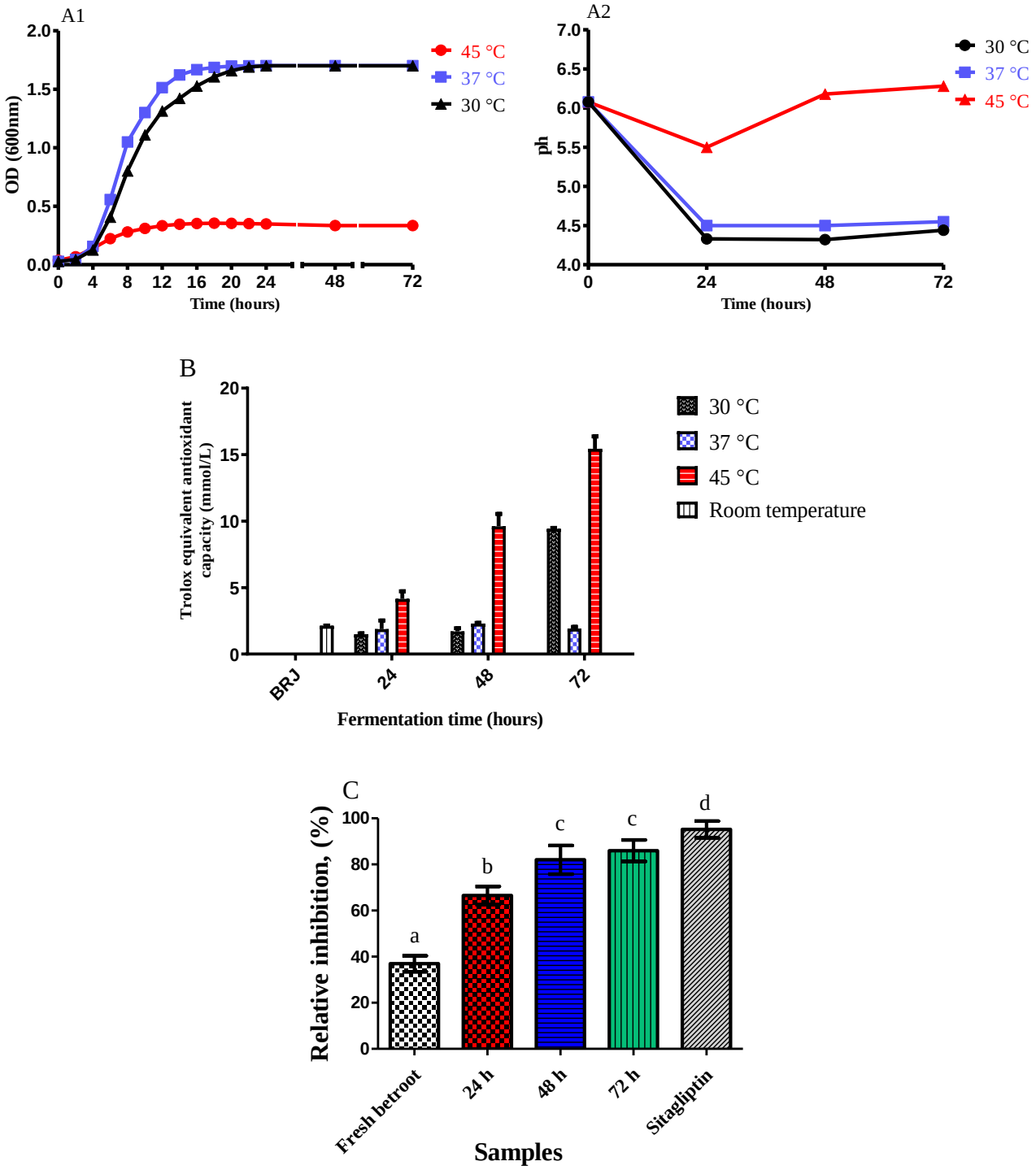


Fig.3.5. Effects of fermentation temperature and time on antidiabetic potentials. (**A1** and **A2**) Microbial growth kinetics under different temperature. (**B**) Total antioxidant capacity of beetroot

fermented under different temperature and time. (**C**) DPP-IV inhibitory ability of beetroot samples fermented at 45 °C for 24, 48 and 72 h.

3.6. Bacteria identification

The identification of PN39 culture was done by 16s RNA sequencing and later confirmed by WGS . Using Cosmos ID bacteria database for comparative analysis showed that core genome coverage (71.9 %) of PN39 culture was closest to *Latilactobacillus curvatus* and formed a separate cluster with *Lacticaseibacillus curvatus_ZJUNIT8_GCF_003254785.1* (Fig.3.6). WGS provides more valuable results then 16s rRNA, therefore isolated strain was named as *Lacticaseibacillus curvatus PN39.*

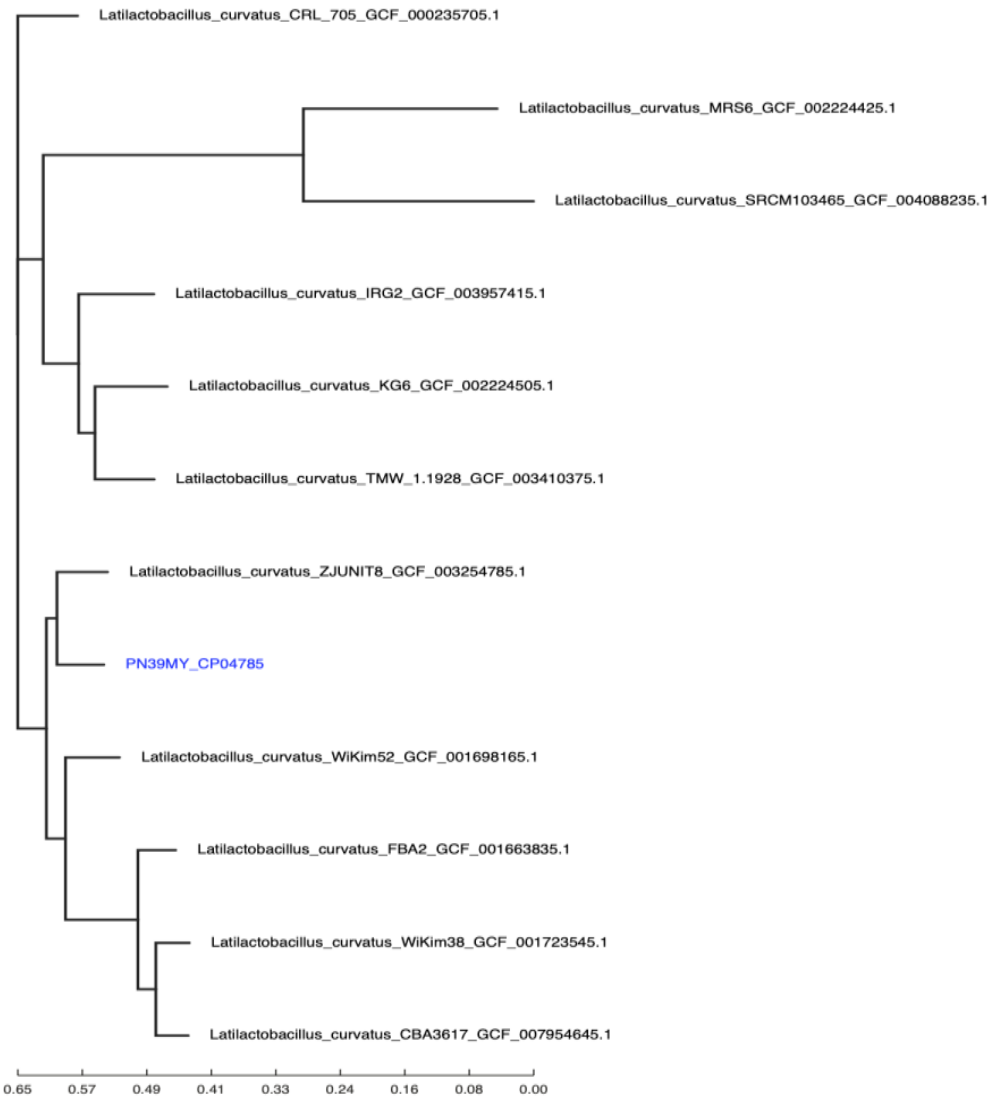
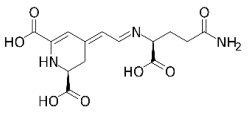
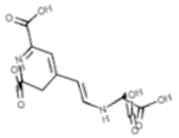
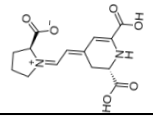
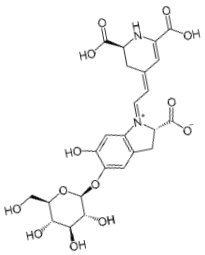
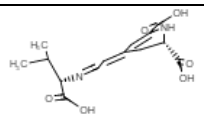
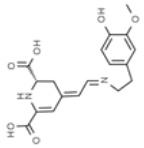
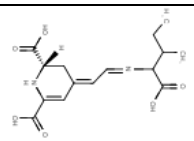
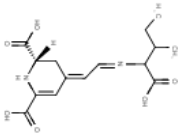
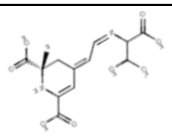
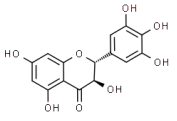
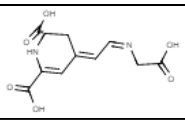


Fig.3.6. Phylogenetic tree of PN39 bacteria after WGS. SNP Tree based on Core Genome Phylogeny.

3.7. Effects of high temperature fermentation on beetroot betalains

Generally, seven betaxanthins *(*Gulgaxanthins I, Glutamic acid-betaxanthin (vulgaxanthins II), indicaxanthin, Valine-isobetaxanthin, 3-methoxytyramine-betaxanthin, Isoleucine-betaxanthin and Leucine-isobetaxanthin) and two betacyanins *(*betanin and isobetanin) were detected in the fresh beetroot with Vulgaxanthin I being the most abundant followed by betanin (Table.3.1). After fermentation with *Lacticaseibacillus curvatus* PN39, theonine-betaxanthin and glycine-betaxanthin were the only betaxanthins present in the fermented samples while betacyanins were not detected. This might have happened because during the fermentation process compounds might have been converted into other molecules (Choińska et al., 2022). Meanwhile, the loss of betanin in the fermented sample could be due to the high fermentation temperature. This insight agrees with prior research indicating that subjecting beetroot to the process of fermentation at elevated temperatures results in a considerable decrease in betalain content, often reaching a reduction of approximately 88%. (Choińska et al., 2022; Czyżowska et al., 2006; Sawicki & Wiczkowski, 2018). Meanwhile, the most abundant compound in the fermented sample was dihydromyricetin which is known to strongly inhibit ROS, DPP-IV (Wu et al., 2022). Undoubtedly, other study has established the significant anti-hyperglycemic effects of dihydromyricetin in T2DM animal models (Yao et al., 2021) and hence, the presence of dihydromyricetin in the fermented sample may have contributed to the strong antidiabetic potentials displayed in *in vitro* study.

Table.3.1. Chromatographic and mass spectrometric data of analyzed pigments in BRJ before and after fermentation with *Lacticaseibacillus curvatus* PN39.

Peak No.	Compounds	Retention time (min)	UV–vis maximum (nm)	*m/z* [M + H]+	Peak area	Structure
Unfermented sample						
1	Vulgaxanthin I	3.5	471	340	1819461	
2	Vulgaxanthin I	4.8	471	340	786804	
3	Glutamic acid-betaxan	5.5	471	341	67467	
4	Glutamic acid-betaxan	6.8	468	341	58325	
5	Unknown	8	457	297	25215	
6	Indicaxanthin	9.5	479	309	52930	
7	Betanin	10.5	532	551	1474903	
8	Betanin	11.5	531	551	938603	
9	Betanin	12.5	532	551	245988	
10	Unknown	13	526	539	167580	
11	Valine-isobx	14	472	311	39128	
12	Valine-isobx	14.7	472	311	29305	
13	3-methoxytyramine-b	17	412	361	42810	
14	Isoleucine-isobx	18.6	472	325	54402	
15	Isoleucine-bx / Leucin	19.5	472	325	59165	
LAB PN 39 fermented sample						
1	Threonine-betaxanthin	12	467	313	6140	
2	Unknown	13	476	313	14673	
3	Dihydromyricetin	14.5	476	321	71422	
4	Glycine-betaxanthin	15	461	267	53630	

3.8. Antibiotic resistance test

Antimicrobial resistance occurs when microbes develop mechanisms that protect them from the effects of antimicrobial agents. All classes of microbes can develop resistance, but for industry and medicine it can be dangerous. For that reason PN39 was tested with antibiotic discs. Results revealed that there is resitance to Vancomycin, Kanamycin and Streptomycin (Table.3.2). However, it is possible that resistance due to lack of antibiotic target. Next step: do genome analysis to identify antibiotic resistance genes and plasmids.

Table.3.2. Used antibiotics and inhibition zone measures. Inhibition zone < 10 mm indicates resistance.

Type	Concentration	Inhibition zone
Erythromycin, E	15 µg	25 mm
Vancomycin, VA	30 µg	0
Kanamycin, K	5 µg	0
Penicillin, P	10 U	19 mm
Ampicillin, AMP	10 µg	23 mm
Gentamicin, GEN	10 µg	10 mm
Streptomycin, S	10 µg	0
Tetracycline, TE	30 µg	25 mm
Rifampicin, RIF	5 µg	22 mm

3.9. Search for antimicrobial resistance gene and plasmid

Through the conjugation process, plasmids can be transmitted from one bacterium to another. There is a risk that patogenic bacteria can pick up an individual bacterium's drug resistance and get imunity. In order to analyse the genome data, the contigs were checked against the PlasmidFinder server 2.0 and ResFinder 4.1. Results indicate that bacteria lack antimicrobial resistance genes and is without plasmids (Supplementary figure 1 and supplementary data 1). Identification of secondary metabolite regions antiSMASH 7.0.0 server found two regions (Supplementary figure 2). NODE_32 that have CoA-disulfide reductase which catalyzes the specific reduction of CoA disulfide by NADPH. Other, NODE_67 region had leucocin A/sakacin P family class II bacteriocin. The latter is known to be common with LAB and is safe to use in industry (Zhang et al. 2022). All reasurch indicates that our culture and its product is safe to use for further reasurch.

3.10. Diabetic mice model testing results

Literature and other similar examples indicate that *Mus musculus*, the C57BL/6J mice line, is a good animal model for the induction of diabetes. Because of the similarity in biological development and disease, mice are an excellent model organism for studying human diseases, prevention, and their treatment. Scientists can make mice have a sickness that looks like type 1 diabetes in people by killing certain cells with a chemical compound called STZ (Abdollahi and Hosseini 2014). When STZ is injected into mice, it specifically targets the β cells in the pancreas which are responsible for producing insulin. This leads to these β cells destruction, which decreases the mouse's ability to produce insulin and clear blood glucose. As a result, glucose accumulates in the blood, resulting in hyperglycemia (Furman 2021). This allows them to study the mechanisms of the disease and test potential treatments. Additionally, STZ is relatively easy to administer and has been widely used in diabetic research for many years (Abdollahi and Hosseini 2014). The results of our *in vitro* studies have provided valuable insights into the potential mechanisms on how our fermented samples could affect weight gain and hyperglycemia in mice. Specifically, we have found that our fermented samples exhibit strong *in vitro* abilities such as DPP-IV inhibition and antioxidant ability. DPP-IV – enzyme that is involved in the breakdown of incretin hormones plays a key role in regulating blood glucose levels. *In vitro* studies have shown that compounds found in our PN39 fermented beetroot samples exhibit strong DPP-IV inhibitory activity, which may contribute to their ability to reduce hyperglycemia *in vivo*. Even though it did not have α-Amylase and α-Gliucosidase inhibition that are involved in the breakdown of complex carbohydrates, which can contribute to obesity, means weight gain *in vivo* should not be suppressed. Additionally, our fermented samples exhibit strong antioxidant activity, which can protect cells from ROS formation and inflammation. *In vitro* studies have shown that compounds found in our PN39 fermented beetroot samples exhibit strong antioxidant activity and can contribute to their ability to reduce diabetes-related complications *in vivo* especially the β cells damage and their viability to produce insulin. Taken together, our *in vitro* studies suggest that compounds found in our fermented samples may play key roles in reducing hyperglycemia in mice by inhibiting key enzymes involved in carbohydrate metabolism and regulating blood glucose levels, as well as providing antioxidant protection against ROS formation and inflammation. These findings provide a strong rationale for further study of our fermented samples as potential therapeutic agents for diabetes and related metabolic disorders.

Before starting STZ-induction, last mice experimental group (No. 4) had PN39 fermented product pretreatment for 2 weeks. It will allow to determine if functional food product has any effect on healthy animals and disease development. After STZ injections, diabetic mice were identified by

measuring blood glucose levels after 11 days. Mice were declared diabetic after STZ-injection if their blood glucose levels were significantly higher than the control group and the treatment process has begun.

Effects of PN39 product on weight changes (Table 3.3) indicates that after 2-week pretreatment mice weight decrease to 18.42 g ± 1.07 g. However, there was not significant difference ($p > 0.05$) comparing to any other group. During treatment all mice were gaining weight. Interestingly, due to the everyday injections and stress mice that got insulin treatment had lower weight then other groups 20.55 g ± 0.94 g, 20.79 g ± 0.90 g and 21.20 g ± 0.49 g on day 7, 14, 21 respectively. However, there was no significant difference between groups during treatment. Weight changes identify obesity development that could lead to diabetes appearance. Meanwhile it not always a case, especially then disease development is genetic. Functional food effect could be both ways while ones decreas body mass in obeased/diabetic animals (Kang et al. 2014) others increase in diabetic animasls (Rajasekaran and Kalaivani 2015). Further study is needed to identify how PN39 fermented beetroot product changes blood glucose level.

Table 3.3. Mice body weight (g) measurements during treatment. Before STZ – mice acclimatization to new environment and 4th group pretreatment with PN39 product for 2 weeks; Time 0 – started treatment after deterring diabetic mice in groups * indicates significant difference to control group ($p < 0.05$).

Group Time (days)	**1** **Control**	**2** **Diabetic mice + water**	**3** **Diabetic mice + insulin**	**4** **Diabetic mice + PN39 product**
Before STZ	19.39 ± 0.77	19.02 ± 0.63	19.09 ± 1.03	18.42 ± 1.07
0	21.12 ± 0.97	20.84 ± 0.84	21.04 ± 0.72	21.04 ± 1.57
7	21.48 ± 0.51	21.62 ± 0.64	21.34 ± 0.76	22.00 ± 1.09
14	21.51 ± 0.62	21.20 ± 0.88	21.35 ± 0.27	22.54 ± 1.58
21	22.08 ± 0.70	21.52 ± 0.68	21.04 ± 0.09	22.22 ± 1.48

Blood glucose levels allow to indicate if sugar in the blood stream is too low (hypoglycemia) or too high (hyperglycemia). Diabetes is associated with high glucose levels increasing a risk of damaging

blood vesicles and ROS formation in the cells. That is why it is important to understand if our functional food product could be as effective to lower blood glucose level. Results (Table 3.4) indicate that using fermented product on healthy mice slightly degrease blood glucose level 6.0 mmol/L ± 1.45 mmol/L comparing to control group 7.7 mmol/L ± 1.32 mmol/L and indicating that there is significant difference (p > 0,05) between control and PN39 product pretreated mice. After STZ injections, PN39 product pretreated mice did not have significant difference comparing to control on day 0 and after 21 days treatment. However, groups that have not been treated and insulin injections was used as treatment had significantly higher blood glucose levels. It could be possible that after fermentation the compounds are modulated to more absorbable forms and in the organism, it will start restore β-cell, increase insulin production or lower ROS formation, in addition neutralizing STZ drug effects. Further study should evaluate glucose metabolism GTT in treated mice.

Table 3.4. Mice blood glucose measure (mmol/L) before and after treatment. Before STZ – mice acclimatization to new environment and 4th group pretreatment with PN39 product for 2 weeks; Time 0 – started treatment after deterring diabetic mice in groups * indicates significant difference to control group ($p < 0.05$).

Group Time (days)	**1** **Control**	**2** **Diabetic mice + water**	**3** **Diabetic mice + insulin**	**4** **Diabetic mice + PN39 product**
Before STZ	7.74 ± 1.32	7.45 ± 0.84	6.99 ± 0.89	6.03 ± 1.45 *
0	7.73 ± 1.23	9.91 ± 1.23*	9.61 ± 0.52*	8.8 ± 0.95
21	7.54 ± 1.12	9.32 ± 1.25*	9.51 ± 0.87*	8.59 ± 0.68

GTT is used to evaluate the ability to regulate glucose metabolism. The spike after glucose injection indicates how much glucose is in the system and decrease during period indicates how well glucose is metabolized and absorbed in tissues. Experiment results (Fig.3.9) showed all diabetic mice glucose levels were increasing till 30 min while controls group started decrease after 15 min of glucose injections. Healthy control group and PN39 treated mice had no significant difference in any time point (p > 0.05). Untreated diabetic mice showed significantly higher glucose times 15 min (29.62 mmol/L ± 3.47 mmol/L) to 120 min (9.41 mmol/L ± 2.89 mmol/L), while insulin treated mice had significantly

different blood glucose level from time 45 min (24.05 mmol/L ± 9.79 mmol/L) till 120 min (8.22 mmol/L ± 1.91 mmol/L). This indicates that diabetic and insulin treated mice had difficulties to absorb glucose in tissue increasing a risk of damaging vascular system. Consumption of PN39 fermented beetroot product have decrease mice sensitivity to glucose and increase its metabolism. Simmilar studies show that fermented products like *Moringa oleifera* could imporve GTT in high-fat diet-induced obese mice (Joung et al. 2017). Kim and Ha et.al (Kim and Ha 2013) have showed that fermented *Rhynchosia nulubilis* could significantly reduse hyperglycemia in diabetic rat model (Kim and Ha 2013). It indicates that after fermentation process compounds were converted into other bioactive molecules that are better absorbed through gut, more biologically active, better scavengers for ROS, possibly work as stimulation signal to restore pancreas β cells , increase insulin production or sensitivity to it.

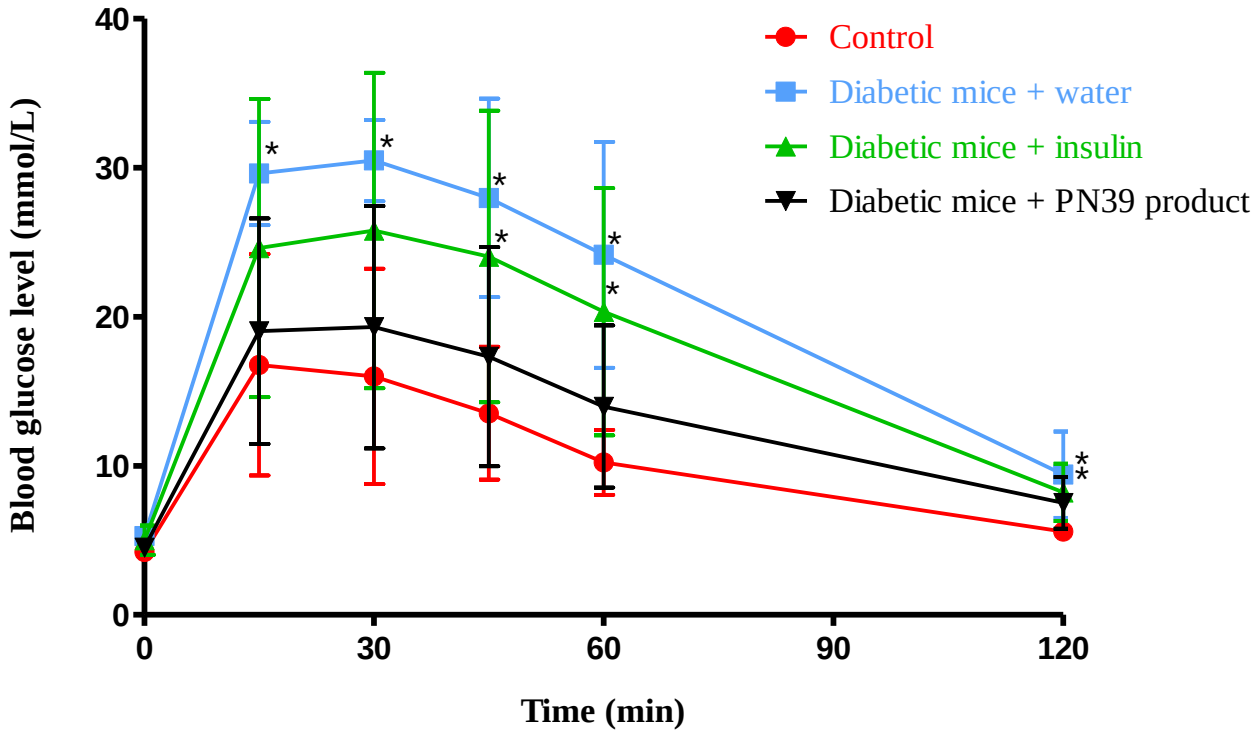


Fig.3.7. GTT after 21 day treatment. * indicates significant difference to control group. .

4. CONCLUSIONS

- LAB cultures can be isolated from Lithuanian fermented vegetables and other fermented products. Among 160 isolates, 22 culture were able to use beetroot as main energy source and could be used to ferment beetroot.
- All 22 tested bacteria cultures had β-Glucosidase activity but only 18 cultures had greater activity then 1000 U/L and were used for a further research.
- 18 bacteria cultures were lacking to inhibit α-Amylase and α-Glucosidase enzymes. Meanwhile, PN39 culture indicated considerable activity to inhibit DPP-IV (48.5 %) and antioxidant ability (3.42 mmol/L), while also having one of the highest β-Glucosidase activity (1580.3 U/L).
- Fermentation with PN39 at 45 °C for 72 h further improved its antidiabetic potentials (DPP-IV inhibition 89.3% and antioxidant capacity 15.44 mmol/L) due to the generation of high amounts of the antidiabetic compound.
- HPLC-MS analysis determined that PN39 fermented sample compound variety is different than BRJ. Identified compound dihydromyricetin in the PN39 fermented extract has the greatest possibility to give anti-diabetic effect to this product.
- 16s rRNA indicated that PN39 belongs to *Lacticaseibacillus paracasei* strain. However, it was not confirmed by WGS that identified culture as *Latilactobacillus curvatus.* WGS provides more valuable results so isolated strain was named as *Lacticaseibacillus curvatus PN39.*
- Though the bacterium showed resistance to some antibiotics, they did not possess antimicrobial resistance genes and plasmids and this indicates that the bacteria were inherently resistant to the antimicrobial compounds. These results confirms that the bacterium is safe to use for further research.
- Animal study indicates that our product have not significant changes to mice weight. PN39 fermented product showed that it can significantly reduce blood glucose level from 7.74 mmol/L to 6.03 mmol/L while used as pretreatment. After 21 days treatment fermented product had notable hyperglycemia reducing effect. GTT indicated that after use of fermented product animal were less sensitive to glucose and were similar to healthy control group.

Future perspective

Further studies are however required to ascertain other metabolites in the fermented samples that may have been involved in the antidiabetic potentials displayed. The fermented product could be tested to identify its effects on gut microbiota modulation. Experiment with tissue could help to identify

fermented product mechanisms, if it increases pancreas β cells viability, promotes β-cell regeneration, increases sensitivity to glucose or insulin, increases insulin production, reduces ROS formation and inflammation.

LIST OF PUBLICATIONS

Contribution to the thesis

- "High temperature lacto-fermentation improves antioxidant and antidiabetic potentials of Lithuanian beetroot"; Eric Banan-Mwine Daliri, **Toma Balnionytė**, Ashwinipriyadarshini Megur, Jonita Stankeviciute, Eglė Lastauskienė, Aurelijus Burokas. Submitted to LWT- "Food Science and Technology".
- "Draft genome sequence of *Latilactobacillus curvatus* PN39MY isolated from fermented vegetables". Eric Banan-Mwine Daliri, **Toma Balnionytė**, Ashwinipriyadarshini Megur, Eglė Lastauskienė, Aurelijus Burokas. Submitted to: "Data in Brief".

Published articles not related to thesis

- "*In-Vitro* Screening and Characterization of LAB from Lithuanian Fermented Food with Potential Probiotic Properties": Ashwinipriyadarshini Megur, Eric Banan-Mwine Daliri, **Toma Balnionytė**, Jonita Stankevičiūtė, Eglė Lastauskienė and Aurelijus Burokas. In review: Frontiers in Microbiology "Food microbiology"

VILNIUS UNIVERSITY
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Toma Balnionytė
Master thesis

Development of an Antidiabetic Functional Product from Beetroot by Lactofermentation

SUMMARY

Each year more people develop diabetes due to different genetic and environment factors. Hyperglycemia increase the risk of developing other chronic conditions such as hypertension, and cardiovascular diseases, which reduce quality of life and increase mortality. That indicates a global problem and therefore calls for diverse therapeutic strategies for mitigating the disease. Diet changes and especially functional food become more popular approach as prevention or disease treatment. Common, cheap, but polyphenolic compound rich vegetable beetroot has great potential to be developed to anti-diabetic product. Due to the lactofermentation betanin could be converted to other bioactive compounds that may offer a natural and effective way to manage the disease and improve the quality of life for those living with diabetes.

In this work, LAB were isolated from different fermented food sources. Among 160 isolated cultures, 18 cultures were able to use beetroot as main source of energy and had great β-Glucosidase activity. *In vitro* test indicated that while 18 bacteria cultures lack inhibition activity of α-Amylase and α-Glucosidase enzymes, PN39 culture indicated considerable activity to inhibit DPP-IV and antioxidant ability. Further work showed that to get the finest product optimal temperature and time for fermentation is 45 ^{o}C for 72 h. HPLC-MS analysis determined that PN39 fermented sample compound variety is different than beetroot and dihydromyricetin has the greatest possibility to give anti-diabetic effect. 16s rRNA analysis and WGS identified that culture is closely related to *Lacticaseibacillus curvatus.* Safety analysis of strain, according to European food safety association standards, showed no antibiotic resistance plasmids harboring in the *Lacticaseibacillus curvatus* PN39. Hence this strain fits all the criteria required for European food association.

In vivo STZ- induced diabetic mice blood glucose levels and GTT allowed to determine that PN39 fermented beetroot has great potential to reduce hyperglycemia.

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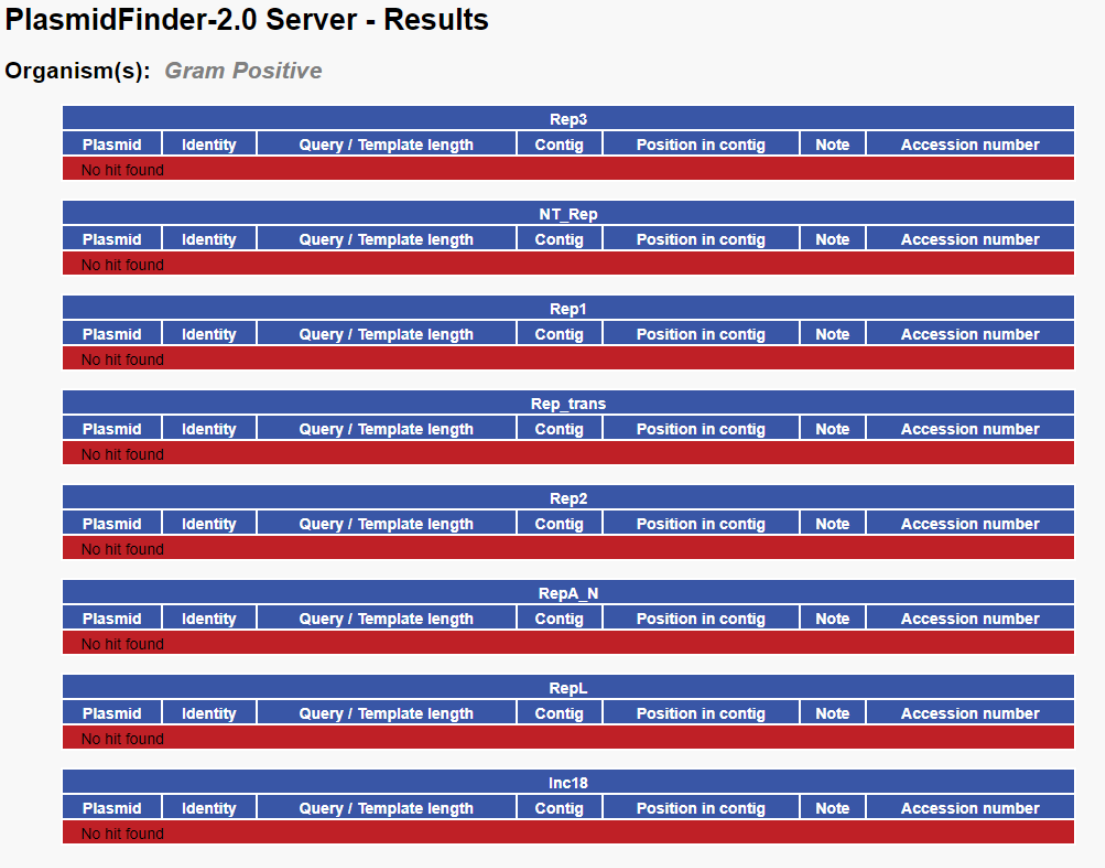
SUPPLEMENTARY MATERIAL

Supplementary table 1. Bacteria strains and their sources.

Strain	Source
LAB3	Fermented tomatoes
LAB5	Fermented tomatoes
LAB18	Sauerkraut
LAB20	Pickles
LAB25	Sauerkraut
39 (b) mix (s)	Pickles
39 (b) mix (b)	Pickles
LAB46	Fermented tomatoes
LAB48	Pickles
LAB55	Sauerkraut
LAB60	Sauerkraut
LAB66	Fermented tomatoes
LAB69	Pickles
LAB6-2	Pickles
A6MI-2	Sauerkraut
A6MI-1	Pickles
PN27.2	Fermented tomatoes
PN36	Pickles
PN38	Sauerkraut
PN38.2	Sauerkraut
PN39	Sauerkraut
PN51	Sauerkraut

Supplementary figure 1



PlasmidFinder-2.0 Server - Results

Organism(s): *Gram Positive*

Rep3						
Plasmid	Identity	Query / Template length	Contig	Position in contig	Note	Accession number
No hit found						

NT_Rep						
Plasmid	Identity	Query / Template length	Contig	Position in contig	Note	Accession number
No hit found						

Rep1						
Plasmid	Identity	Query / Template length	Contig	Position in contig	Note	Accession number
No hit found						

Rep_trans						
Plasmid	Identity	Query / Template length	Contig	Position in contig	Note	Accession number
No hit found						

Rep2						
Plasmid	Identity	Query / Template length	Contig	Position in contig	Note	Accession number
No hit found						

RepA_N						
Plasmid	Identity	Query / Template length	Contig	Position in contig	Note	Accession number
No hit found						

RepL						
Plasmid	Identity	Query / Template length	Contig	Position in contig	Note	Accession number
No hit found						

Inc18						
Plasmid	Identity	Query / Template length	Contig	Position in contig	Note	Accession number
No hit found						

Sup. Fig.1. PlasmidFinder-2.0 results. PN39 do not have plasmid for resitance.

Supplementary data 1

Table 1. ResFinder 4.1 – PN39 dont have antimibrobial resitance genes.

ResFinder phenotype results.
Sample: PN39MY_CP04785_scaffolds.fasta
#
The phenotype 'No resistance' should be interpreted with
caution, as it only means that nothing in the used
database indicate resistance, but resistance could exist
from 'unknown' or not yet implemented sources.
#
The 'Match' column stores one of the integers 0, 1, 2, 3.
0: No match found
1: Match < 100% ID AND match length < ref length
2: Match = 100% ID AND match length < ref length
3: Match = 100% ID AND match length = ref length
If several hits causing the same resistance are found,
the highest number will be stored in the 'Match' column.

# Antimicrobial	Class	WGS-predicted phenotype	Match	Genetic background
azithromycin	macrolide	No resistance	0	
spiramycin	macrolide	No resistance	0	
oleandomycin	macrolide	No resistance	0	
erythromycin	macrolide	No resistance	0	
carbomycin	macrolide	No resistance	0	
telithromycin	macrolide	No resistance	0	
tylosin	macrolide	No resistance	0	
fusidic acid	steroid antibacterial	No resistance	0	
virginiamycin m	streptogramin a	No resistance	0	

quinupristin+dalfopristin streptogramin a No resistance 0

dalfopristin streptogramin a No resistance 0

pristinamycin iia streptogramin a No resistance 0

metronidazole nitroimidazole No resistance 0

vancomycin glycopeptide No resistance 0

teicoplanin glycopeptide No resistance 0

pristinamycin ia streptogramin b No resistance 0

virginiamycin s streptogramin b No resistance 0

quinupristin streptogramin b No resistance 0

fosfomycin fosfomycin No resistance 0

linezolid oxazolidinone No resistance 0

chloramphenicol amphenicol No resistance 0

florfenicol amphenicol No resistance 0

rifampicin rifamycin No resistance 0

temperature heat No resistance 0

ceftiofur under_development No resistance 0

trimethoprim folate pathway antagonist No resistance 0

sulfamethoxazole folate pathway antagonist No resistance 0

hydrogen peroxide peroxide No resistance 0

mupirocin pseudomonic acid No resistance 0

piperacillin beta-lactam No resistance 0

piperacillin+tazobactam beta-lactam No resistance 0

ceftazidime+avibactam beta-lactam No resistance 0

cefixime beta-lactam No resistance 0

amoxicillin beta-lactam No resistance 0

cefepime beta-lactam No resistance 0

imipenem beta-lactam No resistance 0

piperacillin+clavulanic acid beta-lactam No resistance 0

cefotaxime+clavulanic acid beta-lactam No resistance 0

ertapenem	beta-lactam	No resistance	0
ampicillin	beta-lactam	No resistance	0
temocillin	beta-lactam	No resistance	0
ticarcillin	beta-lactam	No resistance	0
amoxicillin+clavulanic acid	beta-lactam	No resistance	0
cefotaxime	beta-lactam	No resistance	0
ceftriaxone	beta-lactam	No resistance	0
cefoxitin	beta-lactam	No resistance	0
ceftazidime	beta-lactam	No resistance	0
cephalotin	beta-lactam	No resistance	0
aztreonam	beta-lactam	No resistance	0
meropenem	beta-lactam	No resistance	0
unknown beta-lactam	beta-lactam	No resistance	0
ticarcillin+clavulanic acid	beta-lactam	No resistance	0
cephalothin	beta-lactam	No resistance	0
penicillin	beta-lactam	No resistance	0
ampicillin+clavulanic acid	beta-lactam	No resistance	0
tiamulin	pleuromutilin	No resistance	0
tigecycline	tetracycline	No resistance	0
doxycycline	tetracycline	No resistance	0
tetracycline	tetracycline	No resistance	0
minocycline	tetracycline	No resistance	0
kasugamycin	aminoglycoside	No resistance	0
netilmicin	aminoglycoside	No resistance	0
unknown aminoglycoside	aminoglycoside	No resistance	0
streptomycin	aminoglycoside	No resistance	0
dibekacin	aminoglycoside	No resistance	0
arbekacin	aminoglycoside	No resistance	0
neomycin	aminoglycoside	No resistance	0

apramycin	aminoglycoside	No resistance	0
tobramycin	aminoglycoside	No resistance	0
sisomicin	aminoglycoside	No resistance	0
isepamicin	aminoglycoside	No resistance	0
fortimicin	aminoglycoside	No resistance	0
lividomycin	aminoglycoside	No resistance	0
kanamycin	aminoglycoside	No resistance	0
paromomycin	aminoglycoside	No resistance	0
butiromycin	aminoglycoside	No resistance	0
gentamicin	aminoglycoside	No resistance	0
butirosin	aminoglycoside	No resistance	0
bleomycin	aminoglycoside	No resistance	0
astromicin	aminoglycoside	No resistance	0
hygromycin	aminoglycoside	No resistance	0
ribostamycin	aminoglycoside	No resistance	0
amikacin	aminoglycoside	No resistance	0
colistin	polymyxin	No resistance	0
fluoroquinolone	quinolone	No resistance	0
ciprofloxacin	quinolone	No resistance	0
nalidixic acid	quinolone	No resistance	0
unknown quinolone	quinolone	No resistance	0
formaldehyde	aldehyde	No resistance	0
spectinomycin	aminocyclitol	No resistance	0
cetylpyridinium chloride	quaternary ammonium compound	No resistance	0
chlorhexidine	quaternary ammonium compound	No resistance	0
ethidium bromide	quaternary ammonium compound	No resistance	0
benzylkonium chloride	quaternary ammonium compound	No resistance	0
lincomycin	lincosamide	No resistance	0
clindamycin	lincosamide	No resistance	0

Supplementary figure 2

Sup. Fig.2. Identified secondary metabolite regions